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Exploring Unique Aspects of Apicomplexan Cell Biology Using Molecular Genetic and Small Molecule Approaches

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**EXPLORING UNIQUE ASPECTS OF APICOMPLEXAN CELL BIOLOGY
USING MOLECULAR GENETIC AND SMALL MOLECULE APPROACHES**

A Dissertation Presented

by

Whittney Dotzler Barkhuff
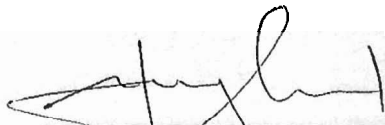
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The Faculty of the Graduate College

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In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Cell and Molecular Biology

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Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, specializing in Cell and Molecular Biology.

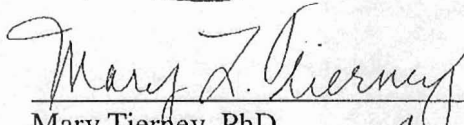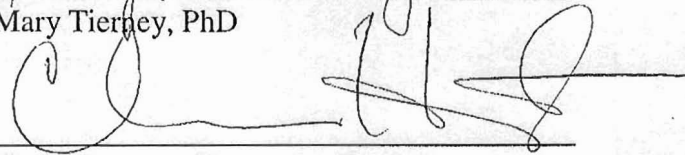Thesis Examination Committee:

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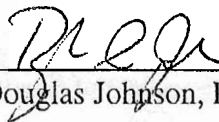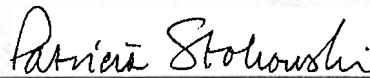Gary Ward, PhD

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ABSTRACT

The Phylum Apicomplexa contains a number of devastating pathogens responsible for tremendous human suffering and mortality. Among these are *Plasmodium,* which is the causative agent of malaria, *Cryptosporidium,* which causes diarrheal illness in children and immuncompromised people, and *Toxoplasma gondii,* which causes congenital defects in the developing fetus and severe disease in immunocompromised people. *T. gondii* also serves as a model organism for other members of this phylum due to the relative ease of parasite culture and manipulation. Although effective treatments exist for some diseases caused by these apicomplexan parasites, drug resistance for others is widespread, perhaps most notably in *Plasmodium* species. Development of new therapeutic agents is needed to combat this resistance and alleviate disease burden. It is important that the drugs target parasitic cell components not conserved in humans in order to minimize side effects and drug toxicity. However, in order to target unique processes, a better understanding of apicomplexan biology must be gained. One approach toward understanding the unique biological processes of apicomplexan parasites is to study proteins conserved among the Phylum Apicomplexa, but not present in other organisms. One such protein, photosensitized INA-labeled protein 1 (TgPhIL1) was identified previously. The work presented in this dissertation describes targeted disruption of this gene in *T. gondii,* which results in parasites with an altered shape and a fitness defect in both tissue culture and a mouse model of infection. Another approach to understanding the unique processes of apicomplexan parasites is to perturb them using small molecules. By identifying the targets of the small molecules, a more detailed understanding of the process can be gained. To this end, a small molecule screen was performed in *T. gondii* in order to identify small molecules that inhibit the apicomplexan-specific and essential process of host-cell invasion. In addition to identifying 24 invasion inhibitors, 6 enhancers were also identified. One of these enhancers, compound 112762, was shown to enhance invasion of other apicomplexan parasites as well. Described herein are attempts to identify the target(s) of this compound. A derivative of this compound was linked to an affinity resin, and TgProfilin was identified as a putative target that may interact covalently with 112762. Additionally, affinity chromatography was used to demonstrate non-covalent binding of a *T. gondii* FK506-binding protein to 112762. Finally, based on a report in the literature of a compound nearly identical to 112762 that inhibits yeast and mammalian protein arginine methyltransferase 1 (PRMT1), it was hypothesized that 112762 might target TgPRMT1 in *T. gondii.* Supportive of this hypothesis, 112762 was shown to inhibit TgPRMT1 *in vitro*, to inhibit parasite protein methylation *in vivo*, and to bind the 112762 affinity resin. TgPRMT1 knockout parasites are being generated in order to determine whether they show resistance to compound 112762. As a result of this work, three potential targets of 112762 in *T. gondii* have been identified. This work opens the door for future studies aimed at understanding and controlling invasion by apicomplexan parasites and other processes specific to the Phylum Apicomplexa.

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CHAPTER 1: LITERATURE REVIEW

GENERAL INTRODUCTION TO *TOXOPLASMA GONDII*

Introduction

The mere mention of the word "parasite" is enough to make most people cringe. It brings to mind images of flesh teeming with worms, emaciated children lying in hospital beds, crying out in the middle of the night in feverish fits, perhaps recent news articles about Bill and Melinda Gates' contributions to the global fight against malaria, and if one is aware of recent medical advancements, maybe a vague recollection of something about a controversial new malaria vaccine on the horizon (Genton, 2008).

For those who study parasites, the allure is two-fold. First, there is the desire to learn more about these organisms so that this knowledge can be used to prevent the morbidity and mortality they cause. The more we understand the inner workings of these tiny unwanted guests, the more precisely and potently we can target and disrupt their essential biological processes, thereby rendering them ineffective as pathogens and alleviating the symptoms and suffering they cause.

At the same time, one can't help but be impressed and amazed by the ways in which parasites bend the fundamental rules of molecular and cellular biology. Elucidating the mechanisms by which they interact with their hosts, survive, replicate, and go on to colonize new hosts is an endeavor in which one must expand one's definition of "normal" and constantly be aware that with these resourceful and dynamic organisms, virtually anything is possible.

It is with these goals and aspirations, both with the hope of uncovering something that could one day be useful in fighting parasitic disease, and with the desire to learn more about these unique life styles, that we take to the bench with pipettor in hand and attempt to unravel the many remaining mysteries that surround the biology of parasites.

Parasitic diseases

The word "parasite" technically refers to any organism that lives on or within another organism, causing it harm. Thus, organisms from any kingdom can be parasites and the word is even used to describe viruses. In order to minimize confusion, however, discussions of parasitic human diseases usually refer to diseases caused by protozoan (unicellular eukaryotes) or metazoan (worm) parasites.

Parasites are responsible for an incredible amount of human suffering and death. Although less common in the developed world, parasitic diseases are a major cause of morbidity and mortality in the developing world. Additionally, they present a major barrier to economic development since people who become sick are often unable to work (Gallup and Sachs, 2001; Sachs and Malaney, 2002). Among the most common parasitic diseases of humans are malaria, leishmaniasis, trypanosomiasis, trichomoniasis, and toxoplasmosis. Their severity ranges from causing asymptomatic, self-limiting infections to being a leading cause of death for certain demographics. Caused by *Plasmodium* species, malaria is particularly devastating, accounting for one in every five childhood

deaths in Africa and putting roughly 40% of the world's population at risk of infection (http://www.cdc.gov/malaria/facts.htm).

A number of unique challenges are posed to one attempting to combat parasitic diseases. When identifying potential drug targets in order to fight infectious diseases, it is helpful to identify targets that are not conserved in mammalian cells in order to minimize unwanted effects on the host. Since parasites are eukaryotes, like us, identification of these unconserved targets in parasites can be more difficult than in bacteria, which are prokaryotes. Additionally, the fact that parasites are eukaryotes means their cellular processes are sophisticated and their ability to evolve complicated drug-resistance mechanisms is great.

Also, parasite life cycles are generally complicated and involve multiple stages. Drugs aimed at treating these diseases may be effective against one stage of the parasite, but not against others. Vaccine development for parasitic diseases has lagged far behind that for other forms of infectious diseases, and this is at least partly due to this issue. Attempts to develop a malaria vaccine, for example, have been incredibly challenging. GlaxoSmithKline has a developed a malaria vaccine called RTS, S that targets the liver stage of the parasite (sporozoite) (Abdulla *et al.*, 2008; Aponte *et al.*, 2007). This vaccine has been shown to provide some protection against malaria, especially in young children and infants. Several other vaccines for malaria are currently being developed, and many of them are stage-specific as well (Kristoff, 2007; Soares and Rodrigues, 1998). Additionally, many parasites have evolved specialized methods of varying antigens

displayed on their surface so that by the time the immune system has identified them, they are no longer present, rendering the immune response ineffective.

The Phylum Apicomplexa

The Phylum Apicomplexa contains over 4,000 organisms (Levine, 1988). All spend at least some part of their lifecycle as intracellular organisms, all have complex life cycles and exist in multiple stages, and many are capable of causing disease in humans and other animals (Levine, 1988).

Members of this phylum include: *Toxoplasma gondii,* which infects roughly a third of the world's population*; Plasmodium* species, which cause malaria; *Cryptosporidium,* which causes diarrheal illness in immunocompromised people and children; and *Eimeria, Neospora* and *Theileria* which are important veterinary pathogens. Among these, *T. gondii* is the most easily studied in the laboratory (Kim and Weiss, 2004).

Toxoplasma gondii as a model organism for the phylum Apicomplexa

The name *T. gondii* is derived from the Greek word "toxo" meaning arc and referring to the half-moon shape of the parasite, and gondii, from gundi, the Tunesian rodent in which Nicolle and Manceaux first identified the parasite in 1908 (Nicolle, 1909). Since it is both an important human pathogen and a useful model organism for studying other apicomplexan parasites, *T. gondii* is an important member of the phylum.

Many apicomplexan parasites are extremely difficult to study in the laboratory. To date, *Cryptosporidium* has not been successfully propagated in tissue culture. In order to acquire the pathogen, investigators must obtain feces containing oocysts and extract the oocysts. *Plasmodium* species provide a number of challenges in the laboratory, for the blood-stage form of *P. falciparum*, called merozoites, cannot be isolated in a manner that preserves their invasiveness. Additionally, merozoites are small, making localization of subcellular structures within the parasite difficult. On top of that, no convenient animal model exists for *P. falciparum,* and *P. falciparum* can only be propagated in tissue culture in red blood cells. Finally, limited molecular genetic tools are available for manipulation of *P. falciparum*.

On the other hand, *Toxoplasma gondii* readily invades virtually all nucleated mammalian cells (Morrissette and Roos, 1998). Human foreskin fibroblasts (HFF's) are routinely used as host cells due to availability and ease of maintenance. Intracellular replication of the fast-growing tachyzoite stage occurs every 4-6 hours. Thus, as long as one has access to a constant supply of easily cultured cells, one can obtain large numbers of *T. gondii* parasites with which to work. Additionally, at 2 μm by 7μm, *T. gondii* is large in comparison to other apicomplexan parasites, notably *Plasmodium* merozoites which are approximately ten times smaller. This makes the visualization of subcellular structures much easier in *T. gondii* than in other apicomplexan parasites.

Another advantage of using *T. gondii* as a model organism is that a number of techniques are available for its genetic manipulation (Gubbels *et al.*, 2008; Mital and Ward, 2008; Roos *et al.*, 1994). Heterologous DNA can be introduced by simple

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electroporation. After being maintained episomally for a brief period, heterologous DNA will insert randomly into the genome. Additionally, sequences can be targeted to specific genomic loci where they insert by homologous recombination. This allows for the generation of both pure and conditional knockout parasites, as well as allelic replacement. It should be noted that although both non-homologous and homologous recombination occur in *T. gondii,* non-homologous recombination occurs much more frequently. This can make generation of knockout parasite lines labor-intensive since constructs designed to integrate at specific loci are much more likely to do so at random. In an attempt to overcome this challenge, it was recently reported that a *T. gondii* recombinase responsible for non-homologous recombination has been knocked out, yielding a strain in which targeted integration is greatly facilitated (Bzik and Fox, personal communication). In addition to the genetic techniques listed above, forward genetic screens have been performed with both temperature-sensitive mutants and insertional mutagenesis (Gubbels *et al.*, 2008).

The tools that are available for genetic manipulation of *T. gondii* make it a useful model organism for other apicomplexan parasites. However, it is most useful as a model organism when studying biological aspects that are conserved across the phylum. Among these are the machinery used to move the parasites across a substrate, certain aspects of the invasion and egress mechanisms, to some degree replication and growth, and many ultrastructural features (Morrissette and Roos, 1998). It is important when using *T. gondii* as a model organism to remain conscious of which aspects of *T. gondii* are conserved across the phylum and will yield useful information about other

apicomplexans. Of course, findings in *T. gondii* must be confirmed in other organisms of interest.

Life cycle

T. gondii is an obligate intracellular parasite with a complex life cycle comprised of both sexual and asexual stages. Within the asexual stage, the parasite exists in one of two forms: tachyzoites and bradyzoites. In comparison to tachyzoites, bradyzoites grow more slowly and more easily evade the host immune system. In contrast, tachyzoites are highly motile, highly invasive, and capable of eliciting a strong immune response. In response to some environmental or immunological cue, parasites in the asexual phase of the life cycle switch back and forth between tachyzoites and bradyzoites (Dubey *et al.*, 1998). When not specified, material in the rest of this thesis deals with the tachyzoite form of *T. gondii,* which is responsible for most pathology in humans and is most easily studied due to its ease of propagation and rapid rate of replication.

For reasons poorly understood, the *T. gondii* sexual cycle occurs only within the intestine of felines and thus is only studied in a handful or laboratories with access to such material. After being shed in cat feces, oocyts, which contain large numbers of infectious organisms, can be ingested by humans and other animals, causing infection. Although ingestion of oocysts is one mode of infection, humans most commonly become infected by eating tissue cysts, consisting of large numbers of bradyzoites, in undercooked pork or beef. When oocysts are ingested, sporozoites are the infectious

form (Tenter *et al.*, 2000). After ingestion, bradyzoites or sporozoites are released in the

lumen of the small intestine. Once free, they invade the enterocytes or intra-epithelial

lymphocytes before differentiating into the tachyzoite, or fast-growing stage. Because *T.*

gondii tachyzoites are highly invasive and replicate quickly, they are then free to rapidly

disseminate throughout the body.

Figure 1: The most common modes of transmission of *T. gondii* to humans.
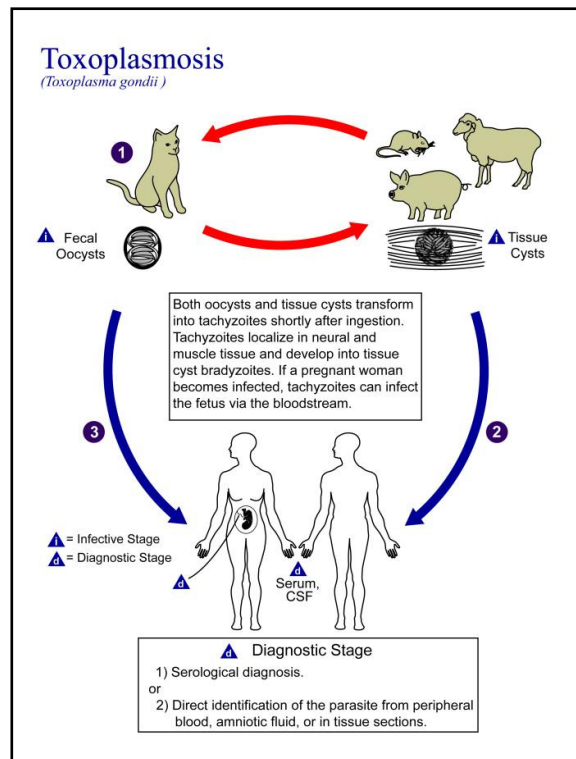
Figure 1: Humans are most commonly infected by *T. gondii* through ingesting tissue cysts in undercooked pork or beef or through ingesting oocysts in contaminated cat feces (www.dpd.cdc.gov).

Medical Relevance

In addition to serving as a model organism for other apicomplexan parasites, *T. gondii* is an important human pathogen in itself, as was alluded to in the previous section. The outcome of an individual's infection with *T. gondii* is related to the individual's immune status.

In the case of an immunocompetent adult host, infection with *T. gondii* is usually asymptomatic and self-limiting, although a small percentage of people experience mild symptoms such as generalized lymphadenopathy, fever, or fatigue (Remington, 1974). In an immunocompetent host, the immune system rapidly detects and begins to fight the infection. Although antibodies are generated against several *T. gondii* antigens, the cellular immune response is thought to play a larger role in controlling the infection than the humoral response. CD4+ and CD8+ T-cells secreting IFN-γ are thought to be particularly important in controlling the infection since mice with deficient T-cell immunity are unable to control *T. gondii* infections and since infection with *T. gondii* results in high levels of IFN-γ expression (Denkers and Gazzinelli, 1998; Filisetti and Candolfi, 2004).

In response to some cue thought to be provided by the immune system, fast-growing tachyzoites begin to differentiate back into slow-growing bradyzoites. Cysts containing bradyzoites begin to develop, and this occurs at a particularly high frequency in the muscles and brain (Weiss and Kim, 2000). It was once thought that such tissue cysts remain latent until the immune system became suppressed, but it has since been

shown that even in an immunocompetent host, cysts periodically rupture. When this happens, bradyzoites differentiate into tachyzoites, undergo a few cycles of replication, and then are triggered to differentiate back into bradyzoites by some unknown environmental or immunological cue (Ferreira da Silva Mda *et al.*, 2008).

In the case of an immunocompromised host, the immune system is either unable to control the initial infection, or if the person becomes immunocompromised after acquiring the infection, is no longer able to control the latent infection. Cysts rupture releasing large numbers of parasites which undergo repeated cycles of host cell invasion, replication, and host-cell lysis. Damage to organ parenchyma is due to a combination of the direct damage caused by these cycles of lysis and to the dramatic inflammation that occurs in response to the presence of the parasite (Hermes *et al.*, 2008).

Because the parasite has a particular tropism for neurons, astrocytes, microglia, and cells of the retina, much of the damage caused by *T. gondii* occurs in the brain and eyes. Obviously, this has serious consequences and results in encephalitis, seizures, characteristic "ring-enhancing" lesions seen by Magnetic Resonance Imaging (MRI), and retinopathy. Toxoplasmic retinopathy is rare but seen in immunocompetent patients as well (Fish *et al.*, 1993). Because 10-40% of HIV-infected people in the US are also chronically infected with *T. gondii*, reactivation of the infection is a serious health problem. Before the advent of Highly Active Antiretroviral Therapy (HAART), 24-47% of *T gondii*-seropositive AIDS patients ultimately developed toxoplasmic encephalitis. Since the introduction of HAART, the incidence in the United States of toxoplasmic

encephalitis among patients diagnosed with AIDS has decreased to 2.2/1000 person-years (Sacktor *et al.*, 2001).

In addition to causing severe disease in immunocompromised people, *T. gondii* can also cause severe disease in the developing fetus. In the case of an immunocompetent pregnant woman, the course of infection is similar to that described above with one major exception: If a woman acquires her primary *T. gondii* infection during pregnancy, the parasite can cross the placental barrier and infect the developing fetus, which can have serious consequences. The earlier in pregnancy this occurs, the more seriously the fetus is likely to be affected by congenital toxoplasmosis. If the immune status of the mother is normal and she has been infected with *T. gondii* before becoming pregnant, reinfection with *T. gondii* is not likely to affect the fetus. As an exception to this rule, a few cases have been reported in which women who acquired a primary *T. gondii* infection within a month of pregnancy delivered babies affected by congenital toxoplasmosis (Remington, 1974; Remington *et al.*, 1995).

Congenital toxoplasmosis is characterized by neurologic abnormalities including seizures, mental retardation, and hydrocephalus. Additionally, the eye is often affected and visual impairment is common. Congenital toxoplasmosis is thought to affect 1 in 10,000 births in the US, but that number is 20 times higher in France, Norway, and Belgium where it is more common to eat raw or undercooked meat (Remington *et al.*, 1995).

Standard treatment for toxoplasmosis in humans is a combination of sulfonamides and pyrimethamine. Additionally, it has been shown that spiramycin has

antitoxoplasmic activity and does not cross the placenta. Thus, spiramycin is a useful drug for the treatment of toxoplasmosis in pregnant women (Elsheikha, 2008; Montoya and Remington, 2008).

Ultrastructure

In order to gain a better understanding of what is known about *T. gondii* biology, let us start with an overview of the parasite's ultrastructure. Apicomplexan parasites contain a number of organelles and cytoskeletal features unique to the phylum. Additionally, many organelles common to other organisms have unique properties in apicomplexan parasites. These topics will be presented here. In the second part of this chapter, ultrastructural features that pertain directly to the material presented in Chapter 2 of this thesis will be described in greater detail.

a) Cytoskeletal features

i) The pellicle—Like all eukaryotic cells, *T. gondii* is surrounded by a plasma membrane. Unique to the phylum Apicomplexa, however, is the presence of an organelle called the inner membrane complex, or IMC, underlying the plasma membrane. The IMC is thought to be derived from either the endoplasmic reticulum or the Golgi apparatus and is composed of a sheet of vesicles, flattened and presumably held together by some sort of molecular sutures that have yet to be identified. The IMC is thought to be analogous to the alveolae of the closely related dinoflagellates and ciliates. Although the

IMC does not extend to the extreme apical end of the parasite, it is otherwise thought to be continuous around the periphery of the parasite (Figure 4), with the exception of a small micropore through which endocytosis has been proposed to occur. Together, the two membranes of the IMC and the plasma membrane are referred to as the pellicle (de Melo and de Souza, 1997).

ii) Conoid—The conoid is a cytoskeletal structure at the apical end of the parasite. It is shaped like a truncated cone and extends and retracts as the parasite moves across a surface. The purpose of this movement is not understood, but it is thought to be essential for both motility and invasion. At one time, the filaments in the conoid were thought to be microtubules. However, they have since been shown to be made of a novel polymer of alpha tubulin (Hu *et al.*, 2002b). Anterior and posterior to the conoid are two sets of polar rings known as the apical and basal polar rings. The basal polar rings are thought to act as microtubule organizing centers (MTOC's) (Russell and Burns, 1984).

iii) Microtubules—Despite the fact that the conoid is not made of microtubules, the parasite is known to have several populations of microtubules. Shown to originate from the extreme apical end of the parasite, two intraconoid microtubules 350 nm in length are oriented along the long axis of the parasite. Additionally, radiating from the posterior polar ring are 22 unusually stable (Stokkermans *et al.*, 1996) subpellicular microtubules that are presumed to be associated with the IMC either directly or indirectly. In addition to the cytoskeletal microtubules, *T. gondii* contains a population of spindle microtubules known to be involved in chromosome separation during mitosis (Hu *et al.*, 2002b; Swedlow *et al.*, 2002).

Figure 2: The conoid, polar rings, and subpellicular microtubules of T. gondii.
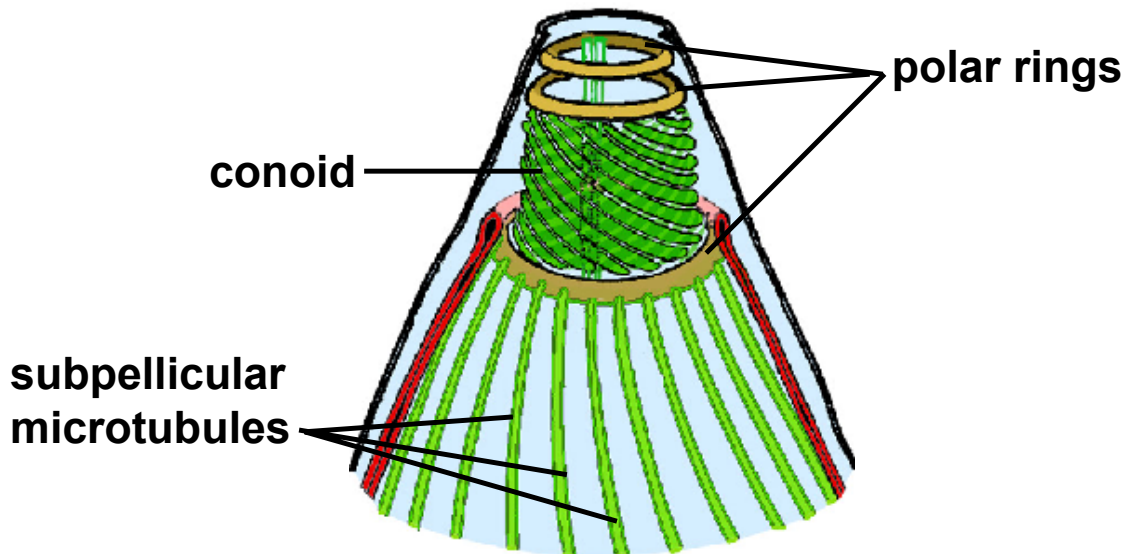
Figure 2: Present at the apical end of the parasite, the conoid is made of a novel polymer of alpha-tubulin arranged in a spiral. The polar rings are present on either side of the conoid, and the 22 subpellicular microtubules can be seen emanating from the posterior polar ring (Nichols, 1987).

iv) Microfilaments—Only one actin gene is present in *T. gondii*, *TgAct1*. It has been shown that 97% of *T. gondii* actin is globular, making visualization of filamentous actin extremely difficult. In fact, actin filaments have only been seen by freeze fracture electron microscopy (Wetzel *et al.*, 2003). Between the plasma membrane and the outer membrane of the IMC are a number of short (100 nm) actin filaments upon which myosin

A walks, generating the mechanical force required for both motility and invasion. *T. gondii* actin is only 83% conserved with mammalian actin, and many of the residues that differ are located on the face with which actin monomers interact with other actin monomers (Sahoo *et al.*, 2006). These differences are thought to be responsible for the differing properties of *T. gondii* and mammalian actin, namely that: a) *T. gondii* actin filaments are less stable than mammalian actin filaments, and b) *T. gondii* actin filaments polymerize at a lower critical concentration than mammalian actin filaments. These properties are thought to explain the presence of short, dynamic actin filaments under the plasma membrane. Actin filaments have not been visualized elsewhere within apicomplexan parasites (Dobrowolski *et al.*, 1997; Gordon and Sibley, 2005; Sahoo *et al.*, 2006; Schatten *et al.*, 2003; Wetzel *et al.*, 2003).

b) Organelles

i) Nucleus, endoplasmic reticulum, and Golgi apparatus—*T. gondii* nucleus is located slightly towards the posterior end of the parasite. As is the case with most organisms, the nuclear envelope is covered with ribosomes and continuous with the rough endoplasmic reticulum. Interestingly, *T. gondii* contains a single Golgi cisternum whose biogenesis has been extensively characterized in an attempt to understand Golgi biogenesis in organisms containing multiple Golgi cisternae (Hartmann *et al.*, 2006; He, 2007; Nishi *et al.*, 2008).

ii) Apicoplast—Four lipid membranes surround the enigmatic apicoplast, an organelle unique to the phylum Apicomplexa. This degenerate plant plastid is thought to

have originated through secondary phagocytosis in which an algae equipped with a symbiont was engulfed by an apicomplexan ancestor (Funes *et al.*, 2002). For those apicomplexans that have retained the apicoplast, it is essential. Interestingly, the apicoplast is absent from *Cryptosporidium* (Zhu, 2004) and the question remains whether *Cryptosporidium* lost it or never had it. Although much remains to be learned about the function of the apicoplast, it is known to be involved in type II fatty acid oxidation (Vollmer *et al.*, 2001). Like the mitochondria, this organelle contains its own DNA, although most proteins found in the apicoplast are encoded by genomic DNA (Marechal and Cesbron-Delauw, 2001; Roos *et al.*, 2002; Soldati, 1999)

iii) Secretory organelles—Three sets of specialized secretory organelles are present in *T. gondii*. These secretory organelles are involved in the processes of motility, invasion, and the development of the vacuole within which the parasite resides in the host cell. These organelles are the micronemes, rhoptries, and dense granules and their contents are discharged during invasion, although constitutive secretion of both the micronemes and the dense granules also occurs. As may be expected, these organelles contain many proteins unique to the Phylum Apicomplexa. A more detailed description of the role of these organelles during invasion will be described in the "invasion" section of this chapter (Dobrowolski and Sibley, 1997; Dubremetz *et al.*, 1993; Dubremetz *et al.*, 1998).

iv) Acidocalcisomes—Another group of organelles present in apicomplexan parasites and worthy of mention are the acidocalcisomes. These are small vesicles around 200 nm in size and one or two of them can be seen typically within a *T. gondii*

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parasite. They have been shown to store intracellular calcium and polyphosphate (Luo *et al.*, 2005; Miranda *et al.*, 2008).

Motility

Apicomplexan parasites possess many unique cytoskeletal structures and organelles, and they move across surfaces using a unique mechanism that involves the use of many of these components. This form of motility is called gliding motility and the mechanism used to generate the force required for gliding motility is also used to generate the force required for invasion (Sibley, 1995; Sibley, 2004; Wetzel *et al.*, 2003). Gliding motility actually refers to a combination of three distinct forms of motility produced by the parasite (Hakansson *et al.*, 1999). The first, circular gliding, refers to a form of motility in which the parasite lies on its side and moves in relatively slow, counterclockwise circles. In the second form, upright twirling, the posterior end of the parasite remains in contact with the substrate and the parasite twirls clockwise like a spinning top. While undergoing the third form of motility, helical rotation, the parasite covers the greatest distance. During helical gliding, the parasite turns in a clockwise manner as it does while twirling, but while lying on its side as in circular gliding. Because of the arc shape of the parasite, it must flip over when it reaches the end of its long axis. It then begins to glide again. In effect, the parasite moves in a straight line for a short period of time and then changes directions slightly after flipping, where it moves in a straight line until once again reaching the end of its long axis and flipping (Hakansson *et al.*, 1999).

The machinery used for gliding motility has been appropriately named the glideosome. The first glideosome component, a type XIV myosin (TgMyoA), is anchored into the outer membrane of the IMC through interactions with two membrane-bound proteins, glideosome-associated proteins 45 and 50 (TgGAP45 and TgGAP50) (Gaskins *et al.*, 2004). Regulated by a myosin light chain that contains an apicomplexan-specific domain (TgMLC), TgMyoA walks along the short actin filaments located directly under the plasma membrane towards the parasite's apical end (Baum *et al.*, 2006; Keeley and Soldati, 2004; Opitz and Soldati, 2002). The polymerization of these actin filaments is known to be tightly regulated and crucial for successful motility since agents that alter actin polymerization disrupt parasite motility (Wetzel *et al.*, 2003).

Bound to these actin filaments are tetramers of the glycolytic enzyme aldolase. Serving a surprising function, aldolase links F-actin to the transmembrane adhesive protein, microneme protein 2 (TgMIC-2). TgMIC-2 interacts with the surface of a host cell or other substrate and is important for providing traction as the parasite moves or invades a cell (Buscaglia *et al.*, 2003; Jewett and Sibley, 2003; Soldati *et al.*, 2001).

Because myosin A is anchored in the stationary IMC, the effect of its walking along the short actin filaments is that TgMIC-2 and TgAldolase are translocated towards the back of the parasite where they must eventually be cleaved or "capped" in order for the parasite to continue along its forward trajectory (Keeley and Soldati, 2004). As mentioned previously, this mechanism is also used to generate the mechanical force required for invasion of host cells which will now be discussed.

Invasion

Invasion requires the coordination of a number of tightly regulated events, and in contrast to many other forms of microbial invasion, is parasite-driven rather than host cell-driven. After moving across a potential host cell, a parasite can be seen prodding the cell via extending and retracting its conoid as if testing some aspect of it in order to determine whether the cell would provide a comfortable environment in which to reside (Carruthers and Boothroyd, 2007). If invasion is to proceed, the parasite must form a more intimate attachment with the cell before invading it. The nature of this intimate attachment is not understood, but the transmembrane microneme protein TgAMA-1 has been shown to be involved (Mital *et al.*, 2005). After intimate attachment, the parasite causes the host plasma membrane to invaginate and begin to surround it. The integrity of the host cell membrane is not compromised during this process (Suss-Toby *et al.*, 1996). At a point called the moving junction that forms between the host cell and the parasite, host-cell transmembrane proteins are excluded from the forming parasitophorous vacuole so that by the time the parasite in the parasitophorous vacuole pinches off from the host membrane, all host-cell transmembrane proteins have been excluded from the vacuole (Mordue *et al.*, 1999). This exclusion of host cell proteins from the vacuole is thought to allow the vacuole containing its resident parasite to avoid fusion with the host endocytic pathway and the demise of the parasite.

As mentioned previously, the micronemes, rhoptries, and dense granules play important roles in invasion and are discharged at different stages of invasion (Dubremetz and Schwartzman, 1993). Micronemes are known to be involved in motility and the

earlier steps of invasion, rhoptries are thought to be involved in invasion and development of the parasitophorous vacuole, and the dense granules are thought to be involved in maintenance of the parasitophorous vacuole (Dubremetz *et al.*, 1993).

Life within the host cell

  a) Association with host organelles

After the parasitophorous vacuole is formed, it moves from the periphery of the host cell to a location more proximal to the host cell nucleus. It is unknown why this occurs since the parasite is able to invade, replicate within, and egress from cytoblasts from which the nuclei have been removed by centrifugation (Romano *et al.*, 2008). The parasitophorous vacuolar membrane (PVM) becomes associated with the host mitochondria and endoplasmic reticulum (Sinai and Joiner, 2001). The association with the mitochondria is known to be mediated by the cytoplasmic tail of rhoptry protein 2 (ROP-2), a transmembrane protein that becomes embedded in the PVM after being secreted from the rhoptries (Sinai and Joiner, 2001). The nature of the association of the PVM with the endoplasmic reticulum has yet to be described, but it has been shown recently that it allows *T. gondii*-derived antigens access to the major histocompatibity complex class I pathway of the host cell (Goldszmid *et al.*, 2009). The PVM has also been shown to interact either directly or indirectly with the host centrosomes and causes reorganization of host microtubules such that they radiate, in effect, from the PVM (Walker *et al.*, 2008) . One could speculate that the parasite uses these microtubules to

traffic vesicles to and from the PVM. Supporting this theory, vesicles moving along host microtubules have been visualized entering the lumen of the parasitophorous vacuole (Coppens *et al.*, 2006). However, the contents of these vesicles and the mechanism by which they move have yet to be determined.

In addition to these associations, the parasitophorous vacuole becomes entangled in host cell intermediate filaments minutes after invasion (Halonen and Weidner, 1994; Halonen *et al.*, 1998). Neither the regulation nor the purpose of this entrenchment in the host cytoskeleton is understood.

b) Nutrient acquisition

While within the host cell, *T. gondii* acquires a number of nutrients and metabolites. Among those things that have been shown to be acquired from the host cell are tryptophan, arginine, cholesterol, glucose, and iron (Daubener *et al.*, 1999; Halonen and Weiss, 2000; Hershko, 1994; Joet *et al.*, 2002; Murray and Teitelbaum, 1992; Saito *et al.*, 2002). *T. gondii* likely scavenges many other substances from the host cell, and much work remains to be done on this subject. Interestingly, the parasitophorous vacuole has been shown to act like a molecular sieve that allows substances up to 1400 Da to freely diffuse into the intravacuolar space, whereas substances larger than 1400 Da are excluded from the vacuole (Schwab *et al.*, 1994). This finding suggests the presence of pores on the parasitophorous vacuole, although no candidate proteins serving this function have been identified to date.

c) Replication

        Replication of *T. gondii* tachyzoites within the parasitophorous vacuole occurs by a process called endodyogeny. The earliest event of endodyogeny that has been observed is the duplication of the centrosomes, represented by the appearance of two foci of TgCen2 (Hu, 2008). Other early events include the assembly of the IMC, which is thought to act as a scaffold upon which the daughter parasites are assembled (Hu *et al.*, 2002a), and the appearance of the subpellicular microtubules which polymerize from the apical to the posterior end of daughter parasites (Swedlow *et al.*, 2002).

        As endodyogeny proceeds, the mother's organelles are duplicated and/or partitioned between the daughter parasites, which continue to grow in size. Finally, the nucleus is divided between the daughter parasites and they overtake the mother, adopting her plasma membrane and leaving behind nothing but a small residual body (Hu *et al.*, 2002a).

Figure 3: Endodyogeny.
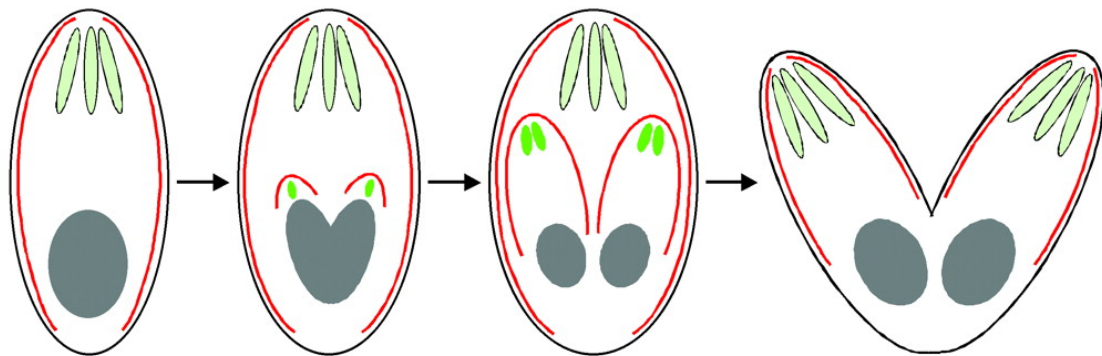
Figure 3: Endodyogeny is the process by which *T. gondii* tachyzoites replicate. Daughter parasites are assembled within the mother parasite using the IMC, shown in red, as a scaffold. The mother's organelles are replicated and distributed to the developing daughters. At the end of endodyogeny, the two daughters bud from the mother, acquire her plasma membrane, and leave behind a small residual body (Carey, 2004).

Although in most cases, tachyzoite replication results in the formation of two daughter cells, a small percentage of replication in *T. gondii* occurs through endopolygeny which results in the formation of three or even four daughter parasites. It has been shown that agents that disrupt spindle microtubules do not cause cessation of parasite replication, suggesting that parasite replication and DNA replication are not tightly linked in *T. gondii* (Hu et al., 2002a).

After undergoing many rounds of replication in the host cell and likely in response to a trigger that has not been identified, the daughter parasites actively egress from the host cells and are once again free to move along and invade fresh host cells.

Summary of General Introduction to *Toxoplasma gondii*

T. gondii is a fascinating organism that is capable of causing human disease and suffering and serves as an important and useful model organism for other apicomplexan parasites. During my graduate work in the Ward lab, I attempted to answer two major questions in order to learn more the biology of apicomplexan parasites. The first was to determine the function of a protein conserved among apicomplexan parasites but with no known homology to non-apicomplexan proteins, TgPhIL1. Our hope was that in gaining an understanding of the function of this protein, we would unveil a process or pathway

unique to the phylum Apicomplexa or learn more about a previously identified process. The second question I aimed to answer was how a small molecule (112762) causes enhancement of *T. gondii* invasion and motility. My hope for this project was that in identifying the target of this enhancer, we would learn more about invasion and motility. The unifying concept behind both of these projects is a desire to learn more about *T. gondii* biology since, as is evident from the review presented above, much remains to be discovered. Additionally, proteins involved in these processes unique to the phylum Apicomplexa are potential drug targets since the processes are not conserved within humans. At this point, a separate literature review will be presented for both projects in order to provide the reader the framework in which to review my work towards answering these questions.

INTRODUCTION TO TgPhIL1

Introduction

Many areas of apicomplexan biology are still poorly understood and several factors contribute to this lack of knowledge. For one thing, many aspects of apicomplexan biology are unique to the phylum and are even unique among members of the phylum. For example, *T. gondii* tachyzoites replicate intracellularly by a process called endodyogeny in which two daughter parasites are assembled within a mother parasite. This process is unique to apicomplexans, but not all apicomplexans replicate by this process. Adding an additional layer of complexity to this, not all stages of *T. gondii*

replicate by this process and it has been shown that even tachyzoites replicate by other means on occasion (Shaw *et al.*, 2001). In some cases, it has been observed that three or four, rather than two, parasites were assembled within the mother parasite in a process known as endopolygeny. Because of this uniqueness and diversity among members of the phylum, even biological processes that are assumed to occur in a predictable manner in other organisms must be dissected experimentally in apicomplexan parasites.

Additionally, many human diseases caused by apicomplexans affect people in the developing world where resources for studying these pathogens are scarce. In addition to a paucity of resources for studying them, apicomplexan parasites pose technical challenges to manipulation in the laboratory, as described previously.

Although dissecting the basic biological processes of these pathogens can be challenging, it is an important step in developing drugs aimed at fighting the diseases they cause. The more original the process, the less likely it is to be conserved with host processes, and the more promising it is as a potential drug target. The fact that apicomplexan parasites contain entire structures and organelles not present in the host offers the opportunity to study whole sets of proteins that may be unique to the phylum. In *T. gondii*, 40% of the annotated proteins are designated as proteins with no homology to non-apicomplexan proteins (Xia *et al.*, 2008), and it is likely that many of these unconserved proteins are to be found in organelles unique to the apicomplexans. Among these unique organelles are the conoid, the specialized secretory organelles (micronemes, rhoptries, and dense granules), the apicoplast, the acidocalcisomes, and the inner

membrane complex. The inner membrane complex is of particular interest because it is involved in a number of important parasite processes.

Functions of the Inner Membrane Complex

The inner membrane complex was briefly described earlier in this thesis and is composed of a sheet of vesicles, flattened, sutured together, and located directly underneath the parasite plasma membrane (Morrissette and Sibley, 2002). Thought to be derived from either the Golgi apparutus or the endoplasmic reticulum, the function of the IMC remains somewhat of an enigma.

One theory is that the IMC is involved in sequestering calcium and releasing it at the parasite periphery in order to generate local increases in calcium concentration. This theory arose from the finding that the closely related *Paramecium tetraurelia* contain structures analogous to the IMC, called the cortical alveoli, that perform such a function. That the cortical alveoli stores and releases calcium was first suggested after the finding was made that a purified fraction of alveoli from *P. tetraurelia* actively pumps calcium (Wright and van Houten, 1990). Using a technique called secondary ion mass spectrometry (SIMS) microscopy, it was then demonstrated that calcium is released at the periphery of the organism in a continuous zone thought to be the alveoli. Additionally, the concentration of calcium in the alveoli of was shown to be 3 to 5 mM which is similar to that in the sarcoplasmic reticulum (Mohamed *et al.*, 2002; Stelly *et al.*, 1995).

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These studies on the cortical alveoli of *P. tetraurelia* raised the possibility that the IMC in apicomplexans serves a similar function, which makes sense in light of the fact that sudden increases in calcium within the parasite are known to occur during *T. gondii* motility and invasion (Arrizabalaga and Boothroyd, 2004). Since the machinery required for motility and invasion are anchored in the IMC, it would be convenient for calcium to be stored in and released from the IMC itself. Also supportive of this theory is the fact that the IMC might be derived from the endoplasmic reticulum, which stores calcium in other systems. Arguing against this hypothesis, however, is the fact that small organelles called alcidocalcisomes have been shown to be the major calcium storage compartment in *T. gondii* (Moreno and Docampo, 2003).

So far, the most well defined role for the IMC is its role in tethering the glideosome at the periphery of the parasite. One member of the glideosome, GAP50, contains a transmembrane domain that likely traverses the outer membrane of the IMC (Gaskins *et al.*, 2004). Another member, GAP45 is both palmitoylated and myristoylated and these modifications are thought to regulate their association with the outer IMC membrane as well (Gaskins *et al.*, 2004). GAP50 is thought to be inserted into the membrane during translation, while GAP45, the myosin light chain, and myosinA are thought to associate with each other after translation in the cytosol and subsequently interact with GAP50 and the IMC at the parasite periphery (Gaskins *et al.*, 2004).

The glideosome is fixed in this membrane and does not move laterally within it. Until recently, it was not known if this was due to an association with the subpellicular microtubules underlying the inner (cytoplasmic) membrane of the IMC or with lipid rafts

in the membrane. Recent studies showed that this fixed localization is dependent on the

presence of cholesterol within the membrane, supportive of the theory that lipid rafts are

responsible for maintaining this localization (Johnson *et al.*, 2007). That the glideosome

is localized in close proximity to the actin filaments under the plasma membrane and

tethered into a membrane through which it cannot move is crucial for generating the

mechanical force required for both motility and invasion.

In addition to this role in positioning and anchoring the glideosome, the IMC is

thought to serve as a scaffold for developing daughter parasites during the process of

endodyogeny. Daughter parasites do not acquire a plasma membrane until they overtake

the mother parasite and usurp hers (Hu *et al.*, 2002a). However, in electron microscopy

performed on replicating tachyzoites, the inner membrane complex is present in daughter

parasites early in the replicative process. Since the true origin of and regulation of

development of the IMC is not understood, it is not possible to disrupt the IMC

pharmacologically and determine whether or not endodyogeny proceeds normally in its

absence. However, the timing of its appearance during development and the fact that a

scaffold is likely needed for daughter cell assembly argue in favor of this role for the

IMC in endodyogeny.

One mystery surrounding *T. gondii* biology and pertaining to the presence of the

IMC is the process of endocytosis. Because the IMC is present and continuous under the

plasma membrane, how or whether endocytosis is able to occur in apicomplexans is a

mystery. The identification of a discontinuity in the IMC which has been termed the

"micropore" may be the answer to this question. A solitary micropore is present in *T.*

gondii towards the apical end of the parasite and is an invagination of the plasma membrane through the inner membrane complex. Suggestive of the fact that endocytosis may occur here are images taken in which small, coated vesicles can be observed at the micropore. In this study, it was also shown that in tachyzoites, extracellular horseradish peroxidase (HRP) used as a tracer can be internalized into vesicles at the micropore (Nichols *et al.*, 1994). The micropore has also been visualized in bradyzoites and is much larger in bradyzoites than in tachyzoites, and the micropore offers a possible explanation to how endocytosis can occur, even in the presence of a continuous IMC (Nichols *et al.*, 1994).

In *Plasmodium gallinaceum* ookinetes, large pores 100 nm in diameter have been visualized in the IMC using cryofracture electron microscopy (Raibaud *et al.*, 2001). Although these pores have never been visualized in *T. gondii*, the possibility that they exist at some stage of the life-cycle remains.

Composition of the IMC, intramembranous particles, and subpellicular network

Many groups have performed studies directed at gaining a better understanding of the structure of the inner membrane complex in apicomplexans. Many of these studies have involved scanning electron microscopy or transmission electron microscopy (SEM or TEM) and they have yielded dazzling images.

Figure 4: The apicomplexan cytoskeleton.
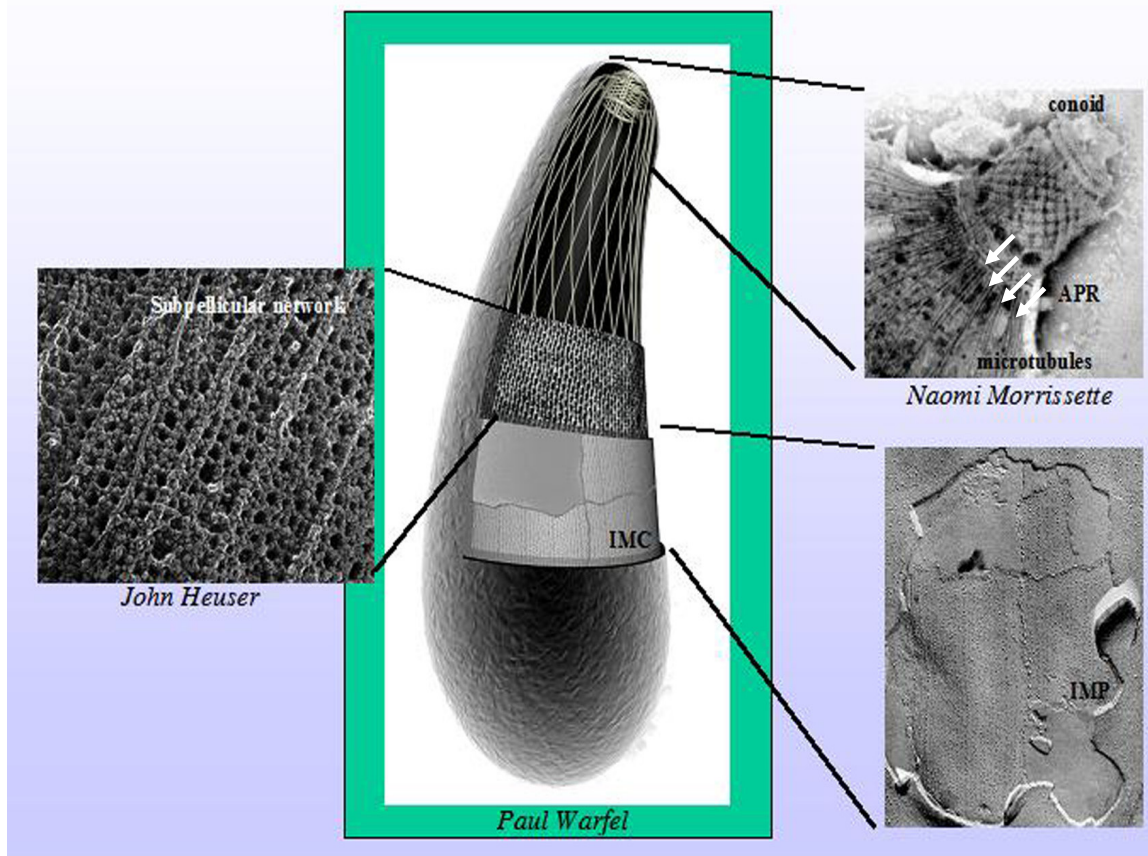
Figure 4: Under the plasma membrane of apicomplexan parasites lies the inner membrane complex, a structure made of flattened vessicles. The associated subpellicular network, intermembranous particles (IMP's), subpellicular microtubules, and apical polar ring (APR) can also be seen in this figure. Subpellicular microtubules are indicated by white arrows (http://www.sibleylab.wustl.edu/).

The appearance of the IMC differs among apicomplexans. For example, in *Plasmodium falciparum*, the IMC is made of one continuous plate that wraps around the parasite and is held together by one long line of sutures (Khater *et al.*, 2004). In contrast, in *T. gondii*, the IMC is made of a truncated cone at the apical end of the parasite called

an apical plate, upon which six rows of plates are attached and extend towards the

posterior end of the parasite. The apical plate starts just posterior to the outer polar ring

and is approximately 1 μm in length. Before reaching the posterior end of the parasite,

the plates end in a gentle spiral, which results in a smooth surface at the very posterior

end of the IMC. Each plate is actually a flattened vesicle, and the distance between the

two membranes of each vesicle is extremely small, on the order of 1.5 nm (de Melo and

de Souza, 1997; Nichols and Chiappino, 1987; Speer *et al.*, 1998).

Both outer faces of the IMC are covered with intramembranous particles

(IMP's), arranged in both double-particle and single-particle lines. The fact that there are

22 double-particle lines and 22 subpellicular microtubules is suggestive of an association

of the subpellicular microtubules with the double-particle lines (Morrissette *et al.*, 1997).

It is possible that the IMP's serve to tether the subpellicular microtubules to the IMC, but

the composition of the IMP's has not been determined and this has not been shown

experimentally. Additionally, it is possible the IMP's serve to stabilize the subpellicular

microtubules which are unusually stable even when isolated (Stokkermans *et al.*, 1996).

Either way, the fact that the IMP's extend to the very posterior end of the

parasite, far past the end of the subpellicular microtubules, suggests the presence of

another cytoskeletal structure upon which the IMP's are laid. This structure has been

identified and is called the subpellicular network (SPN). The SPN is a meshwork of

filaments lying under the IMC, which is thought to confer mechanical stability to the

parasite (Figure 4). The 8-10 nm filaments of the SPN are interwoven into an organized

meshwork and several of their constituent proteins been identified and designated IMC1,

IMC2, IMC3, and IMC4 (Mann and Beckers, 2001). Additionally, four other putative

members of the family have been identified in *T. gondii.* Based on sequence analysis,

putative IMC member have been identified in all apicomplexan parasites examined to

date. These proteins show weak homology to both mammalian intermediate filaments

and the articulin family of proteins in ciliates and dinoflagellates and are predicted to

have coiled-coil conformations (Mann *et al.*, 2002). Their homology with both of these

classes of proteins may be due to the fact that they contain a large number of valines and

prolines (Mann and Beckers, 2001; Mann *et al.*, 2002).

      Arguing in favor of the theory that the SPN confers mechanical stability to and

maintains the shape of apicomplexan parasites is the phenotype observed in *P. berghei*

IMC1 knockout parasites. *P. berghei* contains three isoforms of IMC1, designated

IMC1a, IMCb, and IMCc and expressed at different stages of the parasite life cycle.

When PbIMC1a is knocked out in *P. berghei* sporozoites, the shape of the parasites is

abnormal and they are 20-30% smaller than wild-type parasites. Additionally, they are

more sensitive to osmotic stress than wild-type parasites and their ability to invade host

cells is greatly reduced (Khater *et al.*, 2004). In a similar manner, in *PbIMCb* knockout

ookinetes, the shape of the parasites is altered, their tolerance to osmotic shock is

reduced, and their ability to infect host cells is greatly decreased (Tremp *et al.*, 2008).

      The IMC and associated structues (SPN and IMP's) are of great interest to the

apicomplexan community since they are involved in parasite division, motility, invasion,

morphology, and conferring mechanical stability to the parasites. Many mysteries

surrounding these structures still remain. For example, the identity of the IMP's remains

elusive. Additionally, the nature of association between these structures and the factors contributing to their unusual stability is not understood. Although the IMC itself is an important and fascinating structure, very few proteins of the IMC have been identified. In fact, the members of the glideosome known to associate directly with the outer membrane of the IMC (TgGAP45 and TgGAP50) are the only IMC proteins identified to date.

Why has it been so difficult to identify proteins of the IMC and associated cytoskeletal structures? Identification of both IMC and transmembrane plasma membrane proteins has been severely hindered by the presence of abundant GPI-anchored proteins on the parasite surface. These GPI-anchored proteins are so abundant and immunodominant that they mask other proteins and make their identification extremely difficult. Additionally, the IMC is such a stable structure that it has been difficult to make a purified fraction without stripping it of all associated proteins. Due to these challenges, identification of novel proteins of the IMC and plasma membrane has required the use of more sophisticated techniques.

The use of 5-[^{125}I]iodonapthalene-1-azide (INA) to identify novel proteins of the pellicle

5-[^{125}I]iodonapthalene-1-azide is a highly hydrophobic compound that readily partitions into lipid bilayers. When photoactivated, it causes proteins within close proximity to be labeled with ^{125}I. The basis for this labeling is that photoactivation of INA causes the azide portion of the molecule to convert to a reactive nitrene which then

is free to covalently bind proteins within the membrane, causing them to become radiolabeled. It should be noted that in addition to binding membrane-embedded proteins, INA has been shown to label highly hydrophobic proteins and proteins that contain hydrophobic stretches of amino acids. Additionally, it has been shown to preferentially bind tryptophan and cysteine (Raviv *et al.*, 1987).

INA can be photoactivated by two methods. In the first, called direct photoactivation, short wavelength (314 nm) ultraviolet light is used. Upon exposure to 314 nm ultraviolet light, all INA present within the cell will become activated, which will result in radiolabeling of proteins in all membranes of the cell. A more useful and specific method of INA activation is photosensitization. At wavelengths longer than 370 nm, only INA that has been photosensitized will be activated.

Photosensitization of INA takes place when INA is close enough in proximity to an excited fluorophore that it can collide with the fluorophore, transferring the energy from the fluorophore to INA. When this occurs, INA can become activated at longer wavelengths. The usefulness of this technique lies in using a sensitizing fluorophore that partitions selectively into the bilayer(s) of interest so that only proteins in that bilayer become labeled with [125]I (Figure 5), rather than labeling all membrane proteins of the cell (Raviv *et al.*, 1987).

Figure 5: Photosensitized 5-[^{125}I]iodonapthalene-1-azide (INA) labeling.
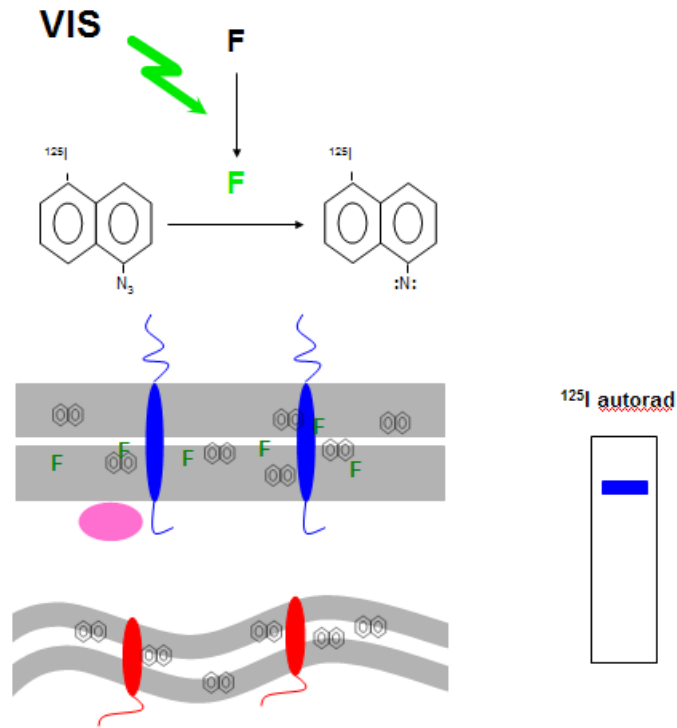
Figure 5: Photosensitized 5-[^{125}I]iodonapthalene-1-azide (INA) labeling involves the use of INA, a hydrophobic compound that partitions into lipid bilayers. In direct INA labeling, photoactivation of INA by UV light causes the compound to covalently bind adjacent proteins, resulting in their being radiolabeled with ^{125}I, which can be detected by audoradiography. In photosensitized INA labeling, longer wavelenths of UV light and a photosensitizing fluorophore are used and only INA molecules in close proximity to the fluorophore will be activated.

This technique was used by Dr. Stacey Gilk, a previous member of the Ward laboratory, in an attempt to identify proteins of the pellicle. These elegant experiments were designed in order to identify proteins other than the abundant GPI-anchored proteins that dominate the surface of the parasite. First, it was determined through the use of fluorescence imaging that C_{16}-eosin can be made to partition selectively within the

membranes of the *T. gondii* pellicle. Additionally, it had been shown previously that C_{16}-eosin could be used to photosensitize INA. It was then shown that when C_{16}-eosin was used as a photosensitizing fluorophore for INA, GPI-anchored proteins did not become labeled. Thus, this technique offered a solution to identifying novel pellicle proteins, despite the presence of highly abundant GPI-anchored proteins (Gilk *et al.*, 2006)

Using a combination of this technique followed by 2 dimensional gel analysis, a number of *T. gondii* proteins were shown to be labeled. When 2D gels containing proteins labeled by photosensitized INA-labeling were compared to those containing proteins labeled by direct INA-labeling, three major spots which showed enhanced labeling by photosensitization were evident. The first two were shown through mass spectrometry analysis to be TgGAP45 and TgGAP50, proteins known to be associated with membranes of the pellicle. The third protein was identified as a previously uncharacterized protein that has come to be known as *T. gondii* Photosensitized-INA-labeled protein-1, or TgPhIL1 (Gilk *et al.*, 2006). Studies on the function of TgPhIL1 will be the subject of Chapter 2 of this thesis. Using the full-length ORF, which was generated by RT-PCR, TgPhIL1 was predicted to be a small protein with a molecular mass of 19 kDa. A polyclonal antibody was generated to the protein and used to show that it runs at 22 kDa on a polyacrylamide gel under reducing conditions (Gilk *et al.*, 2006).

The localization of TgPhIL1 within the parasite, as determined by a number of methods including immunofluorescence with an anti-TgPhIL1 antibody and fluorescence imaging of a YFP-tagged version of the protein, is shown below.

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Figure 6: Localization of TgPhIL1

| DIC | α-IMC1 | PhIL1-GFP | merge |
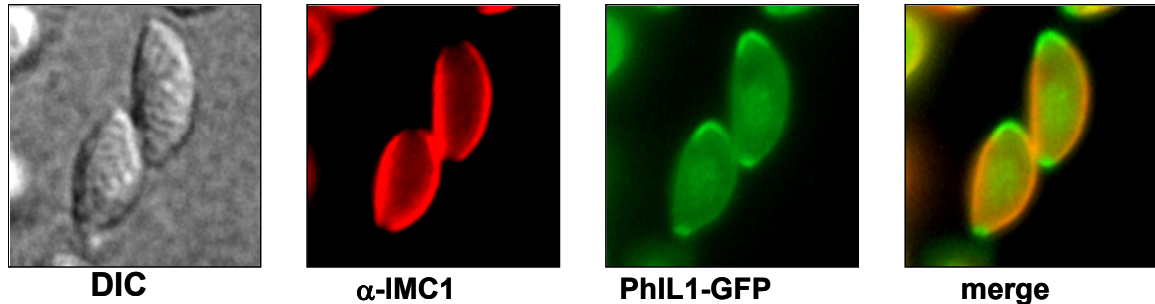
Figure 6: TgPhIL1 is localized to the periphery of the parasite. Additionally, a concentration at the posterior end of the parasite and the apical end of the parasite, just posterior to the conoid, can be observed. TgPhIL1 extends further apically than the marker TgIMC1 which has traditionally been thought to demarcate the inner membrane complex (Gilk *et al.*, 2006).

In addition to being distributed at the periphery of the parasite, a concentration of TgPhIL1 is present at the posterior and apical ends of the parasite. When performing immunofluorescence with anti-TgPhIL1, this apical concentration is more clearly seen after extraction with deoxycholate (DOC) than Triton X-100. When parasites with their apical end pointed towards the observer, the concentration at the apical end has a ring-like distribution. Immunoelectron microscopy with rabbit polyclonal anti-TgPhIL1 confirmed that the protein is indeed located in a ring at the apical end of the parasite, just posterior to the conoid (Gilk *et al.*, 2006).

It was not immediately evident whether TgPhIL1 was located in the plasma membrane or associated with the IMC and underlying cytoskeletal structures since the peripheral localization could be due to either. Upon treatment with *Clostridium septicum* alpha toxin, which causes the *T. gondii* plasma membrane to swell from the IMC, it was

confirmed that TgPhIL1 is likely associated with the IMC or underlying cytoskeletal structures since the staining pattern of TgPhIL1 retains the shape of the parasite in the presence of alpha toxin whereas markers of the plasma membrane do not (Gilk *et al.*, 2006). The precise localization of TgPhIL1 remains a mystery since markers previously thought to demarcate the IMC, such as TgIMC1, fail to completely colocalize with TgPhIL1. Specifically, TgPhIL1 is present both apical to and posterior to the ends of the IMC. This raises the question of whether TgPhIL1 is truly located within or associated with the IMC or whether this novel localization represents a new compartment or association with a previously undescribed structure. Additionally, although TgPhIL1 is thought to be membrane associated, how it associates with membranes remains a mystery since it has neither a transmembrane domain nor a predicted site of lipid modification. It does contain an amphipathic helix at its N-terminus. However, the C-terminal portion of the protein has been shown to be required for localization to the parasite periphery (Gilk *et al.*, 2006), arguing against a role for the amphipathic helix in membrane association.

In addition to the interesting distribution of TgPhIL1 within the parasite, this protein also has intriguing biochemical properties. TgPhIL1 is totally insoluble when parasites are extracted in RIPA buffer, TX-114, 1% DOC, or 1% Triton X-100 (Gilk *et al.*, 2006). In fact, TgPhIL1 was only soluble after boiling in 1% SDS. This extraction profile is similar to that of cytoskeletal proteins, specifically IMC1, TgTubulin, and TgGAP45, suggesting that TgPhIL1 is associated with the cytoskeleton. However, its labeling with INA suggests TgPhIL1 is membrane associated, and this combination of

properties begs the question whether TgPhIL1 might serve as a physical link between the *T. gondii* cytoskeleton and the membranes of the IMC (Gilk *et al.*, 2006).

Sequence analysis of TgPhIL1 shows that the protein is well conserved among members of the Phylum Apicomplexa. Consistent with the fact that homologues exist in other apicomplexans is the finding that in *P. falciparum* sporozoites, staining with anti-TgPhIL1 yields a pattern nearly identical to that in *T. gondii*: peripheral staining with an apical concentration (Gilk *et al.*, 2006).

Outside of the Phylum Apicomplexa, PBLAST analysis of the TgPhIL1 sequence does not identify proteins with significant sequence homology. However, using the Pfam webtool (http://pfam.sanger.ac.uk/), a domain of unknown function (DUF1889) is recognized. This domain is found in a set of hypothetical bacterial proteins including the uncharacterized protein YoaC in *Escherichia coli*. All proteins in this family are hypothetical and contain between 150 and 200 amino acids. At 165 amino acids, TgPhIL1 falls within this range. Given that these bacterial proteins are hypothetical and uncharacterized, no information is available regarding their function.

Since TgPhIL1 has an interesting localization within the parasite, is likely associated with the IMC and/or associated cytoskeletal structures, is highly conserved among members of the Phylum Apicomplexa, and has very little sequence homology outside of the phylum, we became interested in learning more about its function in *T. gondii* and the role it plays in the biology of apicomplexan parasites. With this goal in mind, *TgPhIL1* knockout parasites were generated and characterized. Additionally, experiments aimed at learning more about other proteins associated with TgPhIL1 were

performed in order to elucidate the function of this protein. These experiments are the

subject of Chapter 2 of this thesis and will be described in detail there.

INTRODUCTION TO IDENTIFYING THE TARGET OF INVASION ENHANCER 112762 IN TOXOPLASMA GONDII

Introduction

  Many approaches can be used to explore the biology of apicomplexan parasites.

One approach, which was described previously in this thesis, is to identify proteins that

are interesting for one reason or another and learn as much as possible about them and

other proteins with which they might interact. Undertaking this approach with a novel

protein, the possibility exists of identifying a novel biological process. Another approach

to exploring novel aspects of parasite biology is to perform a screen. In these screens,

one selects a poorly understood biological process, identifies conditions under which the

process is perturbed, and then identifies what precisely caused the process to be

disrupted. For example, one could set-up a chemical mutagenesis screen in *T. gondii*

where parasites are mutagenized and mutants that show altered replication are isolated.

Using one of several available methods, one could then identify the genes that are

mutated and the result would be the identification of a number of proteins involved in *T.

gondii* replication. In fact, this set of experiments has been conducted and has led to the

successful identification of a number of genes involved in replication (Gubbels *et al.*,

2008). The beauty of these screening approaches is that they are free of assumptions,

allowing a process to be explored fully and without the constraints of a previously-held

notion concerning which proteins may or may not be involved.

Small molecule screens

A number of methods are available for disrupting processes in *T. gondii.*

Among those that have been used successfully are chemical mutagenesis, insertional

mutagenesis, and small molecule inhibitors, and each approach offers advantages and

disadvantages. A major advantage of chemical mutagenesis is the ease with which

mutants can be generated and the possibility of generating point mutations rather than

mutations that completely abolish synthesis of the full-length protein, as happens with

insertional mutagenesis. On the other hand, it can be difficult to identify the mutations

responsible for the phenotypes caused by chemical mutagenesis. When insertional

mutagenesis is performed, identifying the disrupted locus is easier than in chemical

mutagenesis. However, the mutations caused by insertional mutagenesis are severe since

large insertions are made into genes.

An alternative to these genetic techniques is small molecule screening. After

identification of small molecules that disrupt a process of interest, the target of the small

molecules must be identified. This target validation step is often the most difficult, but

with the right chemical tools, can yield invaluable information. A major advantage to

small molecule screening in *T. gondii* is that it circumvents a problem with genetic

screening--that *T. gondii* is a haploid organism and disruption of an essential gene will

render the parasites non-viable and impossible to isolate. Techniques such as the

generation of temperature sensitive mutants have been used to circumvent this problem (Gubbels *et al.*, 2008), but this can be technically difficult. When working with small molecules, however, one can add the small molecule during the screen and look for a desired phenotype in its presence, but perform routine culturing of the parasites in its absence.

Within the field of parasitology are many examples of small molecule screens successfully used to identify proteins involved a number of processes. For example, in *P. falciparum*, it was recently shown using a small molecule screen followed by target validation that calcium-dependent protein kinase 1 (PfCDPK1) is required for motility during parasite egress and invasion (Kato *et al.*, 2008). Additionally, in *T. gondii* it was shown through the use of small molecules that cGMP-dependent protein kinase is essential for motility and host cell invasion (Donald *et al.*, 2006), and in *Trypanosoma brucei,* small molecule screening revealed that cysteine proteinases are involved in maintaining normal cell morphology and in cell division (Caffrey *et al.*, 2002).

Using small molecules to study *T. gondii* invasion

Because *T. gondii* and other apicomplexans are obligate intracellular parasites, disrupting the process of invasion is lethal to the parasite. This, coupled with the fact that the process of invasion is conserved across different members of the phylum, makes invasion an attractive process to study in the search for novel therapeutic agents for malaria, toxoplasmosis, cryptosporidiosis, and other diseases caused by these parasites. In addition to the advantages of small molecule screens listed above, another advantage

provided over genetic screens is that the identification of small molecules that inhibit

invasion provides the investigator with a group of compounds that could be used as lead

compounds upon which to develop antiparasitic drugs.

Equipped with this information and eager to identify small molecule inhibitors

of *T. gondii* invasion, our lab performed a small molecule screen. A collection of 12,160

small molecules (ChemBridge's DIVERSet™) was screened using a semi-quantitative

dual-fluorescence assay for an effect on *T. gondii* invasion (Carey *et al.*, 2004).

Compounds that reduced invasion to 20% control levels or increased invasion to 200%

control levels were scored as inhibitors or enhancers, respectively.

As a result of this screen, 65 compounds were shown to inhibit invasion. After

the invasion results were confirmed by a second round of invasion assays, the purity of

the compounds was analyzed, and cytotoxic compounds were excluded. 24 of the 65

compounds identified in the primary screen were shown to be *bona fide* inhibitors of *T.*

gondii invasion (Carey *et al.*, 2004). Unexpectedly, six invasion enhancers were also

identified. Since the process of invasion can be broken into discrete steps, secondary

assays were performed with these 30 compounds in order to determine which steps of

invasion were inhibited by each compound. Among these secondary assays were

constitutive microneme secretion, induced microneme secretion, conoid extension, and

motility (Carey *et al.*, 2004). The results of these assays are shown below. Of interest is

the fact that of all the compounds that inhibited invasion, all but three also inhibited

motility. This may reflect both the fact that invasion and motility require the same

machinery (Meissner et al., 2002b) or that cytoskeletal components, which are known to

be involved in invasion, are particularly prone to interact with and be affected by small

molecules (Peterson and Mitchison, 2002).

Figure 7: Results of secondary assays of small molecule inhibitors and enhancers of T. gondii invasion.

	Invasion	Motility	Secretion: constitutive	Secretion: Ca^{2+}-induced	Conoid
Inhibitors					
INH1		I	I		
INH2			I	I	
INH3		I	I	I	
INH4		I	I	I	
INH5		I		I	
INH6					I
INH7		I	I	I	
INH8		I	I		
INH9		I	I	I	
INH10		I			
INH11		I	E		
INH12		I	I		
INH13		I	I		
INH14		I	I	I	
INH15		I	I		
INH16		I	E		
INH17		I			
INH18		I	E		
INH19		I	I		
INH20		I	I	I	
INH21					
INH22		I	I	I	
INH23		I	I	I	
INH24		I	I	I	
Enhancers					
ENH1	E	E	E		
ENH2	E	E	E		
ENH3	E	E	E		
ENH4	E	E	E		
ENH5	E	E	E		
ENH6	E	E	E		

Figure 7: The steps of *T. gondii* invasion can be broken into separate components and the effect of each inhibitor and enhancer on these steps was analyzed. Interestingly, almost all compounds that affected invasion also affected motility (Carey, 2004).

In addition to secondary assays, the small molecules were screened across

several apicomplexan parasites in order to determine which small molecules affected

processes conserved among multiple organisms (Ward and Carey, unpublished data, see

Figure 8). The 30 compounds were also screened in the bacterium *Salmonella*

typhimurium in order to confirm that these effects are specific to invasion by

apicomplexan parasites and not acting in a way that would affect invasion in an unrelated

organism, suggesting that they act non-specifically or on the host cell.

Figure 8: Effect of invasion inhibitors and enhancers on other apicomplexan parasites.

	T. gondii invasion	T. gondii motility	P. knowlesi invasion	P. berghei gliding	P. berghei invasion	P. berghei LS development	E.tenella growth	C. parvum growth	S. typhimurium invasion
CONTROLS									
DMSO									
CytoD									
PRIORITY COMPOUNDS									
INH-E									
ENH-B									
INH-V									
INH-F									
INH-C									
INH-A									
INH-M									
INH-Q									
INH-N									
INH-K									

= Inhibitor
= No effect
= Enhanced
= Not determined
= Toxic

Figure 8: The 24 inhibitors and six enhancers of *T. gondii* invasion were assayed against a number of other apicomplexan parasites (Ward and Carey, unpublished data). Additionally, it was determined whether they affected *Salmonella typhimurium* invasion, suggesting that they affected invasion in a way that was not specific to the apicomplexans. None of the compounds affected *S. typhimurium* invasion. Several compounds affected invasion and/or motility of multiple parasites. Enhancer B is compound 112762 and identification of the target of this compound is the focus of Chapter 3 of this thesis (Carey, 2004).

Identification of 112762 as a lead compound

      As a result of these experiments, 112762 emerged as a lead compound worthy of

further investigation. In addition to having an effect on *T. gondii* invasion, motility, and

microneme secretion, it enhances *P. knowlesi* invasion and causes odd motility in *P.

berghei*, suggesting that it hits an aspect of the apicomplexan invasion machinery that is

conserved among these parasites. Additionally, based on its structure, it was believed

this compound could be modified in ways that would facilitate target identification

studies. Also, 112762 is particularly interesting because it acts as an enhancer and could

provide valuable insight into invasion and the signals regulating it. For these reasons, we

have attempted to identify the target of compound 112762. The structure of this

compound is shown below.

Figure 9: Structure of Enhancer 112762
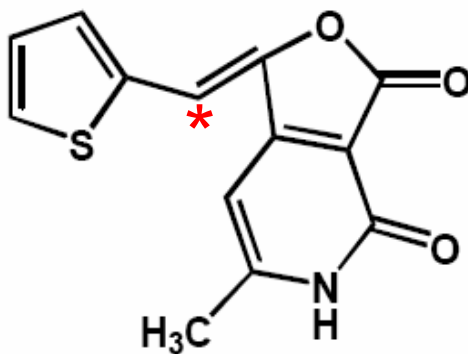
Figure 9: The structure of enhancer 112762. This compound can be modified in several ways and retain its ability to enhance invasion, making it a desirable compound for further study. The red star indicates a double bond which when changed to a single bond, renders the compound totally inactive.

Affinity chromatography

Rachel Morgan in Nick Westwood's laboratory at the University of St. Andrews synthesized a number of derivatives of 112762 (Morgan, 2006a). One derivative, in which the double bond indicated with a star in the figure above is changed to a single bond, has no effect of *T. gondii* invasion or motility, making it an ideal inactive derivative and providing a useful control for subsequent experiments. Rachel was also able to link a modified but still biologically active derivative of the compound to Affigel beads with the goal of performing affinity chromatography experiments in order to identify proteins with which 112762 interacts. The inactive derivative of 112762 was also linked to Affigel beads (Morgan, 2006a) and was used as a control for non-specific binding to the compound. These affinity chromatography experiments and the results they yielded are discussed in detail in Chapter 3 of this thesis.

Arginine methyltransferase screen

In addition to performing affinity chromatography experiments with enhancer 112762, regular searches of the literature were conducted in case new information about its activity became available. In one of these searches, a member of Nick Westwood's group found an article in which a compound almost identical to 112762, compound 112759, was shown to inhibit both yeast and recombinant human protein arginine methyltransferase 1 (PRMT1) (Cheng *et al.*, 2004). The two compounds are structurally identical except for substitution of a sulfur atom in 112762 by an oxygen atom in 112759 (see Figure 7, Chapter 3).

PRMTs, thought at one time to act only upon histones, are emerging as an important regulator of protein-protein interactions, transcription, translation, and intracellular signaling, acting on both nuclear and non-nuclear substrates (Bedford and Richard, 2005). The enzymatic reaction catalyzed by PRMTs involves the addition of a methyl group from S-adenosyl-methionine (SAM) to arginine. This reaction tends to occur in regions of proteins rich in arginines and glycines, called glycine- and arginine-rich (GAR) domains (Tang *et al.*, 1998), although these are difficult to identify and their positive predictive value is fairly low due to the non-specific nature of the sequences. Because the modification does not change the charge of the protein, it is thought to have a more subtle effect than modifications such as phosphorylation or acetylation. It is believed that the additive effect of several of these modifications on hydrophobicity alters protein-protein interactions. Although many arginine methyltransferases have been identified in a number of organisms, PRMT1 is thought to be responsible for the majority of PRMT activity in most cells. Additionally, a PRMT1 homologue has been identified in all organisms examined to date, highlighting its widespread importance (Bedford and Richard, 2005; Fackelmayer, 2005; Pahlich *et al.*, 2006).

In *T. gondii*, five putative PRMTs have been identified by sequence analysis and protein arginine methyltransferase activity has been shown for two of them (Saksouk *et al.*, 2005). All five are expressed in *T. gondii.* These two enzymes, TgPRMT1 and TgCARM1 have been shown to act upon histones proteins H4 and H3, respectively, and this activity has been correlated with transcription of bradyzoite-specific genes and stage

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conversion between tachyzoites and bradyzoites (Saksouk *et al.*, 2005). It was not known

if these or other arginine methyltransferases acted on other proteins in *T. gondii.*.

In the protozoan parasite *Trypanosoma brucei*, five PRMTs have been

identified. Interestingly, the organism contains no histone proteins and non-histone

targets have been identified for several of these enzymes. Among those targets identified

to date are a number of RNA-binding proteins and mitochondrial proteins (Pelletier *et al.*,

2001). In other systems, PRMTs have been shown to be involved in signal transduction,

DNA repair, transcriptional regulation, protein-protein interactions, and protein

trafficking (Pahlich *et al.*, 2006).

Equipped with a variety of molecular tools, we sought to explore the hypothesis

that enhancer 112762 exerts its effects on invasion through an effect on arginine

methylation. In parallel, affinity chromatography experiments were performed with this

compound in order to confirm binding of 112762 to TgPRMT1.

CHAPTER 2: TARGETED DISRUPTION OF TgPHIL1 IN TOXOPLASMA GONDII RESULTS IN ALTERED PARASITE MORPHOLOGY AND A FITNESS DEFECT

Targeted disruption of *TgPhIL1* in *Toxoplasma gondii* results in altered parasite morphology and a fitness defect

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ABSTRACT

The inner membrane complex (IMC), a series of flattened vesicles at the periphery of apicomplexan parasites, is throught to be important for parasite shape, motility and replication. However, very few of the proteins of the IMC that function in these processes have been identified in any apicomplexan. TgPhIL1, a *T. gondii* protein that was previously identified through photosensitized labeling (Gilk *et al.*, 2006), associates with the IMC or underlying cytoskeleton and is concentrated at the apical end of the parasite. Homologs of TgPhIL1 are found in all apicomplexans, but the function of this conserved family of proteins is unknown. To address this question, we generated a *T. gondii* parasite line in which the single copy of *TgPhIL1* was disrupted by homologous recombination. The *TgPhIL1* knockout parasites have a distinctly different shape than wild-type parasites, and normal shape is restored by expression of the wild-type allele in the knockout background. While the knockout parasites show no defect in motility, they are outcompeted in culture by parasites expressing functional *TgPhIL1*. Furthermore, infection with the TgPhIL1 knockout parasites produced a reduced parasite load in the spleen and liver in a mouse model of infection, compared to wild-type parasites. These findings demonstrate a role for TgPhIL1 in the morphology, growth and fitness of *T. gondii* tachyzoites.

INTRODUCTION

The Phylum Apicomplexa contains a number of medically important parasites including *Cryptosporidium,* which causes diarrheal illness in children and immunocompromised patients, *Plasmodium,* which is the causative agent of malaria, and *Toxoplasma gondii,* which causes disease in immunocompromised people and the developing fetus. Other apicomplexan organisms, including *Neospora* and *Eimeria,* cause disease in livestock (Das, 1996; Wallach, 1997). Despite the enormous amount of morbidity, mortality and economic loss caused by these pathogens, a paucity of information exists regarding many aspects of their basic biology.

Apicomplexan parasites contain a number of structures and organelles unique to the phylum. Underlying the plasma membrane in *Toxoplasma* and other apicomplexan parasites are a number of stable cytoskeletal structures (Mann and Beckers, 2001). Among these are the conoid, which is a cone-shaped structure composed of a novel polymeric form of alpha-tubulin (Hu *et al.*, 2002b), polar rings located on either side of the conoid (Hu *et al.*, 2002b), 22 microtubules radiating from the lower polar ring and extending posteriorly two-thirds of the way down the parasite (Nichols and Chiappino, 1987), and the inner membrane complex (IMC), a series of flattened vesicles located directly underneath the plasma membrane (de Melo and de Souza, 1997; Hu *et al.*, 2002a). The plasma membrane and two membranes of the IMC are referred to as the pellicle. Under the IMC is the subpellicular network, a grid of filamentous proteins including TgIMC1, TgIMC2, and TgIMC3, which show weak homology to mammalian intermediate filament proteins (Gubbels *et al.*, 2004; Hu, 2006; Mann and Beckers, 2001; Mann *et al.*, 2002).

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The IMC forms early in endodyogeny, the process by which *T. gondii* tachyzoites replicate, and is thought to provide both a scaffold upon which daughter parasites are assembled during this process, as well mechanical stability to the mature tachyzoites (Gubbels et al., 2004; Hu, 2006; Mann et al., 2002). Additionally, some components of the glideosome, a complex of proteins that provides the mechanical force for motility and host cell invasion, are anchored in the IMC and their localization between the outer membrane of the IMC and the plasma membrane is critical (Baum *et al.*, 2006; Keeley and Soldati, 2004). Despite the important processes in which proteins of the IMC are likely involved, very few proteins of the IMC have been identified. Additionally, the mechanism(s) by which the IMC, subpellicular microtubules, and subpellicular network associate with each other and are organized within the pellicle have yet to be elucidated.

TgPhIL1 (*Ph*otosensitized *I*NA-*L*abeled protein *1*) was identified through photosensitized labeling with 5-[^{125}I] iodonapthaline-1-azide (INA) and localized to the parasite periphery, concentrated at the apical end directly posterior to the conoid (Gilk *et al.*, 2006). When parasites are treated with *Clostridium septicum* alpha-toxin, which causes the plasma membrane to swell away from the IMC, TgPhIL1 remains associated with the IMC and/or associated cytoskeletal structures (Gilk *et al.*, 2006). TgPhIL1 has an interesting biochemical extraction profile, in that it is highly insoluble and extracts much like a cytoskeletal protein; at the same time, labeling with INA suggests a membrane association. TgPhIL1 is highly conserved among members of this phylum

Apicomplexa but shows very little homology to proteins outside of this Phylum, making elucidation of its function based on sequence homology difficult (Gilk *et al.*, 2006).

In order to learn more about the function of TgPhIL1, *TgPhIL1* knockout parasites were generated. Phenotypic analysis of the knockout parasites showed that TgPhIL1 is involved in maintaining the shape of *T. gondii* tachyzoites and that parasites lacking TgPhIL1 are outgrown by wild-type parasites in culture and show a fitness defect in a mouse model of infection.

MATERIALS AND METHODS

Culture of parasites

Toxoplasma gondii RH strain was maintained by serial passage in confluent lines of primary human foreskin fibroblast (HFF) cells in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% heat inactivated fetal bovine serum (FBS), 10 units/ml penicillin G sodium, 10 µg/ml streptomycin sulfate, and 10 mM HEPES buffer as previously described (Roos *et al.*, 1994).

Generation of *TgPhIL1* knockout

All PCR reactions were performed with Pfx polymerase (Invitrogen) unless otherwise indicated. All primers were synthesized by Sigma Genosys and restriction enzymes were purchased from New England BioLabs or Invitrogen.

In order to generate the *TgPhIL1* knockout, PCR was performed on the genomic locus containing the *TgPhIL1* open reading frame (ORF), generating a 6.5 kb fragment (2.9 kb upstream *TgPhIL1* flanking region, 1.3 kb *TgPhIL1* ORF, 3.1 kb downstream *TgPhIL1* flanking region) with the primers PhIL15'KOfor (5'GCTGAGGAGGGGAAGAAGATCGAG3') and PhIL13'KOrevXba1 (5'TGCTCTAGATATCCGCAAATGCTCGCTCGCC3'). The fragment was cloned into pCR-Blunt TOPO (Invitrogen) and inverse PCR was performed with primers KOinvfor (5'AGGAGTGTTCATGTTGTTTGCTG3') and KOinvrev (5'TGAATTCGTCTAATCCAGAGTCTGTC3') to remove the *TgPhIL1* ORF. The ble cassette was digested from pGRA1/ble (Messina *et al.*, 1995) with HindIII and XbaI and the ends were filled-in with Klenow, followed by blunt ligation to the inverse PCR product in order to generate the knockout construct Δ*PhIL1*/ble. The plasmid was linearized with Not1 and transfected into RH parasites which were added to confluent HFF cells and selected with phleomycin as described previously (Messina *et al.*, 1995). The population was screened by immunofluorescence with anti-TgPhIL1 and individual clones were isolated.

Cloning TgPhIL1 under the endogenous promoter for complementation

PCR was performed on *T. gondii* genomic DNA using the primers PhIL1 Endo For XbaI (5'GGGG<u>TCTAGA</u>TGAAAGACTGGAGCATTTCG-3'), which sits 2 kb upstream of the TgPhIL1 ORF, and PhIL1 Endo Rev BglII (5'GGGG<u>AGATCT</u>TCACCGAGAGAAGTCGAGTG-3') containing the 3' end of the

TgPhIL1 ORF, followed by a stop codon. After digestion with XbaI and BglII, the product was ligated into pTubIMC-YFP which was also digested with XbaI and BglII in order to remove the sequence coding for TgIMC1, generating pTgPhIL1Endo/Cat. This places *TgPhIL1* under the control of its endogenous promoter since digestion of the vector with BglII and XbaI removes the tubulin promoter from pTubIMC-YFP. A stop codon at the end of the TgPhIL1 ORF ensures that translation of YFP does not occur.

Western blot analysis

Western blot analysis of parental and *TgPhIL1* knockout lysates was performed on nitrocellulose membranes with rabbit polyclonal anti-TgActin antibody provided by David Sibley (Sibley, 1997) and rabbit polyclonal anti-TgPhIL1 (Gilk, 2005) as described previously, at dilutions of 1:10,000 and 1:5,000 respectively. Following incubation with primary antibody, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG. HRP activity was detected by enhanced chemiluminescence (Amersham Life Sciences).

Immunofluorescence

Immunofluorescence with anti-TgPhIL1 polyclonal rabbit antibody was performed as follows: Parasites were fixed in 4% paraformaldehyde for 10 minutes at 23°C before permeabilization with 10 mM deoxycholic acid in PBS for 30 minutes at 23°C. Fixed and permeabilized parasites were incubated for 15 minutes with anti-TgPhIL1 antibody diluted 1:500 in PBS with 2% BSA. Parasites processed for tubulin immunofluorescence were also extracted with 10 mM deoxycholic acid after fixation with 4% formaldehyde and mouse monoclonal anti-

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alpha-tubulin (Sigma) was used at a 1:400 dilution. Immunofluorescence with anti-TgMORN1, a generous gift from Dr. Marc-Jan Gubbels, and anti-TgIMC1 ((Mann *et al.*, 2002) was performed after fixation in 100% MeOH on ice for 5 minutes. The antibodies were diluted 1:500 and 1:100, respectively. All fixed and permeabilized parasites were then incubated with a 1:1000 dilution of Alexa Fluor-conjugated secondary antibody (Molecular Probes).

Morphometric analysis

Parental RH and *TgPhIL1* knockout parasites in Hanks solution buffered with 10 mM HEPES were fixed to glass coverslips using BD-CellTak (BD Biosciences) and visualized using differential interference contrast (DIC) microscopy. Measurements were taken using ImageJ (url: http://rsbweb.nih.gov/ij/). Results shown are the average of 100 measurements, plus or minus standard error.

Electron microscopy

Pellets of wild-type and *TgPhIL1* knockout parasites were fixed in Karnovsky's fixative for 60 minutes at 4ºC, rinsed 3 times for 4 minutes in Millonig's buffer, and embedded in 2% SeaPrep Agarose for 15 minutes at 4ºC. Cross-linking in Karnovsky's fixative for 15 minutes at 4ºC was performed, followed by washing in the samples in Millonig's buffer and trimming the agarose blocks. 1% OsO_4 was added and the blocks were stored in Millonig's buffer overnight. The following day, the blocks were dehydrated in 35%, 50%, 70%, 85%, 95%, and 100% ethanol before being dehydrated further in propylene oxide and infiltrated with and embedded in Spurr's resin, which was

allowed to polymerize for 12 hours. Ultrathin sections were placed on nickel grids and

contrasted with 2% (wt/vol) uranyl acetate in 50% (vol/vol) EtOH for 6 min and lead

citrate for 4 min. before being analyzed on a JEOL 1210 transmission electron

microscope (Peabody, MA)

Growth competition

Freshly-lysed wild-type, *TgPhIL1* knockout, and *TgPhIL1* complemented clones

(*TgPhIL1;*C5 and *TgPhIL1;*C7) were filtered through a 3-µm Nucleopore syringe filter

(Whatman) and 5 X 10^5 parasites of each strain were added to a 25 cm^2 flask of confluent

HFF cells for a total of 1 X 10^6 parasites per experiment in the combinations indicated

(RH and *TgPhIL1* knockout, *TgPhIL1;*C5 and *TgPhIL1* knockout, *TgPhIL1;*C7 and

TgPhIL1 knockout). Immediately following the lysis of the monolayers (approximately

36 hours post-infection), the parasites were added onto a new monolayer. After every

third passage, immunofluorescence was performed with rabbit polyclonal anti-TgPhIL1

and 500 parasites were scored as either negative or positive for TgPhIL1. The medium

was replaced 12 hours after incubation with a new monolayer to ensure that parasites that

failed to invade were not counted in the analysis. Results shown are the average of the

three experiments, plus or minus standard error.

In vivo **analysis**

Female 6- to 8-wk-old C57BL/6 mice were obtained from Jackson laboratories and

maintained under specific-pathogen-free conditions in accordance with institutional

guidelines. 5 mice per group were infected intraperitoneally with 200μl of PBS containing 10^4 RH or *TgPhIL1* knockout tachyzoites. DNA from spleen and liver sections taken from mice sacrificed on Day 7 post infection was isolated using the High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's instructions. Expression of the *Toxoplasma gondii* gene ToxoB1 was determined with the primers F (5'TCCCCTCTGCTGGCGAAAAGT-3') and R (5'AGCGTTCGTGGTCAACTATCGATTG-3') (Roos *et al.*, 1994) using the AB7500 fast real-time PCR thermal cycler and Power SYBR green reagents (Applied Biosystems). Qiagen-supplied Quantitect primers for mouse housekeeping gene β-actin were used as a normalization control. An unpaired student's t-test was used to determine significant differences, and p-values less than 0.05 were considered significant.

Laser Scanning Cytometry (LSC)-based invasion assay

LCS-based invasion assays were performed with wild-type RH, *TgPhIL1* knockout, and *TgPhIL1* KO;C5 following methods described previously (Mital *et al.*, 2006). Parasites were filtered and washed in Hanks buffer by centrifugation before being resuspended in Hanks buffer containing 1% FBS at a concentration of 1×10^6 parasites/ml. 3×10^6 parasites were added to confluent monolayers of HFF cells grown on 25 mm^2 round glass coverslips and invasion was allowed to proceed for 1 hour at 37°C, followed by washing the coverslips 3 times in PBS. The coverslips were then fixed with 3.1% paraformaldehyde/0.06% gluteraldehyde in PBS for 30 minutes at 23°C, washed three times with PBS, and blocked for 10 minutes with 2% BSA/PBS. Samples

were incubated with 0.5 µg/ml mouse monoclonal anti-TgSAG1 (Argene) in 0.5% BSA/PBS for 15 minutes, washed three times in PBS, and incubated for 15 minutes with goat anti-mouse R-phycoerythrin (DAKO, Carpenteria, CA) at a 1:400 dilution in 0.5% BSA/PBS. Permeabilization was performed with 0.25% Triton X-100 in PBS for 30 minutes before the samples were washed 3 times, incubated once again with 0.5 µg/ml mouse monoclonal anti-TgSAG1 in 0.5% BSA/PBS for 15 minutes, washed 3 times in PBS, and incubated for 15 minutes with goat Alexa 647-conjugated anti-mouse IgG (Molecular Probes). After 3 final washes, the coverslips were inverted and mounted on slides. Samples were analyzed on a CompuCyte Laser Scanning Cytometer (CompuCyte, Cambridge) and data acquisition was performed using Wincyte 3.4 Software (CompuCyte).

Trail assay

Freshly lysed and filtered parasites were harvested, centrifuged at 10,000 x g for four minutes, and resuspended in Hanks buffer at a concentration of 1.5 X 10^7 parasites/ml. They were then added to Cell-Tak-coated 8-well chambered coverglasses (Nunc) and incubated at 37°C for 45 minutes. After aspiration of the parasites from the wells, fixation was performed with 2.5% paraformaldehyde for 15 minutes at 23°C, followed by blocking with 2% BSA/PBS for 15 minutes. Trails were then stained with 5 µg/ml mouse monoclonal anti-TgSAG1 in 0.5% BSA/PBS for 15 minutes, washed three times in PBS, and incubated for 15 minutes with Alexa 488-conjugated goat anti-mouse

antibody (Molecular Probes) at a dilution of 1:100, followed by washing and visualization by fluorescence microscopy.

Conoid extension assay

Conoid extension assay was performed as described previously (Mondragon and Frixione, 1996). Freshly-lysed parasites were treated with DMSO or 1 μM ionomycin (Sigma) for 5 minutes in Hanks buffer before being fixed for 1 hour at 23°C in 1% glutaraldehyde, smeared onto a glass slide, and air-dried. 100 conoids for each sample were scored by phase contrast microscopy as either extended or retracted.

RESULTS

Generation of a *TgPhIL1* knockout parasite

To generate a *TgPhIL1* knockout parasite line, the *TgPhIL1* gene was targeted for disruption by a construct encoding *ble,* which confers resistance to the drug phleomycin (Messina *et al.*, 1995), flanked by 5' and 3' sequences from the *TgPhIL1* genomic locus (Figure 1A). Following transfection and three rounds of phleomycin selection, immunofluorescence with an α-TgPhIL1 antibody was performed on a population of extracellular parasites (Figure 1B). 6% of the population was negative for TgPhIL1 staining. Individual parasites in this population were cloned by limiting dilution and two clones that were negative for TgPhIL1 by immunofluorescence were isolated, expanded and analyzed by western blot with the α-TgPhIL1 antibody (Figure

1C), confirming the lack of TgPhIL1 expression. One clone was used for all subsequent experiments.

Figure 1: Generation of a TgPhIL1 knockout.
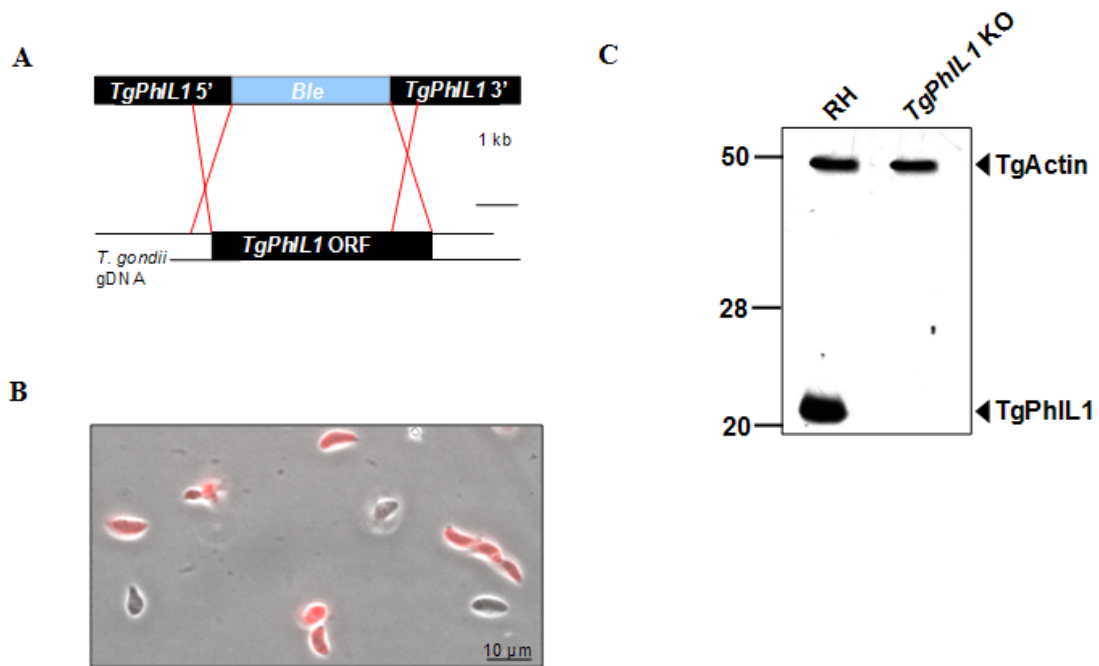
Figure 1: (A) A knockout construct containing a *ble* cassette, flanked by 3 kb of upstream and downstream noncoding sequence from the *TgPhIL1* genomic locus was transfected into wild-type RH parasites, where it was expected to integrate into the *TgPhIL1* locus by homologous recombination. (B) After three rounds of selection with phleomycin, immunofluorescence with an anti-TgPhIL1 antiserum revealed that approximately 6% of the parasites are negative for TgPhIL1, suggesting the ORF was successfully disrupted. A combined phase contrast and immunofluorescence image is shown. Scale bar = 10 μm. (C) Western blotting of individual, cloned parasites confirms the absence of TgPhIL1 expression. The blot was also probed with anti-actin as a loading control. Numbers on the left indicate molecular mass in kDa.

Morphology and complementation of the *TgPhIL1* knockout parasite

Immediately upon generating the *TgPhIL1* knockout parasite line, it was

obvious by light microscopy that the shape of the parasites was altered. *TgPhIL1*

knockout parasites appear rounder and fatter than wild-type parasites (Figures 1B, 2). In

order to quantify this difference, parasites were measured along their long and short axes

at their widest point; these data demonstrate that *TgPhIL1* knockout parasites are indeed

shorter and wider than wild-type parasites (see Figure 2).

In order to confirm that the absence of TgPhIL1 was responsible for this

morphological change, *TgPhIL1* was reintroduced into the *TgPhIL1* knockout parasites in

a construct containing 2kb of *TgPhIL1* upstream genomic sequence, the *TgPhIL1* ORF

and a chloramphenicol acetyltransferase cassette for selection. The construct was

transfected into *TgPhIL1* knockout parasites which then underwent three rounds of

selection with chloramphenicol. Immunofluorescence with α-TgPhIL1 antibody showed

that stable transfectants had been generated and that the expressed TgPhIL1 localizes

properly in *TgPhIL1* knockout parasites (data not shown). Five clones positive for

TgPhIL1 were isolated and analyzed by Western blot analysis with α-TgPhIL1. All five

complemented clones showed similar levels of TgPhIL1 expression, which was

comparable to that of wild-type parasites (data not shown). Two clones, *TgPhIL1* KO;C5

and *TgPhIL1* KO;C7, were arbitrarily chosen for subsequent experiments.

By light microscopy, the shape of the complemented clones appeared to be

similar to that of wild-type parasites (Figure 2). Morphometric analysis of these clones

showed that indeed, complementation of *TgPhIL1* had restored the length and width of

the mutant to that of wild-type parasites (RH=6.52± 0.09μm X 2.36± 0.04μm, *TgPhIL1*

KO=5.38±0.06μm X 2.80±0.04μm , *TgPhIL1* KO;C5=6.37±0.19μm X 2.28±0.11μm)

(Figure 2).

Figure 2: Altered shape and size of *TgPhIL1* knockout parasites.

	RH	*TgPhIL1* KO	*TgPhIL1* KO;C5
length	**6.52±0.09**	**5.38±0.06**	**6.37±0.19**
width	**2.36±0.04**	**2.80±0.04**	**2.28±0.11**
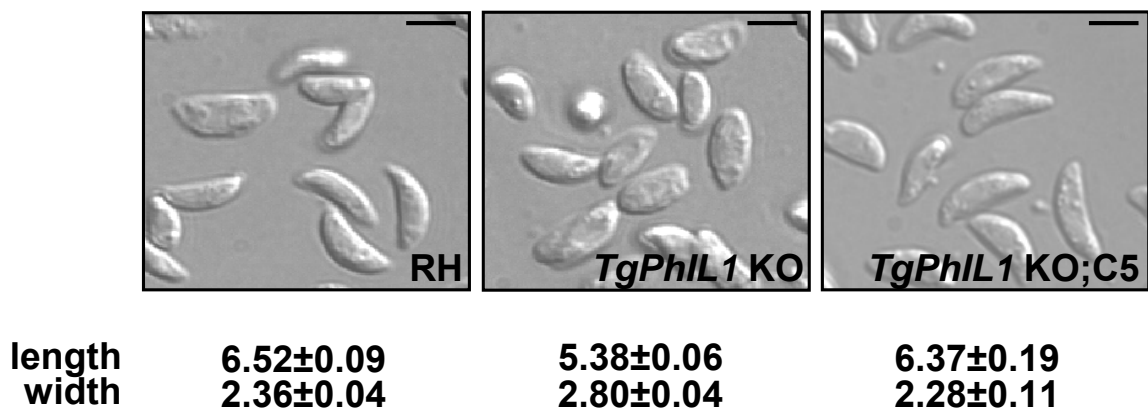
Figure 2: *TgPhIL1* knockout parasites show an alteration in shape compared to wild-type parasites. By DIC microscopy (shown), these parasites appear wider and rounder than wild-type parasites. Numbers indicate the average length and width of 100 parasites, in μm, +/- standard error. Scale bars = 5 μm.

Ultrastructure of the *TgPhIL1* knockout parasites

Given their striking difference in shape, we examined the *TgPhIL1* knockout

parasites by transmission electron microscopy, to see if there were any obvious changes

in their ultrastucture, particularly in the IMC and/or region of the pellicle just posterior to

the conoid, where TgPhIL1 is known to be concentrated. No morphological differences in

the IMC, the spacing between the IMC and the plasma membrane, the conoid or the

subpellicular microtubules were evident (Figure 3).

Figure 3: Electron microscopy of *TgPhIL1* knockout parasites.
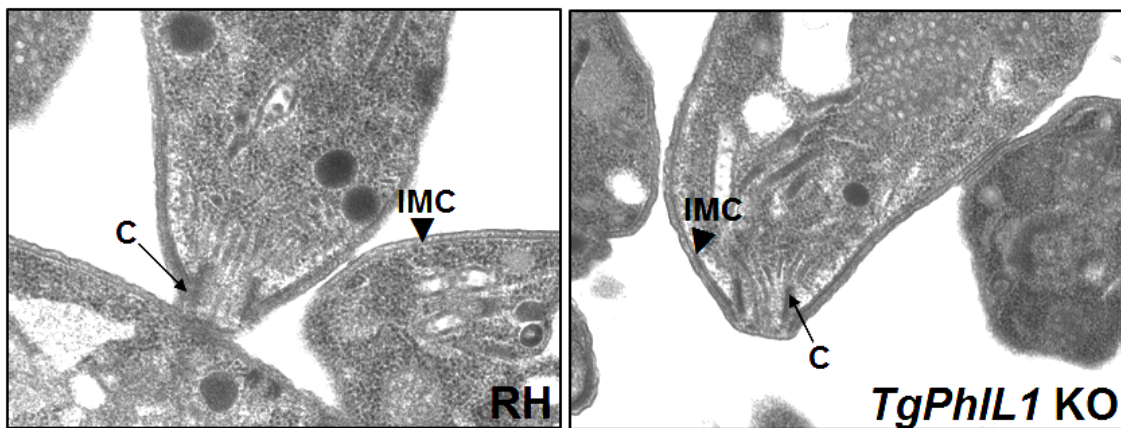
Figure 3: *TgPhIL1* knockout parasites display normal ultrastructure. No morphological defect is seen in the conoid (C) or IMC, and the IMC is spaced appropriately from the plasma membrane.

Conoid extension, motility and invasion of the *TgPhIL1* KO parasites

Since TgPhIL1 forms a ring at the anterior end of the parasite just posterior to

the conoid, its absence might have an effect on the parasite's ability to extend its conoid.

In order to test this hypothesis, wild-type and *TgPhIL1* KO parasites were treated with

1μM ionomycin, which induces conoid extension (Mondragon and Frixione, 1996). In

response to ionomycin treatment, 86±6.3% of wild-type parasites extended their conoid whereas only 14±0.35% of the control parasites (treated with an equivalent volume of DMSO) had their conoids extended. Similarly, in *TgPhIL1* KO parasites, 83±1.8% had extended conoids in response to ionomycin in comparison with 17±2.5% of control parasites (data not shown).

Since the shape of *T. gondii* is thought to be important for gliding motility, any alteration in morphology may have an effect on the parasite's motility. To determine whether this is the case, trail assays were performed. Analysis of trails deposited by *TgPhIL1* knockout parasites revealed that *TgPhIL1* knockout parasites were able to undergo both circular gliding and helical rotation. Additionally, the abundance of the trails deposited by *TgPhIL1* knockout parasites is indistinguishable from those deposited by wild-type parasites (Figure 4A).

To determine whether *TgPhIL1* KO parasites were defective in their ability to invade host cells, quantitative laser-scanning cytometry (LSC)-based invasion assays (Mital *et al.*, 2006) were performed.   Wild-type, *TgPhIL1,* and *TgPhIL1*;C5 parasites showed no show significant differences in their ability to invade host cells in these experiments (Figure 4B).

Figure 4: Conoid extension, motility, and invasion of the *TgPhIL1* knockout parasites.

A
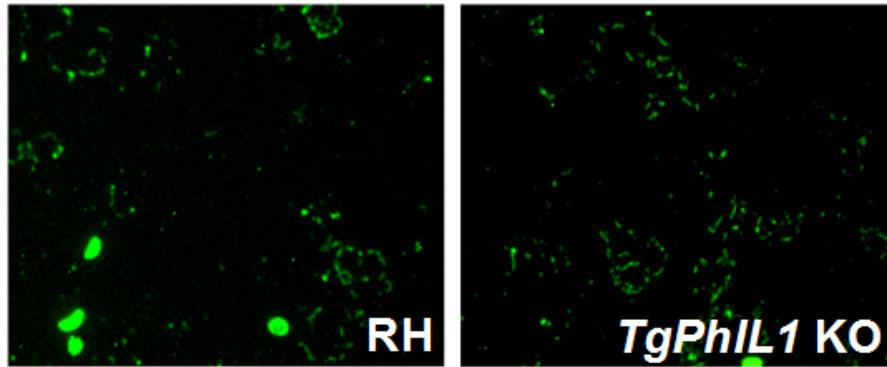
B
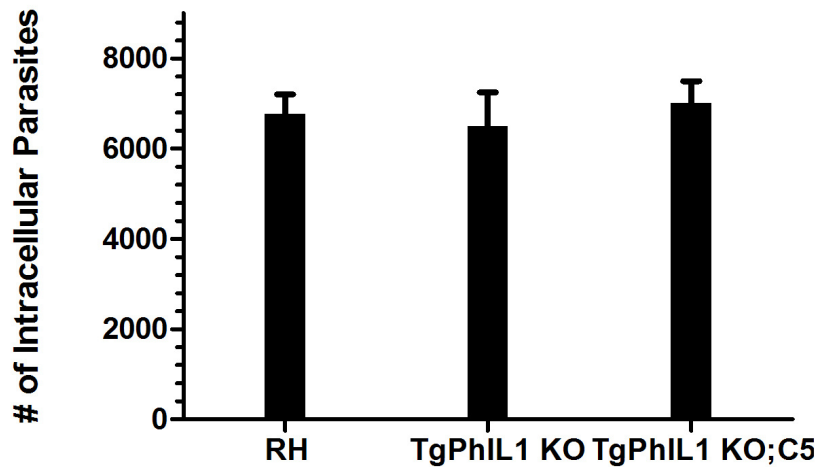
Figure 4: (A) Motility assays were performed on wild-type and *TgPhIL1* knockout parasites. Trails deposited by *TgPhIL1* knockout parasites are indistinguishable from those deposited by wild-type parasites. (B) Laser-scanning cytometry (LSC)-based assays were performed on wild-type, *TgPhIL1* knockout, and *TgPhIL1* KO;C5 parasites. Samples were analyzed in duplicate and the results shown are the average from three independent experiments, although in one experiment a *TgPhIL1* KO and *TgPhIL1* KO;C5 sample was of insufficient quality to be scanned. No significant difference was shown in the ability of the *TgPhIL1* knockout to invade host cells when compared to wild-type or TgPhIL1;C5 parasites.

Other IMC-associated antigens

To determine whether TgPhIL1 influences the localization of alpha-tubulin, inner membrane complex protein 1 (TgIMC1), or membrane occupation and recognition nexus 1 (TgMORN1) (Gubbels *et al.*, 2006), the distributions of these proteins were examined in wild-type and *TgPhIL1* knockout parasites by immunofluorescence microscopy. As shown in Figure 5, the subpellicular microtubules show a similar distribution in wild-type and TgPhIL1 knockout parasites, as does TgIMC1, which is found in a peripheral distribution in growing daughter parasites during endodyogeny. TgMORN1 can be seen forming a ring around the nucleus of both wild-type and *TgPhIL1* knockout parasites during endodiogeny and in a ring at the apical end of both parasite lines, as previously reported (Gubbels et al., 2006; Hu, 2006).

Figure 5: Localization of alpha-tubulin, TgIMC1, and TgMORN1 in *TgPhIL1* knockout parasites.
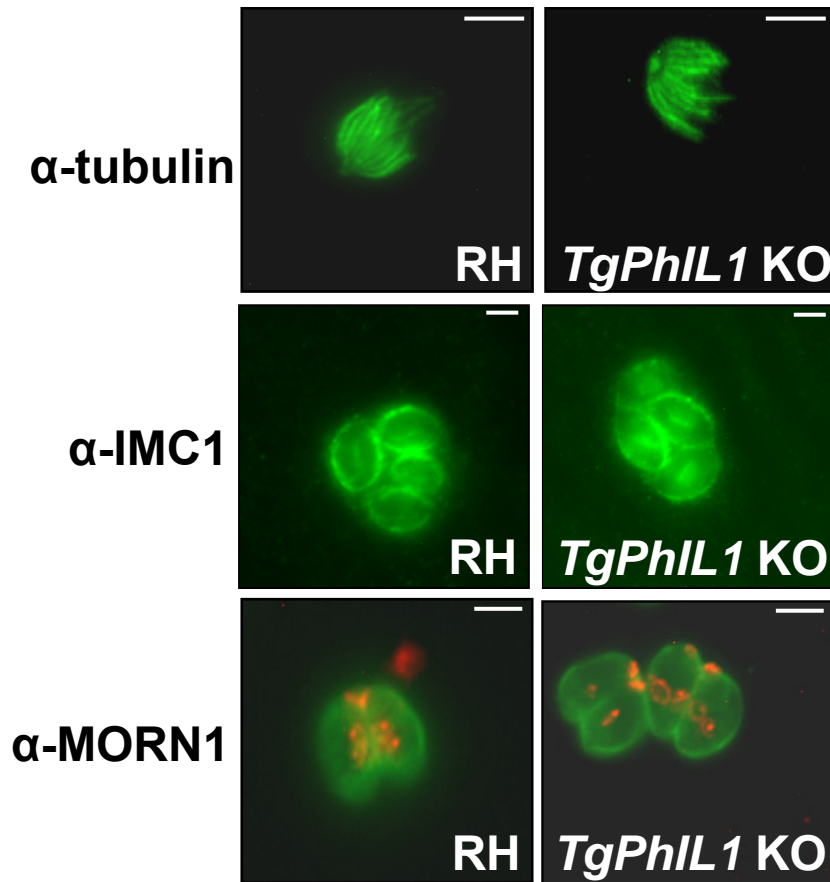
Figure 5: The distribution of alpha-tubulin, TgIMC1, and TgMORN1 was examined in *TgPhIL1* knockout parasites by immunofluorescence microscopy. The splayed out subpellicular microtubules (top panels) appear indistinguishable in the two samples. TgIMC1 localizes to the periphery of growing daughters in both wild-type and *TgPhIL1* knockout parasites during endodyogeny, again in an indistinguishable pattern (middle panels). TgMORN1 is seen as a ring around the nucleus, at the apical end of the parasite, and at the centrocone in both parasites lines (bottom panels). TgMORN1 appears red and TgSAG1 localized to the plasma membrane is shown in green. Scale bars = 5 μm.

Growth competition of *TgPhIL1* KO parasite

To assess the growth of the mutant parasites *in vitro*, wild-type and *TgPhIL1* KO parasites were added to the same flask of confluent HFF cells in equal numbers. Each time the parasites lysed the monolayer, an equal volume of culture supernatant containing the lysed parasites was added to a fresh HFF monolayer. After every third passage (passages 4, 7, and 10), immunofluorescence was performed on the recovered parasites with α-TgPhIL1 and the number ofTgPhIL1 positive and negative parasites determined (Figure 6A). The same experiment was performed with co-cultures of *TgPhIL1* KO vs. *TgPhIL1* KO;C5 and *TgPhIL1* KO vs. *TgPhIL1* KO;C7. On the graph shown in figure 6A each color represents a pair of parasites that were cultured together in one flask. Wild-type parasites had started to outgrow the *TgPhIL1* KO parasites by passage 4 and continued to do so at passages 7 and 10. *TgPhIL1* KO;C5 and *TgPhIL1* KO;C7 parasites also outgrew *TgPhIL1* knockout parasites at all time points. The difference between each pair is significant at all time points ($p < 0.05$).

Dissemination of TgPRMT1 KO parasites *in vivo*

Since the *TgPRMT1* KO parasites were outgrown by wild-type parasites *in vitro*, we were curious to see whether this difference in number of parasites would be reflected *in vivo*. In order to determine this, Female 6- to 8-wk-old C57BL/6 mice were infected intraperitoneally with 10^4 RH or *TgPhIL1* knockout tachyzoites. DNA from spleen and liver sections taken from mice sacrificed seven days after infection was harvested, and RT-PCR was performed in order to determine the amount of parasite DNA present. As shown in Figure 6B, more parasite DNA was present in the livers and spleens of mice

infected with wild-type parasites than with *TgPhIL1* knockout parasites.

Figure 6: Growth competition of *TgPhIL1* knockout parasite and dissemination to organs in a mouse model of infection.

A

B
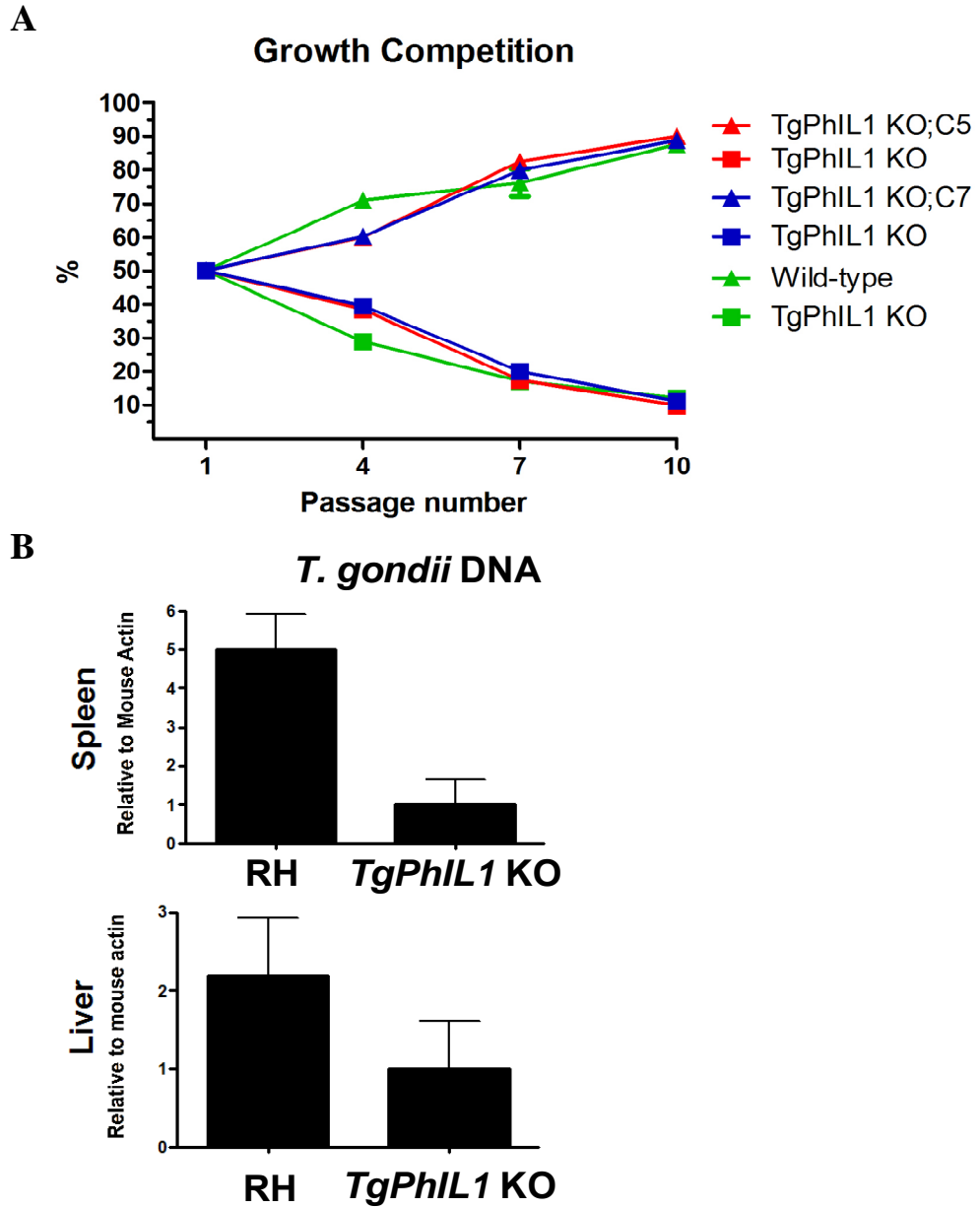
Figure 6: (A) To detect differences in the growth of *TgPhIL1* knockout parasites, equal numbers of wild-type and knockout parasites were mixed and serially passaged through monolayers of HFF cells. After every three passages, immunofluorescence was performed and 500 parasites were counted and scored as either positive or negative for TgPhIL1. This experiment was performed two times in triplicate. Each color (red, green, and blue) indicates a pair of parasite lines passaged together. Wild-type parasites were shown to slowly outgrow the knockout parasites at passages 4, 7 and 10. This experiment was repeated with the two complemented clones, *TgPhIL1* KO;C5 and *TgPhIL1* KO;C7, which also outgrow the *TgPhIL1* knockout parasites. The difference in abundance of each pair is significant at all time points ($p<0.05$). (B) Female 6- to 8-wk-old C57BL/6 mice were infected intraperitoneally with 10^4 RH or *TgPhIL1* knockout tachyzoites. DNA from spleen and liver sections taken from mice sacrificed seven days after infection was harvested, and RT-PCR was performed in order to determine the amount of parasite DNA present. Results shown are the average loads from five mice.

DISCUSSION

TgPhIL1 was originally identified by INA labeling in experiments designed to identify novel proteins of the *T. gondii* pellicle (Gilk *et al.*, 2006). TgPhIL1 is conserved among members of the Phylum Apicomplexa but bears very little sequence homology to any non-Apicomplexan proteins currently in the sequence databases. Formation of a hypothesis regarding the function of proteins which contain no identifiable protein domains or homologs of known function is not an easy task. Based on the limited information known about TgPhIL1, its localization to the IMC, and properties suggestive of both a cytoskeletal and membrane-associated protein (Gilk et al., 2006), we theorized that its cellular function could be to connect the parasite cytoskeleton to the membranes of the IMC. Alternatively, it might be required for proper localization of the subpellicular microtubules, proteins of the subpellicular network, or other cytoskeletal proteins. The fact that TgPhIL1 is highly insoluble complicates both heterologous expression of the protein in order to learn more about its secondary structure and coimmunoprecipitation studies aimed at identifying proteins with which it interacts.

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To begin to elucidate the function of TgPhIL1, we generated a *TgPhIL1* knockout parasite line. Immediately upon generation of the *TgPhIL1* knockout parasites, it was clear that they had a shape defect, appearing rounder and shorter than wild-type parasites. In light of this result, it was hypothesized that these parasites might show a defect in motility. However, motility assays, coupled with observation of these parasites during routine culture, disproved this hypothesis. The knockout parasites also showed no detectable defect in conoid extension or the ability to invade host cells. However, the TgPhIL1 knockout parasites showed consistently slower growth in culture than parasites expressing the wild-type allele. The subtle growth defect observed in culture translated into a decrease in parasite fitness in a mouse model of infection, as the livers and spleens of mice that received IP injections of *TgPhIL1* knockout parasites contained less parasite DNA than mice injected with wild-type parasites.

Although these findings provide some clues as to the function of TgPhIL1, identification of other proteins with which TgPhIl1 interacts would be helpful in determining the precise function of TgPhIL1. Because of the insolubility of this protein, immunoprecipitation of TgPhIL1 in order to identify binding partners is not possible. One alternate method to identification of proteins with which TgPhIL1 interacts is yeast-two-hybrid analysis. Although laborious, this technique has been successful in identifying a number of interacting proteins in many systems. In *Plasmodium falciparum,* a genome-wide yeast-two-hybrid screen has been performed, and the results of this screen are available (www.plasmodb.org). In this screen, the *P. falciparum* homologue of TgPhIL1 was shown to interact with one protein, and a homologue of this

protein exists in *T. gondii* (38.m01070). Although this protein is annotated as another apicomplexan-specific protein with no homology to non-apicomplexan proteins, it was shown in the *P. falciparum* yeast-two-hybrid screen to interact with six other proteins, some of which have been assigned putative functions and appear to have homologues in *T. gondii.* It is possible that confirmation that this protein interacts with TgPhIL1 may provide a handle on the function of TgPhIL1. Alternately, microarray analysis could be performed on the *TgPhIL1* knockout in comparison with wild-type parasites in order to identify transcripts up-regulated in the absence of TgPhIL1. Proteins up-regulated in the absence of TgPhIL1 may by playing a compensatory role in its absence, masking a more dramatic phenotype than that observed.

The precise function of PhIL1 remains unknown, although it clearly contributes to the maintenance of parasite shape, and this may in turn affect some aspect of parasite division or growth. Recently, several other proteins have been identified which are non-essential, but make a clear contribution to parasite growth and fitness. For example, parasites lacking both TgROM1 and TgPP2C have been shown to display subtle growth phenotypes that are only observable when wild-type and knockout parasites are mixed in culture and serially passaged (Brossier et al., 2008; Jan et al., 2007) as is observed with the *TgPhIL1* knockout parasites. Although the *TgPhIL1* knockout parasites display a rather subtle growth defect, it is clear that TgPhIL1 contributes to fitness in *T. gondii,* and that in its absence, the pathogenicity of this organism is compromised.

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CHAPTER 3: IDENTIFYING THE TARGET OF INVASION ENHANCER 112762 IN *TOXOPLASMA GONDII*

ABSTRACT

Apicomplexan parasites including *Plasmodium, Cryptosporidium,* and *Toxoplasma* cause widespread human disease and tremendous suffering, and there is a great need for novel therapeutic agents to combat these parasites. Since apicomplexan parasites are obligate intracellular parasites, compounds that disrupt the ability of these parasites to invade host cells are lethal. Additionally, the machinery used by these parasites to invade host cells is conserved across the phylum, making proteins involved in invasion attractive drug targets. Previously, a small-molecule screen was performed in *T. gondii* in order to identify small molecules which affect *T. gondii* invasion. One small molecule, Enhancer 112762, enhanced *T. gondii* invasion at concentrations as low as 6.2 μM. Additionally, this compound enhanced invasion and motility of other Apicomplexan parasites, and it was shown that the compound could be structurally modified and used to generate affinity resins. Using these resins, I have identified two putative targets of the compound, TgProfilin and a *T. gondii* FK506-binding protein. Additionally, since a compound nearly identical to Enhancer 112762 had been shown to inhibit protein arginine methyltransferases (PRMT's) in other systems, studies were conducted in order to determine whether Enhancer 112762 might target TgPRMT1 in *T. gondii*. Preliminary results suggest TgPRMT1 may be a target of Enhancer 112762. This work describes

methods used to identify and validate these three potential targets of Enhancer 112762 in *T. gondii.*

INTRODUCTION

The Phylum Apicomplexa contains a number of medically important parasites. Members of this phylum include *Plasmodium* species, which cause 300 to 500 million cases of malaria and 700,000 to 2.7 million deaths annually (Filler *et al.*, 2003), *Cryptosporidium,* which causes diarrheal illness in infants and immunocompromised people (Chalmers, 2008), and *Toxoplasma gondii,* which causes congenital toxoplasmosis and toxoplasmic encephalopathy (Porter and Sande, 1992). Due to the widespread and severe human disease caused by these pathogens and the fact that *Plasmodium* species have evolved resistance to many of the available drugs (Barnes *et al.*, 2008) and that no effective drug exists for treatment of *Cryptosporidium* in immunocompromised people, developing therapeutics for these illnesses is of the utmost importance.

Since apicomplexan parasites are obligate intracellular parasites, compounds that block their entry into cells are lethal. Identification of inhibitors of apicomplexan parasite invasion would provide lead compounds whose structures could be modified to be made more "drug-like" and ultimately used to treat these diseases.   Additionally, identification of these compounds, followed by identifying their targets in parasites, would yield information about the process of invasion by apicomplexan parasites. Due to the ease with which it can be manipulated in the laboratory, invasion in apicomplexan

parasites has been studied most extensively in *T. gondii*. From these studies, it is known

that invasion is a parasite-driven process during which the parasite moves across a host

cell, becomes intimately attached to the host-cell at its apical end, and forms a structure

called a "moving junction" at the host cell surface (Baum *et al.*, 2006; Carruthers and

Boothroyd, 2007). Following formation of the moving junction, the parasite causes an

invagination of the host cell to develop and pushes itself into that invagination. During

this process, the host cell membrane remains intact (Suss-Toby *et al.*, 1996). At the

moving junction, host cell proteins are excluded from the forming parasitophorous

vacuole so that by the time parasitophorous vacuole pinches off from the host membrane,

all host-cell proteins have been excluded from the vacuole (Mordue *et al.*, 1999). This

exclusion of host cell proteins from the vacuole is thought to allow the vacuole

containing the parasite to avoid fusion with the host endocytic pathway and the demise of

the parasite (Mordue *et al.*, 1999).

The stages of invasion occur in a sequential manner and can be broken into

discrete steps. Micronemes, present at the apical end of the parasite, are secreted

constitutively and secretion is upregulated early in the process of invasion (Carruthers *et*

al., 1999). The microneme proteins are thought to be involved in the initial interaction of

the parasite with host cells (Meissner et al., 2002a). Rhoptries are large, bulb-shaped

organelles that are discharged during invasion and are thought to be involved in the

establishment of the moving junction and the formation of the parasitophorous vacuole

(Alexander *et al.*, 2005). The signaling events involved in triggering rhoptry secretion

remain enigmatic to date. Dense granules are secreted throughout the parasite life-cycle

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and their contents are thought to be involved in the maintenance of the parasitophorous vacuole (Cesbron-Delauw *et al.*, 1996).

The machinery used by *T. gondii* for invasion is conserved among apicomplexan parasites and is also used by the parasite for motility (Baum *et al.*, 2006). Although much is known about the process of invasion, much remains to be learned. Using molecular genetics to study *T. gondii* invasion is difficult since invasion is an essential process. Disrupting this process pharmacologically circumvents the problem of disrupting an essential process in a haploid organism.

In order to identify small molecules that affect invasion by apicomplexan parasites, a small-molecule screen was performed in *T. gondii* with 12,160 small molecules (ChemBridge's DIVERSet™ collection). Although libraries of more "drug-like" compounds exist, this library was chosen for a number of reasons. For one thing, the compounds in this library are structurally diverse, yet grouped into families of compounds with similar properties. This offers the advantage of providing structure activity relationship (SAR) data immediately upon identification of compounds that affect invasion. Also, this library had been used previously in a number of other screens and had given a relatively high hit rate. We believed the information yielded from other groups using this collection might be useful in trying to identify targets of hit molecules identified in our screen. Additionally, we were able to acquire this collection through our collaborators at the Institute of Chemistry and Cell Biology at Harvard University.

The compounds were screened for an effect on *T. gondii* invasion using a semi-quantitative dual-fluorescence assay as described (Carey *et al.*, 2004). As a result of this

screen, 24 inhibitors of *T. gondii* invasion and six enhancers of invasion were identified. Secondary assays were performed to identify which step of invasion was inhibited. Additionally, the compounds were screened for activity against other apicomplexan parasites, including *Plasmodium knowlesi* and *Plasmodium berghei* (Carey, 2004).

Although the small molecule screen was originally designed to identify inhibitors of invasion, the unexpected identification of invasion enhancers offers a unique angle from which to study the process of invasion. Enhancer 112762 was shown to enhance *T. gondii* invasion and motility at concentrations as low as 6.2 µM. Additionally, it was shown to enhance *P. berghei* invasion and *P. knowlesi* motility, suggesting that it hits an aspect of the invasion machinery that is conserved among apicomplexan parasites. Furthermore, it was shown to be amenable to chemical manipulation (Morgan, 2006b). Enhancer 112762 was selected as a compound warranting further investigation, and this work describes a number of experiments aimed at identifying the target(s) of Enhancer 112726 in *T. gondii*.

MATERIALS AND METHODS

Culture of Parasites

Toxoplasma gondii RH strain was maintained by serial passage in confluent lines of primary human foreskin fibroblast (HFF) cells in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% heat inactivated fetal bovine serum, 10 units/ml penicillin G sodium, 10 µg/ml streptomycin sulfate, and 1 mM HEPES buffer.

Affinity resins

The structural derivatives of Enhancer 112762 were synthesized and linked to Affi-Gel beads as described previously (Morgan, 2006b), and the percentage of N-succinimide esters occupied by compound on each of the eight affinity resins was measured and determined to range from 14% and 19%. The variation in percentages of N-succinimide esters occupied by compound across the resins was taken into consideration in experiments where it was crucial that the amount of compound on the resins be identical. The resins were stored in dimethyl sulfoxide (DMSO) in the dark since 112762 and its structural derivatives are known to be light-sensitive (Morgan, 2006b).

Affinity chromatography aimed at identifying proteins that covalently bind the active resin

6.8×10^8 freshly-lysed tachyzoites were harvested and washed twice in 10 ml phosphate buffered saline (PBS) by centrifugation. After centrifugation, parasites were resuspended in extraction buffer (EB) (1.0% Triton X-100, 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA) in the presence of 1:100 [vol/vol] protease inhibitors (Sigma, P-8340), followed by incubation on ice for 10 minutes and centrifugation at 10,000 x g at 4ºC for 30 minutes to remove insoluble material. The cleared extract was added to 250 µl of a 50% slurry of the active (30-REM-03(15)) and inactive (21-REM-05(15)) resins which had been washed three times in 1 ml EB and incubated for 1 hour at 25°C. After

incubation, the beads were washed three times in EB, two times in 50 mM Tris-HCl pH 7.4, and boiled in 200 µl 1% SDS in 50 mM Tris-HCl. The resins were then washed three more times in 50 mM Tris-HCl. Trypsinization of proteins bound to the resins and analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) were performed as described previously (Morgan, 2006b). Three independent experiments were performed.

Profilin depletion experiment with recombinant TgProfilin

Recombinant TgProfilin was generously provided by Alan Sher at the NIH. 1 µg rTgProfilin was added to 200 µl EB and added to 20 µl of a 50% slurry of the active (30-REM-03(15)) or inactive (21-REM-05(15)) resin or without resin at 4°C for 2 hours. Additionally, 40 µl of rTgProfilin in EB was removed before incubation with the resin to control for minor variations in the amount of rTgProfilin present before incubation with the resins. After incubation, 60 µl of the supernatant was removed and run on an SDS-PAGE gel, followed by silver staining as described previously (Merril, 1990). The amount of compound on the resins was roughly ten times the molar amount of rTgProfilin used.

Profilin depletion experiment with *T. gondii* extracts

5×10^6 freshly-lysed tachyzoites per sample were harvested, washed once in 5 ml PBS by centrifugation, and resuspended in 200 µL EB buffer containing protease inhibitors. After centrifugation at 14000 x *g* to remove insoluble material, the extracts

were incubated with 30 µl active or inactive resin or in the absence of resin. After

incubation for two hours at 23°C, the resins were washed five times in EB and 50 uL of

each supernatant was removed. The supernatant was run on an SDS-PAGE gel, followed

by transfer to nitrocellulose paper. After blocking in 2% milk/TBS-T, western blot

analysis was performed with rabbit anti-rTgProfilin (Plattner *et al.*, 2008) at a dilution of

1:10000 and rabbit anti-GRA8 (Carey *et al.*, 2000) at 1:57000, followed by incubation

with horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig at a dilution of 1:1000

and detection of HRP activity by enchanced chemiluminescence (Amersham Life

Sciences).

Affinity chromatography to identify proteins that bind the active resin non-covalently

Parasites were radiolabeled with 500 µCi ^{35}S in cysteine and methionine-

deficient DMEM media for 16 hours using EasyTag™ EXPRESS^{35}S Protein Labeling

Mix (PerkinElmer). 6.25×10^7 freshly-lysed and radiolabeled tachyzoites per sample

were harvested, washed in 5 ml PBS by centrifugation, and extracted in 200 µl EB

containing protease inhibitors. After extraction and centrifugation at 14000 x *g* to

remove the insoluble material, the soluble extract was added to 50 µl of a 50% slurry of

each of the eight affinity resins. The binding capacity of 50 µl of resin is equal to twice

the amount of protein present in 6.25×10^7 tachyzoites. After incubation of the extract

with resins for 2 hours at 25°C, the resins were washed 5 times in EB, followed by

boiling in SDS sample buffer, which contains 0.1% SDS. The supernatant was removed

after boiling, run on an SDS-PAGE gel, transferred to nitrocellulose paper, and visualized

using autoradiography. For the scaled-up version of this experiment in which

Coomassie-stained bands were isolated for mass spectrometry, the experimental design

was the same with the following exceptions: Tachyzoites were not grown in the presence

of radioactivity and both the amout of resin and the number of parasites were scaled-up

by a factor of 5. Only resins 25-REM-05(10) and 24-REM-05(10) were used in this

experiment. Coomassie staining was performed as described previously (Peterson,

1983). In addition to incubating extract with the active and inactive resins, a third sample

was prepared in which a highly soluble derivative of 112762, 14-REM-05 (Morgan,

2006b) was added to the extract and active resin before washing the samples in order to

determine whether soluble compound could block binding of proteins to the active resin.

The amount of compound used for competition was roughly twice the amount present on

the active resin.

Cloning TgFK506bp-YFP under the tubulin promoter`

  The TgFK506bp ORF was amplified from cDNA using the primers FK506bp

BglII for (5' GGGG<u>AGATCT</u>ATGTTCTACGGTGTCGTTGTCAAGCCTGGC3') and

AvrII rev (5'GGGG<u>CCTAGG</u>TTTCAGCGAGCACACGCCGTTCGAAACATC3').

The product was digested with BglII and AvrII and ligated into pTubIMC-YFP which

was also digested with BglII and AvrII in order to remove the TgIMC1 ORF, generating

pTubFK506bpYFP. The plasmid was electroporated into wild-type RH parasites and

visualized using fluorescence microscopy.

Inhibition of TgPRMT1 methyltransferase activity *in vitro*

Recombinant TgPRMT1 was expressed in and purified from *E. coli* using

plasmids generously provided by Mohamed-Ali Hakimi as described previously

(Saksouk et al., 2005). Recombinant enzyme was incubated in the presence of varying

concentrations of Enhancer 112762 at 21°C for 15 minutes, followed by the addition of

human histone H4 (Upstate) and [^{3}H]-S-adenosyl-methionine (PerkinElmer) and

incubation for 15 minutes at 30°C.   Histone methylation was visualized by SDS-

PAGE/fluorography.

Cloning of myc-TgPRMT1 under the tubulin promoter

TgPRMT1 was amplified from the pMAH plasmid provided by Muhamed-Ali

Hakimi (Saksouk et al., 2005) using the primers myc-TgPRMT1 for

(5'TCATCTCCGAGGAGGACCTGAGTTCCA GCAGCCCCAACTC3'), containing

the *myc* sequence, and myc-TgPRMT1 rev AvrII,

(5'GGGGCCTAGGCTATCGGAGTCTGTAGAACTGGGTGTTTTC3'), followed by

digestion of the product with BglII and AvrII and ligation into pTubIMC-YFP as

described previously for cloning of TgFK506bpYFP. Since the reverse primer contains a

stop codon, YFP is not translated. This plasmid, pTubMyc-TgPRMT, was electroporated

into wild-type RH parasites. Immunofluorescence of myc-TgPRMT1 was performed by

adhering the parasites to glass coverslips using CellTak (BD Biosciences), followed by

fixation in 4% paraformaldehyde in PBS for 30 minutes at 25°C and permeabilization

with 0.25% Triton-X100 in PBS for 30 minutes at 25°C. After blocking in 2%BSA/PBS, the coverslips were washed in PBS and incubated with a 1:500 dilution of mouse monoclonal anti-myc (Green Mountain Antibodies) for 15 minutes at 25°C. After washing, the coverslips were incubated with Alexa 488-conjugated goat anti-rabbit antibody at a 1:1000 dilution, followed by washing the coverslips, mounting them, and visualization by fluorescence microscopy. 5×10^6 myc-TgPRMT1 or wild-type parasites were boiled in sample buffer containing 0.1% SDS and analyzed by SDS-PAGE. Subsequently, western blot analysis with rabbit anti-TgPRMT1 at a 1:2000 dilution and rabbit anti-MIC5 at 1:10000 in TBS-T/0.5% bovine serum albumin (BSA), followed by washing, incubation with HRP-linked goat anti-rabbit secondary antibody, and detection of HRP activity by ECL was performed.

Affinity chromatography with myc-TgPRMT1 parasites

1×10^8 myc-TgPRMT1-expressing parasites were harvested, washed in 5 ml PBS by centrifugation, and extracted in 400 µl EB in the presence of protease inhibitors. After centrifugation at 14000 x *g* to remove the insoluble material, the extract was added to either the active (25-REM-05(10)) or inactive (24-REM-05(10)) resin and incubated for 2 hours at 25°C. After incubation, 5 µl of the supernatant was boiled in sample buffer containing 0.1% SDS. The resins were then washed 5 times in EB and boiled in sample buffer. The supernatant was removed and the samples were run on an SDS-PAGE gel, transferred to nitrocellulose paper, and subjected to western blot analysis as described above. Anti-myc mouse monoclonal antibody (Green Mountain Antibodies) was used at

a 1:500 dilution in 5%BSA/PBS, followed by incubation with HRP-conjugated rabbit

anti-mouse at a 1:1000 dilution and visualization of HRP activity by ECL.

In vivo labeling in the presence of 112762 and 112759

1 X 10^7 wild-type parasites were harvested, washed in 5 ml PBS by

centrifugation, and incubated in 200 µl high potassium buffer (Kafsack et al., 2004) in the

presence of 50 µCi of [^{3}H]-S-adenosyl-methionine (PerkinElmer) for 1 hour at 37 °C.

100 uM 112762, 112759, or an equal volume of DMSO was added and the sample

incubated for an additional 2 hours at 37 °C. The parasites were washed 3 times, boiled

in sample buffer, and subjected to SDS-PAGE/fluorography.

Generation of _TgPRMT1_ KO parasites

T. gondii genomic DNA was isolated using DNAzol® (Talron) according to the

manufacturer's instructions. In order to generate the _TgPRMT1_ knockout parasites, 3 kb

of genomic sequence directly upstream and downstream of the TgPRMT1 ORF was

amplified with Elongase (Invitrogen). The downstream sequence was amplified with

3'TgPRMT1-For-BamHI (5'-AAAA<u>GGATCC</u>GCTCGCGAGAGACGCTTCGC-3') and

3'TgPRMT1-Rev-BamHI (5'-AAAA<u>GGATCC</u>GAGTCCAGATTCGACACAAGGG-3')

before digestion with BamHI and ligation into pGRA1/ble directly downstream of the Ble

cassestte. The upstream sequence was amplified with 5'TgPRMT1-For-HindIII (5'-

GGGG<u>AAGCTT</u>GAACTCTTCACTCGGAAGGAC-3') and 5'TgPRMT1-Rev-HindIII

(5'-GGGG<u>AAGCTT</u>AGGACCAGAAGGACCAGAAGG-3') before digestion with

HindIII and ligation into pGRA1/ble containing the 3' flanking region of TgPRMT1.

This plasmid was transfected into both RH parasites and myc-TgPRMT1-expressing

parasites.

RESULTS

Synthesis and use of affinity resins

The structure of Enhancer 112762 is shown in Figure 7. Active and inactive

derivatives of this compound were synthesized and conjugated to Affi-Gel beads through

N-hydroxysuccinimide ester groups (Figure 1) as described previously (Morgan, 2006b).

This involved replacing the thiophene ring in 112762 with a phenol group before linking

the compounds to the beads. A total of four pairs of active and inactive resins were

synthesized. The active and inactive resins differ only by a single double bond in the

active resin (red arrow in Figure 1). It has been shown previously that increasing the

distance between small molecules and the beads to which they are attached decreases the

non-specific binding of proteins to either the compound or the free N-

hydroxysuccinimide groups on the beads (Furuya et al., 2006). To this end, compounds

were synthesized with longer internal linkers. The active and inactive 112762 derivatives

were coupled with 6-aminohexanoic acid, providing a stable linker that is less likely to

undergo hydrophobic collapse than if a longer carbon chain was added. The resins

containing the 6-aminohexanoic acid extension are 25-REM-05 and 24-REM-05.

Additionally, the length of the linker on the Affi-Gel resin can be increased to achieve further distancing of the small molecule from the resin. The numbers in parentheses in Figure 1 indicate whether a 10- or 15-carbon linker is present on the beads.

Figure 1: Active and inactive derivatives of 112762 bound to Affigel beads
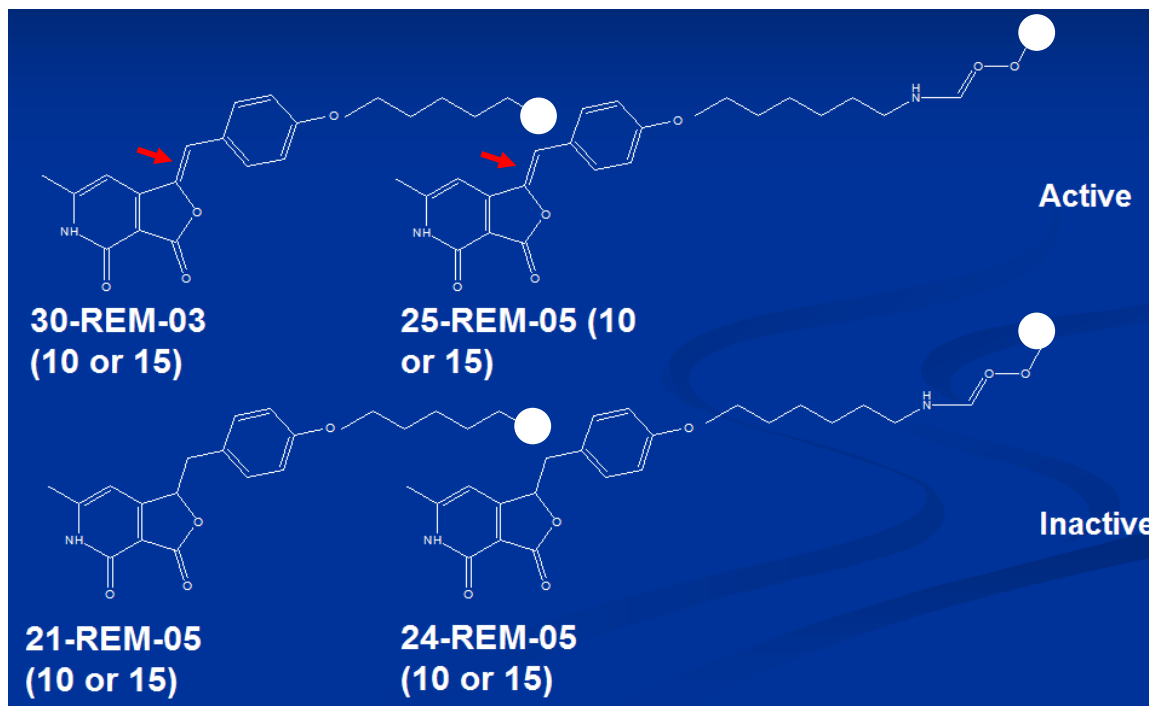
Figure 1: Affinity resins with active and inactive compounds bound to Affigel beads. The active compound is a structural derivative of 112762 shown to enhance *T. gondii* invasion and motility (Carey *et al.*, 2000). The active and inactive derivatives differ only by one double-bond in the active compound (red arrow). The compounds present on resins 25-REM-05 and 24-REM-05 contain 6-aminohexanoic acid in order to increase the distance between the compound and the resin. The compounds were conjugated to both Affi-Gel 10 and Affi-Gel 15 beads.

Attempts to identify a covalent target of 112762

Work with model substrates and 112762 indicates that this compound may interact covalently with proteins (Morgan, 2006b). In order to identify potential covalent targets of 112762, resins 30-REM-03(10) and 21-REM-05(10) were incubated with parasite extract for 1 hour at 23 °C before being washed extensively, boiled in 1% SDS, and washed further in order to remove all proteins not covalently bound (Figure 2). The proteins on the resins were then subjected to trypsinization followed by LC-MS/MS. Three independent experiments were performed.

Figure 2: Affinity chromatography experiments aimed at identifying a covalent target of 112762.
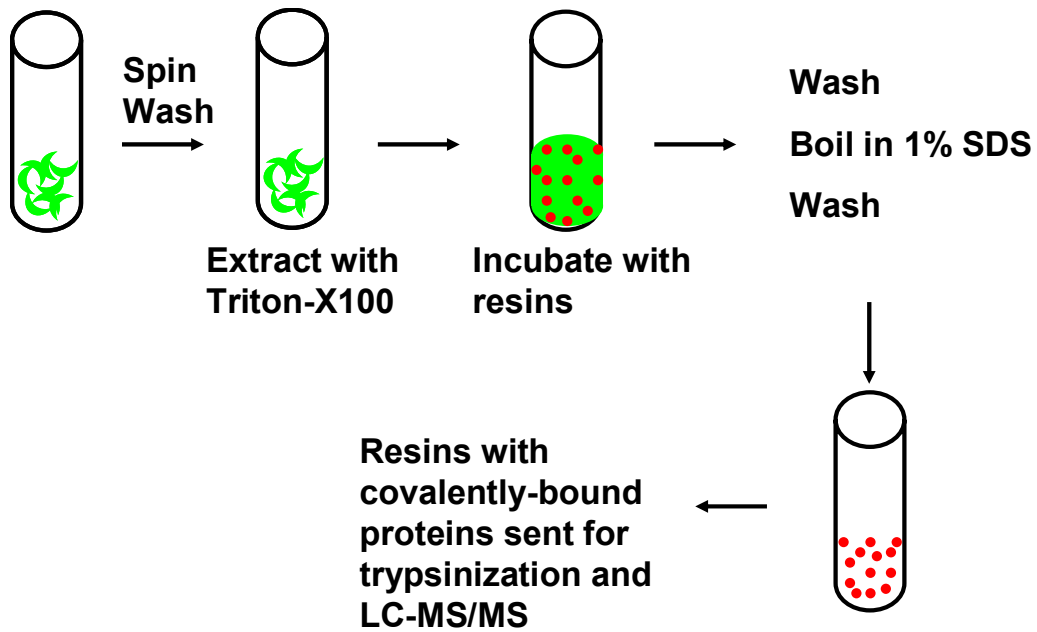
Figure 2: Attempt to identify covalent target(s) of 112762. 6.8 X 10^8 parasites were harvested and filtered. The parasites were then extracted in 1% Triton X-100 and incubated with the active or inactive affinity resins. After incubation for 1 hour at 23°C, the resins were washed extensively and boiled in SDS for 5 minutes to removal all proteins not covalently bound. Finally, the resins with covalently-bound proteins were subjected to trypsinization. Peptides released by trypsinization were identified by LC-MS/MS (Morgan, 2006b).

Interestingly, many more proteins were shown to bind the inactive than the active resins. Table 1 provides a list of proteins that bound the active but not the inactive resin in each experiment.

Table 1: List of proteins released from the active but not the inactive resin by trypsinization.

Experiment 1:
Inflammatory profilin, *T. gondii*
Translation elongation eEF-1 alpha, *Agellomyces*
ENSANGP00000011367, *Anopheles gambiae*
Experiment 2:
Translation elongation eEF-1 alpha, *Agellomyces*
Keratin-1, *Homo sapiens*
Inflammatory profilin, *T. gondii*
Trypsin precursor, pig
Elongation factor-1 alpha, *Mesocyclops edax*
Alcohol dehydrogenase, *Kluyveromyces lactis*
KLAD4 NID, *Kluyveromyces lactis*
Keratin-9 type 1, *Homo sapiens*
Translation elongation factor-1 alpha, *Picea abies*
Hypothetical protein FLJ44603, *Homo sapiens*
Ribonuclease E, *Haemophilus influenzae*
Experiment 3:
Keratin-1, *Homo sapiens*
Keratin-9, type 1, *Homo sapiens*
Inflammatory profilin, *T. gondii*
Translation elongation eEF-1 alpha, *Homo sapiens*
AB104446 NID, *Homo sapiens*

Table 1: Results of three independent experiments aimed at identifying a covalent target of 112762.
Peptides were trypsinized off the resins and identified by LC-MS/MS. Peptides from proteins listed in the table above were identified off the active but not the inactive resin.

The only *T. gondii* protein identified consistently after incubation with the active but not the inactive resin was TgProfilin. Peptides from this protein were identified in all three experiments, suggesting this protein may bind the active but not the inactive compound.

Validation of *T. gondii* inflammatory profilin as a target of 112762

Since TgProfilin is an actin-binding protein and actin polymerization is known to be important for *T. gondii* motility and invasion (Wetzel *et al.*, 2003), TgProfilin was an alluring putative target of Enhancer 112762. In order to validate TgProfilin as a target of 112762, depletion experiments were performed in which recombinant TgProfilin was incubated with the resins in lysis buffer containing 1% Triton X-100. Again, resins 30-REM-03(10) and 21-REM-05(10) were used for these experiments. The amount of compound on the resins was roughly ten times the molar amount of rTgProfilin used. After boiling the samples in 1% SDS, the eluate was run on an SDS-PAGE gel followed by silver staining. The amount of rTgProfilin present after incubation with the active resin does not differ from the amount present after incubation with the inactive resin by silver stain (Figure 3A). Additionally, the amount present after incubation with both resins is similar to the amount present when rTgProfilin is incubated in buffer in the absence of resins, suggesting rTgProfilin does not bind either resin under these conditions.

Although rTgProfilin was not shown to bind preferentially to the active resin, it is possible that this rTgProfilin may be improperly folded, interfering with its ability to

bind the active resin. In order to test whether endogenous TgProfilin binds the active but not the inactive resin, parasites were lysed and the lysate was incubated with the active and inactive resins. After extensive washing of the resins and boiling in 1% SDS, the unbound fraction was run on an SDS-PAGE gel and subject to western blot analysis with anti-TgProfilin and anti-TgGRA8. TgGRA8 is a soluble *T. gondii* protein and was used as a control for non-specific binding to the resins. Western blot analysis with anti-TgProfilin indicates that less TgProfilin remained in the unbound fraction after incubation with the active than the inactive resin (Figure 3B). However, western blot analysis with anti-TgGRA8 showed a similar profile, suggesting that the interaction of TgProfilin with the active resin may be non-specific. It should be noted that although an attempt was made to use identical volumes of different resins, the possibility exists that slight variation occurred in the amount of resins used since the resins are stored as slurries. This variability may explain why variable amounts of TgGRA8 bound the active vs. inactive resins.

Figure 3: Attempt to confirm the interaction between TgProfilin and the active affinity resin.
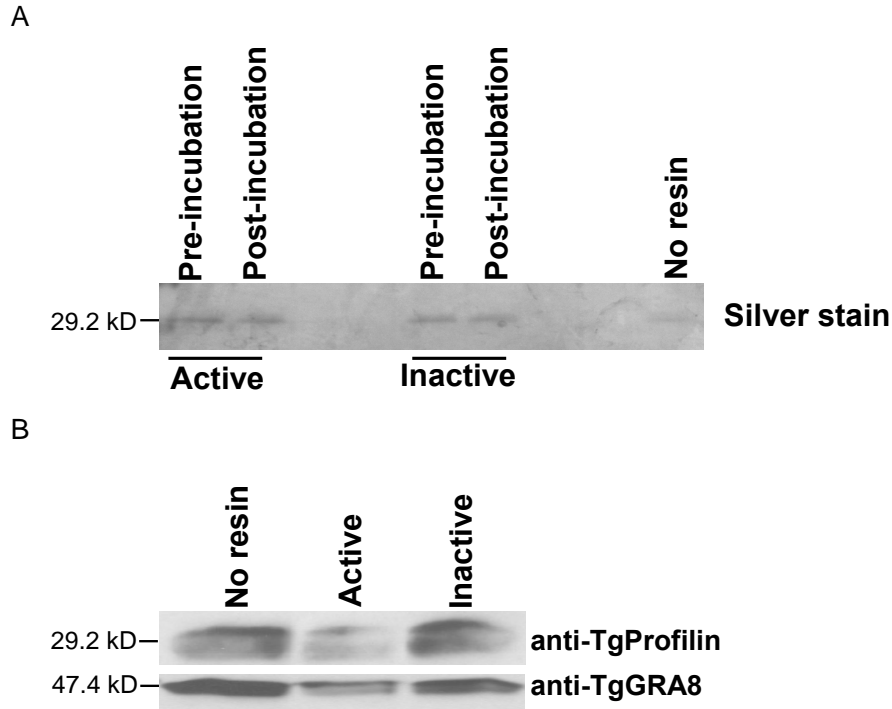
Figure 3: Attempts to validate binding of TgProfilin preferentially to the active affinity resin over the inactive affinity resin. (A) rTgProfilin was incubated with the active 30-REM-03(10) and inactive 21-REM-05(10) affinity resins. Incubation without resin was performed as a control. Silver staining of the supernatant after incubation indicated that the same amount of rTgProfilin was present in the supernatant after incubation with the active and the inactive resin, as well as without incubation with resin. (B) In order to determine whether endogenous TgProfilin present in parasaite extract binds the active resin to a greater extent than the inactive resin, extract was incubated with the active and inactive resins, the resins were washed extensively and boiled in 1%SDS, and the eluate was run on an SDS-PAGE gel and subject to western blot analysis with anti-TgProfilin and anti-TgGRA8. Although more TgProfilin was eluted from the active than the inactive resin, this result was also observed with TgGRA8, suggesting that this binding may be non-specific.

In another attempt to validate binding of TgProfilin to 112762, rTgProfilin was

incubated with 112762 in solution and the protein was subject to MALDI/MS in order to

determine whether a shift of 295 Daltons, the molecular weight of 112762, could be

detected. This shift in molecular weight was not detected (data not shown), suggesting that rTgProfilin and 112762 do not bind one another under these conditions.

Attempts to identify a non-covalent target of 112762

Although the experiment aimed at validating TgProfilin as a target of 112762 did not conclusively rule out this possibility, they did not confirm an interaction between TgProfilin and 112762. At this point, affinity chromatography experiments aimed at identifying a non-covalent target of 112762 were performed. Extracts from ^{35}S-labeled parasites were incubated with the resins, followed by extensive washing of the resins. All eight affinity resins were used in the initial experiments. After washing, the resins were boiled in 1% SDS and the eluate was run on an SDS-PAGE gel, transferred to nitrocellulose, and exposed to film (Figure 4). A prominent ~50 kDa band is present in the samples eluted from two of the active resins but not their inactive counterparts and is indicated by a red arrow. Of note, many more proteins are eluted from the inactive than the active resins from the pairs that do not contain 6-aminohexanoic acid conjugated to the small molecules (first four lanes on the gel). This result is consistent with the mass spectrometry results of tryptic peptides released from 30-REM-03(10) and 21-REM-05(10), where peptides from many more proteins were identified off 30-REM-03(10) than 21-REM-05(10). Also of interest is the fact that the extra ~50 kDa band is present in samples eluted from the Affi-Gel 10 but not Affi-Gel 15 resins. This experiment was performed several times and the extra band was present in every experiment.

Figure 4: ^{35}S-labeled proteins eluted from affinity resins by boiling in 1% SDS.
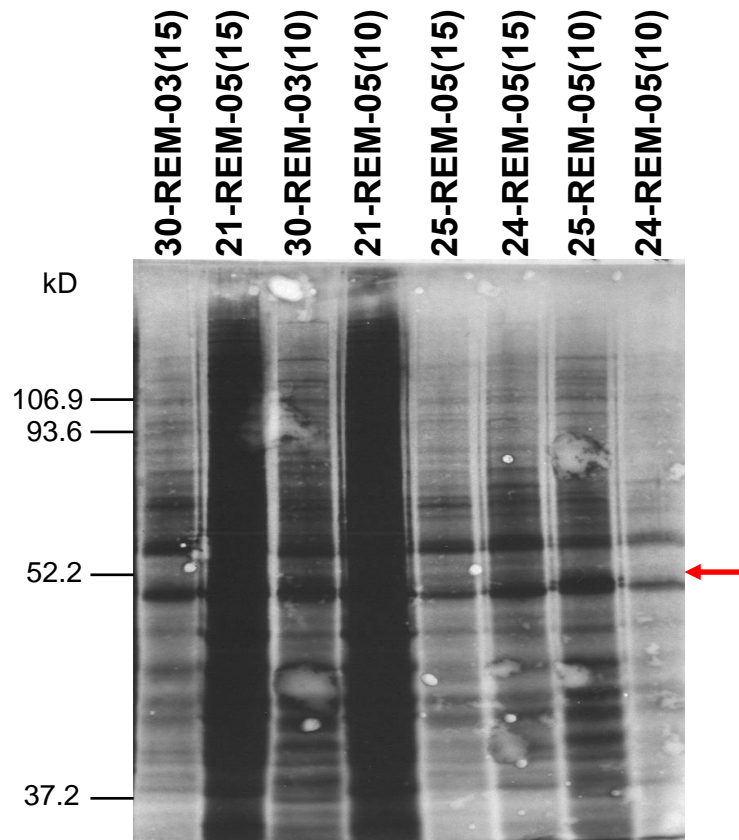
Figure 4: Attempt to identify non-covalent targets of 112762 using affinity resins and extract from ^{35}S-labeled parasites. Extract from parasites grown in the presence of ^{35}S-labeled cysteine and methionine was incubated with all eight resins. After washing the resins and boiling them in 1% SDS, the eluate was run on an SDS-PAGE gel, transferred to nitrocellulose paper, and exposed to film. The eluate off the active resins, 30-REM-04(10) and 25-REM-05(10), contains an extra ~50 kDa band (red arrow) that is not present in the eluate off their inactive counterparts, 21-REM-04(10) and 24-REM-05(10).

Identification of protein preferentially binding active but not inactive resin

In order to identify the protein that appears to preferentially bind the active resin over the inactive resin, it was necessary to excise it from the gel and identify it using mass spectrometry. However, because ^{35}S-labeled proteins cannot be subjected to mass

spectrometry in our facility, another method of detection was needed. The experiment was scaled-up five-fold so that parasites from 5 X T-75's were incubated with 150 µl of 25-REM-05(10) or 24-REM-05(10). In addition to incubation of the extract with the active and inactive resins, a sample was prepared in which a 2-fold excess of soluble compound was added to the parasite extract and resins as competitor. The eluate from all three samples was run on an SDS-PAGE gel and analyzed by Coomassie staining (Figure 5). In addition to the 50 kDa band, five other proteins appear to bind the active but not the inactive resin. The addition of soluble compound does not appear to interfere with the interaction between any of these compounds and the active resin. Still, the relative abundance of these proteins eluting from the active vs. the inactive resins suggests that they may be targets of 112762. Bands were cut from the active, inactive, and competed samples and analyzed by mass spectrometry.

Figure 5: Coomassie stained gel of eluate from active and inactive resin.
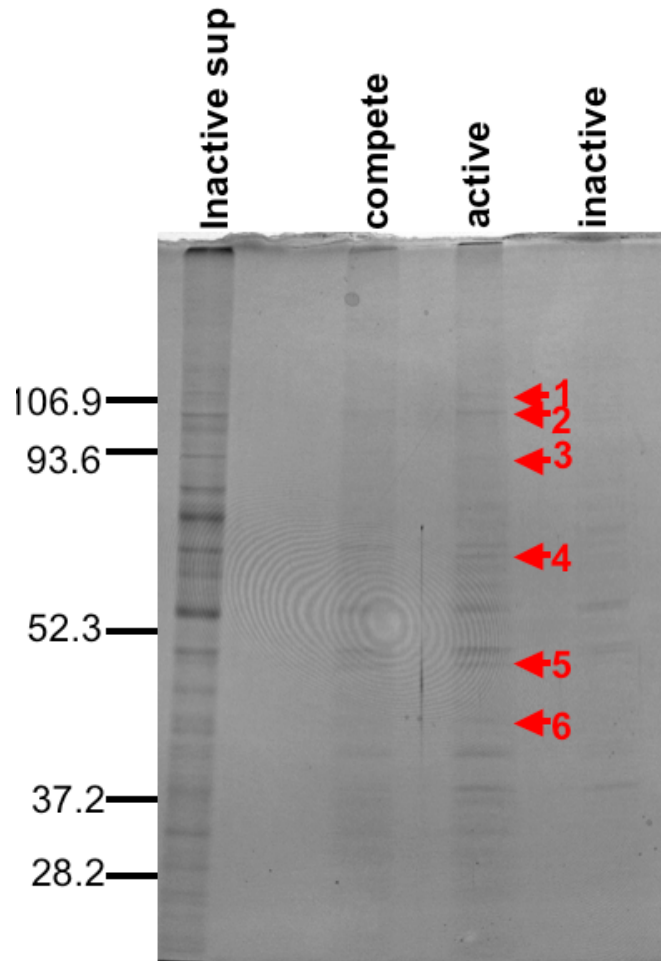
Figure 5: Coomassie-stained gel of proteins eluted from the active and inactive resins by boiling in 1% SDS. Extract containing *T. gondii* proteins was incubated with 25-REM-05(10) (active) or 24-REM-05(10) (inactive). Additionally, a 2-fold excess of soluble compound was added to one 25-REM-05(10) sample (compete). A sample of the supernatant after incubation with the inactive resin was run as a control (far left lane). In addition to an extra band being present in the active but not the inactive sample at approximately 50 kD, five other bands appear to be present in the active but not the inactive samples. Band 5 was cut from the active, competed, and inactive samples and analyzed by mass spectrometry.

The results of this experiment will be focused on band 5 which may correspond to the most prominent band after ^{35}S-labeling (Figure 4). TgThioredoxin peptides were recovered at this position of the gel from the inactive but not the active resin. Conversely, a putative RNA-binding protein was detected in the active resin eluate but not the inactive resin eluate, but only two peptides from this protein were identified and each was only identified one time. Most significantly, three peptides corresponding to a *T. gondii* FK506bp were present in the active but not the inactive sample. This result was reproduced across several experiments.

Localization of TgFK506bp within *T. gondii* tachyzoites

TgFK506bp is an attractive potential target of Enhancer 112762 for a number of reasons. FK506bp's are peptidyl-prolyl isomerases (Gothel and Marahiel, 1999) and are called FK506bp's because they have been shown to bind the immunosuppressive drug FK506 (Tacrolimus). Interestingly, FK506 was shown to inhibit *T. gondii* invasion in a screen performed previously in our lab (data not shown). Additionally, another protozoan parasite, *Trypanosoma cruzi*, has been shown to secrete an FK506bp and secretion of this protein enhances infection of host cells by *T. cruzi* (Moro et al., 1995). Arguing against TgFK506bp as a relevant target of Enhancer 112762, however, is the fact that TgFK506bp has a predicted nuclear localization signal (NLS). Localization to the nucleus does not exclude FK506bp as a potential target of Enhancer 112762. However, it does make it more difficult to explain how an interaction between TgFK506bp and 112762 could lead to enhancement of invasion on the time-scale

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observed. In order to determine the subcellular localization of TgFK506bp within *T. gondii,* TgFK506bp tagged with YFP at its C-terminus (TgFK506bp-YFP) was cloned and transfected into wild-type RH parasites. The localization of this protein was determined by fluorescence microscopy (Figure 6).

Figure 6: Fluorcescence microscopy of TgFK506bp-YFP under the control of the tubulin promoter.

DIC   FK506bp-YFP   merged
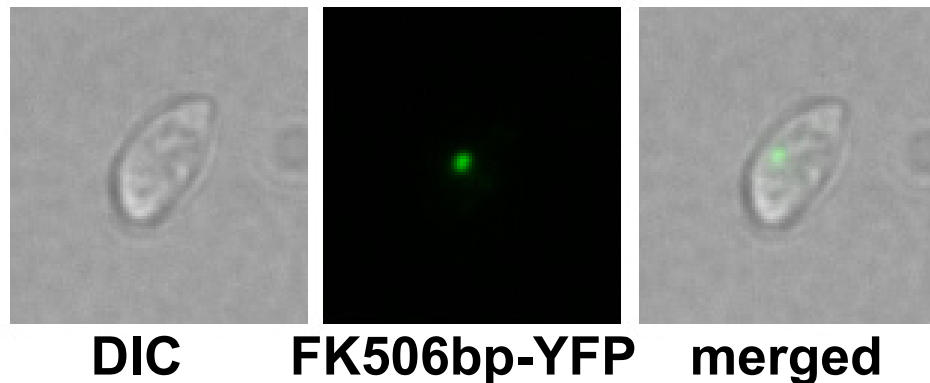
Figure 6: Localization of TgFK506bp-YFP expressed under the tubulin promotor. Fluorescence microscopy of TgFK506bp-YFP reveals that this protein is localized to one discrete, round dot within the parasite. This localization is not coincident with nucleolar or apicoplast staining as determined through the use of DAPI (data not shown).

This fusion protein is localized to a discrete dot within the parasite and the localization is distinct from nucleolar or apicoplast staining as determined by DNA staining using DAPI (data not shown). It is possible this is not the true localization of TgFK506bp and that the localization seen here is actually aggregation due to either expression under the strong tubulin promoter or the presence of a large YFP tag at the C-

terminus of the protein. Either way, TgFK506bp-YFP does not appear to localize to the nucleus.

TgPRMT1 as a potential target of Enhancer 112762

While studies aimed at validatingTgFK506bp as a potential target of Enhancer 112762 were underway, we became aware of a published article in which a compound nearly identical to Enhancer 112762 was shown to inhibit both human and yeast protein arginine methyltransferase-1 (PRMT1) (Cheng et al., 2004). Additionally, it was shown in our lab that 112759 inhibited *T. gondii* invasion (data not shown). The structures of this compound (112759) and Enhancer 112762 are shown below (Figure 7).

Figure 7: Structures of compounds 112759 and 112762.
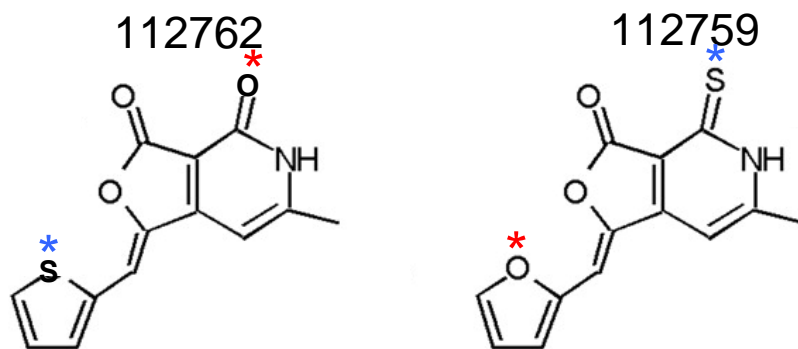
Figure 7: Structures of Enhancer 112762 and protein arginine methyltransferase-1 (PRMT1) inhibitor 112659. The two compounds differ only by the replacement of an oxygen and a sulfur atom. (see asterisks). 112759 was been shown previously to inhibit both human and yeast PRMT1 (Cheng *et al.*, 2004).

Since 112759 had been shown to inhibit PRMT1 in other system, we sought to determine whether 112762 inhibited TgPRMT1. Recombinant TgPRMT1 expressed in *E. coli* was obtained from Muhamed Ali-Hakimi and pre-incubated with varying concentrations of 112672, followed by addition of human histone H4, a known PRMT1 substrate, and [³H]-S-adenosyl-methionine as the methyl donor. SDS-PAGE/fluorography revealed that the level of H4 methylation after incubation with 112762 was decreased compared to that present after incubation with DMSO alone (Figure 8), and the effect of 112762 on rTgPRMT1 was dose-dependent. Also, 112762 was shown to change the methylation profile of *T. gondii* proteins when parasite extracts were incubated with [³H]-S-adenosyl-methionine in the presence of 100 μM of compound (data not shown, experiments performed by Jeralyn Haraldsen).

Figure 8: Inhibition of rTgPRMT1 activity by 112762 *in vitro*.
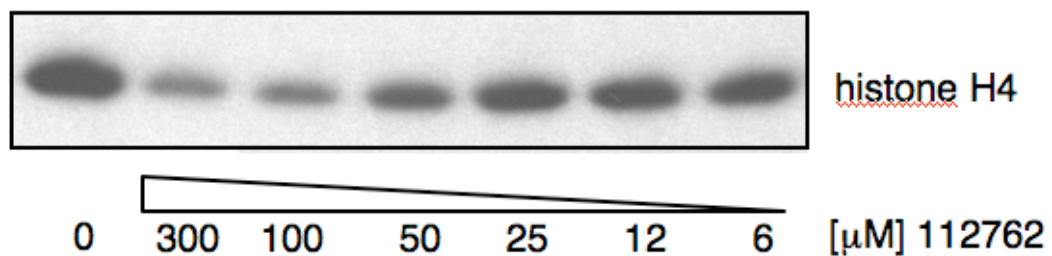
Figure 8: 112762 inhibits rTgPRMT1 in a dose-dependent manner.

Based on these preliminary data, we sought to determine whether parasites that overexpress TgPRMT1 are more resistant to Enhancer 112762 than wild-type parasites. TgPRMT1 containing a myc tag at its N-terminus was cloned under the tubulin promoter and transfected into wild-type parasites. Immunofluorescence performed with anti-myc showed that this enzyme localizes to the cytoplasm, which is consistent with its predicted localization (Figure 9A) (Saksouk et al., 2005). Additionally, myc-TgPRMT1 was immunoprecipitated from tachyzoites using anti-myc antibody and shown to methylate histone H4 (data not shown).

Western blot analysis of wild-type and myc-TgPRMT1-expressing parasites with anti-TgPRMT1 showed that the total amount of TgPRMT1 present in the myc-TgPRMT1-expressing parasites is several fold greater than that present in wild-type parasites (Figure 9B). However the preliminary results of a dual-fluorescence invasion assay showed that myc-TgPRMT1 over-expressing parasites are equally sensitive to 112762 at 50 µM when compared with wild-type parasites (data not shown).

Figure 9: Localization and level of expression of myc-TgPRMT1 in T. gondii.

A

anti-myc

B
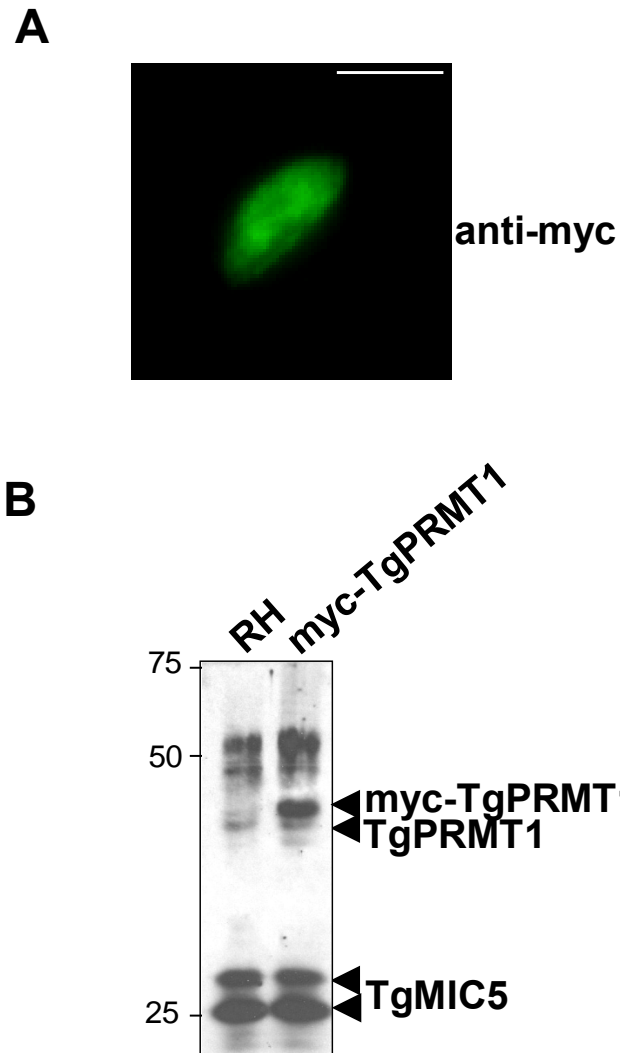
Figure 9: (A) As predicted based on previous studies with TgPRMT1, myc-TgPRMT1 is shown by immunofluorescence with anti-myc to be present in the cytoplasm of tachyzoites. Scale bar = 5 μm. (B) Western blot analysis of equal numbers of wild-type and myc-TgPRMT1-expressing parasites demonstrates nearly equivalent levels of TgPRMT1 the two strains, and a second band corresponding to myc-TgPRMT1 is seen directly above this band in myc-TgPRMT1-expressing parasites. TgMIC5 was used as a loading control and is present as a doublet, representing both the processed and unprocessed form of the protein.

Next, we sought to test whether myc-TgPRMT1 binds the active affinity resin to a greater extent than the inactive affinity resin when the resins are incubated with extract from myc-TgPRMT1-expressing parasites. After incubation with the resins for four hours, washing, boiling, and SDS-PAGE /western blot analysis of the samples with anti-myc, it was shown that a greater amount of myc-TgPRMT1 binds the active resin than binds the inactive resin (Figure 10). Additionally, more myc-TgPRMT1 remains in the supernatant after incubation of extracts with the inactive than the active resin, suggesting that myc-TgPRMT1 preferentially binds the active over the inactive affinity resin.

Figure 10: Affinity chromatography of wild-type and myc-TgPRMT1-expressing parasites followed by western blot analysis with anti-myc.
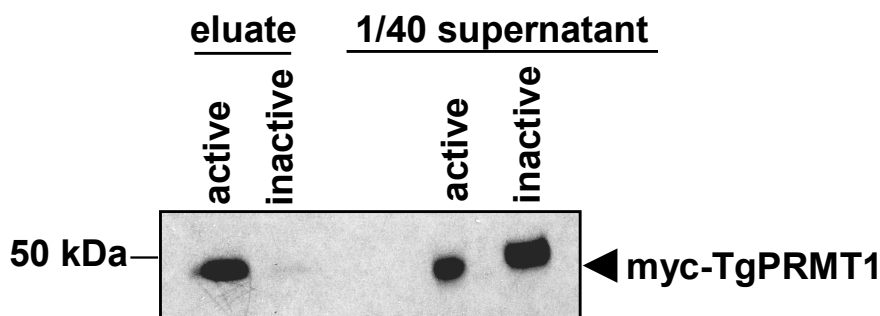
Figure 10: After incubation of extract from Myc-TgPRMT1-expressing parasites with the active and inactive affinity resins, the resins were washed and boiled in the presence of SDS. The eluate, as well as 1/40 of the supernatant, taken before boiling, were run on an SDS-PAGE gel followed by western blot analysis with anti-myc antibody. More myc-TgPRMT1 bound the active than the inactive resin. Additionally, the amount of myc-TgPRMT1 present in the supernatant after incubation with the active resin is less than that after incubation with the inactive resin. Controls (not shown) confirmed equal loading.

***In vivo* methylation of *T. gondii* proteins in the presence of 112762 and 112759**

In order to determine whether 112762 and/or 112759 have an effect on methylation of *T. gondii* proteins, freshly-lysed *T. gondii* tachyzoites were incubated with 112762, 112759, or DMSO in the presence of [^{3}H]-S-adenosyl-methionine for 2 hours before washing the parasites by centrifugation, boiling in 1% SDS, and running the samples on an SDS-PAGE gel followed by fluorography. It is evident that the amount of methylated proteins is less in samples treated with 112762 and 112759 than in samples treated with DMSO (Figure 11).

Figure 11: Inhibition of *in vivo* methylation by 112762 and 112759.
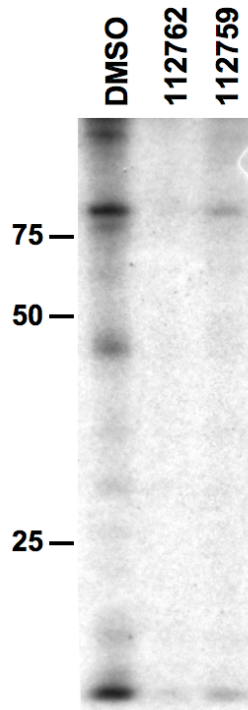
Figure 11: Extracellular parasites were incubated in high potassium buffer for 2 hours at 37°C in the presence of [^{3}H]-S-adenosyl-methionine, followed by the addition of DMSO, 112762, or 112759 (100μM). After an additional hour at 37°C, the samples were boiled in SDS-PAGE sample buffer. SDS-PAGE/fluorography shows decreased levels of methylation in the presence of both 112762 and 112759 compared to DMSO.

Generation of *TgPRMT1* knockout parasites

If parasites lacking TgPRMT1 were shown to have altered sensitivity to 112762 in comparison with wild-type parasites, this would argue strongly in favor of TgPRMT1 as an invasion-relevant target of 112762. Additionally, a *TgPRMT1* knockout could be useful for identification of TgPRMT1 substrates. With these goals in mind, we decided to attempt to generate a *TgPRMT1* knockout. 3 kb of genomic sequence located directly upstream and downstream of TgPRMT1 was cloned into pGRA1/ble such that the *ble* gene was replaced by the TgPRMT1 ORF (Figure 12). In the event that *TgPRMT1* knockout parasites cannot be generated, we will determine whether this is due to the inability of this knockout construct to be used for targeted disruption of *TgPRMT1* by homologous recombination, or whether *TgPRMT1* is an essential gene. The construct will be transfected into both wild-type RH and myc-TgPRMT1-expressing parasites; if the *TgPRMT1* locus can be disrupted in the myc-TgPRMT1-expressing parasites but not in wild-type parasites, this would suggest *TgPRMT1* is an essential gene.

Figure 12: Strategy for generation of *TgPRMT1* knockout parasites.
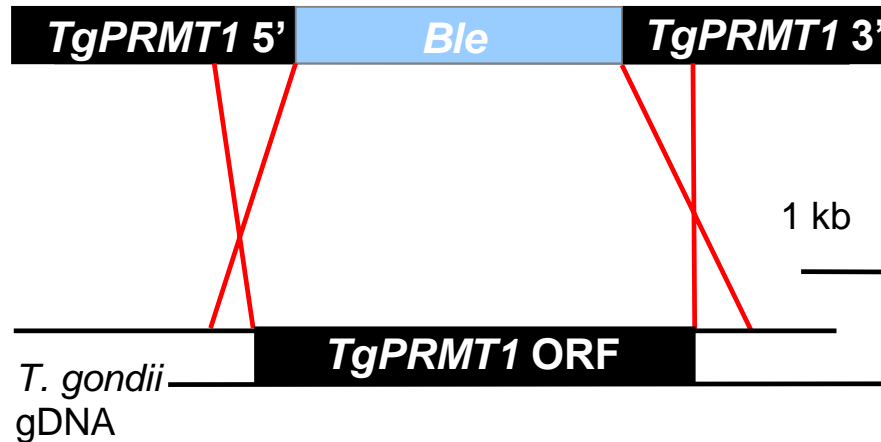
Figure 12: A plasmid has been generated in which the *TgPRMT1* ORF is replaced by *ble*. The construct will be electroporated into wild-type parasites in hopes that double homologous recombination will result in disruption of the *TgPRMT1*ORF with *ble*, which will then be used for selection.

DISCUSSION

The aim of this work was to identify the target(s) of motility and invasion Enhancer 112762 in *T. gondii*. Although the target of this compound has not been identified at this point, several putative targets have been identified. Additionally, much insight has been gained regarding target identification studies. Affinity resins were constructed upon which active and inactive derivatives of Enhancer 112762 were linked through N-succinimide ester linkages to Affi-Gel beads. Several strategies were taken in order to identify proteins that bind the active resin to a greater extent than the inactive resin. Because preliminary work with model substrates and 112762 indicated that this compound may interact covalently with its target (Morgan, 2006b), initial experiments

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were aimed at identifying a covalent target of 112762. After incubation of the active and inactive affinity resins with *T. gondii* extract, boiling the resins to remove all proteins not covalently bound, and trypsinization of peptides from covalently bound proteins on the resins, it was shown that TgProfilin was the only *T. gondii* protein that reproducibly bound the active but not the inactive resin. The possibility that TgProfilin may be a target of 112762 was alluring since actin polymerization is known to be important for invasion and motility and profilin is an actin-binding-protein known to be involved in actin polymerization. However, attempts to validate an interaction between 112762 and TgProfilin did not confirm this interaction.

Attempts to identify potential non-covalent targets of this compound were made by incubating the affinity resins with parasite extract, washing the resins in lysis buffer, and elution by boiling in 1% SDS sample buffer. As a result of this experiment, one strong band was shown to bind the active but not the inactive resin. When this experiment was scaled-up so that the gel containing the extra band could be stained by Coomassie, excised, and subject to mass spectrometry, the protein in this band was identified as TgFK506bp. It should be noted that there is a possibility that this protein is not the protein present in the extra band evident by ^{35}S-labeling parasite proteins; correlation of bands between these two different methods of detection was difficult since the profiles were not identical. Cloning of TgFK506bp-YFP and expression in *T. gondii* revealed that this fusion protein localizes to a discrete focus in the parasites. One focus can be seen in each parasite and the localization is not consistent with localization to the

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nucleolus or apicoplast. It is likely this localization reflects aggregation rather than the true distribution of the protein.

Based on a finding in the literature that a compound nearly identical to 112762 inhibited PRMT1 (Cheng et al., 2004), we sought to determine whether TgPRMT1 may be targeted by Enhancer 112762 using a variety of methods. The results of these experiments were inconclusive, and it this point it was determined that generation of a *TgPRMT1* knockout would be helpful in answering this question. In addition to providing a useful tool for validation of TgPRMT1 as a target of 112762, the *TgPRMT1* knockout parasites could be used to explore arginine methylation in *T. gondii*, a process about which little is known.

Although the target of Enhancer 112762 has not been definitively identified, numerous useful reagents have been generated for target identification of 112762. Additionally, the assumption-free approach of small molecule screening has led us to explore aspects of parasite biology (FK506bp, protein arginine methylation) we would not have pursued otherwise.

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CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS

TgPhIL1

Summary

The identification of TgPhIL1 by Stacey Gilk (Gilk *et al.*, 2006) opened the door for much of my work and left many questions to be answered. In order to learn more about the protein's function, and in the absence of sequence homology to proteins with known functions, a *TgPhIL1* knockout was generated with the hope that some observable phenotype would provide clues to the function of TgPhIL1.

Immediately upon generating the *TgPhIL1* knockout parasite, it was obvious that the shape of these parasites was different that than of wild-type parasites. *TgPhIL1* knockout parasites do not retain the arc-shape of wild-type parasites. Instead, they appear rounder. In order to confirm that this defect in shape was due to the absence of TgPhIL1 and not due to other changes in the parasite due to the integration of the knockout construct, *TgPhIL1* was reintroduced to the knockout parasite line under its endogenous promoter. The shape of this complemented parasite line was restored to that of wild-type parasites. Although the role of the parasite's arc shape in parasite biology is not understood, it is traditionally thought to be important for motility and invasion. In order to determine whether the altered shape of the parasite had an effect on *T. gondii* motility, trail assays were performed in which trails of protein deposited by the parasites were used to track the parasite's path. Trails deposited by *TgPhIL1* knockout parasites looked virtually identical to those deposited by wild-type parasites. It should be mentioned that although helical rotation and circular gliding can be visualized using trail

assays, upright twirling cannot. However, *TgPhIL1* knockout parasites have been seen twirling in culture, which confirms that they are able to perform all three forms of motility.

Next, we sought to determine whether the knockout parasites are able to invade and replicate within host cells to the same extent that wild-type parasites do. In culturing these parasites alongside wild-type parasites, it was obvious that any growth defect present would be subtle. In order to determine whether a slight defect was present, wild-type and knockout parasites were mixed in equal numbers and serially passaged through HFF cells. Immunofluorescence was used to count wild-type and knockout parasites after every three passages, and it was shown that wild-type parasites slowly outgrow *TgPhIL1* knockout parasites.

The fact that wild-type parasite outgrow *TgPhIL1* knockout parasites does not necessarily mean these parasites replicate at a slower rate than wild-type parasites. It is possible they show a defect in invasion, replication, egress, or longevity. We sought to determine whether the parasites show a defect in invasion using a quantitative laser-scanning cytometry-based invasion assay. Although the *TgPhIL1* knockout parasites were not shown to have a significant invasion defect, it is possible they show a very subtle defect that contributes to their being outgrown by wild-type parasites in tissue culture.

It was also shown that when mice are infected with wild-type and *TgPhIL1* knockout parasites, the amount of parasite DNA present in the liver and spleen is reduced compared to wild-type parasites, suggesting the *TgPhIL1* knockout parasite is less

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virulent in a mouse model of infection. Again, whether this is due to a defect in invasion, replication, egress, or other factors remains to be determined. What has been shown is that TgPhIL1 plays a role in maintaining the shape of *T. gondii* tachyzoites, and that parasites lacking this protein are outgrown by wild-type parasites in culture and show decreased fitness in a mouse model of infection.

We performed a number of additional experiments in order to characterize the *TgPhIL1* knockout parasites. We examined the ability of a number of apical and cytoskeletal antigens to localize properly in the *TgPhIL1* knockout parasite line. Our hope was that in identifying antigens that are unable to localize properly in the absence of TgPhIL1, we would uncover something about structures or proteins with which TgPhIL1 interacts. However, of the many antigens examined, all showed the same localization in the wild-type and *TgPhIL1* knockout parasite.

Because TgPhIL1 is thought to be membrane-associated but extracts like a cytoskeletal protein, one hypothesis regarding the function of TgPhIL1 is that it serves to link the inner membrane complex with the subpellicular microtubules or subpellicular network (Gilk et al., 2006). If this were the case, ultrastructural defects may be evident in its absence. In order to determine whether an ultrastructural defect was present in the knockout parasites, electron microscopy was performed on the *TgPhIL1* knockout parasite in parallel with wild-type parasites. No obvious difference was seen in the knockout parasites.

The *TgPhIL1* knockout line provides a valuable tool for determining the function of TgPhIL1 because an unlimited number of assays can be performed in which

the phenotype of wild-type and knockout parasites are compared and putative functions of TgPhIL1 can be ruled out or in based on the results of these assays. However, a limitation of this approach is that an unlimited number of assays can be performed, possibly without discovering differences between the two parasite lines. Other approaches to identification of proteins which interact with TgPhIL1, along with several other experiments aimed at elucidating the function of TgPhIL1 are underway and are described in the appendix. I will now describe several experiments which are not currently being performed, but which may be performed in the future.

Microarray Analysis

DIGE analysis has been performed in an attempt to identify proteins present at different levels or with different post-translational modifications in wild-type vs. *TgPhIL1* knockout parasites (Figure 4, Appendix). However, a major limitation of DIGE is that only the most abundant proteins are visible on 2D gels, so minor proteins present at very different levels in the two samples may not be identified. Microarray analysis is often used alongside DIGE and could provide valuable insight into the function of TgPhIL1. For example, if microarray analysis reveals that several proteins involved in carbohydrate synthesis are upregulated in *TgPhIL1* knockout parasites in comparison to wild-type parasites, *TgPhIL1* knockout parasites may be defective in carbohydrate metabolism and upregulation of these proteins may occur in an attempt to compensate for the absence of *TgPhIL1*.

For microarray analysis, *T. gondii* total RNA would be isolated and analyzed in order to determine the level of host cell RNA contaminating the sample since such contamination might affect the analysis. If the purity and quality of RNA from both wild-type and *TgPhIL1* knockout parasites proved to be acceptable, it would be hybridized to a photolithographic Affymetrix *T. gondii* GeneChip. These microarrays contain 11-fold coverage of the 8000 *T. gondii* protein-coding genes, including genes of the mitochondria and apicoplast. All probes present on the GeneChips have been mapped back to the *T. gondii* genome and this information is conveniently available online (www.toxodb.org).

As a result of these experiments, one would expect to gain information as to the function of TgPhIL1 based on changes in expression levels of other genes with assigned functions. However, one possible result is that no changes in expression are observed, other than an expected change in the expression of TgPhIL1. Even if this were the case, important information would have been gained since it would have been revealed that no compensatory changes take place in the absence of TgPhIL1. A more realistic result would be that changes in the expression levels of many genes would be observed. If these genes code for proteins with diverse functions, it could become difficult to determine which of these changes are actually relevant. One way to circumvent this problem would be to perform this experiment in duplicate or triplicate and focus on changes that are observed across every experiment, suggesting these changes are real and not due to experimental variability. Additionally, one could set a stringent threshold for transcriptional changes that are considered significant. For example, one could set a limit

of a 3- or 4-fold change in expression. The major limitation of these microarray

experiments is that they may result in the acquisition of too much information rather than

too little. However, with the help of a skilled biostatistician, these experiments may

reveal useful information that would not be obtained using other methods.

TgPhIL1 truncation mutants

It has been shown that the C-terminal half of TgPhIL1 localizes properly (Gilk

et al., 2006). However, this portion of the protein is highly insoluble, excluding the

possibility of performing immunoprecipitation with this portion of the protein. On the

other hand, the N-terminal half of the protein remains in the cytoplasm and does not

localize to the periphery or apical end of the parasite. However, this portion of the

protein is soluble. In light of this information, it might be possible to create a truncation

mutant which is both soluble and localizes properly. In order to do this, one could begin

with the N-terminal portion of TgPhIL1 and design primers that generate PCR products

coding for proteins that are 20, 40, 60, (etc.) amino acids longer. It might be possible to

identify the point at which the protein changes from soluble to insoluble, and improperly

to properly localized. Alternately, one could perform the same experiment starting with

the sequence coding for the C-terminal portion of the protein. Although tedious, these

experiments might provide one with a soluble, properly-localized version of TgPhIL1

with which to perform coimmunoprecipitation experiments in order to identify TgPhIL1-

interacting proteins. Additionally, these experiments would yield important information

as to the residues involved in proper localization and solubility for TgPhIL1.

Heterologous expression of TgPhIL1

There is some evidence that TgPhIL1 may interact with microtubules. The finding that its extraction profile mirrors that of a cytoskeletal protein and its localization at the parasite periphery where the subpellucular microtubules are present support this hypothesis. Stronger evidence comes from the fact that in several immunoelectronmicroscopy images, gold-particles appear to speckle the subpellicular microtubules (Gilk et al., 2006).

Biochemical assessment of an interaction between the subpellicular microtubules and TgPhIL1 in *T. gondii* is technically challenging since both proteins are insoluble and the subpellicular microtubules are unusually stable. However, if TgPhIL1 were to interact with microtubules in a heterologous expression system, this would provide stronger evidence that it is indeed a microtubule-associated protein. Additionally, it is difficult to hypothesize about the mechanism by which TgPhIL1 is trafficked to the parasite periphery since virtually nothing is known about trafficking to the IMC and associated structures. However, if TgPhIL1 could be expressed in another system and its localization in that system was studied, it might be possible to infer something about the trafficking of TgPhIL1 or structures with which it may associate. HeLa cells would provide a simple system in which to perform this work due to the ease of transformation and the wide array of tools available for protein expression in this system.

Potential TgPhIL1-interacting protein (38.m01070)

A genome-wide yeast-2-hybrid screen has been performed in *Plasmodium falciparum* and the results of this screen are freely available online (www.plasmodb.org). Although a similar screen has not been performed for *T. gondii*, a homologue of TgPhIL1 is present in *P. falciparum* (PFA0440w). One predicted protein was shown to interact with PfPhIL1 (PFC0325w) in the yeast-2-hybrid screen. Like TgPhIL1, this protein is annotated as a protein of unknown function conserved among apicomplexan parasites. However, a *T. gondii* homologue of this protein does exist (38.m01070). The fact that this protein also appears to be apicomplexan-specific may be both fortunate and unfortunate. On the one hand, it seems likely that TgPhIL1, an apicomplexan-specific protein likely localized to an apicomplexan-specific organelle, would interact with other apicomplexan-specific proteins. On the other hand, working with these apicomplexan-specific proteins has proven extremely challenging. Also adding to the difficulty of working with 38.m01070 is the fact that several conflicting gene models exist for this protein which might make cloning the gene encoding this protein more difficult. However, EST data are available for this protein and using this information in combination with rapid amplification of cDNA ends (RACE), it should be possible to clone the correct coding sequence. A PBLAST search could then be performed against this protein in hopes that the correct sequence would bear resemblance to proteins with assigned functions.

Once the gene has been cloned, a number of experiments could be performed in order to confirm binding of 38.m01070 to TgPhIL1. For example, the yeast-2-hybrid

result could be confirmed in *T. gondii*. Additionally, it may be possible to perform fluorescence resonance energy transfer (FRET) (Kerppola, 2006) or bimolecular fluorescence complementation (BIFC) (Kerppola, 2008) in order to confirm an interaction between these two proteins. However, these experiments are unlikely to work given the fact that no information is available as to the nature of the potential interaction between these two proteins. More preliminary experiments may include determining the subcellular localization of 38.m01070 in order to determine whether it colocalizes with TgPhIL1. This could be performed by expressing a version of the protein tagged with YFP and directly visualizing the distribution of the protein, provided the addition of YFP does not disrupt the solubility or trafficking of this protein. An alternative would be to express this protein with a smaller epitope tag such as myc or $(His)_6$ which is less likely to disrupt the normal trafficking and solubility of the protein. It would be important to tag the protein at both the N- and C-termini to ensure that even these smaller and better-tolerated tags do not alter the localization of the protein. Any localization should also be confirmed by an independent method such as immunofluorescence with serum raised to the antigen. As always, a pre-bleed control should be performed.

In the event that 38.m01070 is shown to interact with TgPhIL1, an attempt would be made to determine the function of this protein as well. A number of experiments would be performed to this end, including an attempt to knock-out this protein. Characterization of the knockout in the case that 38.m01070 is non-essential would be performed in a manner similar to that of the *TgPhIL1* knockout. Additionally, the *P. falciparum* homologue of 38.m01070 was shown to interact with five other

proteins in the genome-wide yeast-2-hybrid screen, and a proposed function is assigned

for several of these proteins. For example, two of the proteins are predicted kinases and

one is predicted to be a phosphatase. It might be possible to use this information in order

to determine the function of 38.m01070 and ultimately, of TgPhIL1. Additionally, it may

be informative to knock this protein out in *T. gondii* and determine whether the

phenotype is consistent with that of *TgPhIL1* knockout parasites. This result may argue

in favor of an interaction between TgPhIL1 and this uncharacterized protein.

Bioinformatics

Although we consider TgPhIL1 to be an apicomplexan-specific protein, it is

hard to imagine that it arose *de novo* and truly shares no homology to non-apicomplexan

proteins. A more likely scenario is that TgPhIL1 is related to proteins present in

organisms for which sequence data is not available. Genomes are being sequenced on an

ongoing basis and this newly acquired sequence information is continuously added to

databases. It might just be a matter of time before sequences are deposited which bear

homology to TgPhIL1. Whether or not these proteins will have predicted functions and

will aid in assigning a function to TgPhIL1 is another matter.

Although the use of bioinformatics in order to predict the function of TgPhIL1 is

not particularly useful at this point due to its extremely weak homology with non-

apicomplexan proteins, I have recently discovered limited sequence similarity between

TgPhIL1 and a conserved domain, that of a metallodependent hydrolase. Many proteins

are considered to be metallodependent hydrolases including DNase, cytosine deaminase,

and magnesium cheletase. Whether TgPhIL1 is truly a hydrolase or metallodependent has yet to be determined. However, this example does provide proof that over time and as more and more sequences are added to databases, patterns may emerge and the function of TgPhIL1 may be revealed. Additionally, the possibility that TgPhIL1 may be related to DNase, which has been shown to bind actin (Morrison and Dawson, 2007), is intriguing since TgPhIL1 is localized to the periphery of the parasite where short actin filaments are known to be present. Arguing against this hypothesis, however, is the finding that rather small perturbations in actin polymerization in *T. gondii* lead to profound changes in motility and invasion (Wetzel et al., 2003). The fact that *TgPhIL1* knockout parasites are devoid of obvious defects in invasion or motility make this hypothesis less likely.

As mentioned in Chapter 1 of this thesis, TgPhIL1 does contain a domain conserved among bacterial proteins of unknown function (http://pfam.sanger.ac.uk/). However, since this domain is only found in hypothetical bacterial proteins with no known function, this information is hardly helpful in assigning a function to TgPhIL1. If however, the function of one of these bacterial proteins is identified, this might point to a potential function for TgPhIL1.

TARGET IDENTIFICATION OF 112762

Summary

Although the target of 112762 has not been identified, several potential targets have been. Additionally, we have gained a great deal of knowledge concerning target identification studies. Originally, we set out to identify the target of 112762 using a technique in which a modified version of 112762, along with an inactive but nearly identical derivative of the compound, was linked to Affigel beads. Parasite extract was incubated with the beads and the resins were washed extensively. In order to identify covalent targets of 112762, the beads were boiled in SDS to remove non-covalent binders, followed by trypsinization of peptides from the resins and mass spectrometry in order to identify the peptides. As a result of these experiments, profilin was identified as a putative target of 112762. However, attempts at validating an interaction between profilin and 112762 did not confirm this result.

Attempts were also made to identify non-covalent targets of 112762. After incubation of the resins with parasite extract and extensive washing of the resins, the resins were boiled in SDS and the eluate was run on an SDS-PAGE gel and visualized. Bands present in the sample incubated with the active compound but not the inactive compound were excised from the gel and sent for mass spectrometry. As a result of these experiments, an FK506-binding protein (55.m00139) was identified as a putative target of 112762. This protein was expressed under the tubulin promoter with a YFP tag at its C-terminus. When parasites stably expressing this construct were observed by fluorescence microscopy, the protein appeared in aggregates within the cytoplasm. It is possible this aggregation is caused by overexpression from the strong tubulin promoter or

by the large YFP tag attached at the C-terminus. No further experiments were conducted in order to validate FK506-binding protein as a target of 112726. Additionally, the supply of affinity resins has been exhausted and will require time-consuming synthesis by our collaborators in Scotland.

In parallel with these affinity chromatography experiments, the hypothesis that 112762 affects protein arginine methylation within the parasite has been explored. This hypothesis is based on the finding that a structurally related compound, 112759, inhibits both human and yeast PRMT1 (Cheng *et al.*, 2004). In order to test this hypothesis, parasites expressing myc-TgPRMT1 have been made and tested for a change in sensitivity to 112762 compared to wild-type parasites. No such change was detected. However, TgPRMT1 was shown by affinity chromatography followed by western blot analysis to bind the active resin preferentially over the inactive resin. Additionally, 112762 was shown to inhibit methylation of several substrates when parasites are treated with tritiated SAM, lysed, and run on an SDS-PAGE gel for analysis. The results of these studies suggest that TgPRMT1 may be a *bona fide* target of 112762. Several experiments aimed at validating TgFK506bp and TgPRMT1, as well as methods that could be used to identify other targets of 112762 will now be described.

Further studies on FK506-binding protein (55.m00139)

FK506-binding protein is predicted to be located in the nucleus (www.toxodb.org). If FK506-binding protein is truly located in this compartment, it is difficult to imagine how its interaction with 112762 could enhance parasite invasion on

the time-scale in which invasion occurs (10-30 seconds). However, if FK506-binding protein is actually localized to a secretory organelle or the cytoplasm, a role for this protein in invasion seems more likely. Because predicting the subcellular localization of *T. gondii* proteins is not an easy task, we sought to confirm the localization of FK506bp experimentally. A version of FK506-binding protein tagged at the C-terminus with YFP was expressed under the strong tubulin promoter and stably expressed in tachyzoites. By fluorescence microscopy, this protein localized to one discrete, round focus within the parasite cytoplasm distinct from the apicoplast or nucleolus. It is likely this distribution reflects aggregation rather than the true localization of the protein, and this could be due either to the presence of the large YFP tag or to expression under the tubulin promoter. In order to confirm the localization of this protein, it could be expressed under its endogenous promoter and with a smaller tag, such as a myc or (His)$_6$ tag, at either its N- or C-terminus. If either tagged version shows a distribution other than a cytoplasmic inclusion, this localization could be confirmed using anti-serum directed at purified, recombinant TgFK506bp and used to determine the localization within the parasite. This would circumvent the problem of mislocalization due to even a small tag. If the localization is shown to be interesting and a role for FK506-binding protein in *T. gondii* seems plausible, this protein would warrant further investigation as a potential target of 112762. Additionally, it would be interesting to determine whether the localization of the proteins changes in response to treatment with Enhancer 112762.

In order to validate FK506-binding protein as a target of 112762, a number of experiments could be performed. Although at this point affinity chromatography resins

are not available, it is possible they might become available in the future. In this event, affinity chromatography with whole parasite extract could be performed, followed by running of the eluate on an SDS-PAGE gel and western blotting with an antibody to TgFK506-binding protein. If a greater amount of TgFK506-binding protein is found to bind the active resin than the inactive resin, this would argue in favor of an interaction between 112762 and FK506bp.

Of course, this interaction would have to be confirmed in a number of different ways and even if it was confirmed, TgFK506bp would have to be confirmed as the invasion-relevant target of 112762. One way to accomplish this would be to generate *TgFK506-bp* knockout parasites and test their sensitivity to enhancer 112762. If these parasites are resistant to 112762 in comparison with wild-type parasites, TgFK506bp is a likely target of 112762. A more stringent way to confirm the relevance of this protein would be to determine the precise residues of TgFK506bp involved in binding to 112762. If these residues are not required for activity of TgFK506bp, it may be possible to mutate these residues so that although the protein retains its function, it cannot bind 112762. If parasites expressing this mutated version of TgFK506bp in place of wild-type FK506bp are resistant to 112762, TgFK506bp will have been confirmed as a relevant target of 112762. The role of this protein in *T. gondii* invasion would then be explored in detail.

Further studies on TgPRMT1

Another putative target of 112762 is TgPRMT1. *TgPRMT1* knockout parasites are being generated and will be tested in an invasion assay in order to determine whether

these parasites are more resistant than wild-type parasites to enhancer 112762. If these

parasites are not more resistant to 112762, it is still possible that TgPRMT1 is a target of

112762, but that in its absence, other protein arginine methyltransferases play a

compensatory role. Since five putative arginine methyltransferases have been identified

in *T. gondii* (Saksouk et al., 2005), knocking out all of these proteins would be a

laborious task as the genes encoding these proteins are not clustered within the genome.

However, in the event that *TgPRMT1* knockout parasites are not resistant to 112762, it

would be important to perform qRT-PCR or microarray analysis to determine whether the

other arginine methyltransferase(s) are upregulated in the absence of TgPRMT1. If this

were shown to be the case, a double-knockout of TgPRMT1 and this other arginine

methyltransferase could be generated and tested for an alteration in sensitivity to

TgPRMT1 in comparison with wild-type parasites.

In the event that arginine methyltransferases are not shown to be likely targets of

112762, the generation of a *TgPRMT1* knockout parasite will provide the field with

useful reagents for learning more about protein arginine methylation in *T. gondii*. For

example, protein methylation could be compared in these parasites vs. wild-type

parasites. These experiments could be performed by incubating wild-type and *TgPRMT1*

knockout parasites with [^{3}H]-S-adenosyl-methionine and comparing the proteins labeled

by SDS-PAGE/fluorography to identify proteins that may be methylated by TgPRMT1.

An alternate approach to this experiment would be to treat parasites with [^{3}H]-

methylmethionine, which in contrast to [^{3}H]-S-adenosyl-methionine, is known to be cell-

permeable (Barth et al., 2003). One drawback of this approach is that when treating

parasites with [3H]-methylmethionine, one must also treat with an inhibitor of protein synthesis so that [3H]-methylmethionine is not incorporated into newly synthesized proteins, confounding the results of these experiments (Barth et al., 2003).

In addition to providing us with a valuable tool with which to study methylation in *T. gondii,* we hope that others in the field will be able to use the TgPRMT1 knockout parasites to study other aspects of parasite biology such as differentiation (Saksouk *et al.*, 2005).

Further work on identifying the target of enhancer 112762

In addition to affinity chromatography, many approaches are available for target identification. Since extensive structure activity relationship (SAR) work has already been performed on this compound, it would be possible to modify this compound in order to facilitate target identification. For example, yeast-3-hybrid (Gallagher et al., 2007) analysis could be performed. Alternately, the compound could be biotinylated, radiolabeled, or modified for click chemistry (Hathaway and King, 2005). Additionally, frequent reviews of the literature and SciFinder searches should be performed in the event that this compound or a structurally-related derivative is shown to be active in an assay performed in another system. This information could point to potential targets of enhancer 112762 in *T. gondii* and ultimately, in other apicomplexan parasites.

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APPENDIX

INTRODUCTION

The generation and characterization of *TgPhIL1* knockout parasites, described in

Chapter 2 of this thesis, was performed in an attempt to gain a better understanding of the

function of TgPhIL1. Our hope was that in performing a number of assays in both wild-

type and *TgPhIL1* knockout parasites, a deficiency or defect in the knockout would be

revealed, and that this phenotype might offer clues to the function of TgPhIL1. As a

result of those experiments, it was shown that TgPhIL1 knockout parasites have an

altered shape and appear rounder than wild-type parasites. Additionally, it was shown

that these parasites are outgrown by wild-type parasites when the two lines are mixed

together in equal amounts and passaged through confluent monolayers. Finally, it was

shown that mice infected with *TgPhIL1* knockout parasites have a lower parasite load in

their livers and spleens than mice infected with wild-type parasites.

These results have provided us with some information regarding the function of

TgPhIL1. We know that the protein is required for normal parasite shape and in order for

the parasite to replicate in cells at the same rate as wild-type parasites. We have

preformed a large number of phenotypic assays comparing wild-type and *TgPhIL1*

knockout parasites, and one approach to learning more about the function of TgPhIL1

would be to continue to perform phenotypic assays. For example, we could determine

whether parasites lacking *TgPhIL1* are defective in glucose uptake or metabolism, ATP

production, lipid synthesis, proteolytic processing of surface adhesins, translation, N-

linked glycosylation, suppression of host-cell apoptosis, vacuole formation, or ability to undergo the sexual cycle within the cat. However, obtaining useful information with this strategy is much like trying to find a needle in a haystack.

In parallel with phenotypic assays, several experiments have been performed in an attempt to identify proteins with which TgPhIL1 might interact. If TgPhIL1 were shown to bind a protein with a known function, this might aid in identifying the function of TgPhIL1. Additionally, an attempt has been made to analyze the proteome of wild-type and *TgPhIL1* knockout parasites in the event that other proteins play compensatory roles in the absence of TgPhIL1. This might explain why TgPhIL1 is a non-essential gene, and why the phenotype of *TgPhIL1* knockout parasites is rather subtle. This section provides a description of phenotypic assays performed on *TgPhIL1* knockout parasites not described elsewhere in this thesis, in addition to a number of other experiments designed to assign a function to TgPhIL1.

MATERIALS AND METHODS

Differential In Gel Electrophoresis (DIGE) analysis

1.5 X 10^7 wild-type and *TgPhIL1* knockout parasites were harvested and resuspended in 50µl lysis buffer (10 mM Tris-HCl), followed by incubation at 25°C for 10 minutes and centrifugation at 12000 X g to remove insoluble material. 0.5 µl CyDye™ DIGE Fluor Cy™5 or CyDye™ DIGE Fluor Cy™3 (GE Healthcare) dye was added to a final concentration of 200 µM and the samples were incubated in the dark for

30 minutes at 25°C. 1µl of 1 mM lysine was added to stop the labeling reaction and the samples were incubated for another 10 minutes at 25°C, followed by mixing of the two samples and addition of 150 µl rehydration buffer. The samples were spun at 14000 x *g* to remove insoluble material, followed by active rehydration overnight at 50V in a Protean IEF Cell (BioRad) and resolved on an 11 cm pH4-7 Immobiline DryStrip (Amersham), followed by SDS-PAGE.

Localization of other antigens in *TgPhIL1* knockout parasites

The distribution of several antigens in the *TgPhIL1* knockout was examined. Immunofluorescence with anti-doublecortin domain containing protein (TgDCXP) was performed on extracellular tachyzoites after permeabilization and fixation with 100% MeOH on ice for 5 minutes. Rabbit anti-TgDCXP was generously provided by Ke Hu. The antiserum was used at a 1:1000 dilution. Anti-SAG1 was also used to identify the parasite plasma membrane. Immunofluorescence with rabbit anti-TgIMC3, generously provided by Marc-Jan Gubbels, was performed as described for anti-TgDCXP except that intracellular rather than extracellular parasites were processed. Anti-TgIMC3 was used at a 1:500 dilution. The *T. gondii* dynein light chain, TgDLC, was cloned previously and plasmids encoding TgDLC-YFP were provided by Ke Hu. This plasmid was electroporated into wild-type (data not shown) and *TgPhIL1* knockout parasites.

Cholesterol distribution

Wild-type and *TgPhIL1* knockout parasites were incubated with 10 μM

cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate

(BODIPY-CE, Molecular Probes) in intracellular buffer (IB) (Krausse et al) for 2 hours at

25°C before being washed in 1ml PBS by centrifugation and visualized by fluorescence

microscopy.

Cloning of TgPhIL1-Nterm and TgPhIL1-Nterm-YFP

DNA sequence encoding the first 80 amino acids of TgPhIL1 was amplified using

pTubPhIL1-YFP as a template and the primers TgPhIL1 BglII For

(5'GGA<u>AGATCT</u>ATGTCGCAGCCAAACCTCC'3)

and TgPhIL 1-80 AvrII Stop rev (5' GGT<u>CCTAGG</u>TCACCCATCTGACAATGGAAC).

The product of this PCR was ligated into pIMC-YFP in place of IMC, which was

removed by restriction digest with BglII and AvrII, forming pPhIL1-Nterm/cat. TgPhIL

1-80 AvrII Stop rev contains a stop codon so YFP is not translated. This construct was

transfected into *TgPhIL1* knockout parasites and cloned by limiting dilution. Clones

shown by immunofluorescence with anti-TgPhIL1 to stably express this construct were

isolated by limiting dilution for further analysis. An identical procedure was followed to

clone TgPhIL1-Nterm-YFP, except the reverse primer used for this cloning was TgPhIL

1-80 AvrII (5'CCCATCTGACAATGGAAC'3) which does not contain a stop codon,

allowing for translation of YFP. This construct was transfected into both wild-type RH

and *TgPhIL1* knockout parasites which were screened for fluorescence and stable clones

were isolated. Western blot analysis and immunofluorescence with ant-TgPhIL1 was

performed as described previously (Chapter 2, Material and Methods).

Immunoprecipitation of TgPhIL1-Nterm-YFP with anti-TgPhIL1 antibody

Wild-type and *TgPhIL1* knockout parasites stably transfected with pTgPhIL1-N-

term-YFP were extracted in 1% Triton-X100 and centrifuged at 14000 x *g* to remove the

insoluble material, followed by incubation of the soluble fraction with rabbit anti-

TgPhIL1 antibody. After incubation with antibody for 1 hour at 25°C, recombinant

Protein A-Sepharose beads (Zymed) were added and incubation was allowed to proceed

for another hour at 25°C. The beads were then washed extensively, boiled in sample

buffer containing 0.1% SDS, and the eluate was run on an SDS-PAGE gel followed by

autoradiography.

RESULTS

DIGE

In order to evaluate whether proteomic changes occur in the absence of TgPhIL1,

total parasite protein extracted from wild-type and *TgPhIL1* knockout parasites could be

run on separate 2 dimensional (2D) gels and compared. A major limitation of this

approach is that correlation of spots between two 2D gels can be difficult since proteins

may resolve in a slightly different manner on the two gels. Additionally, identification of

subtle changes in protein abundance between two gels is not trivial. In order to

circumvent these limitations, a technique known as DIGE has been developed (Unlu *et al.*, 1997)in which two different samples of protein can be visualized on the same gel by labeling proteins from each sample using different fluorophores (Cy3 and Cy5). This experiment was performed with wild-type and *TgPhIL1* knockout parasites (Figure 1) in order to look for proteomic changes present in the absence of TgPhIL1.

Figure 1: Results of DIGE analysis of wild-type and *TgPhIL1* knockout parasites.
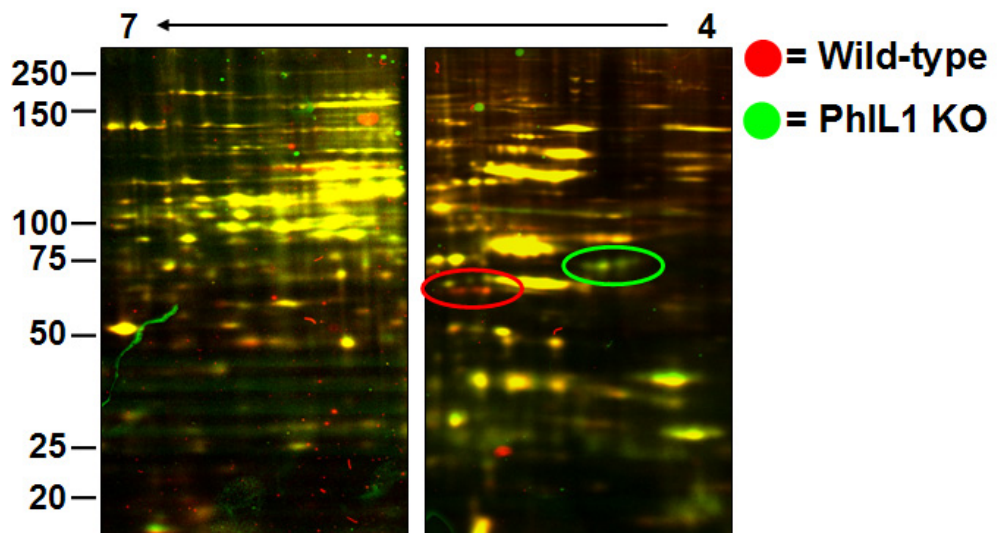
Figure 1: Proteins from wild-type and *TgPhIL1* knockout parasites were solubilized, labeled with either Cy3 or Cy5 dye, and resolved on a 2D gel. Proteins from wild-type parasites appear red and proteins from *TgPhIL1* knockout parasites appear green. Proteins present in equal amounts in both samples appear yellow. Two red spots are present at approximately 60 kD (circled in red) and two green dots are present at 70 kD (circled in green).

Two red spots are visible in the range of 60 kD and two green spots present at approximately 70 kD, suggesting that these proteins are present at different levels in the two samples. Alternately, is possible that the proteins present in the two green spots are the same as those present in the two red spots, but that they are modified differently in the two samples. The appearance of these four spots across multiple DIGE experiments is not consistent. Nonetheless, attempts to repeat these experiments, possibly on a narrower pH range, and to identify the corresponding proteins are underway.

Localization of cytoskeletal antigens in *TgPhIL1* knockout parasites

Since TgPhIL1 is distributed at the periphery and apical end of the parasite, we sought to determine whether the localization of other antigens with similar distributions is altered in the absence of TgPhIL1, suggesting that TgPhIL1 is required for trafficking or targeting of these proteins to their respective locations within the parasite. TgDCXP is a doublecortin domain-containing protein that has been shown to localize to the conoid of developing daughter parasites (Ke Hu, personal correspondence). Both TgDCXP and TgPhIL1 localize to the apical end of developing daughter parasites. Additionally, doublecortin is a microtubule-binding protein (Gleeson et al., 1999) and TgDCXP is though to be associated with the *T. gondii* cytoskeleton. This piqued our interest in examining the localization of TgDCXP in *TgPhIL1* knockout parasites. However, immunofluorescence with rabbit anti-TgDCXP demonstrated normal localization of this antigen in *TgPhIL1* knockout parasites (Figure 2).

Additionally, the localization of TgIMC3, a cytoskeletal protein that shows homology to TgIMC1 and localizes to the IMC of developing daughters (Gubbels et al., 2004), was explored in *TgPhIL1* knockout parasites by immunofluorescence with anti-TgIMC3 and shown to be indistinguishable from that of wild-type parasites. Also, localization of a YFP-tagged version of *T. gondii* dynein light chain, TgDLC-YFP (Hu *et al.*, 2006), was determined by transfecting wild-type and *TgPhIL1* knockout parasites with a plasmid encoding this antigen under the control of the tubulin promoter (pCNATgDLC-YFP, provided by Ke Hu). The localization of this antigen to the extreme apical end of *TgPhIL1* knockout parasites is consistent with that reported in wild-type parasites (Hu et al., 2006).

Figure 2: Localization of cytoskeletal antigens in *TgPhIL1* knockout parasites.
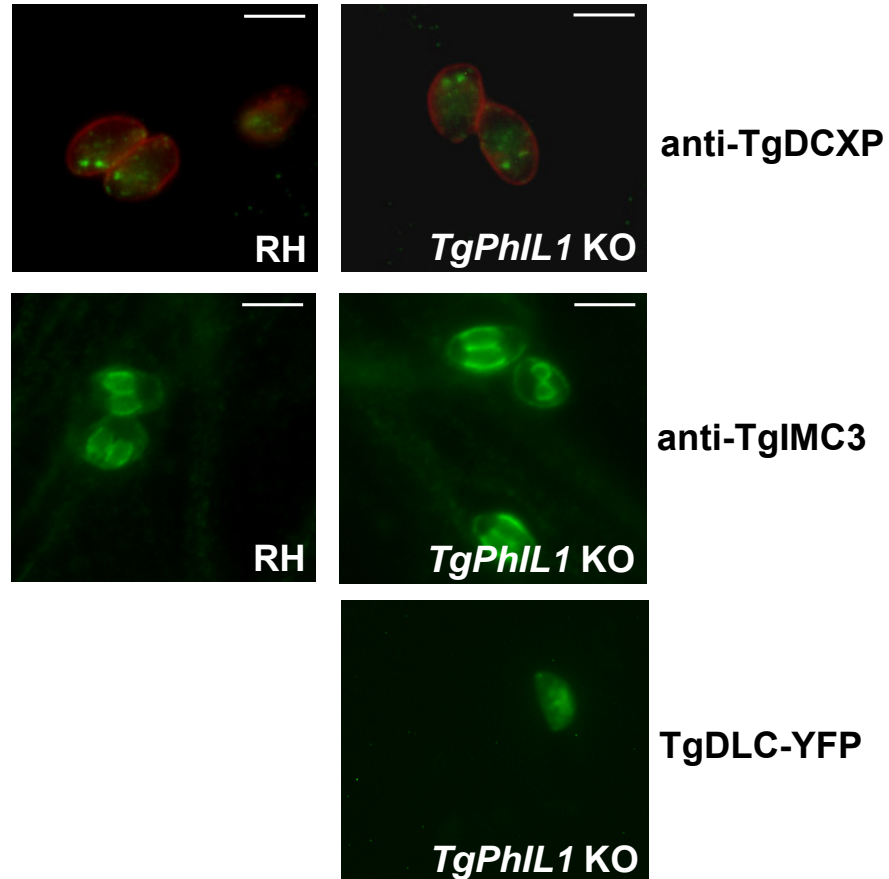
Figure 2: The distribution of several proteins with known patterns of localization were examined in *TgPhIL1* knockout parasites. TgDCXP (top panels), TgIMC1 (middle panels), and TgDLC-YFP (bottom panel) localize properly in the knockout. All cytoskeletal antigens described are shown in green. In the TgDCXP images, TgSAG1, which localizes to the plasma membrane, is shown in red. The distribution of TgDLC-YFP in wild-type parasites is not shown, but is indistinguishable from that reported previously (Hu et al., 2006) and in *TgPhIL1* knockout parasites.

Cholesterol distribution

The distribution of cholesterol in *T. gondii* was shown to be peripheral and

concentrated at the apical end of the parasite by incubating live parasites with BODIPY-

CE (Johnson et al., 2007). Because of the similarity between this distribution and that of

TgPhIL1, the distribution of BODIPY-CE in *TgPhIL1* knockout parasites was visualized

using similar methods. However, the distribution of BODIPY-CE was indistinguishable

in the two strains (Figure 3), suggesting that TgPhIL1 does not affect the distribution of

cholesterol within the parasite.

Figure 3: Distribution of cholesterol in the *TgPhIL1* knockout parasite.
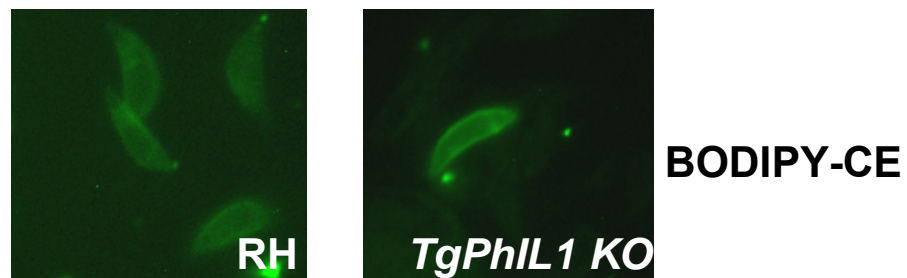
Figure 3: Distribution of cholesterol in wild-type and *TgPhIL1* knockout parasites was examined by treating extracellular parasites with BODIPY-CE in high potassium intracellular buffer. BODIPY-CE was distributed in a similar manner in wild-type and *TgPhIL1* knockout parasites.

TgPhIL1-Nterm-YFP

Since TgPhIL1 is soluble only in 1% SDS and extraction under these conditions

would denature the protein and likely disrupt any interactions between it and other

proteins, coimmunoprecipitation studies with the full-length protein are impossible.

However, the N-terminal portion of TgPhIL1 is soluble (Gilk, 2005), making

coimmunoprecipitation with this portion of the protein technically feasible. The first 80

amino acids of TgPhIL1 fused to YFP were cloned under the tubulin promoter and this

construct was electroporated into *TgPhIL1* knockout and wild-type parasites. Although

this portion of the protein is soluble, it does not properly localize in either parasite line as

determined with an anti-TgPhIL1 antibody (Figure 4A). Rather than being present at the

periphery of the parasite, it is present in the cytoplasm. However, it is possible that

although it mislocalizes, it may still interact with proteins with which it normally

interacts. In order to identify proteins that interact with TgPhIL1-Nterm-YFP,

immunoprecipitation was performed with anti-TgPhIL1 (Figure 4B).

Figure 4: Distribution of and immunoprecipitation with TgPhIL1-Nterm-YFP.

A

TgPhIL1-Nterm-YFP

RH

TgPhIL1 KO

B
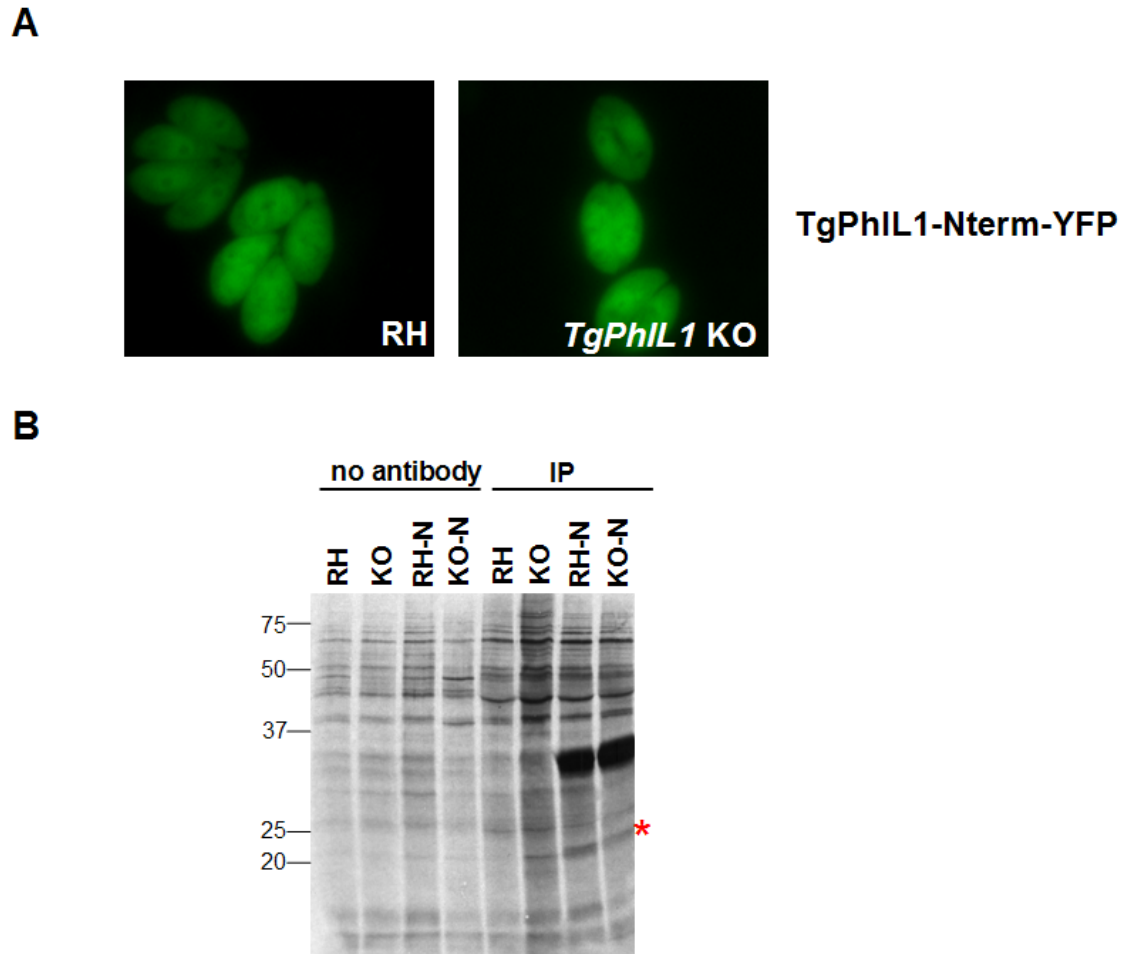
Figure 4: (A) N-TgPhIL1-YFP was expressed in wild-type and *TgPhIL1* knockout parasites and shown to be localized to the cytoplasm in both strains. (B) ^{35}S-labeled wild-type and *TgPhIL1* knockout parasites stably expressing TgPhIL1-Nterm-YFP were extracted in 1% Triton-X100, centrifuged to remove insoluble material, and incubated with anti-TgPhIL1, followed by incubation with recombinant Protein A-Sepharose beads. The beads were washed and then boiled in sample buffer containing 1% SDS, and the eluate was run on an SDS-PAGE gel and visualized by silver stain. The transfected parasites are indicated by RH-N or KO-N (vs. untransfected RH and KO). Controls lacking antibody were performed (left 4 lanes). The red star indicates a protein that appears to be present in the transfected but not the untransfected parasites, suggesting it may interact with TgPhIL1-Nterm-YFP.

The identification of a band present after immunoprecipitation with parasites expressing the soluble TgPhIL1-Nterm-YFP protein is promising. However, the protein may interact with the YFP-tag rather than with the TgPhIL1 portion of the protein. In order to determine whether this is the case, TgPhIL1-Nterm has been cloned without a YFP tag and expressed in wild-type and *TgPhIL1* knockout parasites. If stable transfectants could be generated, we aimed to repeat the immunoprecipitation experiments with these parasites and determine whether the extra band was still visible, suggesting the protein may interact with the N-terminal portion of TgPhIL1 and warrant further investigation. Unfortunately, *TgPhIL1* knockout parasites transiently expressed this protein, but we were unable to obtain stable transfectants. The distribution of TgPhIL1-Nterm-YFP in *TgPhIL1* knockout parasites is cytoplasmic. It is difficult to determine by anti-TgPhIL1 immunofluorescence whether the wild-type parasites express this protein since full-length TgPhIL1 is identified by the antibody. Western blot analysis should be performed on these parasites to determine whether they stably express TgPhIL1-Nterm since they could be used for immunoprecipitation if TgPhIL1-Nterm remains soluble in wild-type parasites.

Figure 5: Transient expression of TgPhIL1-Nterm in wild-type and *TgPhIL1* knockout parasites.
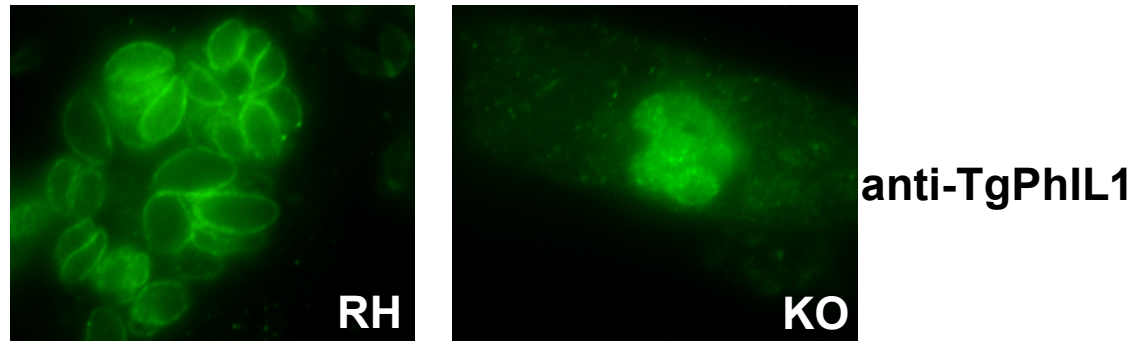
Figure 5: *TgPhIL1* knockout parasites failed to express TgPhIL1-Nterm stably. However, transient transfectants were obtained. TgPhIL1-Nterm was shown to be present in the cytoplasm by immunofluorescence with rabbit anti-TgPhIL1.

Since TgPhIL1-Nterm cannot be stably expressed, the immunoprecipitation experiments shown in Figure 4B should be repeated. Parasites expressing YFP-YFP under the tubulin promoter (Mann *et al.*, 2002) should be used as a control for binding to the YFP tag.

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