STRIATED FIBER ASSEMBLINS IN THE APICOMPLEXA

by

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(Under the Direction of Boris Striepen)

ABSTRACT

The phylum Apicomplexa is a diverse group of intracellular parasites of humans and animals. The invasive stage, the zoite, is found in each species at some point in their life cycle. The zoite is an organized and polar cell with specialized cytoskeletal elements and organelles at the apical end that aid in host cell invasion. Cell division or generation of the zoite occurs using several unconventional mechanisms. Endodyogeny is a form of internal budding, whereby two daughter cells are constructed within the mother following a single round of DNA replication and nuclear division. More complicated forms of Apicomplexan cell division include endopolygeny and schizogony, where dozens of daughters are built within a single mother cell, after multiple rounds of DNA replication with or without consecutive nuclear division. How cells ensure that each daughter receives one copy of the genome and each organelle is a key question that is the focus of this dissertation.

Previous work demonstrated that the centrosomes, from which spindle microtubules originate, associate with organelles during division, and that a striated fiber has been observed near the centrosomes in ultrastructural studies of dividing Apicomplexa. We hypothesize that daughter cells construction is organized by the centrosomes via a physical structure composed of striated fiber assemblin (SFA) proteins.

SFA genes are encoded in the genomes of many Apicomplexa. We visualized these proteins in *T. gondii* tachyzoites expressing epitope tags for immunofluorescence assays (IFA), and observed that SFA proteins have a dynamic localization pattern over the cell cycle. SFA first appears as discrete spots on or near duplicated centrosomes. As daughters are built, SFA forms a curved fiber between the centrosome and the apical end of developing daughter cells. Overexpression of the SFAs results in major division defects as well as mislocalization of other proteins involved in daughter cell formation. Antibodies created against recombinant *T. gondii* SFAs were used for IFA on the related parasite *Sarcocystis neurona*, and label structures near the spindle and apical end of developing zoites. Taken together, these results suggest that the SFA fibers play an important and conserved role in daughter cell formation in the Apicomplexa.

INDEX WORDS: Striated Fiber Assemblin, Cell Division, *Toxoplasma gondii*, Apicomplexa

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DEDICATION

For supporting me in every way possible for over a decade, and for filling every day with laughter and love, I dedicate this dissertation to my amazing husband, Jason.

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who has been instrumental in my development as an education researcher, and will continue to be an important collaborator for years to come. Even in my early days as a graduate student, Peggy always treated me as a colleague, inviting me to events and conferences, introducing me to important members of the field of biology education research, and including me in an ambitious course-redesign project. Marcus Fechheimer is a funny and thoughtful educator who has taught me the importance of careful planning and organization. Marcus has been my most outspoken supporter, conveying faith in my abilities as a teacher to myself and to others, long before I felt I deserved it. I would also like to thank the talented members of the Center for Teaching and Learning, especially Paul Quick and Denise Domizi, who lead the Future Faculty Program, which I was privileged to be a part of.

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS v

CHAPTERS

1 INTRODUCTION 1

2 REVIEW OF LITERATURE 4

2.1 The Apicomplexa 4

2.2 Structure of Invasive Stages 8

2.3 Cell Division in the Apicomplexa 13

2.4 Striated Fiber Assemblins 18

2.5 Figures 23

3 CHARACTERIZATION OF THE STRIATED FIBER ASSEMBLINS IN THE APICOMPLEXA 26

3.1 Abstract 27

3.2 Introduction 28

3.3 Results 31

3.4 Discussion 43

3.5 Materials and Methods 45

3.6 Figures and Tables 50

4 CONCLUSIONS 66

APPENDIX A: BUILDING THE PERFECT PARASITE: CELL DIVISION IN APICOMPLEXA 71

REFERENCES 89

CHAPTER 1

INTRODUCTION

The phylum Apicomplexa is a large and diverse group of protists, all of which are obligate intracellular parasites. Members of the phylum cause important diseases in humans and a wide range of animal species. Dozens of species in the order Eucoccidia are responsible for gastrointestinal maladies in domestic and livestock animals. *Cryptosporidium* species produce severe diarrhea and contribute to chronic wasting syndrome in immunocompromised hosts. Of principle significance are members of the genus *Plasmodium*, which are the causative agents of malaria, a mosquito-borne disease that claims nearly one million lives annually. The species at the center of this dissertation is *Toxoplasma gondii*, a unique and ubiquitous parasite of most every warm-blooded animal. In the US, approximately 11% of the human population is infected with *T. gondii* (Jones et al., 2007). While healthy hosts are usually asymptomatic, certain groups are at risk for serious complications of infection. Pregnant women encountering the parasite for the first time may experience abortion or have a child born with severe birth defects. In addition, acute and re-emergent infections in immunocompromised hosts can result in potentially fatal toxoplasmic encephalitis.

Apicomplexan cells come in many forms and are found in a variety of host environments. Each species must transition through multiple stages and employ both sexual and asexual replication to complete its life cycle. Sexual stages typically occur in intestinal epithelial cells, while asexual reproduction can occur in a variety of cell types. The zoite is an asexual stage found in every Apicomplexan species at some point in its life cycle. Zoites are highly organized

polar cells specialized for host cell invasion. Asexual reproduction in the zoite is achieved using one of three types of cell division, depending on the species: endodyogeny, endopolygeny, or schizogony (reviewed in (Striepen et al., 2007)). All three modes of division use an internal budding mechanism, where daughter cells are built within an intact mother cell and emerge as the final steps of construction are complete. The main difference between the three types of division is the order and number of nuclear replication and division events that occur before cytokinesis. Endodyogeny most closely resembles traditional eukaryotic cell division, where one round of DNA replication is followed by nuclear and cell division. This method generates just two daughter cells per division cycle. In endopolygeny, DNA replication occurs several times before nuclear and cell division begin. This generates a large polyploid nucleus that must be segregated into dozens of budding daughter cells. Schizogony is a blend of these two division mechanisms- multiple rounds of DNA replication and nuclear division occur first, followed by cell division. Again in schizogony, although the nuclei are divided before cell division begins, there is still the complicated task of assigning one nucleus to each daughter cell.

Thus, an intriguing question regarding Apicomplexan reproduction is how cells ensure that each daughter cell receives one set of chromosomes and organelles using such unusual division strategies. It is clear that the centrosome plays an important role in cell division of *T. gondii*- studies have shown that dividing plastids and Golgi bodies maintain an association with the duplicated centrosomes, with one centrosome close by each end of the organelle (Striepen et al., 2000; Hartmann et al., 2006). Recent work from our laboratory has also demonstrated how parasites keep track of duplicated chromosomes. Centromeres are associated with the mitotic spindle through a specialized extension of the nuclear membrane called the centrocone, and this structure and association is maintained continuously throughout the cell cycle (Brooks et al., 2011). One major hypothesis from this work is that the mitotic spindle is always tethered

to the kinetochore of each chromosome, thus providing a mechanism for maintaining organization of the nucleus during complex division processes. If this hypothesis is true, then one question still remains: how is the spindle connected to each developing daughter?

A few years ago, the combination of early transmission electron microscopy and a new paper identifying a conserved set of proteins in Apicomplexa provided the first candidate for the linking structure. Early studies on the ultrastructure of the coccidian *Eimeria* revealed a striated fiber stretching between the centrosome and conoid in developing daughters (Dubremetz, 1975; Dubremetz and Elsner, 1979). Then in 2003, a group of researchers discovered that a protein called striated fiber assemblin (SFA) was encoded in the genomes of several Apicomplexans, including four copies in the genome of *T. gondii* (Lechtreck, 2003). SFAs are self-polymerizing proteins that form a striated fiber structure in green algae associated with microtubule roots that emerge from the basal bodies of flagella (Weber et al., 1993). A single published experiment showed that antibodies generated against a green algal SFA protein stain a structure on or near duplicated centrosomes of *T. gondii* (Lechtreck, 2003). With these data in mind, we set out to explore the role of SFAs as potential connectors between the centrosomes and daughter conoids in dividing Apicomplexan parasites.

This dissertation is divided into 3 parts. Chapter 2 presents a review of the literature, including details of the cell structure and division processes of the Apicomplexan zoite, as well as a thorough description of the SFAs. Chapter 3 describes my work on the characterization of SFA proteins in *T. gondii* and related Apicomplexa, using genetic manipulation of parasites and extensive imaging by epifluorescence microscopy. My conclusions are discussed in Chapter 4, along with suggestions for the future direction of the study of Apicomplexan cell division and the SFAs.

CHAPTER 2

LITERATURE REVIEW

2.1 THE APICOMPLEXA

2.1.1 An Overview

The phylum Apicomplexa is a large group of obligate intracellular parasites of importance in both human and animal medicine. This group contains such notable human pathogens as *Plasmodium*, the causative agent of malaria, a disease which infects 500 million and kills nearly one million people every year, and *Cryptosporidium*, an intestinal parasite that causes chronic wasting in AIDS patients.

Once classified as Sporozoa, the phylum was renamed for the characteristic apical complex of organelles used for host cell invasion (Levine et al., 1980). The Apicomplexa belong to the Kingdom Chromalveolata, a group thought by some to be derived from a single endosymbiosis of a red alga by a heterotrophic eukaryote that subsequently resulted in several phyla containing secondary plastids (Harper and Keeling, 2003; Adl et al., 2005). Within this Kingdom, the Apicomplexa are contained within the superphylum Alveolata, which is defined by the presence of flattened membranes (alveoli) beneath the plasma membrane (Cavalier-Smith, 1991). In this chapter, I will focus on an order of Apicomplexa called the Eucoccidiorida, or the true Coccidia.

Eucoccidia typically infect vertebrate hosts and exhibit three major life cycle transitions (see Appendix Figure 1A). Sexual reproduction (gamogony) occurs in the intestinal tract of the

definitive host, and generates unsporulated oocysts transmitted in the feces. Sporogony occurs outside of the host, as infective sporozoites develop within the oocysts. When sporulated oocysts are ingested by a new host, excystation occurs and sporozoites infect cells and begin asexual reproduction (merogony). The number of asexual divisions varies by species. For example, *Toxoplasma gondii* can continue asexual reproduction indefinitely, while *Eimeria* species can only complete a few cycles before they must generate gamonts. Some Eucoccidia infect only in a single host species, while others use multiple species to complete their life cycle.

This study focuses primarily on *T. gondii*. While sexual stages of *T. gondii* can only occur in a feline host, other life cycle stages can be found in a myriad of animal species. Oocysts passed in feline feces will form eight infective sporozoites following sporulation (Sheffield and Melton, 1970). Ingestion of infective sporozoites in oocysts initiates infection in the intestinal epithelium of the intermediate host. The sporozoite stage is short lived, as parasites convert to the tachyzoite stage just twelve hours after invading a host cell (Dubey et al., 1997). The tachyzoite is the major proliferative stage of the parasite, able to infect most any nucleated cell. The wide infectivity of this stage helps to disseminate the infection into tissues throughout the body of the host. This acute phase of infection is limited by the immune system in most healthy hosts. It is believed that immune stressors induce the conversion from tachyzoite to the slow growing bradyzoites (Bohne et al., 1994). Bradyzoites are enclosed within tissue cysts, and are more docile than the tachyzoite and much less susceptible to attack by host immune cells. They live quietly in skeletal muscle and in immune privileged sites such as the eyes and brain, where they typically remain unnoticed for the life of the host. When a feline ingests bradyzoites in the tissue of a prey animal, they invade intestinal epithelial cells and convert to sexual stages, completing the *T. gondii* life cycle.

Recent serological surveys demonstrate the 11% of native-born US residents are infected with *T. gondii* (Jones et al., 2007), though most will never realize it, since toxoplasmosis is usually asymptomatic in healthy hosts and is rarely diagnosed during the acute phase of infection. However, life-threatening disease can occur in infants infected in the womb and immunocompromised individuals. Toxoplasmosis gained has gained notoriety as a leading cause of death among AIDS patients. Reactivation of latent tissue cysts in the brain leads to toxoplasmic encephalitis in up to 26% of individuals with AIDS (Antinori et al., 2004). In addition, the tachyzoite stage readily crosses the placenta, so women who first become infected during pregnancy can transmit the parasite to the fetus, which may result in abortion or severe congenital defects (Desmonts and Couvreur, 1974).

2.1.2 Tools for the Study of *Toxoplasma gondii*

Of all Apicomplexan species, *T. gondii* is the most thoroughly studied, and for a number of reasons. Unlike most species, the asexual tachyzoite stage of *T. gondii* is a hardy cell that can be perpetually maintained in cell culture (Roos et al., 1994). The tachyzoite reproduces quickly, doubling every 6-8 hours, so large numbers of cells can be obtained in a short period of time (Radke et al., 2001). The genome of *T. gondii* is contained on fourteen chromosomes that have been completely sequenced and well annotated. The complete genome information is publicly available on an easy-to-use online genome database (http://www.toxodb.org/). The genome database contains information on promoter regions and their activity over the cell cycle, expressed sequence tags, and the complete genome of the plastid organelle. Also annotated is a library of large (usually around 40kb) sections of genomic sequence inserted into modified double cos-site plasmids (cosmids) which are available upon request (Gubbels et al., 2008).

Most importantly to current researchers is the fact that *T. gondii* is very amenable to genetic manipulation. While transfection is not currently possible for all Apicomplexa, it is a simple and efficient process in the *T. gondii* tachyzoite (Soldati and Boothroyd, 1993). Researchers can easily insert genes with epitope or fluorescent tags, under the control of a variety of promoters with different expression levels (Striepen et al., 1998). Several drug markers are available for the selection of desired clones, and employing a combination of drugs permit the introduction of multiple changes into a single parasite line (Donald and Roos, 1993; Kim et al., 1993; Messina et al., 1995). Also, the *T. gondii* tachyzoite is haploid, which simplifies the process of genetic modification (Cornelissen et al., 1984).

Several important modified strains have been created and are readily shared among the *T. gondii* research community. For improved efficiency of homologous recombination for knocking out or endogenously tagging genes, the ΔKU80 mutant line can be used. These parasites lack the KU80 protein involved in nonhomologous end-joining, and therefore random integration of targeting plasmids is rare (Fox et al., 2009). A conditional knockout system has also been developed to create mutants of essential genes, where direct knockout is lethal. This system utilizes a parasite strain that contains a tetracycline-dependent transactivator fused to a tet-repressor protein (Meissner et al., 2002). In this strain (TATi), genes under the control of the regulatable promoter Tet7Sag4 are not expressed in the presence of anhydrous tetracycline. By transfecting an additional exogenous copy of a gene under the regulatable promoter before removing the native copy, conditional knockouts can be created for essential genes (Meissner et al., 2002; Mazumdar et al., 2006; van Dooren et al., 2008; Agrawal et al., 2009; Brooks et al., 2010; Lorestani et al., 2010). Alternatively, a regulatable promoter can also be inserted into the genome by site-directed homologous recombination to achieve conditional gene expression (Sheiner, 2011).

2.2 STRUCTURE OF THE TACHYZOITE

Invasive stages of the Apicomplexa are well organized and highly polar cells. Zoites, asexual invasive stages, are present in some form in all Apicomplexan species (see Appendix Figure 1B). In this section I present a description of the cell structures relevant to replication of the *T. gondii* tachyzoite, in order to prepare the reader for understanding the intricacies of its division process. The *T. gondii* tachyzoite is a sickle-shaped cell approximately 2 x 7 µm in size. The anterior pole of the cell is termed the apex or apical end, and the posterior pole is called the basal end.

2.2.1 Apical Structures

The phylum Apicomplexa is characterized by the apical complex of organelles and cytoskeletal structures found in the invasive zoite stage of the life cycle. This complex contains the conoid, preconoidal and apical polar rings, micronemes and rhoptries. Micronemes and rhoptries are secretory organelles with important roles in parasite motility and host cell invasion (Soldati et al., 2001; Dubremetz, 2007). Dense granules comprise another set of secretory organelles important to invasion, but these are not located at the apical end of the parasite. At the apical tip of the *T. gondii* tachyzoite, just beneath the plasma membrane, lie two ring structures: the anterior preconoidal ring and the posterior preconoidal ring (Figure 2.1) (Nichols and Chiappino, 1987). It is presumed that they provide structural stability to the apical end of the parasite, but the specific function of these rings is not known.

Of particular interest for this dissertation is the conoid, a thimble-shaped structure approximately 280 nm in length and 380 nm in diameter, located just posterior to the preconoidal rings (Hu et al., 2002). The conoid is conserved among the coccidia but absent from *Plasmodium* species and related blood-born Apicomplexa. The conoid is composed of fourteen

tightly apposed filamentous subunits that spiral counterclockwise toward the preconoidal rings (Nichols and Chiappino, 1987). The subunits are composed of a unique polymer formed from a curved sheet of nine α-tubulin protofilaments (Hu et al., 2002). The conoid is a highly motile structure, able to extend and retract from its position in the apical end of the parasite in a calcium-dependent manner (Mondragon and Frixione, 1996). Though no direct evidence for its function has been demonstrated, a role in invasion has been frequently suggested, as conoid extrusion occurs prior to and during host cell entry (Mondragon and Frixione, 1996).

Posterior to the extruded conoid sits the apical polar ring. This structure is composed of two thin ring structures apparently attached to each other (Nichols and Chiappino, 1987). Twenty-two microtubules emerge from the polar ring and are separated by 22 blunt projections, giving the structure the appearance of a cog wheel. When the conoid is retracted, it rests within the apical polar ring. Recently, the first marker for this structure was identified in *T. gondii* and given the name RNG1 (Tran et al., 2010). This protein is also encoded in the genomes of related coccidian species. RNG1 is a reliable marker for the apical polar ring in mature cells but does not appear in daughter cells until very late in division.

2.2.2 Microtubules

The *T. gondii* tachyzoite contains three distinct populations of microtubules: subpellicular microtubules, spindle microtubules, and intraconoidal microtubules. Unlike most vertebrate cells, whose cytoskeletal and spindle microtubules emerge from the same microtubule organizing center (MTOC) (Kuntziger and Bornens, 2000), in *T. gondii* each population of microtubules is nucleated from a separate structure (Nichols and Chiappino, 1987). Two of the *T. gondii* tachyzoite's MTOCs are unique to the Apicomplexa: the apical polar ring and the preconoidal ring.

The subpellicular microtubules are nucleated from the apical polar ring (Russell and Burns, 1984). This group contains 22 microtubules that spiral counter-clockwise toward the basal end. They are each 5 µM in length, with the free tip of each strand ending approximately two-thirds of the way down the parasite. This microtubule subset is critically important for maintaining the sickle shape of the tachyzoite. Treatment with the microtubule-disrupting agent oryzalin destroys subpellicular microtubules in dividing cells and results in the loss of cell shape and polarity (Stokkermans et al., 1996; Morrissette and Roos, 1998).

The spindle microtubules are a dynamic population responsible for orchestrating chromosome separation during mitosis. The Apicomplexan spindle is located within a permanent accession of the nuclear membrane, a conical protrusion called the centrocone (Dubremetz, 1973). The protein MORN1 (membrane occupation and recognition nexus) localizes to the centrocone in *T. gondii, S. neurona*, *Plasmodium falciparum* and several *Eimeria* species (Vaishnava et al., 2005; Gubbels et al., 2006). Staining with MORN1 antibodies has shown that the centrocone persists throughout the cell cycle in these parasites, but it is not clear whether the spindle itself is also maintained. Spindle microtubules which pass through an opening in the nuclear envelope at the centrocone are anchored at the centrosome, the MTOC for this microtubule subset. The centrosome contains 2 centrioles arranged in an atypical 9+1 singlet formation, as opposed to the usual 9+0 triplet arrangement (Morrissette and Sibley, 2002). Spindle microtubules are less sensitive than subpellicular microtubules to degradation by oryzalin (Stokkermans et al., 1996; Morrissette and Sibley, 2002).

Two additional microtubules are present within the conoid itself, known as the intraconoidal microtubules (Nichols and Chiappino, 1987). These are nucleated from the anterior preconoidal ring and they extend down through the conoid. They appear to be bound

to each other, are approximately 400 nm in length, and their posterior end extends into the cytoplasm. The function of the intraconoidal microtubules is unknown.

2.2.3 Inner Membrane Complex

The Apicomplexan pellicle is composed of the plasma membrane, subpellicular microtubules and a specialized structure called the inner membrane complex (IMC). As members of the superphylum Alveolata, by definition all Apicomplexa contain alveoli, membrane bound sacs located beneath the plasma membrane of cells. In the Apicomplexa, the IMC is composed of modified alveoli, flattened vesicles located between the plasma membrane and the subpellicular microtubules of parasites (Mann and Beckers, 2001). The IMC contributes to the structural stability of the parasite and plays a role in host cell invasion and in cell division (Mann and Beckers, 2001; Nishi et al., 2008). Freeze-fracture of *T. gondii* tachyzoites show that the IMC is composed of many rectangular plates along the body of the parasite and a single cone-shaped plate at the apex, called the apical cap (Porchet and Torpier, 1977). A recent study identified three proteins specific to sub-compartments of the IMC plates and the apical cap (Beck et al., 2010). In *T. gondii* tachyzoites, ISP1 is localized to the apical cap, ISP2 covers a central section of IMC, and ISP3 is found in both the central and basal regions. Parasites lacking ISP1 show mis-targeting of the other ISP proteins but retain the ability to divide, while ISP2 mutants have defects in daughter cell formation (Beck et al., 2010).

Lining the cytoplasmic face of the IMC is an organized network of 10 nm filaments that covers the entire length of the tachyzoite except for the conoid at the apical tip (Mann and Beckers, 2001). The network connects to the polar ring at the apical end and ends in a cup-shaped structure at the basal end. These filaments are composed of proteins homologous to articulins, proteins that make up the cytoskeleton of many algae and protists (Marrs and Bouck,

1992). The prediction that IMC proteins form coiled-coil structures also suggests a resemblance to intermediate filament proteins. In *T. gondii*, 14 IMC proteins have been identified to date, each containing repeats of an alveolin motif (Anderson-White et al., 2011). The subcellular localization and expression timing of the group is diverse. The majority of IMC proteins are upregulated during daughter budding and downregulated in G1 and S phase and heavily label daughter buds, but a few proteins display the opposite expression pattern and localize exclusively to the cytoskeleton of mature cells (Anderson-White et al., 2011).

In this study, we used an antibody to IMC1 as a marker for the *T. gondii* IMC. This protein is expressed at a high level and localizes to the entire pellicle of both mature and developing cells equally, making it a good choice to use as a reference to identify the cell cycle stage and the position of budding daughters. The IMC in *T. gondii* daughters is relatively unstable and detergent soluble, but proteolytic processing of the C terminus of IMC1 late in budding increases the stability and insolubility of the IMC in mature cells (Mann et al., 2002). *Plasmodium berghei* sporozoites deficient in IMC1 show defects in cell shape and development and are unable to infect host cells (Khater et al., 2004).

2.3 CELL DIVISION IN THE APICOMPLEXA

2.3.1 Modes of Division

Parasites in the phylum Apicomplexa have both sexual and asexual stages in their life cycle. Sexual stages usually occur in the intestinal tract of the definitive host, while asexual replication can happen in a myriad of tissues and hosts. Three main types of asexual reproduction are observed in the Apicomplexa: endodyogeny, endopolygeny, and schizogony

(see Appendix Figure 3). A single species often employs different mechanisms for each life cycle stage.

Arguably the simplest type of cell division found in the Apicomplexa, endodyogeny is used by zoites of some coccidian species, including *T. gondii* (Goldman et al., 1958). Endodyogeny most closely resembles traditional cell division, in that the nucleus replicates once per cell division cycle. Schizogony is the most common mode of division used by Apicomplexan species, observed in most species during some stage of their life cycle. In this process, several rounds of DNA replication and nuclear division occur before daughter budding begins (Bannister et al., 2000). Endopolygeny is perhaps the most complex and least understood type of Apicomplexan cell division. Using this approach, DNA is replicated several times without nuclear division, creating a large polyploidy nucleus (Speer and Dubey, 1999; Vaishnava et al., 2005).

During endogyogeny, two daughter cells are built within an intact and polarized mother zoite, allowing the mother cell to remain invasive throughout much of the replication process. In both schizogony and endopolygeny, the mother cell is a schizont, a non-polar precursor to the polar zoites that will emerge from division. All forms of division in the Apicomplexa use endomitosis, also called closed mitosis, meaning that the nuclear envelope never breaks down. Chromosome condensation during mitosis is minimal, and individual chromosomes are not visible. The nucleolus remains visible throughout the division process (Sheffield and Melton, 1968).

2.3.2 Endodyogeny in *T. gondii*

Replication of the *T. gondii* tachyzoite begins with the duplication of the centrosomes. The details of centrosome duplication are not entirely clear, but one study suggests that the centrosome moves from its normal position apical of the nucleus to the posterior side of the

nucleus, duplicates, and then returns to its starting position as cell division commences (Hartmann et al., 2006). Following duplication, several organelles are associated with the centrosomes in preparation for cell division. The apicoplast, a relict plastid organelle present in the majority of apicomplexan parasites, maintains an association with the centrosome at all times, and prior to division, one centrosome is observed at each end of the organelle (Striepen et al., 2000). Golgi and centrosome duplication and fission occur concurrently, and the divided Golgi resumes contact with the centrosomes before daughter cell construction commences (Hartmann et al., 2006).

After centrosome duplication, replication of the centrocone takes place (Appendix Figure 4A and 4B). A funnel-shaped nuclear extension forms between the two centrosomes, and the spindle microtubules stretch between them, within the bounds of the nuclear membrane (Ferguson, 2007). Conical extensions of the nuclear membrane form between the spindle microtubules and the centrosomes to become the centrocones (Sheffield and Melton, 1968). Kinetochores of replicated chromosomes gather near the nuclear face of the centrocone (Ferguson, 2007). In fact, recent work suggests that the spindle microtubules are in contact with chromosome centromeres constantly throughout the cell cycle (Brooks et al., 2011).

At this point, construction of the daughter cell pellicle begins near the centrosomes. The very early stages of division have been most clearly described during schizogony of *Eimeria* species, but ultrastructural studies of *T. gondii* endodyogeny suggest that the process is very similar for tachyzoites. The pellicle is first seen as a flat IMC vesicle with a few microtubules associated on the side facing the centrosome (Dubremetz and Elsner, 1979). As daughter formation progresses, the IMC grows toward the centrosome and takes on a dome shape, and the conoid and subpellicular microtubules become visible (Sheffield and Melton, 1968; Agop-

Nersesian et al., 2010). From an early stage, an electron dense striated fiber can be seen reaching from the centrosome to the conoid of each daughter cell (Dubremetz, 1975; Dubremetz and Elsner, 1979; Ferguson, 2007). As the conoids move farther away from the centrosomes during daughter growth, the fiber structure elongates between them. It has been suggested that this fiber may function to connect the centrosomes and associated organelles to the cytoskeleton of developing daughters. Examination of serial sections of daughter conoids shows the striated fiber spiraling around the conoid through multiple planes of view (Dubremetz, 1975).

As the daughters develop, their IMC and subpellicular microtubules extend posteriorly in the direction of the centrosomes, eventually containing the centrosomes and all associated organelles within the developing cells. The leading edge of the growing daughter pellicle is capped with MORN1 (Appendix Figure 4C) (Gubbels et al., 2006). The MORN1 ring provides a convenient marker to track the development of a daughter cell, and the size of the ring indicates how far along daughter construction is at any given time. As the daughters fill the mother cytoplasm and duplicated but not yet divided organelles are pulled into daughter cells, the elongated apicoplast and nucleus bend into U shapes (Striepen et al., 2000). This bending is likely due to both the force of the daughter IMC growing into them and the pulling of the organelle ends by associated centrosomes. When the bi-lobed nucleus moves into the daughter cells, the endoplasmic reticulum is carried in along with it.

As the daughter cells near completion, fission of the U-shaped apicoplast occurs simultaneously with the closing of the MORN1 ring (Appendix Figure 4D)(Gubbels et al., 2006). A dynamin-related protein has been shown to be required for apicoplast fission in *T. gondii* (van Dooren et al., 2009). Daughter micronemes and rhoptries are generated de novo while the

mother organelles are degraded (Nishi et al., 2008). The mitochondria, though replicated early in the division process, are the last components to enter the daughters, just before they emerge from the mother cell (Nishi et al., 2008). Once the growing cells have filled the cytoplasm of the mother, the maternal IMC is disassembled, and the daughter IMC comes into contact with the inside of the mother's plasma membrane (Sheffield and Melton, 1968). In places where the daughter IMC is not in contact with the plasma membrane, such as between the two daughters when they are oriented side-by-side, vesicles form and fuse to create new plasma membranes (Sheffield and Melton, 1968; Agop-Nersesian et al., 2010). Any remaining portions of membranes, cytoplasm or organelles not included in the new cells collects in the residual body outside of the basal end of the tachyzoites.

2.3.3 Cell Cycle Control in *T. gondii*

The cell cycle of *T. gondii* varies somewhat from other eukaryotic cells. Parasites do not follow the usual transition from growth (G1) to synthesis (S) to growth (G2) to mitosis (M) to cytokinesis. G2 is entirely absent in *T. gondii*, and there is considerable overlap between S and M phase. In mid S phase, centrosome and Golgi duplication occurs, and daughter cell formation begins when the DNA content reaches 1.8x in late S phase (Gubbels et al., 2008). Although DNA replication is completed and chromosomes are segregated shortly after budding commences, the nucleus does not divide completely until late in daughter cell budding.

Previous studies demonstrating the ease with which cell cycle control was abolished using pharmacological agents led to the hypothesis that Apicomplexa may lack important cell cycle checkpoints (Shaw et al., 2001; Morrissette and Sibley, 2002). However, several key eukaryotic checkpoints are encoded in the genomes of Apicomplexan parasites, though little is known about their role in cell cycle regulation in this phylum. Recently, important strides have

been made in identifying controlling elements of the cell cycle in *T. gondii*. Using a forward genetic screen of temperature-sensitive random mutants, dozens of mutants were found that arrest at crucial stages of the cell cycle (Gubbels et al., 2008). Of particular interest to this work are those who failed to properly segregate DNA or organelles to developing daughters. Several uncoupling mutants were described, that could not successfully segregate the nucleus into daughter cells. Another mutant was found to have a defect in a NIMA-related serine/threonine kinase (Nek) gene, and was unable to complete mitosis or initiate daughter cell budding. In the Nek mutant and an uncoupling mutant, mitotic spindles were disorganized or absent (Gubbels et al., 2008). These observations suggest that the spindle may act as a physical checkpoint controlling chromosome copy numbers, daughter cell budding, and nuclear segregation. This hypothesis is supported by earlier work showing that loss of the spindle microtubules uncouples nuclear division and daughter cell budding in *T. gondii* (Morrissette and Sibley, 2002).

2.4 STRIATED FIBER ASSEMBLINS

2.4.1 Structural and Biochemical Description

Striated microtubule-associated fibers (SMAFs) were first described in the green alga *Oedogonium cardiacum* (Hoffman, 1962), and have since been identified in a number of green algae (Stearns et al., 1976; Melkonian, 1980; Lechtreck and Melkonian, 1991; Geimer et al., 1997). They are found running parallel to microtubule rootlets emerging from basal bodies at the base of flagella. Ultrastructural analysis of SMAFs in *Spermatozopsis similis* showed that the filaments are 2 nm in diameter with regular striations every 30 nm (Figure 2.2)(Lechtreck and Melkonian, 1991). The periodicity of striations in other algal species studied is similar but not exact.

The predominant component of SMAFs is a protein called striated fiber assemblin (SFA) (Weber et al., 1993). SFA proteins range from 30 - 34 kDa in size and have a unique domain structure that has been best analyzed in *C. reinhardtii*, which encodes a single SFA gene (Lechtreck et al., 1996; Lechtreck, 1998). At the N terminus is the head domain, a non-helical segment of 29 amino acids. This domain is rich in serine and threonine and, in the green algae, contains all of the protein's proline residues. Near the end of the head domain an SP repeat is found, often in the context SP(M/T)R, a likely phosphorylation site which may also be a substrate for the cell cycle checkpoint protein $p34^{cdc2}$ kinase (Moreno and Nurse, 1990). The remaining 247 C terminal residues of SFA comprise the rod domain, an alpha-helical structure of repeating coiled-coil forming sequences. Structural predictions based on sequence as well as direct measurements of purified *S. similis* SFA protein indicate that the rod domain is 85% helical (Weber et al., 1993). A 29 residue pattern composed of 4 heptads repeats 8 times, with a skip residue between each repeat. While the amino acid sequence of the repeats varies between SFAs of different species, the length of the repeats is consistent, and the skip residues are highly conserved (Lechtreck and Silflow, 1997; Lechtreck, 2003; Harper et al., 2009).

Analysis of the functions of the head and rod domains as well as the skip residues were conducted by examining the effects of sequence mutations on the structure of resulting SFA fibers (Lechtreck, 1998). Recombinant protein encoding either wild type or mutated *C. reinhardtii* SFA was expressed and allowed to reassemble into fibers. Electron microscopy of the fibers revealed that wild type recombinant proteins and those whose mutations were limited to the first 10 amino acids assembled into fibers with normal periodicity. Removal of more than 10 residues from the N terminus resulted in proteins that failed to form fibers, indicating that the head domain is required for fiber assemblin in *C. reinhardtii*. Substitution of the polar skip

residues with non-polar amino acids altered the pattern of striations in the reassembled fibers. Addition or deletion of one or more rod domain repeats also changed the striation patterns.

SMAFs are likely related to the kinetodesmal fibers of ciliates. Ultrastructural analysis of kinetodesmal fibers in *Paramecium tetraurelia* has shown that the size and striation patterns are similar to SMAFs, as is their location near basal bodies at ciliary roots (Sperling et al., 1991). Similar striated fibers have also been described in *Giardia* species associated with microtubules of the adhesive disk in trophozoites. Isolation and in vitro reassembly of the cytoskeletal protein β-giardin resulted in 2.5 nm fibers with striations every 30 nm (Crossley and Holberton, 1985). While sequence analysis shows only 20% identity between the *C. reinhardtii* SFA and *G. lamblia* β-giardin, they have 42% sequence similarity, and they are the exact same length (Baker et al., 1988; Weber et al., 1993). Their relationship is further supported by structural evidence suggesting that β-giardin has strong alpha helix forming ability caused by eight repeats of 29 residues each (Holberton et al., 1988).

2.4.2 Localization in the Green Algae

Most of the work to characterize the localization of SFA has been completed in *C. reinhardtii*. Antibodies raised against purified *S. similis* SFA was used to visualize the changing position of SFA in *C. reinhardtii* (Lechtreck and Silflow, 1997). Throughout the cell cycle, SFA is associated with the basal bodies near the flagella and the four microtubule roots emerging from them. Two of these microtubule roots are 2-stranded and the other two are 4-stranded. During interphase, SFA is present at the intersection of the four microtubule roots. The SFA structure is cross-shaped, with a bright dot at the center and one fiber extending along each microtubule root. In prophase, the 4-stranded microtubules extend into branching structures, and the associated SFA fibers are disassembled. The 2-stranded microtubules shorten and the SFA fibers

associated with them are reinforced. By the end of prophase, the SFA cross structure has separated into two fibers that are perpendicular to each other. In metaphase and into anaphase, SFA is localized to two punctate structures at each spindle pole. At this point, each spindle has one 2-stranded and one 4-stranded microtubule root, and each root has a spot of SFA associated. In telophase, the 2-stranded root stretches curving across the cell and is covered by a long fiber of SFA, thicker at the base and tapering as it reaches the root end. During cytokinesis, SFA returns to the base of all microtubule roots (four per spindle) as the cell completes division. The 2 cross shapes are positioned in the center of the cell at the cleavage furrow, and are asymmetrically curved outward away from each other.

Based on the position of SFA during cell division in *C. reinhardtii*, researchers have proposed that SFA fibers function to organize and align basal bodies and developing microtubule roots (Lechtreck and Silflow, 1997). Support for this hypothesis includes the observation that newly formed basal bodies are attached to 2-stranded microtubule roots by SFA fibers in *C. reinhardtii* gametes (Weiss, 1984). In addition, it has been shown that microtubule nucleation occurs preferentially at the junction of SFA and microtubule roots on isolated basal bodies of the related green alga *Polytomella agilis* (Stearns et al., 1976).

2.4.3 Conservation in the Apicomplexa

Mining of the available Apicomplexa genome sequences has revealed putative SFA genes in several species. Five *Plasmodium* species were found to encode two distinct SFA genes, termed SFA1 and SFA2 (Lechtreck, 2003). One SFA gene was identified in the piroplasm Apicomplexa species *Theileria annulata* and *Babesia bovis*, as well as in *Cryptosporidium parvum*. Initially, only three SFAs were believed to be encoded in *T. gondii*, but we have identified a fourth. PfSFA2 and TgSFA2 are the closest homologs to the green algal SFA, while

TgSFA4 aligns best with the *G. lamblia* β-giardin. Comparison of the *T. gondii* and *C. reinhardtii* SFAs reveals some clear similarities and differences. The non-helical head domain required for fiber formation in *C. reinhardtii* SFA is absent or poorly conserved in each of the *T. gondii* proteins. However, the rod domain shows high similarity, increasing toward the C terminus. In addition, the skip residues separating 29 bp repeats are highly conserved.

Though four homologs are found in the *T. gondii* genome, we have reason to believe that only three are expressed in the tachyzoite stage. Genome-wide gene expression studies on synchronized populations of *T. gondii* tachyzoites were recently published on the *T. gondii* genome database (http://www.ToxoDB.org/toxo) (Behnke et al., 2010). Analysis of this data for TgSFA2 (Gene ID: TGGT1_063240), TgSFA3 (Gene ID: TGGT1_029850) and TgSFA4 (Gene ID: TGGT1_122350) confirmed that all three genes are expressed in *T. gondii* tachyzoites. Examination of the available data for TgSFA1 (Gene ID: TGGT1_042960) revealed that no mRNA expression was measured. In addition, no expressed sequence tags corresponding to TgSFA1 have been identified and no promoter activity has been measured, suggesting that TgSFA1 is not expressed in tachyzoites of *T. gondii*. Serial analysis of gene expression (SAGE) tagging data indicates that TgSFA1 may be expressed in the conversion to the bradyzoite stage (Radke et al., 2005).

Microarray analysis of mRNA levels throughout the cell cycle demonstrates a dynamic expression profile for each of the *T. gondii* and *Plasmodium* SFAs. In *T. gondii*, the SFAs are upregulated in S phase, reach their peak during mitosis, and are sharply downregulated immediately following cytokinesis (Behnke et al., 2010). An analogous expression pattern is seen in *Plasmodium* species, where the SFAs are upregulated at the onset of schizogony in intraerythrocytic development (Bozdech et al., 2003).

A single experiment has been published that confirms SFA expression in *T. gondii* (Lechtreck, 2003). An immunofluorescence assay was performed on extracellular *T. gondii* tachyzoites using antibodies raised against centrin and SFA proteins purified from *S. similis*. The results show that SFA co-localizes with centrin shortly after centrosome duplication. As the centrosomes move apart, SFA forms a structure adjacent to the centrosomes, initially small and round and transitioning into a short ovoid shape. This finding, combined with observations of striated fibers in developing *Eimeria* zoites, inspired this dissertation research.

FIGURES AND TABLES

Figure 2.1: The apical cytoskeleton of *Toxoplasma gondii*. The apical end of *T. gondii* is an intricate structure composed of cytoskeletal elements, multiple membranes, and invasive organelles. AC- apical cap, ACR- anterior preconoidal ring, CS- conoid subunit, IMC- inner membrane complex, IMT- intraconoidal microtubule, PCR- posterior preconoidal ring, PM- plasma membrane, PR- apical polar ring, SMT- subpellicular microtubules. Modified from [23] with permission of publisher.

Figure 2.2: Ultrastructure of striated fibers. Recombinant *C. reinhardtii* SFA protein self-polymerizes to form striated fibers *in vitro*. Scale bars represent 500 nm (a) and 100 nm (b). Reprinted from (Lechtreck, 1998) with permission of publisher.

Figure 2.3: Localization of SFA structures in *Chlamydomonas reinhardtii*. Immunofluorescence images using anti-tubulin and anti-SFA antibodies demonstrate the changing localization of SFA over the cell cycle. A- interphase, B- prophase, C- metaphase, D- anaphase, E- telophase, F-cytokinesis. Reprinted from (Lechtreck and Silflow, 1997) with permission of publisher.

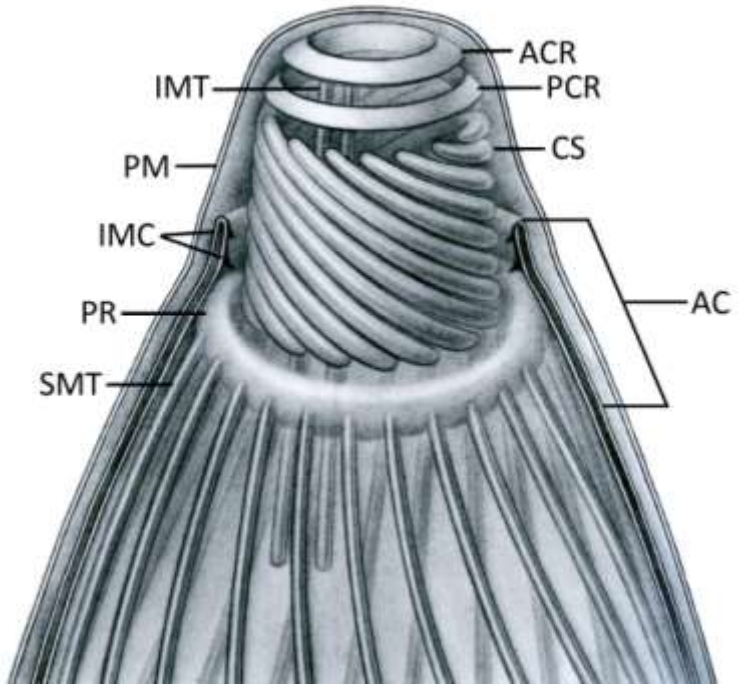

Figure 2.1: The apical cytoskeleton of *T. gondii*.

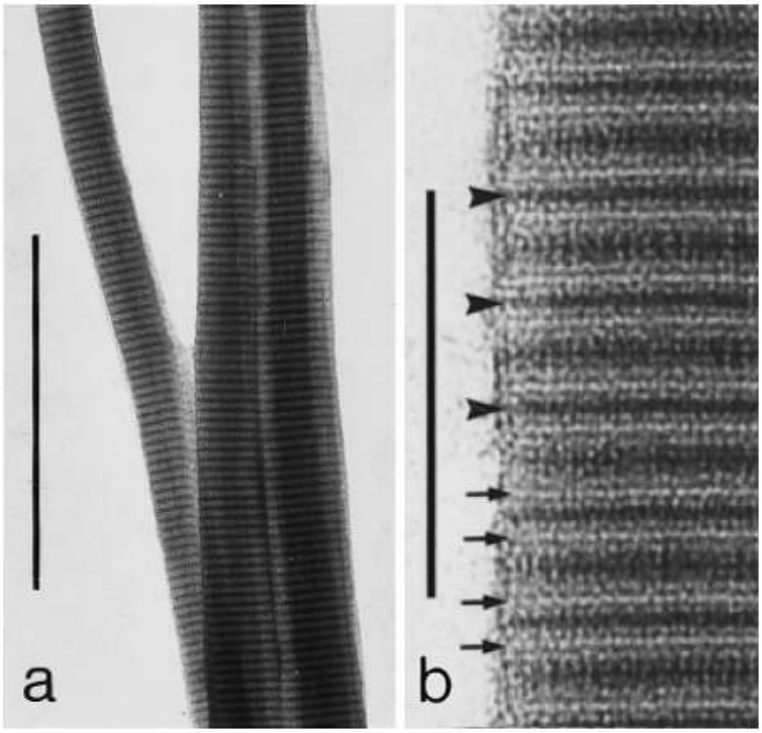

Figure 2.2: Ultrastructure of striated fiber assemblin structures.

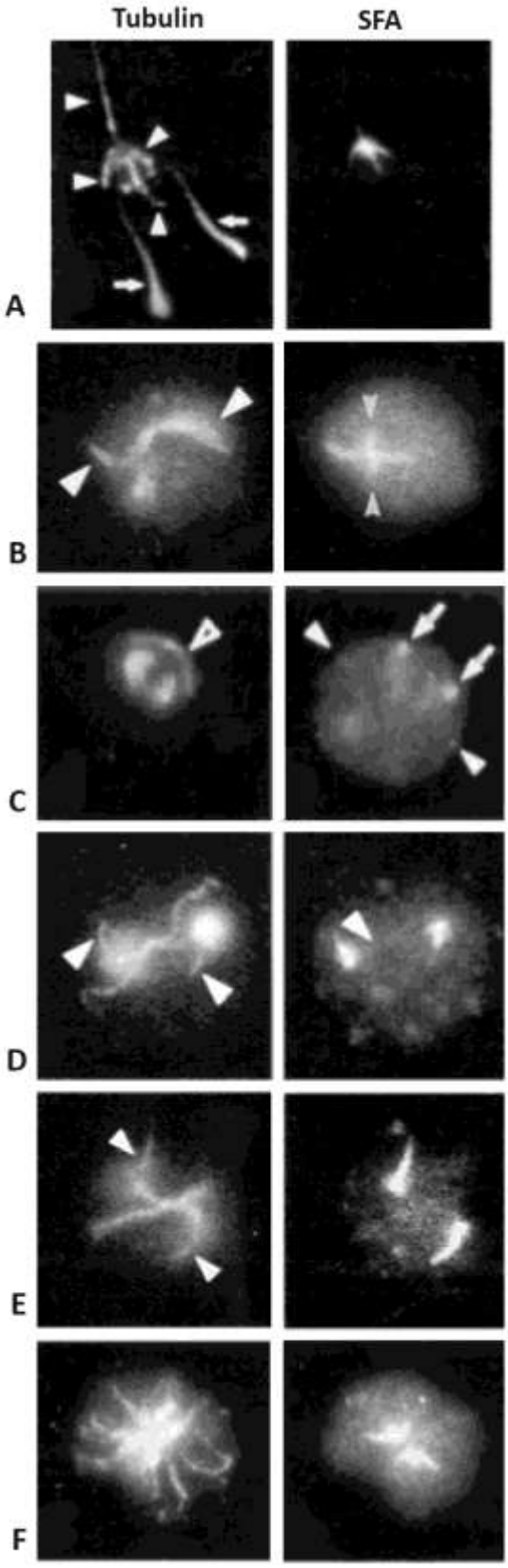

Figure 2.3: Localization of SFA structures in *Chlamydomonas reinhardtii*.

CHAPTER 3

CHARACTERIZATION OF THE STRIATED FIBER ASSEMBLINS IN THE APICOMPLEXA[1]

[1] Jordan, C.N., J.D. Patel, M.E. Francia, J.F. Dubremetz and B. Striepen. To be submitted for publication.

3.1 ABSTRACT

Toxoplasma gondii is a ubiquitous protozoan parasite present in more than 30 million Americans. It is a member of the phylum Apicomplexa, which contains several other important human and animal parasites including *Plasmodium* and *Cryptosporidium* species. *T. gondii* is a highly polar cell that divides by a unique internal budding mechanism called endodyogeny. During this process, two cells are built within the mother cell and the divided nuclei and organelles are segregated to each daughter cell by association with the centrosomes. Striated fiber assemblin (SFA) is a microtubule associated protein believed to stabilize microtubule roots emerging from basal bodies of *Chlamydomonas reinhardtii* and other green algae. We have identified several SFA homologs in *T. gondii* and other Apicomplexan parasites, and in this paper we report the characterization of these proteins in dividing parasites by immunofluorescence microscopy. We show that SFA proteins are dynamic over the cell cycle, first appearing at duplicated centrosomes and then forming fibers connecting the centrosomes to the developing cytoskeleton of daughter cells. Overexpression of the SFAs in *T. gondii* results in dramatic division defects and alteration of key division structures. We propose that SFAs are the link that ties centrosomes to developing daughters, assuring proper segregation of chromosomes and organelles to each new cell.

3.2 INTRODUCTION

Toxoplasma gondii is an intracellular parasite infecting warm-blooded hosts across the globe. Though mostly asymptomatic in healthy hosts, Toxoplasmosis can be fatal for infants that encounter it in utero and for immunocompromised individuals. Closely related to the causative agents of malaria and cryptosporidiosis, as well as several financially important diseases of livestock, the study of *T. gondii* biology may help us understand more about the entire Apicomplexa phylum.

Cell division in the Apicomplexa is a fascinating subject of study. Invasive stages of the Apicomplexa have a unique and highly polar cell organization, with a specialized set of invasion organelles and cytoskeletal structures at the apical end (Dubremetz et al., 1998; Hu et al., 2006). The challenge of rebuilding the apical complex and maintaining proper organization has generated unique cell division mechanisms. Apicomplexan division is also interesting to study from an evolutionary standpoint, as several species contain a remnant algal-derived plastid, the apicoplast, which must be divided and segregated into new cells prior to cytokinesis (McFadden et al., 1996). Our focus in this paper is cell division in the invasive stage of *T. gondii*, the tachyzoite.

Asexual reproduction in the zoite is achieved using one of three types of cell division, depending on the species: endodyogeny, endopolygeny, or schizogony (reviewed in (Striepen et al., 2007)). *T. gondii* divides by perhaps the simplest mechanism found in the Apicomplexa, a type of internal budding called endodyogeny (Sheffield and Melton, 1968). In this process, two daughter cells are constructed within the mother cell, and duplicated organelles are drawn into the developing cells. Budding in *T. gondii* begins in late S phase, when the daughter pellicle, composed of the inner membrane complex (IMC) and subpellicular microtubules, can first be

seen (Gubbels et al., 2008). Nuclear and apicoplast division take place concurrently with daughter cell formation. Daughter cell construction is completed as they emerge from the mother cell, taking her plasma membrane for their own (Morrissette and Sibley, 2002). Two variations on endodyogeny are also present in the Apicomplexa: schizogony and endopolygeny. The main difference between these reproductive mechanisms is the order and number of nuclear replication and division events that occur before cytokinesis. In endopolygeny, DNA replication occurs several times before nuclear and cell division begin. This generates a large polyploid nucleus that must be segregated into dozens of budding daughter cells. In schizogony, multiple rounds of DNA replication and nuclear division occur first, followed by cell division. Again in schizogony, although the nuclei are divided before cell division begins, there is still the complicated task of assigning one nucleus to each daughter cell.

Despite the wealth of research devoted to Apicomplexan cell division, many questions remain unanswered. One issue that has been addressed in several studies is how parasites faithfully segregate all of the necessary cellular components to the daughters. It is widely assumed among Apicomplexan researchers that the answer lies in the centrosome (discussed in (Hartmann et al., 2006; Striepen et al., 2007; Gubbels et al., 2008)). Data suggests that the centrosome is a major organizer of both the chromosomes and organelles during daughter cell formation. Centrosome association during division has been observed for the apicoplast, Golgi and nucleus (Striepen et al., 2000; Vaishnava et al., 2005; Hartmann et al., 2006). In the Apicomplexan zoite, the mitotic spindle is housed in a unique extension of the nuclear envelope called the centrocone, and this structure is observed throughout the cell cycle in both *T. gondii* and the related parasite *Sarcocystis neurona* (Vaishnava et al., 2005; Gubbels et al., 2006). Recent work has also demonstrated that the centromeres are constantly associated with the mitotic spindle in *T. gondii* (Brooks et al., 2011). Based on these observations, we propose that

the mitotic spindle is always tethered to the kinetochore of each chromosome, thus providing a mechanism for maintaining organization of the nucleus during complex division processes. If this hypothesis is true, then one question still remains: how is the spindle connected to each developing daughter?

Striated fibers are found associated with microtubules emerging from basal bodies in green algae, and are known to be composed of a protein called striated fiber assemblin (SFA). SFA was first described in the green algae *Spermatozopsis similis* as a self-polymerizing protein that forms striated filaments alongside the microtubule roots of basal bodies (Lechtreck and Melkonian, 1991). SFAs are ~30 kDa proteins that form polymer fibers associated with microtubules (Lechtreck and Melkonian, 1998). SFA polymers are polar, 2 nm wide, and are thought to be composed of dimers with overlapping N-terminal domains. SFA proteins have been most thoroughly characterized in *Chlamydomonas reinhardtii*, where they are the predominant component of the striated microtubule-associated fibers (SMAF). These fibers run alongside microtubule roots that emerge from basal bodies, and are often observed in a cross pattern of two short and two long fibers (Lechtreck and Silflow, 1997). SMAFs are rigid and biochemically stable, and are believed to act in stabilizing associated microtubules (Lechtreck and Melkonian, 1998).

Some researchers have hypothesized that striated fibers could function in organelle positioning based on their location during division events (Goodenough and Weiss, 1978). Recent work has also demonstrated a relationship between the centrins and SFA proteins. In *C. reinhardtii,* proper localization of SFA and construction of striated fibers is dependent on centrin (Taillon et al., 1992; Schoppmeier et al., 2005). In *T. gondii*, a single published immunofluorescence assay (IFA) using green algal anti-centrin and anti-SFA antibodies label

structures associated with centrosomes following duplication (Lechtreck, 2003). In both *T. gondii* and *Eimeria* species, developing zoites demonstrate an electron dense fiber stretching between the centrosome and conoid (Dubremetz, 1975; Dubremetz and Elsner, 1979; Ferguson, 2007). In high magnification views, the fiber appears to have regular striations (Dubremetz, 1975). Serial sectioning of developing *Eimeria necatrix* merozoites reveals the corkscrew shape of the striated fiber as it curls around the inside of the conoid (Dubremetz, 1975). We hypothesize that this fiber may be the link connecting the mitotic spindle to each daughter cell, and that the structure is composed of SFA proteins.

Four putative SFA homologs have been annotated in the *T. gondii* genome (Lechtreck, 2003). Using a combination of epifluorescence and immuno-electron microscopy, we have thoroughly characterized the localization of these proteins in *T. gondii*. By altering expression levels, we also show disruption of daughter cell formation following overexpression of SFA, which suggests that these proteins play an important role in cell division of *T. gondii*.

3.3 RESULTS

3.3.1 Identification of SFA Genes in *T. gondii*

The *T. gondii* genome contains four SFA homologs, and three are likely expressed in the tachyzoite stage (Figure 3.1A). TgSFA2 (Gene ID: TGGT1_063240) is well conserved, with 24% identity with *C. reinhardtii* SFA. TgSFA2 is named for its close homology (27% identity) to the previously identified *Plasmodium falciparum* SFA2 gene (Lechtreck, 2003). TgSFA3 (Gene ID: TGGT1_029850) is the closest in sequence with the *C. reinhardtii* SFA at 27% identity, despite having a truncated N terminus. TgSFA4 (Gene ID: TGGT1_122350) is most similar (16% identity)

to β-giardin, a related striated fiber protein found in adhesive disks of *Giardia* species (Crossley and Holberton, 1985).

Genome-wide gene expression studies on synchronized populations of *T. gondii* tachyzoites were recently published on the *T. gondii* genome database (http://www.ToxoDB.org) (Behnke et al., 2010). Analysis of this data for TgSFA2, TgSFA3 and TgSFA4 confirmed that all three genes are expressed in *T. gondii* tachyzoites. Furthermore, our analysis demonstrates that the expression of these genes is upregulated in S phase and downregulated promptly following division (Figure 3.1B). Examination of the available data for TgSFA1 (Gene ID: TGGT1_042960) revealed that no mRNA expression was measured. In addition, no expressed sequence tags corresponding to the gene have been identified and no promoter activity has been measured, suggesting that TgSFA1 is not expressed in tachyzoites of *T. gondii*.

3.3.2 SFA2 Localization in *T. gondii* is Dynamic Over the Cell Cycle

A strain of *T. gondii* was recently engineered lacking the Ku80 gene, a component of the non-homologous end joining pathway. As a result, the probability for homologous recombination in this line is greater than in wild type parasites (Huynh and Carruthers, 2009). This strain (ΔKu80) is therefore especially useful for creating endogenously tagged parasites, as random insertion due to non-homologous recombination is rare. Using a plasmid generated by Huynh and Carruthers (2009), we constructed a vector to insert three consecutive hemagglutinin (HA) sequences at the C terminus of TgSFA2 along with a chloramphenicol acetyl transferase (CAT) selectable marker (Figure 3.2 A). We linearized the plasmid in the coding region of TgSFA2 and transfected the DNA into ΔKu80 parasites. We then performed Southern blot analysis to determine if the native locus of TgSFA2 was modified by the insertion of a 3xHA

tag (Figure 3.2 B). We created a probe located in the 3'end of the TgSFA2 coding region, and digested genomic DNA with XhoI. Probing the digested native locus would generate a band of 4.0 kb. Modification of the locus would insert an additional XhoI site after the 3xHA tag, changing the size of the probe-containing region to 1.0 kb. Our results show that the clone E10 has been successfully modified by the 3xHA tag, as indicated by the presence of a 1.0 kb band and the absence of the 4.0 kb band corresponding to the native locus. The Southern blot also shows two additional bands that suggest insertion of the plasmid at random locations. We note again that plasmid transfected contained only a portion of the TgSFA2 coding region, and the only complete coding sequence contained in these additional insertions is the drug selection marker chloramphenicol acetyl transferase (CAT). Western blot using anti-HA antibodies confirms that the only protein labeled has a mass of approximately 33 kDa, the size of the SFA2 with a 3xHA fusion (Figure 3.2 C).

We characterized the localization of SFA2 in these parasites by IFA using anti-HA antibodies to visualize SFA2, in combination with antibodies labeling the centrosomes (Centrin), inner membrane complex (IMC1), centrocone (MORN1), and DNA (DAPI). These antibodies are informative markers for observing cell cycle progression and the growth of daughter buds. MORN1 (membrane occupation recognition nexus) is an essential protein of *T. gondii*, and is highly conserved in all Apicomplexa for which genome data is available (Gubbels et al., 2006). Throughout the cell cycle, MORN1 is found in ring structures at both the apical and basal ends of the cell, as well as in the centrocone (Gubbels et al., 2006). At the onset of mitosis, MORN1 forms a ring around each duplicated centrosome and associated SFA structure, and this ring follows the leading edge of the inner membrane complex (IMC) in daughter buds as they grow. (Hu, 2008). The IMC is part of the pellicle and lies just below the plasma membrane. The IMC is composed of flattened vesicles lined with a filamentous meshwork of proteins. IMC proteins are

among the first recognizable signs of daughter cell formation, and IMC1 clearly labels both the mother pellicle and daughter buds.

Scanning across a field of an unsynchronized population of parasites, several different patterns of SFA2 are observed. SFA2 can be found in small punctate structures, ovoid shapes, and long arced fibers, or can be seemingly absent from the cell altogether. Most cells observed appear to be in G1 or early S phase, as determined by the presence of only one centrocone and one centrosome per parasite. In these cells, no discernable SFA2 labeling is visible without the use of high resolution imaging. Using high resolution deconvolution microscopy along with substantial contrast enhancement, tiny specs of SFA2 are revealed at the conoid and often at the micropore of the cell (Figure 3.3, G phase). At mid S phase, defined by centrosome and centrocone duplication, two clear SFA2 structures appear. Initially, these small punctate structures are perfectly colocalized with the centrin label, when the centrosomes are still very close together (Figure 3.3, Mid S phase). As the centrosomes begin to separate, the SFA2 labeled structures show their own distinct position, immediately next to the centrosomes. As the centrosomes move further apart in S phase, the SFA2 structures elongate, transforming into small ovoid shapes.

In late S phase, when daughter IMC buds first appear, the SFA2 structures begin their transformation into fibers (Figure 3.3, Late S phase). When the distance between daughter buds and centrosomes is short, the SFA2 fibers appear straight. As the daughters grow, and more separation occurs between daughter conoid and mother centrosomes, the SFA2 fiber shows itself to be an arced structure with a spiraling hook shape at its apical end (Figure 3.3, Mitosis). The curved fiber maintains its shape and continues to elongate as the daughter cell grows larger and the conoid and centrosome move farther apart. Once the daughter cell is almost completely

formed, the fiber structure is no longer seen. In its place are two ovoid structures, one at the daughter conoid and one at the centrosome. Careful observation of the different SFA2 patterns along with other cell cycle markers (Centrin, IMC1 and MORN1) has revealed a clear transition of the SFA2 structures over the cell cycle. Quantification of cell cycle stages of SFA2-HA parasites using the markers listed above revealed no significant difference as compared to un-transfected Ku80 cells, and no significant difference in cell cycle timing as compared to published data (Radke et al., 2001) (data not shown).

3.3.3 The SFA2 Fiber Terminates at the Apical Cap of Daughter Cells

Having observed that the SFA2 fiber extends into developing cells, we next wondered what structure the fiber could be associated with in the apical end of daughter buds. The apical end of *T. gondii* contains several cytoskeletal components, in addition to the apical complex of invasion organelles. Using IMC1 as a marker, we observed that the SFA2 fibers extended beyond the apical end of the daughter inner membrane complex (Figure 3.3). In an attempt to more precisely determine where the apical end of the SFA2 fiber terminates, we performed IFAs using antibodies against cytoskeletal structures at the apical end of daughter cells (Figure 3.4). We examined three markers that label structures at the apical end of the cells: ISP1, an IMC sub-compartment protein which makes up the apical cap (Beck et al., 2010); RNG1, a component of the apical polar ring (Tran et al., 2010); and alpha-tubulin, which labels daughter cell microtubules. We observe that the apical end of the SFA2 fiber consistently extends beyond the RNG1 ring (Figure 3.4 A) and the end of tubulin staining (Figure 3.4 B). However, tubulin antibodies often fail to label the conoid (Schwartzman et al., 1985), as does the antibody used in these images. The SFA2 fiber appears to terminate in the apical cap of developing daughters, and does not extend beyond the ISP1 labeled structure (Figure 3.4 C).

To confirm the subcellular localization of SFA2 in *T.* gondii, we performed immuno-electron microscopy on SFA2-HA parasites using gold-labeled anti-HA antibodies. In the earliest stages of cell division, we observe labeling of a linear structure near the site of centrocone duplication, in the approximate location of the centrosome (Figure 3.5 A). Also seen in this image are two small membrane segments near the lower limit of the labeled structure, which may be the first vesicles of IMC appearing at the site of daughter bud construction. As daughter buds are formed, SFA2 labeling is present between the centrocone and apical end of developing cells (data not shown). In larger daughters, a distinctly curved structure is labeled in the apical end of the buds (Figure 3.5 B). These results support the localization of SFA2 described in our IFA studies, and confirm the presence of a curved fiber reaching from the centrosomes to the apical end of daughter cells.

3.3.4 SFA2, SFA3 and SFA4 are Components of the Same Structure in *T. gondii*

C. reinhardtii contains a single SFA protein, while the *T. gondii* tachyzoite expresses three similar SFA proteins. In order to answer the question of whether all three proteins localize to the same structure, we created antibodies to two of the *T. gondii* SFAs. The full open reading frames of TgSFA2 and TgSFA3 were cloned into a bacterial expression vector with an N terminal 6xHis tag and expressed in *Escherichia coli* (Figure 3.6A). SFA2 was most readily soluble using denaturing conditions, while we could purify SFA3 under native conditions. Purified recombinant SFA2 and SFA3 were used to immunize rabbits. Pre- and post-immune serum was tested by IFA on wild type intracellular parasites to select antibodies with the greatest specificity.

Western blot analysis using the RH strain of *T. gondii* demonstrated that both SFA antibodies label a protein of the expected size of an SFA monomer as well as several larger

proteins (Figure 3.6B). Previous work in *S. similis* has shown that purified SFA protein readily self- assembles into high molecular weight complexes visible on protein gels, even under denaturing conditions (Lechtreck, 1998), suggesting that the larger bands we see may be dimers and polymers.

In wild type *T. gondii* tachyzoites, both the SFA2 and SFA3 antibodies display the same localization pattern as was described for the SFA2-HA parasites (data not shown). The only difference we observed was that micropore staining is brighter using SFA antibodies versus HA antibodies, likely due to differences in antibody sensitivity. To examine the localization of SFA2, SFA3 and SFA4 simultaneously, we imaged combinations of endogenously HA tagged parasites, GFP tagged parasites, and antibodies created against the *T. gondii* SFAs. The data shows that SFA3 and SFA4 follow the same expression and localization patterns as SFA2 (Figure 3.7). A slight shift is sometimes observed between the localization of SFA proteins, but the distance is very small and may be due to chromatic aberration.

3.3.5 Antibodies to *T. gondii* SFAs Label the Spindle and Conoid of *Sarcocystis neurona*

Besides *T. gondii*, several other Apicomplexan parasite genomes also contain one or more SFA genes. Previous genome analysis demonstrated that *Theileria annulata, Cryptosporidium parvum* and all *Plasmodium* species examined encode at least one copy of a putative SFA protein (Lechtreck, 2003). Since that report, the genomes of *Eimeria maxima* and *Sarcocystis neurona* have also been sequenced, and we have searched for SFA sequences in both organisms (Figure 3.8). The *S. neurona* genome contains putative orthologs of TgSFA2, TgSFA3 and TgSFA4. An ortholog found on scaffold00084 is 85% identical to TgSFA2, an ortholog on scaffold00029 has 76% identity to TgSFA3, and an ortholog on scaffold00271 is 62% identical

to TgSFA4. The *E. maxima* genome also encodes orthologs of TgSFA2 (62% identity) and TgSFA3 (61% identity) on contig01630 and contig03220, respectively.

We were interested to see if the antibodies we created in *T. gondii* would cross-react with structures in *S. neurona*. Since *S. neurona* divides by the more complex process of endopolygeny, we were also curious about the potential role of SFA fibers in cell division in this species. To visualize the SFA proteins in *S. neurona*, we performed IFAs on intracellular schizonts using the antibodies created against *T. gondii* SFA2 (Figure 3.9 A) and SFA3 (Figure 3.9 B). There is no specific SFA staining prior to the onset of nuclear segregation of the schizont. Once nuclear segregation has begun, tubulin staining reveals the formation of 32 pairs of ring-shaped spindles. SFA appears in punctate structures within the ring of each spindle (Figure 3.9 A, inset). As DNA segregation is completed and daughter buds begin to form, the tubulin antibody shows one spot of staining at each spindle and the beginnings of daughter buds. At this time, SFA is observed in 2 discrete spots just apical to each spindle. As the daughter buds grow larger, an additional spot of SFA appears and co-localizes perfectly with the spindle tubulin. Although no fiber structure is formed, the path between the three discrete SFA structures is arc-shaped in a similar fashion as *T. gondii* fibers (Figure 3.9 B, inset). Once daughter cell construction is nearly completed, SFA is seen at the conoid of each sporozoite. The same localization pattern is observed for both antibodies, with one exception. When the mature sporozoites are released from the host cell, they maintain considerable SFA2 staining across the apical end of the parasite, but SFA3 staining is not observed. The pattern of staining we see in *S. neurona* mimics that observed in *T. gondii*, in both timing of expression and in subcellular localization.

3.3.6 Overexpression of SFA3 and SFA4 is Detrimental in *T. gondii*

Based on the observation that the SFAs are differentially expressed and localized throughout the cell cycle, we hypothesized that timing and level of expression would be important for SFA function. Moreover, analysis of expression levels of the *T. gondii* SFAs showed that levels of mRNA expression during the life cycle correlate with the generation of specific cell structures. Specifically, the expression of the *T. gondii* SFAs changes with the cell cycle, peaking in S phase and decreasing sharply following cytokinesis (Figure 3.1 B). To examine the effect of overexpression on the *T. gondii* SFAs, generated a construct with the TgSFA3 and TgSFA4 minigenes expressed with an N terminal GFP fusion under the control of the strong, constitutive tubulin promoter.

tubGFP-SFA3 was transfected into wild type parasites multiple times and selection never resulted in a stable population. Examination of transient transfectants showed dramatic effects on the parasite cytoskeleton. tubGFP-SFA3 parasites did not survive beyond 48 hours post transfection, and atypical IMC1 and MORN1 localization was observed in cells as earlier as 24 hours post transfection. Most parasites observed at the 24 hour time point were in the two cell vacuole stage, though with normal division they would have been expected to reach the four or eight cell stage by this point. In many examples, there were one or two large spots of SFA3 staining in each parasite. Centrosome localization resembled wild type, with one or two small spots of centrin per cell, usually near the spots of SFA3. At 30 hours post transfection, IMC1 localization identified a disorganized structure approximately 6 µm x 10 µm, nearly five times the size of a typical tachyzoite (Figure 3.10 A). No structures resembling daughter buds were observed in transfected parasites. MORN1 localization was throughout the cells in bright spots or long curved structures, but no MORN1 rings were observed. Also at 30 hours, several

round SFA3 structures had formed within the cells, and smaller spots were scattered throughout. Moderate cytoplasmic accumulation was also apparent. Cell cycle stages could not be identified due to aberrant localization of MORN1 and IMC1.

To assess whether position of the fluorescent tag had an effect on the phenotype, parasites were engineered with a C terminal YFP instead of the N terminal GFP. These parasites were also not selectable. To explore the possibility that the fluorescent tag and not the promoter strength was causing the lethal effects, we also created parasites with tubulin-expressed TgSFA3 that lacked any tag. This experiment resulted in parasites demonstrating the same phenotype as observed for tubGFP-SFA3, which also failed to survive selection with CAT (data not shown).

Like the SFA3 overexpression parasites, tubGFP-SFA4 parasites also demonstrated division defects, though these parasites survived longer, up to 64 hours. Division defects were not observed until 36-42 hours, but after that time point, parasites demonstrated similar morphology and developmental defects as the tubSFA3-GFP parasites (Figure 3.10 B).

Because we observed such marked mislocalization of MORN1 in the overexpression mutants, we were interested to examine the effect of MORN1 expression on the localization of *T. gondii* SFAs. Previous work from our lab has resulted in the creation of an inducible MORN1 knockout line, whose MORN1 gene is under the control of an inducible tetracycline promoter. When treated with anhydrous tetracycline (ATc), MORN1 expression is turned off. Treated parasites undergo several rounds of replication and daughter cell construction but are unable to complete cytokinesis (Lorestani et al., 2010). We performed IFA on iMORN1 parasites with and without ATc treatment using the SFA antibodies. Untreated iMORN1 parasites displayed normal SFA and MORN1 localization (Figure 3.11). Following treatment with ATc, MORN1 antibodies

failed to label any structures. SFA2 and SFA3 antibodies both showed diffuse cytoplasmic or peripheral staining, but normal SFA structures were absent in treated cells (Figure 3.11). These observations indicate a relationship between expression of MORN1 and SFA proteins in *T. gondii.* Future experiments will explore the possibility that MORN1 and SFA proteins form important complexes involved in daughter cell budding.

3.3.7 Attempts to Create SFA Null Mutants in *T. gondii*

To define the precise role of the SFA proteins in daughter cell formation, we made several attempts to create null mutants of TgSFA2 in *T. gondii*. We first tried to knockout the gene directly replacing the coding region of TgSFA2 with a drug resistance marker. A genomic library has been created in a modified double cos-site plasmid (cosmid) yielding thousands of cosmids containing large regions of *T. gondii* genomic sequence (Gubbels et al., 2008). We selected a cosmid from this library that contained the TgSFA2 gene in addition to 45 kb of surrounding genomic sequence. Then, we used bacterial recombination to modify the cosmid (Brooks et al., 2010), replacing the TgSFA2 locus with the CAT gene expressed under a strong *T. gondii* specific promoter. We transfected the modified cosmid into ΔKu80 parasites and obtained a CAT resistant population, but screening of parasites did not reveal any knockout clones.

Based on this result, we hypothesized that TgSFA2 is an essential gene, and therefore direct knockout would not be possible. We attempted to circumvent this issue by instead creating a conditional mutant. By first adding an additional copy of TgSFA2 under control of a regulatable tetracycline promoter (t7s4), followed by replacement of the endogenous gene with the drug resistance cassette, genes can be conditionally regulated using Atc. To allow for control of the t7s4 promoter by Atc, a parasite line was created that expresses the tetracycline

transactivator machinery (TAti) (Meissner et al., 2002). This approach has worked well for the study of other essential genes in *T. gondii* (Meissner et al., 2002; van Dooren et al., 2008; Agrawal et al., 2009; Brooks et al., 2010; Lorestani et al., 2010). We engineered parasites with an exogenous copy of TgSFA2 under the t7s4 promoter with a C terminal HA tag (t7s4-SFA2-HA). We transfected the same modified cosmid into this genetic background and selected for a CAT resistant population, but again failed to identify any knockout clones.

Finally, we tried a method to control protein degradation by inserting a destabilization domain (DD) with an HA tag into the locus. The DD tag targets proteins for degradation, but the activity of the destabilization domain is reversibly blocked by the addition of the ligand Shld1 (Banaszynski et al., 2006). We used a plasmid generated by Huynh and Carruthers (2009) to create a vector that would insert two consecutive HA sequences followed by a DD tag at the C terminus of TgSFA2 and transfected the plasmid into ΔKu80 parasites. However, screening by IFA using anti-HA antibodies showed no parasites expressing the tag.

Recently, a new method was developed to insert a regulatable promoter directly into the upstream region of a gene of interest (Sheiner, 2011). Again, we used cosmid modification to create a construct containing the t7s4 promoter and a single HA tag upstream of the TgSFA3 locus, flanked on both sides by large regions of genomic sequence (Figure 3.12 A). We transfected the modified cosmid into ΔKu80/TAti parasites, and double homologous recombination resulted in the insertion of the t7s4 promoter between the native promoter and start codon of TgSFA3. Amplification of a DNA fragment reaching from the t7s4 promoter to the coding region of TgSFA3 confirms the successful insertion (Figure 3.12 B). Future work to characterize the phenotype of the inducible SFA3 mutants may reveal the precise function of the SFA fiber in *T. gondii* cell division.

3.4 DISCUSSION

We propose that the SFA fibers create a physical link between the centrosome and the daughter buds. Thorough imaging of dividing parasites has shown that SFA is cell-cycle regulated, and that a fiber of SFA forms between the centrosome and conoid of developing daughter cells (Figure 3.13). SFA first appears at the centrosomes immediately following duplication. As the first pieces of daughter IMC are assembled at the new conoid, the SFA structure begins to elongate into a short oval shape. As daughter cell development progresses and the conoid and centrosome move farther apart, the SFA structure grows between the two and reveals a curved end near the conoid of the daughter bud. When budding is nearly completed and the nucleus and other organelles are drawn well into the daughter cells, SFA is observed as 2 structures, one at the centrosome and one at the conoid. Following cytokinesis, when expression levels of the SFAs are sharply downregulated, labeling at the centrosome disappears but persists at the conoid. We observed a similar expression pattern and localization in dividing *S. neurona* parasites, but were surprised that we did not observe a fiber structure in these cells. However, it is possible that a division fiber does exist in this species, but that SFA only decorates the structure and is not the sole component, a hypothesis supported by the linear arrangement of SFA staining in budding cells.

Electron micrographs of dividing Apicomplexa show a striated fiber structure with ends at the centrosome and conoid of *Eimeria necatrix* (Dubremetz, 1975). Serial sections of the apical end of the *E. necatrix* daughter demonstrate that the conoid is encircled by this fiber, and we also observed the SFA fiber to curve at the conoid end. The spiraling end of the striated fiber mimics the spiral orientation of conoid microtubules, and one might speculate that perhaps SFA is associated with conoid microtubules, and may be involved in its biogenesis (Hu et al., 2002).

The fact that SFA protein remains at the conoid throughout the cell cycle in *T. gondii* supports the idea of a tight association between these two structures. Characterization of the TgSFA3 conditional mutant is underway and will be essential in testing these hypotheses.

Studies of SFA proteins in green algae have shown that SFA fibers are always associated with microtubules (Lechtreck and Melkonian, 1998). In *C. reinhardtii* and *S. similis*, SFA fibers are closely apposed with microtubule roots emerging from the basal bodies of flagella (Lechtreck and Melkonian, 1991; Lechtreck and Silflow, 1997). Due to their location and sturdy biochemical nature, it has been suggested that the SFAs are microtubule-stabilizing proteins. Though no microtubule binding assays or other biochemical analysis of this idea has been presented, it is an attractive hypothesis. In both *T. gondii* and *S. neurona*, SFA structures exist at microtubule organizing centers of developing cells, a localization pattern that supports the idea that the SFAs form microtubule associated fibers. Attempts to examine this hypothesis in *T. gondii* using cytoskeletal extraction (Tran et al., 2010) in order to visualize SFA2 staining along isolated microtubules were not successful, since this technique does not preserve daughter cell cytoskeletons. Microtubule binding assays like those conducted for the recently described *T. gondii* ICMAP1 microtubule binding protein could determine if the SFAs are biochemically bound to microtubules or just positioned near them (Heaslip et al., 2009).

The SFA proteins in *T. gondii* likely interact with other important cytoskeletal components of the division machinery as well, such as MORN1 and the IMC proteins. We have collected several pieces of evidence that SFA, MORN1 and IMC proteins may be dependent on each other for proper localization and function in *T. gondii*. Overexpression of SFA proteins causes mis-localization and occasionally absence of MORN1, as well as defects in assembly of the IMC cytoskeleton. Multiple attempts to generate parasites stably expressing fluorescent

fusions of both an SFA protein and either MORN1, Centrin1 or IMC1 yielded no stable transgenics. Time lapse imaging of stable SFA3-YFP parasites co-transfected with IMC1-tomato showed a defect in cytokinesis (data not shown). In addition, examination of MORN1 inducible knockout parasites (Lorestani et al., 2010) revealed that in the absence of MORN1 expression, SFA proteins are mis-targeted and fail to form discrete structures or fibers (Figure 3.10). Future studies examining the biochemical relationship between these proteins by co-immunoprecipitation or two-hybrid assays could confirm that SFA, MORN1, IMC and centrin proteins form important complexes involved in *T. gondii* cell division.

The strong conservation of the SFAs in the Apicomplexa and the analogous SFA structures observed in both *T. gondii* and *S. neurona* suggest that these proteins may serve an important role in zoite construction across the phylum. Future studies to determine the exact function of these proteins and their relationships with other cell division players may finally provide answers to long standing questions about the complex process of building these intricately beautiful cells.

3.5 MATERIALS & METHODS

Parasites and Host Cells

Wild type RH strain *Toxoplasma gondii* parasites were used throughout the study. Parasites were maintained in human foreskin fibroblast (HFF) cells as previously described (Roos et al., 1994).

Sarcocystis neurona strain Sn3 used for immunofluorescence assays were maintained in bovine turbinate (BT) cells as previously described (Vaishnava et al., 2005). BT cells were grown

in RPMI 1640 media with L-glutamine (Cellgro) supplemented with 10% heat inactivated fetal bovine serum (HyClone),110 µg/ml sodium pyruvate, 5 U/ml penicillin, 10 µg/ml streptomycin, and 1 ml/l fungizone (Invitrogen), and 2 mM L-glutamine.

Plasmids and Transgenic Parasites

To tag the endogenous copy of TgSFA2 with a 3xHA tag, a 585 bp fragment ending before the stop codon of the open reading frame was amplified from the *T. gondii* RH strain genome using primers 1 and 2 (see Table 3.1 for all primer sequences used in this study). This fragment was cloned via ligation independent cloning (LIC) (Alexandrov et al., 2004) into the pLIC-HA-CAT vector to create an in-frame fusion to 3 copies of the HA protein. This plasmid was integrated into the endogenous locus in a Ku80 knock-out line as previously described (Huynh and Carruthers, 2009). Stable SFA2-HA transgenic parasite clones were obtained by chloramphenicol selection. Clones were screened for proper integration by Southern blotting. Parasite DNA was isolated from SFA2-HA clones by standard phenol chloroform extraction. Clones were screened for proper integration by Southern blotting using the restriction sites from Figure 3.2, as previously described (van Dooren et al., 2008).

To generate N terminal GFP fusion plasmids for TgSFA2, TgSFA3, and TgSFA4, coding sequences including stop codons were amplified by primers 7 & 8, 9 & 10, and 11 & 12, respectively. The SFA2 fragment was digested with XmaI and PstI and inserted into the plasmid tubGFP-CAT using the same restriction sites. The SFA3 fragment was digested with HpaI, treated with T4 DNA polymerase, and the resulting fragment was digested with XmaI. The final SFA3 fragment was ligated into tubGFP-CAT plasmid that had been digested with PstI, treated with T4, and then digested with PstI. The SFA4 fragment was digested with NsiI, treated with T4 DNA polymerase, and then digested with XmaI. The final half-blunt SFA4 fragment was inserted into

tubGFP-CAT plasmid that had been digested with PstI, treated with T4, and then digested with PstI. Stable tubGFP-SFA2 transgenic parasite clones were obtained by chloramphenicol selection. Stable populations of tubGFP-SFA3 and tubGFP-SFA4 were not able obtained because these plasmids resulted in a lethal phenotype.

To create the IMC1 (Gene ID: TGGT1_116030) tandem tomato plasmid (tubIMC1-dT), the parent plasmids tub-dT-CAT (aka $pCTR_{2T}$)(van Dooren et al., 2008) and tubIMC1-mCherry were utilized. Both plasmids were digested with BglII and AvrII, which removed the IMC1 coding region without the stop codon, and opened tub-dT-CAT at the multiple cloning site for N terminal fusion. The resulting plasmid was transfected into the LIC-SFA3-YFP parasite clone for live cell imaging. Selection with chloramphenicol did not result in a stable population.

Protein Expression & Antibody Generation

The complete coding regions minus introns and stop codons of TgSFA2 and TgSFA3 were amplified from the *T. gondii* RH strain genome with flanks for insertion into the pAVA-421 6xHis tag vector (Alexandrov et al., 2004) using primers 3 & 4, and 5 & 6, respectively. Recombinant fusion proteins were purified on Ni^{2+}-NTA resins (Qiagen, Hilden, Germany) as previously described (Agrawal et al., 2009). Rabbits were initially immunized with 100 µg of purified protein and boosted at weeks 4 and 8 with 50 µg and at week 12 with 100 µg. At 14 weeks, serum containing polyclonal antibodies was collected (Cocalico Biologicals, Reamstown, PA, USA).

Microscopy

For immunofluorescence assays, host cells were inoculated onto coverslips and infected with parasites. Coverslips were fixed 24-72 hours after infection with 3% paraformaldehyde and permeabilized with 0.25% Triton X-100 in PBS. Coverslips were then blocked in 3% bovine serum

albumin (BSA) in phosphate-buffered saline (PBS) for at least 1 hour. The primary antibodies used were mouse anti-alpha tubulin mAb 12G10 (Jerka-Dziadosz et al., 1995) at a dilution of 1:20 (gift of Jacek Gaertig, University of Georgia), anti-CAT at 1:3000 (Abcam), rabbit anti-Centrin1 at 1:1000 (gift of Iain Cheeseman, Massachusetts Institute of Technology), mouse anti-GFP at 1:1000 (Torry Pines Biolabs), rat anti-HA at 1:200 (clone 3F10, Roche Applied Science), mouse anti-IMC1 mAb 45.15 (Wichroski et al., 2002) at 1:500 (gift of Gary Ward, University of Vermont), rabbit anti-IMC3 (Gubbels et al., 2004) at 1:500, mouse anti-ISP1 mAb 7E8 (DeRocher et al., 2008) at 1:500 (gift of Peter Bradley, University of California, Los Angeles), rabbit anti-MORN1 (Gubbels et al., 2006) at 1:250, mouse anti-RNG1 at 1:1000 (gift of Naomi Morrissette, University of California, Irvine), and rabbit anti-SFA2 and anti-SFA3 at 1:1000 (generated in this study). The secondary antibodies used were AlexaFluor 350, AlexaFluor 488, and AlexaFluor 546 (Invitrogen), at a dilution of 1:2000. Images were collected on an Applied Precision DeltaVision inverted epifluorescence microscope, and images were deconvolved using Softworx software.

For cryo-electron microscopy, intracellular SFA2-HA parasites were fixed in 4% paraformaldehyde/0.05% glutaraldehyde for 1.5 hours at room temperature. The sample was then blocked with 1% fetal bovine serum (FBS) in PBS for 1 hr at room temperature, followed by overnight infiltration in 2.3 M sucrose/20% polyvinyl pyrrolidone at 4°C. Samples were frozen in liquid nitrogen and sectioned with a Leica UCT cryo-ultramicrotome. Fifty to seventy nanometer sections were blocked with 1% FBS for 30 min and subsequently incubated with rat anti-HA primary antibody at 1:100. This was followed by incubation with rabbit anti-rat secondary antibody at 1:400. Finally, sections were incubated with 10 nM colloidal gold conjugated protein A, for 30 min. Sections were washed extensively in 0.1% FBS in PBS followed by a water rinse, and stained with 0.3% uranyl acetate/2% methyl cellulose. Samples were viewed with a JEOL

1200 EX transmission electron microscope. Controls, omitting the primary antibody, were consistently negative at the concentration of colloidal gold conjugated protein A used.

Western Blotting

Western blotting was performed as previously described (van Dooren et al., 2008). We used anti-HA antibodies (same as described for immunofluorescence assays), at a dilution of 1:100, anti-HSP60 antibodies (Toursel et al., 2000) at a dilution of 1:2000, and anti-SFA antibodies at a dilution of 1:1000. Horseradish peroxidase (HRP)-conjugated anti-rat and anti-rabbit antibodies (Pierce) were used at a dilution of 1:10,000.

Genome Analysis

All DNA sequences used for gene cloning and alignments were retrieved from publicly available genome database sites (*T. gondii*- www.toxodb.org/toxo; *S. neurona*- sarcodb.ctegd.uga.edu/SarcoDB.shtml; *E. maxima*- www.genomemalaysia.gov.my/emaxdb). The coding regions of *S. neurona* and *E. maxima* were annotated by hand from genomic sequences identified by BLAST using *T. gondii* SFA sequences. Multiple sequence alignment of coding sequences of the Apicomplexa SFAs was conducted using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2).

3.6 FIGURES AND TABLES

Figure 3.1: SFA proteins are encoded and expressed in the Apicomplexa. A) Multiple sequence alignment of the predicted protein sequence of *T. gondii* SFAs and *C. reinhardtii* SFA demonstrate the conservation of the SFAs. While the N terminus is poorly conserved, the C terminus and the skip residues (underlined) show considerable homology. **B)** Microarray data of expression levels over the cell cycle show similar patterns among the three SFA genes expressed in *T. gondii* tachyzoites. In general, expression of the *T. gondii* SFAs peaks during S phase, corresponding to the onset of daughter budding, and declines sharply following cytokinesis.

Figure 3.2: *T. gondii* tachyzoites were engineered with an endogenous 3xHA tag on the C terminus of SFA2. A) Single cross-over between the NruI linearized LIC-SFA2-3xHA plasmid (top) and the genomic locus (middle) placed a 3xHA tag at the C terminus of SFA2 and inserted a chloramphenicol acetyl transferase (CAT) cassette downstream (bottom). Two XhoI sites were present near the native locus, and modification inserted an additional XhoI site between the HA tag and CAT cassette, allowing for analysis of potential modified clones by southern blot. **B)** Southern blot analysis following digest with XhoI showed that the native locus of SFA2 was disrupted as predicted in LIC-SFA2-3xHA clone E10. Two additional bands in the clone indicate that non-homologous integration occurred twice elsewhere in the genome. **C)** Western blot using an anti-HA antibody confirm that the HA tag is expressed on a protein of the proper size. Also included for comparison is a cloned parasite line expressing an ectopic copy of SFA2 with a single HA tag under the tetracycline regulatable promoter (t7s4-SFA2-HA).

Figure 3.3: SFA2 localization is dynamic over the cell cycle of *T. gondii*. IFAs of various cell cycle stages showing the position of SFA2-HA compared to IMC1, Centrin and MORN1. **A, B)** Just after centrosome duplication occurs in mid S phase, SFA2 is seen as small spots perfectly co-localized

with centrin and found on either side of the MORN1-labeled spindle. **C, D)** Later in S phase, SFA2 forms small ovoid structures adjacent to the centrosomes, contained within MORN1 rings. **E, F)** During mitosis, SFA2 forms arced fibers within the daughter buds. The straight end of the fiber is associated with the centrosome **(E)**, and the curved end extends through the apical most IMC1 staining (**F)**. In large daughter buds the fiber appears to have split, with one SFA2 spot at the centrosome and one at the apical end of the cell (F, bottom parasite). One small spot of SFA2 is also visible at the micropore of each daughter cell. **G)** In interphase cells, the only SFA2 observed is at the conoid and micropore of the mature cell. This staining is very weak, so contrast enhancement was employed to demonstrate co-localization with MORN1 in these structures. Scale bars = 5 µm.

Figure 3.4: SFA2 fibers terminate at the apical cap of developing daughter cells in *T. gondii*. Staining for various cytoskeletal markers shows the apical end of the SFA2 fiber. **A)** The SFA2 fiber extends through the apical polar ring structure labeled with antibodies to RNG1. **B)** The curved end of the SFA2 fiber appears to curl around the apical most tubulin staining. **C)** The ISP1 antibody labels a protein in the apical cap, where the SFA2 fiber seems to terminate. Scale bars = 5 µm.

Figure 3.5: Ultrastructural examination of *T. gondii* confirms SFA2 localization. Cryo-ImmunoEM was performed on SFA2-HA parasites using gold-labeled anti-HA antibodies. **A)** A section through the nucleus reveals a funnel-shaped extension of the nuclear envelope during centrosome duplication, with spindle microtubules visible within the structure. Gold particles label a linear structure to the left of the nucleus (arrow).Two small portions of membrane are seen near the staining, which may be the first sections of new daughter IMC. **B)** Two daughter

buds are visible in this section of a dividing tachyzoite. Gold particles stain a curved structure in the apical end of the daughter bud on the right (arrow). Scale bars = 200 nm.

Figure 3.6: SFA2 and SFA3 proteins were expressed for antibody generation. A) The full length proteins of SFA2 and SFA3 were expressed and purified from *E. coli*. Purified proteins were inoculated into rabbits to generate antibodies. **B)** Both the SFA2 and SFA3 antibodies show faint bands near the expected size of the proteins (~30 kDa). Several additional larger bands appearing in both samples may be dimer and polymer forms of the SFA proteins.

Figure 3.7: The three SFA proteins are co-localized in *T. gondii*. Each panel shows IFA images of SFA2-HA parasites transiently transfected with tubGFP-SFA4 and fixed at 18 hours post-transfection. **A)** Two small spots of each SFA protein are seen colocalized at the presumed position of the centrosomes before SFA fiber elongation. The SFA3 antibody also labels the conoid of one of the parasites (arrow). **B)** SFA2 and SFA4 colocalize in two fibers per parasite. Both SFAs are also found at the micropore of each mother cell (arrowheads). **C)** SFA2 and SFA3 stain two structures in the parasite, and SFA3 also labels the micropore (arrowhead). Scale bars = 5 μm.

Figure 3.8: SFA orthologs are encoded in the genomes of related Apicomplexa. Sequence identity between Eucoccidian species (*T. gondii*, *S. neurona*, and *E. maxima*) is remarkably high. Both *S. neurona* and *E. maxima* contain nearly identical copies of TgSFA2 and TgSFA3.

Figure 3.9: SFAs are expressed in *S. neurona* parasites during division. A-B) *Sarcocystis neurona* parasites were examined by IFA using the *T. gondii* SFA antibodies created in this study. Localization patterns of SFA2 and SFA3 were identical in intracellular parasites. Early schizonts (A, left) of *S. neurona* had faint diffuse SFA staining, but no discernible structures were observed. At the completion of DNA replication but before daughter budding begins (A, right),

spindles exist in pairs of ring structures, each with a spot of SFA inside. As daughter buds begin to form (B, left) 2 spots of SFA are seen apical to each daughter spindle. As daughter cells grow larger and the nucleus is divided (B, right), SFA is found in 3 linearly arranged spots, one on each spindle, and two apical to that. Finally, when complete daughters begin to emerge (A, center), SFA is seen as a single structure at the apical point of each cell. Scale bars = 10 µm.

Figure 3.10: Overexpression of SFA3 and SFA4 in *T. gondii* results in lethal division defects. Transfection of *T. gondii* with plasmids containing SFA driven by the tubulin promoter is lethal for SFA3 and SFA4. Imaging of parasites from 24 to 48 hours post- transfection reveals dramatic defects in the structure of the IMC and mislocalization of MORN1. Parasites tagged with a C-terminal GFP also demonstrate accumulation of SFA in large rounded structures. **A)** GFP-SFA3 parasites 30 hours post-transfection. **B)** GFP-SFA4 parasites 48 hours post-transfection show similar defects in MORN1 and IMC1 localization. Scale bars = 10 µm.

Figure 3.11: SFAs structures are not formed in *T. gondii* tachyzoites the absence of MORN1. Immunofluorescence assays were performed using the SFA2 and SFA3 antibodies on inducible MORN1 parasites, with or without anhydrous tetracycline (Atc). SFA and MORN1 localization is normal in parasites not treated with Atc. Following 2 days incubation with Atc, MORN1 expression is ablated, and SFA proteins are targeted to the cell periphery (SFA2) or diffused through the cytoplasm (SFA3). Scale bars = 5 µm.

Figure 3.12: SFA3 promoter replacement in *T. gondii.* A) Double cross-over between a modified cosmid (top) and the genomic locus (middle) inserted a regulatable t7s4 promoter and HA tag at the N terminus of SFA3 (bottom). PCR screen primers and expected sizes are noted at positions A, B, and C. **B)** PCR analysis of a transgenic clone demonstrates the presence of the t7s4

promoter upstream of SFA3 (A) and the absence of the native locus (C). The product shown in (B) indicates an additional insertion of the cosmid at a random location in the genome.

Figure 3.13: Model of *T. gondii* SFA localization over the cell cycle. A) During interphase, SFA exists as tiny punctate structures at the conoid and micropore of tachyzoites. Following centrosome duplication, SFA is initially recruited directly to the centrosomes. **B)** At the onset of daughter conoid construction, SFA forms small ovoid structures between the centrosome and conoid of developing daughters. **C)** As daughter buds grow and the conoid and centrosomes move apart, the SFA structure stretches into a curved fiber formation, with the apical end spiraling around the daughter conoid. **D)** When budding is nearly complete, the SFA fiber appears broken, and discrete structures remain at the conoid and centrosome of each daughter. (SFA- yellow, MORN1- green, microtubules- red, IMC- light blue, nucleus- light teal)

Table 3.1: Primer sequences used in this study. Primer numbers correspond to those given in Materials & Methods. Restriction sites used for cloning and ligation independent cloning sites are underlined. Primers that used restriction sites also contain 3-4 bp of random sequence before the cut site to assure proper binding of the restriction enzyme to the resulting PCR fragment.

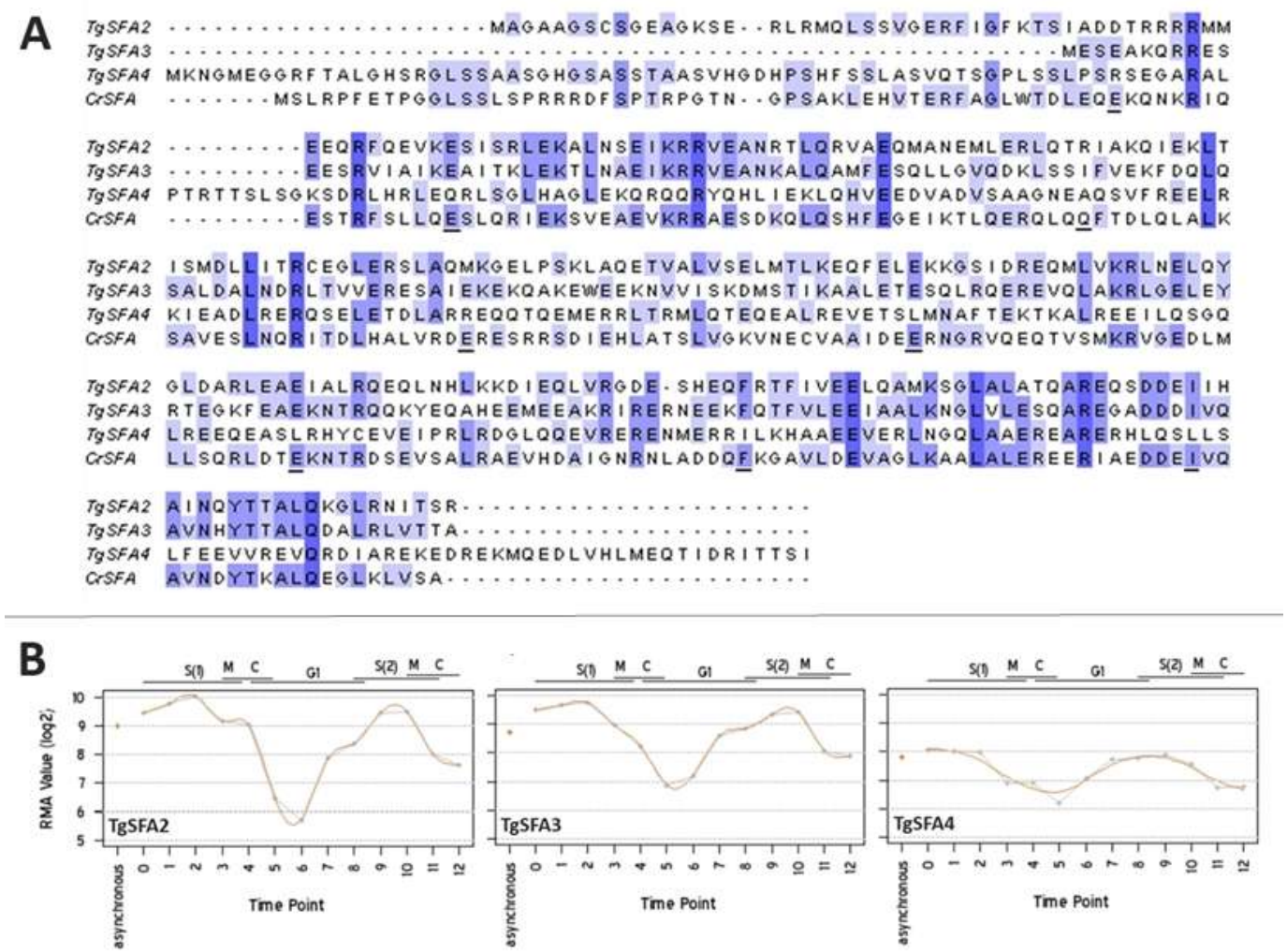

Figure 3.1: SFA proteins are encoded and expressed in the Apicomplexa.

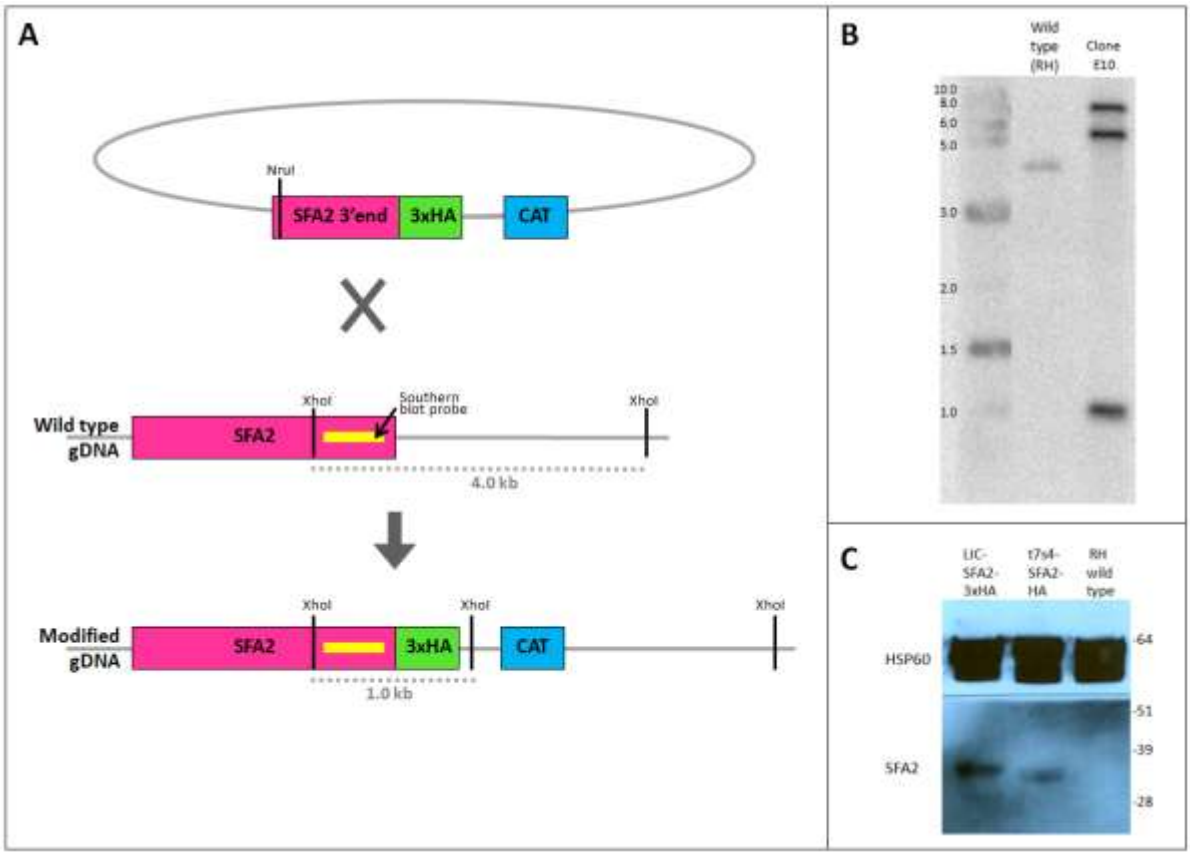

Figure 3.2: *T. gondii* tachyzoites were engineered with an endogenous 3xHA tag on the C terminus of SFA2.

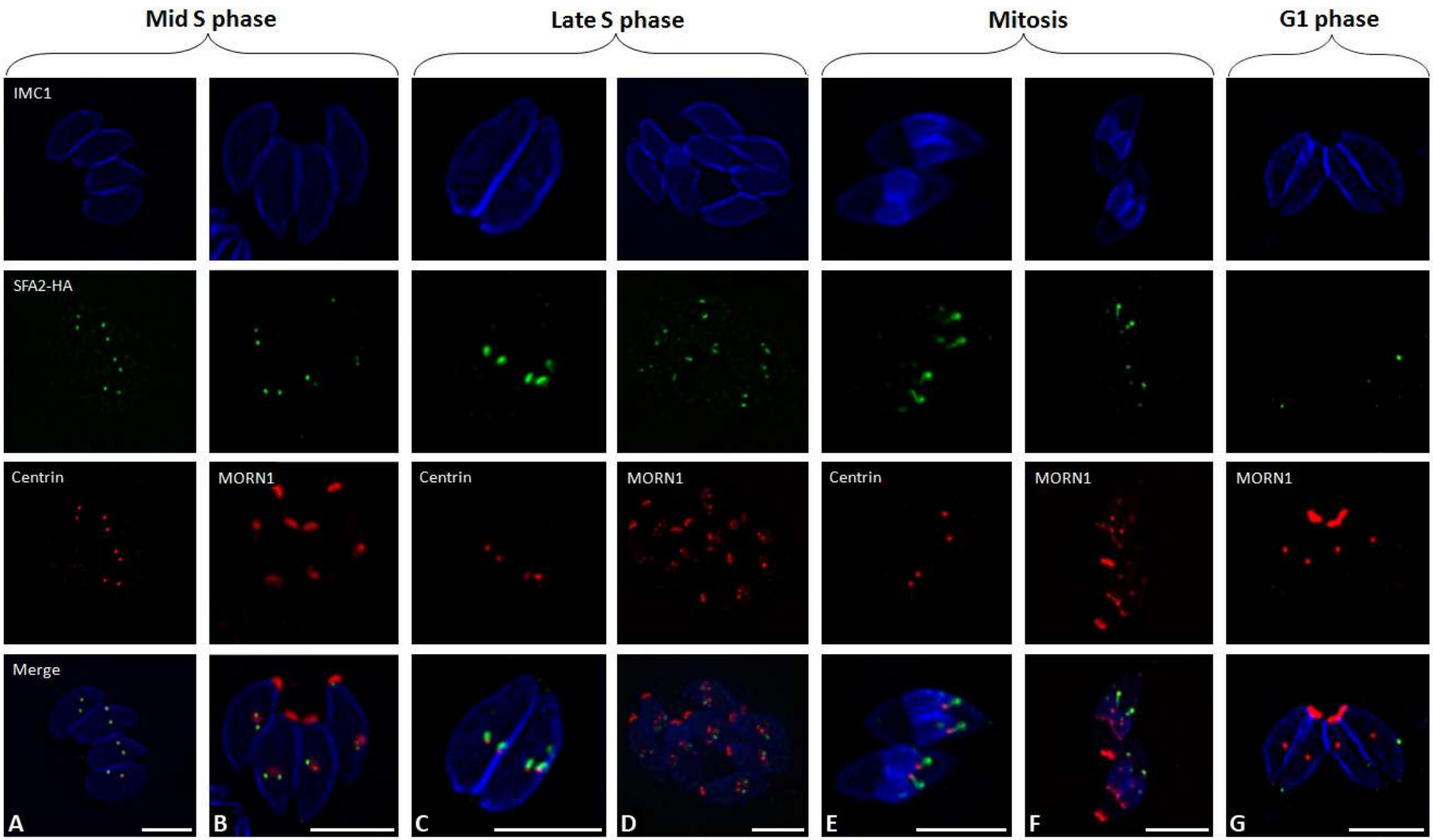

Figure 3.3: SFA2 localization is dynamic over the cell cycle of *T. gondii.*

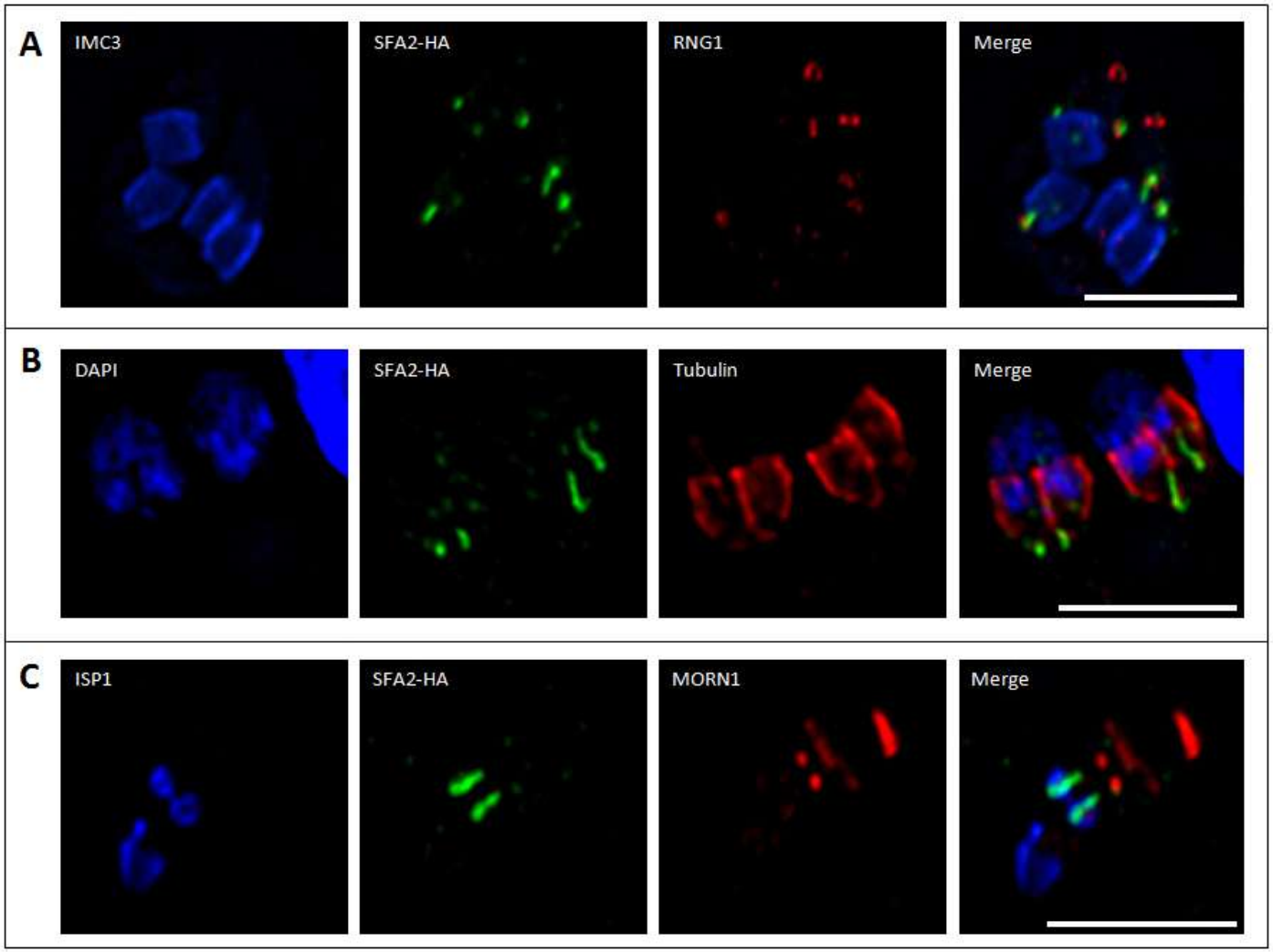

Figure 3.4: SFA2 fibers terminate at the apical cap of developing daughter cells in *T. gondii*.

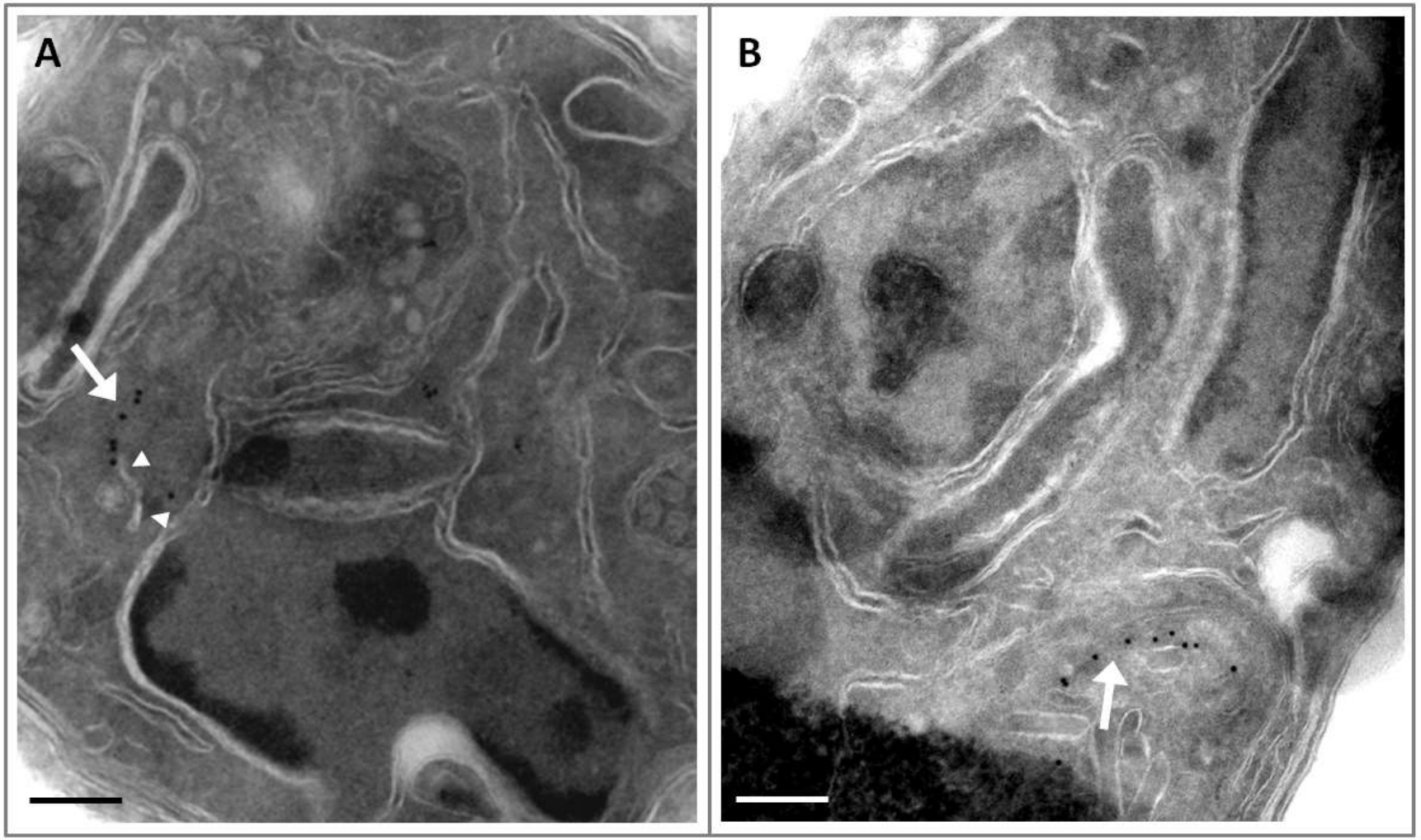

Figure 3.5: Ultrastructural examination of *T. gondii* confirms SFA2 localization.

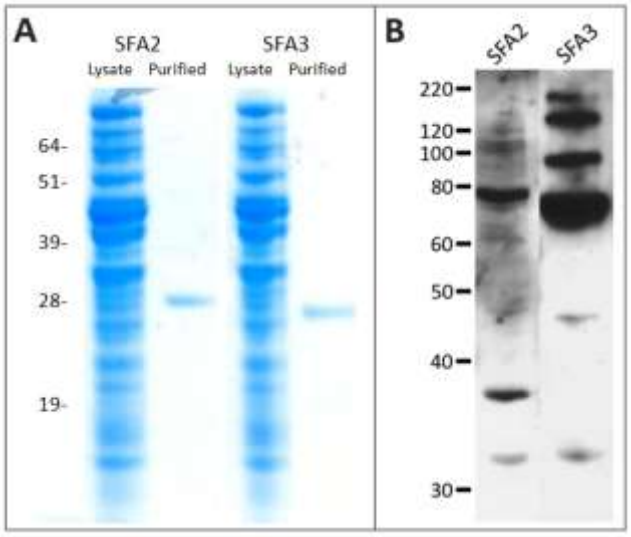

Figure 3.6: SFA2 and SFA3 proteins were expressed for antibody generation.

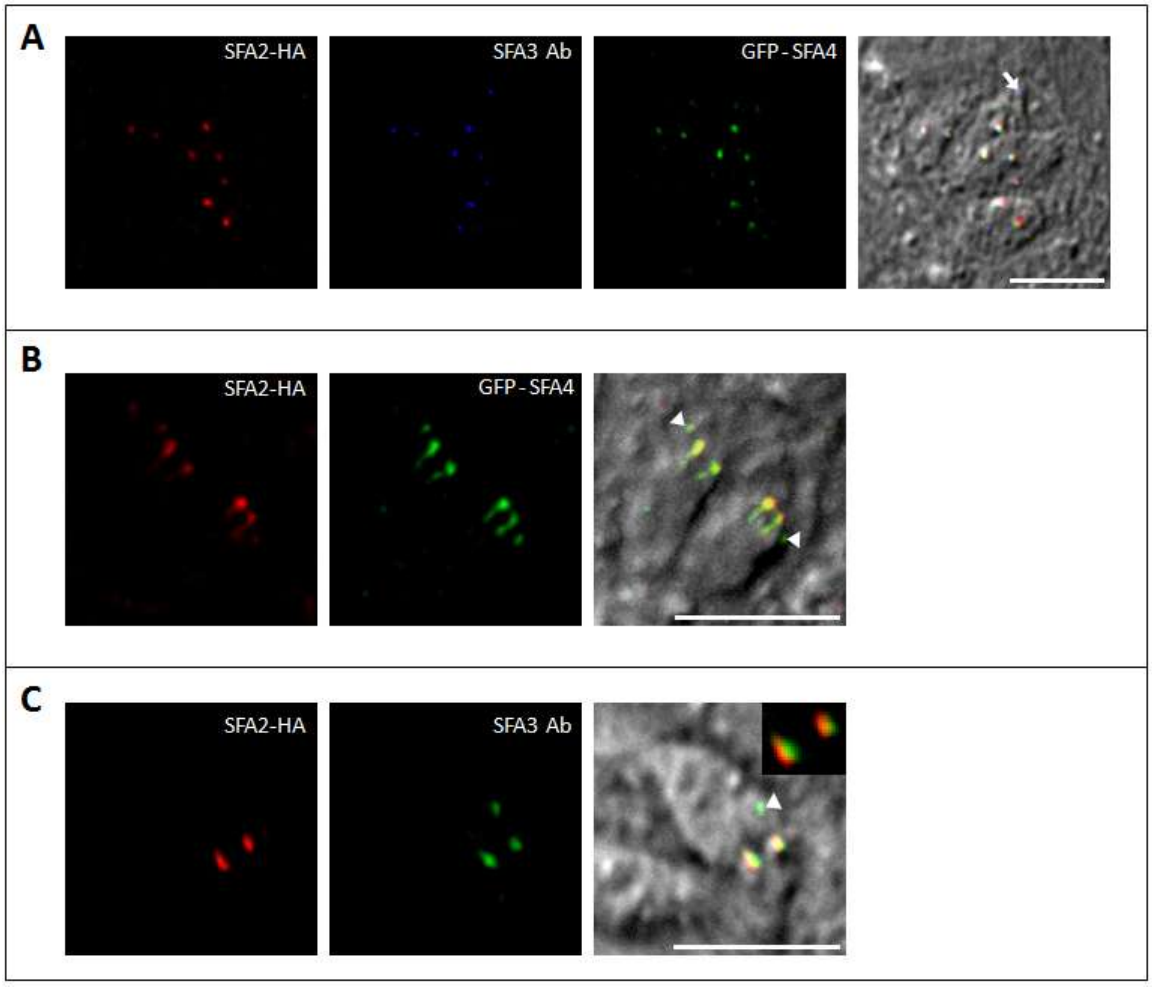

Figure 3.7: The three SFA proteins are co-localized in *T. gondii*.

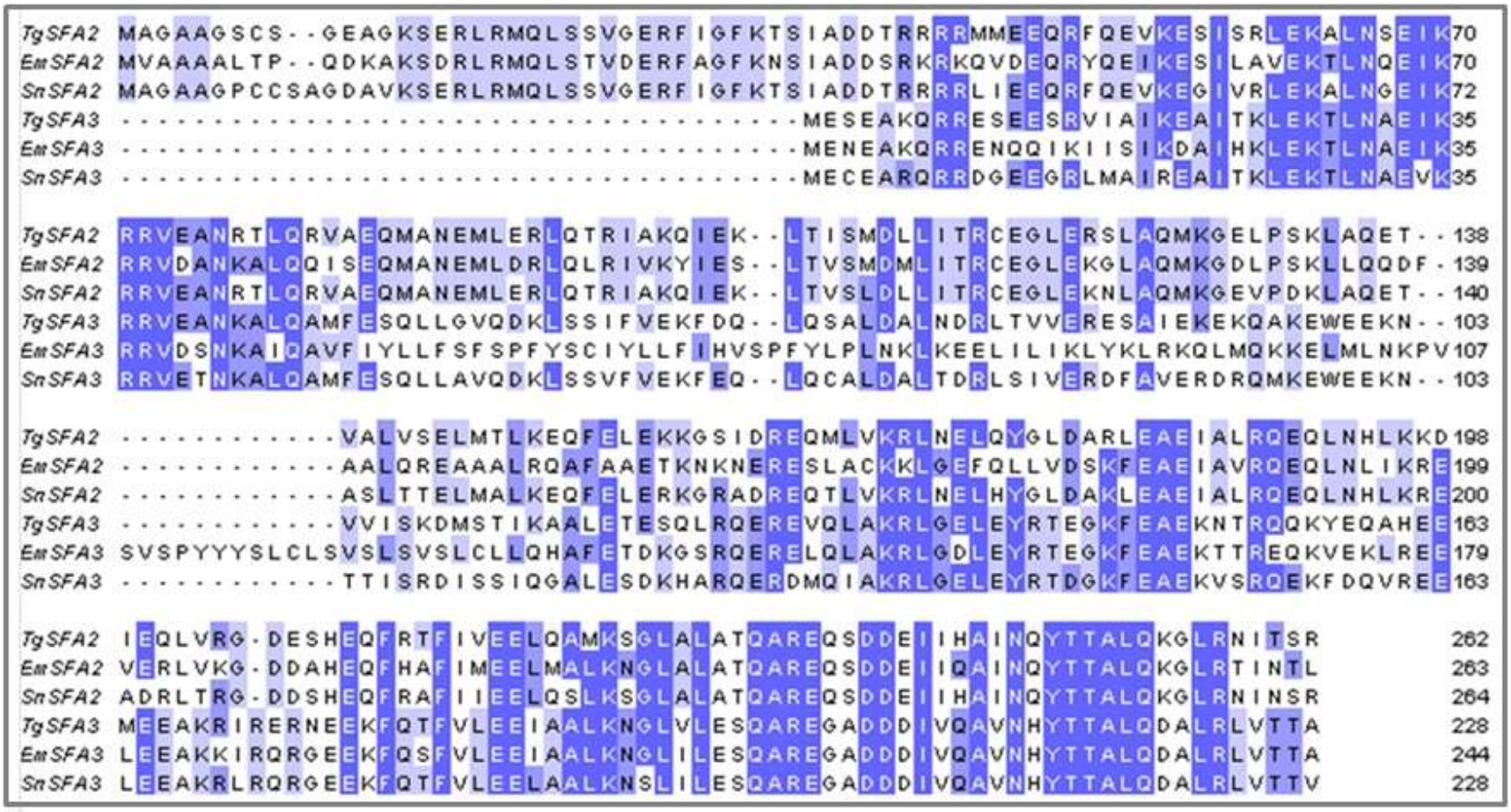

Figure 3.8: SFA orthologs are encoded in the genomes of related Apicomplexa

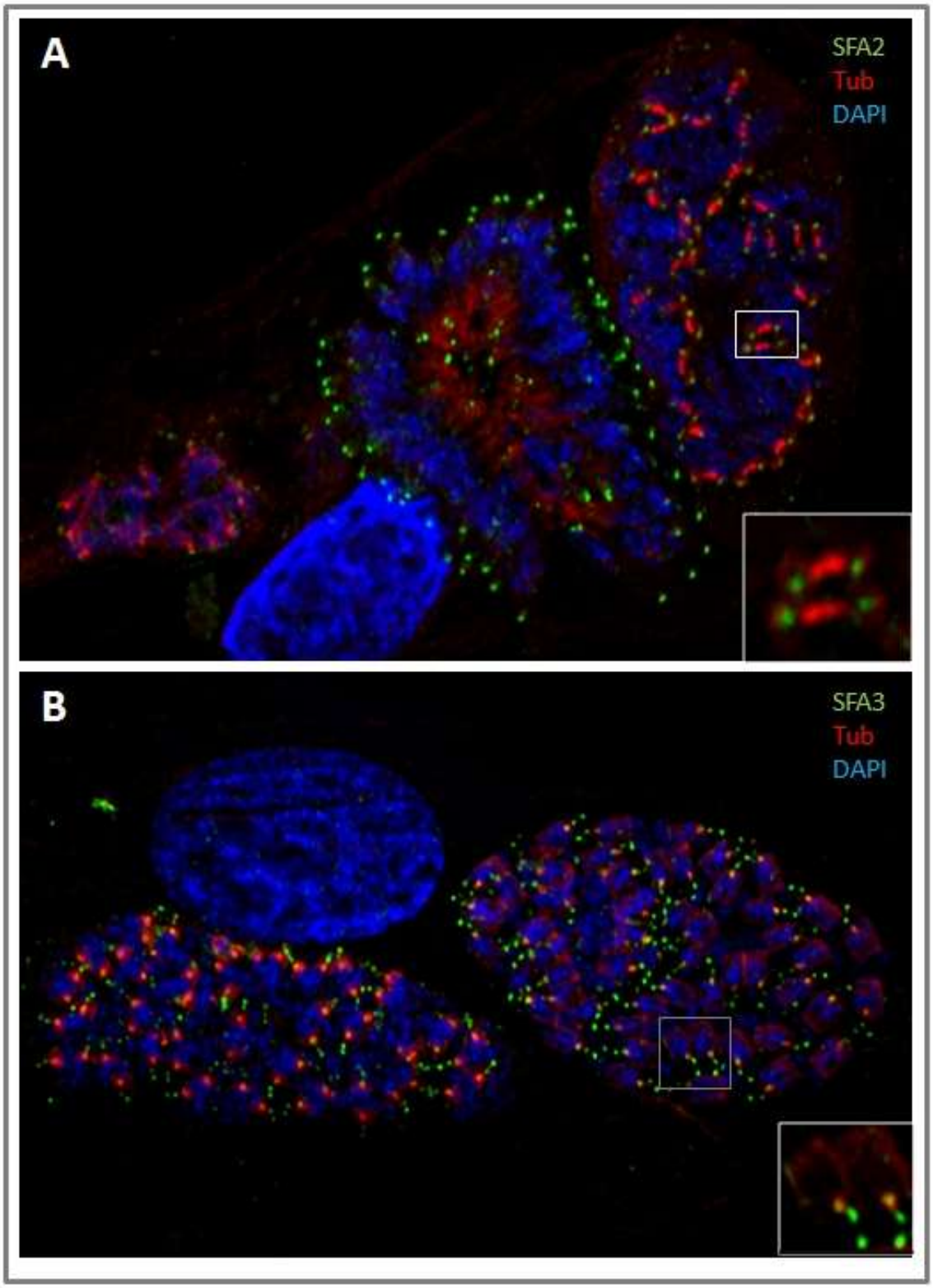

Figure 3.9: SFAs are expressed in *S. neurona* parasites during division.

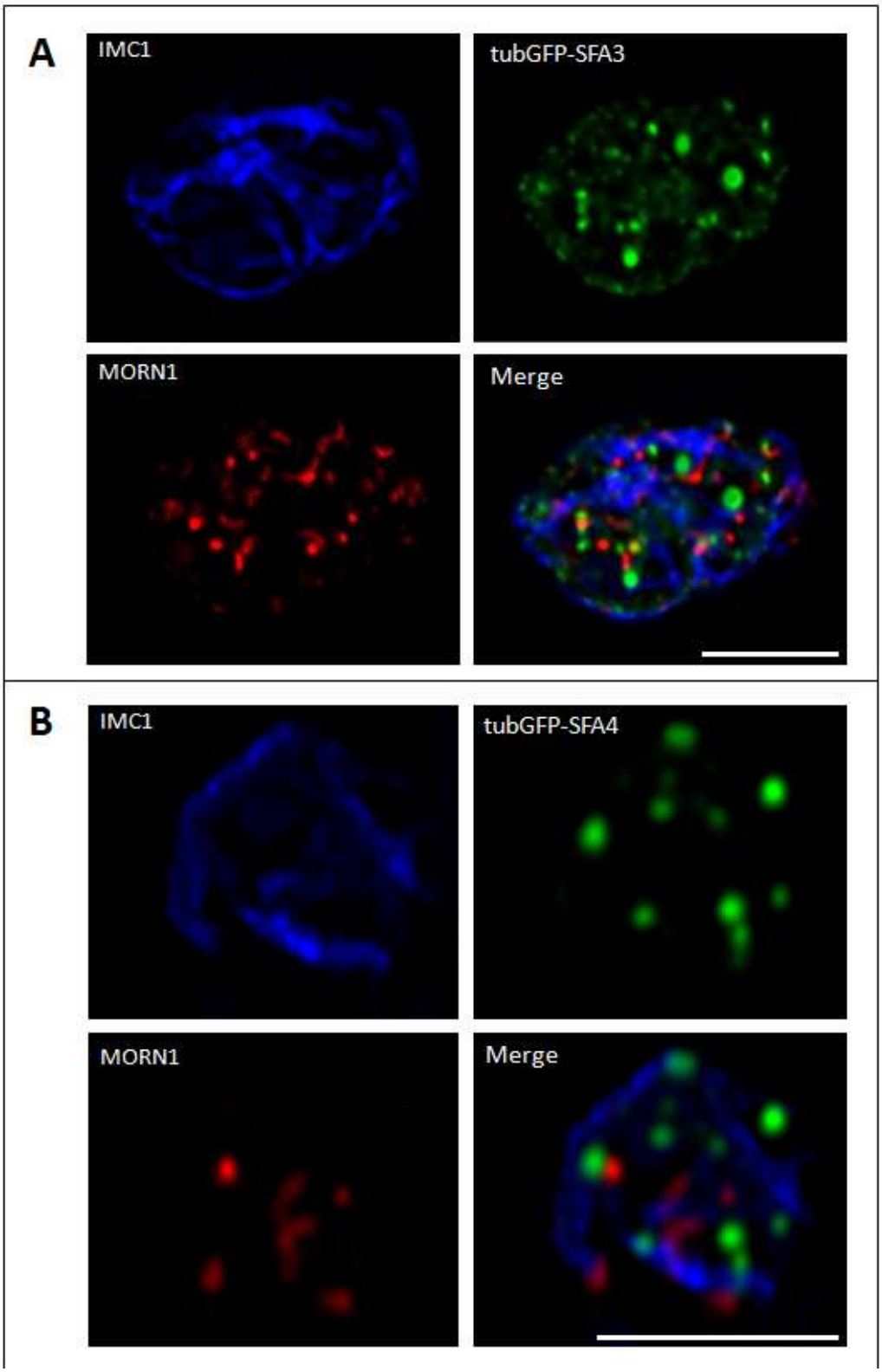

Figure 3.10: Overexpression of SFA3 and SFA4 in *T. gondii* results in lethal division defects.

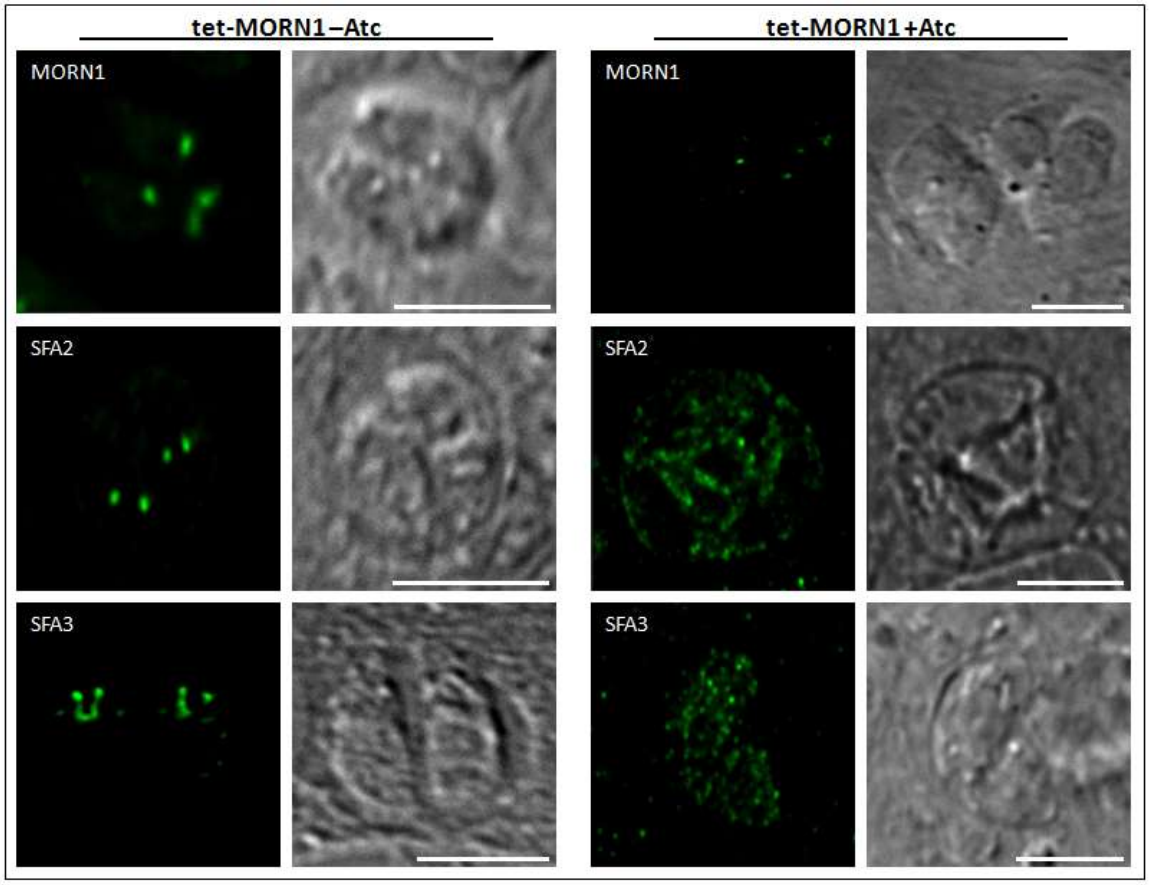

Figure 3.11: SFAs structures are not formed in *T. gondii* tachyzoites in the absence of MORN1.

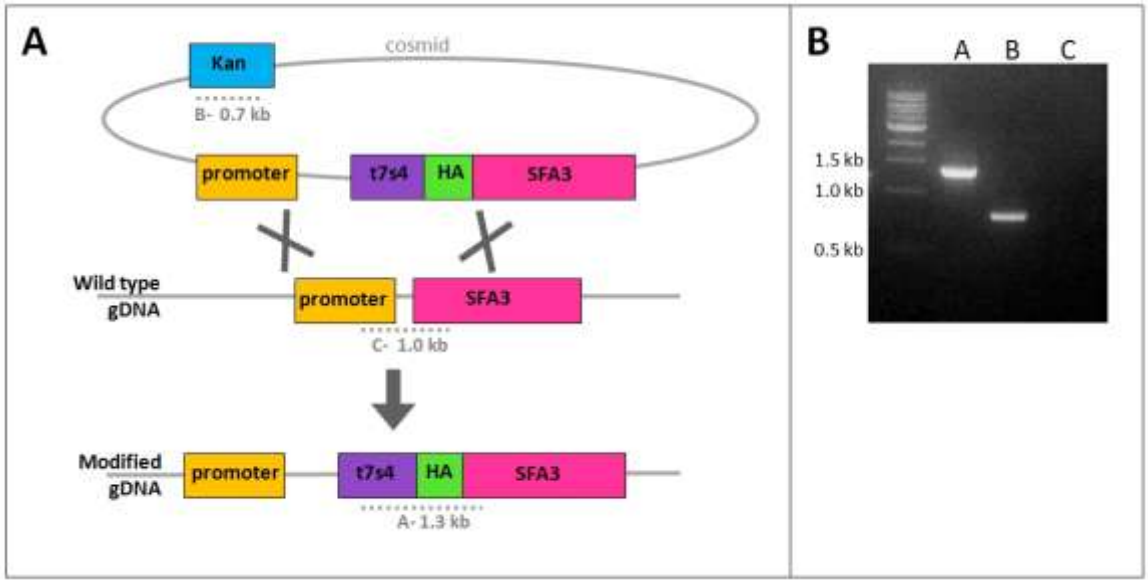

Figure 3.12: SFA3 promoter replacement in *T. gondii.*

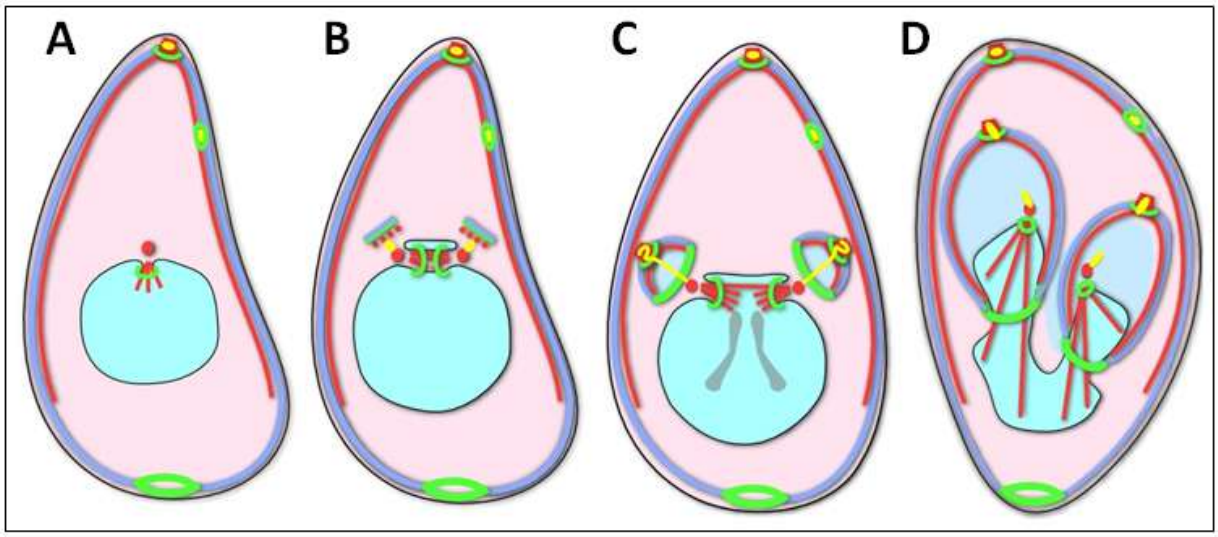

Figure 3.13: Model of *T. gondii* SFA localization over the cell cycle.

Table 3.1: Primer sequences used in this study.

Primer #	Primer sequence 5'-3' (restriction or LIC sites underlined)
1	tacttccaatccaatttcctatgcatGCTACTGTGGTTCTATGGGGCAAGG
2	tcctccagttccaattttagcGCGACTTGTTATATTTCGAAGGCCC
3	gggtcctggttcgATGGCGGGGGCTGCAGGGTC
4	cttgttcgtgctgtttaTTAGCGACTTGTTATATTTC
5	gggtcctggttcgATGGAGTCTGAGGCCAAGCA
6	cttgttcgtgctgtttaTCAGGCTGTCGTGACGAGA
7	cagtcccgggATGGCGGGGGCTGCAGGGT
8	tgcactgcagTTAGCGACTTGTTATATTTCGAAGG
9	atcgcccgggAAAATGGAGTCTGAGGCCAAGCA
10	tcaggttaacTCAGGCTGTCGTGACGAGACGCA
11	cagtcccgggATGAAGAACGGAATGGAA
12	tcagatgcatTCATATAGATGTTGTGATGCG

CHAPTER 4

CONCLUSIONS

Cell division in the Apicomplexa occurs by complex and unusual methods not fully understood. The question that spawned the work presented here is an important one: how do parasites faithfully segregate chromosomes and organelles into daughter cells? We have hypothesized that the mitotic spindle is always connected to chromosomes, and that a physical link exists between the centrosomes and developing daughters. This idea is based on several key observations. First, we know that the centrocone, the structure containing the mitotic spindle, is always present and in constant association with the centromeres (Gubbels et al., 2006; Brooks et al., 2011). Second, multiple studies have observed the centrosomes associating with the ends of duplicated organelles as they move into new daughter cells (Striepen et al., 2000; Hartmann et al., 2006). Finally, electron micrographs show a dense striated fiber stretching from the centrosome to the conoid of developing zoites of *Eimeria* species and *Toxoplasma gondii* (Dubremetz, 1975; Dubremetz and Elsner, 1979; Ferguson, 2007). This last piece of information led us to explore the striated fiber assemblin (SFA) proteins as a possible component of a linking structure.

SFA FORMS A FIBER BETWEEN THE CENTROSOME AND CONOID OF DEVELOPING *T. GONDII* TACHYZOITES.

To visualize SFA proteins in *T. gondii*, we engineered parasites with epitope or fluorescent tags on each of the three SFAs. We observed that SFA first appears as punctate

spots colocalized with the centrosomes immediately following their duplication. As the centrosomes separate, the SFA structures take on an oval shape and move adjacent to the centrosomes. When daughter buds begin to appear, SFA extends into fibers spanning from the centrosome to the conoid of developing daughters. As the buds grow larger, the fibers elongate and reveal a corkscrew shape where they meet the daughter conoids. Late in daughter budding, SFA appears as 2 spots in each new zoite: one at the centrosome and one at the conoid. We predict that when daughters grow sufficiently large, the fiber snaps, leaving remnants at each point of contact. SFA is often seen at the micropore in both mature and daughter cells, but while mature cells retain a small amount of SFA at the conoid, centrosome labeling disappears following division. Our observations of tagged SFA in *T. gondii*, combined with the knowledge that SFA forms striated fibers in green algae (Lechtreck and Melkonian, 1991), lead us to conclude that the striated fiber observed in electron micrographs of forming zoites is composed of SFA.

PROPER EXPRESSION OF SFA IS NECESSARY FOR CELL DIVISION IN *T. GONDII*.

Microarray analysis of gene expression across the cell cycle has shown that each of the *T. gondii* SFAs are upregulated in S-phase when budding initiates and downregulated immediately after division is complete, a pattern supported by our immunofluorescence observations. To examine the effect of alteration of SFA expression levels, we created parasites constitutively expressing the SFAs at a high level under the tubulin promoter. Overexpression of SFA3 or SFA4 was severely detrimental in *T. gondii*. Parasites and nuclei grew very large but daughter cells were never formed.

Parasites overexpressing SFA3 or SFA4 also demonstrated massive mislocalization of both MORN1 and IMC proteins. We examined the relationship between SFA expression and

MORN1 localization further using the inducible MORN1 knockout line (Lorestani et al., 2010). Parasites lacking MORN1 showed diffuse SFA localization throughout the cells, but no distinct SFA structures or fibers could be seen. In addition, multiple attempts to generate stable parasites expressing both a tagged SFA under its native promoter and a tagged tubulin expressed copy of either MORN1 or IMC1 were unsuccessful, even though each plasmid individually was shown to be non-toxic and selectable. These findings suggest that the SFAs, MORN1 and IMC proteins may interact in a mutually dependent manner, and that alteration of one protein affects the localization and function of the others, causing defects in daughter cell development.

SFA LABELS THE SPINDLE OF DEVELOPING ZOITES IN RELATED APICOMPLEXA

SFA homologs are found in the genomes of several Apicomplexa, and sequence conservation within the phylum is very high. To visualize potential homologs in other Apicomplexan parasites, we performed immunofluorescence assays using antibodies generated against recombinant *T. gondii* SFA proteins. In *S. neurona*, SFA clearly stained the spindle and conoids of developing daughters. Though complete fibers were not observed in *S. neurona*, three distinct spots of SFA were arranged linearly in dividing parasites, thus it may be possible that SFA is decorating a fiber of unknown composition in those cells. Comparison of our observations to published images of MORN1 labeling in *S. neurona* suggests that SFA and MORN1 are colocalized at the conoid in budding sporozoites (Gubbels et al., 2006). These observations confirm that SFA homologs are not only encoded but are expressed in a variety of Apicomplexan parasites. In addition, these results suggest that the role of SFA proteins in cell division is not limited to the process of endodyogeny, but is likely important in all mechanisms of zoite formation, as *S. neurona* uses endopolygeny.

FUTURE STUDIES

There are a few interesting ideas for examination of the SFAs from a biochemical standpoint that would further our understanding of these proteins. It is known that the green algal SFA proteins are able to form striated fibers *in vitro*, and that mutation of certain amino acids or domains alters the striation pattern of resulting fibers (Lechtreck, 1998). However, the head domain that is known to be responsible for self-polymerization in the green algae is poorly conserved in Apicomplexan SFAs and missing some key conserved amino acids. It would be interesting to know if the recombinant proteins we created can also self-assemble into fibers *in vitro*, or if some specific co-factors may be required for fiber formation in the Apicomplexa. Based on their localization at microtubule rootlets of green algae, it has been suggested that the SFAs may function to stabilize microtubules, though no direct evidence for this hypothesis has been reported. If recombinant fibers were easily formed *in vitro*, a simple microtubule-binding assay could provide added support for this hypothesis.

We would also like to confirm our hypothesis that the SFAs, MORN1, and IMC proteins interact during daughter cell formation. Immunoprecipitation assays could provide more evidence for this idea. The HA tagged SFA2 protein could be pulled down using commercially available reagents. Probing the eluate with antibodies to potentially interacting structures could reveal protein complexes important in *T. gondii* cell division. Some proteins of interest for which antibodies are available include: MORN1 (mitotic spindle), centrin (centrosomes), ISP1 (apical cap), and several IMC proteins.

Finally, we hope to confirm the essential role of the SFAs in *T. gondii* cell division by characterizing the phenotype of the inducible knockout we have created for TgSFA3. We predict that parasites lacking SFA would have defects in nuclear segregation into daughters, or may be

unable to construct the daughter pellicle at all. Characterization of the SFA3 mutant could be the final step in answering the question of how chromosomes and organelles are segregated into developing zoites.

APPENDIX A

BUILDING THE PERFECT PARASITE: CELL DIVISION IN APICOMPLEXA[1]

[1] Striepen, B., **C.N. Jordan**, S. Reiff and G.G. van Dooren. 2007. *PLOS Pathogens*. 3: e78. Reprinted here with permission from publisher.

ABSTRACT

Apicomplexans are pathogens responsible for malaria, toxoplasmosis, and crytposporidiosis in humans, and a wide range of livestock diseases. These unicellular eukaryotes are stealthy invaders, sheltering from the immune response in the cells of their hosts, while at the same time tapping into these cells as source of nutrients. The complexity and beauty of the structures formed during their intracellular development have made apicomplexans the darling of electron microscopists. Dramatic technological progress over the last decade has transformed apicomplexans into respectable genetic model organisms. Extensive genomic resources are now available for many apicomplexan species. At the same time, parasite transfection has enabled researchers to test the function of specific genes through reverse and forward genetic approaches with increasing sophistication. Transfection also introduced the use of fluorescent reporters, opening the field to dynamic real time microscopic observation. Parasite cell biologists have used these tools to take a fresh look at a classic problem: how do apicomplexans build the perfect invasion machine, the zoite, and how is this process fine-tuned to fit the specific niche of each pathogen in this ancient and very diverse group? This work has unearthed a treasure trove of novel structures and mechanisms that are the focus of this review.

A LEAN AND MEAN INVASION MACHINE

A wide variety of prokaryotic and eukaryotic pathogens have evolved the ability to invade and replicate within the cells of their hosts. Few have developed the level of sophistication and control exerted by the members of the Apicomplexa (Sibley, 2004). Upon contact with a suitable host cell, apicomplexans can invade within seconds, with minimal apparent disturbance of the infected cell (Figure 1). This process is dependent on actin and myosin and is driven by parasite and not host motility (Dobrowolski and Sibley, 1996; Meissner et al., 2002). Tightly associated with host cell penetration is the secretion of three distinct parasite organelles: rhoptries, micronemes, and dense granules. Secretion is timed in succession, and secreted proteins play key roles in adhesion, motility and formation, and elaboration of the parasitophorous vacuole, a new cellular compartment established during invasion that the parasite occupies during its intracellular development [see (Cowman and Crabb, 2006; Carruthers and Boothroyd, 2007) for detailed reviews of this process in *Toxoplasma* and *Plasmodium,* respectively].

The cellular structure of the zoite, the non-replicative extracellular stage, appears streamlined towards one goal: finding and invading the next host cell. Zoites are found at various stages of the apicomplexan life cycle and are the product of asexual as well as sexual replication processes (see Figure 1A for a simplified apicomplexan life cycle). The zoite is highly polarized, with the apical tip containing the organizing center for the subpellicular microtubles that run along the longitudinal axis of the parasite (Morrissette and Sibley, 2002). This axis also polarizes the cell's motility, driving the parasite into host cells with its apex first. In some species, the tip is further elaborated by the conoid, a cytoskeletal structure that is built from a unique, tightly wound tubulin polymer and is extended during invasion and motility (Hu et al.,

2002). Importantly, the apical end is also the site for rhoptry and microneme secretion, with these organelles tightly packed into the anterior portion of the cell. While the anterior of the zoite is focused on invasion, the rest of cell carries the genetic material and tools to grow and develop once in the host cell, including a nucleus and a single mitochondrion, plastid, and Golgi.

DIVIDE AND CONQUER

While invasive zoites are similar across the phylum, intracellular stages differ dramatically in size, shape, and architecture (see Figure 2 for a selection of micrographs). The basis for this diversity lies in the flexibility of the apicomplexan cell cycle. Apicomplexans are able to dissociate and variably mix and match three elements that follow each other invariably in most other cells: DNA replication and chromosome segregation, nuclear division, and, lastly, cytokinesis or budding (see Figure 3 for a schematic). While *Toxoplasma* completes all elements of the cycle after each round of DNA replication, *Plasmodium* and *Sarcocystis* forgo cytokinesis and/or nuclear divisions for multiple cycles, forming stages that are multinucleate or contain a single polyploid nucleus (these division modes are also known as endodyogeny, schizogony, and endopolyogeny (Sheffield and Melton, 1968; Speer and Dubey, 1999; Bannister et al., 2000). Dramatic differences in the division mode also occur between different life cycle stages in a single species; asexual stages of *Toxoplasma* in the cat intestine, for example, divide by endodyogeny and endopolygeny (Speer and Dubey, 2005). In each case, however, the development will culminate in the emergence of multiple invasive zoites, which seek new host cells to invade. Apicomplexans of the genus *Theileria* are a surprising exception to this divide and conquer scenario. *Theileria* sporozoites remain in the lymphocyte that they initially invade, where they amplify in numbers without resorting to leaving the shelter of the host cell. The key to this trick lies in this parasite's ability to transform the host cell through manipulation of the

NFκB pathway. The parasite assembles and activates a mammalian IKK signalosome on its surface, promoting unchecked host cell replication (Heussler et al., 2002; Dobbelaere and Kuenzi, 2004). *Theileria* also interacts with host cell microtubules, enabling these parasites to migrate to, and apparently latch onto, host cell centrosomes. This results in partitioning of parasites into forming daughter cells of the host, exploiting the host's mitotic spindle [see Figures 2 and 3; (Shaw et al., 1991; Dobbelaere and Kuenzi, 2004); and D. Dobbelaere, personal communication].

CHECKPOINTS AND MASTER SWITCHES

Initial work using inhibitors of DNA synthesis (e.g., aphidocolin) and microtubule disrupting agents suggested that classical cell cycle checkpoints might be lacking in apicomplexans (Shaw et al., 2000; Shaw et al., 2001), pointing to potentially novel mechanisms of control over their complex cell cycles. However, studies using different blocking agents (thymidine, pyrrolidine dithiocarbamate) and characterization of a series of temperature-sensitive mutants have found that the *Toxoplasma* cell cycle can be halted at what appear to be specific points, including the G1/S and S/M boundaries (Radke et al., 2001; White et al., 2005; White, 2007). Furthermore, genomic and experimental surveys for proteins commonly associated with cell cycle checkpoints have identified numerous candidates, including cyclins and cyclin-dependent kinases in *Plasmodium* and *Toxoplasma* (Khan et al., 2002; Kvaal et al., 2002; Ward et al., 2004; Chen et al., 2006). An attractive model could suggest the presence of developmentally regulated sets of cell cycle factors resulting in different cell division types, which are in turn controlled by master switches. For example, we could hypothesize that *Toxoplasma* tachyzoites contain master switches to promote nuclear division following DNA synthesis, and cell division following mitosis. Down-regulation of the nuclear division master

switch would result in the multiple rounds of DNA synthesis observed during *Sarcocystis* endopolygeny, while down-regulation of the cytokinesis master switch would lead to the multinucleated schizonts observed in other stages of the *Toxoplasma* life cycle and in *Plasmodium* blood stages. Some initial support for this idea has begun to emerge. A series of homologs of the centrosome-associated NIMA kinase (which, in fungi, controls entry into mitosis and spindle formation) have been shown to be essential for cell cycle progression and survival in *Plasmodium* by gene targeting studies (Dorin et al., 2001; Khan et al., 2005; Reininger et al., 2005; Lye et al., 2006) and in *Toxoplasma* by analysis of temperature-sensitive parasite mutants (M. Gubbels and B. Striepen, unpublished data). NIMA genes appear to be differentially expressed over the *Plasmodium* life cycle. Nek4, for example, is specifically expressed in the female gametocyte and is required for the initial chromosome duplication in the ookinete (zygote) preceding meiosis (Khan et al., 2005; Reininger et al., 2005), but is dispensable in other stages.

COUNTING CHROMOSOMES

A fascinating question when considering the various forms of apicomplexan cell division is, how do parasites keep track of their chromosomes in polyploid stages, and how do they know how many zoites to make upon cytokinesis? The following two observations might be important to consider: the final budding of zoites is invariably associated with a last round of DNA replication and nuclear division, and studies that have used high doses of microtubule disrupting agents have found this to lead to a catastrophic breakdown of the coordination of nuclear division and budding in a variety of species (Shaw et al., 2000; Morrissette and Sibley, 2002; Vaishnava et al., 2005; Fennell et al., 2006). This suggests that the mitotic spindle, or its organizing center, controls the number of daughter cells and the site where they are to be

formed. Apicomplexans use an intranuclear spindle and maintain the nuclear envelope throughout mitosis. The spindle resides in a dedicated elaboration of the nuclear envelope, the centrocone ((Dubremetz, 1973); Figure 4A), and interacts with the cytoplasmic centrosome through an opening of the envelope. Interestingly, recent studies in *Toxoplasma* and *Sarcocystis* using antibodies to tubulin and MORN1 (a protein that localizes to the centrocone; see below) have shown that the centrocone is maintained throughout the cell cycle (Vaishnava et al., 2005; Gubbels et al., 2006). Persistence of the spindle, and persistent kinetochore attachment of chromosomes to the spindle microtubules, would provide a mechanism to maintain the integrity of chromosomal sets through polyploid stages (Vaishnava et al., 2005); however, this hypothesis requires experimental validation. While centrocone-like structures have been identified in *Plasmodium* during mitosis and budding (Bannister et al., 2000; Bannister et al., 2004), it is currently not clear if these persist (developing reagents to the *Plasmodium* homolog of the MORN1 protein should quickly resolve this question).

BUILDING THE ZOITE SCAFFOLD

Apicomplexans preassemble zoites as buds either internally in the cytoplasm *(Toxoplasma)* or directly under the surface membrane *(Plasmodium).* The scaffold for bud assembly and the outline of the new daughter cells is provided by the pellicle, which consists of subpellicular microtubules and the inner membrane complex (IMC). The subpellicular microtubules emerge from an apical microtubule organizing center associated with the polar rings and run along the longitudinal axis of the cell (Russell and Burns, 1984; Morrissette et al., 1997). The IMC is a system of flattened membrane cisternae stabilized by a membrane-associated protein meshwork facing the cytoplasm. Several of the protein components of this meshwork have been characterized and they share weak similarity with articulins, filament

proteins found in ciliates (Mann and Beckers, 2001; Mann et al., 2002; Gubbels et al., 2004). Several IMC proteins show dynamic regulation, with their expression timed to coincide with budding [29]. Some IMC proteins also undergo proteolytic processing, and it has been suggested that this process confers increased rigidity to the IMC following its deposition (Hu et al., 2002; Mann et al., 2002). Recently, proteins integral or tightly associated with the outer IMC membrane have been identified. GAP50, together with GAP45, serve as internal anchors of myosin A and the associated gliding motility machinery (Gaskins et al., 2004; Baum et al., 2006). However, the function of PHIL1, which forms a ring structure at the apical tip of the bud, remains to be elucidated (Gilk et al., 2006).

Following mitotic separation of the chromosomes, budding initiates in the direct vicinity of the centrosomes. The first identifiable sign of the bud is a flattened vesicle associated with a small number of evenly spaced microtubules (Sheffield and Melton, 1968; Dubremetz, 1973; Dubremetz, 1975; Bannister et al., 2000). This structure is further elaborated into a cup, with the conoid at its apex and microtubules extending from the conoid to posterior ring, delimiting the bud. Genetic and proteomic studies in *Toxoplasma* have identified a number of proteins associated with these early processes, and fluorescent protein tagging and live cell microscopy has painted a highly dynamic picture of their localization and function. The Toxoplasma gondii genome encodes several centrin genes, with centrin 1, 2, and 3 having been localized by GFP fusion (Hartmann et al., 2006; Hu et al., 2006; Nagamune and Sibley, 2006). While centrin 1 and 3 appear to be focused at the centrosome, centrin 2 additionally labels the conoid and a peculiar group of punctate structures in the apex of the cell (Hu et al., 2006; Nagamune and Sibley, 2006). Dynein light chain, a component of the minus end-directed microtubular motor dynein, has been detected near the centrosome and the conoid, and may be involved in conoid and centrosomal movements. MORN1 has been particularly informative as a marker for budding, as

it labels both the centrocone and spindle and the apical and posterior ends of the bud [Figure 4; (Gubbels et al., 2006; Hu et al., 2006)]. The precise chronology of assembly—especially in the very early phase of bud development—remains to be elucidated, and would benefit from the generation of mutants for the various steps involved. Early electron microscopic studies have implicated a striated fiber as an organizing element (Dubremetz, 1975); interestingly, proteins similar to algal striated fiber assemblins have been identified recently in apicomplexans and have been shown to localize to the centrosomal region during budding (Lechtreck, 2003). Once the bud is assembled it grows rapidly, most likely driven by microtubule growth. This process runs opposite to spindle extension and effectively partitions the nucleus and much of the cytoplasm. Toward the end of bud development, the MORN1 ring at the posterior end of the bud shows pronounced contraction (see Figure 4J and 4K), which likely aids in organellar division (see below) and cytokinesis. Several observations are consistent with an association of this ring with myosin B/C (Delbac et al., 2001; Gubbels et al., 2006); however, the actin-destabilizing drug cytochalasin D does not interfere with parasite division (Shaw et al., 2000).

COMPLETING PARASITE ASSEMBLY

A fully formed apicomplexan parasite requires a multitude of organelles and intracellular structures that will enable it to carry out the next task of its life cycle—to egress from the host cell and invade a new one. Rhoptries, micronemes, and dense granules form de novo during budding, anterior to the nucleus, endowing each daughter cell with the apical secretory organelles necessary for invasion. Expression of rhoptry and microneme proteins is regulated at the transcriptional level and timed to coincide with budding (Baldi et al., 2000; Brown et al., 2000; Bozdech et al., 2003; Hoane et al., 2003). The apicomplexan secretory pathway is highly polarized, with an endoplasmic reticulum (ER) exit site localized on the apical

face of the nucleus adjacent to the centrocone (Hager et al., 1999; Bannister et al., 2000; Pfluger et al., 2005). Here, proteins are loaded into coated vesicles that travel to the Golgi and on to several (still poorly characterized) trans-Golgi, pre-rhoptry, and pre-microneme compartments (Hager et al., 1999; Ngo et al., 2000; Harper et al., 2006). The Golgi is associated with the centrosome(s), which play an important role in its duplication (He, 2007). Golgi duplication is among the earliest events of budding (Pelletier et al., 2002; Hartmann et al., 2006). In *Plasmodium,* the Golgi divides multiple times during intracellular development, and upon zoite formation, a single Golgi is associated with each bud (Struck et al., 2005). The spatially fixed line-up of ER exit site and Golgi and their association with the nucleus and centrosome likely acts as a highly effective cellular "funnel", directing the flow of proteins and membranes into the growing buds. IMC proteins, including the N-glycosylated GAP50 (Gaskins et al., 2004), probably derive from the Golgi, suggesting that membranes of the IMC form from Golgi-derived vesicles. This would explain the necessity for early division of the Golgi during budding, and suggests that Golgi positioning by the centrosome is critical in mediating deposition of the IMC.

Apicomplexans harbor two endosymbiont-derived organelles, the mitochondrion and the apicoplast, both of which perform a broad array of metabolic functions and are essential for intracellular parasite development (Jomaa et al., 1999; Ralph et al., 2004; Mazumdar et al., 2006; van Dooren et al., 2006). These organelles carry their own genomes (Vaidya et al., 1989; Feagin, 1992; McFadden et al., 1996; Wilson et al., 1996; Kohler et al., 1997) and therefore cannot be formed de novo, but must undergo division followed by segregation into buds. Genomic analyses in apicomplexans have identified proteins commonly involved in mitochondrial division, like dynamin-related proteins (van Dooren et al., 2006). However, the FtsZ-based division machine found in a wide variety of chloroplasts has been lost in apicomplexans (Osteryoung and Nunnari, 2003; Vaishnava and Striepen, 2006). Instead of

relying on their ancestral prokaryotic division ring, it would appear that apicoplasts have developed novel means of division. One model suggests that the force for apicoplast division is provided by association of the apicoplast with the mitotic spindle (Striepen et al., 2000). Dynamic association between the centrosome(s) and the apicoplast has been demonstrated in *Toxoplasma* and *Sarcocystis* and provides a likely means by which these organelles are properly segregated into forming buds (Striepen et al., 2000; Vaishnava et al., 2005). In both organisms, fission of the organelle into daughter plastids is tightly associated with budding, and the constrictive MORN1 ring found at the posterior end of each bud provides an attractive candidate for a fission mechanism [see Figure 4C; (Gubbels et al., 2006; Vaishnava and Striepen, 2006)]. A second model suggests that apicoplast fission is independent of cytokinesis and relies on a medial division ring formed by yet-to-be identified components (Ferguson et al., 2005). The development of the plastid in organisms dividing by schizogony, like *Plasmodium* and *Eimeria,* is not fully understood (van Dooren et al., 2005; Ferguson et al., 2007). While centrosome association is likely to be involved in the segregation into daughters, it is unclear if such association occurs in earlier stages. In *Plasmodium,* mitochondria and apicoplasts form a physical association shortly before budding (van Dooren et al., 2005), suggesting that segregation of these organelles into daughter buds is tightly linked. Nevertheless, better in vivo markers (especially for the centrosome) are needed to identify mechanisms of organellar division and segregation in these organisms.

OUTLOOK

The advent of reverse genetics for a variety of apicomplexans has led to a renaissance in the study of the cell biology of these parasites. A number of exciting new structures and mechanisms have been discovered in this process. Not unlike the study of host cell invasion,

exploring the intracellular development of apicomplexans has brought out conserved themes at the mechanistic level, suggesting significant similarity between different species within the phylum. The “post-genomic” era of apicomplexan cell biology offers powerful experimental avenues that will undoubtedly drive our understanding of cell division and zoite formation. Gene expression profiling using microarrays, now available for several systems, has identified large groups of candidate genes that are expressed during budding. Comparative genomic analysis can be used to further narrow the list of candidates. The ever improving forward and reverse genetics tool box offers robust experimental avenues to test the function of essential genes, and genetic analysis will be critical for establishing the sequence of events during budding (Striepen, 2007). The coming years will likely reveal an increasingly detailed and mechanistic picture of these tiny diabolical, yet fascinating, invasion machines.

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AUTHOR CONTRIBUTIONS

All authors contributed to writing the paper.

FIGURES AND TABLES

Table 1: Apicomplexan Parasites.

Figure 1: Apicomplexa Are Intracellular Parasites. (A) Highly simplified apicomplexan life cycle. Apicomplexans are haplonts, and meiosis (sporogony) immediately follows fertilization. Fertilization might occur within a host cell or extracellularly, giving rise to an oocyst or, less frequently, an invasive stage zygote (ookinete). (B) Schematic representation of a zoite (not all structures are present in all apicomplexans). AP, apicoplast; AR, apical rings; CC, centrocone; CE, centrosome; CO, conoid; DG, dense granule; ER, endoplasmic reticulum; G, Golgi; IMC, inner membrane complex; MI, mitochondrion; MN, microneme; MT, subpellicular microtubule; NU, nucleus; RH, rhoptry. (C) Zoites actively invade the cells of their hosts, establishing a specialized parasitophorous vacuole (PV) (in some species the parasite lyses the vacuole and develops freely in the cytoplasm).

Figure 2: The Diversity of Intracellular Development in Apicomplexans. (A) In *T. gondii,* two daughters are formed during budding. IMC1, red; MORN1, green (reproduced with permission from [32]). (B) *T. gondii.* Histone H2, red; IMC3, green (reproduced from [71]). (C) In *Plasmodium falciparum* liver schizont, budding results in massive numbers of zoites. Image courtesy of Volker Heussler. (D) *T. gondii,* phase contrast image of parasitophorous vacuole harboring multiple tachyzoites. (E and F) P. falciparum late erythrocyte schizont. Acyl carrier protein (plastid), green. RBC, red blood cell. (G–I) *Sarcocystis neurona.* Two intracellular stages with polyploid nuclei, one in interphase and one during mitosis. Tubulin, red. (J) S. neurona budding. IMC3, green. (K) A *Theileria* schizont divides in association with its host cell. Polymorphic immunodominant molecule (parasite surface), green; γ-tubulin (host centrosomes), red. HN,

host nucleus. Image courtesy of Dirk Dobbelaere. The DNA dye DAPI is shown in blue throughout. Not to scale.

Figure 3: The Flexibility of Apicomplexan Cell Division. Schematic outline of cell division by *Toxoplasma* (endodyogeny), *Plasmodium* (schizogony), and *Sarcocystis* (endopolygeny). The *Theileria* schizont is divided in association with host cell division (HN, host nucleus). DNA, grey; IMC, purple; centrosome, red. Note that a centriole as center of the spindle plaque body has not been clearly demonstrated in P. falciparum. Both *Sarcocystis* and *Theileria* develop directly in the host cell cytoplasm, while *Toxoplasma* and *Plasmodium* are contained within a parasitophorous vacuole (light blue).

Figure 4: The Mechanics of Apicomplexan Mitosis and Budding. (A–C) Schematic representation of the nucleus during interphase (A), mitosis (B), and mid-stage budding (C). Smaller type abbreviations refer to organelle-specific marker proteins in T. gondii (most are available as fluorescent protein in vivo tags, see text for further details and references). AP, apicoplast; AR, apical rings; CC, centrocone; CH, chromosome; CO, conoid; CT, centromere; EX, ER exit site; MT, subpellicular microtubule; NE, nuclear envelope; PR, posterior ring; SP, spindle. (D–K) Time lapse series of nuclear division in T. gondii reproduced from [32]. The nucleus is labeled in red (Histone H2b-RFP) and MORN1 in green (MORN1-YFP).

Table 1

Pathogen	Disease	Host	Transmission
Plasmodium falciparum	Malaria	Human	Mosquito bite
Toxoplasma gondii	Encephalitis	Human, variety of animals	Meat, water
Cryptosporidium parvum	Enteritis	Human, cattle	Water
Theileria parva	Lymphoproliferation	Cattle	Tick bite
Eimeria tenella	Enteritis	Chicken	Fecal-oral
Sarcocystis neurona	Myeloencephalitis	Horse, variety of animals	Water, fecal-oral

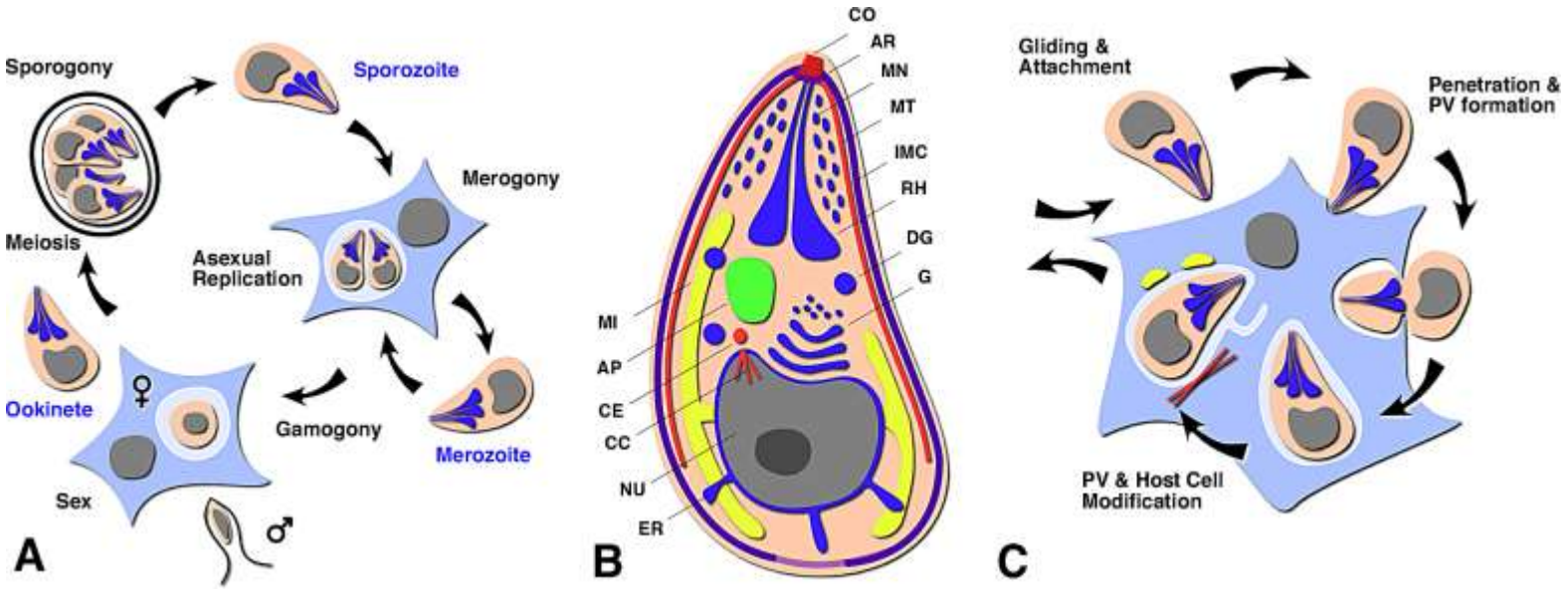

Figure 1: Apicomplexa Are Intracellular Parasites.

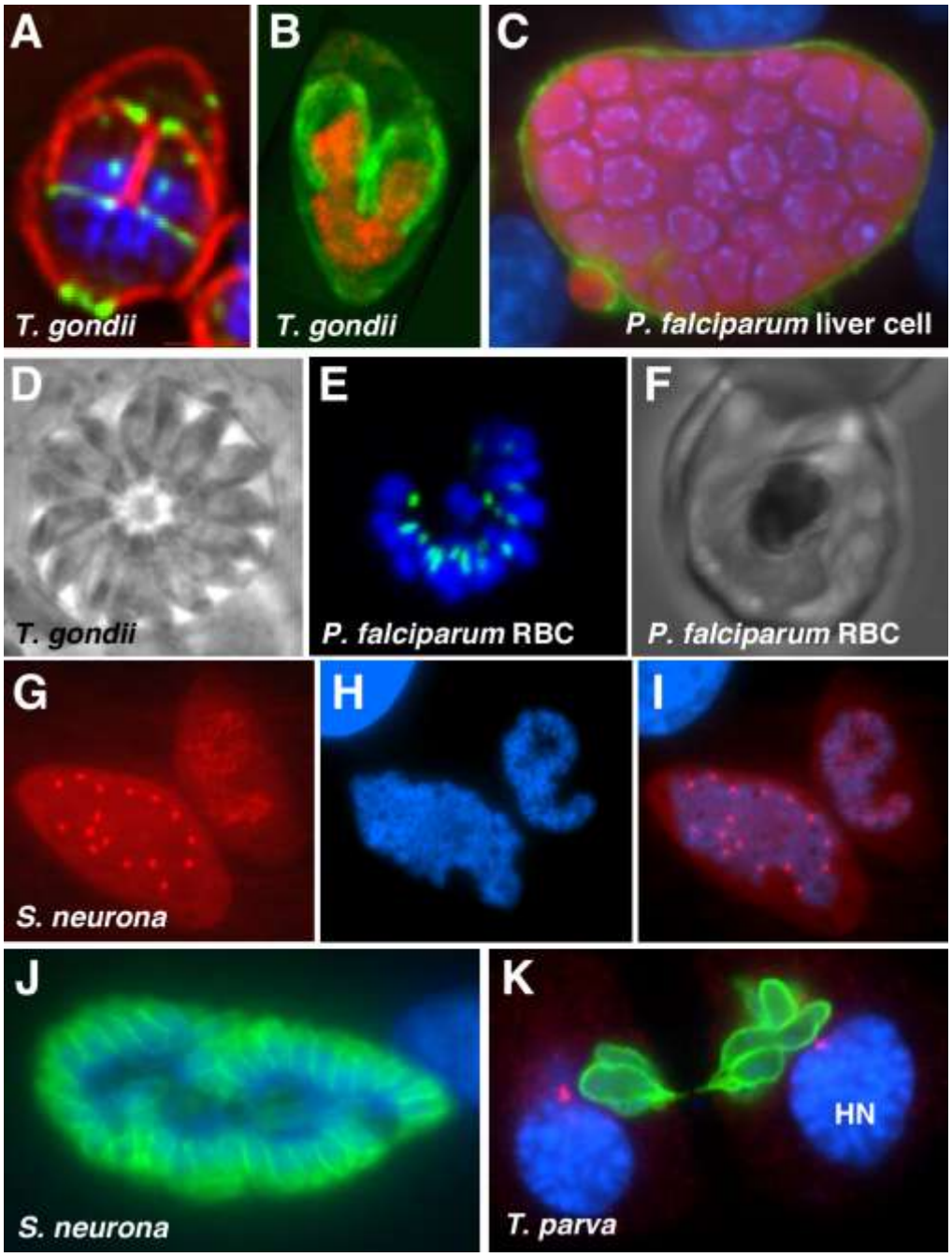

Figure 2: The Diversity of Intracellular Development in Apicomplexans.

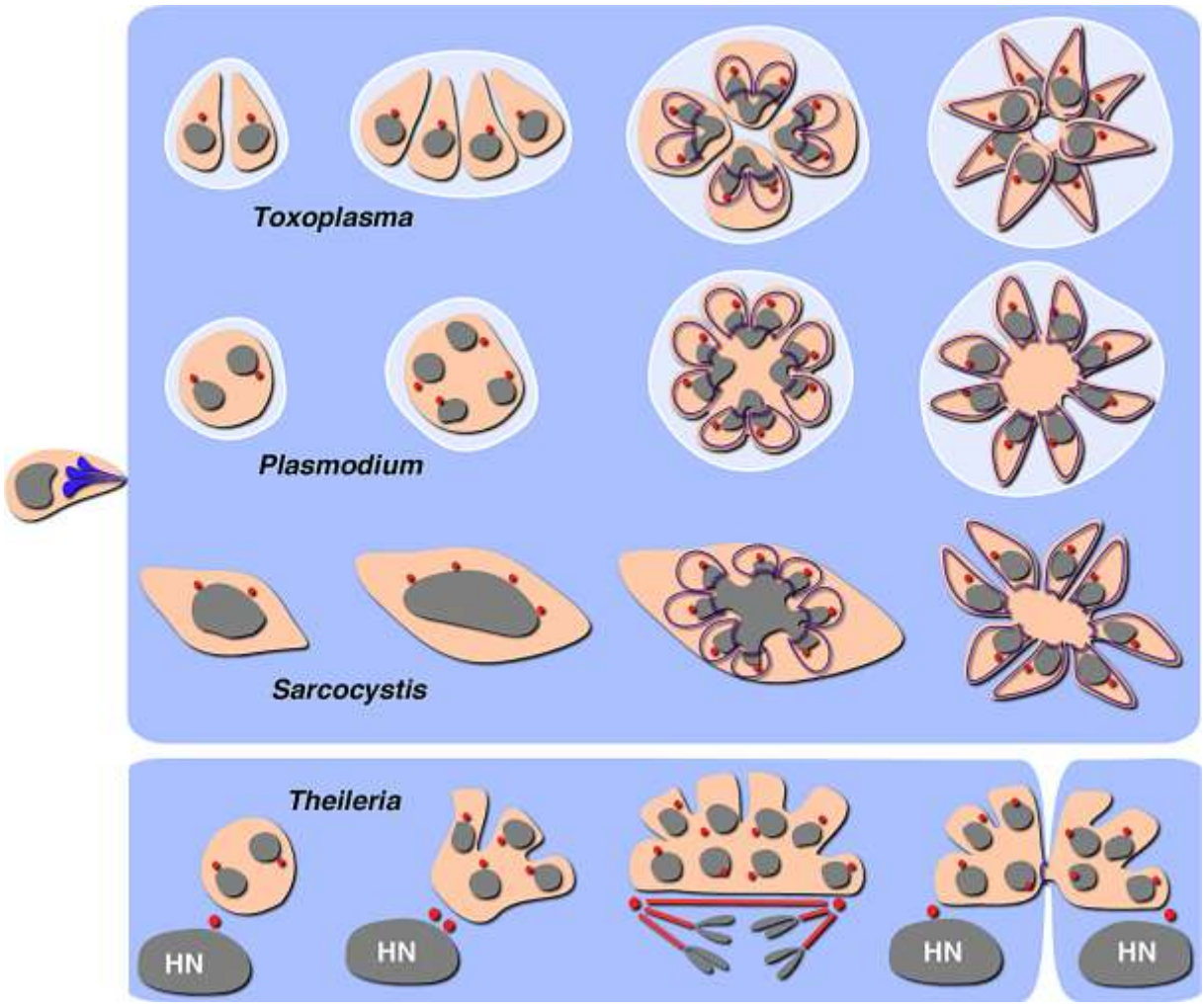

Figure 3: The Flexibility of Apicomplexan Cell Division.

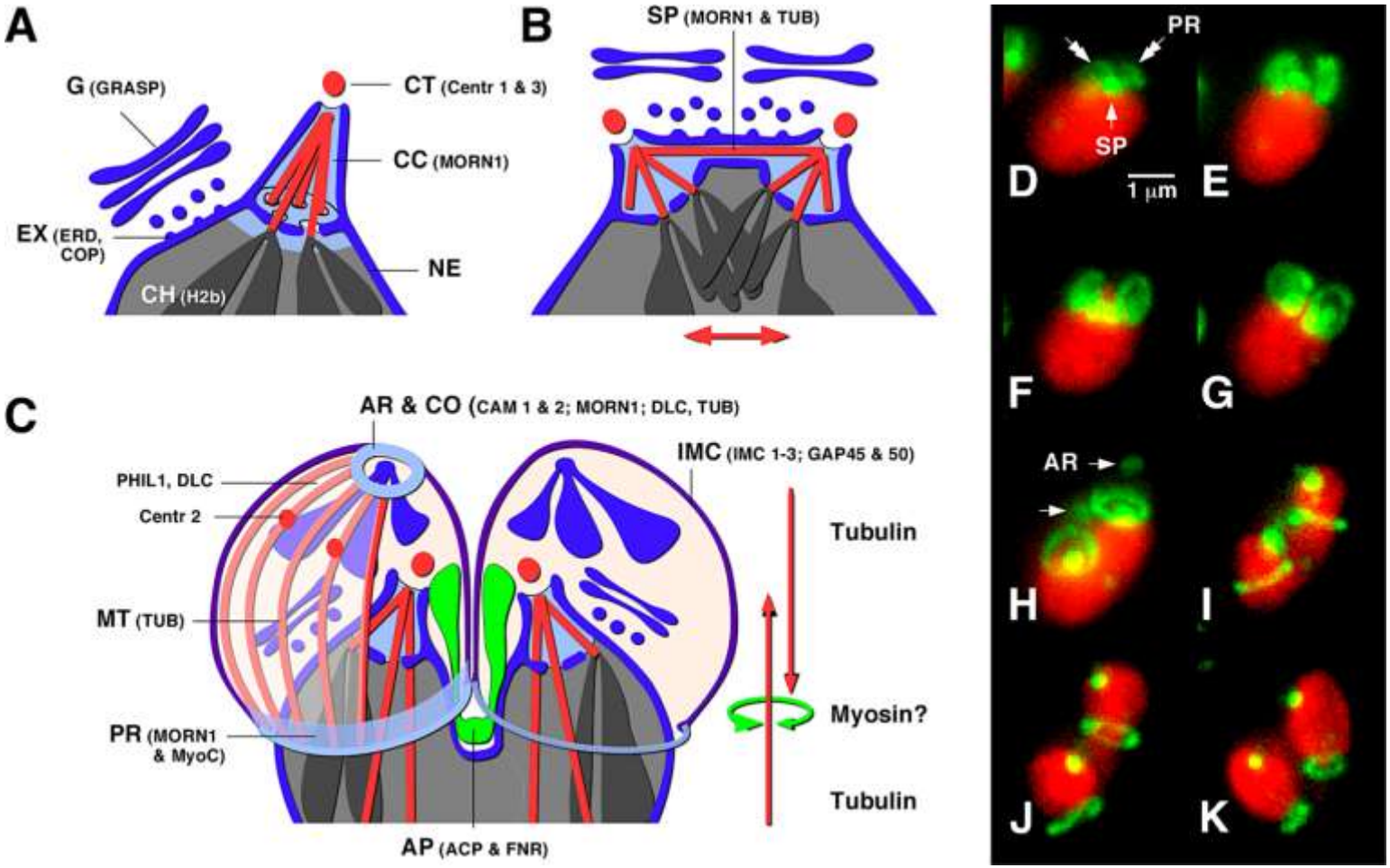

Figure 4: The Mechanics of Apicomplexan Mitosis and Budding.

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