Characterization and Knockdown of TATA-Binding Protein 1 in Toxoplasma gondii

Malorie R. Nitz

University of New Hampshire, Durham

Follow this and additional works at: https://scholars.unh.edu/thesis

Recommended Citation
https://scholars.unh.edu/thesis/1568

This Thesis is brought to you for free and open access by the Student Scholarship at University of New Hampshire Scholars' Repository. It has been accepted for inclusion in Master's Theses and Capstones by an authorized administrator of University of New Hampshire Scholars' Repository. For more information, please contact Scholarly.Communication@unh.edu.
Characterization and Knockdown of TATA-Binding Protein 1 in *Toxoplasma gondii*

By

Malorie Nitz
B.S. Forensic Science, B.S. Biology, University of New Haven 2020

THESIS

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Master of Science
in
Genetics

May, 2022
This thesis was examined and approved in partial fulfillment of the requirements for the degree of Master of Science in Genetics by:

Victoria Jeffers, PhD, Assistant Professor, Molecular, Cellular, and Biomedical Sciences
Sarah Walker, PhD, Assistant Professor, Molecular, Cellular, and Biomedical Sciences
Matthew MacManes, PhD, Associate Professor, Molecular, Cellular, and Biomedical Sciences

On May 4th, 2022

Approval Signatures are on file with the University of New Hampshire Graduate School.
ACKNOWLEDGEMENTS

I would like to express thanks to my mentors, professors, family, and friends who have helped me during my degree progress. First and foremost, I want to thank my advisor Dr. Vicki Jeffers for her mentorship, guidance, and for teaching me to think critically and address all possible outcomes of an experiment. Thank you for your generosity, kindness, and for pushing me to be a better scientist. I would also like to sincerely thank my postdoc Dr. Krista Fleck for being there for me during troubleshooting, optimizing, and to answer all of my nitpicky questions. You have made a huge difference in my experience, and you are an absolutely amazing mentor. Sam Swartz, thank you so much for your help with the knockdown project! I would also like to acknowledge all past and current members of the Jeffers lab.

I would like to thank Krisztina Varga and Logan Brown for their experimental help with protein expression and purification. I would also like to thank Dr. Aiofe Heaslip at UConn for providing several plasmid backbones for my project.

I want to express my deepest gratitude to Joy O’Brien for being an amazing friend and supporter of my work; you have made my graduate experience unforgettable, and I appreciate your guidance, listening, and encouragement. Lastly, I would like to thank my family and friends for the continuous support through my life and academic career. You all remind me to never give up!
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS....................................................................................................................iii
LIST OF TABLES..................................................................................................................................vi
LIST OF FIGURES.................................................................................................................................vii
ABSTRACT...............................................................................................................................................viii
CHAPTER I: INTRODUCTION..............................................................................................................1
  *Toxoplasma gondii*, a eukaryotic pathogen.................................................................1
  *Toxoplasma* Life and Lytic cycle.........................................................................................2
  Clinical Presentation of *Toxoplasma*..................................................................................6
  Transcription Initiation and Regulation...............................................................................8
  TATA-Binding Proteins.........................................................................................................11
  TBPs in *Toxoplasma*........................................................................................................14
  Hypothesis and Research Aims...........................................................................................15
CHAPTER II: METHODS..................................................................................................................16
  Host cell and parasite culture............................................................................................16
  Plasmid construction............................................................................................................16
  Transfection and Drug Selection.......................................................................................19
  Parasite cloning....................................................................................................................21
  Immunofluorescence assays (IFA).....................................................................................22
  Western Blot..........................................................................................................................23
  qRT-PCR.................................................................................................................................24
  Protein Expression and Purification...................................................................................25
  Parasite nuclear extract preparation..................................................................................26
  In-vitro binding assays (EMSA)..........................................................................................26
  Parasite phenotyping assays.............................................................................................27
  TBP DNA Binding Domain alignment..............................................................................29
  Modeling of TgTBP1 and TgTBP2 DNA Binding Domain...............................................29
  Phylogenetic Analysis........................................................................................................30
Statistical Analysis...........................................................................................................30

CHAPTER III: RESULTS........................................................................................................31
- Conservation, protein modeling, and phylogeny of TgTBP1 and TgTBP2..................31
- DNA binding capability of TgTBP1 and TgTBP2.........................................................33
- Determining the interactome of TgTBP1 and TgTBP2................................................35
- Tetracycline Inducible Knockdown of TgTBP1..........................................................36
- TgTBP1 knockdown is lethal to parasites....................................................................38
- TgTBP1 knockdown does not affect cell division or morphology.............................40
- TgTBP1 knockdown affects tgbp2 expression.............................................................41

CHAPTER IV: DISCUSSION.................................................................................................42
- Conservation and phylogeny of TBPs in Toxoplasma...............................................42
- DNA binding capability of TgTBP1.............................................................................42
- TgTBP1 is critical for intracellular infection in Toxoplasma....................................43
- Interactome of TgTBP1..............................................................................................45
- Summary and Future Directions..............................................................................46

APPENDIX.........................................................................................................................47

REFERENCES...................................................................................................................48
## LIST OF TABLES

Table 1. PIC orthologs across humans, yeast, and *Toxoplasma gondii*…………………………...9
Table 2. Antibodies used for Western blotting and immunofluorescence assays……………….24
Table 3. Primers utilized for plasmid construction, integration confirmation, and qRT-PCR…..47
Table 4. Biotin-labelled probes utilized for EMSA studies…………………………………………47
LIST OF FIGURES

Figure 1. Life cycle of *Toxoplasma gondii* .................................................................3
Figure 2. Asexual life stages of *Toxoplasma gondii* .......................................................4
Figure 3. Lytic cycle of *Toxoplasma gondii* ..................................................................6
Figure 4. Transcription preinitiation complex formation .................................................9
Figure 5. TBP DNA binding domain alignment and structural modeling .....................32
Figure 6. TBP and TLF phylogenetic tree ..................................................................33
Figure 7. Recombinant TgTBP1 and electrophoretic mobility shift assays ...................34
Figure 8. TgTBP1-TurboID-V5-HA integration and qRT-PCR ......................................36
Figure 9. Tetracycline inducible knockdown of TgTBP1 .................................................37
Figure 10. Intracellular plaque assays upon TgTBP1 knockdown .................................38
Figure 11. Parasite replication assays upon TgTBP1 knockdown .................................39
Figure 12. Parasite invasion assays upon TgTBP1 knockdown ....................................40
Figure 13. Cell division and morphology upon TgTBP1 knockdown ...........................41
Figure 14. Effect of *tgtbp1* loss on *tgtp2* expression ..................................................41
ABSTRACT

CHARACTERIZATION AND KNOCKDOWN OF TATA-BINDING PROTEIN 1 IN

TOXOPLASMA GONDII

by

Malorie Nitz

University of New Hampshire, May, 2022

Toxoplasma gondii is a single celled eukaryotic parasite that causes the disease toxoplasmosis, which infects approximately thirty to fifty percent of the world’s population. Current treatments for toxoplasmosis are lacking and immunocompromised individuals are at risk of a reactivation of acute infection. Tight control of transcription initiation is critical for maintaining proper infection for the parasite. TATA-binding proteins (TBPs) are critical transcription factors for recognizing and binding to the TATA-box and other motifs in eukaryotes to recruit the transcription preinitiation complex. Toxoplasma contains two TBPs, TgTBP1 and TgTBP2, but TATA-boxes or other conserved motifs have not yet been identified in its promoter regions. Little is known about the role of TBPs in Toxoplasma. I hypothesize that TgTBP1 and TgTBP2 bind to specific sequences within promoters, interact with the TFIID complex, and are essential for parasite viability.

To investigate the role of TgTBP1 in Toxoplasma, I replaced the endogenous tgtbp1 promoter with a tetracycline regulatable promoter to knockdown tgtbp1. After successfully testing the knockdown system, parasite phenotyping assays were performed. Intracellular plaque assays show that TgTBP1 knockdown is lethal to parasites. Parasite doubling assays show that loss of
TgTBP1 results in a significant delay in replication; however, immunofluorescence assays showed that knockdown does not affect cell morphology or halt cell cycle progression, demonstrating that TgTBP1 loss results in slowed progression through the cell cycle. Parasite invasion assays exhibited a defect in host cell invasion upon TgTBP1 knockdown. As tgtbp1 expression decreases, preliminary data shows that tgtbp2 expression increases, providing evidence that TgTBP2 may be attempting to compensate for loss of TgTBP1. With this information, we begin to further narrow down the role of TgTBP1 in transcription initiation and regulation in Toxoplasma gondii.
CHAPTER I: INTRODUCTION

*Toxoplasma gondii,* a eukaryotic pathogen

The obligate intracellular parasite *Toxoplasma gondii* has the ability to infect virtually all nucleated cells in warm blooded animals, including humans. *Toxoplasma* is a part of the phylum Apicomplexa, which consists of medically relevant pathogens such as *Plasmodium* spp., the causative agent of malaria, and *Cryptosporidium* spp., the causative agent of cryptosporidiosis. Apicomplexans are most notably characterized by the apical complex, an instrumental structure for host cell invasion, and once invaded survive inside a parasitophorous vacuole. These organisms contain secretory organelles including rhoptries, micronemes, and dense granules to aid in host cell adhesion and invasion (1).

Because *Toxoplasma* can infect most nucleated cells in mammalian organisms, the pathogen is one of the world’s most common parasites. Worldwide seroprevalence of *Toxoplasma* can vary from 10% to 98% depending on a country’s socioeconomic and environmental status (2). Approximately 60 million Americans are chronically infected with the parasite and are at risk of a reactivation of acute infection. *Toxoplasma* is highly genetically diverse, however the main lineages found in humans include type I, type II, and type III strains, with type II being the most predominant in human and livestock infections (3). Type I strains are highly virulent compared to type II and III, but cannot readily form bradyzoites, the chronic form of *Toxoplasma* infection. Type II and III can maintain chronic infection and revert back to acute infection upon immune system suppression (4).
*Toxoplasma* Life and Lytic Cycle

Life Cycle

As an obligate intracellular parasite, *Toxoplasma* must always reside within a host cell to survive. Although the parasite can infect any warm-blooded animal, the definitive hosts of *Toxoplasma* are felines, meaning that sexual reproduction can only occur in the intestines of cats. Felines lack the delta-6-desaturase enzyme in their intestines, which is required for linoleic acid metabolism, causing an excess of linoleic acid and therefore promoting *Toxoplasma* sexual reproduction (5). Transmission occurs when felines ingest prey containing *Toxoplasma* tissue cysts; following ingestion, a proteolytic enzyme will dissolve the cyst walls, releasing bradyzoites. The parasites progress through five stages of schizogony and eventually convert to merozoites, which are responsible for invading the intestinal epithelium of the cat (6). Once the parasites invade, they begin to differentiate into one of two forms of gametes: microgametes or macrogametes. The fusion of a microgamete and macrogamete form an oocyst, which is released into the intestinal lumen of the feline. These oocysts are shed from the cat within its feces; once it matures in the environment, it can infect intermediate hosts through ingestion (Figure 1) (6).
The asexual component of *Toxoplasma*’s life cycle occurs within intermediate hosts, which are any warm-blooded animal including humans (Figure 2). When an intermediate host ingests an oocyst, sporozoites are released and infect the intestinal epithelium of the intermediate host. The sporozoites will differentiate into tachyzoites, which comprise the acute infection of the parasite. Tachyzoites rapidly replicate inside a host cell, egress from the cell, and infect a new one (7). In an immunocompetent host, the immune system will efficiently prevent the replication of tachyzoites; this pressures tachyzoites to differentiate into bradyzoites, which comprise the chronic infection of *Toxoplasma* in the form of tissue cysts (Figure 2) (7). Tissue cysts are prevalent in the central nervous system, eyes, and muscle tissue. Tissue cysts contain hundreds of bradyzoites and are less susceptible to proteolytic enzymes. They will remain in host cells throughout the life of the host without causing an inflammatory response (8). At a slow rate, bradyzoites can spontaneously differentiate back into tachyzoites. In a healthy host, the immune system can eliminate these activated cells. However, in an immunodeficient host, a reactivation
of acute infection can result in deleterious effects on the central nervous system, ocular tissue, and muscle tissue (9).

The two main routes of infection with *Toxoplasma* are oral and congenital (10). One possible method of transmission of *Toxoplasma* in humans can occur through the ingestion of tissue cysts found in undercooked meat, where the parasite is common in many animals used for food (Figure 1) (11). The parasite can survive for years within animals as tissue cysts, and therefore pose a threat for human infection. Humans can also become infected through ingestion of oocysts through contaminated water or feline feces, which then differentiate into the asexual form of the parasite in the host (11). Congenital toxoplasmosis occurs when a pregnant woman has a primary infection with the parasite, where tachyzoites cross the placental wall and infect the fetus (Figure 1). Depending on the gestational age, congenital toxoplasmosis can cause damage of varying severity (9). *Toxoplasma* poses a serious threat to immunocompromised and pregnant individuals.

**Figure 2.** Asexual stages of *Toxoplasma gondii*, found in intermediate hosts. The acute stage consists of tachyzoites, which rapidly replicate throughout the host. Upon immune system pressure, they differentiate into chronic tissue cysts, which remain throughout the life of the host. These can spontaneously differentiate or differentiate upon immune suppression, reactivating acute infection.
Lytic Cycle

The parasite proceeds through a complex lytic cycle to attach, invade, replicate, and egress host cells to maintain infection (Figure 3). When *Toxoplasma* finds a host cell to invade, the surface antigen protein SAG1, found on the protein coat of the parasite, recognizes proteoglycans on the surface of the host cell (12). Once recognition occurs, microneme proteins are secreted from the apical end of the parasite, which aid in forming an attachment to the host cell and providing gliding motility to the parasite for host cell entry (13). A moving junction is formed to mediate interactions between the parasite and the host cell. Once this is established, rhoptry proteins are excreted from the apical end and invaginates the host cell plasma membrane. As *Toxoplasma* enters the cell, a parasitophorous vacuole (PV) is formed, where the parasites reside during replication (Figure 3). Rhoptry and dense granule proteins are secreted to alter the PV membrane (PVM) to adapt to the host cytoplasm (14). Additionally, dense granule proteins can enter the host cell from the parasite to modify the host cells response to infection (7).

Replication of *Toxoplasma* tachyzoites occurs by endodyogeny, where two daughter cells are formed within a mother cell (15). Typically, tachyzoites will replicate within six to eight hours (Figure 3). After several rounds of replication, egress is triggered by various mechanisms, including a decrease in intracellular K⁺ and pH, and an increase in abscisic acid (ABA) (16). *Toxoplasma* parasites rupture the PV and egress into the extracellular environment, where they use gliding motility to continuously invade other host cells, repeating the lytic cycle (Figure 3). Invasion, replication, and egress are complex components of parasite infection requiring different molecular, cell, and biochemical processes; very tight regulation of gene expression is required to maintain these processes and progress through *Toxoplasma* infection.
Clinical Presentation of Toxoplasma

In immunocompetent individuals, symptomatic infection of *Toxoplasma* is uncommon; the immune system can quickly eliminate tachyzoites, and the parasites that differentiate into latent bradyzoites will remain throughout the life of the host. Individuals who are symptomatic may have muscle pain, headaches, or fever. Tissue cysts containing bradyzoites can develop in organs such as the liver and lungs but are most prevalent in the eyes and central nervous system, including the brain (8). While there are obvious effects of acute toxoplasmosis, less is known about the long-term impact of chronic toxoplasmosis on human health. Studies on the implications of chronic toxoplasmosis have found correlations between chronic infection and behavior and mental disorders (17). *Toxoplasma* seropositivity has been associated with disorders such as schizophrenia, epilepsy, and neurodegenerative diseases. However, there has been no clear experimental evidence of a direct effect of *Toxoplasma* chronic infection on neurological function (9).
While asymptomatic infection is common in healthy individuals, *Toxoplasma* infection in immunocompromised people can be severe. When an individual becomes immunocompromised, latent bradyzoites can differentiate back into tachyzoites, causing a reactivation of acute infection in organs where tissue cysts were residing. This can cause effects such as toxoplasmic encephalitis, where parasites encysted in the central nervous system can cause great tissue damage and inflammation (18). Other common symptoms include myocarditis, fever, ataxia, or seizures (19). Ocular toxoplasmosis is a common presentation of reactivated acute infection, which can cause damage to the ocular tissue and permanent eye damage (20). Reactivation of *Toxoplasma* tachyzoites poses a major threat to immunodeficient individuals. In cases of congenital toxoplasmosis, as the parasites infect the body, tachyzoites infect the uterus and cross the placental wall, eventually infecting the fetus (10). This can have numerous serious consequences, such as premature birth, eye infections, swollen organs and glands, or miscarriage (21).

Drug treatments for *Toxoplasma* infection only treat the acute form of infection; there are no available treatments for removal of chronic tissue cysts. The primary treatment for toxoplasmosis includes a combination of sulfadiazine and pyrimethamine, which target the dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) enzymes in the folic acid synthesis pathway, respectively (22). DHFR is present in both humans and the parasite; pyrimethamine has a slightly higher affinity for parasite DHFR, however it can still cause toxicity in humans. Therefore, this combined treatment is typically supplemented with folinic acid to reduce side effects of toxicity (22). Due to the toxic nature of pyrimethamine, pregnant women are typically administered spiramycin as a nontoxic alternative that does not cross the placental wall (22). There is an urgent need for better treatments for the acute and chronic form
of *Toxoplasma* infection that are more effective and safer for patients. Studying the biology of *Toxoplasma* can reveal novel and unique pathways that can be targeted for therapeutic drugs.

**Transcription Initiation and Regulation**

Synthesis of a protein begins with transcription, the process of reading a DNA molecule and creating an RNA transcript which then used for translation into a protein. Transcription of protein-coding genes is performed by RNA polymerase II. The initiation of transcription occurs in the promoter of a gene, an upstream region of a gene that contains binding sites for regulatory elements and transcription factors (TF). RNA polymerase II transcription requires the presence of several basal TFs, which recognize and bind to specific motifs in the promoter and form the transcription preinitiation complex (PIC). In most eukaryotes, general TF’s TFIID, TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH form the PIC in a stepwise manner (Figure 4) (23). The first step of initiation involves the recognition and binding of the TATA-binding protein (TBP) to a motif in the promoter, which then recruits the rest of TFIID, TFIIA, and TFIIB (Figure 4). This is followed by the recruitment of RNA polymerase II, TFIIF, TFIIE, and TFIIH, completing PIC assembly (Figure 4). To proceed with RNA synthesis, the PIC must melt the promoter to insert the template DNA strand, a process that is reliant on TFIIH to separate the strands, allowing RNA polymerase access to the template (24). Once initiation is complete, elongation and termination occur. Many components of the PIC are conserved across humans and yeast; *Toxoplasma* possesses numerous PIC components; however, several proteins are not present (Table 1). Little is known about the proteins that actively contribute to PIC formation in *Toxoplasma*. 
Figure 4. Transcription preinitiation complex formation. TBP recognizes the TATA-box or other initiator motifs in the promoter, directly interacts with DNA with several amino acid residues and bends the DNA 80°, recruiting the TFIID complex. TFIID then recruits the TFIIA and TFIIB complexes, followed by TFIIE, TFIIF, RNA polymerase, and TFIIH in a stepwise manner.

Table 1. PIC orthologs across humans, yeast, and Toxoplasma gondii. Protein sequences from Uniprot were compared to the Toxoplasma ME49 strain using BLAST (NCBI) in ToxoDB (25).

<table>
<thead>
<tr>
<th>Transcription Factor</th>
<th>Homo sapiens</th>
<th>Saccharomyces cerevisiae</th>
<th>Toxoplasma gondii</th>
<th>Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFIIA</td>
<td>TFIA subunit 1 α</td>
<td>TOA1</td>
<td>hypothetical protein</td>
<td>TGME49_259580</td>
</tr>
<tr>
<td></td>
<td>TFIA subunit 2 α/ Γ</td>
<td>TOA2</td>
<td>--</td>
<td>TGME49_232210</td>
</tr>
<tr>
<td></td>
<td>TFIIA Γ</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TFIIB</td>
<td>TFIIB</td>
<td>TFIIB (SUA7 gene)</td>
<td>TFIIB</td>
<td>TGME49_292010</td>
</tr>
<tr>
<td>TAF1</td>
<td>TAF1/145</td>
<td>TAF1/250</td>
<td>TAF1/250</td>
<td>TGME49_276180</td>
</tr>
<tr>
<td>TAF2</td>
<td>TAF2/150</td>
<td>TAF2/150</td>
<td>TAF2/150</td>
<td>TGME49_214240</td>
</tr>
<tr>
<td>TAF3</td>
<td>TAF3/47</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TAF4</td>
<td>TAF4/48</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TAF5</td>
<td>TAF5/90</td>
<td>TAF5/90</td>
<td>TAF5</td>
<td>TGME49_318260</td>
</tr>
<tr>
<td>TAF6</td>
<td>TAF6/60</td>
<td>TAF6</td>
<td>TAF6</td>
<td>TGME49_295410</td>
</tr>
<tr>
<td>TAF7</td>
<td>TAF7/17</td>
<td>TAF7</td>
<td>TAF7</td>
<td>TGME49_309170</td>
</tr>
<tr>
<td>TAF8</td>
<td>TAF8/65</td>
<td>TAF8</td>
<td>TAF8</td>
<td>TGME49_313810</td>
</tr>
<tr>
<td>TAF9</td>
<td>TAF9/17</td>
<td>TAF9</td>
<td>TAF9</td>
<td>TGME49_233400</td>
</tr>
<tr>
<td></td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TAF9B</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TAF10</td>
<td>TAF10/25</td>
<td>TAF10/25</td>
<td>TAF10/25</td>
<td>TGME49_315120</td>
</tr>
<tr>
<td>TAF11</td>
<td>TAF11/40</td>
<td>TAF11/40</td>
<td>TAF11/40</td>
<td>TGME49_244160</td>
</tr>
<tr>
<td>TAF12</td>
<td>TAF12/61-68</td>
<td>TAF12</td>
<td>TAF12</td>
<td>TGME49_231010</td>
</tr>
<tr>
<td>TAF13</td>
<td>TAF13/19</td>
<td>TAF13/19</td>
<td>TAF13/19</td>
<td>TGME49_203358</td>
</tr>
<tr>
<td>TAF14</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TAF15</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TBP</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TFIIIE</td>
<td>TFIIIEα/ GTF2E1</td>
<td>TFA1</td>
<td>GTF2E1</td>
<td>TGME49_281950</td>
</tr>
<tr>
<td>TFIIIEβ</td>
<td>TFA2</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TFIIIF</td>
<td>TFIIIFα</td>
<td>TFG1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>TFIIIFβ</td>
<td>TFG2</td>
<td>membrane protein</td>
<td>TGME49_269660</td>
</tr>
<tr>
<td>TFIIH helicase subunit XPD</td>
<td>SSL2</td>
<td>TFIIH helicase subunit XPD</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
Gene regulation is a critical process that works with transcription factors to activate or repress transcription. Regulation of gene expression primarily occurs at the initiation level. Activator and repressor proteins regulate the rate of transcription of most genes (26). Activator proteins bind to specific sites termed enhancers to induce transcription. Repressors work in a similar manner but inhibit transcription from occurring. Eukaryotes such as humans and yeast contain the Mediator, a large protein complex that is required for transcription in many RNA polymerase II promoters. The Mediator acts similarly to a general TF, interacting with the PIC as a coactivator to successfully begin RNA synthesis (27). There is no established Mediator complex in Toxoplasma, although some parasite proteins with homology to certain subunits of Mediator have been identified in the genome. An additional layer of gene regulation is chromatin structure; DNA is tightly wrapped around an octamer of histones, forming the nucleosome, the basic unit of chromatin (28). Changes in the structure of chromatin alter the availability of a DNA template for transcription (26). Additionally, histone modifications such as acetylation,
methylation, and phosphorylation mark genes for activating or repressing transcription, and help recruit transcription complexes (28).

Regulation of gene expression is an integral process to maintain eukaryotic cells and organisms. In protozoan pathogens, gene regulation has been shown as an essential pathway for development and pathogenesis. Regulation of gene expression in *Toxoplasma* is largely influenced by epigenetic mechanisms and by ApiAP2 APETELA plant-like proteins (AP2), a family of transcription factors found in Apicomplexa (29).

The PIC binds to specific sequences upstream of the transcription start site known as motifs. The most well-known motif is termed the TATA-box, containing a consensus sequence of “TATAAWWR”, where “W” can be an adenine or thymine base, and “R” can be an adenine or guanine base. The TATA-box is typically located approximately 30 base pairs upstream of the transcriptional start site. The TATA-box is highly conserved amongst higher eukaryotes (30). Other common motifs include the initiator element (Inr), which directly overlaps the transcriptional start site, and the downstream promoter element, which is found following the transcriptional start site (31). While these are the most commonly recognized motifs, each eukaryotic organism can contain many varying consensus motifs within their promoters.

**TATA-Binding Proteins**

A vital protein for the formation of the PIC is TBP, whose role is to bind to the TATA-box or other initiator motifs to begin recruiting the PIC (32). TBP is a small, saddle-shaped protein containing minor and major grooves whose structure is highly conserved. TBP is a part of the TFIID complex, which includes several TBP-associated factors (TAFs). TBP contains a C-terminal domain that helps recognize and bind DNA in a sequence-specific manner; several
amino acid residues contribute to DNA binding, bending the DNA 80° and allowing the recruitment and stabilization of the PIC and RNA polymerase (33). The concave region of TBP binds to DNA and the convex portion is available for cofactor binding (34). TFIIA and TFIIB directly interact with TBP to stabilize the DNA-TBP complex at promoters (35). Most eukaryotes contain a single TBP that is constitutively active in all cell types.

Since TBPs are essential for PIC formation, recruitment of TBP to template DNA could be a rate-limiting step in transcription initiation. In humans and yeast, TBP is largely present as a dimer when not bound to DNA. Dissociating from the dimer is a slow kinetic process, which is another regulating mechanism of gene expression (36). Dimerization of TBPs competes with DNA binding, suggesting that TBP homodimers are a mechanism for negatively regulating DNA binding activity. Additionally, there is a relationship between TBP and the SAGA complex, found in humans and yeast. The SAGA complex is a multi-protein chromatin remodeling complex and is associated with activation of transcription. The Spt3, Spt7, and Spt8 subunits of the SAGA complex bind to and deliver TBP to promoters to nucleate formation of the PIC, regulating the initiation of gene expression (37,38). A conserved SAGA complex has not been identified in Toxoplasma (39). Negative cofactor 2 (NC2) is a negative regulator of basal transcription through direct TBP interaction. NC2 inhibits transcription through binding of TBP, blocking the recruitment of TFIIA and TFIIB and thereby inhibiting PIC assembly (40). An NC2 ortholog does not exist in Toxoplasma. TAF1, a factor in the TFIID complex, can also negatively regulate transcription via interaction with TBP. The TAF1 N-terminal domain (TAND), which is comprised of TAND1 and TAND2, interact with the concave and convex surfaces of TBP, respectively. TAND1 interacts directly with TBP, blocking TBP-DNA binding to the promoter; TAND2 interacts with TBP to inhibit TFIIA-TBP interaction, prohibiting recruitment of the PIC
A TAF1 protein exists in *Toxoplasma*, but its ability to bind TBP and regulate transcription is unknown.

TBPs have been identified and studied in several protozoan pathogens including *C. parvum*, *P. falciparum*, and *Entamoeba histolytica* (35). *C. parvum* contains a TBP protein that binds DNA in vitro; electrophoretic mobility shift assays (EMSA) with recombinant CpTBP have shown that the protein can bind a bacterial TATA-box consensus sequence. Furthermore, CpTBP interacts with multiprotein bridging factor type 1 (MBF1), a transcriptional coactivator that is known to enhance gene expression (42). *P. falciparum* contains a single TBP protein that has been shown to recognize a TATA-box sequence and TATA-box-like sequence (TGTAA) in two *Plasmodium* promoters via EMSA and chromatin immunoprecipitation (ChIP) experiments (43,44). However, PfTBP is bound to DNA in transcriptionally inactive genes, suggesting that PfTBP may be pre-assembled at DNA and other cofactors are needed for PIC formation (45). *E. histolytica* contains two TBPs, EhTBP1 and EhTBP2; EhTBP2 is identical to EhTBP1 and is silenced by genomic DNA methylation (46). *E. histolytica* also contains a TBP-related factor 1 (EhTRF1) that shares significant similarity to EhTBP1. *E. histolytica* contains several promoter motifs including the TATA-box and GAAC-box. It has been shown that EhTBP1 and EhTRF1 bind to the TATA-box, several TATA-box variants, and the GAAC-box in vitro (47,48). While the DNA-binding capability of TBPs in several protozoans in-vitro is known, little is known about TBPs in *Toxoplasma.*
TBPs in *Toxoplasma*

While many eukaryotes contain a single universal TBP, *Toxoplasma* contains two TBPs: TgTBPl and TgTBP2. While this is peculiar, it is even more interesting to note that there have been no TATA-box consensus sequences found in *Toxoplasma*. Additionally, *Toxoplasma’s* Type I RH strain genome has a relatively high GC-content of 52%, making it less likely for a TATA-box sequence to appear in the genome (49). Studies have shown that while there are upstream elements that are required for several genes’ expression, no consensus initiation motifs throughout *Toxoplasma’s* entire genome have yet been defined (50). Certain motifs have been described for specific gene subsets; for example, the transcription factor BFD1 binds to a CACTGG motif in many bradyzoite-specific genes (51). The GAGACGC motif has been identified in 20.4% of tachyzoite stage-specific gene promoters, but a binding transcription factor has not yet been identified. Little is known about *Toxoplasma* TBPs DNA binding properties, their overall function, and their role in parasite fitness and survival. Relative fitness scores of TgTBPl and TgTBP2 calculated from a genome wide CRISPR/Cas9 screen are -4.81 and -3.73 respectively, strongly suggesting that these proteins are essential for parasite viability\(^1\) (52).

It is unknown if and how TBPs are regulated in *Toxoplasma*. The lack of a SAGA complex and Mediator suggests that the parasite may rely more heavily on TFIID for recruitment of the PIC. It is crucial to gain more information about these proteins, how they function, and how they are regulated to determine if they play a significant role in transcription initiation in *Toxoplasma*. Understanding the basal transcriptional machinery is vital for fully understanding

\(^{1}\) This study utilized genome wide CRISPR/Cas9 to assess the contribution of each gene in *Toxoplasma* during infection. Scores range from -6.98 to 2.96, with lower values conferring to higher essentiality for parasite fitness.
how the parasite expresses its genes; detecting unique components in the initiation process could be useful for identifying anti-parasitic drugs for treating toxoplasmosis. I hypothesize that TgTBP1 and TgTBP2 binds to specific sequences within promoters, interacts with the TFIID complex, and is essential for parasite viability. I will address this hypothesis with the following aims: **Aim 1.** Determine the DNA binding capability of TgTBP1 and TgTBP2. **Aim 2.** Determine the impact of TgTBP1 and TgTBP2 expression on tachyzoite fitness. **Aim 3.** Determine the interactome of TgTBP1 and TgTBP2.
CHAPTER II: METHODS

Host cell and parasite culture

Primary human foreskin fibroblast (HFF) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat inactivated fetal-bovine serum (FBS). *Toxoplasma* Type I RH strain parasites lacking ∆Ku80 (∆Ku80) and Type I RH strain parasites containing a tetracycline-controlled transactivator (TATi) were grown in confluent monolayer of HFF cells in DMEM supplemented with 1% heat inactivated FBS. Host cell and parasite cultures were maintained at 37 °C and 5% CO₂.

Plasmid construction

To produce recombinant TgTBP1 protein in *E. coli*, an expression plasmid construct was created with *tgtbp1* fused to an N-terminal GST tag. The protein was expressed in *E. coli* and purified using FPLC. In order to clone the coding region of *tgtbp1* into the plasmid construct, RNA was isolated and complimentary DNA (cDNA) was produced. Fully lysed ∆Ku80 parasites from a T-25 flask were harvested, spun down, and washed with 1X PBS. RNA was isolated from the pellets using phenol:chloroform. Briefly, TRI Reagent® (Sigma-Aldrich) was added to the parasite pellet and incubated at room temperature. Chloroform was added to the solution, incubated at room temperature, then centrifuged at 16,000xg for fifteen minutes at 4 °C. The clear aqueous layer was transferred to a new tube, isopropanol was added to the solution, incubated at room temperature, then centrifuged at 16,000xg for ten minutes at 4 °C. The supernatant was removed, and the RNA was washed with 70% ethanol and centrifuged at 7500rpm for five minutes at 4 °C. The RNA pellet was dried, and the TURBO DNA-free™ Kit (Invitrogen) was used to remove any DNA contamination from the sample. Complimentary
DNA (cDNA) was generated with \textit{tg}tbp1-specific primers (primers 1 and 2, Table 3) using the Omniscpt RT Kit following manufacturer’s instructions (Qiagen). The DNA binding domain of \textit{tg}tbp1 was PCR amplified in two separate pieces using primers 3 and 4 for the first half and 1 and 5 for the second half of the sequence (Table 3) because amplification of the entire sequence was unsuccessful. Assembly PCR was performed to amplify both pieces of the DNA binding domain of \textit{tg}tbp1 together using primers 6 and 7 (Table 3) containing a 14 base pair sequence of homology to the target site of the pGEX-4T-1 plasmid on either end. Digestion of the pGEX-4T-1 plasmid was performed overnight at 37 °C with EcoRI to linearize the plasmid before cloning and was gel purified using the Monarc® DNA Gel Extraction Kit (New England BioLabs). The \textit{tg}tbp1 DNA binding domain sequence was inserted into the linearized pGEX-4T-1 plasmid at the EcoRI site (939) using the NEBuilder® HiFi DNA Assembly Cloning Kit (New England BioLabs) for expression of TgTBP1 fused to an N-terminal GST tag according to manufacturer’s instructions.

To construct a plasmid with TgTBP1 fused to the enzyme TurboID, \textit{tg}tbp1 genomic DNA (gDNA) was cloned into a plasmid containing a TurboID-V5-HA tag to integrate into the parasite genome. gDNA was extracted from fully lysed ∆Ku80 parasites using the DNeasy Blood & Tissue Kits (Qiagen) following manufacturer’s guidelines. \textit{tg}tbp1 gDNA was PCR amplified using primers 8 and 9 (Table 3) to amplify a 1.5kb C-terminal piece of \textit{tg}tbp1. The pTKO2-II-3-MyoF-Turbo-V5-HA plasmid (a gift from Dr. Aoife Heaslip, UConn) was digested overnight at 37 °C using AvrII and BglII for linearization and gel purified using the Monarch® DNA Gel Extraction Kit (New England BioLabs). The \textit{tg}tbp1 sequence was inserted into the linearized pTKO2-II-3-Turbo-V5-HA plasmid at the AvrII and BglII site (2844) using the NEBuilder®
HiFi DNA Assembly Cloning Kit (New England BioLabs) for expression of \textit{tgtp1} fused to TurboID-V5-HA according to manufacturer’s instructions.

To construct a plasmid for regulation of \textit{tgtp1} expression using a tetracycline inducible knockdown system, \textit{tgtp1} gDNA was cloned into a plasmid containing a tetracycline-regulatable promoter and myc tag. gDNA was isolated from fully lysed \textit{ΔKu80} parasites using the DNeasy Blood & Tissue Kits (Qiagen) according to manufacturer’s instructions. A 1.5kb N-terminal piece of \textit{tgtp1} gDNA was PCR amplified using primers 10 and 11 (Table 3). The DHFR-tetO7Sag4-myc-TgBDP1 plasmid was linearized overnight at 37 °C with NotI and BglII and was gel purified using Monarch® DNA Gel Extraction Kit (New England BioLabs). The \textit{tgtp1} sequence was inserted into the linearized DHFR-tetO7Sag4-myc plasmid at the NotI and BglII site (685) using the NEBuilder® HiFi DNA Assembly Cloning Kit (New England BioLabs) according to manufacturer’s guidelines.

The recombined products of each cloned plasmid construct were transformed into NEB® DH5-α competent \textit{E. coli} cells (New England BioLabs). The competent cells and DNA mixture were incubated on ice for thirty minutes, heat shocked to create pores in the membrane of the bacteria allowing the plasmid DNA in, then returned to ice. The cells were grown for one hour in SOC Outgrowth Media (New England BioLabs), then plated in a lawn on an LBA plate and grown overnight at 37 °C.

Isolated colonies of the transformed bacteria were picked and grown in LBA broth overnight at 37 °C. To identify a clone containing the integrated plasmid construct, small scale plasmid purifications were performed. First, the bacteria were pelleted, and the media was removed; the pellet was resuspended in a buffer containing 50mM Tris-Cl, 10mM EDTA, RNase
A, pH 8.0. The bacteria were lysed in a buffer containing 200mM NaOH and 1% SDS, then neutralized in 3M potassium acetate, pH 5.5 to separate the insoluble material. The sample was spun down, and the liquid soluble material was transferred to isopropanol and incubated at room temperature. The DNA was spun down and washed with 70% EtOH and dried at room temperature. Purified plasmid DNA was resuspended in double deionized water.

Plasmid construct integration was confirmed by restriction digests and Sanger sequencing from GeneWiz. Primers flanked the insert and alignments were performed on the sequenced plasmid and the designed plasmid construct to confirm integration. Primers 12 and 13 were used to confirm integration in pGEX-4T-1, primers 14 and 15 were used to confirm integration in pTKO2-II-3-TurboID-V5-HA and primers 16 and 17 were used to confirm integration in DHFR-tetO7Sag4-myc (Table 3). Once integration was confirmed, large scale plasmid purifications were performed using the GenElute™ HP Plasmid Maxiprep Kit (Sigma-Aldrich) with some changes. The spin format was followed according to the manufacturer’s protocol. After eluting the DNA, 0.3M sodium acetate and 100% ethanol was added to the DNA, then incubated at -20°C overnight. The DNA was centrifuged at 5,000xg for thirty minutes, the supernatant was discarded, and the precipitated DNA was washed with 70% ethanol. After centrifugation, the DNA pellet was dried then resuspended in 200-400 μL of water.

Transfection and drug selection

To prepare plasmid constructs for transfection, plasmids were linearized using a restriction enzyme for insertion into the Toxoplasma gene locus using single-crossover homologous recombination. The pTKO2-II-3-TgTBP1-Turbo-V5-HA plasmid was linearized using CspCl for integration of the construct at the C-terminal end of the tgtbp1 gene locus. The
DHFR-tetO7Sag4-myc-TgTBP1 plasmid was linearized using NheI for integration of the construct at the \( tgtbp1 \) gene locus to replace the endogenous \( tgtbp1 \) promoter with the tetracycline-regulatable Sag4 promoter. Five to ten micrograms of DNA was linearized overnight at 37 °C. DNA purification of the linearized plasmid was performed by adding an equal amount of phenol:chloroform:isoamyl alcohol (IAA) to the digested DNA, vortexing, and centrifuging. The top layer was placed into a new tube and an equal volume of chloroform was added, centrifuging once again. This was repeated a total of three times, and the DNA transferred to a new tube after the third spin was precipitated by adding 2x volume of 100% EtOH and 0.1 volumes of 3M sodium acetate. The tube was incubated at -20 °C overnight. The DNA was then centrifuged for thirty minutes at 4 °C. The supernatant was removed, and the DNA pellet was washed with 70% EtOH and centrifuged. The DNA pellet was dried in the flow hood, then resuspended in 20 uL of P3 Primary Cell Solution (Lonza).

Type I RHΔKu80 parasites were used for transfection of pTKO2-II-3-TgTBP1-Turbo-V5-HA containing a disruption in the Ku80 gene to promote homologous recombination (53). Type I RHΔKu80 TATi parasites were used for transfection of DHFR-tetO7Sag4-myc-TgTBP1 containing a tetracycline-controlled transactivator (TATi) to bind to the tetO7 promoter at the \( tgtbp1 \) locus (54). One mL of extracellular parasites was pelleted down, the media was removed, and the resuspended DNA in transfection buffer was added to the pelleted parasites. The DNA-parasite mix was placed into a 16-well strip and the 4D-Nucleofector® X Unit (Lonza) was used for transfection with settings FI 115. Once transfection was complete, the parasites were added to a T-25 flask containing an HFF monolayer 1% FBS DMEM.

Drug selection was commenced 24-48 hours after transfection to allow parasites to recover and begin expressing the selection marker. The DHFR-tetO7Sag4-TgTBP1 parasite line
was supplemented with 1uM pyrimethamine. This line contained the DHFR cassette within the plasmid construct, which makes the parasite line resistant to pyrimethamine treatment. The DHFR cassette contains three point mutations conferring resistance to pyrimethamine, which normally kills parasites (55). The pTKO2-II-3-TgTBP1-Turbo-V5-HA parasite line was supplemented with 25ug/uL mycophenolic acid (MPA) and 50ug/uL xanthine in 100uM KOH. This line contains a hypoxanthine phosphoribosyl transferase (HXGPRT) cassette within the plasmid construct, which makes the parasite line resistant to MPA/Xanthine treatment. The HXGPRT cassette allows parasites to bypass inosine-5’-monophosphate dehydrogenase pathway inhibition and convert xanthine to xanthosine monophosphate (56). Genomic PCR and immunofluorescence assays were used to confirm integration of the plasmid construct into the \textit{tgthbp1} gene locus.

\textbf{Parasite cloning}

Following several rounds of drug selection, uncloned parasite populations were diluted to isolate single parasite clones to identify ones with correct genomic construct integration. Parasites were counted using a hemocytometer. Calculations were performed to determine the volume of a 1:1000 dilution of parasites needed to obtain one parasite per well in a 96-well plate. Parasites were diluted 1:1000 in 10 mL of 1% FBS DMEM media and the calculated volume was added to 20mL 1% FBS DMEM media containing the parasites corresponding drug needed for selection. Two hundred uL of media was added to each well and cultured, undisturbed for five to seven days at 37 °C and 5% CO2. After incubation, each well was checked using light microscopy to identify wells with one plaque; clonal parasites from the wells containing one plaque were screened for correct plasmid integration using the Phire™ Tissue Direct PCR
Master Mix (Thermo Scientific) following manufacturer’s protocol with uncloned positive populations and ΔKu80 parasite gDNA as positive and negative controls, respectively.

**Immunofluorescence assays (IFA)**

Parasites were inoculated and grown in confluent HFF monolayers on coverslips in 12-well plates for approximately twenty-four hours. To detect biotinylation in the pTKO2-II-3-TgTBPl-Turbo-V5-HA parasite line, cells were incubated with 1% FBS DMEM media containing 150uM biotin for one hour before fixing. Infected cells were fixed with 4% paraformaldehyde in 1X PBS, then washed with 1X PBS three times. Cells were permeabilized with 0.2% Triton X-100 in 3% BSA then washed with 1X PBS. Blocking was done with 3% BSA for at least one hour at room temperature or overnight at 4 °C. Cells were incubated in primary antibody in 3% BSA for two hours at room temperature or overnight at 4 °C (Table 2). For the TgTBPl-Turbo-V5-HA parasite line, rat anti-HA was used. For the tet-myc-TgTBPl parasite line, mouse anti-myc was used. To detect acetylated-α-tubulin, mouse anti-acetylated-α-tubulin was used (Table 2). After primary antibody incubation, cells were washed three times in 1X PBS. Cells were incubated in secondary antibody and DAPI to stain the nucleus at 1:1000 dilution in 3% BSA for one hour at room temperature in the dark. For cells containing an anti-HA primary antibody, they were incubated in anti-rat Alexa Fluor 594. For cells containing an anti-myc or anti- acetylated-α-tubulin primary antibody, they were incubated in anti-mouse Alexa Fluor 594. To detect biotinylation in TgTBPl-Turbo-V5-HA parasites, cells were incubated in Streptavidin Alexa Fluor 488 (Table 2). After secondary antibody incubation, cells were washed with 1X PBS. Coverslips were mounted face down onto a microscope slide with VECTASHIELD Antifade Mounting Medium and viewed under a fluorescent microscope.
Western Blot

To produce protein lysate for Western blotting, a heavily infected monolayer of parasites was scraped to release parasites from host cells, pelleted, and the media was removed. Parasites were resuspended in RIPA buffer (50mM Tris HCl, pH 7.9, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 1X protease inhibitor cocktail (PIC). Parasite lysates were kept on ice and sonicated for two minutes at 50% amplitude, then stored at -20 °C. Protein quantification was performed using the Pierce™ BCA Protein Assay Kit according to manufacturer’s instructions using the microplate procedure (Thermo Scientific). Fifty to one hundred μg of sample lysate were added to each Western Blot in equal concentrations across each sample. Samples containing protein lysate, 4X NuPAGE™ LDS Sample Buffer, and β-mercaptoethanol were run on denaturing NuPAGE™ 4-12% Bis-Tris polyacrylamide gels (Invitrogen). Gels were transferred to a nitrocellulose membrane and stained with Ponceau S to verify complete protein transfer. Blots were blocked in 5% BSA or 5% non-fat milk overnight at 4 °C. HA-tagged proteins were probed with a rat anti-HA primary antibody (Table 2). After primary antibody incubation, blots were washed three times with Tris-buffered saline Tween 20 (TBST). Myc-tagged proteins were probed with mouse anti-myc horseradish peroxidase (HRP). Anti-rat antibody conjugated to HRP (GE NA935, 1:2000) was used as a secondary antibody for anti-HA blots (Table 2). To probe for biotinylation of proteins, blots were incubated in streptavidin-HRP conjugate (Table 2). Secondary antibodies were incubated for one hour at room temperature, then washed three times with TBST. Blots were visualized using the Chemiluminescence Western Blot Substrate (Pierce) and imaged using the BioRad V3 Chemidoc.
Table 2. Primary and secondary antibodies used for IFA, Western blotting, and invasion assays.

<table>
<thead>
<tr>
<th>Primary Antibodies</th>
<th>IFA Antibodies</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat anti-HA (Roche 27573500)</td>
<td>1:2000 in 3% BSA</td>
<td></td>
</tr>
<tr>
<td>Mouse anti-myc (Invitrogen 132500)</td>
<td>1:2000 in 3% BSA</td>
<td></td>
</tr>
<tr>
<td>Mouse anti-acetylated-α-tubulin (Sigma T7451)</td>
<td>1:1000 in 3% BSA</td>
<td></td>
</tr>
<tr>
<td>Mouse anti-P30/SAG1 (Invitrogen MA183499)</td>
<td>1:2000 in 3% BSA</td>
<td></td>
</tr>
<tr>
<td>Rabbit anti-Toxoplasma (Invitrogen PA17252)</td>
<td>1:1000 in 3% BSA</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary Antibodies</th>
<th>IFA Antibodies</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-rat Alexa Fluor 594 (Thermo Scientific A11007)</td>
<td>1:5000 in 3% BSA</td>
<td></td>
</tr>
<tr>
<td>Goat anti-mouse Alexa Fluor 594 (Thermo Scientific A11005)</td>
<td>1:5000 in 3% BSA</td>
<td></td>
</tr>
<tr>
<td>Streptavidin Alexa Fluor 488 (Invitrogen S32354)</td>
<td>1:1000 in 3% BSA</td>
<td></td>
</tr>
<tr>
<td>Goat anti-mouse Alexa Fluor 488 (Thermo A11001)</td>
<td>1:5000 in 3% BSA</td>
<td></td>
</tr>
<tr>
<td>Goat anti-rabbit Alexa Fluor 594 (Thermo A11012)</td>
<td>1:5000 in 3% BSA</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Western Blot Antibodies</th>
<th>Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Antibodies</td>
<td>Rat anti-HA (Roche 27573500)</td>
<td>1:2000 in 5% non-fat milk</td>
</tr>
<tr>
<td>Secondary Antibodies</td>
<td>Mouse anti-myc-HRP (Santa Cruz sc-40)</td>
<td>1:100 in 5% non-fat milk</td>
</tr>
<tr>
<td></td>
<td>Anti-rat-HRP (GE NA935)</td>
<td>1:2000 in 5% non-fat milk</td>
</tr>
<tr>
<td></td>
<td>Streptavidin-HRP (Invitrogen S911)</td>
<td>1:2000 in 5% BSA</td>
</tr>
</tbody>
</table>

qRT-PCR

RNA was isolated using phenol:chloroform as previously described above. RNA was denatured at 70 °C for five minutes then chilled on ice for at least five minutes. cDNA was synthesized using the OmniScript RT Kit according to manufacturer’s protocols. Real-time PCR reactions were prepared using the Power SYBR® Green PCR Master Mix (Applied Biosystems). To detect expression of tgtbp1, primers 18 and 19 were used. To detect expression of TurboID, primers 20 and 21 were used. To detect expression of tgtbp2, primers 22 and 23 were used. α-tubulin was used as an endogenous control with primers 24 and 25 (Table 3). Reactions were set up and cycled according to manufacturer’s protocol for MicroAmp 0.1ml Fast Optical 96-well
plates. The plate was heated to 52 °C for twenty seconds, then held at 95 °C for ten minutes. The plate was then cycled at 95 °C for fifteen seconds and cooled to 60 °C for one minute for forty cycles. The melt curve step included heating the plate back to 95 °C for fifteen seconds, then held at 60 °C for one minute, then heated back to 95 °C for thirty seconds. Finally, the plate was cooled to 60 °C for fifteen seconds. The Applied Biosystems 7500 Fast Real-Time PCR System was used to perform qRT-PCR.

**Protein Expression and Purification**

The pGEX-T-1-TgTBP1 plasmid was transformed into BL21 (DE3) competent (Thermo Scientific EC0114) *E. coli* cells and plated as a lawn on an LBA plate overnight at 37 °C. A single colony was picked and grown in LBA broth overnight at 37 °C until turbid. Preculture was added to 500mL fresh LBA at a 1:250 dilution and grown to an OD<sub>600</sub> of 0.6-0.8 and incubated in 100mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for fifteen hours at 15 °C to induce expression of GST-TgTBP1 protein. The bacteria were pelleted at 20,000xg for twenty minutes at 4 °C; the media was removed, and the pellets were stored at -80 °C for at least one hour. The pellets were resuspended in a lysis buffer containing 150mM HEPES, 150mM NaCl, pH 7.6 with 1X protease inhibitor cocktail and benzonase. The lysate was mechanically lysed three times at 1750 psi using a French Press G-M® (Glen Mills) with a 40kpsi cell. The lysate was spun for twenty minutes at 20,000xg at 4 °C and the soluble material was removed for purification.

GST-TgTBP1 protein was purified using fast protein liquid chromatography (FPLC) with Pierce™ Glutathione Chromatography Cartridges. The protein was washed using a buffer containing 50mM Tris, 150mM NaCl, pH 8.0. The protein was eluted using a buffer containing 50mM Tris, 150mM NaCl, 10mM glutathione, pH 8.0. Buffer exchange was performed to
remove glutathione from the purified protein sample using the Zebra Spin Desalting Column (Thermo Scientific).

Protein quantification was performed using the Pierce™ BCA Protein Assay Kit according to manufacturer’s instructions using the microplate procedure (Thermo Scientific). Standards were made using the GST wash buffer previously described above.

**Preparation of parasite nuclear extracts**

Nuclear extracts were prepared by harvesting ∆Ku80 parasites and washing them with cold PBS. The pellet was resuspended in four mL of nuclear extract buffer A containing 10mM HEPES pH 7.9, 1.5 mM MgCl2, 10mM KCl, 0.5mM DTT, 0.1mM EDTA, 0.65% NP-40, 0.5mM PMSF. Parasites were incubated on ice for ten minutes, then centrifuged at for ten minutes at 1,500xg at 4°C. The supernatant containing cytoplasmic extracts was removed and the nuclear pellet was resuspended in 400 μL of nuclear extract buffer B containing 20mM HEPES pH 7.9, 1.5mM MgCl2, 420mM NaCl, 0.2mM EDTA, 0.5mM DTT, 25% glycerol, 0.2mM PMSF. This was incubated on ice for fifteen minutes, then centrifuged at 14,000xg for ten minutes at 4°C. The supernatant contained nuclear extracts and was stored in aliquots at -80°C. Protein quantification performed using the Pierce™ BCA Protein Assay Kit according to manufacturer’s instructions using the microplate procedure (Thermo Scientific). Standards were made using the nuclear extract buffer B.

**In vitro binding assays (EMSA)**

The LightShift™ Chemiluminescent EMSA Kit was used to perform electrophoretic mobility shift assays (Thermo Scientific). Five to fifteen micrograms of recombinant TгTBP1 protein or five micrograms of nuclear extract were combined with 200fmol of biotin-labelled
double-stranded oligonucleotides (Table 4), 10X binding buffer, and Poly(dl·dC). Provided control reactions were performed with each assay using EBNA extract and biotin-labelled DNA. Binding reactions were incubated at room temperature for twenty minutes, then run on a pre-run 6% polyacrylamide gel. The gel was transferred to a nylon membrane and the DNA was crosslinked to the membrane using 254nm UV light. The membrane was incubated in blocking buffer for fifteen minutes, then incubated in a streptavidin-HRP conjugate in blocking buffer for fifteen minutes. The blot was then washed with 1X wash buffer, then incubated for five minutes in a substrate equilibrium solution. The membrane was visualized using equal parts of the provided working solutions for five minutes and imaged using the BioRad V3 Chemidoc.

**Parasite Phenotyping Assays**

To measure the parasites’ ability to successfully invade, replicate, and egress from host cells upon TgTBP1 knockdown, plaque assays were performed. A heavily infected monolayer of intracellular parasites in a T25 flask was syringe lysed and counted. Parasites diluted to approximately 100 parasites per mL were added to a confluent monolayer of HFFs in a 12-well plate. Media contained pyrimethamine for drug selection. Parasites were grown with and without anhydrotetracycline (ATc) in triplicate. The plates grew undisturbed for 5 days at 37 °C in 5% CO₂. Plates were then washed with PBS and fixed with cold methanol. Wells were stained with Crystal Violet to view plaques then washed with PBS. Plates were imaged with the BioRad Chemidoc V3. Plaque assays were performed in triplicate.

To measure parasite replication following TgTBP1 knockdown, doubling assays were conducted. Tet-myc-TgTBP1 and TATi parasites were grown with and without ATc in separate T25 flasks for twenty-four hours. Extracellular parasites were removed, and intracellular
parasites were harvested by scraping, resuspended in media, and syringe lysed. Five μL of parasites were added to three wells in a 12-well plate. The parasites were incubated at 37 °C in 5% CO₂ for two to four hours to invade host cells, then the media and extracellular parasites were removed, and the monolayer was rinsed with media and replaced with media containing pyrimethamine, and either with or without ATc. Individual wells were grown then fixed with cold methanol after twelve, twenty-four, and thirty-six hours for each population. Staining of parasites was performed using the Hema 3™ Manual Staining System (Fisher Healthcare), and the number of parasites in 100 vacuoles was counted for each population at each timepoint using light microscopy. Doubling assays were performed in triplicate.

To assess the parasites’ ability to invade host cells after loss of TgTBP1, invasion assays were performed. Tet-myc-TgTBP1 and TATi parasites were pretreated with and without ATc in separate T25 flasks for thirty-six hours. Extracellular parasites were removed, and intracellular parasites were harvested by scraping, resuspended in media, and syringe lysed. Parasites were counted using a hemocytometer, then diluted to 1x10⁶ parasites/mL in chilled DMEM media containing 1% FBS with or without ATc. Parasites were inoculated into wells containing HFF cells on coverslips and incubated on ice for fifteen minutes. The plate was incubated in a 37°C water bath for one minute, then incubated for two hours at 37°C and 5% CO₂. The media was removed, and the wells were washed with 1X PBS. The cells were fixed with 3% paraformaldehyde for fifteen minutes, then blocked for one hour in 3% BSA in 1X PBS. Wells were incubated with mouse anti-P30/SAG1 antibody for one hour, then washed with PBS (Table 2). Cells were permeabilized with 0.2% Triton X-100 in 3% BSA then washed with 1X PBS. Next, cells were incubated with rabbit anti-Toxoplasma antibody for one hour at room temperature, then washed with 1X PBS (Table 2). Wells were incubated with goat anti-mouse
Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 594 (Thermo A11012, 1:5000) for one hour in the dark at room temperature. Wells were washed with 1X PBS, then mounted face down onto a microscope slide with VECTASHIELD Antifade Mounting Medium and viewed under a fluorescent microscope. The number of parasites were counted in ten random fields of view. Dual colored parasites were counted as extracellular, and red parasites were counted as intracellular. One thousand total parasites were counted for each treatment. The efficiency of invasion was calculated as the percentage of intracellular parasites out of the total number of parasites. Invasion assays were performed in triplicate.

**TBP DNA Binding Domain Alignment**

Protein alignment of the TBP DNA binding domain was conducted with Clustal Omega (version 1.2.4) multiple sequence alignment (57). Amino acid sequences used for protein alignment of the TBP DNA binding domain are as follows: *Homo sapiens* TBP (Uniprot entry P20226, aa 162-338), *Saccharomyces cerevisiae* TBP (Uniprot entry P13393, aa 64-239), *Plasmodium falciparum* TBP (PlasmoDB entry PF3D7_0506200, aa 150-325), *Toxoplasma gondii* TBP1 (ToxoDB entry TGGT1_291080, aa 66-280) and TBP2 (ToxoDB entry TGGT1_258680, aa 299-547).

**Modeling of TgTBP1 and TgTBP2 DNA Binding Domain**

The predicted structure of the *Toxoplasma* TBP1 and TBP2 DNA binding domains were obtained using I-TASSER using the default setting without any restraints or templates (58,59). Results were visualized using UCSF Chimera (60).
**Phylogenetic Analysis**

TBP and TBP-like factor amino acid sequences were obtained from UniProt and VEuPathDB (25). Phylogenetic history was inferred using the Maximum Likelihood method and JTT matrix-based model (61). Evolutionary analysis was conducted in MEGA11, with branch lengths measured in the number of substitutions per site (62). The tree with the highest log likelihood was utilized.

**Statistical Analysis**

ImageJ was used to analyze plaque assay data. To do this, threshold color was used to fill in all white plaques, and the total plaque number and plaque area were calculated for each well. The average plaque area was used to determine percent host cell lysis. RStudio was used to prepare graphical images. Student’s paired t-tests were conducted for replication and invasion assays with \( \alpha=0.05 \).
CHAPTER III: RESULTS

Conservation, protein modeling, and phylogeny of TgTBP1 and TgTBP2

TgTBP1 is predicted to be a 31.5 kDa size protein possessing a C-terminal DNA-binding domain consistent with TBPs but containing a gap within the domain. TgTBP2 is predicted to be a 58 kDa size protein also containing a C-terminal DNA-binding domain consistent with TBPs. TgTBP1 and TgTBP2 contain 19.5% sequence similarity. TBP uses a string of positively charged lysine and arginine residues to interact with the phosphate groups on DNA, and four phenylalanine residues to bend the DNA. As the DNA becomes bent, the DNA-protein interaction becomes enhanced. Firstly, our goal was to determine if TgTBP1 and TgTBP2 possess these important residues and share structural similarities with other TBPs.

The DNA binding domains of TgTBP1 and TgTBP1 were compared to TBP DNA binding domains from *H. sapiens* (Uniprot ID P20226, amino acids 162-338), *S. cerevisiae* (Uniprot ID P13393, amino acids 64-239), and *P. falciparum* (PlasmoDB ID PF3D7_0506200, amino acids 150-325) (Figure 5A). TgTBP1 possesses 0 of 3 arginine, 1 of 2 lysine and 4 of 4 phenylalanine residues, and TgTBP2 contains 2 of 3 arginine, 1 of 2 lysine, and 3 of 4 phenylalanine residues which are important for direct DNA-protein interaction. While TgTBP1 and TgTBP2 are missing some important residues, structural modeling predicts saddle-like structures similar to other TBPs (Figure 5B).
A phylogenetic tree was constructed based on homology on TBPs and TBP-like factors (TLF) from metazoans, nematode, yeasts, Kinetoplastids, and Apicomplexans using the Maximum Likelihood method (Figure 6). Many Apicomplexan and Kinetoplastid TBPs relate more closely to canonical TBPs, including TgTBP2, which has close relation with TBP homologs in Coccidians (Figure 6). TgTBP1 and its homologs in Apicomplexans diverge from TBPs and TLFs. These proteins contain residues found in TBPs as well as TLFs, but do not distinctly relate to one specific group of proteins (Figure 6). TLFs typically possess 1 of 4 phenylalanine residues, 1 of 3 arginine residues, and 0 of 2 lysine residues important for DNA interaction in canonical TBPs. TgTBP1 possesses all four phenylalanine residues and one of two lysine residues significant for DNA binding.
DNA binding capability of TgTBP1 and TgTBP2

To assess the DNA binding capability of TgTBP1, a plasmid construct was made to express a recombinant TgTBP1 protein fused to an N-terminal GST tag. PCR-amplification of TgTBP2 cDNA was attempted to express recombinant TgTBP2 protein but was unsuccessful. PCR-amplification of *tgtp1* cDNA containing the coding region was successful and cloned into the pGEX-4T-1 expression plasmid. The plasmid was transformed into BL21 (DE3) *E. coli*, and
GST-TgTBP1 protein expression was induced using IPTG. The recombinant protein was purified by FPLC for use in EMSAs (Figure 7A). Probes used in initial EMSA experiments included regions of the dense granule protein *gra1* and surface antigen protein *sag1* promoters, approximately 40 base pairs upstream of their transcriptional start sites, and a human TATA-box sequence from the histone H2B gene. A random DNA sequence was included as a control. Gel shift assays with recombinant TgTBP1 and these probes yielded no DNA-protein binding (Figure 7B). In our next set of EMSA experiments, four tiled probes in region of the GRA1 promoter were used in EMSA experiments surrounding the transcriptional start sites. These experiments yielded no DNA-protein binding. Additionally, nuclear extracts were isolated from ΔKu80 parasites and combined with the tiled GRA1 DNA oligonucleotides in EMSA experiments. No binding was observed with nuclear extracts, indicating that no nuclear proteins bound to our chosen *gra1* tiled probes. (Figure 7C).

**Figure 7.** (A) Purified recombinant TgTBP1 protein on a Coomassie stained gel, shown with an arrow at 52kDa. (B) Gel-shift assay with EBNA control reactions (left) and TgTBP1 recombinant protein with GRA1, SAG1, H2B, and random (RDM) probes. (C) Gel-shift assays with EBNA control reactions (left) and nuclear extract with tiled GRA1 and RDM probes.
Determining the interactome of TgTBP1 and TgTBP2

To determine the interactome of TgTBP1, a parasite line was generated with TurboID fused to TgTBP1 at its gene locus. The C-terminal end of \textit{tgtbp1} gDNA was cloned into a plasmid construct containing a TurboID-V5-HA tag and an HXGPRT selection cassette. The plasmid was linearized and transfected into parasites to fuse a C-terminal TurboID-V5-HA tag at the endogenous \textit{tgtbp1} gene locus by single crossover homologous recombination (Figure 8A). The 3’ end of \textit{tgtbp1} in the plasmid construct matches the \textit{tgtbp1} endogenous gene and integrates along with the TurboID-V5-HA tag upstream of the genes stop codon.

TurboID is a biotin ligase engineered from directed evolution of the \textit{E. coli} biotin ligase BirA (63). When a protein is fused to TurboID, and with the addition of biotin to the medium, TurboID converts biotin and ATP to biotin-AMP, a reactive intermediate which covalently labels any proteins in proximity to the protein with biotin. The TurboID-HA-V5 tag was successfully integrated at the \textit{tgtbp1} gene locus with validation using qRT-PCR. However, the HA tag could not be detected by IFA or Western Blot. Additionally, TgTBP1-TurboID-HA-V5 parasites were incubated in biotin and blotted with streptavidin-HRP to detect biotinylated proteins with Western Blotting. However, no significant biotin labelling was detected by Western Blot. Transcript levels of \textit{tgtbp1} does not change between parental ΔKu80 and TgTBP1-TurboID-HA-V5 parasites, but a 500-fold change increase of TurboID expression is observed in TgTBP1-TurboID-HA-V5 parasites compared to ΔKu80 parasites (Figure 8B). This signifies that there was successful integration of TurboID at the \textit{tgtbp1} locus and no copies of \textit{tgtbp1} are present. The TurboID-HA-V5 tag may be cleaved upon TgTBP1 protein production.
To produce a tetracycline inducible knockdown of TgTBP1, a parasite line was generated replacing the tgtbp1 promoter with a tetO7Sag4 promoter. To do this, the N-terminal end of tgtbp1 gDNA was cloned into a plasmid containing an upstream tetO7Sag4 promoter, N-terminal myc tag, and a DHFR selection cassette. Using single crossover homologous recombination, the promoter was integrated at the tgtbp1 locus replacing the endogenous promoter (Figure 9A). The 5’ end of tgtbp1 sequence in the plasmid construct matches the beginning of the endogenous tgtbp1 sequence, integrating the tetracycline-regulatable promoter and myc tag at the tgtbp1 gene upstream of the stop codon. Integration of the tetracycline-regulatable promoter at the tgtbp1 locus was confirmed by genomic PCR (Figure 9B).

Next, the inducible knockdown system was tested to determine when TgTBP1 expression and protein production was decreasing. In normal growth conditions, a tetracycline-controlled trans activator (TATi), present in the TATi parental parasite line, binds to the TRE, promoting normal expression of TgTBP1. When anhydrotetracycline (ATc), a derivative of tetracycline, is
added to the parasites, it binds to TATi, therefore blocking expression of TgTBP1 (Figure 9D). ATc was added to parasites over a course of forty-eight hours. Western blotting, IFA, and qRT-PCR were performed to determine the loss of TgTBP1 expression and protein levels upon addition of ATc. The myc-tag signal could not be detected by IFA or Western blot with or without ATc. qRT-PCR showed a significant fold change decrease in TgTBP1 expression upon 48 hours of incubation with ATc (Figure 9C), demonstrating loss of TgTBP1. The myc tag could have been cleaved upon protein production.

**Figure 9.** (A) Promoter replacement strategy to replace the endogenous *tgibp1* promoter with a tetracycline-regulatable promoter. A plasmid construct was made with the 5’ end of *tgibp1* gDNA fused to a tetracycline-regulatable promoter and myc tag at the N-terminal end. The promoter was replaced by single crossover homologous recombination. (B) Confirmation of integration of the tetracycline-regulatable promoter in tet-myc-TgTBP1 parasites by genomic PCR. (C) Fold change expression of *tgibp1*+/− ATc, showing a significant decrease in *tgibp1* upon the addition of ATc by qRT-PCR determined by paired students t-test. Data normalized to -ATc parasites, across three biological replicates. (D) Tetracycline inducible knockdown system. In normal growth conditions, a tetracycline controlled transactivator (TATi) binds to the tetracycline response element (TRE) in the promoter, promoting normal expression of *tgibp1*. Upon addition of ATc, it binds to the TATi and not the TRE, inhibiting *tgibp1* expression.

**TgTBP1 knockdown is lethal to parasites**

To determine the effect of TgTBP1 loss on parasite growth, plaque assays were conducted, which analyze the overall parasite lytic cycle. Extracellular parasites invade host
cells, replicate, then lyse host cells as they egress. Plaques form when host cells are lysed. Plaque assays were performed with parental controls and knockdown. Parasites do not form plaques with loss of TgTBP1, showing that TgTBP1 is essential for parasite growth (Figure 10A). Average plaque area data shows no significant difference between controls; a significant decrease in percent host cell lysis is observed upon TgTBP1 knockdown (Figure 10B).

![Figure 10](image)

**Figure 10.** (A) Intracellular plaque assay of parental TATi parasites +/- ATc and tet-myc-TgTBP1 +/- ATc, demonstrating no plaque growth upon TgTBP1 knockdown. HFF cells stained with Crystal Violet. Representative of one biological replicate. (B) Average percent host cell lysis from intracellular plaque assays of TATi parasites +/- ATc and tet-myc-TgTBP1 parasites +/- ATc. Representative of three biological replicates. Images were analyzed using ImageJ.

To determine how parasite replication is affected by loss of TgTBP1, parasite replication assays were conducted. *Toxoplasma* tet-myc-TgTBP1 knockdown and control parasites were pretreated with and without ATc for twenty-four hours, then were incubated in four wells each and fixed every twelve hours for an additional forty-eight hours. The number of parasites in 100 separate vacuoles were counted for each treatment. Loss of TgTBP1 resulted in slower parasite replication over 48 hours of growth (Figure 11A). There is a significant difference in average parasites per vacuole at 24 and 48 hours of replication across three biological replicates, showing a delay in parasite replication (Figure 11B).
Invasion assays were performed to determine the effect of TgTBP1 knockdown on host cell invasion. Tet-myc-TgTBP1 control and knockdown parasites were incubated without and with +ATc respectively for 36 hours then inoculated into fresh host cells with a coverslip. Parasites were allowed to invade for two hours, then were fixed, blocked, and probed with an anti-SAG1 antibody with a green fluorophore to target extracellular parasites. The cells were permeabilized, then probed with an anti-Toxoplasma antibody with a red fluorophore to target both extracellular and intracellular parasites. Dual-colored parasites were counted as extracellular, and red colored parasites were counted as intracellular for 1000 total parasites. Control parasites showed a normal rate of invasion, and upon TgTBP1 knockdown, a 55% decrease in invasion is observed, demonstrating a significant defect in host cell invasion upon loss of TgTBP1 (Figure 12).

**Figure 11.** (A) Parasite replication assay of tet-myc-TgTBP1 parasites +/- ATc at 12, 24, 36, and 48 hours of growth, representative of one biological replicate. The number of parasites in 100 vacuoles was counted for each treatment. (B) Average parasites per vacuole of tet-myc-TgTBP1 parasites +/- ATc in three biological replicates, demonstrating a significant decrease in replication at 24 and 48 hours upon TgTBP1 knockdown determined by a student’s t-test.
TgTBP1 knockdown does not affect cell division or morphology

To provide further insight on the role of TgTBP1 in *Toxoplasma*, cell division and morphology upon TgTBP1 knockdown was investigated. Tet-myc-TgTBP1 parasites were incubated with and without ATc for thirty-six hours and IFAs were performed on acetylated-α-tubulin, which marks the surface of microtubules. Four stages of the *Toxoplasma* cell cycle were observed: interphase, S phase, mitosis, and cytokinesis (Figure 13) (64). No morphological differences were observed in *Toxoplasma* parasites upon TgTBP1 knockdown. Additionally, there was no significant halt in cell cycle progression with addition of ATc (Figure 13).

**Figure 12.** Parasite invasion upon TgTBP1 knockdown. One thousand vacuoles were counted for each treatment and each replicate. Percent invasion is normalized to tet-myc-TgTBP1 parasites -ATc. Across three biological replicates, a significant decrease in invasion is observed upon TgTBP1 knockdown determined by a student’s paired t-test.
TgTBP1 knockdown affects *tgtp2* expression

To determine how *tgtp2* expression is affected by loss of TgTBP1, qRT-PCR was performed on samples incubated with and without ATc for 48 hours. RNA was isolated and cDNA was prepared, then qRT-PCR was performed using *tgtp2*-specific primers. Preliminary data shows an increased fold change in *tgtp2* expression upon knockdown of TgTBP1 for 48 hours compared to control parasites, whereas *tgtp1* fold change expression decreases (Figure 14).

**Figure 13.** (A) Four stages of *Toxoplasma* cell division: interphase, where parasites are about to begin dividing, S phase, where daughter cells begin to form within the mother cell, mitosis, where daughter cells are actively beginning to split apart, and cytokinesis, where the two daughter cells separate. (B) Quantification of each cell cycle stage with (+ATc) and without (-ATc) TgTBP1 knockdown. Fifty vacuoles were counted for each treatment, and the cell phase was noted for each vacuole.

**Figure 14.** Preliminary qRT-PCR results demonstrating a decrease in *tgtp1* fold change expression and an increase in *tgtp2* fold change expression upon incubation with ATc for 48 hours. Representative of one biological replicate.
CHAPTER IV: DISCUSSION

Conservation and phylogeny of TBPs in Toxoplasma

The goal of this research was to gain information on the essentiality, DNA binding capabilities, and protein interactions of TgTBP1 and TgTBP2. Structural predictions of TgTBP1 and TgTBP2 predicted structures similar to the canonical TBP; additionally, many of the residues needed for direct DNA interaction in TBPs are present in TgTBP1 and TgTBP2 (Figure 5). Although a few key residues are missing in TgTBP1 and TgTBP2, this is also the case for organisms such as P. falciparum, whose TBP has been shown to bind DNA by EMSA and ChIP experiments (44,45). In fact, TgTBP2 contains the same DNA-interacting residues as P. falciparum, making it likely that TgTBP2 also has DNA binding capability. Additionally, phylogenetic analyses of TgTBP1 and TgTBP2 compared to TBPs and TBP-like factors (TLF) suggest that TgTBP2 is more closely related to canonical TBPs (Figure 6). TgTBP1 diverges with a subset of other TBP1 orthologs in select apicomplexans separately from canonical TBPs and TLFs. The function of TLFs are not well understood, but they are known to be involved in transcription initiation in other eukaryotes (65). TgTBP1 possesses residues present in TBPs and TLFs but diverges from both of these sets of proteins, which may reflect functional differences in TgTBP1. Since TgTBP1 and TgTBP2 contain many of the important residues required for DNA binding and relate to TBPs and TLFs, I evaluated the DNA binding capability, interactome, and essentiality of these proteins in Toxoplasma.

DNA binding capability of TgTBP1

To evaluate the DNA binding properties of TgTBP1 and TgTBP2, I aimed to perform EMSAs on recombinant TgTBP1 and TgTBP2. PCR-amplification of tgbp2 to create

42
recombinant TgTBP2 protein was unsuccessful. Recombinant TgTBP1 was expressed and purified in *E. coli* and EMSAs were performed against sequences within two promoter regions in highly expressed genes in tachyzoites, *gra1* and *sag1*, approximately forty base pairs upstream of the TSS. A TATA-box sequence from the *H. sapiens* H2B gene, and a random DNA sequence were also included. No DNA binding occurred with these probes (Figure 7B). To further explore the DNA binding capability of TgTBP1, several probes were designed to tile the *gra1* promoter over an approximately 150 base pair range around the TSS. No DNA-protein binding was observed with recombinant TgTBP1. To ensure that our recombinant protein was not the factor prohibiting DNA binding, nuclear extracts were prepared and combined with the tiled *gra1* probes in gel shift assays. No protein binding was observed with the nuclear extracts (Figure 7C). Due to the lack of DNA binding with our nuclear extract preparation, it is possible that TgTBP1, TgTBP2, or another transcription factor bind to the *gra1* promoter further upstream from the transcriptional start site to recruit the PIC. TgTBP1 may not bind promoter regions but other regions of DNA, or does not bind DNA at all, whereas TgTBP2 may be the core DNA binding element in *Toxoplasma* based on phylogenetic analyses (Figure 6). Future approaches for this aim will involve creating transgenic parasite lines with TgTBP1 and TgTBP2 fused to a 3xHA tag. These will allow us to perform CUT&Tag on TgTBP1 and TgTBP2 to determine their localization throughout the *Toxoplasma* genome in vivo. Determining the binding sites of transcription factors in promoter regions could reveal motifs in the *Toxoplasma* genome.

**TgTBP1 is critical for intracellular infection in *Toxoplasma***

As transcription factors likely involved in initiating gene expression in *Toxoplasma*, I hypothesized that TgTBP1 and TgTBP2 are essential for parasite viability. To assess the essentiality of these proteins in *Toxoplasma*, a transgenic parasite line was created replacing the
*tgtbp1* promoter with a tetracycline-regulable promoter. The regulatable promoter was successfully integrated at the *tgtbp1* locus (Figure 9B) confirmed by genomic PCR and knockdown of *tgtbp1* expression was confirmed by qRT-PCR (Figure 9C). However, the N-terminal myc tag could not be detected by IFA or Western blot; therefore, it is likely that the myc tag was cleaved upon protein production, or the tag is inaccessible for antibody binding. I confirmed that TgTBP1 is essential for parasite growth through intracellular plaque assays, which assess the overall lytic cycle of the parasite and how it is able to successfully invade, replicate, and egress from host cells (Figure 10). To look deeper into the role of TgTBP1 in the parasite lytic cycle, parasite doubling assays were conducted to determine the effect of TgTBP1 loss on parasite replication in host cells. I observed a delay in parasite replication at 12, 24, and 36 hours of growth, but replication did not completely halt (Figure 11). To gain more information about cell cycle progression and cell morphology upon TgTBP1 knockdown, IFAs were performed probing the surface of microtubules of *Toxoplasma* parasites before and after knockdown. Four stages of cell division were observed for each sample. There was no significant morphological or cell cycle differences in parasites upon loss of TgTBP1 (Figure 13). Parasites could still successfully divide upon knockdown but take longer to progress through the cell cycle. Since the parasites could still successfully divide properly upon TgTBP1 knockdown, I analyzed their ability to invade host cells upon loss of TgTBP1; if the parasites could successfully replicate and egress from host cells, they could have a defect in host cell invasion, resulting in parasite death. Invasion assays confirmed that loss of TgTBP1 results in a defect in invasion; a significant decrease in invasion is observed upon addition of ATc (Figure 12).

Rapid parasite death within several hours TgTBP1 knockdown was not observed, so it is possible that TgTBP2 is compensating for loss of TgTBP1. To determine if *tgtp2* is upregulated
to compensate for loss of TgTBP1, I performed qRT-PCR to quantify *tgthp2* transcript levels during *tgthp1* knockdown. Preliminary data shows that upon loss of *tgthp1*, *tgthp2* expression increases, indicating that TgTBP2 may be attempting to offset for loss of TgTBP1 (Figure 14). Additional replicates will be performed to confirm the increase in *tgthp2* expression. TgTBP1 and TgTBP2 are both predicted to be essential for tachyzoite fitness based on a genome wide CRISPR/Cas9 screen, suggesting that these proteins do not have redundant functions. This is supported by different domain architectures and key residues important for DNA interaction in TgTBP1 and TgTBP2 (Figure 5). In addition, the formation of dimers by transcription factors is a common regulatory mechanism of gene expression in eukaryotes (66). TBPs in humans and yeast form dimers; it is possible that TgTBP1 and TgTBP2 form heterodimers to regulate transcription initiation and modulate DNA-binding specificity (36). Further investigation into the protein interactions of these two proteins are needed to determine their regulatory potential.

**Interactome of TgTBP1**

As essential proteins likely involved in transcription initiation in *Toxoplasma*, I sought to identify the proteins directly interacting with TgTBP1 and TgTBP2, predicting that they interact with factors in the TFIID complex. To do this, I aimed to tag TgTBP1 and TgTBP2 with the biotin ligase TurboID, which covalently labels any nearby proteins with biotin. The TurboID-V5-HA tag was successfully integrated at the *tgthp1* gene locus (Figure 8B). However, the tag could not be detected by Western blot or IFA. Additionally, TgTBP1-TurboID-V5-HA tagged parasites were incubated with biotin and checked for biotinylation of proteins by Western blotting and IFA; there was no significant biotin labelling observed. The TurboID-V5-HA tag could have been cleaved upon protein production. Further optimization of the tag will be needed to use proximity biotinylation labelling for identifying the interactome of TgTBP1. Future
approaches for this aim will include utilizing the 3x-HA tagged TgTBP1 and TgTBP2 parasite lines to perform co-immunoprecipitations and mass spectrometry to identify the factors interacting with the TBPs in *Toxoplasma*. This will allow us to gain insight into how TgTBP1 and TgTBP2 potentially regulate transcription and recruit the PIC to the promoter.

**Summary and Future Directions**

Taken together, I show that TgTBP1 is essential for intracellular infection in *Toxoplasma* and is important for proper host cell invasion and replication. Future studies on TgTBP1 will further investigate its DNA binding abilities, interactome, and its role in regulating gene expression in Toxoplasma. An RNA-sequencing experiment will be performed before and after knockdown of TgTBP1 to determine its role in global gene expression in the parasite and to identify the subsets of genes that are regulated by TgTBP1. With this information, we may narrow down the potential role of TgTBP1 in gene expression and regulation in *Toxoplasma*. Additionally, further investigation into the function of TgTBP2 is needed. Future studies will analyze the interactome, genomic localization, and essentiality of TgTBP2 on parasite viability. Determining how these two proteins function will provide insight into how RNA polymerases are recruited to promoters and how gene expression is regulated in *Toxoplasma*. With this information, we can identify unique features of these processes that could potentially be targeted with therapeutic drugs to treat toxoplasmosis.
### APPENDIX

**Table 3.** Primers utilized in this study for plasmid construction, integration confirmation, and qRT-PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
<th>Forward/Reverse?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CGTGACGCAGATAGTGTTC</td>
<td>Forward</td>
</tr>
<tr>
<td>2</td>
<td>GTTAATGGATGGCGCTGC</td>
<td>Reverse</td>
</tr>
<tr>
<td>3</td>
<td>GCTGATTCTGAAATCCCTTG</td>
<td>Forward</td>
</tr>
<tr>
<td>4</td>
<td>CCTCGCGATCTTCTTCTCATCG</td>
<td>Reverse</td>
</tr>
<tr>
<td>5</td>
<td>CGATGAAGAAGATCGCGAGG</td>
<td>Reverse</td>
</tr>
<tr>
<td>6</td>
<td>GGTTCGCCGCTGGATCCCCGAATTCGAAAGGAGGAAGGCGAAG</td>
<td>Forward</td>
</tr>
<tr>
<td>7</td>
<td>CGGCCTCGCGATTCACCAGCGAATTCGAAACGCGTAGATGT</td>
<td>Reverse</td>
</tr>
<tr>
<td>8</td>
<td>AGCGCGCCCACCGCGTGGGCTAGGCAAGTCGCTTCTCAT</td>
<td>Forward</td>
</tr>
<tr>
<td>9</td>
<td>GAGGCACAGTATTGTCTTTAGATCTACTTGCCGTTCGCAAC</td>
<td>Reverse</td>
</tr>
<tr>
<td>10</td>
<td>AACCTCCGAGGAAGACTGGAAGCTCGACACACTCGTACAGCA</td>
<td>Forward</td>
</tr>
<tr>
<td>11</td>
<td>TGGAGCTTCACCAGCGTGGCGCCGCTGTGAGGACCCCGTCTGG</td>
<td>Reverse</td>
</tr>
<tr>
<td>12</td>
<td>GGCGCTGCAACGACACCTTTGGTG</td>
<td>Forward</td>
</tr>
<tr>
<td>13</td>
<td>CGCGGAGCGATCGCATGTCAGAGG</td>
<td>Reverse</td>
</tr>
<tr>
<td>14</td>
<td>GATTAAGTTGGTAAAGCCAG</td>
<td>Forward</td>
</tr>
<tr>
<td>15</td>
<td>CTGTTGCCACTATGGAAC</td>
<td>Reverse</td>
</tr>
<tr>
<td>16</td>
<td>GAGTTGTAACCTCGAGTGC</td>
<td>Forward</td>
</tr>
<tr>
<td>17</td>
<td>ACTGCGCGCTGTTTACA</td>
<td>Reverse</td>
</tr>
<tr>
<td>18</td>
<td>AAGAGCAGATCGACACAAACAG</td>
<td>Forward</td>
</tr>
<tr>
<td>19</td>
<td>CCGTAGAGAAAAGCTGGAGTG</td>
<td>Reverse</td>
</tr>
<tr>
<td>20</td>
<td>GATTTAAGCTGGCTATGAGG</td>
<td>Forward</td>
</tr>
<tr>
<td>21</td>
<td>CAGAGATATTCTCTGTCCAG</td>
<td>Reverse</td>
</tr>
<tr>
<td>22</td>
<td>GATTCTTCGAGTCAGAAACC</td>
<td>Forward</td>
</tr>
<tr>
<td>23</td>
<td>TCCCGTAATCACAACCTTG</td>
<td>Reverse</td>
</tr>
<tr>
<td>24</td>
<td>GATGCGCTCTGACGAAGACC</td>
<td>Forward</td>
</tr>
<tr>
<td>25</td>
<td>CATCTCCTTCCCCGCTGATC</td>
<td>Reverse</td>
</tr>
</tbody>
</table>

**Table 4.** Biotin-labelled oligos used in EMSA experiments. Probes denote the 5’ to 3’ sequence on the sense strand; a biotin-labelled reverse complimentary sequence for each probe was annealed for all oligos.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence 5’ to 3’ (Sense strand)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRA1-1</td>
<td>GTGACCAACCCCCACTTCGATTGCGCAAATCCGTAACCCATACACCTTT</td>
</tr>
<tr>
<td>SAGI</td>
<td>CTGTAGTCAGTCTTGACTGCTGTTCACCAGCGCTGACGACC</td>
</tr>
<tr>
<td>H2B TATA-Box</td>
<td>CTGAAAGCGATTCTATATAGAAAAAGCACCAGCTTGCTAC</td>
</tr>
<tr>
<td>GRA1-2</td>
<td>CGATATGAAAAAGTAGAATGTATTGCGCTTTACCCGATTGCGCCAATGCGAAG</td>
</tr>
<tr>
<td>GRA1-3</td>
<td>CTGGCGCTGCGGATTTCAACCGTAAAAATGGAATTTAGTTTGGCGCTTT</td>
</tr>
<tr>
<td>GRA1-4</td>
<td>AGACAGACACAAAAACCCGGAAGCGCCGCTGCGGACGCTTGATAGAAATG</td>
</tr>
<tr>
<td>GRA1-5</td>
<td>TAACTGATAAAAAAGGCTGAGAAGACACAGCACCCAAAAACGCAGCTGCTGCG</td>
</tr>
<tr>
<td>Random Sequence</td>
<td>CGAACCTCAGTGGCCTACATCTACCTTGAGTGCTGCC</td>
</tr>
</tbody>
</table>
REFERENCES


54. Meissner M, Krejany E, Gilson PR, de Koning-Ward TF, Soldati D, Crabb BS. Tetracycline analogue-regulated transgene expression in Plasmodium falciparum blood


