Localization of Five Target Proteins in Tachyzoites of Toxoplasma gondii

Abigail M. Kaiser

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Localization of Five Target Proteins in Tachyzoites of Toxoplasma gondii

by

Abigail M. Kaiser

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Public Health with a concentration in Global Communicable Diseases

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And to my family and friends, thank you for your support. All the love and patience you showed really kept me moving forward on the path to reaching my dreams.
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ABSTRACT

Five target genes were selected in Toxoplasma gondii tachyzoites for localization studies. These five genes, detected through proteomics studies, included TgME49_227450, TgME49_223080, TgME49_262390, TgME49_230940, and TgME49_269620. Localization of these five target proteins is a first step to confirm their interaction with TgCrk2 and understand their function and role in TgCrk2 regulation of the tachyzoite cell cycle. Gene models for the targets were analyzed using ToxoDB and ApE analysis tools. Endogenous tagging constructs were created for each target. Transgenic parasites were created. Finally, localization analysis of the target proteins in tachyzoites was completed using immunofluorescent microscopy following. One protein was found to be nuclear, two were located in the cytoplasm, and two were unable to be analyzed. Future research should be completed in order to prove these putative interactors are really correlated with TgCrk2 through co-immunoprecipitation.
CHAPTER ONE:
INTRODUCTION

Lifecycle

*Toxoplasma gondii* is an apicomplexan parasite that can infect most warm-blooded mammals, including humans, but immunocompromised individuals are most vulnerable. The definitive hosts of *T. gondii* are members of the Felidae family that includes domestic cats and their wild counterparts. Oocysts are shed by cats and sporulate in the environment. Intermediate hosts such as rodents ingest the oocysts containing sporozoites which then transform to tachyzoites (Tenter, Heckeroth, & Weiss, 2000). Tachyzoites multiply in various tissues (acute toxoplasmosis) before moving to neural and muscle tissues where they develop into bradyzoites (chronic toxoplasmosis) and form tissue cysts (Tenter, Heckeroth, & Weiss, 2000). Felidae ingest infected intermediate hosts, which initiates *T. gondii* sexual development and completes the life cycle. Humans may be infected with *T. gondii* by consuming undercooked meat, contaminated foods, receiving a contaminated blood transfusion/organ transplant, or across the placenta from mother to fetus (Tenter, Heckeroth, & Weiss, 2000). In humans, the parasites form cysts in muscle tissues, brain, and the eyes and may remain encysted, thus, hidden from the host immune system for the lifetime of the host.

Disease and Symptoms

The disease caused by the *T. gondii* parasite is called toxoplasmosis and it impacts people differently. Healthy individuals infected with *T. gondii* often do not display symptoms because their immune systems are able to keep the parasite from causing illness (Howe & Sibley, 1995). If these
individuals do show symptoms, those symptoms are usually mild and mimic flu-like symptoms that may last a few weeks to months (Machala, et al., 2013). The parasite will remain encysted in the body, so if at any time an individual becomes immunocompromised, he or she is at risk for symptomatic recurrence. In people that are immunocompromised, symptoms of toxoplasmosis may be severe and include dizziness, confusion, fever, seizures, and nausea (Howe & Sibley, 1995). While mothers that were infected long before becoming pregnant may have antibodies that protect the unborn child, if a woman becomes infected during or just before pregnancy, the child may be severely impacted (Machala, et al., 2013). Those infants infected before birth may show no symptoms at birth, but may develop vision loss, mental disabilities, and seizures later in life because of at birth clinical problems (Wilson, 1990). Potential results of a toxoplasmosis infection during pregnancy include a miscarriage, stillbirth, hepatosplenomegaly, and microcephaly or macrocephaly (Inagaki, et al., 2012).

**Current Treatment**

The current treatment of toxoplasmosis in adults is a combination of pyrimethamine, sulfadiazine, and leucovorin (Rajapaske, et al., 2013). These medications can have severe side-effects, especially when the patients are immunocompromised. Common side effects of pyrimethamine include vomiting or loss of appetite. Sulfur drugs, such as sulfadiazine, have many side effects that make them a poor choice for treatment of toxoplasmosis, including itching, skin rash, blistering of skin, difficulty swallowing, yellowing of the skin or eyes, as well as many others (Rajapaske, et al., 2013).

*T. gondii* is an opportunistic pathogen, so these side effects can be especially hazardous. Because some of these individuals, such as those with AIDS, may have to take the above-mentioned drugs for life, it is important to understand the long-term side effects of these drugs. The side ef-
fects of these medicines have been well studied, but a better alternative has yet to be formally produced. In 1988, a study on the efficacy of long-term continuous therapy of central nervous system toxoplasmosis in patients with AIDS was conducted and found that it was necessary to continue lifelong therapy to ensure a relapse does not occur (Leport, et al.). There is also evidence that the parasite may be developing drug-resistance to sulfa compounds (Sims, 2009). There is a replacement for the sulfa compounds called clindamycin (Cmn), but it has yet to be proven more effective than the current standard of care. The combination of Cmn and pyrimethamine is not advised as a long-term treatment option (Sims, 2009). The resistance of the parasite to the chemotherapeutic agents makes understanding \textit{T. gondii} replication vital.

**Parasite Structure (Tachyzoite Phase)**

**Organelles**

\textit{T. gondii} is a unicellular eukaryotic parasite, but because of the specialized life cycle it has many specific compartments. As any eukaryote, \textit{T. gondii} has nucleus with nucleolus wrapped in endoplasmic reticulum (ER), with adjacent Golgi stacks, a developed endosomal system and a long tubular mitochondrion. Among unique structures, the invasion machinery stands out. The apical end is the structure that is used for invasion into host cells. The conoid, rhoptries, and micronemes are all responsible for host cell invasion (Dubey, Lindsay, & Speer, 1998). The conoid is composed of microtubules and allows the parasite to get through the cell membrane of the host to begin replication (Gomez de Leon, et al., 2014). Rhoptries are organelles that secrete enzymes that break down the host cell membrane and help the parasite to breach the membrane of the host cell (Gomez de Leon, et al., 2014). Micronemes also aid in host cell invasion through the secretion of adhesin that
allows the parasite cell membrane to adhere to the host cell surface just prior to invasion (Gomez de Leon, et al., 2014).

The *T. gondii* surface is protected by the pellicle consisting of a plasma membrane and a layer of flattened ER/Golgi-derived vesicles, called the inner membrane complex (IMC) (Santos et al., 2009). The plasma membrane contains many transporters, among them is a single adenosine transporter that also transports hypoxanthine, inosine, and adenine (Schwab, et al., 1995). The glideosome, located between the parasite plasma membrane and the IMC, is used for motility and invasion (Graindorge, et al., 2015). A large set of interacting proteins forms a bridge from the parasite and the host, establishing attachment within the host allowing for the locomotive force created by the glideosome to be transferred from the parasite to the host (Boucher & Bosch, 2015). The IMC is a bimembranal structure consisting of the plasma membrane and a layer of ER/Golgi-derived longitudinal flattened vesicles (Gomez de Leon, et al., 2014; Santos et al., 2009). Attached to the conoid is a polar ring that holds 22 sub-pellicular microtubules (Gomez de Leon, et al., 2014). These sub-pellicular microtubules, connected to the cytosolic side of the IMC, extend helically along more than half of the parasite (Dubremetz & Torpier, 1987).

**Replication**

Apicomplexan parasites, including *T. gondii* divide in a manner that is quite distinct from their hosts. They have three distinct replication mechanisms- endodyogeny, schizogony, and endopolyge-ny and use semi-closed mitosis during chromosome segregation (Tenter, He克eroth, Weiss, 2000; Francia & Striepen, 2014; White & Suvorova, 2018). Budding occurs at the parasite surface (endodyogeny and endopolygeny) or within the cytoplasm producing two to several thousand progenies (schizogony) (Francia & Striepen, 2014). *T. gondii* divides by endodyogeny in intermediate host tissues, producing two internal daughters in a single round of DNA replication and segregation (Ten-
ter, Heckeroth, & Weiss, 2000). Unlike its host, T. gondii progeny are assembled inside of the mother cells (Francia & Striepen, 2014).

**Endodyogenic Cell Cycle**

The endodyogenic cell cycle consists of a distinctive G1 phase, overlapping S and M phases, and cytokinesis (budding). The G2 phase may be absent (Francia & Striepen, 2014). The G1 phase is devoted to parasite growth and preparation for genome replication. The transition from G1 to S phase called START is marked by centrosome duplication. In the S phase, chromosomes replicate and are distributed to the daughter cells in mitosis (Francia & Striepen, 2014).

**Regulatory Roles of Cyclins and Kinases**

In eukaryotes, progression through the cell cycle is highly regulated through the use of checkpoints. These checkpoints operate the activity of cyclin-dependent kinases (Cdks) and cyclins (Francia & Striepen, 2014). Cdk-related kinases (Crks) become known as Cdks once they are proven to be activated by cyclin. Cyclin waves are the result of changes in transcription and degradation (Francia & Striepen, 2014). In T. gondii, there are seven atypical cyclins and ten Crks, some of which work together to regulate cell cycle progression. TgCrk2 was shown to be essential to tachyzoite growth and is expressed throughout the cell cycle (Alvarez & Suvorova, 2017). Loss of TgCrk2 results in G1 phase arrest. Since apicomplexan parasites lack canonical G1 regulators and downstream effectors, the “guilt by association” approach is an option to identify putative interactors. To determine a network of newly discovered Toxoplasma G1 kinase TgCrk2, the laboratory of Dr. Suvorova performed a proteomic search of the kinase partners and substrates using above-mentioned approaches. Localization studies of TgCrk2 were also completed by Suvorova and colleagues, as seen in Figure 4.
Proteomics

Proteomics is the study of proteins which allows for identification, often through the use of protein purification and/or mass spectroscopy. There are multiple ways to identify putative protein interactors; however, those used in this study include Phospho-proteomics, proteomic analysis of protein complexes and BioID proteomics. Protein complexes can be isolated using a specific epitope tag and analyzed by mass-spectrometry (Mayya & Han, 2010). BioID proteomics detects a biotin moiety placed on every protein that comes close to or in contact with a target protein tagged with BioID polypeptide (Varnaite & MacNeill, 2016). P-Proteomics identifies changes in phosphorylation status of the proteins depending on the presence of a kinase (Mayya & Han, 2010). Increases or decreases in phosphorylation of a specific site means that the protein harboring this P-site is a possible substrate for the kinase.

Genetic Studies of *Toxoplasma gondii*

A Toxoplasma model is used because of genetic tractability, growth, and because the knowledge gained is often applicable to other apicomplexan parasites including *Plasmodium spp*, the causative agent of malaria.
Ku80 and Ligation-Independent Cloning

Ku80 is an ATP-dependent DNA helicase involved in DNA strand repair and non-homologous DNA end joining recombination. In its absence, random integration is eliminated which allows for the constructs to be inserted, with homologous sequences, into specifically targeted loci (Huynh & Carruthers, 2009). This means that the ΔKu80 strain will allow the DNA fragment with significant length homology to be inserted efficiently into the genome of the parasite during transformation. It is often used in combination with ligation independent cloning that uses the exonuclease activity of the T4 DNA polymerase. This creates long, sticky ends on the vector and the DNA fragments to be inserted. The sticky ends are non-complementary at the vector ends, which does not allow for circularization of the vector. The plasmids are used for transformation (Chalampos & Jong, 1990).

HXGPRT

*Toxoplasma gondii* HXGPRT (hypoxanthine-xanthine-guanine phosphoribosyl transferase) gene is often used as a selectable marker. This enzyme is not necessary for tachyzoite survival. Toxoplasma tachyzoites with the HXGPRT activity can be selected for in a medium containing mycophenolic acid (MPA) and xanthine (Pfefferkorn & Borotz, 1994). In the parasite, AMP (adenosine monophosphate) is converted to IMP (inosine monophosphate) by AMP deaminase. IMP is then converted to XMP (xanthine monophosphate) by IMP dehydrogenase with the addition of MPA. Finally, XMP is converted to GMP (guanosine monophosphate) with the assistance of the HXGPRT enzyme giving hypoxanthine and guanine. MPA is used to block IMP dehydrogenase, responsible for the conversion of IMP to XMP, the cells become dependent on an active HXGPRT to make it...
GMP from xanthine (Pfefferkorn & Borotz, 1994). Parasites lacking the HXGPRT gene will not survive selection because Toxoplasma does not have a purine salvage pathway. This means the parasites surviving selection have the selectable marker.

**HA Epitope Tag**

The HA tag is derived from a human influenza virus surface glycoprotein called human influenza hemagglutinin (HA). The tag functions as a general epitope tag in many expression vectors. The HA tag allows for the localization of the target protein, as well as purification and isolation (Sino Biological, 2017). It has been successfully used for years in various research endeavors because it does not affect the activity of the recombinant proteins.

**Aims**

The overall goal of this project was to validate five TgCrk2 partners detected in the proteomics studies. Specifically, to (1) to explore and confirm gene models for the target factors using ToxoDB and ApE analysis, (2) build endogenous tagging constructs for each target, (3) to create transgenic parasites, and (4) to localize the target proteins in tachyzoites by IFA. Localization of these five target proteins is a first step to confirm their interaction with TgCrk2 and understand their function and role in TgCrk2 regulation of the tachyzoite cell cycle.
CHAPTER TWO: MATERIALS AND METHODS

Primer Design

Primers were designed with careful consideration of primer length, melting temperature, annealing temperature, GC content, and secondary structures. Preliminary targets were analyzed using ToxoDB and APE software.

Polymerase Chain Reaction (PCR) Amplification

PCR was used to amplify a single copy of DNA generating thousands to millions of copies of the DNA sequence. A DNA polymerase, Phusion, was used to enable selective and repeated amplification. DMSO was added as an enhancer of the Phusion polymerase. There are three phases of PCR—denaturation, annealing, and elongation. PCR reaction mixture and conditions can be seen below in Table 1. PCR products were separated on a 0.8% agarose gel, with 4uL SYBR orange dye, in 1x TBE buffer for approximately 20 minutes at 140V. PCR products were purified using StrataPrep PCR Purification Kit and protocol.
Ligation Independent Cloning

Cloning was completed using the Clontech In-fusion® HD Cloning Kit. Protocol I in section VI was followed. Genomic fragments encompassing the 3’-end of the gene of interest were PCR amplified and incorporated into plasmid LIC-HA3X-HXGPRT, via ligation independent cloning (InFusion, Clontech). Ampicillin plates were used for spreading the cloning product.

Colony PCR

Colony PCR was performed on eight colonies yielded from the plates spread after cloning. A colony was selected, touched to an Ampicillin stab plate, and placed in a PCR tube with the same reaction mixture seen in Table 1. Electrophoresis of the amplicons was done to verify positive colonies using the previously described protocol.

<table>
<thead>
<tr>
<th>Table 1. PCR Reaction Mixtures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>H20</td>
</tr>
<tr>
<td>5x HF Buffer</td>
</tr>
<tr>
<td>DMSO</td>
</tr>
<tr>
<td>dNTPs</td>
</tr>
<tr>
<td>10uM F Primer</td>
</tr>
<tr>
<td>10uM R Primer</td>
</tr>
<tr>
<td>gDNA Template</td>
</tr>
<tr>
<td>Phusion</td>
</tr>
</tbody>
</table>
Purification of Plasmid

The plasmids were purified using the QIAGen Miniprep kit and Protocol.

DNA Linearization through Digestion

Appropriate enzymes were used to linearize the purified plasmid DNA. Approximately 1µg of DNA was cut. A 50µL scale digest was also performed once sequencing of the insert was complete.

Sequencing

Samples were sent to GenScript for sequencing. Sequences were confirmed and matched to the gene models.

Tissue Culture

Parasites were grown in human foreskin fibroblasts (HFF) and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin (Life Technologies). Parasites were cultured in HFF host cells using the same medium used for HFF culture using standard techniques (Weiss & Kim, 2014). Transgenic lines used in this study were produced in the RHΔku80Δhxgprt strain.
Transfection by Electroporation

DNA preparation

Linearized constructs were ethanol precipitated (Table 2), dried and resuspended in 100μl cytomix (120mM KCl, 0.15 mM CaCl₂, 10mM H₂HPO₄ pH 7.6, 25nM HEPES pH 7.6, 2 mM EGTA, 5 mM MgCl₂).

<p>| Table 2. Ethanol Precipitation Reaction |</p>
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>glycogen (carrier)</td>
<td>1</td>
</tr>
<tr>
<td>ammonium acetate</td>
<td>1:10 total vol.</td>
</tr>
<tr>
<td>ethanol</td>
<td>2.5x original vol.</td>
</tr>
</tbody>
</table>

Tachyzoite Preparation

Parental RHΔku80 parasites were grown in non-selection medium for 32-40h. Infected monolayers were scraped, needle passed, filtered and collected by centrifugation. To quantify parasites, 10μl of the filtered culture was placed in the hemocytometer. Four large squares of parasites were counted using a hemocytometer. The number of cells in each square was multiplied by 40,000. The number of parasites from squares one through four were added and multiplied by the amount of media. This gives the total number of parasites. The total number of parasites was divided by 20 million (the goal for transfections) and multiplied by 300μL. The answer was then converted to mL of cytomix used to dilution the parasites to the 20 million.
**Electroporation**

Electroporation is a technique that applies an electrical field to cells in order to increase the permeability of the cell membrane allowing the uptake of DNA. Parasites were diluted in cytomix (20M per 300ul) and put into an electroporation cuvette. DNA in cytomix was also added to the cuvette. The cuvette was connected to a power supply and an electric pulse was applied. Settings for electroporation were 1500 V, 25 ohms, and 25 uF. The cells were allowed to recover in regular 2.4% media overnight.

**Selection and Cloning**

24h post-electroporation, media was replaced with HX- selection media. The HXGPRT (hypoxanthine-xanthine-guanine phosphoribosyl transferase) gene is often used as a selectable marker. Genes of interest were selected for using mycophenolic acid (MPA). MPA blocks the parasites from being able to produce GMP, as Toxoplasma does not have a purine salvage pathway. Those parasites that were successfully electroporated will survive this selection process because the HxG-PRT gene that was added into the genome donates a hypoxanthine and guanine to create GMP, allowing the parasite to bypass the normal method for GMP production. Those parasites without the gene will not survive selection. Those surviving the selection were then transferred to 96-well plates of HFF cells and cloned. Approximately one HX-resistant parasite was placed in each well of 3 96-well plates. The plates were filled with 100uL HX- media and 100uL parasites diluted in HX- media. The parasites were left in the incubator at 37C for 7-10 days to allow plaque formation. Clones were then analyzed using PCR and IFA.

**Immunofluorescent Microscopy**

IFA was completed to allow for localization analysis of the tagged proteins. HFF cells grown on coverslips were infected with *T. gondii* and incubated for 48h. The media was then aspirat-
ed from the cells. Infected host cells were rinsed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 5 min at room temperature. Fixed cells were treated with PBS containing 0.25% Triton X-100 for 20 min to permeabilize cell membranes. Cells were washed with PBS twice and blocked with 1% bovine serum albumin (BSA) in PBS for 20 min at room temperature. Antibodies (Anti-HA Rat and Anti-IMC Rabbit) were diluted in the same blocking buffer and incubated for 60 min at 37°C followed by washing twice with PBS. These antibodies bind to the tagged protein and the parasite’s inner-membrane complex, respectively. Secondary antibodies (Goat Anti-Rabbit 594 and Goat Anti-Rat 488) were added with PBS and 4′,6-diamidino-2-phenylindole (DAPI) and incubated for 10 minutes. DAPI is used to stain the parasite nuclei. After 3 washes with PBS allowing 15 minutes between washes, the coverslips were mounted with Aquamount (Thermo Scientific) and viewed on a Zeiss Axiovert microscope equipped with a 100x objective.
CHAPTER THREE:

RESULTS

Aim 1. Search for Suitable Target Genes

Since apicomplexan parasites lack canonical G1 regulators and downstream effectors, the “guilt by association” approach was taken to identify protein interactions and putative substrates through proteomics and P-proteomics. From a pre-organized proteomics spreadsheet of identified protein interactors/substrates, five targets were selected and analyzed using ToxoDB. Targets were chosen based on level of specificity for TgCrk2-HA (versus TgCrk6-HA and TgCycL-HA), completion of the sequence with few unknown regions, and molecular weight. These targets were then analyzed using ToxoDB in order to check for completion of the genome sequence (few unknown regions) and C-terminal signal peptides because a C-terminus 3xHA tag will be added later. Chosen restriction sites were also analyzed for methylation sensitivity. Selected genes are shown in Table 3.

<table>
<thead>
<tr>
<th>ToxoID</th>
<th>MW (kDa)</th>
<th>Crk2-HA</th>
<th>Crk6-HA</th>
<th>Cycl-HA</th>
<th>Specificity</th>
<th>Expression %</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGME49_223080</td>
<td>307</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>High</td>
<td>60</td>
</tr>
<tr>
<td>TGME49_230940</td>
<td>155</td>
<td>12</td>
<td>0</td>
<td>21</td>
<td>Low</td>
<td>80</td>
</tr>
<tr>
<td>TGME49_269620</td>
<td>59</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>High</td>
<td>60</td>
</tr>
<tr>
<td>TGME49_227450</td>
<td>217</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>Medium</td>
<td>80</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ToxoID</th>
<th>MW (kDa)</th>
<th>Phospho-site Ratio</th>
<th>Specificity</th>
<th>Expression %</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGME49_262390</td>
<td>155</td>
<td>8.888</td>
<td>High</td>
<td>55</td>
</tr>
</tbody>
</table>
Aim 2. Building Target Protein Constructs

If a gene had positive characteristics and a reasonable size for cloning, it was chosen for further analysis. Our strategy for endogenous tagging was gene knock-in tagging with 3xHA epitope. The 5’ end of each target gene was analyzed using APE software Enzyme Selector for mid-insert enzyme cuts that are absent in the vector (pLIC-3xHA-HXGPRT). Gene specific primers with LIC-extensions to provide recombination sites with linearized LIC-vector were designed, as seen in Table 4, below.

<table>
<thead>
<tr>
<th>Table 4. LIC Construct Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene of Interest</td>
</tr>
<tr>
<td>TgME49_227450</td>
</tr>
<tr>
<td>TgME49_223080</td>
</tr>
<tr>
<td>TgME49_262390</td>
</tr>
<tr>
<td>TgME49_230940</td>
</tr>
<tr>
<td>TgME49_269620</td>
</tr>
<tr>
<td>Forward Primer Tag</td>
</tr>
<tr>
<td>Reverse Primer Tag</td>
</tr>
</tbody>
</table>

PCR amplification of the 3’ genomic fragments of the target genes was completed using the conditions shown in Table 5, below.
Three of the five target proteins were amplified and the results can be seen in Figure 6 below. Product size was compared to expected size in order to verify correct product amplification.

![Figure 2. PCR products on 0.7% agarose gel](image)

### Table 5. Optimized PCR Conditions

<table>
<thead>
<tr>
<th>Name (TgME49_))</th>
<th>227450</th>
<th>223080</th>
<th>262390</th>
<th>230940</th>
<th>269620</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>denaturation</strong></td>
<td>98°C for 1 min</td>
<td>98°C for 1 min</td>
<td>98°C for 1 min</td>
<td>98°C for 1 min</td>
<td>98°C for 1 min</td>
</tr>
<tr>
<td><strong>annealing (32x)</strong></td>
<td>98°C for 10 sec</td>
<td>98°C for 10 sec</td>
<td>98°C for 10 sec</td>
<td>98°C for 10 sec</td>
<td>98°C for 10 sec</td>
</tr>
<tr>
<td></td>
<td>61°C for 15 sec</td>
<td>63°C for 15 sec</td>
<td>60°C for 15 sec</td>
<td>60°C for 15 sec</td>
<td>61°C for 15 sec</td>
</tr>
<tr>
<td></td>
<td>72°C for 45 sec</td>
<td>72°C for 1 min</td>
<td>72°C for 45 sec</td>
<td>72°C for 45 sec</td>
<td>72°C for 45 sec</td>
</tr>
<tr>
<td><strong>elongation</strong></td>
<td>72°C for 3 min</td>
<td>72°C for 3 min</td>
<td>72°C for 3 min</td>
<td>72°C for 3 min</td>
<td>72°C for 3 min</td>
</tr>
</tbody>
</table>

Two of the target genes were unable to be amplified. Any number of errors can result in no PCR product, which will further be analyzed in the discussion. After PCR amplification, absorbances were recorded as seen in Table 8.
Table 7 shows the linearization of the vector (LIC-3xHa-HxGPRT) on the large scale.

<table>
<thead>
<tr>
<th>Name (TgME49?)</th>
<th>Dilution</th>
<th>Absorbance A260/A280</th>
<th>Ratio</th>
<th>Concentration (ng/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>227450</td>
<td>1:50</td>
<td>0.0463/0.0241</td>
<td>1.92</td>
<td>116</td>
</tr>
<tr>
<td>223080</td>
<td>1:20</td>
<td>0.0989/0.0735</td>
<td>1.33</td>
<td>99</td>
</tr>
<tr>
<td>262390</td>
<td>1:20</td>
<td>0.0487/0.0258</td>
<td>1.89</td>
<td>49</td>
</tr>
<tr>
<td>230940</td>
<td>1:20</td>
<td>0.0316/0.0152</td>
<td>1.98</td>
<td>32</td>
</tr>
<tr>
<td>269620</td>
<td>1:50</td>
<td>0.0339/0.0171</td>
<td>1.98</td>
<td>85</td>
</tr>
<tr>
<td>LIC-3xHa-HxGPRT Vector</td>
<td>1:20</td>
<td>0.3206/0.1778</td>
<td>1.80</td>
<td>321</td>
</tr>
</tbody>
</table>

LIC-3xHa-HxGPRT vector contains LIC sequences on both sides of the PacI site.

The vector digested with the PacI enzyme is treated with T4 polymerase in the presence of dGTP and dCTP. T4 polymerase has exonuclease activity which allows it to create large, sticky ends on the vector that can bind with the LIC sequences of the forward and reverse primers, seen in Table 4. Using ligation independent cloning (LIC) the PCR product is inserted into the vector. Our LIC-vector also contained a 3xHA tag that will allow for specific recognition of the target protein. The end
product is a plasmid with a 3’end of the gene of interest fused with 3xHa tag, and also contains a drug-selection gene HXGPRT, as explained in Materials and Methods. The final LIC constructs can be seen in Figure 7.

Cloning was completed using the Clontech In-fusion® HD Cloning Kit. Protocol I in section VI was followed. Ampicillin plates were used for spreading the transformed bacteria, after transformation described in Materials and Methods. Colony PCR was run to identify positive colonies using the original cloning primers. These products were then run on 0.7% agarose gel electrophoresis. If a PCR product of the predicted size was detected, then, corresponding bacterial clone was chosen to isolate plasmid DNA. From 3 ml of overnight culture grown in LB with
amipicillin. An analytical digest (1ug DNA) of each construct as described in Materials and Methods was performed. The plasmids, both cut and uncut, were analyzed using 0.7% agarose gel electrophoresis. If a plasmid had been successfully linearized, plasmid DNA was sent for sequencing to verify correct insert.

**Aim. 3. Creation of Transgenic Parasites**

The plasmids were linearized in order to create two ends which induce repair mechanisms within the plasmid. When repair takes place, the linear plasmid is inserted into the genome based on homology provided by the 3’ end of the cloned plasmid. Successful *T. gondii* transfections require ~10-20ug of DNA. Electroporation involves a single recombination event resulting in the introduction of the whole plasmid into the genome at the 3’ end of the gene of interest.

The reactions for each digestion can be seen below in Table 8, and the results of linearization can be seen below in Figure 8.

<table>
<thead>
<tr>
<th>Name</th>
<th>Purified DNA (uL)</th>
<th>10x Cutsmart Buffer (μL)</th>
<th>Water (μL)</th>
<th>Enzyme (μL)</th>
<th>Enzyme Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>227450</td>
<td>7</td>
<td>1</td>
<td>1.5</td>
<td>0.5</td>
<td>PstI</td>
</tr>
<tr>
<td>223080</td>
<td>7</td>
<td>1</td>
<td>1.5</td>
<td>0.5</td>
<td>MfeI</td>
</tr>
<tr>
<td>262390</td>
<td>4</td>
<td>2</td>
<td>13.5</td>
<td>0.5</td>
<td>SrgAI</td>
</tr>
<tr>
<td>230940</td>
<td>7</td>
<td>1</td>
<td>1.5</td>
<td>0.5</td>
<td>BglIII</td>
</tr>
<tr>
<td>269620</td>
<td>7</td>
<td>1</td>
<td>1.5</td>
<td>0.5</td>
<td>Eco47III</td>
</tr>
</tbody>
</table>
After linearization was completed, HFF cells were electroporated following the previously discussed protocol. Parasites were selected using mycophenolic acid (MPA). Drug-resistant polyclonal populations were cloned in 96-well plates as described in Material and Methods. Cloning of parasites with the genes of interest was completed in 96-well plates. Serial dilutions were completed to ensure as close to one parasite per 100μL occurred during cloning. For TgME49_227450, there was an estimated 1.25 parasite/100mL, for TgME49_223080 there was approximately 1.1 parasites/100 μL, and for TgME49_269620 there were approximately 0.94 parasites/100 μL. Plates were incubated at 37ºC to allowing formation and expansion of parasite plaques formed by sequential lysis of the host cells. Wells with a single plaque were selected.

**Aim. 4. Analysis of the Target Protein Localization Through IFA**

Once transgenic clones were expanded into 96-well plates, cells were transferred and expanded to 24-well plates, then to T25 flasks for storage. An Immunofluorescence Assay (IFA) was completed to visualize the localization of the target protein. As previously discussed, the cell structure of Toxoplasma is well known. To distinguish sub-cellular compartments, we co-stained parasites
with nuclear DAPI stain (blue), α-IMC1 antibody that recognize surface alveolar protein (red) and α-HA antibody (green) to visualize our target protein. The results of the IFA analysis can be seen in Figure 8. TgME49_227450 was observed to be a cytoplasmic protein, since there is no or very little overlap between red α-HA and blue DAPI staining. TgME49_223080 was observed to be a nuclear protein, because it shows strong co-localization with nuclear DAPI stain. TgME49_269620 was observed to be a cytoplasmic protein with perinuclear accumulation, because the protein was excluded from nucleus (no overlap with blue DAPI stain) however, there was a higher concentration of the protein at the apical end of the nuclei. This may suggest an accumulation of this protein in the Golgi stacks (arrow).

Figure 5. Localization of three target proteins
CHAPTER FOUR: 
DISCUSSION

All four aims of the project were successfully completed. We have tagged and localized three out of five candidate TgCrk2 interactors. Alvarez and Suvorova (2017) previously found that the Cdk-related kinase TgCrk2 localizes in both the cytoplasm and the nucleus. Therefore, we would expect to find true interacting proteins localized also to cytoplasm, nucleus or both compartments. TgME49_227450 was found to be a cytoplasmic protein and thus, could form a complex with TgCrk2 in the cytoplasm. TgME49_227450 was suspected to be a NUDIX (nucleoside diphosphate linked to another moiety, x) family protein as a hydrolase, which explains its location in the cytoplasm. These hydrolases are responsible for removing biochemical pathway intermediates within the cell. These enzymes can be found throughout the cell and are often thought to be cytosolic proteins (Ogawa, Ueda, Yoshimura, & Shigeoka, 2005). Functionality of the other two proteins of interest were previously unknown.

TgME49_223080 was found to localize in the nucleus, while TgME49_269620 was a cytoplasmic protein with perinuclear accumulation. Both these open reading frames are annotated as hypothetical. Endogenous tagging and IFA analysis confirms abundant protein expression in tachyzoites. The cellular function of these proteins is unknown, but our localization studies of TgME49_269620 and TgME49_223080 may give some insight into their function in cell cycle regulation.

In conclusion, three T. gondii proteins were studied for which expression and localization had not been previously studied. Our findings on TgME49_227450, TgME49_223080 and Tg-
ME49_269620 proteins correlate with predicted participation in the complex with TgCrk2. Localization of these five target proteins is a first step to confirming interaction with TgCrk2 and understanding their function and role in TgCrk2 regulation of the tachyzoite cell cycle. Future research should be completed in order to prove these putative interactors are really correlated with TgCrk2 through Co-immunoprecipitation. More consideration should also be given to the protein Tg-ME49_227450 in proving its function as a NUDIX family protein. Once proven to be TgCrk2 interactors, the function and role in cell cycle regulation of these targets of interest can be further analyzed.
REFERENCES


