Title
Functional dissection of molecular mechanisms underlying host invasion and replication in the obligate intracellular pathogen Toxoplasma gondii

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Publication Date
2012

Peer reviewed|Thesis/dissertation
Functional dissection of molecular mechanisms underlying host invasion and replication in the obligate intracellular pathogen *Toxoplasma gondii*

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Microbiology, Immunology and Molecular Genetics

by

Joshua Ryan Beck

2012
ABSTRACT OF THE DISSERTATION

Functional dissection of molecular mechanisms underlying host invasion and replication in the obligate intracellular pathogen *Toxoplasma gondii*

by

Joshua Ryan Beck

Doctor in Philosophy in Microbiology, Immunology and Molecular Genetics

University of California, Los Angeles, 2012

Professor Peter J. Bradley, Chair

Apicomplexans are a large group of obligate intracellular parasites including *Plasmodium falciparum*, the causative agent of human malaria, and *Toxoplasma gondii*, an important pathogen of immunocompromised individuals. *T. gondii* is capable of replicating in a broad range of host cells and establishes its infection by actively invading its host cells. This invasion process is mediated by the rhoptries, unique apical secretory organelles that inject a complex of proteins into the host cell to facilitate penetration through formation of a tight-junction interface between the parasite and host called the moving junction (MJ). During the invasion process, a parasitophorous vacuole is created within which the parasite resides, evading destruction by host lysosomes. Sequestered within this vacuole, the parasite is free to divide by a unique process of
internal budding known as endodyogeny wherein two daughter parasites form within a mother cell. Following repeated rounds of replication, the host is disrupted and the parasites egress out to re-invade neighboring cells, beginning this lytic cycle anew. A better understanding of these unique processes of host cell invasion and parasite replication are needed as the pathology caused by *T. gondii* and other apicomplexans is dependent on these processes.

In the first section of this dissertation, I describe the discovery and functional analysis of a *Toxoplasma* palmitoyl acyl transferase (TgDHHC7) that localizes to the surface of the rhoptries. Remarkably, conditional disruption of this enzyme results in a loss of apical tethering of the rhoptries and a complete block in their function, allowing for a definitive establishment of their role in invasion but not replication or egress. Palmitoylation by membrane-resident PATs is a well-characterized mechanism for recruiting proteins to target membrane systems. Therefore, it is likely that TgPAT1 facilitates apical tethering of rhoptries by recruiting one or more proteins to the cytosolic face of the rhoptry membrane which then serve to mediate docking. Indeed, knockdown of the palmitoylated rhoptry armadillo-repeat protein TgARO recapitulates loss of TgDHHC7, showing this protein is also required for rhoptry tethering and strongly suggesting it is a target of TgDHHC7.

During invasion, a complex of the rhoptry neck proteins RON2/4/5/8 localizes to the MJ where it is thought to provide a stable anchoring point for host penetration. This complex is also believed to serve as a molecular filter that restricts access of host plasma membrane proteins to the nascent parasitophorous vacuole, protecting it from lysosomal fusion. During the initiation of invasion, the preformed MJ/RON complex is injected into the host cell where RON2 spans the plasma membrane while RON4/5/8 localize to its cytosolic face. While an important interaction between a parasite surface-bound adhesin, AMA1, and RON2 outside of the host cell has been
elucidated, little is known about the interactions and role of the MJ/RONs present within the host cytosol. In the second section, I provide a comprehensive analysis of RON5. Using a conditional knockdown approach, I show RON5 is critical for the organization of the MJ RON complex and that disruption of this complex results in a block in rhoptry secretion and host invasion, demonstrating the importance of MJ RONs for host entry. Furthermore, domain analysis of RON5 using functional complementation reveals that a C-terminal region of RON5 is critical for RON2 stability and invasion, defining the first functionally important domain in RON5.

Apicomplexan parasites undergo complex life cycles, employing unique forms of internal budding for their replication. The simplest example of these is the *Toxoplasma* binary division system known as endodyogeny, wherein two daughter parasites are assembled within an intact mother cell. Internal budding is facilitated through the *de novo* construction of an inner membrane complex (IMC), a series of flattened vesicles and underlying cytoskeletal features that provides the scaffold for daughter cell assembly within the cytosol. Little is known regarding the molecular mechanisms that orchestrate internal budding. The final section of this work identifies the apicomplexan-specific IMC Sub-compartment Protein (ISP) family of IMC proteins in *Toxoplasma*. These proteins are organized into distinct sub-compartments within the IMC and this arrangement was found to depend on coordinated myristoylation and palmitoylation of conserved N-terminal residues in each protein as well as a unique hierarchical targeting mechanism. Interestingly, while a replicating parasite typically produces two daughters per round of division, disruption of ISP2 results in the assembly of aberrant numbers of daughters with a corresponding loss in parasite fitness indicating a role for this family in the control of budding. Together, these studies provide valuable new insights into host cell invasion and parasite division in this important opportunistic pathogen.
The dissertation of Joshua Ryan Beck is approved.

Patricia J. Johnson

Kent L. Hill

Feng Guo

Peter J. Bradley, Committee Chair

University of California, Los Angeles

2012
DEDICATION

For my wife Rochelle, who supported and encouraged the work herein described with great patience and care.
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LIST OF ABBREVIATIONS

A – adenine
aa – amino acids
ABA – abscisic acid
ADP – adenosine diphosphate
AIDS – Acquired Immuno-Deficiency Syndrome
AMA1 – apical membrane antigen 1
ATF6β – activating transcription factor 6 beta
ATP – adenosine triphosphate
BALB/c – albino laboratory strain of house mouse
BLAST – Basic Local Alignment Search Tool
bp – base pairs
BSA – bovine serum albumin
C – cytosine
cytD – cytochalasin D
DHHC-CRD – aspartic acid/histidine/histidine/cysteine - cysteine-rich domain
DMEM – Dulbecco’s modified eagle medium
DMSO – dimethyl sulfoxide
DNA – deoxyribonucleic acid
DOC2 – double C2-like domain-containing protein
DrpB – Toxoplasma gondii dynamin related-protein B
EM – electron microscopy
ER – endoplasmic reticulum
G – guanine
GAP – glideosome associated-protein
GDP – guanosine diphosphate
GFP – green fluorescent protein
GPI – glycosylphosphatidylinositol
GRA – dense granule protein
GTP – guanosine triphosphate
HA – hemagglutinin
HAART – highly active anti-retroviral therapy
HBSS – Hank’s buffered saline solution
HFF – human foreskin fibroblast
HXGPRT, hpt – hypoxanthine-xanthine-guanine phosphoribosyl transferase
ICAM1 – inter-cellular adhesion molecule-1
IF – intermediate filament
IFA – immunofluorescence assay
IFNγ – interferon-gamma
ixB – i-kappa-B regulator
IL – interleukin
IMC – inner membrane complex
IMP – intramembrane particle
IP – immunoprecipitation
IPTG – isopropyl-beta-D-thiogalactopyranoside
IRG – immunity-related GTPase
ISP – IMC Sub-compartment Protein
kDa – kilodalton
KU80 – Ku heterodimer subunit 80
LC – liquid chromatography
LD₅₀ – lethal dose for 50% of sample population
LD₁₀₀ – lethal dose for 100% of sample population
M2AP – MIC2-associated protein
mAb – monoclonal antibody
MAPK – mitogen-activated protein kinase
MIC – microneme protein
MJ – moving junction
ml – milliliters
MLC – myosin light chain
mM – millimolar
µm – micrometer
MNN – membranous network of nanotubules
MOI – multiplicity of infection
MS/MS – tandem mass spectrometry
MTOC – microtubule organizing center
MyoA – myosin A
NCBI – National Center for Biotechnology Information
NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells
ng – nanogram
Ni-NTA – nickel-nitrilotriacetic acid
nm – nanometer
NMT – N-myristoyl transferase
NTPase – nucleotide triphosphate hydrolase
ORF – open reading frame
PAGE – polyacrylamide gel electrophoresis
PAN – plasminogen apple, nematode I
PAT – palmitoyl acyl transferase
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PFAM – protein family database
PLA2 – phospholipase A2
PLP1 – perforin-like protein-1
PP2C-hn – protein phosphatase 2 C host nucleus
PV – parasitophorous vacuole
PVM – parasitophorous vacuolar membrane
Rab – Ras-associated binding protein
RHA – T. gondii type I isolate ΔHXGPRT
RIPA – radioimmunoprecipitation
ROM– rhomboid-like protease
RON – rhoptry neck protein
ROP – rhoptry bulb protein
SφXE – serine/hydrophobic residue/any amino acid/glutamic acid cleavage site
SAG – surface antigen
SDS – sodium dodecyl sulphate
SFVE – serine/phenylalanine/valine/glutamic acid cleavage site
SFQE – serine/phenylalanine/glutamine/glutamic acid cleavage site
SILAC – stable isotope labeling by amino acids in cell culture
spp – several species
SRS – SAG related sequence
STAT – signal transduction and transactivator
SUB2 – subtilisin-like serine protease 2
T – thymine
TEM – transmission electron microscopy
Tg, T. gondii – Toxoplasma gondii
TM – transmembrane
TX-100 – Triton X-100 detergent
6X His tag – hexahistidine tag
ACKNOWLEDGEMENTS

I am indebted to my advisor, Dr. Peter Bradley, whose enthusiasm, encouragement, availability and friendship have contributed vastly more to my scientific development and success than any other single factor. I also thank my committee, Dr. Patricia Johnson, Dr. Kent Hill and Dr. Fung Guo who provided encouragement and direction throughout my graduate tenure. In particular, Dr. Johnson has provided extensive support and guidance during my time at UCLA for which I am grateful. Former members of the Bradley lab, including Dr. Kurtis Straub, Dr. Bettina Hajagos and Dr. Jay Turetzky, were indispensable in my training and provided stimulating interaction and support. I am thankful to Connie Fung, whom I had the pleasure of overseeing during her undergraduate research in the lab. Additionally, Dr. Naomi Morrissette has been at times like a second mentor and I appreciate her support and guidance. I am also thankful to the many individuals who demonstrated the widely regarded collegiality of the Toxoplasma field though shared reagents and supportive interactions: John Murray, Brook Anderson-While, Marc-Jan Gubbels, Ke Hu, Gary Ward, Michael White, My-Hang Huynh, Vern Carruthers, Lilach Sheiner, Boris Striepen, John Boothroyd and Michael Reese. My work was supported for several years by the NIH Microbial Pathogenesis Training Grant #T32-AI07323.

I would not have had the opportunity to pursue my interests in molecular life science were it not for my parents, Paul and Bonnie Beck, who have unendingly supported and encouraged my interests. Finally, I am deeply grateful for the care and support of my lovely wife Rochelle who has patiently tolerated my fascination with very small things.
Chapter 2 is a version of:

Author contributions: JRB and PJB conceived and designed the experiments. JRB, FC, KWS, IC and AAV performed the experiments. JRB, JAW and PJB analyzed the data. JRB and PJB wrote the paper.

Chapter 3 is a version of:
Beck, J.R. and Bradley, P.J. RON5 is critical for organization and function of the moving junction complex. Manuscript in preparation.

Author contributions: JRB and PJB conceived and designed the experiments. JRB performed the experiments. JRB and PJB analyzed the data. JRB and PJB wrote the paper.

Chapter 4 is a version of:

No permissions required. Author contributions: JRB and PJB conceived and designed the experiments. JRB, IARF and JCdL performed the experiments. JRB, NSM and PJB analyzed the data. JRB, MHH, VBC, NSM and PJB contributed reagents and/or materials. JRB, NSM and PJB wrote the paper.
Chapter 5 is a version of:

Fung, C.*, Beck, J.R.*, Robertson, S.D., Gubbels, M.J., and Bradley, P.J. (2012). *Toxoplasma* ISP4 is a central IMC sub-compartment protein whose localization depends on palmitoylation but not myristoylation. Molecular and biochemical parasitology 184, 99-108. (*Authors contributed equally to this work.)

It is reprinted here by permission from Elsevier. Author contributions: CF, JRB and PJB conceived and designed the experiments. CF and JRB performed the experiments. CF, JRB and PJB analyzed the data. SDR and MJG contributed reagents and/or materials. CF, JRB and PJB wrote the paper.
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Publications


Fung, C.*, Beck, J.R.*, Robertson, S.D., Gubbels, M.J., and Bradley, P.J. (2012). Toxoplasma ISP4 is a central IMC sub-compartment protein whose localization depends on palmitoylation but not myristoylation. Molecular and biochemical parasitology 184, 99-108. (*Authors contributed equally to this work.)


Presentations

Beck JR, Tonkin ML, Coppens I, Boulanger MJ and Bradley PJ. The Toxoplasma ISP family proteins play an important role in daughter cell formation. 23th Annual Molecular Parasitology Meeting. Marine Biological Laboratory, Woods Hole, MA. Sept 22-26, 2012 (talk).


Chapter 1

Introduction
Toxoplasma gondii among apicomplexan parasites

Obligate intracellular pathogens avoid clearance by host immune processes through sequestering themselves within their host cells. Entrance into this intracellular compartment provides a shelter from immune assault as well as a bounty of materials to support nutrition and growth. Access to this privileged niche is achieved through a variety of mechanisms by both prokaryotic and eukaryotic pathogens. While many organisms induce their uptake through manipulation of existing host pathways (e.g., endocytosis), apicomplexan parasites employ their own sophisticated machinery to actively invade their host cells, generating a tailor-made vacuole in the process within which they are free to replicate. A number of remarkable innovations unique to this phylum of organisms support these specialized biological processes and underlie the pathology of an array of devastating diseases caused by this large group of parasites.

The apicomplexan phylum is a vast group of obligate intracellular eukaryotic parasites containing about 6,000 described species (although a total of 1.2 - 10 million species are estimated to exist) (Adl et al., 2007; Levine, 1988). The phylum is comprised of three major clades: the Gregarina (parasites of invertebrates), the Coccida (mainly parasites of vertebrates) and the Hematozoa (parasites of both vertebrates and invertebrates)(Figure 1-1). The most notorious members of the phylum are the Hematozoan malarial parasites (Plasmodium spp, including P. falciparum). Astoundingly, 3.3 billion humans are currently at risk for infection by Plasmodium spp, resulting in ~225 million infections and nearly a million deaths annually, mostly among children under five in sub-Saharan Africa (WHO, 2011). Other agriculturally prominent Hematozoans include Babesia spp and Theileria spp, which cause severe disease in cattle and other livestock (Bock et al., 2004; CFSPH, 2009). Toxoplasma gondii, the most eminent member of the Coccida and focus of this dissertation, chronically infects 30% of the
human population and causes severe neurological disorders and death in immunocompromised persons and neonates (Hill et al., 2005). *Neospora caninum*, a close relative of *T. gondii* which causes abortions in cattle, is a prime concern for the dairy and beef industries while infection by *Eimeria* spp results in an estimated $1.5 billion in annual revenue losses in the poultry industry (Dubey et al., 2007; Sharman et al., 2010). *Cryptosporidium* spp, another key apicomplexan group of human pathogens distinct from the three principal clades, causes potentially life threatening diarrheal disease in immunocompromised individuals (Ayuo, 2009).

**Historical overview and significance**

*Toxoplasma gondii* was first discovered by Nicolle and Manceaux in Tunisia in the tissue of the North African rodent *Ctenodactylus gundi* and named for its morphology (*L. toxon* = bow or arc, *plasma* = life; the specific epithet was taken from a mistaken spelling of the host) (Nicolle and Manceaux, 1908, 1909). Splendore, working independently in Brazil, simultaneously described the parasite in rabbit tissue but did not name the organism (Splendore, 1908). Sabin and Olitsky established the obligate intracellular nature of the parasite in 1937 and an understanding of the complete life cycle was achieved by 1970 through the combined work of numerous investigators (Dubey, 2009; Sabin and Olitsky, 1937). In 1948, Sabin and Feldmen introduced a serological diagnostic for identifying infected individuals and by the 1950s it began to be appreciated through epidemiological surveys that the *Toxoplasma* host range and distribution was enormous, with the prevalence in the global human population approaching 30% and exceeding 90% in certain populations (Hill et al., 2005; Sabin and Feldman, 1948; Silveira et al., 1988; Tenter et al., 2000). Analysis of field isolates over the past century coupled with
increasingly sophisticated genetic techniques have led to a general acceptance that *T. gondii* is the only member of the genus, which maintains an unexpected population structure (Tenter et al., 2000). In Europe and North America, the population is dominated by three genotypes (Type I, II and III) with most cases of human toxoplasmosis caused by Type II strains (Howe et al., 1997; Howe and Sibley, 1995; Khan et al., 2005). In South America, the situation differs markedly with the population containing a large number of exotic strains not belonging to the Type I, II or III clonal lineages and an increased incidence of human infection with Type I strains (Khan et al., 2006).

A growing understanding of the burden of *T. gondii* infection fueled extensive characterization efforts by electron microscopy (EM) during the 1960s. The simultaneous examination of several different apicomplexan parasites in addition to *Toxoplasma* showed a conserved apical complex of cytoskeletal structures and putative secretory organelles in these parasites, providing the first indication that these organisms constituted a unified taxonomic group (a feature for which the phylum was later named: Apicomplexa = apical complex) (Levine et al., 1980; Scholtyseck and Mehlhorn, 1970). These studies yielded a description of the unique cellular processes that were behind the remarkable biology of *T. gondii*, providing a stepping stone to single-molecule analysis with the establishment of molecular genetic and proteomic disciplines (Dubremetz and Ferguson, 2009).

*Toxoplasma* was recognized as an important opportunistic pathogen with the development of immunosuppressive therapies and the advent of the AIDS epidemic during the 1970s and early 1980s (Cohen, 1970; Luft et al., 1984; Ruskin and Remington, 1976). Importantly, although most infections are asymptomatic, the encysted, chronic state of the infection cannot be cleared by the host immune system or therapeutics (Tenter et al., 2000).
Thus, the high prevalence of *Toxoplasma* infection imposes a major risk for reactivation of latent infection and resulting severe disease in individuals who become immunocompromised (Luft and Chua, 2000). Indeed, during the 1990s it was reported that globally, 40% of AIDS patients develop severe Toxoplasmic encephalitis with a 10-30% mortality rate (Tenter et al., 2000). As a result, the development of highly active anti-retroviral therapy (HAART) is arguably the most significant medical development for the control of Toxoplasmosis (Boothroyd, 2009).

Nonetheless, despite significant advances in the past century, *Toxoplasma* remains a major global health burden with no commercially available human vaccine (the potential efficacy of an vaccination strategy for actual disease control is a topic of current debate) and no available therapeutic options capable of clearing the chronic infection (Ferguson, 2009; Furtado et al., 2011; Kur et al., 2009).

Due to its relative ease of propagation in tissue culture and genetic tractability, *T. gondii* is widely recognized as the model apicomplexan (Kim and Weiss, 2004). Indeed, studies in *T. gondii* have led the way in many of the major advances in the molecular understanding of apicomplexan biology and disease in the past three decades (Boothroyd, 2009). Finally, it is worth noting that the apicomplexan phylum is part of a larger Alveolata infrakingdom that includes two other phyla of free-living protists, the Ciliates and Dinoflagellates (the specific evidence for this taxonomic grouping is discussed further in Chapter 4) (Keeling et al., 2005). Thus, study of apicomplexans, including *Toxoplasma*, allows for comparative analysis of the means by which related organisms facilitate differential lifestyles of parasitism and free-living predation.
Life cycle and transmission

*Toxoplasma* undergoes a complex, heteroxenous life cycle with feline species serving as the definitive host while essentially any warm-blooded animal (mammals and birds) can be infected as an intermediate host (Figure 1-2) (Tenter et al., 2000). Asexual development in the intermediate host involves both rapid and slow proliferating forms of the parasite known as tachyzoites and bradyzoites, respectively (Gr. *tachy*=rapid; *brady*=slow). During the acute stage following primary infection, tachyzoites propagate by invasion of a host cell, replication within this intracellular niche and eventual egress resulting in disruption of the host cell. As a cell-mediate immune response develops, tachyzoites are either cleared or differentiate into bradyzoites, which form tissues cysts inside a host cell (Bohne et al., 1994). Sheltered within the thick tissue cyst wall, bradyzoites are refractory to immune clearance and slowly replicate, persisting for years within the host. The parasite shows a tropism for neural and muscular tissue and the major tissue cyst burden is found within the central nervous system and retina as well as skeletal and cardiac muscle. Cysts periodically reactivate with bradyzoites converting to tachyzoites, which begin to rapidly propagate once again. In immune competent individuals, a swift cell-mediated immune response keeps such reactivation in check, with tachyzoites either being killed or differentiating again to bradyzoites. However, this cyst reactivation can lead to recrudescence in hosts that have entered an immunocompromised state and can no longer maintain immune response necessary for control of tachyzoite proliferation.

When infectious forms of the parasite are ingested by a cat, the parasite initiates its sexual cycle. Asexual replication in the epithelium of the cat small intestine produces gametes, which then fuse and undergo a process of oocyst generation. Unsporulated oocysts are released into the intestinal lumen and shed in the feces (Frenkel et al., 1970). Once in the environment, the
sporulation occurs resulting mature oocysts which harbor two sporocysts, each of which contain four sporozoites. Mature oocysts are highly infectious and persist in the environment for years owing to their strong cyst wall. Ingestion of oocysts by felines or other animals initiates the sexual or asexual cycle anew.

Infection by *Toxoplasma* can occur via both the tachyzoite and bradyzoites forms of the parasite, resulting from the asexual propagation of the organism, as well as from the sporozoites generated within oocysts following the sexual cycle (Tenter et al., 2000). Natural horizontal transmission of *Toxoplasma* occurs in two ways: ingestion of tissues cysts through carnivorism and ingestion of oocysts shed in cat feces by any animal. These two mechanisms are responsible for the vast majority of infections, although in the human population the importance of either of these mechanisms varies in according to behavioral patterns within cultures. Vertical transmission can also occur following primary infection of the mother during pregnancy. In such case, when protective immunity has not previously been established, the tachyzoite is able to cross the placenta during the acute stage of the infection resulting in congenital Toxoplasmosis (Remington et al., 2006).

In these ways, transmission may occur directly between intermediate hosts (carnivorism and congenital infection), directly between definitive hosts (oocysts and congenital infection), from definitive to intermediate host (oocysts) or from intermediate to definitive host (carnivorism). In addition to the normal infection routes, transmission between hosts appears to be supported by parasite-mediated behavioral changes, in particular the conversion of an innate fear of cat urine into an attraction to cat urine in rodents chronically infected with *Toxoplasma* (Vyas et al., 2007). This has raised the interesting hypothesis that transmission from intermediate rodent hosts to the definitive feline host is enhanced through highly specific behavior
modification of the host by the parasite. Behavioral modification in human hosts has been suggested by cohort studies (Flegr, 2007). Additionally, a link between *T. gondii* infection and schizophrenia has been suggested based primarily on the observation of a higher incidence of infection in schizophrenics; however, more extensively controlled studies are needed to clarify the reason for this association (Yolken et al., 2009).
Ultrastructure and cell biology of the *Toxoplasma* tachyzoite

The *Toxoplasma* tachyzoite is a crescent-shaped cell measuring ~2x8 μm. Ultrastructural characterization by TEM during the 1960s revealed a highly polarized cellular arrangement and a number of unique organelles (Sheffield and Melton, 1968; Vivier, 1970). Pertinent aspects of the tachyzoite structure are described below and complemented with more recent insights from cell biology and molecular genetic studies (Figure 1-3). As the work described in this dissertation was carried out exclusively in tachyzoites, “*Toxoplasma*” will hereafter refer to this stage except where specifically noted otherwise.

**Nucleus and genome** *Toxoplasma* possesses a ~65 Mb genome which encodes ~8,000 genes, many of which represent hypothetical proteins with no clear similarity to characterized genes outside the apicomplexa (www.toxodb.org). The 14 chromosomes that comprise the genome are kept in an uncondensed state during karyokinesis and the nuclear envelope is maintained throughout the cell division process (Khan et al., 2005; Morrissette and Sibley, 2002). The parasite is haploid in all stages except those that immediately follow gamete fusion during sexual reproduction (Kim and Weiss, 2004). The genomes of representative isolates of the three major strains have been sequenced and sequencing of several exotic strains is now in progress.

**Cytoskeleton** The parasite cytoskeleton is an elaborate and highly organized structure composed of tubulin, intermediate filament-like proteins and actin. Together, these structures define the form and polarity of the *Toxoplasma* cell, provide strength to its structure, create a scaffold for cell division events and enable a unique form of gliding motility. Similar to other eukaryotes, a microtubule spindle, emanating from the centriole, is assembled within the nucleus to facilitate karyokinesis. Unlike other organisms, however, the centriole is located on the cytoplasmic face.
of the nuclear envelope and the microtubules are organized through an elaboration of the nuclear
envelope known as the centrocone (Dubremetz, 1973). Additionally, the spindle microtubules
maintain physical connection between the centriole and the kinetochore of each chromosome
throughout the cell cycle and this appears to be a mechanism for monitoring genome number and
ensuring proper segregation of replicated genomes at the end of the internal budding process (see
“replication” below) (Brooks et al., 2011).

Tubulin also plays a critical role in the cortical structure of the parasite, traditionally referred to
as the pellicle. A second major population of microtubules are assembled from a unique
microtubule organizing center (MTOC) at the cell apex called the apical polar ring (Russell and
Burns, 1984). In the *Toxoplasma* tachyzoite, 22 subpellicular microtubules extend from this
structure and spiral about two-thirds down the length of the parasite (Nichols and Chiappino,
1987). These microtubules are key in determining cell polarity as cells in which these
microtubules have been pharmacologically eliminated loose their polarity (Stokkermans et al.,
1996). In addition, the apical-to-posterior growth of daughter bud scaffolds is likely driven by
polymerization of the subpellicular microtubules (see “replication” below) (Shaw et al., 2000).

An unorthodox tubulin structure known as the conoid is present at the extreme apex of
the parasite. The conoid is a tightly-wound cylinder with the appearance of a truncated cone
(hence the name) composed of a unique tubulin polymer (Hu et al., 2002b). A pair of
preconoidal rings cap the apical face of the conoid and the entire structure is positioned just
below the polar ring but extends and retracts beyond this ring when the parasite becomes motile,
a phenomenon than can be induced by calcium flux (Mondragon and Frixione, 1996; Nichols
and Chiappino, 1987). A pair of short (~350 nm), intra-conoidal microtubules are positioned
within the conoid and have been hypothesized to function as a track to facilitated conoid motility
or exocytosis of apical secretory organelles (see “rhoptries” below) (Carruthers and Sibley, 1997; Nichols and Chiappino, 1987). Interestingly, the conoid is only present within coccidial species of the apicomplexa and thus may play a role in penetrating the epithelium of the gut during intestinal stages of infection; however, clear evidence supporting any specific conoid function is lacking (Morrissette and Sibley, 2002).

Unlike other eukaryotes, the *Toxoplasma* actin cytoskeleton is almost entirely composed of globular actin and short microfilaments that assemble under the plasma membrane while longer filaments are absent (Dobrowolski et al., 1997b). The actin cytoskeleton functions primarily to support parasite motility and polymerization of actin microfilaments and myosin activity are critical to this process (see “gliding motility” below) (Dobrowolski et al., 1997a; Dobrowolski and Sibley, 1996). Finally, a family of intermediate filament-like proteins form a meshwork that contributes strength to the pellicle and may serve a variety of other functions related to cell division (Anderson-White et al., 2011). These features are part of a cortical membrane system known as the inner membrane complex (IMC), discussed in the following section.

**IMC** The IMC is a series of flattened vesicles and underlying protein meshwork positioned just beneath the entirety of the parasite plasma membrane except for a small gap at the extreme apex and base. This structure provides a morphological framework for the gliding motility essential in host invasion as well as a scaffold for daughter cell construction. The meshwork is composed of a 14-member family of membrane skeleton proteins (IMCs) that assemble to form a network of intermediate filaments (Anderson-White et al., 2011). A patch-work arrangement of flattened vesicles, known as alveoli, is assembled on top of this protein meshwork (Porchet and Torpier, 1977). These alveoli are roughly rectangular in shape and tightly sutured together at their
borders. In coccidian species like *Toxoplasma*, a unique cone-shaped alveolus, known as the apical cap, resides at the apex of the IMC. Intimate connection between the IMC and other pellicle features are clearly visible by freeze-fracture EM analysis, which reveals numerous intramembrane particles (IMPs) that, in part, trace an imprint of the underlying subpellicular microtubules (Morrissette et al., 1997). *De novo* assembly of the IMC during daughter bud formation occurs by vesicle traffic from the Golgi that is mediated by Rab11b, a small GTPase conserved among alveolates (Agop-Nersesian et al., 2010). Collectively, ultrastructural analysis of the IMC indicates a highly ordered structure with varying molecular composition between different sub-compartments. A detailed analysis of molecular compartmentalization within the IMC and function in daughter formation is provided in Chapters 4 and 5.

**Endosymbiotic organelles: the apicoplast and mitochondrion** A curious feature of apicomplexan protists is the presence of a non-photosynthetic plastid called the apicoplast (apicomplexan plastid) (McFadden and Roos, 1999). This organelle is present in all apicomplexans evaluated to date except for the *Cryptosporidia* and one Eugregarine species (Figure 1-1) and is essential for parasite viability owing to key roles in cellular metabolism, such as fatty acid and isoprenoid biosynthesis (Lang-Unnasch et al., 1998; Mazumdar et al., 2006; Toso and Omoto, 2007; Yeh and DeRisi, 2011; Zhu et al., 2000). Four membranes surround the apicoplast, which contains a circular, 35 Kb genome encoding prokaryotic translation machinery (Kohler et al., 1997). However, many of the proteins that localize to the apicoplast are encoded within the parasite genome and imported across the four membranes of the organelle using standard plastid translocation machinery, although components similar to the endoplasmic reticulum-associated degradation system may also be involved (Agrawal et al., 2009; van Dooren et al., 2008). Division of the apicoplast proceeds through a dynamin-mediated fission event (van
Dooren et al., 2009). Based on the presence of a quadruple membrane together with phylogenetic analysis of several plastid genomes, this organelle is hypothesized to be the product of a secondary endosymbiotic event involving a red algae (Janouskovec et al., 2010; Vaishnava and Striepen, 2006). The critical importance of this prokaryote-like organelle to parasite viability has made the apicoplast a key focus for development of novel drug targets (Fichera and Roos, 1997). *Toxoplasma* also possesses a single mitochondrion harboring a 6 Kb genome which wraps the nucleus and displays all the standard features of mitochondrial biology (Melo et al., 2000).

**ER and Golgi** The endoplasmic reticulum (ER) surrounds the nucleus and is primarily concentrated on its posterior side although projections do extend out into the cell in other directions (Joiner and Roos, 2002). The overall volume of the ER is reduced compared to other higher eukaryotes and the nuclear envelope itself constitutes a major portion of the ER. A stack of 3-5 Golgi cisternae are positioned on the apical side of the nucleus/ER and coated vesicles bud exclusively from the apical side of the nuclear envelope/ER in transit to the Golgi (Hager et al., 1999). The simplicity of this secretory pathway has been exploited to address outstanding questions, including Golgi biogenesis, which occurs by lateral extension and medial fission in *Toxoplasma*, consistent with a template-based process for replication (He, 2007; Pelletier et al., 2002). In sum, the parasite possesses a highly polarized secretory pathway that serves to deliver protein cargo a variety of sub-cellular compartments including the IMC and three distinct secretory organelles: the rhoptries, micronemes and dense granules.

**Micronemes** The micronemes are small, rod-shaped secretory organelles that line the apical third of the parasite periphery. These organelles harbor an array of adhesin molecules that are critical for gliding motility and invasion. Microneme secretion is stimulated by calcium release from storage compartments within the parasite including the mitochondrion, acidocalcisomes.
and the ER (Nagamune et al., 2008b). This intracellular calcium flux activates the calcium-dependent protein kinase 1, which governs microneme exocytosis (Lourido et al., 2010). Discharge occurs at the apex of the parasite, however the specific site of microneme release is not known. Fusion with the duct-like rhoptry necks (see “rhoptries” below) has been proposed as a possible mechanism, although clear evidence for such an event is lacking and more recent observations suggest a microneme release point at the extreme apex of the cell just below the conoid (Dubremetz, 2007; Paredes-Santos et al., 2011). Recently, a homolog of the mammalian double C2-like domain-containing protein (DOC2), which recruits membrane fusion machinery in a calcium-dependent manner, was found to be required for microneme exocytosis and further investigation of this mutant may clarify the specific site of fusion (Farrell et al., 2012).

**Rhoptries** The rhoptries are club-shaped secretory organelles (G. *rhop*=club) ~2 µm in length that are bundled together at the anterior pole of the cell. A single *Toxoplasma* cell contains 6-14 individual rhoptries, each comprised of a larger, bulbous body that tapers to a thin, electron dense neck that is tethered at the extreme apex of the cell, providing a conduit for secretion of the rhoptry contents (Dubremetz, 2007). Proteomic analysis of the rhoptries identified many of the organellar contents, which were found to be differentially localized within distinct compartments in either the rhoptry body (ROPs) or the neck (RONs) (Bradley et al., 2005). RON proteins are typically conserved across the apicomplexan phylum and play key roles in host cell penetration while ROP proteins are highly divergent within the phylum and appear to serve tailored roles in modulating infection of the host(s) specific to each species. Freeze-fracture EM analysis reveals organized tracks of IMPs in the rhoptry membrane, and a few transmembrane proteins have been discovered to localize to this membrane, including a Na+/H+ exchanger (Karasov et al., 2005; Lemgruber et al., 2010). TEM sectioning of the rhoptry lumen reveals
honey-combed appearance that may contain a tightly packed crystalline array of cargo, possibly facilitated by the high cholesterol content of the organelle (Foussard et al., 1991). Oddly, later studies failed to recapitulate the reportedly high cholesterol density in the rhoptries and indicate that the rhoptry cholesterol can be depleted with no obvious consequence to organelle function (Coppens and Joiner, 2003; Lemgruber et al., 2010). The acidic nature of the rhoptry lumen and the conspicuous absence of a clear lysosomal compartment in the parasite led to the proposal that rhoptries are a form of secretory lysosomes (Ngo et al., 2004; Norrby et al., 1968; Shaw et al., 1998). However, the more recent discovery of a plant-like vacuole with clear functional analogy to the plant and yeast equivalent of the mammalian lysosome indicates that rhoptries are not the only compartment with features of an endolysosomal system (Miranda et al., 2010; Parussini et al., 2010).

Rhoptries are assembled de novo during daughter cell formation and are first visible as roughly oval, post-Golgi compartments called pro-rhoptries that label with antibodies that specifically recognize pro-regions of immature rhoptry proteins (Carey et al., 2004). By late stages of daughter cell formation, pro-rhoptries elongate into their mature form. Expression of proteins destined for the rhoptry compartment is tightly regulated with peak transcription occurring in a narrow, ~1 hour window during rhoptry biogenesis (Behnke et al., 2010). Biogenesis of both rhoptries and micronemes proceeds through a shared pathway that requires the sortillin-like transmembrane cargo receptor TgSORTLR, which binds microneme and rhoptry proteins in a mannose-6-phosphate independent manner within the lumen of secretory vesicles and recruits endocytic sorting machinery to the vesicle surface (Sloves et al., 2012). Additionally, the alveolate-restricted dynamin-like protein DrpB is involved in generation of the apical secretory organelles and conditional disruption of TgSORTLR or DrpB results in ablation
of both micronemes and rhoptries (Breinich et al., 2009). How discernment between micronemal and rhoptry secretory cargo is made during later stages of endocytic traffic to ensure delivery to the proper organelle is not currently understood. Targeting information for many rhoptry body proteins is contained within an N-terminal pro-domain, which is sufficient for trafficking reporter molecules to the rhoptries (Bradley and Boothroyd, 2001). These pro-domains are typically removed by proteolytic processing at a consensus serine/hydrophobic residue/any amino acid/glutamic acid (SΦXE) cleavage site during maturation of the organelle, although mutants which abolish this processing do not appear to impact targeting or function of rhoptry proteins in which they have been generated (Bradley et al., 2002; Hajagos et al., 2012; Turetzky et al., 2010). TgSUB2, a subtilisin-like serine protease targeted to the rhoptry lumen, is likely to be the responsible maturase as it undergoes autoprocessing at the consensus site and directly interacts with ROPs known to be processed (Miller et al., 2003).

Rhoptry exocytosis appears to occur exclusively during host penetration and exposure to host cells is the only known trigger for release of the organelle (Carruthers and Sibley, 1997). The limited interior volume of the conoid permits only two or three rhoptry necks from occupying this space simultaneously and in keeping with this, only a few rhoptries release their contents during invasion (Dubremetz, 2007). Such conservative deployment of the rhoptry arsenal may ensure the tachyzoite remains competent for invasion of a new cell should the immediate host cell become compromised early in the infection before parasite replication can occur. Work in \textit{P. falciparum} indicates that binding of the micronemal protein EBA175 to its receptor on the erythrocyte surface transduces a signal that results in restoration of basal Ca\textsuperscript{2+} concentration levels within the parasite cytosol and subsequent rhoptry exocytosis (Singh et al., 2010). While a EBA175 ortholog is not encoded by \textit{T. gondii}, the \textit{Toxoplasma} micronemal
protein MIC8 is required for host penetration and deletion of the luminal, C-terminal tail of this protein arrests the invasion process just prior to rhoptry secretion, suggesting a key signal may be transmitted by binding of MIC8 to a receptor on the host surface (Kessler et al., 2008). Apparent exocytosis has been visualized by cryoEM and appears to involve a porosome-like structure positioned between the anterior end of the rhoptry neck and the plasma membrane, however more work is needed to clarify the signaling pathways and molecular events that mediate rhoptry secretion (Jena, 2009; Paredes-Santos et al., 2011). As a step toward this goal, Chapter 2 presents data demonstrating that the polarized tethering of the rhoptries is dependent on protein palmitoylation and this apical arrangement is a prerequisite for rhoptry release, which mediates host penetration.

**Dense granules** Dense granules are spherical secretory organelles ~200 nm in diameter that are distributed along the length of the cell body in the invasive stages of apicomplexan parasites. Their name is derived from their highly electron-dense appearance when visualized by TEM and in the case of the *Toxoplasma* tachyzoite, ~ 20 of these organelles are present in each cell, although this number varies considerably between different species (Mercier et al., 2005). Dense granules are the default, constitutive secretion pathway in *Toxoplasma*, although a transient burst of secretion is observed shortly following host invasion indicating that constitutive secretion is subject to regulation as certain points (Coppens et al., 1999; Karsten et al., 1998). The contents of the dense granules are released into the PV through fusion with the parasite plasma membrane at sub-apical locations (Dubremetz et al., 1993). Ultrastructural analysis has led to the speculation that dense granules may access the PM at alveoli junction points in the IMC, however little is known about this process (Leriche and Dubremetz, 1990; Paredes-Santos et al., 2011).
The tachyzoite lytic cycle is central to the pathology associated with *Toxoplasma* infection

During the asexual phase of the *Toxoplasma* life cycle, a tachyzoite invades a permissive host cell to create an intracellular niche. Once secluded within the host cell, the parasite divides through a process of internal budding called endodyogeny wherein two daughter parasite buds are assembled within an intact mother cell. Successive rounds of replication result in a geometric expansion of parasites, filling the host cell. Eventually, the host cell is disrupted and the parasites egress out of the host cell to invade neighboring cells (Figure 1-4). An increasing number of parasite effectors have been recognized to play important roles in host-pathogens interactions during *Toxoplasma* infection with important implications for disease outcome. However, the pathology associated with Toxoplasmosis is exclusively the consequence of necrotic tissue damage in sensitive tissues and the associated host immune response resulting from unhindered parasite progression through the lytic cycle described above (Black and Boothroyd, 2000). As an introduction to the work described in this dissertation, the current understanding of key aspects of this lytic cycle are reviewed below.

**Invasion**

Invasion in *Toxoplasma* and related apicomplexans is a highly coordinated process that involves the sequential discharge of three distinct secretory organelles: the micronemes, rhoptries and dense granules (Carruthers and Sibley, 1997). The invasion process can be separated into the key steps of attachment, penetration and vacuole remodeling and the contribution of the secretory organelles to each of these steps is described below. Entry into the host cell is an active process on the part of the parasite that results in the generation of a non-fusogenic vacuole within which the parasite resides and replicates for the duration of the infection (Morisaki et al., 1995).
A unique form of gliding motility generated by the parasite is critical for transit from one host cell to the next and supplies the motive force that powers host cell penetration (Sibley, 2010). Micronemal proteins are critical for gliding motility as well as attachment to the host cell while rhoptries discharge proteins that mediate a tight-junction interface between parasite and host membranes during penetration as well as subversion of host signaling pathways and protection from innate host immune defenses (Boothroyd and Dubremetz, 2008). Finally, dense granules generally serve to remodel the vacuole formed during invasion, although a role in modulation of host signaling has also been recently described for a protein secreted from these organelles (Mercier et al., 2005; Rosowski et al., 2011).

**Attachment**

The act of invasion begins with parasite attachment to the host cell, a multi-step process of increasingly intimate association that culminates in penetration (Carruthers and Boothroyd, 2007). No single, definitive receptor for host binding has been identified. The extensive host range and ability of *Toxoplasma* to infect any nucleated cell within distinct host species suggests that such a receptor would need to be broadly conserved, or that the parasite possesses the ability to recognize a wide range of unique receptors between different species and cell types. The later is more likely the case, as accumulating evidence points to a scenario wherein a number of carbohydrates and other molecules on the host surface are recognized by a diverse array of parasite lectins and adhesins during the attachment process (Friedrich et al., 2010a). These receptor-ligand attachment steps precede a penultimate commitment to invasion that occurs through insertion of a rhoptry-derived protein complex into the host plasma membrane, which effectively serves as a final receptor.
The surface of the parasite is dominated by a family of GPI-anchored surface antigens (SAGs) and SAG-related sequence (SRS) proteins that support initial attachment (Lekutis et al., 2001). This has been most directly shown through antibody blocking of SAG1 and genetic ablation of SAG3, both of which decrease attachment (Dzierszinski et al., 2000; Mineo and Kasper, 1994). Manipulation of carbohydrates on the host surface diminishes parasite attachment and this effected SAG-receptor repertoire is constituted at least in part by glucosaminoglycans as SAG3 binds to heparin and other sulfated sugars (Carruthers et al., 2000a; Ortega-Barria and Boothroyd, 1999). In keeping with the homogenous distribution of SAGs on the parasite surface, attachment at this initial stage can occur in any orientation. These interactions are thought to be low-affinity, permitting initial binding to the host prior to engagement of adhesins stored within the micronemes (Carruthers and Boothroyd, 2007).

The micronemes contain an array of modular proteins (MICs) composed of a diverse variety of adhesive domains. Following initial attachment, these adhesins are released apically onto the parasite surface by microneme exocytosis. These adhesins form stronger interactions with the host surface and their apical location induces reorientation of the parasite, positioning the cell apex proximal to the host cell membrane (Carruthers and Boothroyd, 2007). Most micronemal adhesins exist as oligomeric complexes containing a type I TM protein and one or more associated non-TM proteins (Carruthers and Tomley, 2008). Key adhesins critical for advancement through the parasite lytic cycle include the MIC2/MIC2-associated protein (M2AP) and MIC3/8 complexes as well as apical membrane antigen 1 (AMA1).

MIC2 is the Toxoplasma homolog of the Plasmodium thrombospondin-related anonymous protein (TRAP), one of the earliest micronemal adhesins identified and a critical player in gliding motility and hepatocyte invasion by Plasmodium sporozoites (Kappe et al.,
Both TRAP and MIC2 contain an integrin-like A-domain that facilitates binding to heparin (Harper et al., 2004; McCormick et al., 1999). Similar to defects seen following ablation of TRAP, conditional disruption of MIC2 results in the loss of helical gliding motility and catastrophic defects in host attachment (Huynh and Carruthers, 2006). Invasion is severely compromised in parasites depleted of MIC2, likely owing to the major defect in host attachment. Like MIC2, conditional knockdown of MIC8 also indicates it is essential for parasite invasion and survival. However, while MIC2 is critical for gliding motility and host attachment, MIC8 acts at a later stage in the invasion process and is necessary for establishing a moving junction (see below) to initiate host penetration but not for motility or attachment (Kessler et al., 2008). Following secretion from the micronemes and dimerization, the MIC3/8 complex binds to host cells, but the identity of the involved receptor(s) on the host surface is not known (Cerede et al., 2002). In contrast to the key contribution of both MIC2 and MIC8 to invasion, both these proteins are dispensable for egress. As microneme secretion is required for egress, this indicates that distinct microneme proteins differentially mediate these two processes (Farrell et al., 2012).

In addition to heparin and related glycosaminoglycan-like carbohydrates, host sialic acid serves as another receptor for *T. gondii* (Friedrich et al., 2010a). MIC1, a soluble component of the MIC1/4/6 complex, binds to sialic acid and this interaction has been characterized in detail (Blumenschein et al., 2007; Friedrich et al., 2010b; Garnett et al., 2009). Deletion of *MIC1* results in a modest decrease in invasion and virulence. Loss of MIC3, a component of the MIC3/8 complex with a modular structure that is completely unrelated to MIC1, produces no significant defects. However, disruption of *MIC3* together with *MIC1* produces a major loss of virulence showing that unrelated adhesins in distinct complexes can act synergistically to
promote invasion (Cerede et al., 2005). This synergistic effect underscores the idea that 
*Toxoplasma* invasion is not accommodated by a single host receptor but rather that a large 
repertoire of host surface molecules mediate attachment and eventual internalization of the 
*Toxoplasma* tachyzoite. Invasion by *Plasmodium* merozoites operates through a similar set of 
distinct “invasion pathways” that rely on various erythrocyte surface molecules and the 
extremely broad host range of *Toxoplasma* may represent an expansion of this multi-pathway 
approach to host invasion (Duraisingh et al., 2008).

Another critical molecule for invasion, and a current micronemal adhesin of great 
interest, is AMA1. This molecule was initially identified as the target of an invasion-inhibitory 
monoclonal antibody in *P. knowlesi* and subsequently emerged as a leading vaccine candidate for 
malaria prevention due to this fact and the finding that immunization with a variety of AMA1 
derivatives induces protective immunity against the malaria erythrocytic stage in animal models 
(Deans et al., 1982; Remarque et al., 2008; Thomas et al., 1984). AMA1 is a monomeric, type I 
transmembrane protein with a large N-terminal ectodomain exposed to the extracellular 
environment. Structural analysis, first in *Plasmodium* and later in *Toxoplasma*, revealed this 
ectodomain is composed of a pair of adhesive Apple/PAN (plasminogen, apple, nematode) 
domains that are important for receptor binding in other systems (Carruthers and Tomley, 2008; 
Crawford et al., 2010; Pizarro et al., 2005). Knockdown of *AMA1* gene expression demonstrated 
a critical role in invasion (Mital et al., 2005). In this conditional knockdown mutant, AMA1 
levels are decreased to < 0.5% of that observed in the parental strain. Following loss of AMA1, 
parasites still exhibit normal gliding motility and initial attachment, but do not progress to later 
stages of more intimate association with the host surface and fail to reorient their apex to the host 
surface for invasion. Remarkably, the receptor for AMA1 is not a host molecule but the parasite
rhoptry neck protein RON2, which is inserted into the host membrane at the onset of penetration (Alexander et al., 2005). Binding of these two partners is thought to mediate a tight-junction interface between parasite and host surfaces during invasion but could alternatively play roles in signaling during invasion (see below) (Tyler et al., 2011).

**Gliding motility**

While apicomplexan gametes possess flagella, all other life stages lack this motility apparatus and depend instead upon a unique form of gliding motility (Morrissette and Sibley, 2002). In the *Toxoplasma* tachyzoite, this gliding motility, produced by a molecular apparatus termed the “glideosome” is necessary for entry and exit of host cells as well as transit from one host cell to the next. Both circular and helical forms of gliding are exhibited by the parasite, the later of which involves a combination of gliding and twirling to generate productive forward motion (Hakansson et al., 1999). Gliding requires actin polymerization and is powered by a unique class XIV myosin (MyoA) restricted to apicomplexans and related members of the Alveolata (Dobrowolski and Sibley, 1996; Foth et al., 2006; Meissner et al., 2002). A complex of gliding associated proteins (GAPs) unique to apicomplexans anchors the MyoA motor in the IMC. This complex consists of two integral membrane proteins, GAP40 and GAP50, which are immobilized in the IMC, providing a firm foundation against which molecular torque can be applied to generate forward motion (Gaskins et al., 2004; Johnson et al., 2007). GAP45, another motor complex member, binds MyoA and its associated light chain MLC1 and recruits them to the IMC through interaction with GAP40/50. Conditional knockdown of GAP45 eliminates parasite motility, indicating that this association is critical for glideosome function (Frenal et al., 2010). GAP70, a homolog of GAP45, localizes exclusively to the apical cap of the IMC and
serves a similar role in recruiting the motor complex to this IMC sub-compartment (Frenal et al., 2010).

Studies aimed at identifying the molecular components that link surface adhesins to the motor resulted in the surprising discovery that the glycolytic enzyme aldolase forms a bridge between actin filaments and the C-terminal tail of MIC2 (Jewett and Sibley, 2003). This provided a model for motility generation wherein force applied to actin filaments by MyoA is transduced through the aldolase bridge to MIC2, which is bound to the substrate on which the parasite is positioned. With the motor complex immobilized in the IMC, this force results in rearward translocation of surface-bound MIC2, propelling the parasite forward (Opitz and Soldati, 2002). Aldolase binding has since been demonstrated in vitro for recombinant versions of a number of other adhesins, including AMA1 (Sheiner et al., 2010). In addition to its structural bridging role, the parasite aldolase is enzymatically active and this glycolytic activity is critical for glideosome function. The adaptation of a glycolytic enzyme as a structural bridge provides a convenient method of energy production to power the motor (Starnes et al., 2009).

Regulation of gliding motility is facilitated through Ca²⁺ signaling at multiple levels. First, the exocytosis of adhesins from the micronemes onto the parasite surface upon release of Ca²⁺ stores is critical to motility (Carruthers et al., 1999). Additionally, Ca²⁺-dependent phosphorylation is observed on GAP45, MLC1 and MyoA, indicating that a calcium-controlled kinase activity (or activities) modulates the glideosome complex (Nebl et al., 2011). Furthermore, a calmodulin-like protein was recently shown to complex with MyoA-MLC1, suggesting that the glideosome can directly sense and bind Ca²⁺ for its regulation (Nebl et al., 2011). Surprisingly, a lysine methyltransferase is also required for activation of the glideosome (Heaslip et al., 2011). While lysine methytransferases are typically present in the nucleus, this
enzyme is localized to the apical complex but rapidly redistributes to the cell body upon intracellular Ca$^{2+}$ flux. Finally, control of actin polymerization at the cell cortex also contributes to the control of gliding motility. While *Toxoplasma* does not encode the classical Arp2/3 actin-nucleating complex, it does possess a number of formins, which serve to regulate actin dynamics (Daher et al., 2010).

**PM-IMC cohesion and spacing are established by the glideosome.** At a terminal stage of daughter cell formation, Rab11a, a small GTPase highly conserved among apicomplexans, mediates GAP45-MyoA-MLC1 delivery to the PM-IMC nexus on forming daughter buds, a process required for completion of IMC development and daughter pellicle formation (Agop-Nersesian et al., 2009). Linkage to the plasma membrane is achieved by coordinated myristoylation and palmitoylation of the GAP45 N-terminus while the C-terminus of GAP45 is anchored to the IMC such that the protein spans the distance between these two membrane systems (Frenal et al., 2010). Knockdown of GAP45 not only eliminates motility function, but also results in irregular spacing between the plasma membrane and IMC, indicating that in addition to its role in recruiting MyoA to the IMC, GAP45 also functions in determining the spacing between the PM and IMC and maintaining pellicle cohesion. Interestingly, complementation of GAP45 knockdown with a version of GAP70 designed to target to the entire IMC rescues parasite motility but results in an increase in the distance between the plasma membrane and IMC. This differential spacing is thought to be mediated by the length of a coiled-coil domain, which is longer in GAP70 than GAP45. An emerging theme in IMC biology is the expansion of IMC protein families in coccidian apicomplexan species. This expansion correlates with the presence of the apical cap IMC sub-compartment in these species, which often encode IMC protein homologs that are specifically targeted to apical cap. In the case of the
case of the glideosome, the significance of GAP70 function in maintaining a larger PM-IMC
spacing in the apical cap than in the rest of the IMC may reflect beneficial morphological
constraints in the curvature of the parasite apex (Frenal et al., 2010). However, the importance of
this differential spacing appears subtle as GAP45 migrates into the apical cap in a GAP70
knockout with little or no consequence \textit{in vitro}.

\textbf{Surface adhesin shedding.} Propulsion is achieved via translocation of adhesins in an apical to
posterior fashion by the immobilized motor complex (Dubremetz and Ferreira, 1978; Russell and
Sinden, 1981). To support continual forward motion, adhesin are eventually shed from the
parasite surface and deposited as substrate-bound “trails” that can be visualized by IFA (Sibley,
2004). The importance of shedding for motility-dependent processes is illustrated by expression
of a non-cleavable MIC2 mutant increases host cell attachment but severely impedes invasion
(Brossier et al., 2003). Shedding is accomplished by an intramembrane cleavage event, implying
that rhomboid proteases are responsible (Carruthers et al., 2000b). Indeed, two of the six
rhomboids encoded within the \textit{Toxoplasma} genome (ROM4 and ROM5) traffic to the plasma
membrane (Dowse and Soldati, 2005). ROM4 is localized evenly across the entire parasite
surface while ROM5 is concentrated at the posterior end of the parasite, suggesting a role in
release of adhesins as they migrate to the rear of the cell surface (Brossier et al., 2005). \textit{In vitro}
analysis showed that ROM5 displays broad substrate specificity; however, no activity was
detected for ROM4 in similar assays (Brossier et al., 2005; Dowse et al., 2005). Nonetheless,
conditional suppression of ROM4 revealed a role for this protease in shedding of MIC2, MIC3
and AMA1 (Buguliskis et al., 2010). Furthermore, parasites depleted of ROM4 are unproductive
in gliding motility and inhibited in invasion. This mutant also attaches better to host cells than
wild type but exhibits a defect in apical re-orientation, which may be the source of the invasion defect.

Unexpectedly, shedding of surface adhesins may also function as a signal to begin replication. Parasites presumably monitor their extracellular/intracellular status to coordinate replication, as extracellular tachyzoites do not initiate new rounds of division. Using a dominant negative strategy, Santos et al. discovered that ROM4-mediated proteolytic release of the AMA1 C-terminal tail in the parasite cytosol provides a signal to proceed in replication (Santos et al., 2011). The authors observe a major block in replication when AMA1 cleavage is inhibited through overexpression of a dominant negative version of ROM4 and this block can be rescued by exogenous expression of the AMA1 C-terminal tail but not unrelated peptides. However, these results were disputed by the generation of a non-cleavable AMA1 mutant that inhibits invasion but not replication and this remains a point of controversy (Parussini et al., 2012).

Host penetration and subversion

The moving junction. Following attachment and apical reorientation, rhoptry secretion is triggered and host penetration begins. During the late 1970s, detailed analysis of P. knowlesi merozoite invasion of erythrocytes by TEM revealed the establishment of a circumferential zone of attachment between the parasite and host cell surface reminiscent of a tight-junction interface (Aikawa et al., 1978). This tight-junction was observed to form following apical reorientation of the parasite and then migrate down the length of the parasite as ring-shaped point of intimate attachment, eventually closing behind the parasite following complete host cell penetration. While the tight-junction is stably fixed at the erythrocyte surface, it appears to slide along the surface of the parasite during penetration and was thus named the “moving junction” (MJ). Loss
of the merozoite surface coat following passage beyond this tight-junction suggested this was a site of molecular modification during the invasion event. This was further confirmed by freeze-fracture EM studies which showed that intramembranous particles (IMPs) on the P face of the merozoite plasma membrane were absent beyond the MJ (Aikawa et al., 1981). These studies also revealed an intriguing rhomboidal array of particles at the MJ suggestive of a highly organized molecular structure at this site.

**Molecular components of the MJ: AMA1 and the RON2/4/5/8 complex.** Attempts at identification of interacting partners led to the surprising finding that AMA1 is able to immunoprecipitate the previously identified rhoptry neck proteins 2 and 4 from *Toxoplasma* lysates (Alexander et al., 2005). Subsequent IFA analysis of the localization of RON4 during host penetration revealed that this protein is secreted from the rhoptry neck into a discrete, ring shaped structure that corresponds with the point of parasite constriction at the MJ and migrates with the MJ down the length of the parasite during invasion (Alexander et al., 2005; Lebrun et al., 2005). A similar re-localization from the rhoptry neck into the MJ was later shown for RON2 during invasion (Tyler and Boothroyd, 2011). The identity of two additional bands immunoprecipitated by AMA1, migrating at 110 and 45 kD respectively, was found to match a predicted ~178 kD protein (Ts4705) previously discovered in proteomic analysis of the rhoptries, although the localization of this protein to the rhoptries had not been confirmed (Bradley et al., 2005). Mass spectrometric peptides from the 110 kD band mapped exclusively to the N-terminal portion of the Ts4705 while the peptides form the 45 kD band mapped to an adjacent portion of the C-terminal region of the protein, consistent with a proteolytic processing event to separate this protein into N and C-terminal fragments (Alexander et al., 2005). Pulse-chase analysis of Ts4705 confirmed that the N and C fragments were translated as a single polypeptide which
undergoes proteolytic processing to yield mature N and C fragments (Besteiro et al., 2009).

Furthermore, antibodies raised independently against the recombinant N- or C-terminal portions of Ts4705 both produced an IFA signal in the rhoptry neck and labeled the MJ during host invasion and this protein was thus named RON5 (Straub et al., 2009). Further crosslinking experiments during parasite invasion confirmed the biological relevance of this AMA1 interaction with the RON2/4/5 complex, hereafter referred to as the MJ RON complex (Alexander et al., 2005).

RON8, an additional member of the MJ RON complex, was independently identified by more extensive immunoprecipitation analysis of the AMA1-MJ RON complex and by a monoclonal antibody in Neospora (Besteiro et al., 2009; Straub et al., 2009). Unlike RONs 2/4/5, RON8 is not broadly conserved among apicomplexans and appears restricted to the Coccidia. Using a combination of pulse-chase labeling and immunoprecipitation, Besteiro et al. showed that the MJ RON complex is pre-formed in the rhoptry necks prior to interaction with AMA1 and that differential transcriptional regulation between the MJ RONs and AMA1 likely prevents their premature association within the secretory pathway prior to host invasion (Besteiro et al., 2009). Subsequent work in other species, particularly P. falciparum, revealed that the AMA1-MJ RON complex and its role in invasion is conserved across the phylum (Alexander et al., 2006; Cao et al., 2009; Collins et al., 2009; Richard et al., 2010). Interestingly, paralogs of AMA1 and RONs 2 and 4 are encoded by Toxoplasma and other coccidans while non-coccidans, such as Plasmodium spp, lack these isoforms (Besteiro et al., 2011). No paralogs of RON5 are encoded by any apicomplexan.

**RON complex topology at the MJ.** Among the five components of the Toxoplasma MJ RON complex, AMA1 and RON2 are confirmed membrane proteins while RON4 and RON8 are
soluble. RON5 may contain a transmembrane domain within its putative, N-terminal pro-
domain, although the confidence for this prediction is weak (Besteiro et al., 2009). The
identification of parasite proteins at the MJ raised the exciting possibility that the MJ RON
complex plays a role in tight-junction formation. Since AMA1 was known to be secreted onto
the parasite surface, the presence of transmembrane domains in RON2 suggested that this protein
might be inserted into the host plasma membrane. To resolve this possibility, a series of topology
experiments using differentially permeabilized cells or host cells pre-loaded with antibodies
against specific regions of each MJ RON were carried out and showed that RON4, RON5N,
RON5C and RON8 as well as the N-terminal portion of RON2 are all present on the cytosolic
side of the host plasma membrane at the MJ during invasion (Besteiro et al., 2009; Straub et al.,
2009). In contrast, a C-terminal portion of RON2 was found to be exposed to the extracellular
environment, suggesting that this region of the protein is the interaction domain linking the MJ
RON complex to AMA1 on the parasite surface (Lamarque et al., 2011).

The AMA1-RON2 interaction. Using the conservation of different regions of RON2 as a guide,
Tyler and Boothroyd pinpointed a highly conserved, ~50 amino acid region in the RON2 C-
terminus as a putative AMA1-interaction domain (Tyler and Boothroyd, 2011). A recombinant
GST fusion to this peptide was able to immunoprecipitate AMA1 from parasite lysates and bind
AMA1 on the surface of extracellular parasites, indicating a specific interaction with AMA1.
Furthermore, pretreatment of parasites with the same recombinant protein was found to inhibit
invasion but not rhoptry secretion, indicating this interaction is indeed important for host
invasion. Independently, Lamarque et al. identified the same interaction domain in the RON2 C-
terminus and demonstrated that a recombinant portion of this domain was able to bind
recombinant AMA1 in ELISA assays or the AMA1 ectodomain displayed on the surface of
mammalian cell through heterologous expression (Lamarque et al., 2011). Interestingly, this domain from both *Toxoplasma* and *P. falciparum* RON2 were each shown to interfere with invasion, but not in a cross species fashion, indicating that the interaction between AMA1 and RON2 is conserved across the Apicomplexa, but that specific features of the interaction have diverged in different species. A co-crystal structure of a RON2 peptide bound to the AMA1 ectodomain has been solved for both *Toxoplasma* and *P. falciparum*, revealing a strong interaction resulting from the RON2 peptide burying a large amount of surface area in a hydrophobic groove on the surface of AMA1 (Tonkin et al., 2011; Vulliez-Le Normand et al., 2012). These structural studies also clarify the species-specific nature of the interaction.

Collectively, these results led to the proposal of a model where the MJ RON complex would be inserted into the host membrane at the onset of invasion with RON2 spanning the host PM to form a critical link with AMA1 on the parasite surface. This AMA1-RON2 interaction has been hypothesized to serve as a link to the host cortex to generate a stable point of traction at the MJ, which the parasite uses to pull itself into the host during penetration by engagement of AMA1 with the glideosome motor (Tyler et al., 2011). In support of this model, a second copy of AMA1 harboring a point mutation in its cytoplasmic tail which disrupts binding of the recombinant AMA1 tail to aldolase is unable to rescue invasion following knockdown of endogenous AMA1 (Sheiner et al., 2010). However, it should be noted that an interaction between AMA1 and aldolase in the parasite could not be demonstrated as it has for MIC2, although this may be due to a limited window of this interaction during invasion.

Alternatively or in addition to functioning in traction, the AMA1-RON2 interaction may sense MJ formation and transduce a signal to initiate host penetration. This scenario is supported the following observations in *Toxoplasma* and *P. falciparum*: (i) cytochalasin D treated parasites
attach, apically reorient, form an MJ and secrete rhoptry bulb proteins but cannot penetrate the host cell due to a block in motility in the absence of actin polymerization (Aikawa et al., 1981; Hakansson et al., 2001; Miller et al., 1979); (ii) conditional knockdown of AMA1 results in a loss of secretion of rhoptry body proteins, (Mital et al., 2005); (iii) treatment of parasites with RON2 peptides encompassing the AMA1-binding site mimics AMA1 engagement of RON2 by binding AMA1 and blocks interaction between AMA1 and RON2 as well as host cell penetration (Lamarque et al., 2011; Srinivasan et al., 2011; Tyler and Boothroyd, 2011); (iv) under these circumstances, secretion of rhoptry bulb proteins still occurs as does engagement of the motor, resulting in merozoites pushing unproductively against the erythrocyte surface (Richard et al., 2010; Riglar et al., 2011; Srinivasan et al., 2011; Tyler and Boothroyd, 2011); (v) antibodies that bind near but not in the RON2 binding site on AMA1 block interaction between AMA1-RON2 and secretion of rhoptry body proteins (Srinivasan et al., 2011). Collectively, these data suggest AMA1 engagement of RON2 may transmit a signal for secretion of rhoptry body proteins. Additionally, the AMA1 cytoplasmic tail is phosphorylated in both Toxoplasma and Plasmodium and studies in P. falciparum found that mutants which abolish these phosphorylation events interfere with invasion but not with binding aldolase (Leykauf et al., 2010; Solyakov et al., 2011; Treeck et al., 2011; Treeck et al., 2009). Thus, it is tempting to speculate that phosphorylation of the cytoplasmic tail of AMA1 is a requisite step in a signaling cascade that actives rhoptry secretion and penetration.

While a large amount of evidence now supports the importance of AMA1 participation in the MJ and in host invasion, recent studies have surprisingly revealed this protein is dispensable during certain stages of the Plasmodium life cycle. Using a stage-specific Flp/FRT mediated recombination event, Giovannini et al. ablated expression of the AMA1 gene in P. berghei
sporozoites (Giovannini et al., 2011). Remarkably, these AMA1-null sporozoites were still able to efficiently invade hepatocytes; however, merozoites derived from these sporozoites fail to propagate, indicating AMA1 is critical for invasion in the merozoite, in agreement with earlier work. RON2 and AMA1 isoforms are variably expressed in the tachyzoite and sporozoites life stages of *Toxoplasma*, suggesting that unique stage-specific MJ RON complexes exist (Besteiro et al., 2011). However, no AMA1 or MJ RON isoforms are encoded within the genomes of the *Plasmodia*. Experiments by Giovannini *et al.* using a *Toxoplasma* AMA1 conditional knockdown mutant suggest that AMA1 may not be required to form a RON4-positive MJ but that AMA1 is important in initial attachment and rearrangement of parasites prior to MJ formation. This later finding is disputed by a considerable body of earlier work (discussed above) and clear resolution of the controversy surrounding the role of AMA1 during invasion awaits future functional studies.

**Roles for cytosolic RONs.** While the interaction between AMA1 and RON2 on the parasite surface is now characterized, the bulk of the MJ RON complex, present at the cytosolic face of the host plasma membrane during invasion, is less well studied. Using the same-stage specific recombination strategy discussed above, Giovannini *et al.* were also able to eliminate some expression of RON4 in *P. berghei* sporozoites which resulted in a decrease in invasion, indicating RON4 plays an important role in this process (in contrast to the dispensability of AMA1 at this stage) (Giovannini et al., 2011). This study indicates MJ RONs are vital for invasion but stops short of providing insight into the reason for this importance of RON4.

A more extensive functional analysis of the coccida-restricted MJ component RON8 was achieved through disruption of the *RON8* gene in *Toxoplasma*, showing that loss of RON8 results in a ~70% decrease in host penetration and a more than five log reduction in virulence.
Interestingly, \textit{ΔRON8} parasites that fail to invade were found to detach from the host rather than stall at the attachment stage, suggesting a possible failure by the parasite to secure a firm grip on the host and commit to invasion. When exogenously expressed in mammalian cells, RON8 traffics to its site of action at the cytosolic face of the plasma membrane, suggesting it facilitates a role in stabilizing penetration through direct interaction with one or more features of the host cell cortex (e.g., the cortical cytoskeleton) \citep{Straub2009}. While the host has traditionally been viewed as passive during the invasion process, recent work provides evidence for polymerization of host actin at the MJ during invasion \citep{Gonzalez2009}. Observations in \textit{Toxoplasma} and \textit{P. berghei} suggest that the parasite actives host Arp2/3 to induce rapid F-actin polymerization at the MJ, which the parasite may exploit for an anchor point to pull its way into the host cell. An exciting possibility is that this anchor is generated by interaction of the MJ RON complex with the host cortical cytoskeleton.

In the absence of RON8, association between the remaining MJ RONs is unaffected and invading parasites still form a RON4-positive MJ. However, a possible defect in MJ closure was observed with trails labeled by RONs 2, 4 and 5 extending behind 15\% of invaded parasites. Collectively, these results reveal that RONs 4 and 8 are import for invasion. However, functional conclusions about the RON2/4/5 complex, conserved throughout apicomplexans, is limited by technical challenges in the \textit{P. berghei} system and the lack of functional data on RON5 in any species. To aid this situation, Chapter 3 presents a comprehensive analysis of \textit{Toxoplasma} RON5 demonstrating that this protein is crucial for organization of the MJ complex and providing the first clear genetic demonstration that the RON2/4/5 complex plays an indispensable role in invasion.
**Parasitophorous vacuole formation.** As the parasite penetrates the host cell, a parasitophorous vacuole (PV) is created within which the parasite resides and replicates until disruption of the host and egress. The PV membrane (PVM) is largely derived from the host plasma membrane (PM) through invagination of the PM during host penetration (Suss-Toby et al., 1996). A spike in the conductance of the host PM is observed upon apical attachment of the parasite to the host and thought to represent a transient breach in the host membrane that may correspond with initial secretion of the rhoptries and insertion of the MJ RON complex into the host membrane. At the end of penetration, separation of the PVM from the host membrane occurs by pinching off a fission pore. Apparent defects in PVM fission were observed in Δron8 parasites following invasion, suggesting that the MJ RON complex may mediate this fission event (Straub et al., 2011). Whether other parasite or host factors similar to known components of membrane fission machinery (such as dynamins) play a role in this process is not known.

**Molecular sieving at the MJ.** A point of great interest is the fact that following invasion, the parasite does not enter the host endocytic pathway or fuse with host lysosomes (Jones and Hirsch, 1972; Mordue et al., 1999b). This endocytic escape is dependent on the active invasion process as vacuoles resulting from phagocytic uptake of parasites acquire endocytic markers and ultimately fuse with host lysosomes (Mordue and Sibley, 1997). Subsequent studies determined that the host membrane is modified as it “flows” past the MJ such that certain host membrane components are selectively excluded from the forming PVM and this is thought to be the mechanism by which the parasite avoids destruction by host lysosome fusion (Mordue et al., 1999a). The nature of this molecular sieving at the MJ has been evaluated by determining the ability of a number of host membrane constituents (both lipids and proteins) to access the PVM. Host lipids are allowed access to the PVM, as are proteins affixed to the membrane by lipidation.
(GPI-anchors on the extracellular leaflet and fatty acylation on the cytoplasmic leaflet). In contrast, type I transmembrane proteins are not allowed to enter the nascent PVM during invasion. Additionally, proteins anchored to the extracellular leaflet by lipidation that are components of larger multimeric complexes do not pass the MJ. Many of the transmembrane proteins found to be excluded from the PVM are linked to the host cytoskeleton (ICAM1, CD44, Na\(^+\), K\(^-\)-ATPase and \(\beta_1\)-integrin). In the case of the ICAM1, restriction from the PVM was found to require a cytosolic domain necessary for anchoring the protein to the host cytoskeleton (Mordue et al., 1999a). However, association with the cytoskeleton is not a sufficient criteria for sieving as hemagglutinin, which is not linked to the cytoskeleton, is also unable to pass the MJ.

An investigation of the role of lipid rafts in sieving at the MJ showed that sequestration of proteins within these membrane microdomains is neither necessary nor sufficient for passage beyond the MJ but may sometimes favor incorporation into the PVM (Charron and Sibley, 2004). As perturbation of the cytosolic tail of ICAM1 enable this protein to enter the PVM, the mechanism of filtration may reside at the cytosolic face of the host PM. The fact that the bulk of the MJ RON complex is positioned at this site in the MJ during invasion raises the possibility that this complex may be the sieving machine. A similar sieving process occurs during \textit{P. falciparum} merozoite invasion of erythrocytes, which also involves the MJ RON core complex of RON2/4/5 (RON8 is not present in \textit{Plasmodium} spp) (Murphy et al., 2007; Murphy et al., 2004). In summary, the process of molecular sieving which occurs at the MJ is highly complex and a more thorough understanding of this activity awaits further molecular characterization of the invasion event.
**Contribution of rhoptries to the PV.** At the onset of invasion, likely corresponding with the transient breach in the host membrane observed by patch-clamp experiments (see above), a burst of rhoptry secretion occurs injecting multilamellar vesicles containing rhoptry proteins into the host cell. These structures fuse with the PV following parasite invasion and were thus termed evacuoles since they display PV-like properties but do not contain an actual parasite (Hakansson et al., 2001). In this way, rhoptry derived lipids and rhoptry body proteins can enter the PV (as in the case of ROP1) or associate with the cytoplasmic face of the PVM (as in the case of ROP2, which associates with the PVM via a series of amphipathic helices) (Reese and Boothroyd, 2009). In addition to fusing with the forming PV, evacuoles traffic away from their site of injection in a host-microtubule dependent fashion to discrete regions of the host cell, including the ER and mitochondria (Hakansson et al., 2001). Rhoptry secretion appears to continue following the establishment of the MJ and the beginning of penetration, resulting in the accumulation of rhoptry proteins such as ROP1 within the forming PV (Figure 1-5C). Thus, the rhoptry contents likely contribute to modification of the PV lumen, although the functional significance of this is poorly understood.

**Host subversion by rhoptry body proteins is critical for virulence.** Evaluating F₁ progeny from a genetic cross between a Type II and Type III strain, Saeij *et al.* identified a number of key virulence loci within the parasite genome (Saeij et al., 2006). Remarkably, each of these loci was found to encode a kinase (ROP16 and ROP18) or pseudokinase (ROP5) that is injected from the rhoptry body into the host cell at the onset of invasion. The first of these to be characterized was ROP16, which traffics to the host nucleus following injection and directly phosphorylates the transcription factors STAT3 and STAT6, resulting in down regulation of pro-inflammatory cytokines IL-6 and IL-12 (Ong *et al.*, 2010; Saeij *et al.*, 2007). The importance of this host
immune modulation is underscored by the major difference in parasite virulence resulting from a single amino acid polymorphism between the ROP16 alleles harbored by Type I and Type II stains (Yamamoto et al., 2009). This powerful difference may be due in part to the fact that host modulation is extended to cells that never incur an actual infection as Koshy et al. found that Toxoplasma can inject rhoptry effectors into cells it does not productively invade with the same outcome in host manipulation (Koshy et al., 2012). Remarkably, in a chronic mouse infection, cells that have been injected with rhoptry effectors but are not infected by parasites substantially outnumber those that are infected in multiple tissues, including the brain.

Another key virulence locus encodes ROP18, a kinase which localizes to the surface of the PVM following injection into the host cell where it acts to phosphorylate immunity-related GTPases (IRGs) (Fentress et al., 2010; Fentress et al., 2012). In the absence of parasite-mediated inhibition, IRGs are loaded onto PVM, switch to an active GTP-bound state and multimerize, resulting in rupture of the PV and parasite death (Martens et al., 2005; Zhao et al., 2009). Phosphorylation by ROP18 inactivates IRGs to prevent their loading onto the vacuole membrane and subsequent destruction of the PV, enabling parasite survival and virulence (Steinfeldt et al., 2010). ROP18 has also been reported to interact with the ER membrane-bound transcription factor ATF6β to induce its degradation, thereby modulating the host immune response (Yamamoto et al., 2011).

The third and most prominent virulence locus encodes a family of pseudokinases collected referred to as ROP5 (Reese et al., 2011). ROP5 directly binds IRGs to induce a conformation change to an inactive, GDP-state that also exposes threonine residues targeted by ROP18 (Fleckenstein et al., 2012). In this way, ROP5 serves as a co-factor for ROP18, inhibiting
IRG switching to the GTP-bound activation state while simultaneously enabling phosphorylation by ROP18 to permanently inactivate the IRG target.

Phylogenomic analysis indicates that ROP kinases constitute large, 44 member family in *Toxoplasma* and one additional member of this family, ROP38, has been evaluated and shown to down regulate MAPK signaling during in vitro infection of human cells (Peixoto et al., 2010). Studies focused on parasite virulence have exclusively made use of the mouse model of infection, however the remarkably wide *Toxoplasma* host range suggests that many of the rhoptry proteins injected during invasion may be tailored to modulate specific hosts but non-discriminately secreted during invasion of any cell. Indeed, IRGs are not present in humans and virulent alleles of ROP5 and ROP18 do not aid parasite survival in IFNγ-activated human cells (Niedelman et al., 2012). In addition to the large *Toxoplasma* kinase family, a number of other rhoptry body proteins have been identified, including protein phosphatases, multiple classes of proteases and the actin-binding protein toxofilin and some of these have been confirmed to be secreted into the host cell (Bradley et al., 2005; Gilbert et al., 2007; Hajagos et al., 2012; Lodoen et al., 2010). However, the functional consequence of delivering the majority of these putative effectors into the host is largely unknown. Recent evidence does suggest toxofilin promotes local actin depolymerization at the site of invasion to aid in passage through the host cortical cytoskeleton, presumably a major hurdle to host entry for the parasite (Delorme-Walker et al., 2012). However, deletion of toxofilin produces only a minor defect in invasion kinetics, indicating that the cortical actin cytoskeleton is either not a major barrier or more likely that other factors are involved.
Contribution of dense granules to the PV and host subversion. In contrast to the transient secretion of rhoptries that accompanies invasion, dense granules undergo a short burst of secretion shortly after invasion followed by constitutive secretion over the course of the intracellular residence of the parasite (Dubremetz et al., 1993). While the dense granule proteins are poorly characterized, most appear to reside in the PV or PVM after their exocytosis and serve to modify this compartment to accommodate the intracellular lifestyle of Toxoplasma (Mercier et al., 2005). A number of changes occur within the PV and PVM during the first hour following invasion. Initially, at the time of PV formation, the PVM is devoid of IMPs, which appear during the hour following invasion and are likely formed from proteins secreted into the vacuole by the rhoptries and dense granules (Porchet-Hennere and Torpier, 1983). Additionally, a membranous network of nanotubules (MNN) is formed during this hour, beginning at the posterior end of the parasite and eventually filling the entire PV (Sibley et al., 1995). Initiation of the MNN requires GRA2, which contains a pair of amphipathic alpha-helices that enable membrane association and induction of membrane curvature (Mercier et al., 2002). Tubule extension further requires the transmembrane protein GRA6. While the MNN is suspected to play roles in nutrient acquisition or delivery of parasite products into the host cell, the network is severely degenerate or completely eliminated in a ΔGRA6 or ΔGRA2 parasite line, respectively, without any obvious ramifications for parasite growth in tissue culture, although the ΔGRA2 mutant does display decreased virulence during a mouse infection (Mercier et al., 1998).

A number of GRA proteins with no clear homology to other characterized proteins have been identified and characterized, but clear functions remain elusive with the exception of GRA2 and GRA6. In addition, several dense granule proteins with obvious homologies have been discovered, including cyclophilins, nucleoside triphosphate hydrolases (NTPases), and serine
protease inhibitors (Bermudes et al., 1994; High et al., 1994; Morris et al., 2002). All of these proteins are secreted into the PV and the NTPases have been shown to be enzymatically active in vitro, but a clear establishment of function within the PV is still outstanding (Asai et al., 1995).

In addition to the role of dense granule proteins in PV generation, a recently identified dense granule protein, GRA15, was shown to be secreted into the host cytosol where it activates the NF-κB pathway resulting in suppression of the host immune response in general and IL-12 production in particular. While modulation of host signaling by rhoptry kinases (see below) has been well characterized, this finding demonstrates that important effectors for determining virulence and the outcome of infection are stored in the dense granules as well (Rosowski et al., 2011).
Replication

Apicomplexans display a remarkable flexibility in the ordering of key cell division steps of DNA replication, nuclear division and cytokinesis (Figure 1-6) (Gubbels et al., 2008). Replication generally occurs by a unique form of internal budding wherein de novo construction of new IMCs provides the scaffold for daughter cell assembly within the cytosol. In the *Toxoplasma* tachyzoite, division proceeds by a process of endodyogeny with two new daughter parasites being assembled within the cytosol of an intact mother cell. The beginning of replication is signaled by the migration of the centriole to the base of the nucleus, where it divides before returning the apical face of the nucleus (Nishi et al., 2008). Bud nucleation begins when the nucleus has reached a DNA content of ~1.8N (Hu et al., 2002a). The specific factors responsible for nucleation of the IMC scaffold are unknown, but this role may be accomplished by IMC15, a member of the intermediate-filament family of IMC proteins, which appears at a very early stage in bud generation and also associates with the centriole (Anderson-White et al., 2011). Once formed, the posterior end of each bud is labeled by the membrane occupation and recognition nexus protein MORN1, which is also present in the centrocone (Gubbels et al., 2006). As the buds extend, a process likely driven by polymerization of the subpellicular microtubules, DNA replication finishes and the 2N nucleus is drawn up into each bud, adopting a horseshoe shape until an eventual fission event resolves the nucleus into two separate 1N allotments (Shaw et al., 2000). Other organelles are also replicated through a fission based process (Golgi, mitochondrion and apicoplast) or synthesized de novo (micronemes and rhoptries) and packaged into the daughter buds (Nishi et al., 2008). After the each daughter has received the full allotment of organelles, the base of each growing IMC contracts, an event that requires MORN1 and is likely powered by the calcium-binding contractile protein TgCentrin2.
(Heaslip et al., 2010; Hu, 2008; Lorestani et al., 2010). At this stage, the daughter buds occupy nearly the entire maternal cytosol. In a terminal step, the daughters acquire a plasma membrane through a combination of Rab11a-mediated synthesis and adoption of the maternal membrane (Agop-Nersesian et al., 2009). At the completion of the process, a small residual body containing remnants of the mother is discarded in the PV.

In contrast to the endodyogeny division pathway employed during the tachyzoite phase, asexual replication within the intestine of the feline host occurs through endopolygeny, where multiple rounds of DNA replication and nuclear division precede a final round of DNA replication and nuclear division during which IMC scaffolds are assembled and packaged with organelles (Figure 1-6). Interestingly, previous studies observed that tachyzoites sometimes (< 1% of the time) divide in an endopolygeny-like fashion, generating three or more buds per mother cell during a single round of replication (Hu et al., 2002a). The frequency of this phenomenon in tissue culture was found to be enhanced by certain stresses but invariably return to normal levels (< 1%) (Hu et al., 2004). The signals that orchestrate this process and determine the division path taken during replication are currently unknown. While the IMC has long been known to be the scaffold within which new daughter parasites are assembled, data is presented in Chapter 4 suggesting that proteins localizing to the IMC also play a role in counting daughters during replication. Thus, the IMC may be a critical signaling platform in determination of the cell division pathway through which the parasite proceeds.
Egress

Following repeated replication events, the PV is filled with new parasites and occupies nearly the entire host cytosol. Egress of the parasites then occurs, destroying the host cell and liberating the parasites to invade neighboring cells and reinitiate the lytic cycle. Abscisic acid (ABA), an important plant signaling hormone, is a key host exit cue rapidly produced by *Toxoplasma* just before egress (Nagamune et al., 2008a). The spike in ABA causes production of cyclic ADP-ribose, which triggers release of parasite Ca\(^{2+}\) stores resulting in microneme secretion, motility activation and egress. In addition, external potassium ion concentration is a critical gauge for host cell integrity and a drop in K\(^+\) concentration in the host cytosol triggers release of parasite Ca\(^{2+}\) stores resulting in microneme secretion, motility activation and egress (Moudy et al., 2001). Loss of host plasma membrane integrity, possibly through mechanical stress at the host cortex after the host cell is swollen with replicated parasites or through immune attack, results in a decrease in cytosolic K\(^+\) levels stimulating egress (Persson et al., 2008). Thus, K\(^+\) sensing appears to serve as an emergency monitoring system for escaping from the host in dire circumstances while ABA signaling is a critical signal during normal circumstances.

A number of barriers must be crossed by the parasite to escape the host during egress, including the PVM, host organelles and cytoskeletal material recruited to the surface of the PV and finally the host cortical cytoskeleton and plasma membrane (Roiko and Carruthers, 2009). Activation of gliding motility is key for traversing these barriers as the block of motility by pharmacological manipulation or gene disruption prevents host exit (Black et al., 2000; Heaslip et al., 2011). However, motility itself is not sufficient for the parasite to exit the host and egress also requires TgPLP1, a perforin-like protein secreted from the micronemes, which disrupts the PVM (Kafsack et al., 2009). Interestingly, this central requirement for microneme secretion in
Toxoplasma host escape is not paralleled erythrocyte escape by P. falciparum as a conditional block in microneme exocytosis through knockdown of PfDOC2.1 does not inhibit merozoite egress from red blood cells (Farrell et al., 2012). Host factors have also been found to contribute to the egress process as knockdown of the Ca\(^{2+}\)-dependent host proteases calpain-1 and -2 inhibits egress (Chandramohanadas et al., 2009). It was hypothesized that these proteases may be critical for disassembly of the host cytoskeleton as calpains are known to play important roles in cytoskeletal rearrangements.

A number of similarities between the invasion and egress processes have been noted including their rapid nature and dependence on Ca\(^{2+}\) fluctuation, microneme secretion and active parasite motility. Live imaging and scanning EM analysis of induced parasite egress revealed remarkable similarities to the invasion process with parasites crossing the PVM and host plasma membrane at discrete sites through points of constriction reminiscent of the MJ (Hoff and Carruthers, 2002). Additionally, although clear rhoptry secretion is not observed during egress, Alexander et al. did observe RON4-positive moving junction ring formation during this process, further suggesting that the MJ RON complex or other rhoptry derived structures may be critical for host exit (Alexander et al., 2005). However, a definitive contribution of the rhoptries to egress remains unclear as no mutants that specifically ablate rhoptry function have been reported. This problem is addressed in Chapter 2.
Preface

The focus of my dissertation is an understanding of the molecular mechanisms that undergird host cell invasion and subversion as well as intracellular replication in *Toxoplasma*. This has largely been pursued through the characterization of parasite-specific organelles (the rhoptries and IMC) and the processes facilitated by these organelles that are critical for parasite biology and thus for virulence. A large portion of genes within the *Toxoplasma* genome encode hypothetical proteins and many of the unique activities within this phylum of pathogens are likely to be carried out or supported by proteins with uncharacterized functionalities. Indeed, of the dozen or so proteins that were the specific focus of my graduate research, only one of these (TgPAT1) is a clearly defined member of an enzyme family and most contain no detectible sequence features that would offer any clue to their function. Despite the inherent difficulties associated with the study of such hypothetical proteins, these molecules hold promise not only to reveal fascinating novel biology in divergent protozoan species, but also for understanding the molecular basis of host-parasite interactions in the Apicomplexa and ultimately for the development of novel control strategies to combat the devastating diseases caused by these organisms.

My graduate work on *Toxoplasma* began with the characterization of an IMC protein that was discovered through a panel of monoclonal antibodies. This led to the identification of a family of apicomplexan-specific proteins (the ISPs) discretely localized within several sub-compartments within the IMC. At the outset of these studies, only a handful of IMC proteins had been identified and factors responsible for organization of the IMC were unknown. Work aimed at elucidating the trafficking and function of the ISP family (described in Chapters 4 and 5)
showed the critical importance of palmitoylation in protein trafficking to the IMC revealed an important role for these proteins in endodyogeny.

During this time, I became interested in the host cell invasion process and the parasite molecules that are involved. Simultaneously, a number of new technologies became available for genetic manipulation of the parasite and I was able to apply these to the study of the rhoptry neck protein RON5, described in Chapter 3. These studies showed that RON5 is critically involved in organizing the MJ RON complex, enabled a clear genetic demonstration of the critical importance this complex in host penetration and provided a domain analysis of RON5 to begin defining key regions of the protein involved in MJ complex function.

As a follow-up to my initial work on the ISPs, I set out to identify palmitoyl acyl transferases localized to parasite-specific organelles and characterize the functional importance of this lipid modification in unique parasite processes. While a PAT resident within the IMC was discovered, this work also led to the identification of a PAT localized to the rhoptries (TgPAT1) and the discovery that palmitoylation activity is responsible for tethering the rhoptries at the cell apex. Additionally, conditional disruption of TgPAT1 provided the first mutant that specifically disrupts the rhoptries but not other secretory organelles, allowing for a clear description of importance of this organelle in invasion but not intracellular replication or egress. This work is described in Chapter 2.

In this dissertation, I have presented these studies in an order that follows the sequence of events during the lytic cycle (invasion, replication and egress) rather than in the chronological order in which they were performed. Finally, in Chapter 6, I draw conclusions from this work,
layout goals for further analysis of *Toxoplasma* invasion and replication and offer perspectives on apicomplexan cell biology and the development of new control strategies.
Figure Legends

Figure 1-1 – Phylogeny of the Apicomplexa.

Tree showing hypothetical phylogeny tree for the apicomplexan phylum. The three principal apicomplexan clades are the Gregarina (parasites of invertebrates), the Coccida (parasites of vertebrates, including *Toxoplasma gondii*) and the Hematozoa (parasites of both vertebrates and invertebrates, including *Plasmodium* spp). Distinctive features between groups include: the absence of the apicoplast in the cryptosporidians and eugregarines and the absence of the conoid in the hematozoans. Free-living organisms hypothesized to be related to the apicomplexans are indicated in grey at the base of the tree. Branch thickness represents the number of described species in each group (shown in white ovals). Adapted from (Šlapeta and Morin-Adeline, 2011).

Figure 1-2 – The *Toxoplasma* life cycle.

*Toxoplasma* undergoes a heteroxenous life cycle. Feline species serve as the definite host where the sexual cycle proceeds in the small intestine (top left), resulting in the generation of unsporulated oocysts that are shed in the feces. In the environment, sporulation occurs yielding mature, highly infectious oocysts each containing eight sporozoites. Ingestion of sporulated oocysts by virtually any animal results in the asexual cycle. Passage through the stomach induces breakdown of the oocyst and release of sporozoites, which convert to rapidly dividing tachyzoites, initiating the asexual cycle. Conversion of tachyzoites to slow growing bradyzoites leads to generation of persistent tissue cysts, predominantly in muscle tissue and the central nervous system, and chronic infection. Ingestion of these tissue cysts results in transmission of
either the asexual phase, in the case of intermediate hosts, or the sexual cycle, in the case of felines. Infection of intermediate hosts, including humans, can occur through ingestion of tissues cyst, oocysts or vertically during pregnancy. Adapted from (Hunter and Sibley, 2012).

Figure 1-3 – Ultrastructure of the *Toxoplasma* tachyzoite.

Diagram of a *Toxoplasma* tachyzoite and accompanying TEM image showing relevant features of the parasite ultrastructure. The pellicle is composed of the plasma membrane and underlying IMC, which is further under girded by a set of subpellicular microtubules (not shown). The conoid, a truncated cone composed of tubulin, resides at the extreme apex of the cell. Also featured at the apex are the microneme (M) and rhoptry (R) secretory organelles. Dense granules (DG), an additional class of secretory organelle, are scattered throughout the cytoplasm. The parasite possesses a polarized secretory pathway with clearly discernible ER and Golgi (G) compartments. A central nucleus (N) is visible. Following invasion, the parasite resides in a parasitophorous vacuole bounded by a membrane (VM) and filled with network of nanotubules (VN). Adapted from (Joiner and Roos, 2002; Lingelbach and Joiner, 1998).

Figure 1-4 – The lytic cycle of the *Toxoplasma* tachyzoite.

Tachyzoite propagation begins through contact with and invasion of a permissible host cell. During the invasion process, a parasitophorous vacuole is created within which the parasite begins to divide by a unique form of internal budding known as endodyogeny. Repeated rounds of replication result in a geometric expansion of the parasite, eventually leading to disruption of
the host cell out of which the parasite egresses to reinitiate the cycle through invasion of new host cells. Successive rounds of this lytic cycle results in tissue damage that, together with the ensuing host immune response, produces the pathology characteristic of Toxoplasmosis.

**Figure 1-5 – Host cell invasion by the Toxoplasma tachyzoite.**

(A) Diagram of key steps in host cell invasion by Toxoplasma. Secretion of adhesins from micronemes (blue) onto the parasite surface facilitates attachment and gliding motility. Following apical reorientation, complex of RON proteins (RON2/4/5/8) are injected from the rhoptry necks (red) into the host membrane. This RON complex interacts with the micronemal protein AMA1 on the parasite surface to form a tight-junction interface between the host and parasite called the moving junction (red). The moving junction is thought to severe an anchoring point through which the parasite pulls itself using gliding motility to enter the host. Shedding of surface adhesins supports forward movement. In the process, a parasitophorous vacuole (PV) is created through invagination of the host plasma membrane. During penetration, the moving junction also serves as a molecular sieve, selectively limiting access of host transmembrane proteins into the forming PV, resulting in a modified vacuole that is non-fusogenic with the host endolysosomal system. As the parasite penetrates, rhoptry body proteins (green) are injected into the host cytosol and the forming PV. The moving junction closes behind the parasite as it enters the host and eventually pinches off from the host membrane. Following invasion, dense granules (orange) secrete to modify the PV environment. (B) Rhoptry body (green) and neck (red) compartments labeled by immunofluorescence with antibodies against ROP13 and RON5. The parasite IMC is detected by antibodies against IMC1 (blue). (C) Visualization of rhoptry
secretion during host penetration. RON4 (red) is injected into the moving junction while ROP1 (green) is secreted into the forming PV. Overly with a phase-contrast image shows the parasite squeezing through a point of constriction at the RON4-positive moving junction to enter the host cell within a PV.

**Figure 1-6 – Apicomplexan modes of cell division by internal budding.**

Apicomplexan parasites divide by a unique process of internal budding wherein new daughter cells are assembled within the mother cytosol. Internal budding occurs in a variety of forms dictated by the ordering of the key division steps of DNA replication, nuclear division and cytokinesis, which involves synthesis of daughter IMC scaffolds, segregation of organelles into these scaffolds and adoption of the plasma membrane onto each daughter cell. (A) Most apicomplexans divide by a process called schizogony wherein multiple rounds of DNA replication and nuclear division precede a final step of DNA replication and nuclear division accompanied by daughter bud (IMC) assembly. Buds are assembled just beneath the maternal plasma membrane and bud out through the membrane in the final stage. (B) The *Toxoplasma* tachyzoite divides by endodyogeny, generating two daughters within the maternal cytosol, which mature before another round of endodyogeny begins. (C) Asexual stages of *Toxoplasma* division occurring within the intestinal epithelium of the definite host employ endopolygeny where successive rounds of DNA replication and nuclear division proceed a final round of DNA replication and nuclear division during which daughter IMCs are assembled in the cytosol, into which organelles are packaged. (D) An additional variation of the endopolygeny process is observed in *Sarcocystis* sp., where multiple rounds of DNA replication yield an unsegregated,
polyploid nucleus. Division of this polyploid nucleus then occurs simultaneously with daughter IMC generation and organelle packaging. Adapted from (Ferguson et al., 2007).
Figure 1-2

The diagram illustrates the life cycle of a parasite, starting with the ingestion of oocysts by a host. These oocysts are sporulated in the gut and produce sporozoites. Sporozoites infect the liver, resulting in tissue cysts. Further development leads to the shedding of oocysts, which can be ingested by a new host. The cycle continues with infection and tissue development in intermediate hosts, such as pigs and sheep, and potentially in humans through food or waterborne transmission.
Figure 1-4

1. Invasion

2. Division

3. Host Disruption & Egress

Host

Cell
Figure 1-5

A
Attachment
Micronemes

B
Phase

C
Penetration
Rhoptries
Shedding of adhesins

Remodeling
Dense granules

RON5 ROP13 BODY

5μ

RON1

1μ

Host lysosome
Vesicle (PV)

Moving Junction

Nascent

58
Figure 1-6

a. Schizogony

b. Endodyogeny

c. *Toxoplasma* Endopolygeny

d. *Sarcocystis* Endopolygeny
References


gondii with similar composition and function to the plant vacuole. Molecular microbiology 76, 1358-1375.


Chapter 2

A *Toxoplasma* palmitoyl acyl transferase and the palmitoylated armadillo-repeat protein TgARO govern apical rhoptry tethering and reveal a critical role for the rhoptries in host cell invasion but not egress
Abstract

Apicomplexans are obligate intracellular parasites that actively penetrate their host cells to create an intracellular niche for replication. Commitment to invasion is thought to be mediated by the rhoptries, specialized apical secretory organelles that inject a protein complex into the host cell to form a tight-junction for parasite entry. Little is known about the molecular factors that govern rhoptry biogenesis, their subcellular organization at the apical end of the parasite and subsequent release of this organelle during invasion. We have identified a *Toxoplasma* palmitoyl acyltransferase, TgDHHC7, which localizes to the rhoptries. Strikingly, conditional knockdown of TgDHHC7 results in dispersed rhoptries that fail to organize at the apical end of the parasite and are instead scattered throughout the cell. While the morphology and content of these rhoptries appears normal, failure to tether at the apex results in a complete block in host cell invasion. In contrast, attachment and egress are unaffected in the knockdown, demonstrating that the rhoptries are not required for these processes. We show that rhoptry targeting of TgDHHC7 requires a short, highly conserved C-terminal region while a large, divergent N-terminal domain is dispensable for both targeting and function. A catalytically inactive TgDHHC7 fails to rescue apical rhoptry tethering, demonstrating that tethering of the organelle is dependent upon TgDHHC7 palmitoylation activity. We tie the importance of this activity to the palmitoylated rhoptry armadillo-repeat containing protein TgARO by showing that conditional knockdown of TgARO recapitulates the dispersed rhoptry phenotype of TgDHHC7 knockdown. The unexpected finding that apicomplexans have exploited protein palmitoylation for apical organelle tethering yields new insight into the biogenesis and function of rhoptries and may provide new avenues for therapeutic intervention against *Toxoplasma* and related apicomplexan parasites.
Author Summary

Apicomplexans possess a highly polarized secretory pathway that is critical for their ability to invade host cells and cause disease. This unique cellular organization enables delivery of protein cargo to specialized secretory organelles called micronemes and rhoptries that drive forward penetration into the host cell. The rhoptries are tethered in a bundled at the apex of the parasite, but how these organelles are organized in this manner is unknown. In this work, we identify a rhoptry-localized palmitoyl acyl transferase (named TgDHHC7) that functions to properly affix the rhoptries at the apical end of the parasite. Conditional disruption of TgDHHC7 results in a failure to tether the rhoptries at the cell apex and a corresponding loss of rhoptry function. We exploit this mutant to clearly demonstrate a critical role for the rhoptries in host invasion but not attachment or egress. Additionally, we find that TgDHHC7 must be catalytically active to function and that knockdown of the candidate substrate TgARO produces an identical phenotype to loss of TgDHHC7. The finding that Toxoplasma employs protein palmitoylation to position the rhoptries at the cell apex provides new insight into the molecular mechanisms that underlie apicomplexan cell polarity, host invasion and pathogenesis.
Introduction

Apicomplexans are a large phylum of globally important parasites that cause substantial disease in their human and animal hosts. Species of particular interest to human health include *Toxoplasma gondii*, which infects one-third of the world’s human population and causes disease in immunocompromised individuals and neonates, and *Plasmodium falciparum*, which causes malaria resulting in 1-2 million deaths annually (Miller et al., 2002; Tenter et al., 2000). Most apicomplexans have obligate intracellular life cycles and the disease they cause is dependent upon their ability to actively invade their host cells, replicate within an intracellular niche and ultimately egress from the host. These key aspects of apicomplexan biology are critically linked to unique parasite-specific organelles and the remarkable polarized organization of the apicomplexan cell (Joiner and Roos, 2002).

Host cell invasion is a highly coordinated process of attachment and penetration involving two apical secretory organelles, the micronemes and rhoptries (Carruthers and Boothroyd, 2007). The micronemes are small, bar-shaped organelles that line the apical third of the parasite periphery. To initiate the invasion process, an array of molecular adhesins are released from the micronemes onto the parasite surface and facilitate attachment to the host cell. On the cytosolic side of the parasite’s plasma membrane, these adhesins are connected to an actin-myosin motor that is itself immobilized in a double-membrane system called the inner membrane complex (IMC). This motor apparatus (known as the glideosome) is responsible for generating a unique type of gliding motility that is utilized by apicomplexan parasites for host penetration (Keeley and Soldati, 2004).

Following attachment via micronemal adhesins, the parasites reorient to position their apical end towards the host membrane and the rhoptries are then released, an event that
corresponds with the beginning of host penetration (Carruthers and Boothroyd, 2007; Farrow et al., 2011). Rhoptries are club-shaped organelles each consisting of a larger, bulbous body and tapered, duct-like neck (Boothroyd and Dubremetz, 2008). The rhoptry necks are positioned at the extreme apex of the cell, providing a conduit for release of the organellar contents. At the onset of invasion, several proteins contained with the rhoptry necks are injected into the host membrane and localize to a tight-junction structure known as the “moving junction” through which the parasite passes to invade the host (Shen and Sibley, 2012). In contrast, proteins contained within the rhoptry body are injected into the host cytosol where they access various compartments and modulate host functions (Boothroyd and Dubremetz, 2008). As the parasite penetrates the host, a parasitophorous vacuole is formed from invagination of the host membrane within which the parasite resides and replicates (Suss-Toby et al., 1996).

Protein traffic to the rhoptries and micronemes proceeds through a shared pathway until late in the secretory pathway (Sloves et al., 2012). General secretory pathway mutants have been described that eliminate the function of both the micronemes and rhoptries, illustrating the key importance of these organelles for motility, attachment, invasion and egress (Breinich et al., 2009; Sloves et al., 2012). While a mutant in the calcium-binding protein TgDOC2.1 has recently been described that fails to recruit the membrane fusion machinery needed for microneme exocytosis, mutants that specifically disable the rhoptries have not been reported (Farrell et al., 2012). Thus, the precise contribution of this organelle to invasion and egress remains to be clearly addressed. Additionally, while the highly polarized nature of the apicomplexan cell, including apical rhoptry positioning, has fascinated cell biologists for decades, the molecular mechanisms that establish this cell polarity are not understood.
Protein palmitoylation is a widely employed eukaryotic strategy for the spacio-temporal control of protein localization and function (Linder and Deschenes, 2007; Resh, 2006b). Previous work in our lab and others showed that palmitoylated proteins play key roles in the organization and function of the IMC (Beck et al., 2010; Frenal et al., 2010; Fung et al., 2012). Additionally, the armadillo-repeat containing protein TgARO is recruited to the cytoplasmic face of the rhoptries via palmitoylation, indicating that this modification is also involved in rhoptry biology (Cabrera et al., 2012). More broadly, a recently reported “palmitoylome” in *Plasmodium falciparum* revealed that this modification is extensively applied to parasite proteins implicated in many unique processes that are critical for pathogenesis (Jones et al., 2012). Covalent attachment of palmitate to protein substrates is catalyzed by two palmitoyl acyl transferase (PAT) enzyme classes: the membrane bound O-acyl transferase (MBOAT) family and the Asp-His-His-Cys cysteine-rich domain (DHHC-CRD) family. Generally, MBOATs palmitoylate secreted proteins while DHHC-CRD PATs act on non-secreted substrates to recruit them to target membrane systems (Resh, 2006a). The *Toxoplasma* genome encodes three putative MBOAT family homologs. In contrast, 18 DHHC-CRD containing proteins are encoded, a relatively large number for a single-celled organism (mammals encode 23, *S. cerevisiae* encodes 7, *Giardia lamblia* encodes 9 and *Trypanosoma brucei* encodes 12), suggesting that palmitoylation of intracellular proteins has been extensively outfitted to serve the unique organelle systems in this parasite (Emmer et al., 2009; Saric et al., 2009; Smotrys and Linder, 2004). However, it should be noted that other apicomplexans appear to encode fewer DHHC-CRD proteins, such as *P. falciparum* which encodes 12. The localization and function of any of the DHHC-CRD proteins resident within apicomplexan-specific organelles remains to be determined.
In this work, we employ a candidate gene strategy to identify both rhoptry and IMC DHHC-CRD PATs in the model apicomplexan *Toxoplasma*. To explore the role of palmitoylation in rhoptry biology, we perform a conditional knockdown of the rhoptry PAT (named TgDHHC7). Interestingly, elimination of this enzyme results in a loss of apical rhoptry tethering with the organelles instead scattered throughout the cytosol. While the positioning of the rhoptries in this mutant is completely disrupted, the biogenesis, morphology and cargo sorting of the organelles are not impacted. Likewise, other secretory organelles are unaffected by loss of TgDHHC7. Parasites with dispersed rhoptries are completely unable to invade host cells, but motility, attachment and egress are not impacted, revealing the precise role of the rhoptries and demonstrating that their positioning is critical for function. We use mutagenesis to show that the divergent N-terminal region of TgDHHC7 is dispensable for both targeting and function while a short C-terminal region is required for rhoptry localization. A catalytically inactive version of TgDHHC7 fails to rescue rhoptry function, indicating that palmitoylation activity is required for tethering. Finally, we provide strong evidence that TgARO is a key substrate recruited by TgDHHC7 to facilitate rhoptry apical organization by showing that conditional knockdown of TgARO produces the same dispersed rhoptry phenotype that results from knockdown of TgDHHC7. These results demonstrate the importance of protein palmitoylation for apicomplexan biology and provide new insight into the polarization of the *Toxoplasma* cell and rhoptry biosynthesis and function.
Results and Discussion

Identification of palmitoyl acyl transferases targeted to the *Toxoplasma* rhoptries or IMC.

To explore the function of palmitoylation in parasite-specific processes, we sought to identify PATs resident in organelles unique to apicomplexans using the model apicomplexan *Toxoplasma*. Because we are primarily interested in the recruitment of proteins to the cytoplasmic face of organelles (Beck et al., 2010; Cabrera et al., 2012), we focused on the 18 DHHC-CRD homologs encoded within the *Toxoplasma* genome (Table 2-1). The rhoptries and IMC are assembled *de novo* during each round of parasite internal budding with expression of IMC and rhoptry genes peaking together in a narrow window, as shown by the expression profile of prototypical rhoptry and IMC genes (ROP1 and IMC1, Figure 2-1A). Thus, we filtered our candidate gene list by comparing expression-timing data for the 18 putative PATs with these representative genes (Figure 2-1A and Figure 2-S1A). Of the highly transcribed candidate PATs, five genes displayed a rhoptry/IMC expression signature. We attempted to localize the corresponding proteins by introduction of a 3xHA epitope tag at the endogenous C-terminus of each gene and succeeded for two of the five candidate PATs. One of these proteins was found to localize to the rhoptries (TGME49_252200, named TgDHHC7) while the other localized to the IMC (TGME49_293730, named TgDHHC14), validating our approach (Figure 2-1B-C). In this work, we focus on functional characterization of the rhoptry-localized TgDHHC7 to gain insight into the contribution of palmitoylation activity to rhoptry biology.

A comparison of TgDHHC7 distribution with the rhoptry body protein ROP13 and rhoptry neck protein RON11 (TGME49_230350, Figure 2-S2) shows colocalization with both markers, indicating that TgDHHC7 is present along the length of the entire rhoptry in both the bulbous body and duct-like neck (Figure 2-1B). To confirm the coding sequence for this gene,
we cloned and sequenced the TgDHHC7 cDNA. While the resulting sequence agrees with RNAseq data (Reid et al., 2012), the migration of the endogenously tagged protein by SDS-PAGE is inconsistent with the predicted size, leaving ambiguity about the correct start codon, which we resolved with a combination of mass spectrometry and ectopic protein expression in Toxoplasma (Figure 2-S3).

TgDHHC7 is predicted to contain four transmembrane helices with the highly conserved DHHC residues located just upstream of the third of these hydrophobic regions (Figure 2-2A). DHHC-CRD PATs characterized in other systems function to palmitoylate soluble, cytosolic proteins, tethering them to target membranes. Thus, TgDHHC7 likely adopts a membrane topology that positions the DHHC-CRD domain in the cytosol where it would enable anchoring of proteins to the cytosolic face of the rhoptries. In agreement with this expected topology, TgARO was recently shown to traffic to the cytosolic face of the rhoptry membrane via protein palmitoylation (Cabrera et al., 2012).

Orthologs to TgDHHC7 were identified across the apicomplexan phylum, indicating conservation in related organisms possessing rhoptries (Figure 2-S4). Conservation beyond Apicomplexa was also observed, including homology to the yeast PAT Pfa3, which is involved in the recruitment of Vac8p to the yeast vacuole to facilitate homotypic membrane fusion (Smotrys et al., 2005; Wang et al., 2001). Interestingly, Vac8p also contains armadillo repeats and is homologous to TgARO (Cabrera et al., 2012), further suggesting that TgARO may be recruited to the rhoptries by TgDHHC7 and raising the possibility of a role in rhoptry membrane fusion for this putative enzyme-substrate pair. Additionally, the acidic environment of the rhoptry lumen has led to the proposal that rhoptries may be a form of secretory lysosomal granules (Ngo et al., 2004). The fact that both TgDHHC7 and TgARO are homologous to the
Pfa3/Vac8p proteins of the yeast vacuole, the equivalent of a lysosome in this organism, further suggests that rhoptries in apicomplexan parasites resemble specialized lysosomes.

**A conserved C-terminal region is required for rhoptry trafficking of TgDHHC7.**

Although the targeting determinants for a number of soluble rhoptry proteins have been characterized, less is known about the requirements for trafficking of multipass transmembrane proteins to the rhoptries. While the core DHHC-CRD and surrounding TM domains are present in all known PATs, the N- and C-terminal regions outside of the TM domains are highly variable and thought to play roles in subcellular targeting and substrate recognition specific to individual PATs (Mitchell et al., 2006). TgDHHC7 has a long N-terminal region upstream of the first TM domain (residues 1-247) and a shorter C-terminal region downstream of the fourth TM domain (residues 456-537) (Figure 2-2A). The N-terminal region of TgDHHC7 is elongated and divergent relative to orthologs in other apicomplexans (Figure 2-S4). In contrast, the TgDHHC7 C-terminal region appears well-conserved among these orthologs, as well as with yeast Pfa3 (Figure 2-S4). The C-terminal region of TgDHHC7 includes a PaCCT motif (red box, Figure 2-2A), a recently identified motif present in most eukaryotic PATs that is important for targeting in Pfa3 (Gonzalez Montoro et al., 2009). Downstream of the PaCCT motif is a conserved stretch of 21 residues (blue box, Figure 2-2A) followed by a less conserved region at the extreme C-terminus.

To test the importance of these features in rhoptry targeting of TgDHHC7, we expressed a second copy of TgDHHC7 cDNA with a C-terminal HA epitope tag under the control of the promoter for the rhoptry neck protein RON5, which has a similar expression profile to the endogenous TgDHHC7 promoter. This full-length version of TgDHHC7 targets properly to the
rhoptries (Figure 2-2B). We then generated a series of N- and C-terminal truncations in this second copy of TgDHHC7 and evaluated their impact on targeting. A TgDHHC7 truncation mutant lacking the majority of the N-terminal region (residues 1-162) had no defect in rhoptry localization (Figure 2-2C). A more extensive N-terminal truncation (residues 1-206) also targeted properly (data not shown), demonstrating that the N-terminus of TgDHHC7 is not required for targeting.

We next evaluated the importance of the various C-terminal features of TgDHHC7 for rhoptry trafficking. Removal of the non-conserved, extreme C-terminal region (residues 511-537, Figure 2-S4) did not grossly affect targeting (Figure 2-2D), although this mutant was sometimes also seen in a non-rhoptry compartment that localized adjacent to the Golgi marker GRASP55 (arrows, Figure 2-2E). In contrast, truncation of the highly conserved region up to but not including the PaCCT motif (residues 489-537) or including the PaCCT motif (residues 474-537) resulted in a complete loss of rhoptry localization, showing these residues are necessary for trafficking TgDHHC7 to the rhoptries (Figure 2-2F-G). These truncated proteins were found to colocalize with GRASP55, indicating TgDHHC7 fails to traffic beyond the Golgi in mutants lacking these key residues. Together, these results show that the TgDHHC7 C-terminal conserved region (blue, Figure 2-2A) just downstream of the PaCCT motif plays a key role in rhoptry sorting.

Establishment of a TgDHHC7 conditional knockdown mutant.

Localization of TgDHHC7 to the rhoptries indicates that Toxoplasma utilizes protein palmitoylation for specialized parasite secretory functions. To directly assess the role of TgDHHC7 in rhoptry biology, we replaced the endogenous promoter with a tetracycline-
repressible promoter in TgDHHC7-3xHA parasites via homologous recombination to generate a conditional knockdown mutant, which we named TgDHHC7cKO. As expected, exchange of the endogenous promoter with a weaker truncated version of the SAG4 promoter results in lower TgDHHC7 protein levels than those observed in the parental line (Figure 2-3A). Rhoptries are assembled de novo during each round of parasite division and protein traffic to the organelle is restricted to a narrow window during biosynthesis. In agreement with this, we observe some mistargeting of TgDHHC7 under the control of the constitutive SAG4 promoter in the TgDHHC7cKO parasites, which likely corresponds to protein synthesized outside of this rhoptry biosynthesis timeframe (-Atc, Figure 2-3B). Culture of TgDHHC7cKO parasites in the presence of anhydrotetracycline (Atc) results in depletion of TgDHHC7 with protein levels falling below detectable levels by 72 hours (Figure 2-3B-C).

**TgDHHC7 is required for apical docking during rhoptry biogenesis.**

*Toxoplasma* parasites possess 6-14 individual rhoptries each composed of a larger posterior body and tapered anterior neck (Paredes-Santos et al., 2011). To determine the effect of the loss of TgDHHC7 on the rhoptries, we performed IFA analysis on TgDHHC7cKO with an array of antibodies recognizing components of either the rhoptry body (ROPs) or rhoptry neck (RONs). As seen in the parental line, the rhoptries are normally bundled together in the apical end of the cell with the necks of the organelle docked at the extreme apex of the parasite where they are positioned to facilitate exocytosis of their contents (Fig 4A, arrow) (Dubremetz, 2007; Paredes-Santos et al., 2011). TgDHHC7cKO parasites cultured without Atc maintained the apical rhoptry bundle (Fig4B, arrow) but also contained some individual rhoptries present outside of the apical region of the cell (Fig4B, arrowhead), presumably due to the lower levels of
TgDHHC7 produced in this line. In contrast, following culture with Atc, TgDHHC7cKO parasites showed a complete loss of the apical rhoptry bundle resulting in dispersed rhoptries scattered throughout the cell (Fig 4C). Interestingly, these scattered rhoptries appear to retain normal morphology and subdomain organization with each ROP2/3/4 body signal associated with a single, polar RON11 neck signal. Standard morphology and subdomain organization was also observed in these dispersed rhoptries with an array of additional rhoptry markers including the body markers ROP7, ROP13 and PP2Chn and the neck markers RON2 and RON8 (data not shown). In contrast, no effect was observed with IFA markers for the micronemes (Figure 2-S6A), IMC (Fig 4D), dense granules, mitochondrion, apicoplast or plant-like vacuole (data not shown), indicating these organelles are unaffected by loss of TgDHHC7. The protein contents of scattered rhoptries also appeared normal in regard to maturation by proteolytic processing of both ROPs and RONs (Figure 2-S5A).

Rhoptries are synthesized during each round of Toxoplasma replication wherein new daughter cells are assembled inside an intact mother cell (Striepen et al., 2007). To determine the point at which parasites depleted of TgDHHC7 incur rhoptry defects, we examined relevant markers for rhoptry biogenesis by IFA. Late secretory compartments labeled by the dynamin-like protein DrpB that are specifically destined for rhoptry/microneme organelles appeared normal (Figure 2-S5B), as did markers for pro-rhoptries (Fig 4D), indicating that traffic to the dispersed rhoptries proceeds normally (Breinich et al., 2009; Carey et al., 2004). During parasite replication, the nascent rhoptries are detected in early forming daughter parasites, suggesting that tethering may be critical for organelar delivery into daughter cells. However, while mature rhoptries in parasites lacking TgDHHC7 are not docked at these structures, biogenesis of new rhoptries faithfully occurs within assembling daughter buds and not elsewhere in the maternal
cell (Fig 4D). This observation indicates that the position of rhoptry nucleation is likely a consequence of golgi/ER polarization at the base of daughter buds and that tethering at the apex of daughter buds is a later event in rhoptry biogenesis. Taken together, these results suggest that proteins recruited to the rhoptry surface by TgDHHC7 play critical roles in proper rhoptry bundling and apical docking, but not organelle assembly or sorting of rhoptry contents through the secretory pathway.

To better assess the morphology and orientation of rhoptries in the TgDHHC7 knockdown, we analyzed these parasites by transmission electron microscopy (TEM). Apical bundles of rhoptries properly docked within the conoid were easily observed in both the parental line and untreated TgDHHC7cKO parasites while apical rhoptry localization and docking were almost never observed following Atc treatment (Fig 4E). Under these conditions, scattered rhoptries were observed in the knockdown throughout the cytosol distal to the parasite apex (Fig 4F). In agreement with our IFA analysis, these scattered rhoptries appeared morphologically normal with large, mottled bodies and tapered necks (inset, Fig 4F). Other aspects of the cell ultrastructure appeared normal, including the presence and proper positioning of micronemes (Fig 4E-F). Less frequently, some irregular structures could be observed in parasites depleted of TgDHHC7, including the presence of amylopectin granules (observed in <9% of sections) and multi-membranous structures of unclear origin (observed in <7% of sections) (Figure 2-S5C-D). Overall, these results demonstrate that loss of TgDHHC7 causes a severe and specific defect in apical rhoptry docking.
Rhoptry function is critical for host cell invasion but not egress.

Host cell invasion by apicomplexan parasites involves the establishment of a tight-junction interface called the moving junction between the host and parasite surface during penetration (Aikawa et al., 1978). Formation of the moving junction is believed to depend on a complex of rhoptry neck proteins injected into the host cell just before penetration (Shen and Sibley, 2012). While rhoptries in the TgDHHC7 knockdown appear morphologically intact and receive their proper contents, their failure to dock at the cell apex suggests these organelles are unable to secrete their contents and are thus rendered non-functional. Indeed, while parasites depleted of TgDHHC7 grow normally within the host cell (data not shown), they encounter a nearly complete block in invasion of new host cells (Figure 2-5A). The few invasion events observed following TgDHHC7 knockdown are likely the result of residual levels of TgDHHC7 as when the knockdown is extended over several lytic cycles, TgPATcKO parasites are unable to form plaques, demonstrating that proper rhoptry secretion is critical for invasion and thus for survival of the parasite (Figure 2-5B).

Interestingly, the invasion block encountered upon knockdown of TgDHHC7 is not accompanied by a corresponding increase in parasites attached to the host surface (Figure 2-5A, blue bars). To ensure that secretion of micronemal adhesins is not compromised, we further evaluated the integrity and function of this organelle by IFA and gliding motility assays and found no detectable defects compared to wild-type parasites (Figure 2-S6). A similar phenotype is seen upon disruption of the moving junction component RON8 in which initial attachment is unaffected, but parasites that fail to invade subsequently detach and are washed away in the assay (Straub et al., 2011). To determine if this is the case in the TgDHHC7cKO strain, we treated parasites with cytochalasin D to paralyze the actin-myosin motor that powers invasion in
order to separate initial attachment from subsequent invasion events. Under these conditions, no difference in attachment was seen for cells depleted of TgDHHC7, indicating that initial attachment is not impacted and that parasites lacking functional rhoptries detach from the host following failure to invade (Figure 2-5C).

At the onset of invasion, *Toxoplasma* parasites inject a subset of rhoptry body proteins into their host cell in structures called evacuoles, which can be visualized by IFA (Hakansson et al., 2001). To ensure injection was blocked from rhoptries that fail to dock at the cell apex, we evaluated evacuole formation and found these structures were largely abolished in the absence of TgDHHC7 (reduced ~82% in TgDHHC7cKO following Atc treatment), confirming a defect in exocytosis of rhoptry proteins (Figure 2-5D). Collectively, these results indicate that rhoptry secretion and host penetration are dependent on TgDHHC7-mediated apical docking.

Additionally, the specific ablation of rhoptry function without impacting the other regulated secretory organelles provides the first unequivocal demonstration that rhoptry apical secretion is critical for invasion.

In addition to their role in invasion, rhoptries have also been suggested to function in egress as RON4-positive moving junction rings have been observed during host exit (Alexander et al., 2005; Hoff and Carruthers, 2002; Sibley). On the other hand, pharmacological inhibition of phospholipase activity blocks rhoptry secretion and inhibits invasion but not egress; however, these results are difficult to interpret in light of clear pleiotropic effects and a lack of any identified target(s) (Ravindran et al., 2009). To resolve the question of rhoptry contribution to egress, we evaluated the ability of TgDHHC7-depleted parasites to egress and observed no obvious defects in natural egress (data not shown). To more quantitatively assess this question, we induced egress using the calcium ionophore A23187 and again observed no significant
difference in egress efficiency between parasites with or without functional rhoptries (Figure 2-5E), clearly demonstrating that rhoptries are not important for host cell exit.

**TgDHHC7 palmitoyl transferase activity is required for apical rhoptry docking.**

Palmitoylation by membrane-resident PATs is a well-characterized mechanism for recruiting proteins to a target membrane system (Linder and Deschenes, 2007; Resh, 2006b). Therefore, it is likely that TgDHHC7 facilitates apical docking of rhoptries by recruiting one or more proteins to the cytosolic face of the rhoptry membrane which then serve to mediate docking. Alternatively, it is possible that TgDHHC7 directly mediates docking in a manner that is independent of its palmitoylation activity. To distinguish between these possibilities, we generated a catalytically inactive version of TgDHHC7 by mutating the cysteine within the highly conserved DHHC to a serine (TgDHHC7<sub>C371S</sub>). This mutation has been shown to abolish palmitoylation activity in vitro and function in vivo for several characterized PATs, including Pfa3 (Lobo et al., 2002; Roth et al., 2002; Smotrys et al., 2005). We then complemented our TgDHHC7cKO mutant by targeting a second copy of either the wild-type or mutant TgDHHC7 expression cassette to the Toxoplasma uracil phosphoribosyl transferase locus. While both versions of TgDHHC7 localize to the rhoptries (Figure 2-6A), only complementation with wild-type is able to rescue the lethal block in invasion incurred upon knockdown of the endogenous copy of the gene (Figure 2-6B, FL). These results demonstrate that TgDHHC7 catalytic activity is necessary for rhoptry tethering through the recruitment or modification of other factors.

Since truncation of the first 162 residues of TgDHHC7 does not affect rhoptry targeting (Figure 2-2C), we complemented the TgDHHC7cKO strain with TgDHHC7<sub>Δ1-162</sub> and found it is able to functionally complement the loss of endogenous TgDHHC7, as shown by the formation
of plaques in the presence of Atc (Figure 2-6B, Δl-162). A further truncation of TgDHHC7 lacking the first 206 residues (TgDHHC7Δl-206) also targets properly to the rhoptries and rescues invasion upon knockdown of the endogenous TgDHHC7 (Figure 2-6B, Δl-206). As expected, catalytically inactive versions of TgDHHC7Δl-162 and TgDHHC7Δl-206 target to the rhoptries (not shown) but fail to rescue the knockdown defect. These results demonstrate that the N-terminal region of TgDHHC7 (residues 1-206) is not necessary for proper rhoptry targeting or function.

**TgARO is required for apical rhoptry tethering.**

TgARO (TGME49_261440) is an attractive candidate substrate for TgDHHC7 by virtue of its palmitoylation-dependent recruitment to the rhoptries and its homology to yeast Vac8p, the substrate of the TgDHHC7 homolog Pfa3 (Cabrera et al., 2012). In keeping with a potential role in apical tethering during late rhoptry maturation, expression timing of TgARO lags behind other rhoptry proteins (including TgDHHC7) by about one hour (Figure 2-7A). To explore the possibility that TgARO is involved in rhoptry tethering, we first incorporated a 3xHA epitope tag at the endogenous TgARO locus (TgARO-3xHA). As previously reported, TgARO was observed on the entire surface of the rhoptry (Cabrera et al., 2012); however, we also noted that the protein is often concentrated at the apex of the organelle, consistent with a role in apical tethering (Figure 2-7B).

To assess TgARO function, we next generated a conditional knockdown by promoter replacement in the TgARO-3xHA line (TgAROcKO). The TgAROcKO strain showed lower levels of basal TgARO expression (Figure 2-7C) although this decrease was less dramatic than that observed in the TgdHHC7cKO strain (compare with Figure 2-2A). IFA analysis of untreated TgAROcKO parasites revealed an intact apical bundle of rhoptries (arrow, Figure 2-
7D) as well as some rhoptry dispersion (arrowhead, Figure 2-7D), similar to TgDHHC7cKO parasites. Culture of TgAROcKO parasites in the presence of Atc for 48 hours dramatically depleted TgARO levels (Figure 2-7E). Consistent with a role in tethering, knockdown of TgARO was found to recapitulate the phenotype observed upon depletion of TgDHHC7, including the complete loss of apical rhoptry tethering, a major block in host invasion and a failure to form plaques in the presence of Atc (Fig F-H). Interestingly, TgARO is still clearly visible at the apex of dispersed rhoptries in untreated TgAROcKO parasites (arrowhead, Figure 2-7D), suggesting that a threshold concentration of TgARO must be reached to achieve tethering. Collectively, these results demonstrate TgARO is also required for apical rhoptry tethering and strongly suggest that TgARO is recruited to the rhoptry surface by TgDHHC7 to facilitate this process (Figure 2-8).

A possible role for TgARO in membrane fusion during rhoptry exocytosis is suggested by virtue of its homology to Vac8p, which interacts with the yeast cis-SNARE complex and is required for homotypic vacuole fusion. Palmitoylation of Vac8p by Pfa3 anchors the protein in the vacuole membrane and corresponds with its release from the cis-SNARE complex (Veit et al., 2001). In the absence of Vac8p, vacuole docking and formation of trans-SNARE pairs proceeds, but fusion cannot occur (Wang et al., 2001). To evaluate the importance of palmitoylation for TgARO function, we attempted to complement the TgDHHC7cKO and TgAROcKO strains with a version of TgARO engineered to target to the rhoptry surface in a palmitoylation-independent fashion. This was accomplished by fusing TgARO (lacking the palmitoylated N-terminal cysteines critical for rhoptry targeting) to the C-terminus of a catalytically inactive version of TgDHHC7 (Figure 2-S7A-B). While this chimeric protein did target to the rhoptries, it was unable to rescue knockdown of either endogenous TgDHHC7
(Figure 2-S7C) or TgARO (data not shown), indicating that more is required to facilitate apical rhoptry tethering than simple localization to the rhoptry surface. A similar experiment with Vac8p also failed to rescue homotypic vacuole fusion in S. cerevisiae, indicating that palmitoylation may confer functionality beyond targeted membrane recruitment (Subramanian et al., 2006).

Armadillo-repeats are comprised of highly variable, ~42 residue motifs, each composing three α-helices (Tewari et al., 2010). Tandem repeats of this motif fold into a superhelix that generally functions to promote protein-protein interactions. Thus, understanding the precise mechanism of apical rhoptry tethering will be aided by the identification of TgARO interacting partners. One possibility raised by homology to Vac8p is that TgARO may function to dock the rhoptry neck in a pre-fusion state at the parasite apex through interactions with membrane fusion machinery, priming these organelles for rapid exocytosis during host invasion. A docked, pre-fusion arrangement is also observed in trichocyst secretory organelles harbored by ciliates that are reminiscent of rhoptries in their morphology, regulated secretion and docking at the cell cortex (though not exclusively at the apex) (Dubremetz, 2007). A further similarity is the presence of a “rosette” of integral plasma membrane proteins at the docking site of both trichocysts and rhoptries, likely representing the machinery involved in docking and/or fusion (Plattner and Kissmehl, 2003; Porchet and Torpier, 1977). Interestingly, the armadillo-repeat containing protein Nd9p has been shown to play a key role in exocytosis of trichocysts in Paramecium tetraurelia (Froissard et al., 2001). While Nd9p contains a series of armadillo-repeats, it does not possess the N-terminal signal for myristoylation and palmitoylation present in TgARO and Vac8p. Furthermore, while Nd9p is required for rosette formation and exocytosis, it
does not appear to play a role in positioning trichocysts at the cell cortex, indicating clear
differences from the function of TgARO (Bonnemain et al., 1992; Froissard et al., 2001).

The fact that TgARO is recruited to the rhoptry surface in a palmitoylation-dependent
fa\n
cshion and that knockdown of either TgDHHC7 or TgARO produces the same phenotype
strongly suggests that TgARO is a target for TgDHHC7 (inset, Figure 2-8). TgARO is the only
protein known to be palmitoylated on the rhoptry surface and it will be interesting to determine if
additional substrates exist for TgDHHC7 that mediate other functionalities which may be
masked by the severity of the rhoptry dispersion phenotype. Additionally, armadillo-repeat
proteins (including Vac8p) often serve multiple, independent functions and thus TgARO may
have other cellular roles beyond rhoptry tethering (Tewari et al., 2010). Interestingly, a calcium-
dependent phosphorylation event was detected at serine 33 of TgARO, suggesting a possible role
for calcium signaling in rhoptry docking and release (Nebl et al., 2011). However, while calcium
signaling is a well-characterized mechanism for controlling microneme secretion, it is not known
to play a role in rhoptry regulation (Billker et al., 2009). Further work investigating the
biological relevance of this modification will clarify its significance to rhoptry tethering or other
TgARO functions.

Remarkably, loss of TgDHHC7 or TgARO compromises rhoptry localization and
function but not formation, unlike previously reported mutants that abolish rhoptries by
impacting secretory traffic destined for these organelles (Breinich et al., 2009; Sloves et al.,
2012). More importantly, these established secretory mutants ablate both rhoptries and
micronemes while depletion of TgDHHC7 or TgARO produces rhoptry-specific defects,
providing the first clear demonstration that rhoptries are necessary for invasion but not egress.
Together, these findings reveal that PATs play critical roles in unique apicomplexan biology that is foundational to host-parasite interactions and the pathogenesis of these organisms.
Materials and Methods

Toxoplasma and host cell culture

*T. gondii* RHΔhpt strain and modified strains were maintained in confluent monolayers of human foreskin fibroblast (HFF) host cells as previously described (Donald et al., 1996).

Light microscopy and image processing

Fixation and immunofluorescence staining of *T. gondii* were carried out as previously described (Bradley et al., 2005). All cells imaged in this study were fixed with 3.5% formaldehyde in PBS. Image stacks were collected at z-increments of 0.2 µm with an AxioCam MRm CCD camera and AxioVision software on an Axio Imager.Z1 microscope (Zeiss) using a 100x oil immersion objective. Deconvolved images were generated using manufacturer specified point-spread functions and displayed as maximum intensity projections.

Electron microscopy

*T. gondii* strains were cultured -/+ 1.5 µg/ml Atc for 24 hours, then infected into fresh HFF monolayers and allowed to grow for 24 hours -/+ 1.5 µg/ml Atc. The cells were then fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 hr at room temperature and processed as described (Coppens and Joiner, 2003) before examination with a Philips CM120 EM (Eindhoven, the Netherlands) at 80 kV.
Antibodies and fluorescent fusion constructs.

The following *Toxoplasma* primary antibodies were used in IFA or Western blot: rabbit anti-ROP13 (Turetzky et al., 2010), polyclonal rat anti-RON11 (see below and Figure 2-S2), mouse anti-IMC1 mAb 45.15 (Wichroski et al., 2002), rabbit anti-RON2 (Straub et al., 2011), rabbit anti-RON4 (Alexander et al., 2005), mouse mAb anti-ROP2/3/4 (Leriche and Dubremetz, 1991), rabbit anti-SAG1 (Burg et al., 1988), polyclonal mouse anti-ISP3 (Beck et al., 2010), mouse anti-F1-ATPase beta subunit mAb 5F4 (Bradley, unpublished), polyclonal mouse anti-DrpB (Breinich et al., 2009), rabbit anti-proROP4 UVT70 (Carey et al., 2004), mouse anti-MIC2 mAb T3 4A11 (Achbarou et al., 1991), mouse anti-ROP1 mAb TG49 (Schwartzman and Krug, 1989), and polyclonal mouse anti-RON5C (Straub et al., 2009). Hemagglutinin (HA) epitope was detected with mouse mAb HA.11 (Covance), rabbit polyclonal anti-HA (Invitrogen) or rat mAb 3F10 (Roche). FLAG epitope tags were detected with mouse anti-FLAG mAb M2 (Sigma) or rat anti-DYKDDDDK mAb (Stratagene).

For generation of RON11 anti-sera, a portion of RON11 coding sequence comprising the C-terminal 219 residues was cloned from *Toxoplasma* cDNA (primers P1/P2, Table 2-2) into the expression vector pET101/D-TOPO (Invitrogen). The resulting plasmid was transformed into *E. coli* BL-21DE3 cells, recombinant protein was expressed by 1 mM IPTG induction, purified over Ni-NTA agarose (Qiagen), and injected into a rat for anti-sera production.

For detection of GRASP55, the pGRASP55-YFP (Hartmann et al., 2006) plasmid was transfected into parasites allowing for transient expression of the GRASP55-YFP fusion.
Candidate PAT filtering by expression profiling

The expression timing of 18 *Toxoplasma* genes predicted to encode proteins containing a DHHC-CRD domain (Table 2-1) was compared to the periodic expression pattern of the known IMC and rhoptry proteins IMC1 and ROP1 (Behnke et al., 2010) and putative PATs were filtered on the basis of their similar to the rhoptry/IMC expression signature of these genes.

Generation of endogenous epitope tags

For endogenous tagging of TgDHHC7, TgDHHC14 and TgARO, the endogenous tagging vector p3xHA.LIC.DHFR (Konrad et al.) was first modified to replace the DHFR selectable marker cassette with a chloramphenicol acetyl-transferase (CAT) selectable marker between the restriction sites HindIII/XbaI resulting in the plasmid p3xHA.LIC.CAT. A portion of the genomic locus of each gene up to but not including the stop codon was PCR amplified from *Toxoplasma* genomic DNA (TgDHHC7: P3/P4; TgDHHC14: P5/P6; TgARO: P7/P8) and inserted into p3xHA.LIC.CAT by ligation-independent cloning (Huynh and Carruthers, 2009) to generate the vectors pTgDHHC7-3xHA.LIC.CAT, pTgDHHC14-3xHA.LIC.CAT and pTgARO-3xHA.LIC.CAT. These plasmids were linearized with ApaI, MfeI or NheI, respectively, and transfected into the TATiΔTgKu80 parasite line (Sheiner et al., 2011). Following selection with chloramphenicol, parasites were cloned by limiting dilution and a clone expressing the tagged protein of interest was isolated and designated TgDHHC7-3xHA, TgDHHC14-3xHA or TgARO-3xHA.
**Generation and complementation of TgDHHC7cKO and TgAROcKO parasites**

For direct replacement of the TgDHHC7 or TgARO promoter with the conditional TetOSAG4 promoter by homologous recombination, 5’ (TgDHHC7: P9/P10; TgARO: P13/P14) and 3’ (TgDHHC7: P11/P12; TgARO: P15/P16) regions flanking the promoter were PCR amplified from *Toxoplasma* genomic DNA and cloned into the vector pDT7S4myc (van Dooren et al., 2008) between *NdeI* and *BglII/AvrII* sites, respectively. The resulting vectors, pTS4-TgDHHC7-DHFR and pTS4-TgARO-DHFR, were linearized with *ApaI* and transfected into TgDHHC7-3xHA or TgARO-3xHA parasites. Following selection with 1 µM pyrimethamine, parasites were cloned by limiting dilution and genomic DNA from individual clones was analyzed by PCR for promoter replacement (TgDHHC7: P17/P18; TgARO: P17/P19). A clone that had undergone the intended recombination event was designated TgDHHC7cKO or TgAROcKO.

For expression of complementing copies of TgDHHC7, the RON5 promoter was PCR amplified from *Toxoplasma* genomic DNA (primers P20/P21) and inserted into the UPRT targeting vector pUPRT-HA (Reese et al., 2011) between *SpeI* and *BamHI* by blunting both the digested vector and PCR amplicon. A FLAG epitope tag version of this vector was generated by PCR amplifying the 3’ UTR with a forward primer encoding the FLAG epitope sequence (primers P22/P23) and inserting this amplicon between *NotI/EcoRV*, replacing the inframe fusion to a C-terminal HA tag with a FLAG tag (pUPRT-FLAG). To complement TgDHHC7cKO parasites, the TgDHHC7 coding sequence was PCR amplified from *Toxoplasma* cDNA (primers P11/P24) and inserted into this vector between *BglII* and *NotI* to generate the vectors pUPRTKO-TgDHHC7-HA/FLAG. The vectors were linearized with *NruI* and transfected into wild-type (to assess targeting) or TgDHHC7cKO parasites followed by selection with 5 µg/ml 5-
fluorodeoxyuridine to facilitate targeted replacement of the UPRT locus (Donald and Roos, 1995). For complementation with mutant versions of TgDHHC7, the TgDHHC7 coding sequence was cloned into pJet1.2 (Fermentas) and PCR-based mutagenesis was carried out as previously described (Beck et al.) (primers P25/P26). Full length and N-terminally truncated (primers P27/P24 and P28/P24) versions of TgDHHC7 encoding the C371S mutation were expressed in TgDHHC7cKO parasites as described above.

**Palmitoylation-independent targeting of TgARO to the rhoptry surface.**

For targeting of TgARO to the rhoptry surface independent of palmitoylation, the TgARO coding sequence lacking the first six codons that encode the relevant cysteines (P29/P30) and inserted into the vectors pUPRTKO-TgDHHC7-C371SΔ1-162-HA/FLAG at NotI to create the vectors pTgDHHC7-C371SΔ1-162-TgAROΔ1-6-HA/FLAG. As a control, the N-terminally truncated TgARO coding sequence was cloned (P31/P30) into pUPRTKO-TgDHHC7-C371SΔ1-162-HA/FLAG between BglII and NotI to generate the vectors pUPRTKO-TgAROΔ1-6-HA/FLAG. Each vector was linearized with NruI and transfected into wild-type (to assess targeting), TgDHHC7cKO and TgAROcKO parasites followed by selection with 5 µg/ml 5-fluorodeoxyuridine to facilitate targeted replacement of the UPRT locus.

**Generation of TgDHHC7 C-terminal truncations**

For TgDHHC7 C-terminal truncations, TgDHHC7 coding sequence under the control of the RON5 promoter was amplified from the vector pUPRTKO-TgDHHC7-WT-FLAG using the indicated primer pairs (FL: P32/P24, Δ511-537: P32/P33, Δ489-537: P32/P34, Δ474-537: P32/P35) and inserted between Acc65I and NotI in the expression vector pNotI-HA to generate
full length and truncated TgDHHC7 expression vectors with a C-terminal fusion to an HA epitope tag. Vectors were linearized with Acc65I, transfected into parasites and grown in media containing 50 μg/ml mycophenolic acid and 50 μg/ml xanthine to select for stable integration of the vector.

Mass Spectrometry

Excised gel slices containing TgDHHC7 were digested with trypsin, fractionated online using a C18 reversed phase column, and analyzed by MS/MS on a Thermofisher LTQ-Orbitrap XL as previously described (Kaiser and Wohlschlegel, 2005; Wohlschlegel, 2009). MS/MS spectra were subsequently analyzed using the ProLuCID and DTASelect algorithms (Eng et al., 1994; Tabb et al., 2002).

Gliding motility assays

Motility assays were performed as previously described (Dobrowolski and Sibley, 1996). Briefly, parasites were grown 72 hours +/- 1.5μg/ml Atc, monolayers were washed with PBS and intracellular parasites were collected by scrapping and passage through a 27-gauge needle. Equivalent parasite numbers were allowed to glide on FBS-coated glass chamber slides for 30 min before formaldehyde fixation and processing for IFA with rabbit anti-SAG1.

Plaque assays

Parasites were grown 48 hrs +/- 1.5 μg/ml Atc, syringe lysed and infected into 6-well dishes containing fresh, confluent HFF monolayers +/- Atc. Cultures were allowed to grow nine days before fixation with methanol followed by staining with crystal violet.
**Invasion, evacuole and egress assays**

Invasion assays were performed as previously described (Huynh et al., 2003). Briefly, parasites were grown 72 hrs +/- 1.5 µg/ml Atc, monolayers were washed with PBS and intracellular parasites were collected by scrapping and passage through a 27-gauge needle. Equivalent parasite numbers were resuspended in pre-warmed media and allowed to infect HFF monolayers on coverslips for one hour. Monolayers were then washed, fixed with EM-grade 3.7% formaldehyde/PBS (Biosciences, Inc.), blocked with PBS/3%BSA for 30 min and incubated with rabbit anti-SAG1 diluted in PBS/3%BSA for 1 hr. After washing, samples were permeabilized in PBS/3%BSA/0.1% Triton X-100 for 30 min and then incubated with mAb 5F4 diluted in PBS/3%BSA for one hour. Following incubation with secondary antibodies, samples were examined by fluorescence microscopy and parasites were scored as invaded (SAG1-, 5F4+) or attached (SAG1+, 5F4+). Invasion assays were performed in triplicate, five fields were counted on each replicate coverslip and the average number of invaded and attached parasites per field was calculated.

For evacuole assays, parasites were grown +/- 1.5 µg/ml Atc for 36 hours to allow large vacuoles to form and intracellular parasites were collected by scrapping and passage through a 27-gauge needle. Evacuole assays were then performed as previously described (Mital et al., 2005). The number of evacuoles were counted across five fields per coverslip on three independent coverslips per sample and the average number per field was calculated.

Egress assays were performed as previously described (Black et al., 2000). Briefly, parasites were grown +/- 1.5 µg/ml Atc for 24 hours, then infected into fresh HFF monolayers on coverslips and allowed to grow an additional 36 hours +/- 1.5 µg/ml Atc. Coverslips were then washed with PBS and incubated in 1µM calcium ionophore A23187 (Sigma) diluted in Hanks
Balances Salts Solution at 37° before being fixed in methanol and processed for IFA with rabbit anti-SAG1. At least 100 vacuoles per coverslip were counted across five fields on three independent coverslips per sample and scored as egressed or not egressed. For each of the above assays, experiments were repeated at least twice and values from a representative experiment are shown as the mean ± SD.
**Figure Legends**

**Figure 2-1 – Identification of palmitoyl acyl transferases targeted to the Toxoplasma rhoptries or IMC.**

(A) The expression profile of 18 candidate PATs (DHHC-CRD containing genes) was compared to known IMC and rhoptry genes across the *Toxoplasma* cell cycle (Figure 2-S1). Five candidate genes were found to display an expression signature similar to the IMC/rhoptry pattern, peaking in a narrow window during daughter cell budding. (B-C) IFA of two candidate IMC/rhoptry PATs localized by introduction of a 3xHA epitope tag at the endogenous C-terminus. (B) TgDHHC7 was found to localize to the rhoptries, as assessed by colocalization with both the rhoptry body protein ROP13 and rhoptry neck protein RON11. Red: anti-HA antibody detected by Alexa594-anti-mouse IgG. Green: rabbit anti-ROP13 antibody detected by Alexa488-anti-rabbit IgG Blue: rat anti-RON11 antibody detected by Alexa350-anti-rat IgG. (C) TgDHHC14 was found to localize to the IMC, as assessed by colocalization with the IMC protein IMC1. Red: anti-HA antibody detected by Alexa594-anti-rabbit IgG. Green: mouse anti-IMC1 antibody detected by Alexa488-anti-mouse IgG. Blue: Hoechst stain. All scale bars = 5µm.

**Figure 2-2 - A conserved region of the TgDHHC7 C-terminus is required for rhoptry targeting.**

Analysis of determinants for rhoptry targeting of TgDHHC7. (A) Diagram showing different truncation mutants utilized in this study with an expanded view of the C-terminal PaCCT motif (red box) and downstream conserved region (CR, blue box). Homology between TgDHHC7 residues 474-510 and various orthologs is shown (see Figure 2-S3 for full alignment details). (B) A second copy of the full length TgDHHC7 coding sequence with a C-terminal HA epitope tag
was expressed in wild type parasites under the control of the RON5 promoter and found to localize to the rhoptries in the same fashion as the endogenous protein. The localization of several truncations mutants was then evaluated. Red: anti-HA antibody detected by Alexa594-anti-mouse IgG. Green: rabbit anti-ROP13 antibody detected by Alexa488-anti-rabbit IgG. (C) Removal of residues 1-162 had no apparent effect on rhoptry localization ($\Delta_{1-162}$). Red: anti-HA antibody detected by Alexa594-anti-rabbit IgG. Green: mouse anti-ROP2/3/4 antibody detected by Alexa488-anti-mouse IgG. (D) Truncation of residues 511-537 to remove a non-conserved extreme C-terminal region of the protein did not grossly impact targeting of TgDHHC7. Red: anti-HA antibody detected by Alexa594-anti-rabbit IgG. Green: mouse anti-ROP2/3/4 antibody detected by Alexa488-anti-mouse IgG. (E) However, in some parasites TgDHHC7$_{\Delta_{511-537}}$ signal was detected outside the rhoptries adjacent to the Golgi marker GRASP55. Red: anti-HA antibody detected by Alexa594-anti-mouse IgG. Green: GRASP55-YFP. (F-G) In contrast, further C-terminal truncations that remove the CR (residues 489-537) or the CR and PaCCT motif (residues 474-537) completely abrogate rhoptry targeting. These mutants were found to localize to the Golgi, as assessed by co-localization with the GRASP55, indicating the CR is necessary for TgDHHC7 transit from the Golgi to its final rhoptry destination. Red: anti-HA antibody detected by Alexa594-anti-mouse IgG. Green: GRASP55-YFP. Scale bars = 5µm.

**Figure 2-3 – Establishment of a TgDHHC7 conditional knockdown.**

(A) Western blot comparing TgDHHC7 protein levels in parental and TgDHHC7cKO strains without Atc treatment. Exchange of the endogenous TgDHHC7 promoter with a Tet-repressible, conditional promoter results in lower levels of basal TgDHHC7 expression. ISP3 serves as a
loading control. (B-C) Growth of TgDHHC7cKO parasites in the presence of Atc results in a depletion of TgDHHC7. (B) Western blot showing TgDHHC7 levels after 72 hours -/+ Atc. (C) IFA showing TgDHHC7 signal after 48 hours -/+ Atc. Red: anti-HA antibody detected by Alexa594-anti-mouse IgG. Scale bars = 5µm.

Figure 2-4 - TgDHHC7 is required for apical rhoptry docking.

(A-C) IFA detecting the rhoptry body protein ROP2/3/4 and rhoptry neck protein RON11. (A) During normal rhoptry biogenesis, 6-14 rhoptries are generated, each with a polarized morphology consisting of a posterior, bulbous body and tapered anterior neck domain. These individual rhoptries are bundled together with the necks docked at the extreme apical end of the parasite (arrow). (B) TgDHHC7cKO parasites, which express lower levels of TgDHHC7 than the parental line, maintain an apical bundle of rhoptries (arrow) but also contain some individual rhoptries in posterior areas of the cell (arrowhead). (C) Upon depletion of TgDHHC7 by Atc treatment, apical rhoptry bundles are lost with individual rhoptries scattered throughout the cell cytosol. The rhoptry neck and body domains are clearly maintained in each scattered organelle. Red: rat anti-RON11 antibody detected by Alexa594-anti-rat IgG. Green: mouse anti-ROP2/3/4 antibody detected by Alexa488-anti-mouse IgG. All IFA scale bars = 5µm. (D) Rhoptry organization defects incurred by loss of TgDHHC7 occur after pro-rhoptry formation. Mature rhoptries labeled by RON11 are scattered throughout the cell following Atc treatment of TgDHHC7cKO parasites. In contrast, the pro-rhoptry marker proROP4 is only observed within forming daughter buds labeled by IMC1, similar to what is observed in the parental line. Red: anti-IMC1 antibody detected by Alexa594-anti-mouse IgG. Green: rabbit anti-proROP4 antibody detected by Alexa488-anti-rabbit IgG Blue: rat anti-RON11 antibody detected by Alexa350-anti-
rat IgG. (E-F) TEM analysis of parent and TgDHHC7cKO lines. (E) A normal apical bundle of rhoptries (R) are visible in wild-type parasites and in untreated TgDHHC7cKO cells. Following Atc treatment of TgDHHC7cKO parasites, bundles of rhoptries docked at the extreme apex of the cell are no longer observed. Rhoptries are instead scattered, with many no longer oriented in a longitudinal fashion (R). Importantly, a standard apical arrangement of micronemes (M) is still present in these conditions. (F) In TgDHHC7 depleted cells, individual rhoptries are observed scattered throughout the cell cytosol. These scattered rhoptries are morphologically unchanged with obvious body (B) and neck (N) domains. Note the lumen of these rhoptries have a normal, mottled appearance.

Figure 2-5 –TgDHHC7 is critical for host invasion but not egress.

(A-B) Parasites depleted of TgDHHC7 encounter a complete block in invasion. (A) Parental or TgDHHC7cKO parasites were grown for 72 hours -/+ Atc and then allowed to invade into fresh host cells for one hour. Following depletion of TgDHHC7, parasites show a nearly complete block in host penetration (asterisk, p-value < 0.001). A corresponding increase in attached, uninvaded parasites is not seen (blue bars). A minor decrease in penetration is also seen for untreated TgDHHC7cKO, likely due to the lower levels of TgDHHC7 expressed in this strain relative to the parental line. (B) Parasites depleted of TgDHHC7 cannot form plaques in fibroblast monolayers. Parental or TgDHHC7cKO parasites were grown 48 hours -/+ Atc and then infected into fresh fibroblast monolayers at an infective dose of 200 parasites per well and allowed to incubate for nine days. TgDHHC7cKO parasites are unable to form plaques in the presence of Atc, even at an infective dose of 20,000 parasites per well. (C) Initial attachment is not affected upon knockdown of TgDHHC7. Parasites were grown for 60 hours -/+ Atc before
treatment with cytochalasin D to block motility and arrest the invasion process just after attachment. (D) Loss of TgDHHC7 impairs secretion of evacuoles by rhoptries (asterisk, p-value < 0.001). Parasites were grown for 60 hours -/+ Atc before treatment with cytochalasin D to block invasion and allow evacuole formation. Evacuoles were detected by staining for ROP2/3/4. (E) Parasite egress is unaffected by knockdown of TgDHHC7. Parasites were grown 60 hours -/+ Atc and then induced to egress by treatment with calcium ionophore A23187 before fixation and staining for detection with anti-SAG1. The egress efficiency of parasites with defective rhoptries (lacking TgDHHC7) was not significantly different from parental or untreated TgDHHC7cKO parasites.

**Figure 2-6 –TgDHHC7 catalytic activity is necessary for rhoptry function.**

(A) IFA showing localization of C371S mutant version of TgDHHC7. Targeting to the rhoptries is unaffected by the C371S mutation as assessed by co-localization with the rhoptry body protein ROP2/3/4. Red: anti-HA antibody detected by Alexa594-anti-rabbit IgG. Green: mouse anti-ROP2/3/4 antibody detected by Alexa488-anti-mouse. Scale bar = 5µm. (B) Complementation of TgDHHC7cKO assessed by plaque assay. TgDHHC7_{FL,WT} rescues the defect incurred by the knockdown of TgDHHC7 while TgDHHC7_{FL,C371S} does not, failing to form plaques even with an infectious dose of 20,000 parasites per well. TgDHHC7 N-terminal truncations removing the first 162 or 206 residues also rescue the defect while catalytically inactive versions of these truncation mutants do not.
Figure 2-7 – TgARO is required for apical rhoptry tethering and host cell invasion.

(A) Expression of TgARO is delayed about one hour after other rhoptry proteins. Expression of representative genes from several classes of rhoptry proteins are shown including ROP1 (rhoptry body proteins), RON2 (rhoptry neck proteins) and the Na+/H+ exchanger TgNHE2 and TgDHHC7 (multipass integral membrane rhoptry proteins). (B) IFA showing endogenously tagged TgARO-3xHA is distributed along the surface of the entire rhoptry and concentrated at the apex of the organelle. Red: rabbit anti-HA antibody detected by Alexa594-anti-rabbit IgG. Green: mouse anti-ROP2/3/4 antibody detected by Alexa488-anti-mouse IgG. Blue: rat anti-RON11 antibody detected by Alexa350-anti-rat IgG. All scale bars = 5µm. (C) Western blot showing lower levels of TgARO following promoter replacement. ISP3 serves as a loading control. (D) Untreated TgAROCKO parasites maintain an apical rhoptry bundle (arrow) but also display some dispersed rhoptries (arrowhead). TgARO is visible not only in the apical bundle but also at the apex of each of the individual dispersed rhoptries. (E) Western blot showing knockdown of TgARO after 48 hours of growth with Atc. ISP3 serves as a loading control. (F) Following Atc treatment, cells are depleted of TgARO and the apical bundle of rhoptries is completely lost with the rhoptries scattered throughout the cytosol. (G) Parasites depleted of TgARO encounter a complete block in invasion. Parental or TgAROCKO parasites were grown for 48 hours -/+ Atc and then allowed to invade into fresh host cells for one hour. Following depletion of TgARO, parasites show a nearly complete block in host penetration (asterisk, p-value < 0.001). A corresponding increase in attached, uninvaded parasites is not seen (blue bars). A minor decrease in penetration is also seen for untreated TgAROCKO, likely due to the lower levels of TgARO expressed in this strain relative to the parental line. (H) Parasites depleted of TgARO cannot form plaques in fibroblast monolayers. Parental or TgAROCKO parasites were
grown 48 hours +/- Atc and then infected into fresh fibroblast monolayers at an infective dose of 200 parasites per well and allowed to incubate for nine days. TgAROcKO parasites are unable to form plaques in the presence of Atc, even at an infective dose of 20,000 parasites per well.

**Figure 2-8 – Model of TgDHHC7 and TgARO function in rhoptry biogenesis.**

TgDHHC7 is positioned in the rhoptry membrane with the catalytic DHHC-CRD domain in the cytosol where it recruits TgARO to the cytosolic face of the rhoptries by palmitoylation. Rhoptry biogenesis occurs *de novo* during each round of parasite replication and recruitment of TgARO by TgDHHC7 during this process facilitates apical rhoptry docking. The failure to recruit TgARO to the rhoptries following knockdown of TgDHHC7 or direct knockdown of TgARO results in the synthesis of rhoptries that are morphologically intact with respect to ultrastructure and cargo, but scattered throughout the parasite cytosol. Without proper tethering at the cell apex, these rhoptries are unable to secrete their contents. While egress from one host cell and attachment to the next occurs normally, the loss of functional rhoptries results in a block in penetration (likely due to a failure to inject rhoptry neck proteins and form a moving junction) and subsequent detachment from the host cell.
Figure 2-1

A Discovery of *Toxoplasma* Rhopty & IMC PATs
- Identification of DHHC-containing genes by sequence analysis
- Filtration by Rhopty/IMC expression profiling
- Localization by endogenous epitope tagging

B
- Phase
- TgDHHC7
- ROP13
- RON11
- Merge

C
- Phase
- TgDHHC14
- IMC1
- Hoechst
- Merge
Figure 2-2
Figure 2-3

A

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Figure 2-4
Figure 2-5

A. Host Cell Invasion

B. Parent and TgDHHC7cKO cells with and without Atc treatment.

C. Host Cell Attachment

D. Evacuole Secretion

E. Ionophore Induced Egress
Supplemental Material

Supplemental Figure Legends

**Figure 2-S1**
Candidate PAT filtering by cell cycle expression profiling. Notably, IMC and rhoptry genes display a similar pattern with expression levels peaking in a narrow 1-hour window during daughter bud formation (see Figure 2-1A).

**Figure 2-S2**
Identification, localization and initial characterization of the novel rhoptry neck protein RON11. Our earlier proteomic analysis of *Toxoplasma* rhoptries identified several putative rhoptry proteins that have not yet been confirmed, including the gene TGME49_230350 (formerly annotated as TGTWINSCAN_5713) (Bradley et al., 2005). (A) The TGME49_230350 gene model (which we confirmed by cDNA sequencing) predicts a 1254 residue protein with at least four predicted transmembrane domains (black) and a C-terminal EF hand domain (green), suggesting a role for this protein in binding calcium and/or sensing calcium fluctuation. The protein is conserved across the Apicomplexa, including orthologs in *Plasmodium spp.* (Bradley et al., 2005). To localize TGME49_230350, we raised rat anti-sera against a recombinantly expressed portion of the protein corresponding to the C-terminal 219 residues (bracket). (B-C) IFA analysis of parasites using the rat anti-sera raised against TGME49_230350. (B) The antibody was found to stain the neck portion of the rhoptry organelle, as shown by colocalization with RON2, and the protein was thus named RON11. Red: anti-RON11 antibody detected by Alexa594-anti-rat IgG. Green: rabbit anti-RON2 antibody detected by Alexa488-
anti-rabbit IgG. Scale bar = 5µm. (C) Early invasion IFA assay showing a parasite in the act of host penetration. Unlike RON4, RON11 does not relocalize from the rhoptry neck (arrowheads) to the moving junction (arrows) during host invasion. Green: anti-RON11 antibody detected by Alexa488-anti-rat IgG. Red: rabbit anti-RON4 antibody detected by Alexa594-anti-rabbit IgG. (D) Western blot using the rat-anti-RON11 antibody detects a major band at ~130kD in agreement with the predicted size of the protein. In addition, a minor band is detected at >170kD. The large size of this minor band may represent a size shift due to post-translational modification or multimerization of the protein that fails to dissociate during SDS-PAGE.

Figure 2-S3
Determination of the correct TgDHHC7 start codon. (A) TgDHHC7 protein sequence based on cDNA sequencing and RNAseq analysis. Three start codons are possible (boxed) in the first exon with corresponding protein masses predicted at 60, 43, and 38kD. (B) Western blot analysis of our endogenously tagged strain (TgDHHC7-3xHA) shows a single band at ~45kD. Taking into account the size of the 3xHA tag (4.6kD), this indicates the endogenous protein migrates at ~40kD, most consistent with the second start codon. (C) To resolve ambiguity regarding the true start codon, we first performed MS/MS analysis on purified TgDHHC7-3xHA. TgDHHC7-3xHA was immunoprecipitated from parasite lysates and resolved by SDS-PAGE. The resulting Coomassie stained band (arrow) was cut from the gel, digested with trypsin and analyzed by MS/MS. The TgDHHC7 peptides discovered by MS/MS (shown in red in A) eliminate the possibility of M3 as the start codon. (B) To determine whether M1 or M2 is the correct start codon, we then expressed TgDHHC7 cDNAs starting at either the M1 or M2 methonine with a C-terminal 1xHA tag and compared migration of the resulting proteins to the endogenously
tagged TgDHHC7-3xHA. While migration of the endogenously tagged protein is most consistent with the predicted size of M2 as the start codon, a TgDHHC7 cDNA beginning at M2 (TgDHHC7\textsubscript{M2-1xHA}) was found to migrate at ~30kD, 15kD smaller than predicted from primary sequence, indicating that M1 is the correct start codon and that TgDHHC7 migrates faster than expected, as is commonly observed for multi-pass transmembrane proteins (Rath et al., 2009). Indeed, a TgDHHC7 cDNA beginning at M1 (TgDHHC7\textsubscript{M1-1xHA}) was found to migrate slightly faster than TgDHHC7-3xHA, consistent with the 3.3kD difference in size due to the 1xHA vs 3xHA tag in these two proteins. (D) Table summarizing the expected and observed sizes of the various forms of TgDHHC7 examined.

**Figure 2-S4**

Alignment of TgDHHC7 sequence with orthologs in other species identified by BLAST. The highly conserved DHHC (red box), predicted transmembrane domains (black underline), PaCCT motif (red underline) and a C-terminal conserved region (blue underline) are indicated. Species abbreviations and accession numbers: *Saccharomyces cerevisiae* (Pfa3), NP_014073; *Plasmodium falciparum* (Pf), XP_001351838; *Plasmodium vivax* (Pv), XP_001613674; *Theileria annulata* (Ta), XP_952273; *Babesia bovis* (Bb), XP_001611639; *Cryptosporidium muris* (Cm), XP_002141787; *Eimeria tenella* (Et), AET50820; *Toxoplasma gondii* (Tg), AFW99807.

**Figure 2-S5**

Additional rhoptry analysis in TgDHHC7cKO parasites. (A) Analysis of proteolytic processing of rhoptry proteins following knockdown of TgDHHC7. TgDHHC7cKO parasites were grown -
/+ Atc for 48 hours before harvesting parasites. Processing of rhoptry body proteins ROP1 and ROP13 and the rhoptry neck protein RON5C was assessed by Western blot. No difference in the ratio of pro to mature forms of these proteins was observed, indicating proteolytic processing of rhoptry contents proceeds normally in dispersed rhoptries. (B) DrpB localization and dynamics are unaffected by knockdown of TgDHHC7. IFA of untreated parental parasites and TgDHHC7cKO parasites following growth with Atc for 60 hours. Rhoptries are scattered throughout the cell in TgDHHC7cKO parasites following Atc treatment, as assessed by staining for ROP13 and RON11. However, no change was observed in the signal strength or localization pattern of the dynamin-like protein DrpB. Red: anti-DrpB antibody detected by Alexa594-anti-mouse IgG. Green: anti-ROP13 antibody detected by Alexa488-anti-rabbit IgG. Blue: anti-RON11 antibody detected by Alexa350-anti-rat IgG. Scale bars = 5µm. (C-D) Minor abnormalities in TgDHHC7cKO parasites observed by TEM. (C) Parasites lacking TgDHHC7 were sometimes (< 9% of TEM sections) observed to contain amylopectin granules (arrows), which may be a sign of stress. (D) Multi-membranous bodies (arrow) of unclear origin were sometimes (< 7% of TEM sections) observed in parasites lacking TgDHHC7.

**Figure 2-S6**

Microneme biosynthesis and parasite gliding motility are unaffected by TgDHHC7 knockdown. (A) IFA analysis of micronemes in parental and TgDHHC7cKO parasites. Parental parasites were grown – Atc while TgDHHC7cKO parasites were grown 60 hours + Atc prior to fixation and processing. Micronemes are unaffected upon knockdown of TgDHHC7, as assessed by staining for the microneme protein MIC2. In contrast, rhoptries are scattered throughout the cell in TgDHHC7cKO parasites following Atc treatment, as assessed by staining for ROP13 and
RON11. Red: anti-MIC2 antibody detected by Alexa594-anti-mouse IgG. Green: anti-ROP13 antibody detected by Alexa488-anti-rabbit IgG. Blue: anti-RON11 antibody detected by Alexa350-anti-rat IgG. Scale bars = 5µm. (B) Gliding motility, which requires secretion of micronemal adhesions, was assayed as a measure of microneme functionality. No difference was observed in the frequency or length of SAG1 trails deposited by parental or TgDHHC7cKO parasites +/- Atc treatment.

Figure 2-S7

Palmitoylation-independent targeting of TgARO to the rhoptry surface is unable to rescue the defects incurred upon knockdown of TgDHHC7 or TgARO. (A) N-terminal truncation of the first six residues of TgARO removes the myristoylation and palmitoylation signals that are critical for rhoptry targeting, resulting in gross mistargeting throughout the cytosol. Red: mouse anti-HA antibody detected by Alexa594-anti-mouse IgG. Green: rabbit anti-ROP13 antibody detected by Alexa488-anti-rabbit. Scale bar = 5µm. (B) Fusion of this N-terminally truncated version of TgARO to the C-terminus of a catalytically inactive form of TgDHHC7 (TgDHHC7-C371SΔ1-162) restores targeting of TgARO to the rhoptry surface. Red: anti-HA antibody detected by Alexa594-anti-rabbit IgG. Green: mouse anti-ROP2/3/4 antibody detected by Alexa488-anti-mouse. Scale bar = 5µm. (C) Complementation of TgDHHC7cKO assessed by plaque assay. Neither the TgAROΔ1-6 truncation mutant or the chimeric TgDHHC7-TgARO fusion are able to rescue the defect incurred by the knockdown of endogenous TgDHHC7. These complemented strains still fail to apically tether rhoptries (assessed by IFA, data not shown) and cannot form plaques in the presence of Atc. Similarly, complementation of TgAROcKO parasites with the
TgARO_{Δ-6} truncation mutant or the chimeric TgDHHC7-TgARO fusion also failed to rescue knockdown of TgARO (data not shown).
Figure 2-S1
Figure 2-S2
Figure 2-S3

A

B

TgDHHC7-3xHA    TgDHHC7-M1-1xHA    TgDHHC1-M2-1xHA

50                    37

C

D

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Figure 2-S5

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C

D

1μ

0.5μ
Figure 2-S6

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136
Figure 2-S7

A

TgARO_{Δ1-6}

TgARO_{Δ1-6}  ROP13  merge

B

TgDHHC7-C371S_{Δ1-162}

TgARO_{Δ1-6}  HA

Chimera  ROP2/3/4  merge

C

-Atc 200  +Atc 200  +Atc 20,000

TgDHHC7cKO

TgARO_{Δ1-6}

Chimera
Table 2-1. DHHC-CRD containing proteins encoded within the *Toxoplasma* genome. Relevant information was acquired from ToxoDB (release 8.0).

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Table 2-2. Primers used in this study as discussed in text. Restriction sites and mutated bases are shown in lowercase.

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References


Chapter 3

RON5 is critical for organization and function of the moving junction complex
Abstract

Apicomplexans facilitate host cell invasion through the formation of a tight-junction interface between parasite and host plasma membranes called the moving junction (MJ). A complex of the rhoptry neck proteins RON2/4/5/8 and the microneme protein AMA1 localize to the MJ during invasion where they together provide a stable anchoring point for host penetration. During the initiation of invasion, the preformed MJ RON complex is injected into the host cell where RON2 spans the plasma membrane while RON4/5/8 localize to its cytosolic face. While an interaction between AMA1 and RON2 outside of the host cell has been characterized, little is known about the functions of MJ RONs inside the host cell. Here we provide a detailed analysis of RON5 to resolve outstanding questions about complex topology, assembly and function during invasion and egress. Conditional knockdown of RON5 results in complete degradation of RON2 and mistargeting of RON4, indicating that RON5 plays a critical role in the organization of the MJ complex. While RON8 is unaffected by disruption of the RON2/4/5 complex, these parasites are unable to invade, directly demonstrating for the first time that MJ RONs play a critical role in entry (but not egress). Although previous studies suggested the MJ was unnecessary for rhoptry secretion, we find that parasites unable to form a MJ fail to inject rhoptry effectors into the host cytosol. Functional complementation of our mutant reveals that while proteolytic separation of RON5 N- and C-terminal fragments is dispensable, the C-terminal domain is critical for function.
Introduction

Apicomplexa is a large phylum of eukaryotic pathogens comprised of ~6,000 described species which cause extensive disease in humans and other animals (Adl et al., 2007; Levine, 1988). Apicomplexans of particular interest include *Toxoplasma gondii*, which chronically infects approximately one-third of all humans and causes neurological disorders in immunocompromised individuals as well as the human malarial agent, *Plasmodium falciparum*, which is the cause of nearly million deaths annually (Hill et al., 2005; Mackintosh et al., 2004). These parasites have obligate intracellular life cycles and the disease they cause is absolutely dependent upon their ability to invade and replicate within their host cells (Striepen et al., 2007). A better understanding of the parasite molecules and processes that facilitate host cell invasion is needed to aid in development of better therapeutics and control strategies.

Invasion in apicomplexans is a highly coordinated process of attachment and entry that depends on sequential protein secretion events from two different organelles, the micronemes and rhoptries (Carruthers and Sibley, 1997). Initially, secretion from the micronemes releases molecular adhesins onto the parasite plasma membrane, providing a firm attachment to the host surface (Carruthers and Tomley, 2008). Translocation of these adhesins in an apical to posterior direction via an actin-myosin motor within the parasite pellicle generates a unique gliding motility, providing the driving force for host penetration (Sibley, 2010).

After initial attachment, the parasite apex is oriented toward the host cell, followed by discharge of the rhoptry contents (Carruthers and Boothroyd, 2007). Rhoptry secretion corresponds with the formation of a tight-junction interface between parasite and host plasma membranes called the moving junction (MJ) and a commitment to invasion. A complex of the rhoptry neck proteins RON2/4/5/8 and the microneme protein AMA1 localizes to the MJ during
invasion where it is thought to provide a stable anchoring point for host penetration (Alexander et al., 2005; Besteiro et al., 2009; Lebrun et al., 2005; Straub et al., 2009). The MJ is also the site of a molecular sieve that restricts access of host plasma membrane proteins to the nascent parasitophorous vacuole, rendering the vacuole non-fusogenic and protecting the parasite from lysosomal destruction, a function that may be performed by the MJ RON complex (Mordue et al., 1999).

The initial identification and preliminary characterization of MJ components has led to a model in which RON2 and AMA1 mediate tight-junction formation and bridge the invading parasite and host cell surfaces while RONs 4/5/8 are positioned in the host cytoplasm (Besteiro et al., 2009; Lamarque et al., 2011; Straub et al., 2009; Tyler and Boothroyd, 2011). While an important interaction between parasite surface-bound AMA1 and RON2 outside of the host cell has been elucidated, the bulk of MJ RON complex resides inside the host cell during invasion and little is known about the roles of performed by these proteins (Tonkin et al., 2011; Vulliez-Le Normand et al., 2012). In addition, there is limited genetic evidence definitively demonstrating the role of the RONs in invasion. A knockout of the Coccidia-restricted MJ component RON8 shows that while not essential, this protein plays an important role in efficient invasion (Straub et al., 2011). Taken together with the conservation of other MJ RONs across the Apicomplexa, these data suggest a core complex of RON2/4/5 comprises the critical invasion machinery.

Here we provide a comprehensive analysis of Toxoplasma RON5 to resolve outstanding questions about MJ complex topology, assembly and function. Using a conditional knockdown approach, we show that depletion of RON5 results in complete degradation of RON2 and mistargeting of RON4, indicating RON5 is critical for complex organization. In contrast, RON8
targeting and stability are unaffected by disruption of the RON2/4/5 complex. Parasites lacking RON5 egress efficiently but cannot invade new host cells, demonstrating the key importance of the MJ RON core complex in penetration. Injection of rhoptry effectors into the host cytosol requires formation of a moving junction. Finally, complementation of RON5 knockdown with a series of mutants reveals that while proteolytic separation of RON5 N- and C-terminal fragments is dispensable, the C-terminal domain is critical for RON2 stability and MJ function. Together, this work demonstrates that RON5 is crucial for the organization of the MJ complex and provides the first clear genetic demonstration that the MJ RONs play a critical role in invasion. This data also indicates that the highly conserved RONs 2/4/5 constitute a core MJ complex that represents the key invasion machinery that is central for causing disease in these globally important pathogens.
Results

Characterization of RON5 maturation.

During maturation in transit to the rhoptries, RON5 is processed at least twice to separate the protein into three fragments. Antibodies raised independently against the RON5-N and -C fragments demonstrated that both were incorporated into the mature MJ complex and secreted into the MJ during invasion (Besteiro et al., 2009; Straub et al., 2009). However, the fate of the fragment removed by the more N-terminal processing event (residues 34-314 following removal of the signal peptide) remains to be characterized. Several rhoptry body proteins contain N-terminal pro-domains that are critical for organelle targeting and thus this region may constitute a pro-domain with possible functions in RON5 folding or targeting (Bradley and Boothroyd, 2001). Alternatively, this region may be incorporated into the mature complex and function in the MJ during invasion. A hydrophobic region that could form a transmembrane domain is predicted in this region, which would have implications for MJ complex topology and function if validated (Besteiro et al., 2009). To address this unresolved point, we generated a double epitope tagged version of RON5 with an FLAG tag at C-terminus and a HA tag just downstream of the predicted signal peptide cleavage site (Figure 3-1A). This double-tagged version of RON5 was placed under control of the RON5 promoter and the resulting expression cassette was then targeted to the UPRT locus by homologous recombination to allow for expression of this double epitope-tagged second copy of RON5. We then monitored rhoptry maturation in parasites expressing this cassette using an antibody against the pro domain of ROP4 that labels pro-rhoptry compartments. While RON5c-FLAG was present in both pro- and mature rhoptries, HA signal was only detected in proROP4-positive rhoptry compartments, indicating that this N-
terminal region of RON5 is a pro-domain (hereafter referred to as proRON5) not present in mature rhoptries and thus not incorporated into the mature MJ complex (Figure 3-1B).

Establishment of a RON5 conditional knockdown strain.

RONs 2/4/5/8 and AMA1 are the only parasite proteins known to localize to the moving junction and are believed to play an important role in host invasion. We have shown that the Coccidia-restricted RON8 is important but not absolutely required for host invasion, suggesting that the remaining MJ RONs 2/4/5 compose an apicomplexan MJ core complex that constitutes the key invasion machinery employed across the phylum (Straub et al., 2011). While a RON4 paralog and two RON2 paralogs are present in the Toxoplasma genome, RON5 appears to be a single copy gene with no isoforms (Besteiro et al., 2011). Thus, we reasoned that disruption of RON5 was likely to yield unambiguous functional insight into the MJ complex. Attempts to disrupt RON5 in the ∆KU80 parasite strain were unsuccessful, suggesting a critical role in parasite biology (Beck et al., 2010). To explore this possibility further, we first generated a parasite strain containing an endogenous C-terminal RON5 3xMyc tag to improve detection of RON5. We then replaced the endogenous RON5 promoter with a conditional, tet-regulatable promoter (Figure 3-2A). As expected from the truncated tetO-SAG4 conditional promoter, parasites having undergone the desired recombination event (RON5cKO) show a lower level of RON5 expression compared to the parental line (note upshift in migration of RON5c due to the presence of the 3xMyc tag) (Figure 3-2B). Rhoptries are assembled de novo during each round of parasite division and protein traffic to the organelle is restricted to a narrow window during biosynthesis (Behnke et al., 2010). In agreement with this, we observe some mistargeting of RON5c under the control of the constitutive SAG4 promoter in the RON5cKO parasites, which
likely corresponds to protein synthesized outside of this rhoptry biosynthesis timeframe (-Atc, Figure 3-2D). However, the majority of RON5c signal was present in the rhoptry necks, as assessed by co-localization with RON11.

Treatment with anhydrotetracycline (Atc) to repress expression results in a steady loss of RON5c with protein levels dropping below detectibility by 72 hours (Figure 3-2C). No gross effect on rhoptries was observed in parasite lacking RON5 as assessed by IFA with rhoptry body markers ROP2/3/4 and the non-MJ rhoptry neck marker RON11 (+Atc, Figure 3-2D). Additionally, no defect in intracellular replication was detected in parasites lacking RON5 (data not shown).

**RON5 is critical for host invasion and evacuole formation but not egress.**

To test the importance of RON5 in host cell entry, we performed invasion assays on RON5cKO parasites depleted of RON5. In the absence of Atc treatment, a minor decrease in the invasive capacity of these parasites is observed relative to the wild type strain, likely corresponding to the lower levels of RON5 produced in the knockdown strain (red bars, Figure 3-3A). In contrast, a major block in invasion is observed following depletion of RON5 (blue bars, Figure 3-3A), indicating RON5 is critical for host invasion. To better assess this invasion defect over the course of several lytic cycles, we performed plaque assays. While wild type parasites readily formed plaques in the presence or absence of drug treatment, no plaques were formed by the RON5cKO parasites in the presence of Atc, even with high parasite numbers (Figure 3-3B). These results show the critical importance of RON5 for host invasion and suggest RON5 may be essential for this process.
Interestingly, the block in parasite invasion in the absence of RON5 is not accompanied by a simultaneous increase in attached parasites (blue bars, Figure 3-3A). Thus, parasites depleted of RON5 either exhibit an attachment defect, or more likely, detach from their host following a failure to invade as previously observed following disruption of RON8 (Straub et al., 2011). To distinguish between these two possibilities, we treated parasites with cytochalasin D, arresting the invasion process just after apical reorientation but prior to penetration by disabling gliding motility through inhibition of actin polymerization (Dobrowolski and Sibley, 1996). Under these conditions, parasites depleted of RON5 were found to attach to host cells with the same efficiency as untreated or parental line parasites, indicating that initial attachment is not impaired and that a failure to invade in the absence of RON5 is followed by gliding motility-based detachment at which point these parasites are washed away during invasion assay processing (Figure 3-3C).

During invasion, parasites inject a number of key effectors from the rhoptry body into the host cytosol to modulate innate immunity (Hunter and Sibley, 2012). Rhoptry secretion can be visualized by arresting the invasion process with cytochalasin D. Under these circumstances, an early stage MJ is still formed at the point of apical contact between the parasite and host surface and several rhoptry body proteins can be visualized entering the cell in membranous structures called evacuoles (Hakansson et al., 2001). To determine the importance of RON5 for secretion of rhoptry body contents, we monitored the formation of evacuoles in parasites depleted of RON5. Although cytochalasin D parasites lacking RON5 still attach normally (Figure 3-3C), a striking loss of evacuole formation was observed indicating RON5 is critical for rhoptry secretion (Figure 3-3D).
Finally, in addition to the key roles in invasion and rhoptry secretion highlighted above, the MJ has also been suggested to play a role in host cell exit as RON4-positive MJ rings have been reported to form during egress (Alexander et al., 2005; Hoff and Carruthers, 2002; Sibley, 2010). However, we observed no difference in natural egress in parasites lacking RON5. To more quantitatively assess the importance of RON5 in this process, we induced egress using the calcium ionophore A23187. Under these conditions, we again observe no defect in host cell exit as parasites with or without RON5 egressed with the same efficiency (Figure 3-3E). Collectively, these results demonstrate that RON5 plays a critical role in host cell invasion but is dispensable for egress.

**The MJ RON core complex is disrupted in the absence of RON5.**

To determine the impact of the loss of RON5 on the remainder of the MJ complex, we examined these components by western blot. Interestingly, following RON5 knockdown, RON2 signal is also eliminated, indicating that RON5 is critical for maintaining the stability of RON2 (Figure 3-4A). This complete destabilization is specific to RON2 while RON4 and RON8 show only minor levels of perturbation under these conditions. The concomitant loss of RON2 levels closely mimics RON5 levels over a series of time points during RON5 knockdown, further showing the necessity of RON5 for RON2 stability (Figure 3-S1). The dependence of RON2 upon RON5 was also clearly observed by IFA as parasites lacking RON5 also lack RON2 (Figure 3-4B).

In contrast to the destabilization of RON2 in the absence of RON5, RON8 is intact and properly targeted to the rhoptry neck in the absence of RON5 (Figure 3-4B). While Western blot analysis of RON4 indicates that it is largely intact in the absence of RONs 5 and 2, IFA revealed
a targeting defect with RON4 present throughout the rhoptry body but not in the neck (Figure 3-4C). Notably, RON8 signal faithfully targets to the rhoptry neck in these conditions and the loss of colocalization between RON4 and RON8 strongly suggests that these proteins do not directly interact in the absence of RONs 2 and 5. Collectively, these results demonstrate that the RON5 knockdown effectively constitutes a RON5/2 knockdown and shows for the first time directly that moving junction RONs are critical for invasion.

**Establishment of a complementation system to probe RON5 function.**

The effect of RON5 knockdown on the integrity of the MJ complex and on invasion raises the question as to what region of RON5 necessary for maintaining stability of RON2 as well as whether any RON5-specific roles during invasion exit. To explore these questions, we established a functional complementation system by targeting a full-length RON5 expression cassette under the control of its endogenous promoter to the UPRT locus. To distinguish this copy of RON5 from the regulatable copy transcribed from the endogenous locus, we engineered an HA epitope tag at the C-terminus. As expected, this full-length version of RON5 expressed from this locus targets properly to the rhoptry necks, co-localizing with RON11 (Figure 3-5A). Expression of this second copy of RON5 insensitive to Atc fully rescues the stability of RON2 upon knockdown of endogenous RON5 (Figure 3-5B). In addition, complementation with full-length RON5 rescues host invasion to wild-type levels and restores the ability of these parasites to plaque in the presence of Atc (Figure 3-5C-D).
RON5N/C processing is dispensable for MJ function.

We next employed this system to assess the importance of processing of RON5 into RON5N and RON5C. To determine the site of processing, we scanned the RON5 sequence to identify candidate sites that match the consensus sequence characterized in other rhoptry protein processing events (SΦXE) (Bradley et al., 2002). A single match to the consensus was identified (SFVE, residues 1258-1262) within the region where processing is expected to occur based on SDS-PAGE migration of the mature N- and C-terminal fragments and peptide coverage generated from individual mass spectrometric analysis of RON5N and RON5C (Alexander et al., 2005; Straub et al., 2009). However, mutagenesis of all four residues (SFVE>AGDR) quadruple mutation of this site in a second copy of RON5 did not affect migration of the C-terminal fragment in SDS-PAGE relative to the wild-type protein, indicating that this mutant was still processed (SFVE>AGDR, Figure 3-6A).

We have recently shown that processing of the rhoptry protein TLN1 occurs at a non-consensus site in which glutamine is tolerated at the P1 location (Hajagos et al., 2011). An SFVQ site is also present within the region where processing of RON5 is expected to occur (residues 1288-1291). A similar mutagenesis of this site (SFVQ>AGDR) results in a modest upshift of RON5C that does not agree with a block in processing to separate RON5N and C, but is consistent with processing upstream at the SFVE site (SFVQ>AGDR, Figure 3-6A). To test if this was the case, we generated a double mutant at both sites and observe a large upshift to ~180 kD in this mutant, indicating a block in RON5N/C processing (SFVE+SFVQ, Figure 3-6A). These results indicate either that processing of RON5 is favored at SFVQ and shifted to SFVE upon ablation of this site, or that processing occurs at both sites in the endogenous protein.
Despite this block in RON5N/C processing, this mutant was found to target to the rhoptry necks, indicating that this processing event is not important for proper trafficking (data not shown). To assess the functional impact of the failure to separate RON5N/C, we complemented the RON5 conditional knockdown strain with the double processing mutant. Surprisingly, this mutant fully rescued the stability of RON2 (not shown) and host invasion and restored the ability to form plaques upon depletion of endogenous RON5 (Figure 3-6B). Collectively, these results demonstrate that proteolytic separation of RON5N and RON5C is not grossly important for MJ complex integrity, trafficking or function.

**RON5C is required to stabilize RON2.**

Since RON5N/C processing is not required for function, we tested the possibility that RON5C is dispensable all together. The RON5 sequence does not contain any recognizable domains to target for functional analysis. Thus, to guide the design of addition mutants, we generated a homology plot between *Toxoplasma* and *P. falciparum* RON5 sequences to determine conservation hot spots that might encode key regions for interaction with other complex members and function (Figure 3-7A). The plot reveals three general regions of varying conservation between the two species, the highest of these being the C-terminal half of RON5N (residues 897-1257). An intermediate level of conservation is seen for the N-terminal half of RON5N (residues 315-896) while the lowest level of conservation is observed in an area that roughly corresponds to *Toxoplasma* RON5C (residues 1292-1702). Using this information together with secondary structure prediction, we designed a series of C-terminal truncations and expressed each of these mutants from the UPRT locus in the RON5cKO strain (Figure 3-7A). Three of these truncations (∆618-1702, ∆898-1702, ∆1084-1702), each of which removes the
entire RON5C region as well as portions of RON5N, were found to grossly mistarget (Figure 3-7B). In contrast, truncations which remove half (Δ1476-1702) or all (Δ1258-1702) of RON5C continue to target to the rhoptry necks (Figure 3-7B). Interestingly, the majority of RON5Δ1476-1702 signal localized slightly posterior to non-MJ complex markers for the rhoptry neck, although the significance of this slight shift in localization is unclear. These results indicate that RON5C is dispensable for trafficking while the C-terminal region of RON5N is necessary for localization to the rhoptry necks.

We next evaluated the ability of these truncations mutants to rescue the stability of RON2 upon knockdown of endogenous RON5. As expected, the Δ618-1702, Δ898-1702 and Δ1084-1702 truncations, which grossly mistarget, each fail to stabilize RON2 in the absence endogenous RON5 (Figure 3-7C). Additionally, the Δ1258-1702 truncation mutant, which does target to the rhoptry neck, also fails to rescue RON2 stability, demonstrating that although RON5N/C processing is dispensable, RON5C is required for RON2 integrity. In contrast, the Δ1476-1702 truncation lacking the C-terminal 227 residues of RON5C completely rescues RON2 stability (Figure 3-7C). To monitor both the impact on penetration and downstream intracellular survival, we performed invasion and plaque assays using the RON5cKO strain complemented with the Δ1258-1702 or Δ1476-1702 mutants. We found that the Δ1258-1702 mutant was unable to rescue invasion or form plaques upon knockdown of endogenous RON5 while the Δ1476-1702 mutant restored both of these phenotypes (Figure 3-7D-E). Taken together with our analysis of N/C processing, these results identify residues 1292-1475 of RON5 as critical for maintaining RON2 stability and suggest this domain may directly interact with RON2.
**Discussion**

**Organization and function of the MJ complex is dependent upon RON5.**

The establishment of a tight-junction interface between invading apicomplexan parasites and their host cells was first observed over 30 years ago (Aikawa et al., 1978). More recently, the exciting discovery that a complex of rhoptry neck proteins is secreted into this tight-junction provided candidates for understanding the molecular basis for this unique style of host penetration (Alexander et al., 2005). The topology of the protein components of the MJ complex during invasion has been determined and a key interaction between an extracellular portion of RON2 and the parasite-surface bound AMA1 has been mapped (Besteiro et al., 2009; Lamarque et al., 2011; Straub et al., 2009; Tonkin et al., 2011; Tyler and Boothroyd, 2011). However, a number of outstanding questions remain regarding the precise functional role of this interaction, partly owing to the lack of any RON2 mutants. Additionally, the bulk of the MJ complex is present within the host cytoplasm during invasion and little is known regarding the roles that these proteins perform at the cytoplasmic face of the host membrane. We have taken a conditional approach to generate a RON5/2 double knockdown, revealing RON5 is a key organizing factor of the MJ complex and providing the first genetic functional analysis of these core components of the MJ complex.

RON5 is efficiently eliminated in our knockdown mutant without impacting rhoptry structure or parasite replication. Depletion of RON5 does result in complete degradation of RON2 and mistargeting of RON4 while RON8 targeting and stability are not grossly affected. This specific impact on RONs 2 and 4 upon knockdown of RON5 provides further support for the idea that these proteins constitute an MJ core complex in keeping with their conservation across the phylum while RON8 represents a coccidial innovation that contains its own targeting
information to facilitate sorting to the rhoptry neck. We have previously shown that RON8 is important for efficient invasion by *Toxoplasma* (Straub et al., 2011). While loss of RON8 comes at a substantial cost in terms of invasion, and subsequently virulence, the *RON8* gene can nonetheless be disrupted. In contrast, we were unable to generate a *RON5* knockout line and conditional knockdown of RON5 reveals a critical importance for the MJ core complex in host invasion.

The simultaneous loss of RON2 upon RON5 knockdown may be due Endoplasmic Reticulum-Associated Degradation (ERAD) quality control systems that sense misfolded proteins, extract them from the ER and target them to the proteasome (Vembar and Brodsky, 2008). In agreement with this, components of the ERAD system have been identified in *Toxoplasma* (Agrawal et al., 2009). The specific degradation of RON2 but not other MJ complex components in the absence of RON5 suggests that RON5 may directly bind RON2 and ensure its proper folding or mask a RON2-encoded signal for ER retention and degradation. Indeed, components of several protein complexes are subject to ER retention and rapid elimination by ERAD when not incorporated into their larger complex (Vembar and Brodsky, 2008).

In contrast to RON2, the soluble MJ component RON4 is not degraded but fails to target to the rhoptry neck in the absence of RON2/5, indicating that RON4 contains targeting information to enter the rhoptry, but requires interaction with RON2/5 to ultimately reach the neck of this organelle. Little is known about the determinants for sub-domain trafficking within the rhoptries and this is the first example of targeting information that distinguishes between the rhoptry neck and rhoptry body sorting in *Toxoplasma*, providing a lead to further understanding the organization of the rhoptry contents. Interestingly, a reverse scenario was observed for the *P. falciparum* rhoptry body protein RAP1, which mistargets to the rhoptry neck following
truncation of C-terminal residues (Richard et al., 2009). In addition, rhoptry neck targeting of a recently identified RON complex in *Toxoplasma* consisting of RON9 (a predicted transmembrane protein) and RON10 (a predicted soluble protein) requires both partners as gross mistargeting and total loss of rhoptry localization of each protein occurs in the absence of the other. Conservation of this RON pair is restricted to the *Coccidia* and *Cryptosporidia* but the function of these proteins remains unknown (Lamarque et al., 2012).

**The emerging model of MJ complex function during apicomplexan invasion.**

While the localization of the RON2/4/5/8 complex to the MJ immediately suggested roles in host invasion, determination of the precise nature of these functions has been elusive. The tight-junction nature of the interface at the MJ and presumable need for an anchoring point for the parasite to push/pull its way into the host cell suggested the MJ serves as a gripping point. This possibility is supported by the observation of host F-actin dynamics at the MJ, which may represent exploitation of the host cytoskeleton by the parasite to provide a foundation upon which the MJ can be built (Gonzalez et al., 2009). In this scenario, the cytosolic portion of the MJ RON complex would interact with host cortical elements, allowing for a stable structure against which the parasite glideosome could push through the AMA1-RON2 interaction (Tyler et al., 2011). Peptide or antibody blocking of AMA1-RON2 interaction inhibits invasion, supporting such a role (Lamarque et al., 2011; Tonkin et al., 2011; Tyler and Boothroyd, 2011). In addition, this model is supported by binding of the AMA1 C-terminal tail to aldolase *in vitro*, although this AMA1-aldolase interaction has not been demonstrated in the parasite (Sheiner et al., 2010). RON8 may play a role in binding host cortical features, however this is unlikely to be the only MJ RON component that fulfills this role since RON8 is not present outside of the
Coccidia (Straub et al., 2009; Straub et al., 2011). Our finding that disruption of the MJ core complex results in a more severe block in invasion but that RON8 is not affected further supports this idea.

In addition to a role in gripping, the MJ RON complex has also been suggested to be fulfill a molecular sieving activity present at the MJ, which restricts access of certain host transmembrane protein into the forming PV (Shen and Sibley, 2012). Analysis of determinants of this activity suggest that sieving occurs on the cytosolic face of the host membrane, which fits nicely with localization of most of the MJ RON complex at this site (Mordue et al., 1999). However, evidence supporting direct involvement of MJ RONs in sieving is lacking. Future work aimed at generating RON5 mutants for study in our functional complementation system may provide more insight into this activity.

The discovery that phosphorylation of the AMA1 C-terminal tail is crucial for *P. falciparum* invasion together with characterization of the AMA1-RON2 interaction suggested an additional role for the MJ complex in signaling through AMA1 (Treeck et al., 2009). In support of alternative or even dispensable roles for AMA1, stage-specific ablation of AMA1 in *P. berghei* showed that this protein is not required for host invasion by sporozoites but is critical for merozoite invasion of erythrocytes while RON4 appears indispensable for sporozoite invasion (Giovannini et al., 2011). These data indicate that the AMA1-RON2 interaction is not critical for *P. berghei* sporozoites, although antibodies against AMA1 do inhibit *P. falciparum* invasion (Silvie et al., 2004). Sporozoites invasion displays a number of unique features and more work is needed to make sense of the dispensability of AMA1 in *P. berghei* sporozoites (Kappe et al., 2003).
The signals and mechanisms that facilitate rhoptry release represent a significant gap in our understanding of apicomplexan invasion. Recent studies suggest that receptor engagement at the host surface by the micronemal adhesins EBA175 in *P. falciparum* and MIC8 in *T. gondii* may transmit key signals for rhoptry exocytosis (Kessler et al., 2008; Singh et al., 2010). Additionally, engagement of RON2 by AMA1 is important for invasion and has been suggested as a means by which the parasite senses formation of the MJ and signals to advance through the penetration process (Tyler et al., 2011). Interestingly, peptides which mimic this interaction by binding the hydrophobic groove on the surface of AMA1 block interaction with RON2 and invasion but not secretion of rhoptry body proteins (Lamarque et al., 2011; Riglar et al., 2011; Srinivasan et al., 2011; Tonkin et al., 2011; Tyler and Boothroyd, 2011). Additionally, our demonstration that RON2/5 is required for rhoptry secretion suggests that rhoptry secretion proceeds in a stepwise fashion with deployment of the MJ RONs from the rhoptry neck preceding secretion of the rhoptry body contents. Furthermore, antibodies which bind near but not in the RON2 binding site on the AMA1 surface prevent AMA1-RON2 interaction and rhoptry body secretion in *Plasmodium*, suggesting that exocytosis of this compartment is a distinct event from secretion of rhoptry neck proteins (Srinivasan et al., 2011). Thus, it is tempting to speculate that rhoptry neck secretion is triggered by binding of host surface receptors by MIC8 and that subsequent engagement of RON2 by AMA1 signals for rhoptry body release. Future work will exploit the loss of RON2 upon RON5 knockdown to directly address this possibility.
Materials and Methods

Toxoplasma and host cell culture

*T. gondii* RHΔhpt (parental) strain and modified strains were maintained in confluent monolayers of human foreskin fibroblast (HFF) host cells as previously described (Donald et al., 1996).

Antibodies

The following *Toxoplasma* primary antibodies were used in IFA or Western blot: mouse polyclonal anti-RON5C (Straub et al., 2009), polyclonal rat anti-RON11 (Bradley, unpublished), rabbit anti-RON2 (Straub et al., 2011), rabbit anti-RON4 (see below), rat polyclonal anti-RON4 (Alexander et al., 2005), anti-ROP2/3/4 mAb TA7 1A11 (Leriche and Dubremetz, 1991), rabbit anti-SAG1 (Burg et al., 1988), polyclonal mouse anti-ISP3 (Beck et al., 2010), monoclonal mouse anti-F1-ATPase beta subunit 5F4 (Bradley, unpublished), rabbit anti-proROP4 UVT70 (Carey et al., 2004). Hemagglutinin (HA) epitope tags were detected with mouse mAb HA.11 (Covance), rabbit polyclonal anti-HA (Invitrogen) or rat mAb 3F10 (Roche). MYC epitope tags were detected with mouse mAb 3E10 (Neomarkers). FLAG epitope tags were detected with mouse anti-FLAG mAb M2 (Sigma). For generation of rat anti-RON4 sera, a portion of the RON4 coding sequence comprising residues 85-983 was recombinantly expressed in *E. coli* BL-21DE3 cells and purified over Ni-NTA agarose (Qiagen) as previously described (Bradley et al., 2005). The resulting protein was injected into a rat for anti-sera production.
Light microscopy and image processing

Fixation and immunofluorescence staining of *T. gondii* were carried out as previously described (Beck et al., 2010). Image stacks were collected at z-increments of 0.2 µm with an AxioCam MRm CCD camera and AxioVision software on an Axio Imager.Z1 microscope (Zeiss) using a 100x oil immersion objective. Deconvolved images were generated using manufacturer specified point-spread functions and displayed as maximum intensity projections.

Generation of RON5 endogenous epitope tags

The endogenous tagging vector p3xHA.LIC.DHFR (Konrad et al., 2011) was first modified to replace the DHFR selectable marker cassette with a chloramphenicol acetyl-transferase (CAT) selectable marker between the restriction sites *HindIII/XbaI* resulting in the plasmid p3xHA.LIC.CAT. A portion of the genomic locus of RON5 up to but not including the stop codon was PCR amplified from *Toxoplasma* genomic DNA (primers P1/P2) and inserted into p3xHA.LIC.CAT or p3xMyc.LIC.CAT by ligation-independent cloning (Huynh and Carruthers, 2009) to generate the vectors pRON5-3xHA.LIC.CAT and pRON5-3xMyc.LIC.CAT. These plasmids were linearized with *PstI* and transfected into the TATiΔTgKu80 parasite line (Sheiner et al., 2011). Following selection with chloramphenicol, parasites were cloned by limiting dilution and a clone expressing the tagged protein of interest was isolated and designated RON5-3xHA or RON5-3xMyc.

Generation and complementation of RON5cKO parasites

For direct replacement of the RON5 promoter with the conditional TetOSAG4 promoter by homologous recombination, 5’ (primers P3/P4) and 3’ (primers P5/P6) regions flanking the

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RON5 promoter were PCR amplified from *Toxoplasma* genomic DNA and cloned into the vector pDT7S4myc (Sheiner et al., 2011) between NdeI and BglII/AvrII sites, respectively. The resulting vector, pTS4-RON5-DHFR, was linearized with *ApaI* and transfected into RON5-3xMyc or RON5-3xHA parasites. Following selection with 1 µM pyrimethamine, parasites were cloned by limiting dilution and genomic DNA from individual clones was analyzed for RON5 promoter replacement (primers P7/P8). A clone that had undergone the intended recombination event was designated RON5\textsubscript{Myc}cKO or RON5\textsubscript{HA}cKO.

For expression of a complementing second copy of RON5, the RON5 promoter was PCR amplified from *Toxoplasma* genomic DNA (primers P9/P10) and inserted into the UPRT targeting vector pUPRTKO-HA (Reese et al., 2011) between *SpeI* and *BamHI* by blunting both the digested vector and PCR amplicons, resulting in the vector pUPRTKO-RON5-promoter-HA. The full length RON5 coding sequence was PCR amplified from a *Toxoplasma* cDNA library (primers P11/P12) and inserted into this vector between BglII/NotI sites to generate the vector pUPRTKO-RON5-HA. This vector was linearized with *NsiI* and transfected into RON5cKO parasites followed by selection with 5 µg/ml 5-fluorodeoxyuridine to facilitate targeted replacement of the UPRT locus (Donald and Roos, 1995).

**Generation RON5 mutants and double epitope tagged versions of RON5**

For site directed mutagenesis, a portion of the RON5 coding sequence between the restriction sites *SmaI* and *NotI* was digested from the vector pUPRTKO-RON5-HA and inserted into the cloning vector pJet1.2 (Fermentas). Site-directed mutants were generated by Quick Change Mutagenesis (Strategene) with mutagenesis primers as follows (forward primer given, reverse compliment was also used): SFVE>AGDR (P13) and SFVQ>AGDR (P14).
For expression of double tagged RON5 to monitor proRON5, a FLAG epitope tag version of the vector pUPRTKO-RON5-HA was first generated by PCR amplifying the 3’ UTR with a forward primer encoding the FLAG epitope sequence (primers P15/P16) and inserting this amplicon between NotI/EcoRV, replacing the inframe fusion to a C-terminal HA tag with a FLAG tag (pUPRTKO-RON5-FLAG). A portion of the 5’ RON5 coding sequence was PCR amplified (primers P11/P17) and inserted into the cloning vector pJet1.2 (Fermentas). An HA epitope was then inserted into the RON5 coding sequence between residues X and Y using Quick Change Mutagenesis (P18) and this modified coding sequence was inserted into the vector pUPRTKO-RON5-FLAG between BglII/RsrII resulting in the vector pUPRTKO-RON5-PRO-HA-C-FLAG.

For generation of an inframe deletion of proRON5, a portion of the RON5 promoter and 5’ coding sequence was PCR amplified from the vector pUPRTKO-RON5-PRO-HA-C-FLAG with a reverse primer encoding a KasI site (primer P19/P20). This amplicon was inserted between Nhel/AscI. A portion of the RON5 coding sequence was then PCR amplified (primers P21/P22) and inserted between KasI/AscI, resulting in the vector pUPRTKO-RON5Δpro-N-HA-C-FLAG. For generation of C-terminal truncations of RON5, truncated portions of the RON5 coding sequence were amplified (Δ618-1702: P11/P23; Δ898-1702: P11/P24; Δ1019-1702: P11/P25; Δ1258-1702: P11/P26; Δ1476-1702: P11/P27) and inserted into the vector pUPRTKO-RON5-promoter-HA between BglII/NotI to generate the indicated C-terminal truncations.
**Plaque assays**

Parasites were grown 48 hrs +/- 1.5 µg/ml Atc, syringe lysed and infected into 6-well dishes containing fresh, confluent HFF monolayers +/- Atc. Cultures were allowed to grow nine days before fixation with methanol followed by staining with crystal violet.

**Invasion, evacuole and egress assays**

Invasion assays were performed as previously described (Huynh et al., 2003). Briefly, parasites were grown 72 hrs +/- 1.5 µg/ml Atc, monolayers were washed with PBS and intracellular parasites were collected by scrapping and passage through a 27-gauge needle. Equivalent parasite numbers were resuspended in pre-warmed media and allowed to infect HFF monolayers on coverslips for one hour. Monolayers were then washed, fixed with EM-grade 3.7% formaldehyde/PBS (Biosciences, Inc.), blocked with PBS/3%BSA for 30 min and incubated with rabbit anti-SAG1 diluted in PBS/3%BSA for 1 hr. After washing, samples were permeabilized in PBS/3%BSA/0.1% Triton X-100 for 30 min and then incubated with mAb 5F4 diluted in PBS/3%BSA for one hour. Following incubation with secondary antibodies, samples were examined by fluorescence microscopy and parasites were scored as invaded (SAG1-, 5F4+) or attached (SAG1+, 5F4+). Invasion assays were performed in triplicate, five fields were counted on each replicate coverslip and the average number of invaded and attached parasites per field was calculated.

Egress assays were performed as previously described (Black et al., 2000). Briefly, parasites were grown +/- 1.5 µg/ml Atc for 24 hours, then infected into fresh HFF monolayers on coverslips and allowed to grow an additional 36 hours +/- 1.5µg/ml Atc. Coverslips were then washed with PBS and incubated in 1µM calcium ionophore A23187 (Sigma) diluted in Hanks
Balances Salts Solution at 37°C before being fixed in methanol and processed for IFA with rabbit anti-SAG1. At least 100 vacuoles per coverslip were counted across five fields on three independent coverslips per sample and scored as egressed or not egressed. For each of the above assays, experiments were repeated at least twice and values from a representative experiment are shown as the mean ± SD.

**Construction of an inter-species RON5 homology plot**

RON5 coding sequences from *T. gondii*, *Neospora caninum* and *P. falciparum* were aligned in ClustalX and used to calculate the percent identity and similarity for 50 amino acid windows in five amino acid sliding steps across the entire alignment (Curtidor et al., 2011).
Figure Legends

Figure 3-1 – The N-terminus of RON5 is a pro domain that is not incorporated into the mature MJ complex.

(A) Diagram showing RON5 double tagging strategy. An HA tag was inserted immediately after the signal peptide and a FLAG tag was fused to the 3’ end of the coding sequence. This HA-N-RON5-C-FLAG second copy was targeted to the UPRT locus under the control of the endogenous RON5 promoter. (B) IFA showing localization of the double epitope tagged RON5 protein. The N-terminal portion of RON5 tagged with HA (green) is always seen to co-localize with proROP4 (red), indicating it is present in the pro-rhoptry compartment. RON5C-FLAG (blue) is present in the pro-rhoptry as well but also labels the mature rhoptries, visible as a distinct compartment anterior to the pro-rhoptries not containing proROP4. The HA signal is never seen to co-localize with FLAG signal in the absence of proROP4, indicating it is not present in the mature rhoptries. Red: rabbit anti-proROP4 antibody detected by Alexa594-anti-rabbit IgG. Green: rat anti-HA antibody detected by Alexa488-anti-rat IgG. Blue: mouse anti-FLAG antibody detected by Alexa350-anti-mouse IgG. Scale bar = 5µm.

Figure 3-2 – Establishment of a RON5 conditional knockdown mutant.

(A) Strategy for generating RON5cKO parasites through direct replacement of the RON5 endogenous promoter with a tetracycline-repressible element (TRE) by homologous recombination. The TRE consists of the seven tandem tetracycline operator sequences fused to a truncated SAG4 promoter. A C-terminal endogenous 3xMyc tag was introduced for improved detection. (B) Western blot comparing RON5C in wild type and RON5cKO strains without Atc. Exchange of the endogenous RON5 promoter with a Tet-repressible, conditional promoter
results in lower levels of basal RON5 expression. A 5 kD upshift in the RON5cKO strain corresponding to the endogenous 3xMyc tag is also observed. ISP3 serves as a loading control. (C-D) Growth of RON5cKO parasites in the presence of Atc results a depletion of RON5. (C) Western blot showing RON5 levels after 24, 48 and 72 hours of Atc treatment. (D) IFA showing RON5C signal after 48 hours +/- Atc. Some mistargeting of RON5C is seen in untreated parasite due to the replacement of the endogenous, cell cycle regulated promoter with the constitutive Tet-repressible promoter. No gross impact on the rhoptries is observed following depletion of RON5 as assessed by the non-MJ rhoptry neck protein RON11 and the rhoptry body marker ROP2/3/4. Red: rabbit anti-HA antibody detected by Alexa594-anti-rabbit IgG. Green: rat anti-RON11 antibody detected by Alexa488-anti-rat IgG. Blue: mouse anti-ROP2/3/4 antibody detected by Alexa350-anti-mouse IgG. All scale bars = 5µm.

Figure 3-3 – RON5 is critical for host invasion but not egress.

(A-B) A major invasion defect is observed in parasite that lack RON5. (A) Parental or RONcKO parasites were grown for 72 hours +/- Atc and then allowed to invade into fresh host cells for one hour. Following depletion of RON5, parasites show a nearly complete block in host penetration (asterisk, p-value < 0.001). A corresponding increase in attached, uninvaded parasites is not observed (blue bars). A minor decrease in penetration is also seen for untreated RON5cKO parasites, likely due to the lower levels of RON5 expressed in this strain relative to the parental line. (B) Parasites depleted of RON5 cannot form plaques in fibroblast monolayers. Parental or RON5cKO parasites were grown 48 hours +/- Atc and then infected into fresh fibroblast monolayers at an infective dose of 200 parasites per well and incubated for nine days. RON5cKO parasites are unable to form plaques in the presence of Atc, even at an infective dose
of 20,000 parasites per well. (C) Initial attachment is not affected by knockdown of RON5. Parasites were grown for 60 hours -/+ Atc before treatment with cytochalasin D to block motility and arrest the invasion process just after attachment. (D) Loss of RON5 eliminates secretion of rhoptry body proteins as assessed by evacuole formation (asterisk, p-value < 0.001). Parasites were grown for 60 hours -/+ Atc before treatment with cytochalasin D to block invasion and allow evacuole formation. Evacuoles were detected by staining for ROP2/3/4. (E) Parasite egress is unaffected by loss of RON5. Parasites were grown 60 hours -/+ Atc and then induced to egress by treatment with calcium ionophore A23187 before fixation and staining for detection with anti-SAG1. The egress efficiency of parasites depleted of RON5 was not significantly different from untreated RON5cKO parasites.

Figure 3-4 – The MJ RON core complex is disrupted in the absence of RON5.

(A) Western blot analysis of MJ RONs 2, 4 and 8 following RON5 knockdown. RONs 2, 4 and 8 were compared in the RON5cKO strain with or without 72 hours of Atc treatment. RON2 is eliminated in the absence of RON5. In contrast, a minor decrease in RON4 and RON8 levels is observed. ISP3 serves as a loading control. (B) IFA showing RON2 is lost in parasites depleted of RON5 while RON8 levels and targeting are unaffected. Although a minor decrease in RON8 signal was observed by Western blot following depletion of RON5 (A), no change in RON8 was observed between vacuole with or lacking RON5. Red: rat anti-HA antibody detected by Alexa594-anti-rat IgG. Green: rabbit anti-RON2 antibody detected by Alexa488-anti-rabbit IgG. Blue: mouse anti-RON8 antibody detected by Alexa350-anti-mouse IgG. All scale bars = 5µm. (C) IFA showing RON4 is mistargeted to the rhoptry body in the absence of RON5. RON4 normally co-localizes in the rhoptry neck with RON5 and RON8. However, in parasites depleted
of RON5, co-localization between RON4 and RON8 is lost with RON4 signal extended just posterior to RON8 indicating mistargeting to the rhoptry body. Red: rabbit anti-HA antibody detected by Alexa594-anti-rabbit IgG. Green: rat anti-RON4 antibody detected by Alexa488-anti-rat IgG. Blue: mouse anti-RON8 antibody detected by Alexa350-anti-mouse IgG. All scale bars = 5µm.

**Figure 3-5 – Establishment of a RON5cKO functional complementation system.**

(A) IFA showing rhoptry neck targeting of a second copy of RON5 with a C-terminal HA tag. RON5-HA targets properly to the rhoptry neck, as assessed by co-localization with RON11. Red: rabbit anti-HA antibody detected by Alexa594-anti-rabbit IgG. Green: rat anti-RON11 antibody detected by Alexa488-anti-rat IgG. Scale bar = 5µm. (B) Western blot showing rescue of RON2 stability in Atc treated RON5cKO by stable expression of a second copy of RON5. Expression of an HA tagged second copy of RON5 is unaffected by Atc treatment while expression the endogenous copy of RON5 under the control of the tet-regulatable promoter is lost. While repression of endogenous RON5 results in degradation of RON2, expression of the complementing RON5 second copy fully rescues RON2 stability. ISP3 serves as a loading control. A complete rescue of (C) invasion and (D) restored plaque formation is also observed in RON5cKO parasites complemented with a second copy of RON5.

**Figure 3-6 – RON5N/C processing is dispensable for MJ complex function.**

(A) Western blot showing SDS-PAGE migration of RON5N/C processing mutants. To assess the impact of various mutants on RON5N/C process, a second copy of RON5 with a C-terminal HA tag was expressed in parasites. Migration of RON5C in the indicated mutants was compared
with wild type RON5C (also a second copy with a C-terminal HA tag). The SFVE>AGDR mutation has no effect on migration of RON5C. The SFVQ>AGDR mutation results in a small upshift in RON5C migration consistent with a shift in processing to an upstream site. In contrast, the SFVE>AGDR + SFVQ>AGDR double mutant shows a major upshift to ~180 kD, indicating a block in RON5N/C processing. The lower, faint bands in the double mutant lane are likely breakdown products. Complementation of RON5cKO with the RON5N/C processing mutant completely rescues (B) invasion and (C) restores plaque formation following Atc treatment.

Figure 3-7 – RON5C is required to stabilize RON2.
(A) Homology plot showing the level of conservation of RON5 sequence features across the apicomplexan phylum. The percent identity (blue) and similarity (orange) in an alignment of the RON5 sequences from *T. gondii*, *N. caninum* and *P. falciparum* is shown for a five amino acid sliding window. (B) IFA showing localization of indicated RON5 truncation mutants. A series of C-terminal truncations of RON5, each containing a C-terminal HA tag, were expressed from the UPRT locus. Gross mislocalization is seen for the Δ618-1702, Δ898-1702 and Δ1084-1702 truncations. In contrast, the Δ1258-1702 and Δ1476-1702 truncations were found to target to the rhoptry necks as assessed by co-localization with the non-MJ RON11. A slightly more posterior neck localization relative to RON11 was observed for RON5Δ1475. Red: rabbit anti-HA antibody detected by Alexa594-anti-rabbit IgG. Green: rat anti-RON11 antibody detected by Alexa488-anti-rat IgG. All scale bars = 5µm. (C) Western blot assessing the ability of various RON5 truncation mutants to rescue stability of RON2 in the absence of endogenous RON5. RON2 levels were compared following 72 hours of Atc treatment in RON5cKO parasites.
complemented with a series of RON5 truncation mutants. Endogenous RON5C levels were monitored with an anti-Myc antibody to ensure depletion of endogenous RON5. Complete degradation of RON2 is still observed in cells complemented with the Δ618-1702, Δ898-1702, Δ1084-1702 and Δ1258-1702 RON5 truncation mutants. In contrast, complementation with RON5Δ1476-1702 completely rescues the stability of RON2. ISP3 serves as a loading control. (D) Complementation with RON5Δ1258-1702 fails to rescue (D) invasion or (E) plaque formation in the absence of endogenous RON5 while RONΔ1476-1702 provides a complete rescue of invasion and plaque formation.
Figure 1
Figure 2
Figure 3

A. Host Cell Invasion

- Parasites / field
- Red: invaded
- Blue: attached

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B. Parent

- Atc
- +Atc

- Parasites / field

200

C. Host Cell Attachment

- Parasites / field
- Red: -Atc
- Blue: +Atc

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D. Evacuole Secretion

- Evacuoles / field

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E. Ionophore Induced Egress

- % Egress
  - 0 min
  - 1 min
  - 2 min

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Figure 4
Figure 5

(A) Panels showing phase and fluorescence images of RON5c-HA and RON11.

(B) Western blot analysis showing expression of RON5c-HA, RON5c-3xMyc, RON2, and ISP3.

(C) Bar graph depicting host cell invasion under different conditions.

(D) Optical images of RON5cKO and RON5FL-HA under -Atc and +Atc conditions.
Figure 6

A

B

C

SFVE+SFVQ>AGDR

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Figure 7

A

Alignment Position

% Identity / Similarity

PRO  RON5-N  RON5-C

3147  1262/1291

C

\( \Delta 618-1702 \)  \( \Delta 898-1702 \)  \( \Delta 1084-1702 \)  \( \Delta 1258-1702 \)  \( \Delta 1476-1702 \)

RON5C  RON2  ISP3

D

Host Cell Invasion (+Atc)

Parasites / field

Parent  RON5KO  RON5KO  R5 \( \Delta 1258 \)  R5 \( \Delta 1476 \)

E

1257  1475

-Atc  +Atc
Supplemental Material

Supplemental Figure Legends

Figure 3-S1 – RON2 levels closely mimic RON5 levels during RON5 knockdown.

Western blot showing RON2 and RON5C levels after 24, 48 and 72 hours of Atc treatment. RON2 levels closely mimic diminishing RON5C levels showing that RON2 stability is dependent upon RON5.
Table 3-1. Primers used in this study as discussed in text. Restriction sites and mutated bases or inserted bases are shown in lowercase.

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Chapter 4

A Novel Family of *Toxoplasma* IMC Proteins Displays a Hierarchical Organization and Functions in Coordinating Parasite Division

Published as:

Abstract

Apicomplexans employ a peripheral membrane system called the inner membrane complex (IMC) for critical processes such as host cell invasion and daughter cell formation. We have identified a family of proteins that define novel sub-compartments of the *Toxoplasma gondii* IMC. These IMC Sub-compartment Proteins, ISP1, 2 and 3, are conserved throughout the Apicomplexa, but do not appear to be present outside the phylum. ISP1 localizes to the apical cap portion of the IMC, while ISP2 localizes to a central IMC region and ISP3 localizes to a central plus basal region of the complex. Targeting of all three ISPs is dependent upon N-terminal residues predicted for coordinated myristoylation and palmitoylation. Surprisingly, we show that disruption of ISP1 results in a dramatic relocation of ISP2 and ISP3 to the apical cap. Although the N-terminal region of ISP1 is necessary and sufficient for apical cap targeting, exclusion of other family members requires the remaining C-terminal region of the protein. This gate-keeping function of ISP1 reveals an unprecedented mechanism of interactive and hierarchical targeting of proteins to establish these unique sub-compartments in the *Toxoplasma* IMC. Finally, we show that loss of ISP2 results in severe defects in daughter cell formation during endodyogeny, indicating a role for the ISP proteins in coordinating this unique process of *Toxoplasma* replication.
Author Summary

Apicomplexans are the cause of important diseases in humans and animals including malaria (Plasmodium falciparum), which claims over a million human lives each year, and toxoplasmosis (Toxoplasma gondii), which causes birth defects and neurological disorders. These parasites possess a unique cortical system of membrane sacs arranged on a cytoskeletal meshwork, together referred to as the inner membrane complex (IMC). The IMC is the anchor point for the gliding motility machinery necessary for host invasion and also a scaffold around which new parasites are constructed during replication. Here we have uncovered new insights into the organization and function of this structure by identifying and characterizing ISP1-3, a family of proteins that define novel sub-compartments within the Toxoplasma IMC. Residues predicted for myristoylation and palmitoylation are critical in the membrane targeting of these proteins, suggesting that multiple palmitoyl acyltransferase activities reside within the IMC and dictate its organization. Surprisingly, ISP1 is required for proper sub-compartment sorting of ISP2 and 3, revealing a novel hierarchical targeting mechanism for the organization of this membrane system. Disruption of ISP2 results in defects during endodyogeny and a dramatic loss in parasite fitness, revealing that the ISP proteins play an important role in coordinating parasite replication.
Introduction

The phylum Apicomplexa contains numerous obligate intracellular pathogens that are the cause of serious disease in humans and animals, greatly influencing global health and causing significant economic loss worldwide. The phylum includes *Plasmodium falciparum*, the causative agent of malaria which claims 1-2 million human lives annually, and *Toxoplasma gondii*, a pathogen that infects more than thirty percent of the world’s population and causes severe neurological disorders and death in immunocompromised individuals (Hill et al., 2005). Most of the drugs used to treat apicomplexans target metabolic pathways or the chloroplast-derived apicoplast (Gherardi and Sarciron, 2007; McFadden and Roos, 1999; Sonda and Hehl, 2006), but these parasites also possess elaborate and unique structures that are required for replication and invasion and thus represent attractive new targets for therapeutic intervention.

Apicomplexans are grouped with dinoflagellates and ciliates in the alveolata infrakingdom (Keeling et al., 2005). The unifying morphological characteristic of this group is the presence of alveoli: membrane sacs located beneath the plasma membrane. Molecular phylogenetic data supports this grouping, as does the identification of a conserved family of articulin-like membrane skeleton proteins, the alveolins, which associate with alveoli in all three phyla (Gould et al., 2008; Tremp et al., 2008). While the presence of alveoli is conserved, each of these groups has adapted this peripheral membrane structure for different cellular functions to fit their distinct niches. In dinoflagellates, the alveoli sometimes contain cellulose-based plates that function as protective armor (Lau et al., 2007). In contrast, ciliate alveoli are calcium storage devices thought to play roles in regulation of cilia, exocytosis from cortical organelles known as extrusomes, and control of cytoskeletal elements (Plattner and Klauke, 2001; Stelly et al., 1995; Stelly et al., 1991).
In apicomplexans, the alveoli in conjunction with an underlying filamentous network are termed the inner membrane complex (IMC) (D'Haese et al., 1977; Mann and Beckers, 2001). Flattened alveoli underlie the entirety of the plasma membrane except for a small gap at the apex and base of the cell (Porchet and Torpier, 1977). These cisternae are organized into a patchwork of rectangular plates capped by a single cone-shaped plate at the apex of the cell. Freeze-fracture studies of the IMC plates expose a lattice of intramembranous particles (IMPs), an arrangement that suggests an association with proteins of the underlying filamentous network and subtending cortical microtubules (Cintra and de Souza, 1985; Dubremetz and Torpier, 1978; Morrissette et al., 1997). Together, these features of the IMC are the foundation for a unique form of gliding motility used for host cell invasion and also serve as the scaffold for daughter cell formation during division (Keeley and Soldati, 2004; Striepen et al., 2007).

*Toxoplasma* tachyzoites replicate by endodyogeny, a process of internal cell budding that produces two daughters within an intact mother parasite. Following centriole duplication, daughter cell formation begins with the concurrent assembly of an apical and basal complex (Hu et al., 2006). Although these two structures consist of cytoskeletal components that will eventually cap opposite ends of the mature parasite, they are initiated in close spatial and temporal proximity. IMC construction then proceeds by the extension of the basal complex away from the daughter apical complex, generating a bud into which replicated organelles are packaged. Parasite division is completed by a number of maturation steps terminating with the adoption of the maternal plasma membrane (Gubbels et al., 2008).

The apical, cone-shaped cisterna is unique in form and presumably the earliest membrane component deposited into the nascent IMC (Striepen et al., 2007). A number of cytoskeletal IMC markers localize to a region at the parasite apex thought to correspond to this apical-most IMC
A GFP fusion of the dynein light chain, TgDLC, can be detected in an apical cap region but predominantly localizes to the conoid and is also found in the basal complex, spindle poles and centrioles. TgCentrin2, the most divergent of the three *Toxoplasma* centrin homologues, labels the preconoidal rings and a peripheral ring of ~6 annuli located at the lower boundary of the TgDLC cap. It has been suggested that these annuli lie at the juncture between the apical cap plate and the flanking set of IMC plates (Hu et al., 2006). Additionally, PhIL1, a cytoskeletal IMC protein of unknown function, is detected throughout the IMC but strongly enriched in the apical cap and basal complex (Gilk et al., 2006). Only a few proteins are known to directly associate with the IMC membranes. These include a number of proteins associated with gliding motility (Bullen et al., 2009; Gaskins et al., 2004; Rayavara et al., 2009), as well as the heat shock protein Hsp20 (de Miguel et al., 2008) and one isoform of the purine salvage enzyme hypoxanthine-xanthine-guanine phosphoribosyltransferase (Chaudhary et al., 2005). Thus, despite the central role of this conserved membrane system in apicomplexan biology, little is known of its composition, organization, and construction.

We present here a family of proteins unique to the Apicomplexa that localize to three distinct sub-compartments of the *Toxoplasma* IMC. ISP1 localizes to a region corresponding to the apical cap, ISP2 occupies a central IMC region, and ISP3 resides in both the central IMC region and a basal IMC compartment. ISP1 and 3 are early markers for bud formation and label previously unobserved daughter IMC structures in the absence of parasite cortical microtubules, indicating that microtubules are not required for initial assembly of IMC membranes. We show that the ISPs are initially targeted to the IMC by conserved residues predicted for coordinated myristoylation and palmitoylation in the extreme N-terminus of each of these proteins. Interestingly, deletion of *ISP1* results in the relocalization of ISP2 and 3 to the apical cap,
demonstrating an interactive, hierarchical targeting among this family of proteins to these distinct sub-compartments of the IMC. Finally, disruption of \textit{ISP2} results in a severe loss of parasite fitness and dramatic defects in daughter cell formation. Although the ISP2 knockout parasites ultimately compensate for these defects, this data shows an important role for these proteins in the coordination of daughter cell assembly.
Results

Monoclonal antibody 7E8 labels the apical cap of Toxoplasma

We previously generated a panel of monoclonal antibodies against a mixed fraction of *T. gondii* organelles (DeRocher et al., 2008). One of the antibodies, 7E8, stains a cone-shaped structure at the periphery of the apical end of the parasite (Figure 4-1A). This staining pattern extends from a gap at the extreme apex (Figure 4-1A, arrow) ~1.5µm along the length of the parasite, a localization suggestive of the apical IMC plate observed by electron microscopy (Porchet and Torpier, 1977). Colocalization with TgCentrin2 shows that 7E8 staining is delimited at its apex and base by this apical cap marker, indicating that 7E8 does indeed detect a protein associated with the anterior-most IMC plate (Figure 4-1D).

During early endodyogeny, 7E8 staining is visible in daughter parasites as a pair of small rings within each mother parasite (Figure 4-1B, arrows). As daughter formation proceeds, this structure enlarges and extends to form the apical cap seen in mature tachyzoites (Figure 4-1C). The association with forming daughter scaffolds together with the extreme apical gap further suggests that 7E8 labels the apical sub-compartment of the IMC. We also frequently observe 7E8 staining a single dot near the basal border of the cone (Figure 4-1C, arrow) which is distinct from TgCentrin2 annuli (Figure 4-1D, inset).

Identification of ISP1, the Toxoplasma protein recognized by mAb 7E8

Western blot analysis of *Toxoplasma* lysates with mAb 7E8 revealed a single band at ~18 kDa (Figure 4-1E). We used the 7E8 antibody to isolate its target protein by immunoaffinity chromatography. The isolated protein was separated by SDS-PAGE (Figure 4-1F), digested with trypsin, and seven peptides were identified by mass spectrometry corresponding to the
hypothetical *T. gondii* protein TGGT1_009340 (Figure 4-1G). EST and cDNA sequencing confirmed that the gene model is correct. Due to its unique localization, we named this protein IMC Sub-compartment Protein 1 (ISP1).

Examination of the 176 amino acid sequence of ISP1 reveals that it contains a high number of charged residues (~30%). While there are a relatively large number of ESTs encoding ISP1, the protein lacks conserved domains that could suggest its function. The protein contains a glycine at position two, which is predicted to be myristoylated (Maurer-Stroh et al., 2002) as well as a pair of cysteines at positions seven and eight strongly predicted to be palmitoylated (Ren et al., 2008). Since ISP1 lacks a predicted signal peptide or transmembrane domain, these residues suggested a mechanism for IMC membrane association. BLAST analysis of the ISP1 sequence revealed orthologues across the apicomplexan phylum, including *Neospora*, *Theileria*, *Cryptosporidia*, *Babesia*, and *Plasmodium* (Figure 4-S1). Orthologues were also found in *Eimeria* by BLAST against EST libraries (data not shown). ISP1 also showed significant homology in its C-terminal region to CP15/60, a poorly characterized putative surface glycoprotein in *Cryptosporidia* (Jenkins et al., 1993; Tilley et al., 1991). No ISP1 orthologues were identified outside of the phylum indicating that this protein is restricted to the Apicomplexa.

**Identification and localization of ISP2 and ISP3**

BLAST analysis of the *T. gondii* genome using the ISP1 sequence identified two additional hypothetical proteins with considerable sequence similarity to ISP1, which we named ISP2 (TGGT1_058450) and ISP3 (TGGT1_094350) (Figure 4-2A). The greatest degree of sequence similarity between these three proteins exists within the C-terminal two-thirds of their
sequences. The N-terminal regions of the proteins are more divergent, but each contain a conserved glycine at position two as well as a pair of conserved cysteines predicted to be myristoylated and palmitoylated, respectively (Figure 4-2A, boxed residues). ISP2 additionally contains a third cysteine at position five predicted to be palmitoylated. Similar to ISP1, these proteins are highly charged and have a relatively large number of corresponding ESTs.

OrthoMCL analysis of the ISPs indicates two ortholog groups within Apicomplexa. ISP1 and ISP2 segregate with one group while ISP3 segregates with another (Figure 4-S1). The Toxoplasma genome may encode a fourth ISP family member (TGGT1_063420), although it does not segregate with any OrthoMCL group. This predicted protein lacks the conserved glycine and cysteine residues present in the N-termini of other ISP proteins. Only a single EST is present for TGGT1_063420, indicating that it is poorly expressed relative to the other ISPs, and thus it was not investigated further.

To localize ISP2 and ISP3 in T. gondii, we expressed each gene under the control of its endogenous promoter with a C-terminal HA epitope tag. Intriguingly, ISP2 localizes to a previously unrecognized central sub-compartment of the IMC, which begins at the base of the ISP1 apical cap and extends approximately two-thirds the length of the cell. The apical boundary of this compartment is delineated by the TgCentrin2 annuli (Figure 4-2B). The posterior boundary has a jagged edge suggesting it corresponds to discrete IMC plates (Figure 4-2D, arrows). While the ISP2 signal terminates near the end of the subpellicular microtubules, the termini for these two structures are not identical (Figure 4-S3, WT). Antisera raised against recombinant ISP2 confirmed this central IMC sub-compartment localization, ensuring that exclusion of ISP2 from the apical cap and basal IMC is not an artifact of epitope tagging (Figure 4-S2A).
Similar to ISP2, ISP3 stains the central section of the IMC. However, ISP3 staining extends to the posterior end of the complex, identifying a third sub-compartment of the IMC (Figure 4-2C). A small gap in ISP3 staining is observed in the posterior region similar to that seen for other IMC proteins (Gaskins et al., 2004). Antisera raised against recombinant ISP3 gave a poor signal by IFA, but was sufficient to confirm localization to both the IMC central and basal sub-compartments (Figure 4-S2B). As with ISP1, ISP2 and ISP3 are visible in forming daughter parasites. Whereas the maternal signals of ISP1 and ISP2 appear to remain stable throughout endodyogeny, the maternal ISP3 signal rapidly attenuates with the onset of endodyogeny while it concentrates in daughters (Figure 4-2E). Attenuation of ISP3 in mothers and enrichment in daughters was also observed with our polyclonal antibody, indicating this is not the result of a C-terminal processing event that removes the HA epitope tag (Figure 4-S2C). Thus, ISP3 provides an excellent marker for bud initiation, growth, and maturation during endodyogeny (Figure 4-2E).

The ISPs are associated with the IMC at the cell periphery but are not embedded in the IMC protein meshwork

The observations that the ISPs are visible at the periphery of forming daughters prior to adoption of the maternal plasma membrane and that gaps are present at the extreme apex and base suggests an association with the IMC. To confirm IMC association, we treated extracellular parasites with Clostridium septicum alpha-toxin. This vacuolating toxin causes a dramatic separation of the plasma membrane and the underlying IMC, enabling differential localization of these closely apposed membrane systems (Wichroski et al., 2002). In toxin-treated parasites, the
ISP proteins segregate with the IMC and not with the plasma membrane, confirming that the ISPs are indeed IMC proteins (Figure 4-3A-B).

To ascertain if the ISPs are embedded in the IMC protein meshwork that includes the articulin-like protein IMC1, we performed detergent extractions of extracellular parasites in 0.5% NP-40. In these conditions, each ISP was solubilized similar to the control protein ROP1, while IMC1 remained in the insoluble pellet fraction (Figure 4-3C). This extraction profile demonstrates that the ISPs are not embedded in the detergent resistant protein meshwork that underlies the IMC membranes.

**ISP1 and ISP3 localize to nascent daughter buds in the absence of microtubules**

We disrupted microtubules in intracellular parasites to assess whether the underlying microtubules influence ISP localization. Apicomplexan microtubules are selectively susceptible to disruption by dinitroanilines, such as oryzalin (Stokkermans et al., 1996). After 40 hours of 2.5 µM oryzalin treatment, all tubulin is unpolymerized and dispersed. Without spindle microtubules (mitosis) and subpellicular microtubules (budding), productive daughter formation repeatedly fails resulting in an undivided, amorphous mother cell with a polyploid DNA content (Morrissette and Sibley, 2002) (Figure 4-4A). Intriguingly, we observe ISP1 labeling numerous small rings that are centrally located within oryzalin-treated parasites (Figure 4-4A, inset) of approximately the same dimensions as ISP1 early daughter buds in untreated, replicating parasites (compare with Figure 4-1B, arrows). Since polymerization of subpellicular microtubules is essential to drive bud extension, these rings likely represent failed attempts to build new daughter buds (Shaw et al., 2000). A larger peripheral patch of ISP1 with a central hole is also observed, likely representing the original parent apical cap (Figure 4-4A, arrows).
While ISP2 was not observable in these early bud rings (Figure 4-4B), we did detect ISP3 in these structures within oryzalin-treated parasites (Figure 4-4C, inset arrows), suggesting that both the apical cap and remaining IMC sub-domains are formed independently of microtubules at a very early stage of bud development. While membrane skeleton proteins are likely candidates for providing the foundation for these structures, we were unable to detect the articulin-like protein IMC1 in these early bud rings, even at lower oryzalin concentrations (0.5µM) that only disrupt cortical microtubules (Figure 4-4D).

**An N-terminal region is sufficient for sub-compartment targeting of ISP1 and ISP3 but not ISP2**

The greatest sequence similarity within the ISP family is present in the C-terminal two-thirds of the proteins while the N-terminal region is more divergent (Figure 4-2A), thus we reasoned that the unique targeting of each ISP family member might be controlled by its N-terminal region. To test if the N-terminal region of ISP1 is necessary for targeting, we eliminated the first 63 residues to create a truncated protein fused to YFP. ISP1_{64-176}-YFP does not target to the IMC but is instead distributed throughout the cytoplasm and nucleus, showing that this N-terminal region is necessary for apical cap targeting (Figure 4-5A). To determine if the ISP1 N-terminal region is sufficient for targeting, we fused the first 65 residues of ISP1 (containing the putative acylation sequence and divergent N-terminal region) to YFP and expressed this construct in Toxoplasma. The ISP1_{1-65}-YFP fusion traffics to the apical cap in an identical fashion to endogenous ISP1 (Figure 4-5B). To further narrow the N-terminal region required for apical cap targeting, we generated an additional fusion of the first 29 residues of ISP1 (containing the putative acylation sequence) to YFP. This fusion also traffics in a manner...
identical to full length ISP1 (Figure 4-5C), demonstrating that this N-terminal domain is both necessary and sufficient for apical cap targeting.

To assess targeting of ISP2 and ISP3, we also created fusions of their N-terminal regions (residues 1-41 and 1-36 respectively) to YFP. The ISP31-36-YFP fusion targets to the central and basal sub-compartments of the IMC but is restricted from the apical cap (Figure 4-5D), showing that this region is sufficient for proper sub-compartment targeting. In contrast, ISP21-41-YFP localized to the entire IMC, overlapping with endogenous ISP1 in the apical cap and extending into the basal IMC sub-compartment (data not shown). To ensure this change in targeting for ISP21-41 was not an artifact of the YFP fusion, we replaced YFP with an HA tag (shown to have no effect on the targeting of full length ISP2, Figure 4-5E). The ISP21-41-HA protein also localized throughout the IMC (Figure 4-5F), demonstrating that the N-terminal domain of ISP2 is sufficient for targeting to the IMC, but not for correct sub-compartment localization.

**Conserved N-terminal residues predicted for acylation are critical for ISP targeting**

Protein myristoylation occurs co-translationally through the action of an N-myristoyl transferase (Resh, 2006). This modification is sufficient to promote transient association with membranes for otherwise cytosolic proteins. This weak membrane affinity can then be stabilized by addition of one or more palmitoylations through the action of a palmitoyl acyltransferase (PAT), effectively locking a protein into a target membrane system in a mechanism known as “kinetic trapping”. The ISPs each contain a second position glycine followed by cysteines within the first 10 residues that are predicted to be myristoylated and palmitoylated, respectively (Figure 4-2, boxed residues). We mutated the glycine and cysteine residues in HA epitope tagged ISP constructs to examine their effect on targeting. As predicted by the kinetic trapping model,
mutation of the second position glycine to an alanine abolished IMC targeting in each family member (Figure 4-6 and Figures S3-4, G2A), resulting in proteins distributed throughout the cytoplasm. Mutation of the cysteine residues to serine was performed individually and together. While only minor defects in targeting were observed with individual cysteine mutations, mutation of both cysteines abolished ISP1 and ISP3 targeting (Figure 4-6 and Figure 4-S4). In the case of ISP2, targeting was only abolished when all three cysteines were coordinately mutated (Figure 4-S3). While coordinated cysteine mutants of the ISPs are distributed in the cytoplasm similar to G2A mutants, we also often observed perinuclear staining that is especially concentrated just apical of the nucleus (arrows, Figure 4-6 and Figure 4-S3-4). Presumably, myristoylation of these proteins still occurs, but without palmitoylation, these mutants are left to transiently sample the different membrane systems within the cell and therefore may appear concentrated as they associate with the ER and Golgi membranes present in this region. These results demonstrate that these residues are essential to ISP sorting and indicate that coordinated acylation of the ISPs is responsible for IMC membrane targeting.

Disruption of ISP1 results in relocalization of ISP2 and ISP3 to the apical cap

To assess the function of ISP1, we disrupted the ISP1 gene by homologous recombination (Figure 4-7A). We identified clones which lacked ISP1 expression by IFA and Western blot (Figure 4-7B-C), indicating successful disruption of the ISP1 locus and demonstrating that ISP1 is not necessary for in vitro propagation of T. gondii. Disruption of ISP1 did not result in any gross defect in parasite growth. However, we were surprised to find that both ISP2 and ISP3 were relocalized in the Δisp1 strain. In the parental strain, ISP2 staining terminates sharply at the ring of TgCentrin2 annuli bordering the base of the apical cap (Figure
4-7D, arrowheads). However, in Δisp1 parasites, ISP2 staining extends past this border, relocating to the apical cap sub-compartment of the IMC (Figure 4-7D). Apical cap relocation is also observed for ISP3 in the Δisp1 strain (Figure 4-7E). To ensure the ISP2 and ISP3 relocation to the apical cap is truly a result of the absence of ISP1, we reintroduced the ISP1 gene with a C-terminal YFP fusion into the Δisp1 strain. This fusion protein targets correctly to the apical cap and, importantly, reestablishes the wild-type localization of ISP2 (Figure 4-8A, insets) and ISP3 (data not shown), excluding them from the apical cap. Thus, ISP1 exhibits a gate-keeping effect on ISP2 and 3, preventing access to the apical cap and establishing a hierarchy of protein targeting among these IMC sub-compartments. To determine if ISP1 performs a broader scaffolding function within the apical cap, we evaluated the localization of TgDLC1 using a GFP fusion; however, we observed no change in the localization of this protein in the absence of ISP1 (data not shown).

**An ISP1 C-terminal domain is necessary for exclusion of ISP2 and ISP3 from the apical cap**

Given the ability of ISP1 to exclude other family members from the apical cap, we exploited our ISP11-65-YFP construct to determine whether or not the N-terminal region that is sufficient for apical cap targeting also plays a role in exclusion from this compartment. Expression of this construct in Δisp1 parasites does not result in exclusion of ISP2 (Figure 4-8B) or ISP3 (data not shown) from the apical cap, demonstrating that distal sequences present in the more conserved regions of ISP1 (residues 66-176) are necessary for exclusion. To further assess whether the C-terminal region from another ISP family member could substitute for the ISP1 C-terminal domain and function in exclusion, we constructed a hybrid protein containing the N-
terminal 65 amino acids of ISP1 and the C-terminal region of ISP2 (residues 43-160) fused to YFP. Similar to the ISP1$_{1-65}$-YFP construct, the ISP1$_{N/2c}$-YFP chimera targets to the apical cap but does not exclude ISP2 (Figure 4-8C) or ISP3 (data not shown). These results demonstrate that the exclusion activity of the C-terminal region of ISP1 is specific to this family member and cannot be replaced by the complementary region from ISP2.

We created an additional chimera consisting of the N-terminal region of ISP2 (residues 1-41) fused to the C-terminal region of ISP1 (residues 67-176). While the N-terminal region of ISP2 alone targets YFP or HA throughout the IMC (Figure 4-5F), inclusion of the C-terminal region of ISP1 restricts the localization to the apical cap and central regions of the IMC (Figure 4-8D, see discussion). In parasites expressing this chimera, ISP2 and 3 are mostly relocalized into the base portion of the IMC (Figure 4-8E-F, brackets). The fact that the ISP1 C-terminal region is able to exhibit exclusion activity against the other ISPs when artificially targeted to other domains of the IMC strengthens the conclusion that the ISP1 C-terminal region constitutes an ISP exclusion domain.

**Disruption of ISP2 results in a severe loss of parasite fitness and division defects in daughter cell formation**

To further investigate the function of the ISP proteins, we disrupted the genes encoding ISP2 and ISP3 by homologous recombination. To accomplish this, we employed a recently developed Δku80 parasite strain that is highly efficient at homologous recombination (Huynh and Carruthers, 2009). We first removed HPT from the Ku80 locus by homologous recombination and negative selection using 6-thioxanthine, creating Δku80Δhpt strain parasites. We then used
this strain to disrupt *ISP2* or *ISP3* and confirmed these deletions by IFA (not shown) and Western blot (Figure 4-9A and Figure 4-S5).

In contrast to our findings for Δ*isp1* parasites, localization of other ISP family members was unchanged in both Δ*isp2* and Δ*isp3* strains (data not shown). While no gross phenotype was seen in Δ*isp3* parasites, the Δ*isp2* strain parasites were obviously defective in growth as the knockout was rapidly lost from transfected populations and its isolation required cloning early following transfection. To assess this loss in fitness, we performed competition growth assays between parent and Δ*isp2* parasites by mixing these strains in culture and monitoring the culture composition at each passage. The parental strain rapidly out competed the Δ*isp2* parasites, confirming a severe fitness loss in these parasites (Figure 4-9B). Further analysis by IFA revealed that Δ*isp2* parasites display a number of defects in parasite division. Most frequently, we observed the construction of >2 daughters per mother cell in each round of endodyogeny with some parasites assembling as many as 8 daughters (Figure 4-9C). To quantify this defect, we stained for ISP1, an early marker for bud formation during endodyogeny, and counted vacuoles containing parasites undergoing endodyogeny and assembling >2 buds. As expected, we saw a dramatic increase in the number of parasites producing more than two daughters in the Δ*isp2* strain (Figure 4-9D). Neither Δ*isp1* or Δ*isp3* parasites showed any aberration in daughter cell assembly compared to wild-type parasites (data not shown).

Assembly of >2 daughters in Δ*isp2* parasites sometimes occurred around a single polyploid nucleus with karyokinesis accompanying budding (bottom left parasite, Figure 4-9C) while other parasites assembled the spindle apparatus and underwent karyokinesis without budding, resulting in a mother parasite with two nuclei (Figure 4-9E). We also observe parasites
containing two discrete nuclei in the process of budding > 2 daughters (outlined parasites, Figure 4-9F).

Less frequently, we observed a catastrophic failure of \( \Delta isp2 \) parasites to appropriately segregate nuclei, resulting in anucleate zoids and nuclei extruded in the vacuole (Figure 4-9G). These vacuoles also show major defects in apicoplast segregation with a few cells receiving both a nucleus and an apicoplast while some received only an apicoplast and others received neither. Finally, some vacuoles with nuclear segregation defects contained many immature buds within the vacuole (Figure 4-9H). These buds appear to be outside of any intact parasite and it is unclear if they were initiated within a mother cell and then somehow liberated into the vacuolar space or if they were the result of a budding event that was initiated within the vacuolar space itself. In these vacuoles, several elongated apicoplasts are strung throughout the vacuolar space, associated with the extracellular buds and nuclei.

Surprisingly, the \( \Delta isp2 \) parasites recovered from both the fitness and replication defects after approximately two months of culture (data not shown), preventing complementation by genetic rescue. To ensure these phenotypes are specific to the disruption of \( ISP2 \) and not the consequence of any off target effects, we generated a second independent \( \Delta isp2 \) line. This line displayed the same loss of fitness and cell division defects, indicating these phenotypes are specifically linked to disruption of the \( ISP2 \) locus (data not shown).
Discussion

The IMC in apicomplexan parasites

Alveoli are the unifying morphological feature among ciliates, dinoflagellates and apicomplexans where these unique membrane stacks have been adapted to suit these divergent organisms in vastly different niches. In apicomplexans, the membrane stacks (the IMC) have been exploited to provide unique and critical roles in parasite replication, motility and invasion. Freeze-fracture studies reveal a highly sophisticated arrangement of IMC plates with dissimilar organization of IMPs in the apical versus lower plates indicating compositional differences between these regions (Porchet and Torpier, 1977). Identification of the ISPs clearly demonstrates that the protein constitution of the membrane cisternae is not uniform. The ISP compartments have sharp boundaries (Figure 4-2B-D), suggesting that they correspond to discrete cisterna or groups thereof (Figure 4-10A).

ISPI localizes to the apical cap compartment that is delimited by TgCentrin2 and thus represents the first membrane associated protein of this apical-most IMC plate. Previously, the cytoskeleton-associated proteins PhIL1 and TgDLC1 were shown to localize in part to the apical cap region (Gilk et al., 2006; Hu et al., 2006). The C-terminal half of PhIL1 is sufficient for apical cap localization and also for retaining cytoskeletal association. This portion of the protein lacks predicted transmembrane domains or acylation signals, indicating that it links directly to a sub-domain of the cytoskeleton independent of the membrane stacks. Electron micrographs of detergent-extracted parasites show substantial differences in the cytoskeletal filaments in this region (e.g. thicker filaments and a parallel instead of interwoven arrangement), indicating that distinct sub-domains exist in both the IMC membranes and underlying network (Mann and Beckers, 2001).
Localization of ISP2 and 3 revealed two additional sub-compartments of the IMC that have not been previously observed: a central compartment labeled by ISP2 and a basal compartment labeled by ISP3. The abutment of ISP2 and ISP3 staining against the posterior end of the apical cap likely corresponds to the junction between the apical cap and the rectangular plates constituting the remainder of the IMC. The presence of TgCentrin2 annuli at this border is striking as centrins are calcium-binding contractile proteins known to play a role in the duplication of microtubule organizing centers (Salisbury, 1995). While the ISP3 sub-compartment clearly terminates at the posterior end of the IMC, it is unclear what accounts for the basal boundary of the ISP2 sub-compartment which lies approximately two-thirds down the length of the parasite. One possibility is an association with the cortical microtubules that also terminate in this region (Nichols and Chiappino, 1987). However, the microtubules and ISP2 signal do not consistently terminate at the same point. Alternatively, the signal termination may correspond to another junction of IMC plates and the exclusion of ISP2 from the basal region of the IMC may reflect another point of hierarchical targeting, as we discovered for ISP1 in the apical cap.

While ISP1 and 2 are both retained in mother parasites during endodyogeny, ISP3 maternal staining dissipates as daughter parasites form. The strong ISP3 signal in early buds along with the rapid attenuation of ISP3 signal in the mother during endodyogeny provides an unhampered view of the membranes of the daughter buds (Figure 4-2E). Expression of IMC proteins is tightly regulated during the cell cycle including the ISPs, which show an expression profile similar to that of IMC1 (Michael White, personal communication). Thus, the bright ISP3 staining in daughters and concomitant loss of signal in mother cells could be due to synthesis in daughters and degradation in mothers. Alternatively, since palmitoylation is a reversible lipid
modification, recycling by de-palmitoylation at the parent IMC and re-palmitoylation at daughter IMCs could account for the ISP3 dynamics observed.

**ISP proteins and daughter bud initiation**

ISP1 and 3 are localized to numerous ring structures in oryzalin-treated parasites, indicating that initiation of bud IMC assembly repetitively occurs under these conditions and is not dependent on microtubules. Microtubule polymerization is essential for cell division and cortical microtubule extension is thought to drive bud growth, explaining why buds in parasites lacking microtubules never elongate (Shaw et al., 2000). ISP1 and 3 are localized to distinct compartments in forming daughter cells, demonstrating that IMC sub-compartmentalization is established early during endodyogeny. The ISP1 and 3 signals are not always perfectly overlapping in oryzalin-treated cells, suggesting that IMC membrane specialization may be established even in these early bud rings, although the rings are too small to clearly visualize distinct sub-domains. The absence of ISP2 from these rings may indicate later recruitment to daughter buds or simply be a consequence of drastic perturbation of the cell under these conditions.

Some nucleating scaffold element must provide a foundation for these early IMC membrane bud rings. The earliest signs of daughter bud formation observed by electron microscopy are a dome-shaped vesicle and associated microtubules (Sheffield and Melton, 1968). The basal complex protein TgMORN1 is the earliest protein marker of bud generation, forming a pair of rings around the centrioles after their duplication at approximately the same time daughter conoids are assembled (Hu, 2008). In oryzalin-treated parasites observed during the first few hours following drug addition, initial TgMORN1 ring formation still occurs and can
be followed until cells attempt to bud, at which point the inability to polymerize new microtubules results in drastic loss of parasite morphology. After 24 hours of oryzalin treatment, TgMORN1 localizes in patches sparsely associated with peripheral sheets of IMC membrane skeleton marker IMC1 but does not label anything resembling the bud rings observed for ISP1 and 3 (Gubbels et al., 2006; Hu et al., 2006). In our study, IMC1 did not localize to ISP1-labeled bud rings in oryzalin-treated parasites, demonstrating that it is not required for bud initiation. Furthermore, TgMORN1 has been disrupted and shown to be non-essential for parasite growth (Heaslip et al.). Future studies with ISP1 and 3 will enable the discovery of the critical nucleating factors that mediate bud initiation.

**Acylation of conserved N-terminal residues confers ISP targeting**

Protein acylation is a widely employed eukaryotic mechanism to mediate membrane association of proteins that lack a transmembrane domain. Our mutation of conserved N-terminal residues that are predicted to be myristoylated and palmitoylated indicates that these modifications are responsible for IMC membrane targeting. These mutagenesis studies also agree with our deletion analysis demonstrating that the N-terminal regions of ISP1 and 3 are sufficient for correct targeting. Together, these data suggest a kinetic trapping model for ISP localization in which ISP proteins are first co-translationally myristoylated in the cytosol enabling sampling of membranes, then recognized and palmitoylated by a unique PAT (or PAT activity) that is present in each sub-compartment, thus locking the protein into the appropriate membrane sub-compartment (Figure 4-10B).

For ISP1 and 3, this multiple PAT model agrees with our deletion analysis showing that N-terminal regions of the proteins are sufficient for sub-compartment localization. Recognition
of each ISP protein as a substrate would be determined by the context of the sequences immediately surrounding the residues required for myristoylation and palmitoylation. Indeed, additional deletion analysis showed that the first ten residues of ISP1 mostly retain apical cap targeting (data not shown). In contrast, while the N-terminal region of ISP2 is sufficient for general IMC membrane association, deletion of the C-terminal region or its substitution in the ISP2n/1c chimera alters sub-compartment specificity. These structural changes to ISP2 may remove important information for establishing stringent PAT specificity, permitting incorporation into other IMC sub-compartments. A similar effect was recently discovered for the palmitoylated protein Vac8 in \textit{Saccharomyces cerevisiae}. While palmitoylation of wild-type Vac8 was only catalyzed by one of the five \textit{S. cerevisiae} PATs tested, truncation of the Vac8 C-terminus resulted in its palmitoylation by all five PATs (Nadolski and Linder, 2009).

Alternatively, it is possible that palmitoylation of the ISP family is facilitated by a single PAT that is localized throughout all three IMC compartments and regulated by additional cofactors. Modulation of PAT activity against certain substrates by additional protein cofactors has been shown in both yeast and mammalian systems (Swarthout et al., 2005; Zhao et al., 2002).

The presence of a Asp-His-His-Cys-cysteine-rich domain (DHHC-CRD) is the hallmark of PAT activity and has allowed for the identification of several PATs in other systems, including 7 in \textit{S. cerevisiae} and 23 in mammalian genomes (Smotrys and Linder, 2004). Within the \textit{Toxoplasma} genome, 18 DHHC-CRD containing proteins are predicted to be encoded, a relatively higher number among protists (\textit{e.g.} the \textit{Giardia lamblia} and \textit{Trypanosoma brucei} genomes are predicted to contain 9 and 12 PATs, respectively (Emmer et al., 2009; Saric et al., 2009)), indicating a more extensive PAT network may be present to accommodate protein sorting within the numerous unique membrane systems in apicomplexans. Future work localizing
Hierarchical targeting of the ISP family within the IMC

Relocalization of the other ISP family members into the apical cap may explain the lack of any gross phenotype in \(\Delta isp1\) parasites. Whereas targeting to the apical cap is mediated by the N-terminal region of ISP1, relocalization of other family members into this sub-compartment is dependent on the C-terminal portion of this protein. Both the ISP1\(N/2_c\) and ISP2\(N/1_c\) chimeras support the conclusion that this gate-keeping is specific to ISP1 and directed against ISP2/3. Interestingly, while distal sequences of ISP2 are also required for its exclusion (as shown by ISP2\(1_{41}\)-HA), this is not the case for a comparable truncation of ISP3.

Perhaps the simplest explanation for the mechanism of ISP2/3 exclusion from the apical cap is provided by our multiple PAT model (Figure 4-10B). This model would suggest that in wild-type parasites, the presence of ISP1, either directly or indirectly via other proteins, modulates PAT activity in the apical cap, thus preventing recognition of ISP2 and 3. In the absence of the ISP1 C-terminal domain, ISP2 and 3 are able to be recognized as substrates of the apical cap PAT and also localize to this compartment. This model would also suggest that the exclusion insensitivity of truncated ISP2 (Figure 4-5F), as compared to truncated ISP3 (Figure 4-5D), may simply result from a change in the ability of PATs to specifically recognize and act upon this altered molecule (discussed in the previous section). Alternatively, deletion of ISP1 may result in relocalization of a central sub-compartment PAT into the apical cap, thus enabling ISP2 and 3 to localize to this membrane region.

Finally, it is also possible that ISP1 exclusion is the result of a receptor in the apical cap,
which the C-terminal domain of ISP1 binds with a higher affinity than ISP2 or 3. The absence of
the ISP1 C-terminal domain would then allow binding of the similar regions of ISP2 and 3 to the
receptor in the apical cap. However, the variable exclusion observed in C-terminal truncations of
ISP2 and 3 argues against this scenario. We have attempted to identify ISP1 binding partners by
immunoprecipitation under gentle conditions but have had no success, indicating that if partners
do exist, they are not strongly interacting. Regardless of the precise mechanism, the targeting of
the ISP family demonstrates that organization of the *Toxoplasma* IMC is an interactive, complex
process. To our knowledge, this hierarchical targeting is a completely unprecedented mechanism
for sorting of palmitoylated proteins in any membrane system. It will be interesting to see if
similar mechanisms of membrane organization are present in other members of the eukarya.

**ISP2 is important in daughter cell formation**

Disruption of *ISP2* results in defects in daughter cell formation, indicating that ISP2 is
important for proper coordination of daughter parasite assembly. Our observation that ~5% of
wild-type parental strain vacuoles assemble >2 daughters is in agreement with previous studies
(Hu et al., 2002). *Toxoplasma* populations have been reported to undergo flux in the percentage
of parasites displaying this trait due to certain stresses (Hu et al., 2004), however the dramatic
(~60%) effects on daughter parasite assembly in the ∆isp2 strain vastly exceed these previous
reports. Furthermore, the severe fitness loss in these parasites indicates this failure to properly
coordinate cell division has serious consequences for parasite biology. This could be due to
abortive replication events, as we do observe ultrastructural and organelle partitioning defects
that are likely terminal (e.g. parasites lacking a nucleus or apicoplast and immature daughter
buds within the vacuole, Figure 4-9G-H). However, many of the ∆isp2 progeny produced in
parasites assembling >2 siblings appear viable as they seem to properly assemble the IMC and
cortical cytoskeleton and also receive nuclear DNA, an apicoplast and a mitochondrion (data not
shown). In these cases, poor control over the number of daughter cells being assembled may also
render a fitness cost on parasites during the normally efficient proliferative tachyzoite life stage.

The increase in the number of daughter parasites per mother cell results in several
outcomes. In some parasites, DNA replication and karyokinesis occur prior to bud formation
(Figure 4-9E-F), while in others, multiple rounds of DNA replication appear to occur without
karyokinesis, resulting in large nuclei that are segregated in a single step among multiple
daughters (Figure 4-9D). In either case, mother parasites that produce greater than 2 daughters
are no longer performing endodyogeny, but instead replicating by one form or another of
endopolygeny (Ferguson et al., 2008; Hu et al., 2002; Striepen et al., 2007). The presence of
replication abnormalities in Δisp2 parasites reminiscent of division in other Toxoplasma life
stages and other apicomplexan species suggests this protein plays a role in coordinating progress
along the proper cell division pathway in tachyzoites and that this coordination is needed to
maintain parasite fitness.

It is unclear how Δisp2 parasites ultimately recover from these defects and return to
normal growth and replication. In both of the independent ISP2 knockouts performed months
apart, the defects in growth and daughter formation were stable for at least two months.
Recovery may be due to compensation via the other ISP proteins or may instead involve other
players. It will be interesting to determine whether double knockouts of the ISP proteins, or even
a triple knockout, will yield a more severe and stable phenotype. These functional implications
for ISP2 underscore the idea that apicomplexan-specific processes are likely tied to the many
hypothetical genes encoded within these parasites, some of which will provide novel therapeutic
targets. The conservation of this family throughout the phylum suggests that the unique ISP targeting mechanism is conserved and raises the possibility that these proteins are more broadly involved in coordinating the various pathways of cell division that are critically important to the pathogenesis of apicomplexan parasites.
Materials and Methods

Toxoplasma and host cell culture

*T. gondii* RHΔhpt (parental) strain and modified strains were maintained in confluent monolayers of human foreskin fibroblast (HFF) host cells as previously described (Donald et al., 1996).

Generation of monoclonal antibody 7E8

Monoclonal antibodies (mAb) were generated against a mixed fraction of organelles from *T. gondii* (DeRocher et al., 2008). For immunization, ~100µg of purified organelles (Bradley et al., 2005) were injected in RIBI adjuvant into a BALB/c mouse. Following four injections, the spleen was isolated, hybridoma lines were prepared, and supernatants from individual clones screened for antibody reactivity.

Antibodies

The following primary antibodies were used in IFA or Western blot: rabbit polyclonal anti-tubulin (Morrissette and Sibley, 2002), rabbit polyclonal anti-SAG1 (Burg et al., 1988), anti-IMC1 mAb 45.15 (Wichroski et al., 2002), anti-ROP1 mAb TG49 (Schwartzman and Krug, 1989), and anti-ATrx1 mAb 11G8 (DeRocher et al., 2008). Hemagglutinin (HA) epitope was detected with mAb HA.11 (Covance) or rabbit polyclonal anti-HA (Invitrogen).

Light microscopy and image processing

Fixation and immunofluorescence staining of *T. gondii* were carried out as previously described (Bradley et al., 2005). All cells imaged in this study were formaldehyde-fixed except
parasites in Figure 4-S2B, which were fixed with methanol. Image stacks were collected at z-increments of 0.2 µm with an AxioCam MRm CCD camera and AxioVision software on an Axio Imager.Z1 microscope (Zeiss) using a 100x oil immersion objective. Deconvolved images were generated using manufacturer specified point-spread functions and displayed as maximum intensity projections.

Identification of ISP1 by immunoaffinity purification with mAb 7E8

The protein recognized by monoclonal antibody 7E8 was isolated from 5x10^9 T. gondii RH tachyzoites lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.5% NP-40, 0.5% sodium deoxycholate). Insoluble material was removed from the lysate by centrifugation at 10,000 x g for 30 min after which the remaining soluble lysate fraction was incubated with mAb 7E8 cross-linked to protein G-Sepharose beads (Amersham) using dimethylpimelimidate as previously described (Harlow and Lane, 1988). After washing in RIPA buffer, the bound protein was eluted using high pH (100 mM triethylamine, pH 11.5) and the eluate was separated by SDS-polyacrylamide gel electrophoresis (PAGE). Coomassie staining identified a single 18-kDa band, which was excised and trypsin digested before analysis by mass spectrometry at the Vincent Coates Foundation Mass Spectrometry Laboratory, Stanford University Mass Spectrometry (http://mass-spec.stanford.edu).

Expression of epitope-tagged and fluorescent fusion proteins

YFP-αTubulin and mRFP-TgCentrin2 were expressed in parasites using previously described plasmids (Hu et al., 2006; Striepen et al., 2000). HA epitope-tagged lines and YFP
fusions pISP1/2/3-HA/YFP were generated by cloning the genomic loci of ISP1 (primers P1/P2), ISP2 (primers P3/P4) or ISP3 (primers P5/P6) into the expression plasmids pNotI-HA-HPT or pNotI-YFP-HPT using the restriction sites HindIII/NotI. These vectors contain a C-terminal HA tag or YFP fusion and selectable marker HPT driven by the DHFR promoter (Rome et al., 2008). The ISP1_{1.65} truncation was generated by cloning YFP (primers P7/P8) at the restriction sites EcoRV/PacI in pISP1-YFP. The ISP2_{1.41} truncation was generated by cloning YFP (primers P9/P8) at the restriction sites Rsrl/NotI in pISP2-YFP. The ISP3_{1.36} truncation was generated by cloning the ISP3 promoter and residues 1-36 (primers P10/P11) at the restriction sites Pmel/AvrII in the previously described vector ptubYFP-YFP/sagCAT (Gubbels et al., 2003). The ISP1_{64-176} truncation was generated by cloning the ISP1 promoter and start codon (primers P1/P12) at the restriction sites HindIII/EcoRV in pISP1-YFP. The ISP1_{N/2C} chimera was generated by cloning ISP2_{43-160} (primers P13/P4) at the restriction sites EcoRV/NotI in pISP1-YFP. The ISP2_{N/1C} chimera was generated by cloning ISP1_{67-176}-YFP (primers P14/P8) at the restriction sites Rsrl/Pacl in pISP2-HA. For expression, 1.6x10^7 parasites were transfected with 30 µg of plasmid and then analyzed by IFA as specified in figure legends.

**Alpha-toxin treatment**

Separation of the parasite IMC and plasma membrane was achieved by treatment with *C. septicum* alpha-toxin as previously described (Wichroski et al., 2002). Briefly, freshly lysed, extracellular parasites were washed and incubated 4 hrs in serum free media with or without 20 nM activated alpha-toxin. Following treatment, cells were fixed in 3.5% formaldehyde, allowed to settle on glass slides and analyzed by IFA.
**Disruption of the Cortical Cytoskeleton of *T. gondii***

Tachyzoites were allowed to infect HFF monolayers on coverslips in media containing 0.5 or 2.5 μM oryzalin (Sigma). Parasites were allowed to grow 30-40 hrs post-infection and then fixed and examined by IFA.

**Generation of ISP2 and ISP3 Antisera**

The coding sequences for ISP2 (primers P15/P16) and ISP3 (primers P17/P18) were PCR amplified from *T. gondii* cDNA and cloned into pET101/D-TOPO (Invitrogen). Constructs were transformed into *E. coli* BL21DE3 cells, grown to A₆₀₀ of 0.6–0.8 and induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside (Sigma) for 5 hrs at 37°C. Recombinant ISP2 and ISP3 were purified over Qiagen Ni-NTA agarose under denaturing conditions and eluted with a low-pH buffer as per the manufacturer's instructions. Eluted proteins were dialyzed against PBS and ~75 μg was injected per immunization into BALB/c mice (Charles River) on a 21 day immunization schedule. Polyclonal antiserum was collected from mice after the second boost and screened by IFA and Western blot analysis.

**Detergent extraction of ISP Proteins**

For detergent extraction experiments, 3x10⁷ freshly lysed parasites were washed in PBS, pelleted and lysed in 1 mL TBS (50mM Tris-HCl [pH 7.4], 150mM NaCl) containing 0.5% NP-40 and complete protease inhibitors (Roche) for 15 min at 4°C and then centrifuged for 15 min at 14,000 x g. Equivalent amounts of total, supernatant and pellet fractions were separated on a 15% gel, transferred to nitrocellulose and blotted using anti-IMC1, anti-ROP1, mAb 7E8, polyclonal anti-ISP2, and polyclonal anti-ISP3.
Site Directed Mutagenesis

Mutations were generated by Quick Change Mutagenesis (Strategene) using HA-tagged, wild-type ISP1, 2 or 3 with mutagenesis primers as follows (forward primer given, reverse compliment was also used): ISP1: G2A (P19), C7S (P20), C8S (P21), C7,8S (P22). ISP2: G2A (P23), C5S (P24), C8S (P25), C9S (P26), C8,9S (P27), C5,8,9S (P28). ISP3: G2A (P29), C6S (P30), C7S (P31), C6,7S (P32). PCR amplified products were treated with DpnI to digest wild-type template and transformed into E. coli.Recovered clones were sequenced to confirm mutations.

Disruption of ISP1

The deletion of the ISP1 gene was accomplished by double homologous recombination using a construct derived from the pMini-GFP.ht knockout vector (Karasov et al., 2005) which contains the selectable marker hypoxanthine-xanthine-guanine phosphoribosyltransferase (HPT) and also contains the green fluorescent protein (GFP) as a downstream marker to distinguish homologous and heterologous recombinants. The 5’ flank (3,147 bp) and 3’ flank (3,042 bp) of ISP1 were amplified from strain RH genomic DNA using primer pairs P33/P34 and P35/P36, respectively. These genomic flanks were then cloned into pMini-GFP.ht upstream and downstream of HPT, resulting in the vector pISP1-KO-HPT.

After linearization with NheI, 30 µg of pISP1-KO-HPT was transfected into RHΔhpt parasites and selection for HPT was applied 12 hours post-transfection using 50 µg/ml mycophenolic acid and 50 µg/ml xanthine. Surviving parasites were cloned by limiting dilution eight days post-transfection and screened for GFP by fluorescence microscopy. GFP-negative clones were assessed for absence of mAb 7E8 staining by IFA. Western blot analysis was carried
out on whole-cell lysates of Δisp1 clones and parental strains using mAb 7E8 and anti-ROP1 antibody as previously described (Bradley et al., 2005). The HPT gene was removed from RHΔisp1 + HPT by a second round of double homologous recombination. The pISP1-KO-HPT vector was digested by EcoRV/NheI to remove the HPT gene and then blunted using Klenow enzyme and re-circularized by ligation. The resulting vector was linearized by EcoRI and transformed into RHΔisp1 + HPT, followed by selection for the absence of HPT on 200 µg/ml 6-thioxanthine (Sigma). After 3 weeks of selection, parasites were cloned and screened for the absence of GFP expression. Clones that were GFP-negative were then assessed for the inability to grow in mycophenolic acid and xanthine, indicating loss of HPT. One such clone was chosen and deletion of the ISP1 locus was confirmed by PCR. This clone was designated Δisp1.

**Generation of Δku80Δhpt strain parasites**

The HPT selectable marker was removed from the Ku80 locus of the previously described Δku80 strain (Huynh and Carruthers, 2009). Briefly, 10µg of a PCR fusion construct containing a 5' Ku80 flank (primers P37/P38) fused to a 3' Ku80 flank (primers P39/P40) was transfected into RHΔku80-HPT parasites. Selection against HPT with 6-thioxanthine and confirmation of marker loss were carried out as described above.

**Disruption of ISP2 and ISP3**

For disruption of ISP2, a knockout vector was generated by cloning ~3kb 5’ (primers P41/P42) and 3’ (primers P43/P44) genomic flanks into a modified version of pMiniGFP.ht in which HPT was replaced by the selectable marker DHFR-TSc3, yielding the vector pISP2KO-DHFR-TSc3. After linearization by NotI, 30µg of this vector was transfected into Δku80Δhpt
parasites and selection was applied 12 hours post-transfection using 1 μM pyrimethamine. Parasites were cloned and confirmed to lack ISP2 as described above. For disruption of ISP3, the vector pISP3-KO-HPT was generated by cloning ~3kb 5’ (primers P45/P46) and 3’ (primers P47/P48) genomic flanks into pMiniGFP.ht. After linearization by KpnI and transfection into the Δku80Δhpt strain, parasites were selected for HPT, cloned and confirmed to lack ISP3 as described above.

**Competition growth analysis of Δisp2 parasites**

Freshly lysed parental and Δisp2 parasites were counted and mixed in desired ratios before infection of 3.3x10⁶ parasites into a T25 flask of confluent HFFs. Parasites were allowed to disrupt the monolayer before passing into a fresh T25. At initial infection and at each passage, samples of the mixed culture were infected into coverslips and allowed to grow 32 hours before fixation and staining with polyclonal anti-ISP2 and rabbit polyclonal anti-tubulin as a co-marker to monitor mixed culture composition. At least 500 vacuoles were counted from each of 4 coverslips per passage. Values represent mean ± 3 standard deviations for a representative experiment.

**Quantification of Aberrant Numbers of Daughter Parasite Assembly**

Parental line and Δisp2 parasites were infected onto coverslips and allowed to grow 18-24 hours before fixation and staining with mAb 7E8 as a marker for daughter buds and rabbit polyclonal anti-tubulin as a co-marker. Fifty vacuoles containing parasites undergoing bud formation were counted from each of 3 coverslips per sample. Vacuoles containing one or more
parasites assembling >2 daughters were scored as aberrant. Values represent the mean ± SD from a representative experiment.
Figure Legends

Figure 4-1. mAb 7E8 stains an apical cap structure in mature *Toxoplasma* tachyzoites and forming daughter parasites.

A-C. IFA labeling with 7E8 and anti-tubulin displaying parasites before the onset of endodyogeny (A), early in endodyogeny (B), and late in endodyogeny (C). (A) 7E8 labels a peripheral cone-shaped structure at the parasite apex ~1.5µm in length. A gap in staining exists at the apex of the cone (arrow). (B) During early endodyogeny, 7E8 labels small rings with a central hole at the apex of forming daughter parasites (arrows). (C) As endodyogeny proceeds, the 7E8 rings enlarge and elongate into the apical cap structures seen in mature tachyzoites. 7E8 also labels a single spot in many parasites, which resides near the base of the apical cap but is clearly distinct (arrow). This spot is present through the cell cycle as seen in (A) and (B). We denote it here in late endodyogeny to demonstrate that it is a distinct structure and not merely an early daughter bud. Red: mAb 7E8 detected by Alexa594-anti-mouse IgG. Green: anti-tubulin antibody detected by Alexa488-anti-rabbit IgG. Scale bar = 5µm.

D. 7E8 staining is delimited at both its apex and base by TgCentrin2, which labels the preconoidal rings as well as a series of annuli further down the cell periphery in the apical end of the parasite. The 7E8 apical spot does not colocalize with TgCentrin2 annuli (inset arrows). TgCentrin2 also localizes to the centriole and the basal complex. Red: 7E8 antibody detected by Alexa488-anti-mouse IgG (pseudo-colored red for consistency in the color scheme). Green: mRFP-TgCentrin2 (pseudo-colored green).

E. Western blot analysis of *Toxoplasma* lysates by 7E8 detects a single band at ~18kD.
F. The 7E8 immunoaffinity purified 18kD protein visualized by Coomassie-staining in an SDS-PAGE gel. The band was excised from the gel, digested by trypsin and the resulting peptide fragments identified by mass spectrometry.

G. The 176 amino acid sequence of ISP1, the protein recognized by 7E8. Boxed regions indicated 7 tryptic peptides identified by MS/MS. Arrowheads denote exon boundaries.

Figure 4-2. ISP2 and ISP3 define two additional novel sub-compartments of the IMC.

A. BLAST analysis within the T. gondii genome identified two paralogs of ISP1 we denoted ISP2 and ISP3. The greatest sequence homology is present within the C-terminal portion of this family of proteins beginning at residue 71 of ISP1 while the N-terminal portion of each protein is more divergent. ISP1 and ISP2 show a higher sequence similarity with each other compared to ISP3. All family members have a conserved glycine at position two predicted to be myristoylated and a pair of conserved cysteines predicted to be palmitoylated within the first 10 residues (boxed). ISP2 contains an additional cysteine at position 5 that is predicted to be palmitoylated (boxed). The gene models for ISP1-3 were confirmed by cDNA sequencing. Nucleotide sequences are available in GenBank under the accession numbers HQ012577-HQ012579.

B-C. ISP2 and ISP3 were expressed with a C-terminal HA epitope-tag under the control of their endogenous promoter in parasites expressing mRFP-TgCentrin2. (B) ISP2 localizes to a novel, peripheral sub-compartment beginning at the basal border of the ISP1 apical cap and extending approximately two-thirds the length of the parasite. ISP2 is not found in the basal third of the parasite periphery. (C) ISP3 staining overlaps with ISP2 but extends further to the base of the parasite where there is a small gap in staining, indicating an association with the IMC. The boundary between the ISP1 apical cap and the sub-compartments labeled by ISP2 and ISP3 is
occupied by a ring of TgCentrin2 annuli. Blue: anti-HA antibody detected by Alexa350-anti-rabbit IgG. Red: 7E8 antibody detected by Alexa488-anti-mouse IgG (pseudo-colored red). Green: mRFP-TgCentrin2 (pseudo-colored green).

D. The base of the ISP2 compartment terminates in a jagged edge (arrows). A trace of the ISP2 compartment boundary (solid line) was performed to illustrate this feature within the whole parasite (dashed line). Red: anti-HA antibody detected by Alexa594-anti-mouse IgG.

E. Different stages of daughter budding were observed in parasites stably expressing ISP3-HA. With the beginning of endodyogeny, the maternal ISP3 signal decreases as the signal increases in daughter parasites. By mid-endodyogeny, ISP3 has disappeared completely from the maternal cell periphery. The parasites used in these images are Δisp1, thus ISP3 targets throughout the IMC, including the apical cap (see Figure 4-7). Red: anti-HA antibody detected by Alexa594-anti-mouse IgG. Green: anti-tubulin antibody detected by Alexa488-anti-rabbit IgG.

All scale bars = 5μm.

**Figure 4-3. ISPs associate with the IMC but are not imbedded in the underlying protein meshwork.**

A-B. Extracellular parasites expressing ISP2-HA (A) or ISP3-HA (B) were incubated 4 hrs with or without 20 nM *Clostridium septicum* alpha-toxin. In untreated cells, the plasma membrane marker SAG1 cannot be resolved from the IMC membranes. Following alpha-toxin treatment, a dramatic swelling of the plasma membrane occurs, separating it from the underlying IMC and enabling resolution of these two membrane systems. Each ISP family member clearly segregates with the cell body and not the distended plasma membrane, indicating an association with the IMC. Red: 7E8 antibody detected by Alexa594-anti-mouse IgG. Green: anti-HA antibody
detected by Alexa488-anti-rat IgG. Blue: anti-SAG1 antibody detected by Alexa350-anti-rabbit IgG.

C. Parasites were extracted with 0.5% NP-40 and separated into total (T), soluble (S) and pellet (P) fractions. Extracts were subjected to SDS-PAGE, blotted and probed with antibodies as indicated. As expected, the detergent resistant IMC protein meshwork containing IMC1 remains in the pellet under these conditions. In contrast, ISP1-3 are resolved into the soluble fraction, similar to the soluble control protein ROP1, demonstrating that these proteins are not embedded in the protein meshwork of the IMC.

**Figure 4-4. ISP1 and ISP3 are targeted to early daughter buds in the absence of parasite microtubules.**

A. Parasites were treated with 2.5 µM oryzalin for 40 hrs. In the absence of microtubules, ISP1 labels numerous ring structures (inset) within the center of the cell reminiscent of early daughter buds. Unpolymerized parasite tubulin is dispersed throughout the cytoplasm. Red: mAb 7E8 detected by Alexa594-anti-mouse IgG. Green: YFP-αTubulin.

B. Parasites expressing ISP2-HA were treated with 2.5 µM oryzalin for 40 hrs. ISP2 is localized in patches at the cell periphery and does not appear to associate with the ISP1 labeled rings (inset). Red: mAb 7E8 detected by Alexa594-anti-mouse IgG. Green: anti-HA antibody detected by Alexa488-anti-rabbit IgG.

C. Parasites expressing ISP3-HA were treated with 2.5 µM oryzalin for 40 hrs. ISP3 is also localized to the ring structures labeled by ISP1 (inset arrows), although ISP1 and 3 rings do not always perfectly colocalize. Red: mAb 7E8 detected by Alexa594-anti-mouse IgG. Green: anti-HA antibody detected by Alexa488-anti-rabbit IgG.
**D.** Parasites expressing ISP1-HA were treated with 0.5 µM oryzalin for 30 hrs. IMC1 labels partially formed parasites and sheets of IMC1 at the cell periphery and ISP1 apical cap staining can be observed at the apex of some of these structures. However, the majority of ISP1 signal is still localized to rings within the center of the cell under these less stringent conditions. These rings do not associate with IMC1 stained structures (insets). Red: anti-HA antibody detected by Alexa594-anti-rabbit IgG. Green: anti-IMC1 antibody detected by Alexa488-anti-mouse IgG. Scale bars = 5 µm. Inset scale bars = 1 µm.

**Figure 4-5. An N-terminal domain is sufficient for IMC sub-compartment targeting of ISP1 and 3 but not ISP2.**

**A.** The C-terminal portion of ISP1 (residues 64-176), which bears the greatest sequence homology with other ISP family members, was expressed with a C-terminal YFP tag. The ISP1\textsubscript{64-176}-YFP protein is dispersed throughout the cytosol and nucleus, demonstrating that the first 63 residues of ISP1 are necessary for IMC apical cap targeting. Green: ISP1\textsubscript{64-176}-YFP. Red: anti-IMC1 antibody detected by Alexa594-anti-mouse IgG.

**B-C.** Residues 1-65 (containing the putative acylation sequence and divergent N-terminal region) or 1-29 (containing the putative acylation sequence) of ISP1 were expressed with a C-terminal YFP fusion. Both proteins target in an identical manner to endogenous ISP1, demonstrating that the first 29 residues are sufficient for IMC apical cap targeting (cap shown in inset). Green: ISP1\textsubscript{1-65}-YFP or ISP1\textsubscript{1-29}-YFP.

**D.** Residues 1-36 of ISP3 were expressed with a C-terminal YFP fusion. The ISP3\textsubscript{1-36}-YFP protein targets in an identical manner to endogenous ISP3, including exclusion from the apical cap demonstrated by non-overlapping signal with ISP1 (inset), showing that these residues are
sufficient for proper ISP3 sub-compartment targeting within the IMC. Green: ISP31-36 YFP. Red: mAb 7E8 detected by Alexa594-anti-mouse IgG.

E. Targeting of full length ISP2-HA is restricted to the central IMC sub-compartment identical to endogenous ISP2 as shown by non-overlapping signal with ISP1 in the apical cap (inset) and lack of signal in the basal IMC sub-compartment (bracket).

F. Residues 1-41 of ISP2 were expressed with a C-terminal HA tag. The ISP21-41-HA protein targets to all three sub-compartments of the IMC, as shown by overlap with endogenous ISP1 in the apical cap (inset) and signal within the basal IMC sub-compartment (bracket). A small gap is visible at the extreme apex and base of the ISP21-41-HA staining, indicating this protein is still targeting to the IMC. Identical results were seen using YFP in place of HA (data not shown). Green: anti-HA antibody detected by Alexa488-anti-rabbit IgG. Red: mAb 7E8 detected by Alexa594-anti-mouse IgG.

**Figure 4-6. Mutation of ISP1 residues predicted for acylation results in ISP1 mistargeting.**

Mutations of residues predicted for myristoylation or palmitoylation were generated in an HA epitope-tagged copy of ISP1 and expressed in parasites under the endogenous promoter. Wild-type (WT) ISP1-HA targets in an identical fashion to endogenous ISP1. A severe targeting defect occurs in ISP1(G2A) with the mutant protein dispersed throughout the cell in a punctate fashion. Mutation of individual cysteines predicted for palmitoylation (C7S and C8S) produces no significant defect in targeting, but coordinated mutation of these cysteines results in gross mistargeting of ISP1(C7,8S) throughout the cell in a punctate fashion with a signal accumulation just apical of the nucleus (arrows). Red: anti-HA antibody detected by Alexa594-anti-mouse IgG. Green: anti-tubulin antibody detected by Alexa488-anti-rabbit IgG. Blue: Hoechst stain.
Figure 4-7. ISP2 and ISP3 are relocalized to the apical cap in the absence of ISP1.

A. Schematic showing the ISP1 knockout strategy. Double homologous recombination results in the replacement of ISP1 with the selectable marker HPT and the loss of the downstream marker GFP. An additional round of homologous recombination removes HPT to exclude any polar or selectable marker effects.

B. Loss of ISP1 is demonstrated by the absence of 7E8 staining by IFA in Δisp1 parasites. Red: mAb 7E8 detected by Alexa594-anti-mouse IgG. Green: anti-tubulin antibody detected by Alexa488-anti-rabbit IgG.

C. Western blot analysis detects ISP1 in parental strain but not in Δisp1 parasites. ROP1 serves as a loading control.

D. ISP2 localization in wild-type parasites is non-overlapping with ISP1 and ends sharply at the basal boundary of the apical cap normally occupied by ISP1. A ring of TgCentrin2 annuli resides at this boundary (arrowheads). In Δisp1 parasites, ISP2 relocalizes above the TgCentrin2 boundary, filling the apical cap.

E. ISP3 is also relocalized to the apical cap in Δisp1 parasites as assessed by the co-marker TgCentrin2. Red: mRFP-TgCentrin2. Green: anti-HA antibody detected by Alexa488-anti-rabbit IgG.

Figure 4-8. Exclusion of ISP2 and ISP3 is mediated by the C-terminal domain of ISP1.

A. Full length ISP1 with a C-terminal YFP fusion was expressed in Δisp1 parasites together with ISP2-HA. ISP1-YFP targets correctly to the apical cap and reestablishes normal localization of ISP2, excluding it from this region of the IMC (insets). Green: ISP1-YFP. Red: anti-HA antibody detected by Alexa594-anti-mouse IgG.
**B.** ISP1<sub>1-65</sub>-YFP was expressed in Δisp1 parasites together with ISP2-HA. While ISP1<sub>1-65</sub>-YFP targets correctly, ISP2 continues to relocalize to the apical cap in the presence of this truncated ISP1 protein (insets), demonstrating that the ISP1 C-terminal region (residues 66-176) is necessary for exclusion of ISP2 from the apical cap. Green: ISP1<sub>1-65</sub>-YFP fusion. Red: anti-HA antibody detected by Alexa594-anti-mouse IgG.

**C.** A chimeric protein consisting of the ISP1 N-terminus (residues 1-65) and the ISP2 C-terminus (residues 43-160) fused to YFP was expressed in Δisp1 parasites together with ISP2-HA. This chimeric protein targets to the apical cap but does not prevent relocalization of ISP2-HA into the cap, as seen by the overlap of the two signals (insets). Green: ISP1<sub>N</sub>/2<sub>C</sub>-YFP. Red: anti-HA antibody detected by Alexa594-anti-mouse IgG.

**D.** A chimeric protein consisting of the ISP2 N-terminus and the ISP1 C-terminus fused to YFP (ISP2<sub>N</sub>/1<sub>C</sub>-YFP) was expressed in wild-type parasites. This chimeric protein targets to the apical cap and central IMC compartments. Green: ISP2<sub>N</sub>/1<sub>C</sub>-YFP. Red: anti-tubulin antibody detected by Alexa594-anti-rabbit IgG.

**E-F.** The exclusion activity of the ISP1 C-terminal domain against ISP2/3 can function in other IMC sub-compartments. (E) ISP2<sub>N</sub>/1<sub>C</sub>-YFP was transiently expressed in Δisp1 parasites stably expressing ISP2-HA. ISP2 is relocalized to the base sub-compartment of the IMC in parasites expressing this chimera. ISP2 is not present in the base IMC sub-compartment in parasites that are not expressing the chimeric protein (brackets). (F) ISP2<sub>N</sub>/1<sub>C</sub>-YFP was transiently expressed in Δisp1 parasites stably expressing ISP3-HA. In the presence of the chimeric protein, ISP3 is concentrated in the base sub-compartment of the IMC (brackets). ISP3 is evenly distributed throughout the IMC of parasites that are not expressing the chimeric protein. Green: ISP2<sub>N</sub>/1<sub>C</sub>-YFP. Red: anti-HA antibody detected by Alexa594-anti-mouse IgG.
All scale bars = 5µm.

Figure 4-9. Disruption of ISP2 results in a severe loss of parasite fitness and defects in daughter cell formation.

A. Western blot analysis using polyclonal anti-ISP2 confirms the loss of ISP2 in Δisp2 parasites. ROP1 serves as a loading control.

B. A competition growth assay reveals a severe fitness loss in Δisp2 parasites. Parent and Δisp2 parasites were mixed in culture and passaged. At each passage, the composition of the mixed culture was evaluated by IFA. Although Δisp2 parasites initially comprised >80% of the culture, they were rapidly outcompeted by the parental strain and essentially lost from the culture within four passages. Values represent means ± 3 standard deviations.

C. Parasites lacking ISP2 assemble >2 daughters per round of endodyogeny. ISP1 was used as a marker for daughter buds. The top left parasite in this vacuole is assembling four daughters while the other three parasites are assembling five daughters each. While the top left parasite has divided its nucleus and is now budding two daughters around each of two separate nuclei, the other three parasites appear to each be budding five daughters around a single polyploid nucleus. Red: mAb 7E8 detected by Alexa594-anti-mouse IgG. Green: anti-tubulin antibody detected by Alexa488-anti-rabbit IgG. Blue: Hoechst stain.

D. Quantification of the >2 daughters phenotype in Δisp2 parasites. Parasites undergoing endodyogeny were counted and scored for the percentage of vacuoles in which parasites were assembling >2 daughters. Most vacuoles contain one or more parasites assembling >2 daughters in the Δisp2 strain. Values represent means ± SD, n=3, from a representative experiment.

*P<0.001
**E-F.** Parasites lacking ISP2 can perform karyokinesis before budding. (E) ISP1 is an early marker for bud formation visible before nuclear segregation during endodyogeny. No daughter ISP1 signal is visible in these parasites although the spindle apparatus is assembled and has already separated the chromosomes into two nuclei, showing that karyokinesis can precede budding in Δisp2 parasites. (F) After undergoing a round of karyokinesis without budding, Δisp2 parasites can bud around each of the segregated nuclei. This vacuole contains two parasites (dashed outlines) that have undergone karyokinesis prior to budding and are now assembling two daughters around each individual nucleus. Red: mAb 7E8 detected by Alexa594-anti-mouse IgG. Green: anti-tubulin antibody detected by Alexa488-anti-rabbit IgG. Blue: Hoechst stain.

**G-H.** Parasites lacking ISP2 display catastrophic replication defects. Parasites were stained for the apicoplast thioredoxin-like protein 1 (ATrx1), which labels the apicoplast, as well as tubulin and DNA. (G) In the vacuole shown, a few parasites have received both a nucleus and an apicoplast (arrow) while others contain only an apicoplast (double arrowhead) and some contain neither (arrowhead). Several nuclei have been extruded into the vacuole along with one or more apicoplasts. (H) Other vacuoles containing extruded nuclei also contained several daughter buds that appear to be outside of an intact mother parasites (~18 in this vacuole visible by tubulin). Many nuclei and elongated apicoplasts are present in the vacuole and appear associated with the forming buds. For clarity, a dashed line indicates the boundary of the parasitophorous vacuole. Red: anti-ATrx1 detected by Alexa594-anti-mouse IgG. Green: anti-tubulin antibody detected by Alexa488-anti-rabbit IgG. Blue: Hoechst stain.

All scale bars = 5µm.
Figure 4-10. Model for ISP sorting within the parasite IMC.

A. Alveoli, flattened membrane sacs, rest just under the plasma membrane atop a filamentous network in the *Toxoplasma* IMC. These alveoli are arranged as a patchwork of rectangular plates with a unique cone-shaped plate capping the apex. A small gap is present at the extreme apex and base of the IMC. The ISPs reveal three distinct sub-compartments within the IMC: the apical cap (red, ISP1), central IMC (light blue, ISP2 and ISP3), and basal IMC (dark blue, ISP3). Colors indicate speculative arrangement of sub-compartments within discrete alveoli or combinations thereof.

B. Model for ISP sorting within the IMC. (1) ISP1/2/3 are co-translationally myristoylated within the cytosol at a conserved second position glycine by the action of an N-myristoyl transferase (NMT). (2) This initial acylation allows the ISPs to transiently associate with the various membrane systems within the cell, including the cisternae of the IMC. (3) Different PATs (or PAT activities) located within the three distinct sub-compartments of the IMC specifically recognize and palmitoylate their unique ISP substrates, locking them into the appropriate sub-compartment. An apical cap PAT with specificity for ISP1 locks it into the cap while a central IMC PAT is able to recognize and palmitoylate both ISP2 and ISP3 within the central IMC sub-compartment. ISP3 is stably localized to the IMC base sub-compartment by the action of a basal IMC PAT. (4) The presence of ISP1 in the apical cap provides an additional level of sorting by preventing the localization of ISP2 and 3 into this sub-compartment. While the N-terminus of ISP1 is sufficient for its sorting to the apical cap, the ISP1 C-terminal domain is required to prevent the localization of other ISP family members into the cap, possibly through the modulation of the apical cap PAT specificity for ISP2 and 3.
Figure 4-1

A

Phase

7E8

Tubulin

merge

5 μ

B

Phase

7E8

Tubulin

merge

C

Phase

7E8

Tubulin

merge

D

Phase

7E8

TgCentrin2

merge

E

F

G

MGAVSSCVAEEEERQMVQENAGTKGEKSRYYYYGD
KSGRSPSHRASHAASAPQVTAADIEDLHRILLAGMAYLV
LLQDGLRLQICILHYNEADSSLSISCEDKVRIPLSDIKAL
LHTRDQLQRVTKANLVDDESCVALLLESGNCIPRLFDG
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Figure 4-6

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</table>

Legend:
- WT: Wild Type
- G2A: Mutant G2A
- C7S: Mutant C7S
- C8S: Mutant C8S
- C7,8S: Mutant C7,8S

Notes:
- Phase: Phase contrast
- ISP1: Immunostaining of ISP1
- Tubulin: Immunostaining of Tubulin
- Hoechst: DAPI staining
- Merge: Composite image of all channels
Figure 4-7

A

B

C

D

E

245
Figure 4-8

A

\[\text{ISP1} \quad \text{YFP} \]

B

\[\text{ISP1 1-65} \quad \text{YFP} \]

C

\[\text{ISP1 1-65} \quad \text{ISP2 43-160} \quad \text{YFP} \]

D-F

\[\text{ISP2 1-41} \quad \text{ISP1 67-176} \quad \text{YFP} \]

**A**

Phase

ISP1

ISP2

merge

**B**

Phase

ISP1_{1-65}

ISP2

merge

**C**

Phase

ISP1_{N/2C}

ISP2

merge

**D**

Phase

ISP2_{1C}

Tubulin

merge

**E**

Phase

ISP2_{N/1C}

ISP2

merge

**F**

Phase

ISP2_{N/1C}

ISP3

merge
Figure 4-9

A

B

C

D

E

F

G

H

Figure 4-9

A

B

C

D

E

F

G

H

Figure 4-9
Figure 4-10
Supplementary Material

Figure 4-S1. ISP ortholog groups.

OrthoMCL DB (www.orthomcl.org) was utilized to identify ortholog groups for the ISP family. ISP1 and 2 belong to one OrthoMCL group (OG4_23348) (A) while ISP3 belongs to another group (OG4_34375) (B). The ISP1 and 2 group contains proteins from all apicomplexans available in the OrthoMCL DB while the ISP3 group contains only proteins from *Neospora caninum*, *Plasmodium* species, and *Babesia bovis*. EuPathDb (www.eupathdb.org) accession numbers are given for each protein. The *P. berghei* protein PB301233.00.0 and *P. yoelii* protein PYO2085 each lack a start methionine, indicating incomplete N-termini in the annotation of the gene models associated with these proteins. The related CP15/60 protein from *Cryptosporidia* forms a separate OrthoMCL group (OG4_74892, data not shown).

Figure 4-S2. Antibody confirmation of sub-compartment localizations for endogenous ISP2 and ISP3.

A. ISP2 antisera confirms the localization of endogenous ISP2 to the central IMC sub-compartment in a fashion identical to the HA epitope-tagged ISP2 shown in Figure 4-2B. Endogenous ISP2 is clearly absent from the apical cap (brackets) and basal portion of the IMC. Red: polyclonal anti-ISP2 detected by Alexa594-anti-mouse IgG. Green: anti-tubulin antibody detected by Alexa488-anti-rabbit IgG.

B-C. ISP3 antisera functions poorly by IFA. The staining is, however, sufficient to (B) confirm the localization of endogenous ISP3 to the central and basal IMC shown by HA epitope-tagged ISP3 in Figure 4-2C and (C) confirm the attenuation of maternal ISP3 signal and enrichment of ISP3 in daughter parasites during endodyogeny as shown in Figure 4-2E. Red: polyclonal anti-
ISP3 detected by Alexa594-anti-mouse IgG. Green: anti-tubulin antibody detected by Alexa488-anti-rabbit IgG.

**Figure 4-S3. Mutation of ISP2 residues predicted for acylation results in ISP2 mistargeting.**

Mutations of residues predicted for myristoylation or palmitoylation were generated in an HA epitope-tagged copy of ISP2 and expressed in parasites under the control of the endogenous promoter. A severe targeting defect occurs in ISP2 (G2A) in which ISP2 signal is dispersed throughout the cell in a punctate fashion. The ISP2 (C5S) mutant shows an intermediate localization defect with some mistargeting and some proper localization. Mutation of the conserved cysteine pair residues individually or together does not grossly mistarget ISP2 (C8S), (C9S), or (C8,9S). A serious targeting defect occurs when all three N-terminal cysteines are coordinately mutated in ISP2 (C5,8,9S). While ISP2 (C5,8,9S) is distributed throughout the cytosol, signal concentration is observed perinuclear and just apical of the nucleus (arrows). Red: anti-HA antibody detected by Alexa594-anti-mouse IgG. Green: anti-tubulin antibody detected by Alexa488-anti-rabbit IgG. Blue: Hoechst stain.

**Figure 4-S4. Mutation of ISP3 residues predicted for acylation results in ISP3 mistargeting.**

Mutations of residues predicted for myristoylation or palmitoylation were generated in an HA epitope-tagged copy of ISP3 and expressed in parasites under the control of the endogenous promoter. A severe targeting defect occurs in ISP3 (G2A) with the mutant protein dispersed throughout the cell in a punctate fashion. Individual cysteine mutants ISP3 (C6S) and (C7S) show no gross defect in targeting. Coordinated mutation of these cysteines results in gross mistargeting of ISP3 (C6,7S) throughout the cell in a punctate fashion. As seen in ISP1 and ISP2
coordinated cysteine mutants, a concentration of signal is observed just apical of the nucleus (arrows). Red: anti-HA antibody detected by Alexa594-anti-mouse IgG. Green: anti-tubulin antibody detected by Alexa488-anti-rabbit IgG. Blue: Hoechst stain.

**Figure 4-S5. Disruption of ISP3.**

Western blot analysis using polyclonal anti-ISP3 confirms the loss of ISP3 in Δisp3 parasites. ROP1 serves as a loading control.
Figure 4-S2
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Table 4-1. Primers used in this study as discussed in text. Restriction sites and mutated bases are shown in lowercase.

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References


Chapter 5

*Toxoplasma* ISP4 is a central IMC sub-compartment protein whose localization depends on palmitoylation but not myristoylation

Published as:

Fung, C.*, Beck, J.R.*, Robertson, S.D., Gubbels, M.J., and Bradley, P.J. (2012). *Toxoplasma* ISP4 is a central IMC sub-compartment protein whose localization depends on palmitoylation but not myristoylation. Molecular and biochemical parasitology 184, 99-108. (* Authors contributed equally to this work)
Abstract

Apicomplexan parasites utilize a peripheral membrane system called the inner membrane complex (IMC) to facilitate host cell invasion and parasite replication. We recently identified a novel family of *Toxoplasma* IMC Sub-compartment Proteins (ISP1/2/3) that localize to sub-domains of the IMC using a targeting mechanism that is dependent on coordinated myristoylation and palmitoylation of a series of residues in the N-terminus of the protein. While the precise functions of the ISPs are unknown, deletion of *ISP2* results in replication defects, suggesting that this family of proteins plays a role in daughter cell formation. Here we have characterized a fourth ISP family member (ISP4) and discovered that this protein localizes to the central IMC sub-compartment, similar to ISP2. Like ISP1/3, ISP4 is dispensable for the tachyzoite lytic cycle as the disruption of *ISP4* does not produce any gross replication or growth defects. Surprisingly, targeting of ISP4 to the IMC membranes is dependent on residues predicted for palmitoylation but not myristoylation, setting its trafficking apart from the other ISP proteins and demonstrating distinct mechanisms of protein localization to the IMC membranes, even within a family of highly-related proteins.
Introduction

The phylum Apicomplexa consists of approximately five thousand known species of obligate intracellular parasites, many of which cause serious diseases in humans and animals worldwide. Apicomplexans of major importance to human health include the malaria parasite *Plasmodium falciparum*, which accounts for approximately one million deaths annually (Hay et al., 2010), and *Toxoplasma gondii*, a pathogen that chronically infects about one-third of the human population (Hill et al., 2005). Although infected individuals with an intact immune system are typically unharmed, *Toxoplasma* is capable of causing severe neurological disorders and even death in immunocompromised patients (Tenter et al., 2000). In addition, infants that become infected with *Toxoplasma* congenitally can suffer from severe birth defects, ranging from ocular disorders to hydrocephalus (Martin, 2001).

Apicomplexans possess a number of unique cellular structures that compartmentalize parasite-specific functions and also represent new potential therapeutic targets. One of these is a peripheral membrane system called the inner membrane complex (IMC). The IMC is a double membrane structure composed of flattened vesicles called alveoli that underlie the plasma membrane and are linked to a supporting cytoskeletal meshwork which faces the cytoplasm (D’Haese et al., 1977; Mann and Beckers, 2001). Freeze-fracture studies of the IMC reveal that the flattened alveoli are organized into a patchwork of tightly sutured rectangular plates. In the cyst-forming coccidal sub-group of apicomplexans, this structure is capped by a single cone-shaped plate at the apical end of the cell (Porchet and Torpier, 1977). An actin-myosin motor embedded in the IMC produces a form of gliding motility critical for extracellular transit, host cell invasion, and egress (Hakansson et al., 1999; Keeley and Soldati, 2004). In addition to supporting parasite motility, the IMC serves as a scaffold for the assembly of new daughter buds.
during parasite replication via an internal budding process known as endodyogeny (Striepen et al., 2007). While a number of IMC protein constituents have been identified, these probably represent only a small fraction of the total proteins and corresponding activities that are present in this organelle.

Recently, we identified a family of IMC Sub-compartment Proteins (ISP1/2/3) in *Toxoplasma gondii* and showed that it plays a role in coordinating cell division. While the ISP family is conserved across Apicomplexa, different species maintain varying numbers of ISP proteins (for example, *Plasmodium* species appear to possess only two family members) (Beck et al., 2010). The ISPs contain no identifiable domains and appear to be restricted to this phylum, likely representing specialized apicomplexan functions as indicated by the dysregulation of endodyogeny upon disruption of ISP2 (Beck et al., 2010). While ultrastructural observations showed that the IMC is non-contiguous, a higher degree of compartmentalization was appreciated by the discovery that ISP1/2/3 localize to distinct membrane plates or groups of plates within the IMC. ISP1 targets to the cone-shaped apical cap, while ISP2 localizes to a central region of the IMC which begins at the base of the apical cap and extends about two-thirds the length of the cell. ISP3 is found in the central IMC region but also extends to the basal end of the parasite (Beck et al., 2010). Coordinated acylations are responsible for IMC membrane targeting, suggesting a “kinetic trapping” model in which the ISP proteins are first myristoylated in the cytosol to enable transient sampling of membranes, followed by palmitoylation at the IMC, which locks the proteins into the appropriate membrane compartment. Disruption of either myristoylation or palmitoylation signals in ISP1/2/3 completely ablates IMC targeting and results in a cytoplasmic localization. In addition, ISP1 performs a gate-keeping function that
excludes ISP2 and ISP3 from the apical cap region, revealing a hierarchical targeting within this membrane system that is just beginning to be understood (Beck et al., 2010).

BLAST analysis of the *T. gondii* genome using the ISP1-3 sequences identified a potential fourth ISP family member (TGGT1_063420, www.toxodb.org), but this protein was not previously characterized due to poor expression levels and an uncertain gene model (Beck et al., 2010). Here, we demonstrate that TGGT1_063420 (denoted ISP4) localizes to the central IMC sub-compartment, similar to ISP2. Disruption of *ISP4* did not result in any apparent replication or growth defects, suggesting that other family members may substitute in its absence. Finally, we show that ISP4 targets to the IMC by a mechanism distinct from the other three family members. While trafficking of ISP1/2/3 to the IMC is dependent on both myristoylation and palmitoylation (Beck et al., 2010), ISP4 targeting is only contingent upon residues predicted for palmitoylation. Together, these experiments provide new molecular insight into the organization and construction of the IMC, a unique membrane structure critical to *Toxoplasma* pathogenesis.
Results

Identification of ISP4

We previously identified a fourth putative ISP family member (TGGT1_063420) in the *Toxoplasma* genome. However this protein was not examined further due to an uncertain gene model and its low expression level relative to the other ISPs (Beck et al., 2010). Analysis of expression timing data for TGGT1_063420 reveals a periodic pattern with peak transcription levels consistent with a cell-cycle regulated protein (Behnke et al., 2010) (Figure 5-1A). Interestingly, a comparison with ISP1-3 as well as other characterized IMC proteins shows that peak expression of TGGT1_063420 lags behind ISP1-3 and most of the known components of the IMC protein meshwork (IMC1, 3-6, 8-11, 13 and 15) by 1 hour, similar to the IMC meshwork protein IMC14 (Anderson-White et al., 2011). Taken together with the importance of ISP2 in parasite division, these observations provided an impetus for further characterization of TGGT1_063420.

The gene model for TGGT1_063420 indicates the protein lacks the conserved myristoylation and palmitoylation signals in other ISP family members. However, only a single EST is available for TGGT1_063420 which covers the C-terminal two-thirds of the protein, leaving uncertainty about the N-terminus of the gene model. To determine the correct coding sequence, we cloned and sequenced cDNAs for this gene. This showed that the predicted first exon of the gene model is inaccurate and revealed the correct first exon, which agrees with a recently generated RNAseq data set (Reid AJ, 2012). Surprisingly, the corrected protein sequence also lacks the conserved myristoylation signal required for targeting other ISP family members to the IMC membranes, although two cysteine pairs predicted for palmitoylation are
present further into the sequence (Figure 5-1B). Despite these differences, sequence similarity to ISP1-3 suggests that TGGT1_063420 is an ISP family member.

Characterization of ISP4

To determine whether TGGT1_063420 localizes to the IMC, we raised an antibody against a recombinant portion of the protein. The resulting anti-sera detected a band at ~22 kD by Western blot of Toxoplasma lysates, the expected size for the protein (Figure 5-2A). However, the anti-sera did not give any signal by immunofluorescence assay (IFA) under a variety of fixation conditions (data not shown). To resolve the localization of TGGT1_063420, we integrated a 3xHA endogenous tag at the C-terminus of the protein (Figure 5-2B). Western blot analysis with our anti-sera shows an ~5 kD up-shift in the target band corresponding to the triple HA fusion, validating the specificity of the antibody (Figure 5-2C). A band of the same size is also seen with anti-HA antibodies in the tagged strain but not the parent (Figure 5-2C), confirming that this clone contains an endogenous tag of the gene. Although signal strength was poor (likely due to low expression levels), IFA using an anti-HA antibody shows that this protein targets to the central region of the IMC and is not found in the apical cap or basal IMC sub-compartments, similar to the localization of ISP2 (Figure 5-2D). To more clearly evaluate localization, we expressed a second copy of the gene from the stronger tubulin promoter and observed clear co-localization with ISP2 (Figure 5-2E-F). Thus, we named this protein ISP4.

While co-localization with ISP2 suggests that ISP4 is associated with the IMC membranes, this data does not rule out the possibility of an association with the plasma membrane or cytoskeletal meshwork of the IMC. Because localization to the forming daughter buds precludes a plasma membrane association, since buds at this stage of division have not yet
adopted the maternal plasma membrane, we examined whether ISP4 could be detected in dividing parasites similar to other ISP family members. We were able to detect the protein in forming daughter parasites in both the endogenous tagged strain (Figure 5-3A) and in parasites expressing a second copy of ISP4 under the control of the tubulin promoter (Figure 5-3B). This data indicates ISP4 is a component of the IMC and not the plasma membrane.

To address whether ISP4 associates with the cytoskeletal meshwork, we conducted a detergent extraction experiment with ISP4-3xHA strain parasites. In this experiment, ISP4 is completely solubilized by detergent extraction, similar to the soluble control protein ROP1, and does not fractionate with IMC1, a component of the insoluble IMC network (Figure 5-3C). These results indicate that ISP4 is not embedded in the cytoskeletal meshwork of the IMC, similar to ISP1-3.

Previously, we described a hierarchical membrane targeting system within the ISP family whereby ISP2/3 are excluded from the IMC apical cap compartment by an ISP1-dependent mechanism (Beck et al., 2010). To assess whether this gate-keeping function of ISP1 is also responsible for exclusion of ISP4 from the apical cap, we expressed HA-tagged ISP4 under the control of the tubulin promoter in wild-type and Δisp1 parasites. In wild-type parasites, the central IMC sub-compartment localization of ISP4 terminates at a ring of TgCentrin2 annuli known to delineate the boundary between the ISP1 apical cap and the remainder of the IMC (Beck et al., 2010; Hu et al., 2006) (Figure 5-4). In Δisp1 parasites, however, ISP4 is no longer excluded from the IMC apical cap but instead relocalizes into the cap region that is normally occupied by ISP1 (Figure 5-4). These results confirm that ISP4 is also subject to the ISP1-dependent hierarchical targeting system that acts on ISP2/3.
**ISP4 is dispensable for the tachyzoite lytic cycle and endodyogeny**

We have previously shown that ISPs 1-3 are not individually essential in *T. gondii*, but that parasites lacking ISP2 are substantially less fit and frequently switch from endodyogeny to an endopolygeny-like replicative mode (Beck et al., 2010). To assess the consequence of the ablation of *ISP4*, we carried out gene disruption by homologous recombination in the ISP4-3xHA strain (Figure 5-5A). Disruption of *ISP4* was confirmed by loss of HA staining by Western blot and IFA in a clonal ∆isp4 line (Figure 5-5B-C). Using competitive growth assays and IFA, we examined the ∆isp4 parasite strain for changes in fitness and daughter cell formation but found no apparent defects compared to the parental line (data not shown). These results demonstrate that *ISP4* is not essential and suggest a possible functional redundancy among certain members of the ISP family. Additionally, the knockout provides a background lacking ISP4 to study the biosynthesis and trafficking of the protein to the IMC.

**Determination of the correct ISP4 start methionine**

The first exon of ISP4 contains two potential start methionines within the first eleven amino acid residues. As we could not determine which of these residues constitutes the correct start codon from the size of the protein on a gel, we engineered two untagged ISP4 cDNA expression vectors, one which contains both and the other which only contains the second methionine (designated M1 and M2, respectively, Figure 5-6A). Because detection of ISP4 expressed from its endogenous promoter is difficult, we drove expression of these constructs from the more robust tubulin promoter. We expressed each construct in ∆isp4 parasites and compared their SDS-PAGE migration to the endogenous protein in wild-type parasites. Expression of ISP4 from the M1 construct resulted in a doublet with the top band corresponding
with the size of endogenous ISP4. Expression of the M2 construct resulted in a single band that migrates at the same position as the lower band of the M1 doublet (Figure 5-6B). Together, these data indicate that endogenous ISP4 is primarily translated from the M1 start site and that the second start site is likely also used when driven from the tubulin promoter. A faint band at the M2 size is also detected for endogenous ISP4, suggesting that M2 may serve as a less frequent translation start site in the endogenous context, although we cannot exclude the possibility that this band is the result of degradation.

**Palmitoylation, but not myristoylation, is critical for ISP4 trafficking**

Both myristoylation and palmitoylation are required to anchor the ISP1-3 proteins to the IMC membranes. This suggests a model wherein the second position glycine is myristoylated cotranslationally by an N-myristoyltransferase, allowing ISP1-3 to transiently associate with the IMC. At the IMC membranes, the proteins then encounter a palmitoyl acyl transferase (PAT) which palmitoylates the conserved cysteine pair to strengthen and stabilize the association with the IMC (Beck et al., 2010).

The absence of a position two glycine in ISP4 suggests that membrane association occurs via a mechanism distinct from the other ISPs. ISP4 does however contain two cysteine pairs that are predicted to be palmitoylated (Figure 5-1B, residues 26,27 and 72,73, red boxes). To assess their role in ISP4 trafficking, we mutated these cysteines pairwise into serines in our ISP4-HA expression construct. Mutation of the cysteine pair at position 26,27 abrogates IMC targeting, resulting in ISP4 localized throughout the parasite cytosol. In contrast, mutation of the cysteine pair at position 72,73 had no gross effect on the localization of ISP4 (Figure 5-7).
Some proteins lacking a second position glycine are known to undergo N-myristoylation post-translationally following a protein cleavage event that exposes an internal glycine residue at the N-terminus of the processed protein (Martin et al., 2011). Thus, while ISP4 does not contain a position two glycine, any glycine residue upstream of the critical cysteine pair at 26,27 could facilitate N-myristoylation following proteolysis. A single glycine present at position 15 fits these qualifications (Figure 5-1B, yellow box). To rule out the possibility of myristoylation in ISP4 targeting, we mutated this residue to alanine and found no targeting defects in this mutant (Figure 5-7). Together, these results indicate that ISP4 targeting is dependent upon cysteine residues 26,27, which are predicted for palmitoylation, but not on myristoylation, demonstrating a new means of targeting for this protein family.
Discussion

**ISP4 is a additional member of the Toxoplasma ISP family**

The *Toxoplasma* IMC is a critical organelle for processes of cell division and parasite motility. The ISPs are a family of IMC membrane-anchored proteins conserved throughout Apicomplexa but not present in other eukaryotes. Here, we have shown that the *Toxoplasma* ISP family contains a fourth member that targets to the central sub-compartment of the IMC and displays features common to other family members, including lipid-based anchoring to the IMC membranes and ISP1-dependent exclusion from the apical cap. Expression timing of IMC proteins generally fits into three categories which peak early in budding, late in budding, or outside of budding during late G1 (Figure 5-1A). The periodic expression timing observed for ISP4 fits the second category, lagging behind ISP1-3 by about one hour, similar to IMC14. Interestingly, while ISP4 is observed in forming buds (Figure 5-3A), IMC14 is only seen in mature parasites (Anderson-White et al., 2011), suggesting that additional factors beyond timing of expression guide trafficking of these proteins to either the maternal or daughter IMC.

Similar to ISP1 and 3, ISP4 is dispensable and deletion of *ISP4* produces no major changes in fitness or endodyogeny. In contrast, loss of ISP2, which also localizes to the central IMC sub-compartment, results in major fitness defects and replication errors. Interestingly, after serial passage for several months, Δisp2 parasites largely recover from replication and fitness (Beck et al., 2010), raising the possibility that changes in the regulation of *ISP3* or *ISP4* expression might compensate for the loss of ISP2 since they each localize to the central sub-compartment. Thus, it will be interesting to determine if coordinate disruption of multiple *ISP* genes will produce more severe defects in division. Considering the absence of identifiable domains in these apicomplexan-specific proteins, definition of the precise functional roles of the
ISP family will benefit from combining these genetic approaches with future studies aimed at structural analysis.

**ISP4 targeting is contingent on residues predicted for palmitoylation but not myristoylation**

Eukaryotes often catalyze the addition of fatty acyl groups to proteins that lack transmembrane domains to mediate their association with lipid membranes. Targeting of ISP1/2/3 to the IMC appears to function via kinetic trapping (Resh, 2006) where initial myristoylation in the cytosol permits sampling of the IMC membranes, during which the proteins encounter a PAT that catalyzes their stable association with the IMC by palmitoylation (Beck et al., 2010). As both modifications are strictly required for ISP1/2/3 targeting, we were surprised to find that ISP4 lacks the N-terminal myristoylation signal conserved in other family members. We further eliminated the possibility of a targeting-dependent myristoylation event at glycine 15. Similar to ISP1/2/3, targeting of ISP4 depends on a pair of cysteines (positions 26,27) predicted to be palmitoylated, although these resides are recessed further into the protein than the critical palmitoylation signals in ISP1/2/3. An additional pair of cysteines in ISP4 (positions 72,73) are predicted for palmitoylation but dispensable for targeting. A model of ISP protein sorting to the IMC membrane sub-compartments is presented in Figure 5-8.

The finding that myristoylation does not play a role in ISP4 targeting is unexpected and in light of our kinetic trapping model, raises the question of how ISP4 is brought into close enough proximity to an IMC-resident PAT to become palmitoylated. It is possible that an accessory factor associates with ISP4 and delivers it to an IMC-resident PAT for acylation and future studies aimed at identification of ISP4 interacting partners will explore this possibility.
However, many IMC proteins only contain palmitoylation signals (see below) and thus the necessity of myristoylation for ISP1/2/3 trafficking may represent an exception to a more general palmitoylation-only mechanism of membrane association. In addition, the fact that ISP4 is subject to the same ISP1-dependent apical cap exclusion as ISP2/3 indicates that myristoylation is not required for this hierarchical component of ISP sub-compartmentalization.

**Palmitoylation is a critical lipid modification for IMC assembly and function**

Palmitoylation is a unifying feature in the targeting of all four ISP family members and represents an emerging key activity for assembly and organization of the IMC. In addition to the ISPs, several other IMC proteins with diverse functions are known to undergo palmitoylation. The glideosome-associated protein GAP45 is localized throughout the IMC and tethered to the both the alveoli membranes and the cytosolic face of the plasma membrane. Plasma membrane association is accomplished by myristoylation and palmitoylation of the GAP45 N-terminus, while the C-terminus of this protein associates with the IMC, possibly via additional C-terminal palmitoylations (Frenal et al., 2010; Rees-Channer et al., 2006). GAP70, a coccidian-specific homolog of GAP45, similarly bridges the plasma membrane and IMC via acylations, but localizes only to the apical cap. An additional member of the glidesome complex, myosin light chain 1, is predicted for palmitoylation at several N-terminal cysteines. Mutation of these cysteines simultaneously abolishes IMC association and GAP45 binding, suggesting that they are palmitoylated and important for IMC association, although this could not be separated from GAP45 binding. In addition to glideosome components, an isoform of the purine salvage enzyme hypoxanthine-xanthine-guanine phosphoribosyltransferase is localized to the IMC via palmitoylation but not myristoylation (Chaudhary et al., 2005), showing that palmitoylation is
also employed for recruiting metabolic activities to the IMC, although the purpose of targeting such an enzyme to the IMC remains unclear. Finally, several other IMC proteins contain residues that are predicted for palmitoylation but the importance of these residues for IMC targeting has not yet been directly tested. These include TgHSP20, which associates with the outer leaflet of the IMC membranes and plays a role in the regulation of gliding motility, the glidesome component myosin light chain 2, and several members of the intermediate filament-like IMC meshwork proteins, which may associate with the cytoplasmic face of the IMC membranes via this lipid modification (Anderson-White et al., 2011; de Miguel et al., 2008; Montagna et al., 2012; Polonais et al., 2011). Considering the prevalence of IMC protein palmitoylation, identification of the enzyme(s) responsible for this modification will be an important step in unraveling the biology of this parasite organelle.

At present, no PATs have been reported on in the Apicomplexa. BLAST analysis of the *T. gondii* genome with known PAT sequences from *S. cerevisiae* identified eighteen putative PAT homologs, all of which contain the hallmark Asp-His-His-Cys-cysteine-rich domain (DHH-CRD) (Beck et al., 2010; Smotrys and Linder, 2004). This relatively high number suggests that PATs may play an extensive role in the sorting of proteins to the unique and specialized membrane systems within *Toxoplasma* (Emmer et al., 2009; Saric et al., 2009). The identification and localization of IMC PATs will provide a better understanding of ISP protein sorting within the IMC and open new avenues for biochemical analyses of the enzymatic activities that are critical to the organization of this unique membrane structure.
Materials and Methods

Toxoplasma and host cell culture

*T. gondii* RHΔhpt (parental) strain and modified strains were grown on confluent monolayers of human foreskin fibroblast (HFF) host cells in DMEM supplemented with 10% fetal bovine serum, as previously described (Donald et al., 1996).

Transcriptional profile analysis of ISP4

Expression data for ISP4 and other indicated IMC proteins was acquired from a previously described genome-wide microarray expression dataset (Behnke et al., 2010).

Antibodies

The following previously described primary antibodies were used in immunofluorescence (IFA) or Western blot assays: rabbit polyclonal anti-tubulin (Morrissette and Sibley, 2002), anti-IMC1 mAb 45.15 (Wichroski et al., 2002), anti-ISPI mAb 7E8 (Beck et al., 2010), mouse polyclonal anti-ISPI mAb 45.15 (Wichroski et al., 2002), anti-ISPI mAb 7E8 (Beck et al., 2010), and anti-ROP1 mAb TG49 (Schwartzman and Krug, 1989). The hemagglutinin (HA) epitope was detected with mouse mAb HA.11 (Covance), rabbit anti-HA (Invitrogen), or rat mAb 3F10 (Roche).

For production of polyclonal mouse anti-ISPI (TGGT1_063420), the coding sequence for residues 60-181 of ISP4 was PCR-amplified from a *Toxoplasma* cDNA library (primers P1/P2) and cloned into pET101/D-TOPO (Invitrogen). Constructs were transformed into *E. coli* BL-21 and protein expression was induced with 0.5 mM IPTG. ISP4<sub>60-181</sub> was purified over Ni-NTA agarose (Qiagen) and injected into a BALB/c mouse (~200 μg per immunization). Sera was collected from the mouse following each boost and screened by IFA and Western blot.
For production of rabbit anti-TgCentrin1 antibody, the full length ORF of TgCentrin1 was PCR amplified from cDNA (primers P3/P4), inserted by ligation independent cloning into plasmid pAVA0421 (Alexandrov et al., 2004) and purified as described previously (Gubbels et al., 2006) to generate a His₆-tagged N-terminal fusion protein. Briefly, recombinant protein was expressed in *E. coli* BL21 STAR (DE3)pLys (Invitrogen) by induction with 0.5 mM IPTG and purified over TALON Metal Affinity Resin (Clontech). Polyclonal antiserum was generated by rabbit immunizations (Covance, Denver, PA) and affinity purified against the recombinant protein cross-linked to cyanogen bromide Sepharose 4B (Sigma).

**Immunofluorescence assays (IFA) and Western blot**

For IFA, HFFs were grown to confluency on coverslips and infected with *Toxoplasma gondii*. After 18-24 hours, the coverslips were fixed and processed for indirect immunofluorescence as previously described (Bradley et al., 2005). The coverslips were mounted in vectashield (Vector Labs) and viewed with an Axio Imager.Z1 fluorescent microscope (Zeiss) as previously described (Beck et al., 2010).

For Western blot, parasite lysates were separated by SDS-PAGE and transferred overnight onto nitrocellulose filter paper. Target proteins were detected with the indicated primary antibodies followed by secondary antibodies conjugated to horse radish peroxidase as previously described (Bradley et al., 2002).

**Endogenous Tagging and Second-Copy Expression of ISP4**

For endogenous tagging of *ISP4*, we first replaced the DHFR-TSc3 selectable marker with the HPT selectable marker in the plasmid p3xHA-LIC-DHFR (Konrad et al.) to generate the
plasmid p3xHA-LIC-HPT. A 3’ portion of the ISP4 gene was PCR-amplified (P5/P6) and inserted into p3xHA-LIC-HPT using a ligation-independent cloning approach to generate a triple HA-epitope tag fusion just before the stop codon (Huynh and Carruthers, 2009). 25 μg of the construct was linearized with EcoRV and transfected into Δku80Δhpt parasites (Beck et al., 2010). The parasites were selected in MX media (50 μg/ml mycophenolic acid and 50 μg/ml xanthine), cloned by limiting dilution, and screened by Western blot and IFA against the HA tag. A clone that had undergone the intended recombination event was selected and designated ISP4-3xHA.

To generate an ISP4 expression vector, the ISP4 gene was PCR-amplified (primers P7/P8) from a Toxoplasma cDNA library with a REV primer designed to create an in-frame HA tag fusion at the C-terminus. This PCR product was cloned into the vector pTub-YFP,YFP (Gubbels et al., 2003) between BglII/Ascl, resulting in the plasmid pTubISP4HA.

**Detergent extraction of ISP4**

For detergent extraction experiments, 3x10^7 ISP4-3xHA parasites were washed in PBS, pelleted and lysed in 1 mL TBS (50mM Tris-HCl [pH 7.4], 150mM NaCl) containing 0.5% NP-40 and Complete Protease Inhibitor Cocktail (Roche) for 15 min at 4°C. Lysates were centrifuged for 15 min at 14,000 x g. Equivalent amounts of total, supernatant, and pellet fractions were separated by SDS-PAGE and analyzed by Western blot.

**Disruption of ISP4**

5’ and 3’ flanking regions of the ISP4 gene were PCR-amplified (primers P9/P10 and P11/P12, respectively) from wild-type genomic DNA and inserted into the plasmid
pMiniGFP.ht-DHFR (Beck et al., 2010) at *NotI* and *ApaI*, respectively. 50 μg of the knockout vector was linearized with *EcoRV* and transfected into ISP4-3xHA parasites. The parasites were selected with 1 μM pyrimethamine for three passages and cloned by limiting dilution. Plaques were screened for GFP fluorescence, GFP nulls were selected, and loss of ISP4 was confirmed by IFA and Western blot. A knockout clone was isolated and designated Δisp4.

**Determination of the correct ISP4 start methionine**

ISP4 cDNA sequences were PCR-amplified from a *Toxoplasma* cDNA library either beginning from the position one methionine codon (primers P7/P13, designated ISP4 M1) or truncating the first 10 codons and beginning at the position eleven methionine codon (primers P14/P13, designated ISP4 M2). These fragments were inserted into the plasmid pTub-YFP,YFP between *BglII/AscI* (Gubbels et al., 2003). 50 μg of each vector was linearized with *PmeI* and transfected into Δisp4 parasites and selected with MX media. These transfected populations (designated ISP4 M1 and ISP4 M2) were screened by Western blot using our mouse ISP4 antisera.

**Site-directed mutagenesis**

PCR-based mutagenesis of ISP4 was carried out in the pTubISP4HA construct using the following primers (forward primer given, reverse complement was also used): C26,27S (P15), C72,73S (P16), and G15A (P17). Mutations were sequenced-verified and 25 μg of the wild-type and each mutagenized vector were linearized with *PmeI* and transfected into RHΔhpt parasites. The transfected populations were selected with MX media and screened by IFA against the HA tag.
Figure Legends

Figure 5-1. *Toxoplasma* TGGT1_063420 is a fourth member of the ISP family.

A. The expression profile of TGGT1_063420 throughout the *Toxoplasma* cell cycle was compared to the profiles of ISP1-3 and the intermediate filament-like IMC proteins of protein meshwork. Three general classes of periodic transcription profiles are observed for IMC proteins with peaks early in budding (ISP1-3, IMC1), late in budding (IMC14), or outside of budding during late G1/S (IMC7 & 12). TGGT1_063420 expression also adopts a periodic pattern indicating is it regulated in a cell-cycle dependent manner with peak expression lagging behind ISP1-3 by one hour, similar to IMC14.

B. A sequence alignment of TGGT1_063420 with ISP1-3 shows the highest degree of homology within the C-terminal two-thirds of the proteins, while the N-terminal regions are more divergent. Notably, ISP1/2/3 each contain a conserved glycine at position two, which is myristoylated, and a pair of cysteines within the first ten residues, which are palmitoylated (black boxes). However, these conserved residues, which are essential for IMC targeting, are not present within the TGGT1_063420 protein sequence. TGGT1_063420 does contain two pairs of cysteines at positions 26,27 and 72,73, which are predicted to be palmitoylated (red boxes). A single glycine residue is present upstream of the first cysteine pair at position 15 (yellow box). ISP1-3 sequences are available in GenBank under the accession numbers HQ012577-HQ012579. The TGGT1_063420 (ISP4) sequence is available under the accession number JX082399.

Figure 5-2. TGGT1_063420 (ISP4) localizes to the central sub-compartment of the IMC.

A. Western blot analysis of *Toxoplasma* lysates with anti-TGGT1_063420 detects a single band at ~20 kD, which is the predicted size of the protein.
B. Schematic showing integration of a 3xHA epitope tag at the C-terminus of the endogenous TGGT1_063420 locus.

C. Western blot analysis of parental and TGGT1_063420-3xHA lysates. Anti-TGGT1_063420 detects a band at ~20 kD in the parental strain and a band with a ~5 kD upshift (corresponding to the addition of the 3xHA tag) in TGGT1_063420-3xHA parasites (upper panel). Anti-HA detects a band with the same upshift in TGGT1_063420-3xHA parasites and nothing in the wild-type lane (lower panel), confirming that the TGGT1_063420-3xHA strain contains the expected endogenous tag of TGGT1_063420.

D. TGGT1_063420-3xHA signal co-localizes with ISP2 in the central IMC sub-compartment and thus the protein was named ISP4. Like ISP2, ISP4 signal is not detected in the apical cap or basal sub-compartments of the IMC. Green: anti-HA antibody detected by Alexa488-anti-rabbit IgG. Red: anti-ISP2 antibody detected by Alexa594-anti-mouse IgG.

E. Schematic showing the expression of a 1xHA-tagged, second copy of ISP4 under the control of the tubulin promoter.

F. Expression of ISP4-HA under the control of the tubulin promoter results in higher protein levels and better signal detection. Clear co-localization is observed between ISP4 and ISP2. Green: anti-HA antibody detected by Alexa488-anti-rat IgG. Red: anti-ISP2 antibody detected by Alexa594-anti-mouse IgG. All scale bars = 5µm.

**Figure 5-3. ISP4 localizes to the IMC in forming daughter parasites and is not imbedded in the underlying protein meshwork.**

A. IFA of parasites late in endodyogeny. ISP4 is detected in forming daughter parasites, indicating an association with the IMC and not the plasma membrane. Red: anti-HA antibody
detected by Alexa594-anti-mouse IgG. Green: anti-tubulin antibody detected by Alexa488-anti-rabbit IgG.

B. IFA of tubulin promoter-driven ISP4-HA parasites at the midpoint of endodyogeny. ISP4 signal is seen in both daughters and in the central IMC sub-compartment of mature parasites. Red: anti-HA antibody detected by Alexa594-anti-mouse IgG. Green: anti-tubulin antibody detected by Alexa488-anti-rabbit IgG. Scale bars = 5µm.

C. ISP4 is not embedded in the IMC protein meshwork. ISP4-3xHA parasites were extracted with 0.5% NP-40 and separated into total (T), soluble (S), and pellet (P) fractions. While IMC1, which is part of the detergent-resistant IMC protein meshwork, remains in the pellet fraction, ISP4 and the soluble control protein ROP1 are found in the soluble fraction.

Figure 5-4. ISP4 is relocalized to the apical cap in the absence of ISP1.

ISP4 is subject to ISP1-dependent exclusion from the apical cap. To detect the borders of the apical cap IMC sub-compartment occupied by ISP1, we utilized a pan-centrin antibody that recognizes TgCentrin1 in the centriole as well as TgCentrin2 annuli (arrowheads), which delimit the boundary between the apical cap and the rest of the IMC. In wild-type parasites (WT), ISP4 localization ends sharply at the base of the apical cap. However, in Δisp1 parasites, ISP4 is relocalized anterior to the centrin annuli into the apical cap. Red: anti-HA antibody detected by Alexa594-anti-mouse IgG. Green: anti-TgCentrin1 antibody detected by Alexa488-anti-rabbit IgG. Scale bars = 5µm.
Figure 5-5. Disruption of the ISP4 gene.

A. ISP4 knockout schematic. The 5’ and 3’ genomic flanks of ISP4 were amplified and cloned into a knock-out construct containing the selectable marker DHFR-TSC3 and a downstream GFP cassette. Transfection of the knockout vector into ISP4-3xHA parasites allows for the replacement of ISP4-3xHA with the selectable marker DHFR-TSC3 via homologous recombination. The GFP cassette provides a convenient method for identification of non-homologous recombinants.

B. Western blot probed with mouse anti-HA. ISP4-3xHA was detected in the parental strain but not in Δisp4 parasites. ROP1 serves as a loading control.

C. IFA of parental and Δisp4 parasites. ISP4-3xHA is no longer detected in Δisp4 parasites. Red: anti-IS1 antibody (mAb 7E8) detected by Alexa594-anti-mouse IgG. Green: anti-HA antibody detected by Alexa488-anti-rabbit IgG.

Figure 5-6. Determination of the correct ISP4 start methionine.

A. Schematic of M1 and M2 ISP4 expression constructs under the control of the tubulin promoter.

B. M1 and M2 constructs were expressed in Δisp4 parasites and compared to WT parasites by Western blot using anti-IS1 antibody. Expression of the M1 version of ISP4 results in a doublet with the upper band running at the same size as the endogenous ISP4 band in WT parasites. Expression of M2 produces a single band which runs at the same size as the lower M1 band, indicating the M1 methionine is the correct ISP4 start codon. A faint lower band is also seen in WT parasites that corresponds with the M2 band and lower band of M1 doublet. These lower bands suggest that the M2 methionine can serve as an alternate start codon under the
control of the tubulin promoter and less frequently in the endogenous context, but does not exclude the possibility that the lower band in WT parasites results from degradation.

**Figure 5-7. IMC targeting of ISP4 is dependent on residues predicted for palmitoylation but not myristoylation.**

Mutations of indicated residues predicted for palmitoylation were generated in an HA epitope-tagged copy of ISP4 and expressed in parasites under the control of the tubulin promoter. Wild-type (WT) ISP4-HA targets to the central sub-compartment of the IMC, similar to the endogenous ISP4 protein. Mutations of the cysteine pair at position 26,27 into serines (C26,27S) results in gross mistargeting of ISP4 throughout the cytosol of the cell, while mutation of the cysteine pair at position 72,73 (C72,73S) produces no significant effect on targeting. Mutation of the N-terminal glycine at position 15 (G15A) into an alanine also produces no significant effect on ISP4-HA targeting.

**Figure 5-8. Model of ISP sorting within the Toxoplasma IMC.**

Schematic showing the model for ISP sorting within the IMC. (1) ISP1/2/3 are myristoylated at a conserved position two glycine by an N-myristoyl transferase (NMT). (2) Myristoylation allows for ISP1/2/3 to transiently sample various membranes within the cell. (3) Once ISP1/2/3 associate with the IMC, different PATs or PAT activities located in the three IMC sub-domains recognize and palmitoylate their respective ISP substrates, locking ISP1/2/3 into specific sub-compartments. (4) ISP4 is targeted to the IMC central sub-compartment independent of a myristoylation event. (5) ISP2/3/4 are excluded from the apical cap in an ISP1-dependent manner. Modified from (Beck et al., 2010).
Figure 5-3
Figure 5-4
Figure 5-5

A

Δisp4

ISP4 KO plasmid

ISP4 3'

ISP4 5'

 DHFR

ISP4-3xHA

ISP4 3

ISP4 5

GFP

B

Parental

Δisp4

ISP4-3xHA

ROP1

28

55

C

Δisp4

Parental

Phase

ISP1

ISP4

Δisp4 (+DHFR) genomic
Figure 5-6
Figure 5-7

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Figure 5-8
Table 5-1. Primers used in this study as discussed in text. Restriction sites and mutated bases are shown in lowercase.

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Chapter 6

Conclusions and Outstanding Questions
Rhoptry biology and mechanism of invasion

**Determine the precise mechanism of apical rhoptry tethering.** The discovery that apical tethering of the rhoptries is controlled by protein palmitoylation via TgDHHC7 and requires the armadillo-repeat protein TgARO (chapter 2) provides a molecular basis for part of the unique polarization of the apicomplexan cell that has fascinated cell biologists for decades (Dubremetz and Ferguson, 2009). Identification of TgARO interacting proteins should reveal the downstream players in apical rhoptry tethering. Additionally, other targets of TgDHHC7 may exist and the full repertoire of substrates can be investigated using stable isotope labeling by amino acids in cell culture (SILAC) coupled with quantitative proteomic analysis (Martin et al., 2012; Zhang et al., 2008). Importantly, the targeting mechanism of palmitoylation-based recruitment of TgARO to the rhoptry surface is shared between *T. gondii* and *P. falciparum*, suggesting that this mechanism of rhoptry polarization is conserved and central to invasion in diverse apicomplexan species. To the best of my knowledge, this research provides the first example of palmitoylation-based control of organelle positioning. Once the precise mechanism is understood, it will be interesting to determine if other eukaryotes have employed a similar strategy for control of organelle position.

Conditional disruption of TgDHHC7 is the first mutant in *Toxoplasma* (or any apicomplexan) that specifically eliminates rhoptry function without impacting other secretory organelles. This assigns a definitive role for the rhoptries in invasion but not egress. Additionally, the dispensability of the rhoptries (as well as the micronemes) for the intracellular portion of the parasite lytic cycle (e.g., replication) reinforces the fact that the apicomplexan cell is essentially a highly specialized invasion machine with the architecture of the parasite fundamentally built around this process.
**Mechanism of invasion**

The critical importance of the MJ RON complex in host invasion, demonstrated clearly by conditional disruption of RON5 (chapter 3), shows that the indispensable role of the rhoptries in host invasion is fulfilled, at least in part, through deployment of the RON2/4/5 complex. With the development of a conditional knockdown mutant for RON5, the precise roles of the MJ RONs with the host cell can now be explored in more detail. Additionally, the concomitant knockdown of RON2 upon loss of RON5 provides a powerful new tool for exploring the function of the MJ RON complex through interaction with AMA1 on the outside of the host cell.

**Determine the functions of the MJ complex inside and outside of the host cell.** Determining the precise role(s) of the AMA1-RON2 interaction in host invasion will be key to elucidating MJ function during invasion. Is the MJ RON complex largely a structural platform for RON2-AMA1 interaction to mediate tight-junction formation, or does it have other functions? In support of the former possibility, functional analysis of RON8 suggests a role in binding elements of the host cytoskeleton to stabilize penetration (Straub et al., 2011). Thus, identification of the specific interactions that mediate RON8 recruitment to the host cortex will be an important step (Straub et al., 2009). However, the fact that RON8 is not strictly required for invasion in *Toxoplasma* and is absent entirely in hematozoans indicates RONs 2/4/5 may also interact with cortical elements as well and that these interactions may be important for the conserved invasion machinery. The host F-actin dynamics observed at the MJ raise the possibility of an interaction between the RON2/4/5 complex and actin (or actin binding proteins) and investigation of this prospect should be a priority (Gonzalez et al., 2009).
Because the primary sequence of the MJ RONs contains no recognizable domains and provides no clues to function, structural analysis is likely to be a powerful method to assess function. However, such information is likely to be difficult to obtain considering the complex is large and contains transmembrane domains (the *Toxoplasma* MJ RON complex is at least 760 kD, but may be much larger depending upon the stoichiometry of the its components). The emerging technology of CXMS, a combination of chemical crosslinking and quantitative proteomic approaches, is ideally suited to determining inter-protein interactions within the complex (Petrotchenko and Borchers, 2010; Singh et al., 2010). The resulting information would be directly applicable to the design of mutants to complement conditional knockdown of *Toxoplasma* RON5 with the ultimate goal of separating the stability of RON2 from other phenotypic aspects of invasion. CryoEM and cryo electron tomography (cryoET) might eventually be applied to obtain high-resolution 3D structural information, although the transmembrane nature of RON2 and the challenges associated with exogenous expression of a large, secreted complex are certain to present technical hurdles to reaching this goal.

Following deployment, RON2 adopts a topology in the host plasma membrane (N-terminus in the cytosol, C-terminus out) that is difficult to reconcile with its expected topology in the parasite secretory pathway (Lamarque et al., 2011). How do the MJ RONs cross (RON4/5/8) or become inserted (RON2) in the host membrane? Membranous whorls have been observed with in the rhoptries by EM and may be involved in MJ RON storage in the necks and/or injection into the host (Boothroyd and Dubremetz, 2008; Stewart et al., 1985). Additionally, how is the transient breach in the host membrane generated at the onset of invasion and what is the role of this event in injection of MJ RONs and rhoptry release (Suss-Toby et al., 1996)? Phospholipase activity may be involved in this process as it has been implicated in rhoptry
release and this possibility should be explored further (Ravindran et al., 2009; Saffer and Schwartzman, 1991).

The MJ RON ring displays a remarkably dynamic expansion and contraction during penetration and this raises questions about the nature of this structure. Is the complex itself responsible for constriction at the MJ or is this mediated by other factors, such as the physical barrier of the host cytoskeleton? It is possible that the MJ RON complex oligomerizes within the MJ and this could enable contraction of the ring, although a more parsimonious explanation is that constriction is a mere consequence of the strong binding between AMA1 and RON2. Finally, what is the mechanism for molecular filtration at the MJ and does the MJ RON complex accomplish this activity? Again, if RON2-stabilizing mutants of RON5 could be generated, it may be possible to separate and demonstrate the putative MJ roles of anchoring and sieving using the RON5 conditional knockdown. For example, one can envision a RON5 mutant that is able to stabilize RON2, enabling MJ function in gripping and penetration but not sieving. Such a mutant might be expected to ultimately lead to fusion with the host endocytic pathway following invasion. Establishment of similar conditional knockdown systems for investigation of RONs 2 and 4 will aid this process.

**Determine the signaling events and mechanisms that mediate rhoptry release.** Preliminary work points to a role for the C-terminal tail of *Toxoplasma* MIC8 in signaling rhoptry secretion but the precise events that mediate exocytosis remain unknown (Kessler et al., 2008). Identification of the MIC8 receptor would aid efforts to unravel its role in invasion. Additionally, proteomic analysis identified a phosphorylation event on serine 935 in the C-terminal tail of MIC8 and the importance of this residue for invasion should be investigated (Treeck et al., 2011). The C-terminal tail of AMA1 has also been implicated in signaling during
invasion through engagement of RON2 (Riglar et al., 2011; Sheiner et al., 2010; Srinivasan et al., 2011). Furthermore, the phosphorylation status of the AMA1 C-terminal tail is known to be important for invasion but additional studies are needed to determine if these post-translational modifications are connected with engagement of RON2 (Treeck et al., 2009). Quantitative proteomic approaches may be helpful in answering these questions and identifying the downstream effects that result from AMA1 phosphorylation.

If the AMA1-RON2 interaction does signal for rhoptry release, it would require secretion of MJ RONs to precede discharge of other rhoptry proteins. Interestingly, formation of an early stage MJ can be separated from secretion of rhoptry body proteins in Plasmodium merozoites, suggesting that rhoptry secretion may occur in distinct steps (Riglar et al., 2011; Srinivasan et al., 2011). The distinct neck and body sub-compartments of the rhoptry seems to be suited to stepwise release, although this scenario would presumably require multiple mechanisms to permit differential exocytosis of these compartments. Discovery of molecules involved in the fusion events that permit rhoptry discharge will shed light on the nature of the actual exocytosis event and the involvement of the mysterious “porosome” structure at the apex of the cell (Paredes-Santos et al., 2011).
**ISPs and cell division**

**Determine the ultimate basis for IMC sub-compartmentalization.** The elaborate ultrastructure of the IMC has been a longstanding point of interest for electron microscopists and cell biologist. Discovery and characterization of the ISPs (chapters 4 and 5) yielded a new level of molecular insight into the organization of this structure, revealing extensive compartmentalization that is established through a combination of protein palmitoylation and hierarchical constraints. Localization of TgPAT2 within the IMC (chapter 2) provides an excellent lead to study the role of palmitoylation in IMC organization and function. Initial analysis suggests that this enzyme is essential for parasite survival (data not shown) and further functional analysis using conditional approaches will reveal its precise role and provide new insight into the mechanism of ISP localization. How are PATs localized and what else is required besides palmitoylation? Since PAT activity appears to be a key basis for protein distribution in the IMC, characterization of the targeting determinants of TgPAT2 should be undertaken. Additional IMC-localized PATs may exist as several candidate PATs that display an IMC-like expression signature remain to be localized.

More fundamentally, how are the IMC alveoli generated and sutured together? This process seems likely to involve membrane fusion machinery (e.g., SNARE proteins) that has yet to be studied in apicomplexans and candidate gene approaches should be undertaken to begin characterizing this process. Finally, development of techniques for purifying IMC material would permit proteomic analysis, allowing for a better characterization of the molecules that control IMC assembly as well as identifying novel functionalities that are likely to reside within this organelle.
Determine the mechanisms that orchestrate internal budding. Gene disruption studies demonstrated a functional role for ISP2 in cell division by internal budding. Apicomplexan replication is remarkable not only in the uniqueness of the generally mechanism (internal daughter budding) but also in the flexibility it affords for the ordering of key events in the cell cycle. Determining how the parasite counts the number of daughter buds to assemble in a given round of division (e.g., two in endodyogeny vs eight or more in endopolygeny) is a key goal for understanding this process. It is expected that factors controlling this process would act prior to or at the initiation of nascent IMC assembly (Gubbels et al., 2008). Thus, the discovery that proteins which localize to the IMC after it begins to form are critical for determining the number of daughter buds produced in a given round of division came as a surprise. This suggests that the IMC serves as more than just a scaffold for building new parasites during this process but may also play key roles in signaling for specific outcomes in division. However, the possibility that abnormal division pathways occur as an indirect result of some stress created by loss of ISP2 should not be excluded. Determining why loss of ISP2 results in replication and fitness defects will require further exploration of the precise role of this protein, which will be aided by work to identify protein-protein interactions. Additionally, the ability of ISP2 knockout parasites to recover from their defects over time may provide new insight into the role of these proteins by applying microarray analysis or RNAseq approaches to identify the changes in gene expression that underlie this recovery.

Apicomplexans are highly specialized for a parasitic life and consequently, these organisms excel at rising to the challenges presented by host barriers to infection, levying a major burden of disease on the human and animal population as a consequence. Fortunately, the unique adaptations these organisms possess which enable their success as pathogens are absent
from their mammalian hosts and thus hold promise to be exploited for their control. This is particularly true in regard to the parasite lytic cycle, which is the direct cause of pathology in most diseases caused by apicomplexans (e.g., the tachyzoite lytic cycle in neural tissue in *Toxoplasmosis* and the merozoite lytic cycle during the *Plasmodium* blood stage). The combination of genetic systems and powerful “-omics” era approaches is providing enormous amounts of new insight into the critical determinants of parasite biology. Application of the results of these studies, in combination with public health initiatives, has the potential to usher in unprecedented control of the devastating diseases produced by this remarkable group of parasites.
References


