NUCLEAR DIVISION OF *Sarcocystis neurona*

by

DAVID PATRICK MORRISON

(Under the direction of Boris Striepen)

Abstract

The phylum Apicomplexa contains unicellular parasites that cause numerous important diseases in humans and domestic animals. We study fundamental biological processes in these pathogens to identify new targets for disease intervention. This thesis focuses on the unique cell division mechanisms employed by these organisms. Using light and electron microscopy we demonstrate that in the model organism *Sarcocystis neurona* the mitotic spindle persists throughout the entire cell cycle. The interphase mini-spindles in this study could provide a scaffold to organize the many chromosomes in the polyploid nucleus of *S. neurona* by constant kinetochore interaction. Fluorescence in situ hybridization (FISH) analysis demonstrates that chromosomes are distributed through the nucleus in a regular pattern. We also characterized two kinetochore proteins in *Toxoplasma gondii*, CenH3 and Bub3, which will be used in the future to establishing kinetochore markers to further test this model. Lastly we have analyzed the modification status of nuclear histone proteins using a battery of diagnostic antibodies throughout the cell cycle. We found an abundance of euchromatic histone modifications (mH3K4 or aH3K9) and little or no histone modifications associated with condensation (mH3K9 or pH3S10). This data suggests that apicomplexan chromosomes remain in an uncondensed state throughout.
Index Words:

Apicomplexa, *Toxoplasma gondii*, *Sarcocystis neurona*, Cell division, Nuclear division, Endopolygeny, CenH3, Histone Modifications, kinetochore, fluorescence in situ hybridization
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I: Introduction and Literature Review

Apicomplexa

The Phylum Apicomplexa contains many medically important parasites that cause disease and morbidity in humans and agriculturally important animals. These include several *Plasmodium species* the cause of human malaria (Gardiner et al., 2003), *Toxoplasma gondii* which causes encephalitis in AIDS (acute immunodeficiency syndrome) patients (Carruthers, 2002; Dubey et al., 1998), *Cryptosporidium parvum* an agent of severe diarrhea (Enemark et al., 2003), and *Sarcocystis neurona* which causes neurological degeneration in horses (Dubey et al., 2001a; MacKay, 1997).

The life cycles of members of the phylum are complex and usually include asexual reproduction in the intermediate host, but in the definitive host the parasites undergo differentiation into gametes and sexual reproduction. Most apicomplexan parasites are obligate intracellular parasites that grow and replicate only within the cells of their host animal. They all share a highly polarized morphological organization with a tappered apical rounded end. The phylum members also share similar organelles in the apical end involved in motility and adhesion to and invasion of the host cell including rhoptries, micronemes, dense granules, and a conoid. The member also share another common organelle the plastid which is involved in metabolic processes (Black and Boothroyd, 2000; Bray and Garnham, 1982; Dubey et al., 2001a; Tzipori and Griffiths, 1998).

Overview of Cell Division

Mitosis is the common mechanism used by eukaryotes to faithfully duplicate and distribute their DNA during cell division. The precision of the process is crucial to inheritance
and cell survival. Before mitosis the cell undergoes a growth phase called interphase. Interphase consists of three stages: G1, a gap period prior to DNA synthesis, S phase in which the chromosomal DNA is replicated, and G2, a gap period after DNA replication prior to the onset of actual division, M phase or mitosis. After the segregation of chromosomes, the cell splits its cytoplasm and organelles in a process called cytokinesis forming two daughter cells (Hyams and Brinkley, 1989).

M phase has been subdivided into: prophase, metaphase, anaphase, and telophase. Condensation of the chromosomes, a breakdown of the nuclear envelope, and the formation of the bipolar mitotic spindle characterize prophase and prometaphase. The chromosomes begin to interact with the microtubules as the nuclear membrane fully disperses. This prepares the replicated DNA for segregation into the forming daughter cells. Within the next stage, metaphase, the sister chromosomes converge at the equatorial plate in the middle of the cell with microtubules extended from microtubule organizing centers (MTOCs) at either pole of the cell setting up for a precise segregation of the sister chromatids into both the daughter cells. In anaphase, the sister chromosomes separate and move to their respective sides. As the chromosomes reach the opposite poles, the nuclear envelope begins to reform, the chromatin begins to loosen and the mitotic spindle regresses. This process happens during telophase reforming the nucleus. To complete cell division, a cleavage furrow forms pinching the plasma membrane and the cytoplasm into two daughter cells in a process called cytokinesis (Murray and Hunt, 1993).

In order to complete this process of replicating and dividing the cell into daughter cells, both plants and animal cells have evolved a common set of components. Scaffolding with polarity is needed so the chromosomes, organelles, and cellular material are distributed equally
between the daughter cells. The microtubules in the mitotic spindle fulfill this function. The microtubules originate from the MTOC, the centrosome which may contain the centrioles. As the cell develops, the MTOC replicates and divides to form two distinct microtubule centers. This adds an orientation to the internal environment of the cell. The microtubules connect to the cell membrane, various organelles and cell components. To enable precise interaction of the MT with the nucleus DNA, the chromosomes evolved a specific microtubule binding region. An array of proteins, collectively called the kinetochore, connect the microtubules to a distinct area of each chromosome called the centromere. The sister chromatids, the replicated chromosomes, are connected at the centromere through mitosis until anaphase when they separate and move to opposite poles. For movement of the chromosomes, microtubule associated proteins (MAPs) are employed in the kinetochore surrounding the attachment site. MAPs catalyze the process of adding or subtracting to the microtubule plus end attached to the chromosome or movement of the various cellular components (including other microtubules) along microtubules. To facilitate the division of the DNA, the chromosomes are condensed by a series of proteins that wrap the DNA up into nucleosomes and tighter bundles call solenoids. This keeps the DNA more separated and less intertwined, so it will be less likely to break during mitosis (Bryant et al., 1985; Murray and Hunt, 1993).

**Cell Division in Mammalian Cells**

Mammalian cell division has been investigated thoroughly and a detailed mechanistic model has been developed for this process. During interphase, mammalian cells show the typical phases: G1, S, and G2. In preparation for cell division, the chromosomes are duplicated one time in S phase. Replication is a time consuming process. It thought that it is done before entering mitosis to avoid undue time constraints on the process.
In order to organize microtubules for the complex division the centrosomes are replicated. This begins to occur in G1 phase and finishes before prophase (Hinchcliffe et al., 1998; Lange and Gull, 1995; Vorobjev and Nadezhdina, 1987). The centrioles along with its surrounding matrix, called the pericentriolar material (PCM), make up the centrosome which is the MTOC for the mitotic spindle (Bobinnec et al., 1998; Zeligs and Wollman, 1979). The centrioles are a cylindrical structure composed of 9 microtubule triplets with a hollow center and in non-dividing cells they are grouped in pairs (Bornens et al., 1987).

The replicated centrosomes separate in the first phase of mitosis, prophase. During interphase the cell has a single microtubule origination site. As the centrosomes move further apart a bipolar organization is produced with microtubule origination sites at both centrosomes. By late prophase, the cell has two separate microtubule origins at opposite ends of the cell surrounding the condensed chromosomes (Hinchcliffe et al., 1998; Lange and Gull, 1995; Vorobjev and Nadezhdina, 1987). Microtubule motors (a type of MAP) moving microtubules from each spindle pole against each other perform the separation of the centrosomes (Endow, 1999; Heald and Walczak, 1999). The cell now has scaffolding for division of the chromosomes, organelles, and other cellular components.

During prophase, the DNA becomes highly condensed into sister chromatids, chromosome replicated pairs linked at the centromere. This is done to preserve the chromosomes through the process of nuclear division, a process that puts much force on the DNA molecules and could potentially tear or shear tangled molecules. The DNA is already wrapped by the nucleosomes into the “beads on a string” conformation, packaged further by histone H1 linking the nucleosome to produce solenoids and further condensed by nuclear scaffolds organizing the DNA into loops (Felsenfeld and Groudine, 2003; Finch and Klug, 1976). In prophase,
condensins (Hirano, 2005; Hirano and Mitchison, 1994) and cohesins (Michaelis et al., 1997; Weitzer et al., 2003) facilitate the linking of chromosome DNA into coils and binding of the sister chromatids together, respectively.

With microtubules arranged for cell division and the chromosomes condensed, the cell is now ready for the breakdown of the nuclear envelope and the construction of the equatorial plate. The nuclear envelope breaks down into its basic components of phosphorylated lamin proteins, vesicles of nuclear envelope and the subpore complex (Maul, 1977). This is controlled by the phosphorylation state of lamin proteins (Erhardt et al., 2002; Hunter et al., 2003; Likhacheva and Bogachev, 2001). This facilitates the shift from a functional nuclear envelope into dispersed components and the reformation of the nuclear envelope once the nuclear division is completed.

In order to divide sister chromatids they must first be loaded onto the scaffold of microtubules. The chromosomes are exposed to the cellular environment with the breakdown of the nuclear envelope and the microtubules now have access to the centromeres. In prophase and prometaphase (in between prophase and metaphase), microtubules are shorter and shorter lived (more dynamic) (Zhai et al., 1996). They are searching for kinetochores of each chromosome. If they find a kinetochore, they are stabilized acquiring this connection to the plus end of the microtubule for the rest of mitosis (Brinkley et al., 1988; Maiato and Sunkel, 2004). This process is termed “search and capture” by some authors. The microtubules can do this both by a direct hit on the kinetochore or a side hit on the kinetochore followed by movement of the kinetochore to the end of the microtubule. CENP-E is a major protein in the kinetochore that enables the attachment of chromosomes to microtubules (Brown et al., 1996; Yao et al., 2000). Each individual chromosome is connected to microtubules from one MTOC and sister chromatids are connected to microtubules from opposite spindle poles. This arranges the chromosomes on the
microtubule spindle scaffold facilitating the separation of sister chromatids between the spindle poles and subsequent daughter cells.

The next problem encountered by the spindle and chromosome complex is the movement of the chromosomes to the equatorial plane, the area where all the chromosomes line up equal distance between the spindle poles. Many proteins in the kinetochore aid the processes of microtubule movement (Endow, 1999; Kline-Smith and Walczak, 2004). A protein that depolymerizes the microtubules at the plus end is MCAK (mitotic centromere associated kinesin) (Hunter et al., 2003; Walczak et al., 1996; Wordeman and Mitchison, 1995) helping to facilitate the movement the chromosome toward the spindle poles. Cytoplasmic dyneins are also involved in other areas of the spindle apparatus enabling focusing of the microtubules (Karcher et al., 2002; Karki and Holzbaur, 1999).

In vitro, a microtubule spindle can be constructed without the centrosomes and kinetochore (Heald et al., 1996). The nuclear matrix contains a GTP bound GTPase Ran which coordinates the construction of the mitotic spindle at the DNA and cytoplasmic dyneins create the spindle pole focus (Carazo-Salas et al., 1999; Gruss and Vernos, 2004). It is suggested that centrosomes and kinetochores are in place to facilitate a proper construction and stability of the bipolar spindle and as a redundant system (Endow and Glover, 1998).

When all the chromosomes are attached to microtubules, mitosis enters metaphase. Microtubule movement is more dynamic during this phase (Zhai et al., 1995; Zhai et al., 1996). By this point the spindle poles are well formed consisting of kinetochore microtubules attached to the kinetochores of the chromosomes. Polar microtubules running from both centrosomes connect the poles and pushing them apart, and astral microtubules radiate from the area around the centromere to the plasma membrane. The main function of the spindle in metaphase is to
line the chromosomes up at the equatorial line and to prepare for the separation of sister chromatids and their segregation into daughter cells (Inoue, 1996; Lodish and Darnell, 2000).

Once the chromosomes are aligned the sister chromatids separate and begin to move toward opposite poles and the poles move further apart. The cell has entered anaphase. Anaphase consists of two different parts: anaphase A and anaphase B. Anaphase A encompasses the pulling force of each spindle poles’ microtubules on the chromosomes to separate the sister chromatids and move them toward opposite spindle poles (Cande and Hogan, 1989; Lodish and Darnell, 2000). The force originates on the kinetochore end of the microtubules (the plus end). There is a rapid depolymerization of the microtubules on the plus end as the chromosome moves toward the spindle poles facilitated by the MCAK (Walczak et al., 1996; Wordeman and Mitchison, 1995). This force along with kinetochore associated dynein produce the movement of the chromosomes toward the spindle poles (Banks and Heald, 2001; Barton and Goldstein, 1996). CENP-E binds the chromosome to the microtubule as the migration occurs (Lombillo et al., 1995; Yen et al., 1991). As this occurs there is also movement of the spindle poles further away from each other. This is Anaphase B. The astral and polar microtubules are involved in this movement. Bipolar kinesin proteins (another type of MAP) act on the polar microtubules pushing them against each other and separating the spindles from the area where they overlap. Furthermore, there is polymerization on the plus end of these microtubules as an additional force. Astral spindle fibers are pulled toward the plasma membrane by dynein molecules attached to the membrane. These forces together work to separate the forming daughter cells and prepare for cytokinesis (Barton and Goldstein, 1996).

Once the chromosomes reach the spindle poles and are sufficiently separated telophase begins. All the processes to prepare for mitosis now run in reverse. The nuclear envelope
reforms. Chromosomes decondense to their interphase state. Microtubules reform the monopolar spindle organization and organelles reassociate with the spindle (Leblond and El-Alfy, 1998; Thyberg and Moskalewski, 1998).

The final separation of the two daughter cells occurs through the process of cytokinesis. A cleavage furrow is formed in the region of the previous metaphase plate. It is formed by a contractile ring of actin and myosin. This process leads to the separation of the two daughter cells at the cleavage furrow (Gatti et al., 2000; Sanger and Sanger, 2000).

**Cell Division in Plant Cells**

Overall the plant cell division follows the scheme exhibited by animal cells. However, important differences exist. The most pronounced differences are due to restrictions placed on the cell by the cell wall and the ability to grow and divide within this rigid limitation. The plant cells develop different uses for microtubules and the spindle poles. Though the mitotic microtubule spindle have a similar structure to animal cells they are organized differently (Francis et al., 1998).

During G1 and S phase microtubules are found in two distinct areas. One is associated with the nucleus and radiates to the cell cortex (the outer portion of the cell). The other, called cortical microtubules, evenly line the cell surface in the cortex in a circular fashion perpendicular to the cell expansion. The cortical microtubules are involved in the deposition of the cellulose and microfibrils for cell wall synthesis (Cyr and Palevitz, 1995; Goddard et al., 1994). As the cell moves from G2 into mitosis the cortical microtubules disperse and a dense set of microtubules form at the equatorial plane called the preprophase band (PPB). The PPB lines the site of future division of the cell. As the cell moves into mitosis, the PPB microtubules disperse (though some microtubules may persist) and the nuclear associated microtubules organize to
form a bipolar spindle (Mineyuki, 1999; Mineyuki et al., 1991; Vos et al., 2004). The bipolar spindle in plants has similar organization as in animal cells though there are no centrosomes so the spindle displays a broader apex (Smirnova and Bajer, 1992). The chromosomes condense and the nuclear envelope breaks down after the PPB formation (Dixit and Cyr, 2002; Rose et al., 2004). The bipolar spindle separates the chromosomes. During telophase a new arrangement of microtubules begins to form at the equator of the telophase spindle called the phragmoplast. The phragmoplast is a dense set of microtubules that are involved in the formation of the cell plate, the new cell wall that separates the two daughter cells. Cytokinesis proceeds as the phragmoplast and cell plate spread to the cell wall fully dividing the cells (Goddard et al., 1994; Vantard et al., 1990).

The overall organization of the spindle is similar in plant and animal cells. However, the plant spindle poles lack centrioles, spindle pole plaque, or even pericentriolar material (Smirnova and Bajer, 1992). Centrioles are absent in all higher order plants. They are present only in some algae and certain tissues of mosses and ferns. The nuclear surface is the only site shown to display properties of microtubule nucleation (Clayton et al., 1985; Lambert, 1993; Shimamura et al., 2004; Stoppin et al., 1994). The microtubule assembly on the nuclear surface is dynamic as the cell cycle progresses. During G1 there is little microtubules nucleation. The nucleating activity on the nuclear surface is maximized when both the PPB and the spindle are being assembled in G2. (Canaday et al., 2000; Francis et al., 1998)

Both in centrosomes and spindle pole plaques, $\gamma$-tubulin is a main player in the nucleation of microtubules. The $\gamma$-tubulin is mostly contained in the centrosomes and spindle pole plaques and involved with nucleation of mitotic spindle (Horio et al., 1991; Joshi et al., 1992; Li and Joshi, 1995; Oakley et al., 1990). Within plant cells $\gamma$ tubulin is present throughout the
microtubules: the spindle, the phragmosplast, and the PPB. Some studies have suggested there may be a conformational change in γ-tubulin in the plant cell cycle (Dibbayawan et al., 2001; Liu et al., 1994; Liu et al., 1993). Other centrosome-associated proteins are present on the nuclear surface suggesting that this is the microtubule-nucleating center (Lambert, 1993). In vitro studies show that the nuclear surface is capable of assembling microtubules (Stoppin et al., 1994). In the spindle, the γ-tubulin is distributed in a scattered manner, not evenly dispersed along the microtubules, suggesting that it is not part of the microtubule structure. It has been suggested that γ-tubulin could control microtubule dynamics rather than nucleation (Schmit, 2002). This is supported by the discovery of Spc98p, an essential spindle pole body component, localized at the nuclear surface, but not to the microtubules (Erhardt et al., 2002). MAPs have been shown to be able to control the movement of the microtubules and organize the microtubules in much the same manner as in animal cells (Stoppin et al., 1996). It has been suggested that the nucleated microtubules may be detached from the nuclear surface, arranged and organized by the MAPs into the different microtubule assembles (Canaday et al., 2000). The γ tubulin has also been localized to the kinetochore suggesting that it may help to stabilize the microtubule interaction (Binarová et al., 1998).

**Single Cell Eukaryotes**

Investigations into other model organisms have demonstrated interesting deviations from the systems in the plants and animals. The model organisms use components in mitosis and cell division in similar fashions, but skip steps or have additional steps. The organisms evolve mechanisms due to selective pressures in their specific environments (Endow and Glover, 1998; Forsburg and Nurse, 1991; Murray and Hunt, 1993).
Budding yeast, *Saccharomyces cerevisiae*, cell division occurs much like the animal system. However, the budding process constrains the cell division process, resulting in a reorganization of the events of mitosis. The important differences from the previously described systems are that in budding yeast the nuclear envelope stays intact throughout mitosis and the G2 phase is not distinguishable due to constraints of the budding process undertaken by *S. cerevisiae* (Endow and Glover, 1998; Forsburg and Nurse, 1991; Murray and Hunt, 1993). The assembly of the mitotic spindle can occur in S phase. Spindle pole bodies organize the mitotic spindle. Spindle pole bodies (SPB) are MTOCs electron dense multilayer body that resides within the nuclear envelope. They replicate conservatively connected by a bridge, unlike the semi-conservative replication of centrioles (Byers and Goetsch, 1974; Rout and Kilmartin, 1990). The mitotic spindle extends through the nucleus from the nuclear embedded SPB. The spindle assembles during S phase with dome capped microtubules (Byers et al., 1978). The G2 phase, the gap phase between replication of the chromosomes and the beginning of nuclear division, is not obvious. DNA replication occurs during the budding process. The chromosomes condense though not into visually obvious macrostructure exhibited by plant and animal cells (Guacci et al., 1994). Once the chromosomes condense, they are divided as in the mammal system, but the nuclear envelope is intact throughout the process. This limits the metaphase lining up of chromosomes at the equatorial plate, so the nucleus heads straight into anaphase pulling the sister chromatids apart (Straight et al., 1997). The nucleus divides and the daughter cell buds off by cytokinesis.

Another yeast model organism, the fission yeast *Schizosaccharomyces pombe*, divides the nucleus in much the same manner as budding yeast with certain differences revolving around their growth and division styles. The growth and development of the fission yeast is different
from that of budding yeast in that they grow in rod shaped cells as they lengthen. They also have a distinct G2 phase because they don’t have the constraint of budding. As fission yeasts get larger they undergo a symmetrical mitosis producing daughter cells. They divided much like plant cells by laying down a cell wall in the middle of the rod after mitosis (Forsburg and Nurse, 1991; McCully and Robinow, 1971; Murray and Hunt, 1993). As in budding yeasts, the nuclear envelope remains intact throughout mitosis and the cell cycle (McCully and Robinow, 1971) and the chromosomes condense (Toda et al., 1981). The mitotic spindle forms within the nuclear envelope as in budding yeast, but the spindle pole bodies do not continually reside in the nuclear envelope. During interphase, the SPB lies next to the nucleus in the cytoplasm similar to centrioles in animal cells. It replicates similar to budding yeast SPB with a bridge between the duplicate SPB, but the replication occurs external to the nuclear envelope. As the fission yeast enters mitosis the nuclear envelope invaginates and breaks down below the SPBs. The SPBs are inserted into the nuclear envelope and the mitotic spindle is nucleated and coordinated through mitosis. Once mitosis is completed, the SPB leaves the nuclear envelope (Ding et al., 1997). It is also suggested that the SPBs are continuously connected to the centromeres through out interphase and mitosis (Funabiki et al., 1993).

*Tetrahymena* is a model organism belonging to a group of protists, the alveolates, that also contain the Apicomplexans. The microtubules, mitotic spindles and the cell cycle is well studied in these organisms (Asia and Forney, 1999; Orias, 2000).

*Tetrahymena* has a complex nuclear arrangement. It has a micronucleus and a macronucleus that are divided differently during asexual and sexual reproduction. The micronucleus contains a diploid genome of five chromosomes, while the macronucleus contains multiple sub-chromosomal fragments (Altschuler and Yao, 1985; Conover and Brunk, 1986;
Gall, 1986). During asexual reproduction the micronucleus undergoes typical mitosis. The DNA replicates and then it moves out from the indentation in the macronucleus where it normally resides. A mitotic spindle forms and the chromosomes condense. The nucleus divides while it is continually enclosed by the nuclear envelope (Elliott, 1973; Ray, 1956). During this same period, the macronucleus undergoes amitotic division. The nucleus splits into two equal parts forming the macronuclei for the daughter cells. The mother cell divides to form two daughter cells each receiving a macronucleus and a micronucleus. (Elliott, 1973; Gall, 1986)

In the sexual reproduction, there are multiple steps of meiosis and mitosis. The micronuclei divide by meiosis and replicate by mitosis before exchanging micronuclei. The exchange micronuclei fuse with the other micronuclei and this is then replicated by mitosis twice to form 4 micronuclei (Elliott, 1973; Ray, 1956). The old macronucleus is degraded and two new macronuclei are formed from two of the micronuclei (Austerberry et al., 1984; Brunk and Conover, 1985). The cell then undergoes division to form the new daughter cells. This results in an exchange of micronuclei creating new micronuclei, a disintegration of the macronuclei, and a formation of new macronuclei (Gall, 1986).

Visually condensed chromosomes with centromeres have been observed in description of their meiotic events (Elliott, 1973), but not much is known about their structure. Basal bodies and microtubules of Tetrahymena and other ciliates have been used to explore the process of establishing the mitotic spindle (King et al., 1982), but little is known about the construction of the specific mitotic spindle in *Tetrahymena*. The mitosis of the Tetrahymena system is an interesting system, but the mitotic events of the micronuclei are more interesting in comparison to other organisms and mitotic systems.
These systems show interesting deviations from the higher order organisms. Many of the structures are highly conserved: the use of a mitotic spindle, a MTOC for producing the spindle (even if it is not a distinct structure), an attachment to the DNA via kinetochores, and the condensation of the DNA into chromosomes.

**Cell Division of Apicomplexa**

The phylum Apicomplexa contains a wide assortment of single cell eukaryotic parasites. Many members cause diseases in humans such as malaria (*Plasmodium*) and opportunistic encephalitis (*Toxoplasma*). Other members of this phylum that cause diseases in animals and have an economic impact such as theileriosis of cattle (*Theileria*), coccidiosis of chickens (*Eimeria*), and equine protozoal myeloencephalitis (EPM) (*Sarcocystis*).

The typical apicomplexan is an obligate parasite, so the parasitic life cycle is an important aspect of their replication. Typically, the parasites cycle between an intermediate host and a definitive host. The intermediate host is an organism that the parasite can infect, use for asexual reproduction and then be passed on to infect other organisms. The definitive host is an organism in which the parasite undergoes sexual reproduction. The other type of host is an aberrant host, a dead end host. In these organisms, the parasite infects cells, but the newly replicated parasites are not able to be passed out of the cells or organisms to infect other organisms. Parasites may act differently according to what cell type in the host they are affecting (Black and Boothroyd, 2000; Waters and Janse, 2004).

The life of organisms in the phylum Apicomplexa offers challenges to the cell division of the parasites. The members of the phylum have evolved several different schemes to facilitate the production of new generations. All the members use the same machinery, but divide the various cellular components at different points as cell division proceeds.
The members of Apicomplexa do not have mutual exclusion of DNA replication, nuclear division and cytokinesis during their asexual reproduction. DNA replication, nuclear division and cell cytokinesis are not prerequisites for movement into the next replication cycle. While classical cells have nuclear replication and nuclear division followed by cytokinesis to form two daughter cells, some species of apicomplexan replicate the DNA multiple times before proceeding to cytokinesis resulting in many daughter parasites upon cytokinesis.

Consequentially, an array of lytic cycles that all begin and end with the conserved motile phase, the merozoite, are exhibited by closely related species (Figure 1). Even with this major divergence of mechanisms, the cell division of the species share common modes of dividing the cellular components and common proteins and mechanisms involved in the process including the use of the mitotic spindle (Black and Boothroyd, 2000; Dubey et al., 2001a; Dubey et al., 1998; Waters and Janse, 2004).

*Toxoplasma* divides by a sequence similar to typical mitosis called endodyogeny. Cytokinesis directly follows a single DNA replication and nuclear division event (Black and Boothroyd, 2000). The two daughter cells develop within the original mother cell. The centrosomes, including a centriole and the mitotic spindle, are recruited to allow for the separation of the DNA to either daughter cell (Morrissette and Sibley, 2002b). This single nuclear division precedes a single cytokinesis event, which produces two daughter cells contained within the original mother cell’s parasitophorous vacuole. The organelles are also equally divided between the daughter cells in a mechanism not fully understood (Waters and Janse, 2004).

*Plasmodium, Eimeria,* and *Theileria* undergo a form of division that disconnects nuclear division and cytokinesis called schizogony (Bannister and Mitchell, 1995; Dubremetz and
Elsner, 1979; Goldova et al., 2000; Shaw and Tilney, 1992; Waters and Janse, 2004). The chromosomes replicate and the nuclei divide multiple times within the mother parasite before cytokinesis proceeds. The mother nucleus can divide once to form two nuclei, twice to form four nuclei, thrice to form 8 nuclei and so on. After the daughter nuclei divide, they are contained within the single mother cell that increases in size throughout the development process. When the nucleus has replicated a species-specific number of times, the multinucleated mother parasite undergoes cytokinesis where each nucleus splits off of the mother cell simultaneously. The organelles and cytoplasm distribute equally to all the daughter parasites. The mechanisms that control this process are not elucidated.

*Sarcocystis* further convolutes the mitotic events in a process called endopolygeny (Dubey et al., 2001a; Speer and Dubey, 2001). The DNA replicates multiple times, but the nucleus does not divide. The *S. neurona* schizont, the developing intracellular parasite, exhibits a nucleus that increases in size with time as the DNA replicates. The different replications of DNA do not establish independent nuclei with individual nuclear envelopes, but rather develop one large polyploid nucleus contained within a single nuclear envelope. Nuclear replication progresses with the polyploid nucleus increasing in size until 64 daughter nuclei separate from one another and simultaneously undergo cytokinesis producing merozoites. This process is poorly understood.

In each method of the nuclear division, the nuclear envelope remains intact as seen in the two yeast species and in *Tetrahymena* (Bannister and Mitchell, 1995; Black and Boothroyd, 2000; Dubey et al., 2001a; Goldova et al., 2000; Shaw and Tilney, 1992; Speer and Dubey, 2001). Unlike fission yeast and *Tetrahymena* the DNA does not condense into visual chromosomes.
The mitotic spindle forms within the nuclear envelope as exhibited by yeast and *Tetrahymena*. The MTOC is recruited by an invagination of the nuclear envelope like fission yeast (Bannister and Mitchell, 1995; Kelley and Hammond, 1972; Morrissette and Sibley, 2002b). Unlike fission yeast the nuclear envelope does not break down below the MTOC and it is not indicated that it incorporates into the nuclear envelope (Bannister and Mitchell, 1995; Gubbels MJ, 2006; Jura et al., 1983; Morrissette and Sibley, 2002b). The microtubules traverse the cleft from the MTOC to the DNA within the nuclear envelope, but the effect of the microtubules on the division process is not fully elucidated.

The MTOC is the centrosomes, sometimes referred to as spindle pole plaques, which includes a centriole in some species including *Toxoplasma gondii* (Morrissette and Sibley, 2002b). The centriole structure is atypical containing nine singlet microtubules surrounding a single microtubule (Desser, 1980; Dubremetz and Elsner, 1979; Kelley and Hammond, 1972; Morrissette and Sibley, 2002a). This structure is not seen in some other Apicomplexa species such as *Plasmodium* (Bannister and Mitchell, 1995) and *Theileria* (Shaw and Tilney, 1992), but might be obscured within the electron dense centrosome. Theses species are considered to organize the spindle with spindle pole bodies. Other organisms, including *Eimeria*, have a centriole closely related to, but separated from a spindle pole body (Desser, 1980; Dubremetz and Elsner, 1979; Kelley and Hammond, 1972). Some authors have considered this as a possible template for basal bodies in the male gametes (Striepen et al., 2000). There is also evidence that the centriole may work as a “super-organizing center” arranging the organelles of the parasite during cell division (Striepen et al., 2000).

Currently the structures of the apicomplexa centromere and kinetochore are poorly understood. A+T rich (>97%) centromere DNA sequences have been established in
Plasmodium with genomic extrapolation. They are approximately 3kb length in an 11kb non-coding region (Bowman et al., 1999). Theileria also shows similar motifs in its chromosomes (Waters and Janse, 2004). Since the DNA does not fully condense into discrete chromosomes, there is a difficulty in visualizing the spindle microtubules interaction with the chromatin material.

**Nuclear Division in Sarcocystis neurona**

*Sarcocystis neurona* is employed as a model organism to better understand the organization of the proteins and structures involved in the nuclear division process. A better understanding of process of endoploegeny will help to elucidate the cell division process of other members of Apicomplexa.

*S. neurona* causes a debilitating nerve disease in horses called equine protozoal myeloencephalitis (EPM) which is the most diagnosed cause of neurological disorders in horses in the U.S. (Dubey et al., 2001a). The horse is a dead end host of the *S. neurona* where the merozoites and schizont forms of the parasite are present, but the parasite is not passed out of the host. The definitive host of *S. neurona* is the opossum in which the gametes develop and fuse to form sporocysts (Dubey and Lindsay, 1998; Fenger et al., 1997). These sporocysts are ingested by another animal. If ingested by an intermediate host, schizonts (actively infective parasites) and sarcocysts (parasites encysted in muscle tissue) can be found in the infected animal. Many animals from cats, skunks and racoons to armidilo and sea otter have been identified as intermediate host (Cheadle et al., 2001; Dubey et al., 2000; Dubey et al., 2001b; Stanek et al., 2002; Stanek et al., 2003). Infection of Opossums can be induced by ingestion of muscle infected with the sarcocysts. Horses on the other hand are thought to be an aberrant host
(dead end), but this assertion has been challenged as of late (Dubey et al., 2001a; Mullaney et al., 2005).

Currently, there is limited molecular biological knowledge of *Sarcocystis neurona*. The known proteins were discovered for the veterinary importance as indicators of infection and represent mostly surface proteins and microneme proteins (Ellison et al., 2002; Hoane et al., 2003; Howe et al., 2005; Hyun et al., 2003). The genome sequence for *S. neurona* is currently not available, but there are many closely related apicomplexan parasite genomes that can be mined for important protein sequences. An EST (expressed sequence tags) project is under way which can be incorporated to help establish expression of the mRNAs (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=s_neurona) (Howe, 2001).

There is much interest into finding drug targets in Apicomplexa especially surrounding the apicoplast organelle (Fichera and Roos, 1997; McFadden and Roos, 1999). Its origins lie in the plant and algal kingdom and mammalian cells lack any form of plastid (Kohler et al., 1997). This made the organelle of interest. It could contain metabolic processes not present in mammalian cells that could be utilized as a drug target. It has been confirmed that the plastid is maintained and required for the completion of the parasitic cycle and its genome is maintained (He et al., 2001a). As well, several metabolic pathways are confined to the organelle (He et al., 2001b).

The apicoplastid has been observed to associate with the centrosomes during mitosis (Striepen et al., 2000). As a result the plastid is in close contact with the a stable nuclear envelope as mitosis and cytokinesis proceed. It has been hypothesized that centrosome association is the mechanism for plastid division, as apicomplexans lack the plastid division machinery conserved in plant and cyanobacteria proteins (Vaishnava et al., 2005).
The diversity of lytic cycles in Apicomplexa exhibits a variety of distinct plastid morphologies during mitosis. The centrosome association hypothesis was validated with these different lytic cell cycles with a series of experiments in *Sarcocystis neurona* which undergoes endopolygeny with the plastid surrounding the large polyploid schizont nucleus (figure 2E). This model provides excellent microscopy allowing precise localization of the plastid, the nucleus and the mitotic spindle (Vaishnava et al., 2005).

In the course of investigating the morphology of the plastid and its association to the centrosome and the nucleus, an interesting tubulin array was observed (Figure 2). At each centrin stained centrosome a mitotic spindle like structure extended away (figure 2A-C). These intranuclear microtubule bundles were present throughout development. This morphology was consistent with earlier studies of *Toxoplasma gondii*, though, this was not exhibited in every mitotic cell. Another localization of tubulin consisting of small dots associated with the centrosomes was also observed (Figure 2 D-F). These intracellular dots were intensely stained near the nuclear envelope and were displayed throughout the development of the larger endopolygeny nucleus before the division into merozoites. This staining pattern had either one or two dots per centrosome. This number of dots per nucleus corresponded to the amount of DNA contained within the nucleus, so as the DNA replicated the number dots increased exponentially. The number of dots was consistent with successive rounds of replication of the DNA (1 to 2 to 4 to 8 to 16 to 32 to 64). After 6 rounds of replication indicated by 64 intracellular dots, the schizont divided the nucleus and underwent cytokinesis. The spindle pole was also found to be required for plastid segregation. A normal cell cycle would not proceed if the microtubule organization was destroyed with oryzalin (Vaishnava et al., 2005).
Our working model consists of a cycle of active mitosis using extended monopolar spindles and interphase which features persistent minispindles (the dot phase) (Figure 3). The extended intranuclear spindle morphology is thought to be the miotic phase and the intranuclear mini-spindle morphology is interphase phase when synthesis occurs. These two phases cycle, increasing the size of the polyploidy nucleus for 5 rounds (32N) without nuclear division or cytokinesis. The DNA replicates and is separated by centrosomes pulling the DNA along the microtubules as they move apart. With the onset of the sixth round of replication, the nucleus enters a nuclear division phase followed by cytokinesis creating 64 daughter parasites. We hypothesize that the DNA to remain organized for cytokinetic event would be continuously connected to the mitotic spindle even through the interphase when the DNA is replicated.

This led us to an interesting question: how is the DNA in the schizont nucleus organized? This question has many implications. How are the chromosomes organized? Are the tubulin mini-spindle contained within the nucleus? Where are the kinetochores/centromeres during different stages? Is the DNA differentially condensed in the microtubule dot and extended microtubule spindle phases? Or do the parasites have a differential histone modification. This masters of science thesis attempts to answer these questions.
2: Materials and Methods

Cell and Parasite Culture

For *Sarcocystis neurona* culture bovine turbinate (BT) cells, inner nose epithelial cells (kindly supplied by Dan Howe, University of Kentucky), were grown in T-25 culture flask at 37°C under 5% CO₂ atmosphere in Dulbecco’s modified Eagle’s medium (DMEM, HyClone) supplemented with 10% heat inactivated fetal bovine serum (HyClone), 2 mM L-glutamine, 5 U/ml penicillin, 10 µg/ml streptomycin, and 1 ml/l fungizone (Invitrogen). The BT cells were seeded onto circular coverslips in 6 well plates and grown to confluency. BT cells cultures were infected with *Sarcocystis neurona* (Sn3 graciously supplied by Dan Howe, Kentucky University) grown in T-25 flask. The parasites were then grown for 3-4 days prior to fixation.

*Toxoplasma gondii* RH strain was passaged in human foreskin fibroblast (HFF) according to standard protocols (Roos et al., 1994). They are grown for 36-48 hours before fixation.

Electron Microscopy

BT Cells were grown in polystyrene petri dishes to confluency. Cells were then infected with *Sarcocystis neurona*. The infected cells were fixed after 72 hours for 1 hour on ice in 1% Glutaraldehyde and 1% osmium tetroxide in 50mM sodium phosphate buffer at pH 7.2. Washed three time with dH₂O and post fixed with 0.5% uranyl acetate (aq) overnight. Samples were dehydrated in a progressive series of ethanol from 30% to 100% in 10 minute intervals and washed three times with molecularly sieved 100% ethanol. Ethanol was removed with an
increasing series of freshly mixed Epon (DDSA 7.5ml, Epon 12.1ml, NMA 6.7 ml, and 10 drops of DMP-30: Electron Microscopy Sciences) placed in at one hour intervals (1Epon:2Ethanol, 1:1, 2:1, 100% Epon). Pure epon was left on the dishes overnight and replaced in the morning. Samples were polymerized at 60°C for 24-48 hours.

Blocks were cut and trimmed and 60-80nm sections were cut and placed on formvar coated 0.5mm slot grids. Sections were stained with 4% Uranyl Acetate (aq) for 25-30 minutes and 4% Lead Citrate (aq) for 2 minutes. Then samples were viewed with a JEO JEM-100 CX II microscope.

**Immunolabling Electron Microscopy**

BT Cells were grown in T-25 flask and infected with *Sarcocystis neurona* once confluent. *S. neurona* infected cells were fixed in the flask 24-72 hours after infection with 2% paraformaldehyde, 0.1% glutaraldehyde, 50 mM Sodium Phosphate and .5 x PBS at room temperature (pH 7.2) for 20-30 minutes on ice. Cells were washed with dH₂O, scraped and centrifuged. Samples were dehydrated as described above and passaged through a series of LR White resin on ice (2 Ethanol:1 LR white, 1:1, 1:2, pure LR white). The pellet was transferred to a gelatin capsule and the LR white was allowed to infiltrate overnight, followed by polymerizing at 50°C in fresh LR white.

60-80nm sections were cut and recovered onto formvar coated 100 mesh copper grids. In order to label the sections they were place on 50µl drops of the Polyclonal rabbit antiserum against acyl carrier protein (ACP) [provided by G.I McFadden, University of Melbourne (Waller et al., 1998)] diluted 1:400 in PBS with 2% Bovine Serum Albumin (BSA) and on 50µl the secondary antibody of goat anti Rabbit Ig conjugated with 10nm gold (BBinternational) diluted
1:50. The grids were washed with 2% BSA in PBS after each antibody step and with 1xPBS and dH$_2$O after the completion of the labeling procedure.

Sections were stained with 4% Uranyl Acetate (aq) for 45 minutes and 4% Lead Citrate (aq) for 2-3 minutes and washed thoroughly with dH$_2$O. Then samples were viewed with a JEO JEM-100 CX II microscope.

**Fluorescence in Situ Hybridization (FISH)**

This protocol was adapted from a FISH protocol previously described by Teri Lear (Lear et al., 1998). Infected cells (*S. neurona*: three to four days, *T. gondii*: 48 hours post infection) were fixed with methanol and Acetic Acid at a ratio of 3:1 cooled to -20°C. Samples were dehydrated (5 minutes in 70%, 90%, 100% Ethanol) and air dried. The samples were stored at -20°C in an airtight container with desiccation pellets. Before use of the stored samples another dehydration series and air drying was performed. Fixed cells were denatured at 70°C for 2 minutes in a solution of 70% formamide and 2xSSC (3M NaCL, 0.3M sodium citrate, and 1mM EDTA) at a pH of 7.3. The coverslips were then dehydrated and cooled down in a series of ethanol stored at -20°C (2 minutes each: 70%, 95%, 100% ethanol) and then dried on a slide warmer at 42°C.

Digoxegenin probes (Roche Dig Nick Translation kit) or biotin probes (Invitrogen) were prepared according to manufacturer’s protocol with cosmid DNA of two *S. neurona* surface antigens, SnSAG1 and SnSAG2, provided by Dan Howe (Howe et al., 2005). The probe was precipitated (0.1 volume of LiCl and 3.0 volumes of Cold 100% Ethanol for Digoxegenin probes or 0.1 NaAcetate and 2 volumes of Cold Ethanol for Biotin Probes) and incubated for either 30 minutes at -70°C or overnight at -20°C. Samples were centrifuged at 14,400g. The supernatant ethanol was decanted and 50 µl of 70% ethanol was added. The sample was centrifuged, the
supernatant was decanted and the pellet was dried with a Savant Vacuum dryer. The probes were then re-suspended in 50 µl of TE Buffer (10mM Tris-Cl, and 1mM EDTA at pH 7.5) and stored at -20°C. In preparation for hybridization, the probe DNA (100ng) was mixed with salmon sperm DNA (6µg) then precipitated as before. Once dried, the probes were mixed into 5.5µl of Formamide and then 5.5µl of 20% Dextran Sulfate and 4 X SSC (pH 7.0) (final concentration: 50% Formamide, 10% Dextran Sulfate, and 2 X SSC). Once mixed, the probe was denatured in 75°C water bath for 10 minutes and put on ice.

For hybridization, denatured probe (11µl) was placed on the denatured sample, covered with another coverslip, and placed in a humidified chamber at 37°C overnight. The samples were then washed in a solution of 50% formamide and 2xSSC (pH 6.9-7.0) and followed by 2xSSC solution (pH 7.0) in three newly prepared solutions for 5 minutes, 3 minutes, and 3 minutes under agitation at 42°C. The cover slips were then placed in PBD (PBS with 0.1% tween (Omnipur) at room temperature and blocked (10% BSA in PBD for Biotin probes or 1% BSA for Digoxigenin probes) for 25 minutes at room temperature. Immunofluorescence was preformed with Sheep antibodies to digoxigenin (Fab Fragments) with conjugate RhodamineRX (Jackson Immune Research) diluted 5:500 or mouse antibodies to biotin with conjugate FITC (Roche) diluted 2.5:500 and mounted with DAPI III (Vysis).

**Immunofluorescence**

Infected cells on coverslips were fixed in 3% paraformaldehyde in PBS for 20 minutes at room temperature, and permeabilized with 0.25% Triton X100 in PBS for 10 minutes at room temperature. Blocking was performed with 1% BSA in PBS for 30 minutes at room temperature. The primary antibodies were diluted in 1% BSA in PBS and the coverslips were inverted onto 100µl of the diluted solution for 1 hour at room temperature in a humidified chamber. Coverslips
were washed with 3 ml of PBS 3 times for 5 minutes at room temperature. 100 µl of the secondary antibody (Goat anti-mouse Alexa 546, Goat anti-mouse Alexa 488, Goat anti-rabbit Alexa 546, or Goat anti-rabbit Alexa 488) was placed on the coverslips in the same manner for 1 hour. The coverslips were washed in PBS and incubated in 5µg/ml DAPI in PBS. Excess dye was removed and the cells were mounted on slides with GelMount (Biomeda Corporation). The slides were then viewed on a Lecia Scope DMIRBE microscope. Images were captured with a CCD camera (C4742-95; Hamamatsu) and Openlab software (Improvision).

The following anti-histone H3 antibodies used were: Rabbit-anti-histone H3 monomethylated lysine 4 (Abcam), Rabbit-anti-histone H3 dimethylated lysine 4 (Upstate), Rabbit-anti-histone H3 trimethylated lysine 4 (Abcam), Rabbit-anti-histone H3 monomethylated lysine 9 (Abcam), Rabbit-anti-histone H3 dimethylated lysine 9 (abcam), Rabbit-anti-histone H3 trimethylated lysine 9 (Abcam), Rabbit anti histone H3 phosphorylated serine 10 (Upstate), rabbit anti-unmodified histone H3 (Upstate), Rabbit anti-acetylated lysine 9 (Upstate), Rabbit anti-acetylated lysine 14 (Upstate) and Rabbit anti-histone H3 Arginine 17 (Abcam). The anti-α-tubulin antibodies were graciously supplied by Jacek Gaetig (Univeristy of Georgia).

**Western Blot**

*Toxoplasma gondii* were needle passed and filter purified. The parasite concentration was calculated. The samples were prepared per manufacture protocol to preserve the proteins (Inveitrogen) at 0.5 x 10^6 parasites/µl. The samples were loaded on a 4-20% Tris-Bis pre-fabricated gel (Invitrogen) and electrophoreses was preformed at 100V for 2 hours along with molecular mass standards ladder (Invitrogen). The gel was then blotted onto nitrocellulose membrane (Invitrogen) for 2 hours at 100V (or 20V overnight) at 4°C in blotting buffer (25mM Tris, 190mM glycine (Biorad) in 20% methanol). The membrane was blocked for 1 hour at
room temperature (or overnight at 4°C) with 5% milk, 1% BSA, 0.1% Tween (Omnipur), and 1xPBS. Then washed in PBS with 0.1% tween for 10 minutes (if blocked over night washed 3 times with 0.1% Tween-PBS and 2 times with PBS 10 minutes a piece) at room temperature. The primary antibodies were diluted in 0.1% Tween-PBS and exposed to the membrane at room temperature for 1 hour. The membrane was then washed 3 times with 0.1% Tween-PBS and 2 times with PBS 10 minutes a piece at room temperature. The corresponding secondary antibodies (Goat-anti-Rabbit-alkaline phosphatase, Goat-anti-mouse-alkaline phosphatase (Biorad)) were diluted 1:3000 in 0.1% Tween-PBS and exposed to the membrane. The membranes were developed according to the manufacture’s protocol. They were incubate with developing buffer (100mM Tris-Cl, 100mM NaCl, 5mM MgCl2) with Nitroblue tetrazolium (Biorad) and 5-bromo,4 chloro,3-indolylphosphate (Biorad).

**cDNA purification**

RNA was extracted from $1 \times 10^7$ parasites with Trizol reagent (Invitrogen) incubated at 25°C for 5 minutes. 0.2ml of chloroform was added and shaken vigorously. The mixture was incubated for 3 minutes at 25°C and centrifuged for 15 minutes at 10,000g. To the upper aqueous layer 0.5ml isopropanol was added. The solution was incubated for 10 minutes at room temperature and centrifuged for 15 minutes at 10,000g in order to pellet the RNA. The pellet was then washed with 70% ethanol and centrifuged again. The pellet was air dried briefly and dissolved in DEPC treated H2O (Omiipur). RNA was quantified in a SmartSpec 3000 (Biorad).

1µg of RNA was mixed with 10mM dNTP mix and 500µg/ml oligo dT (Invitrogen) and heat inactivated for 10 minutes at 65°C. The RNA sample was mixed with 0.1M dithiothreitol (Invitrogen), 40U/µl Recombinant ribonuclease inhibitor (Promega), and 1x First Strand Buffer (Invitrogen). The mixture was incubated for 2 minutes at 42°C. Superscript II Reverse
Transcriptase (Invitrogen) was added and the mixture was incubated for 50 minutes. The mixture was incubated at 70°C for 15 minutes to stop the reaction. Then 1µl Rnase H (New England Biolabs) was and incubated for 20 minutes at 37°C. The resulting cDNA was used to perform the 5’Race and PCR experiments.

**Polymerase chain reaction (PCR)**

PCR was performed with the previously constructed cDNA. Primers for CenH3: forward primer with BclI site gatctgatcaaaaATGGGCTCGCATCAAGACGAC, reverse primer with AvrII site cagtcctaggGCAAGGAAACGCATGCCGCA. Primers for BUB3: forward primer with BglIII site gtcaagatctaaaATGTCTATCGATCTGCGGCACG, reverse primer with AvrIII site GAGGCCCCAGGCATTGCAGGCACctagggtac.

The samples were denatured at 94°C for 5 minutes. Samples were thermocycled for 30 cycles of 1 minute of 94°C denaturing step, 60-65°C annealing step, and 1-2 minutes at 72°C extension step, and a final step of 72°C at 10 minutes. The samples were presevered at 4°C. 5-10µl of each PCR product was visualized in a 0.8% agarose gel and molecular weight was judged by comparison to 1KB ladder (New England Biolabs) run in a parallel lane.

**Restriction Digest and Ligation**

Restriction digests were performed on the PCR products and the destination vector. PCR product was digested with BclI and AvrII. The destination vector was digested with BglIII and AvrII and purified away from the insert on a 0.8% agarose gel. The PCR product was inserted into the destination vector by a ligation reaction with T4 DNA ligase. The ligation reaction was then transformed into chemically competent *E. coli* DH5α. The bacteria were plated and incubated at 37°C overnight. Colonies were sampled and plasmid DNA was isolated using
standard protocols (Qiagen). Plasmid DNA was restriction digested with BglII and AvrII (there is an internal BglII site in CenH3 at 1700bp) to confirm the insertion.

5’ RACE

The 5’ RACE (rapid amplification of cDNA end) of the CenH3 gene was performed per manufacturer’s protocol with GeneRacer (Invitrogen). The T. gondii cDNA was used and the initial primer was ggtacgcctcaacctgcgcactgcaatg a sequence in a probable centromeric H3 within the typically conserved region, but not corresponding to the conserved H3 sequence. The PCR did not amplify a band, but rather a light smear upon 0.8% agarose gel. A nested PCR was performed with a gene specific primer directly 5’ to the other primer (gtgcatcgctgtgatctgtgagg) and a primer specific to the manufacture universal primer (on each random primer) was supplied by the manufacturer. A 10µl nested PCR sample was run on a 0.8% agarose gel. The product was inserted into the topoTA (Invitrogen) per manufacturer’s protocol. Then insert was sequenced with a plasmid specific forward and reverse primers (Invitrogen) surrounding the insert site.

Identification of CenH3 and BUB3 Genes

Genbank searches for CENH3 resulted in multiple CENH3s for human, mouse, and yeast. The Plasmodium falciparum genomes were searched for homologs of cenH3 by using the BLAST algorithm TblastN with human CenH3 as the query sequence. The top matches (lowest p-value) were parsed with any of the matches that match near exact matches for histone H3 or H3.3. The resulting protein was used as the query sequence in search for homologs in the T. gondii genome with a TblastN algorithm. Again the histone H3 and H3.3 were parsed out of the match and the lowest other p-value (p=7.2e^-15). Primers were designed from highly conserved areas that diverged in this from the Histone H3.
Toxoplasma gondii Bub3 was identified by a text search in Toxodb.org which contained multiple exons, but it was incomplete as compared to the other bub3s in Genbank. Other bub3s were found in Plasmodium flaciparum (plasmodb.org), Cryptosporidium parvum (cyptodb.org), and Eimeria tenella (from Sanger Institute, www.sanger.ac.uk). The E.tenella had the most similar sequence to the TgBub3. Both genomic sequences were used as queries against each other with a TBLASTX algorithm on NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The exons lined up and a complete cDNA of both species was arranged. Primers were designed from the start site and the stop site without the stop site.

Alignments

Alignments for histone H3, CenH3 and bub3 were preformed with clustalW (http://www.ebi.ac.uk/clustalw/). The following H3 used are from Genbank: human H3 (Accession # CAB02546), mouse H3 (Accession # S06743), T. gondii H3 (Accession # AAO23911), and P. falciparum H3 (Accession # AAO23910). The P. falciparum CenH3 displayed is from Genbank (Accession #CAD52476). The T. annulata probable CenH3 found on Contig 13 in from Sager Institute with a genome search with TgH3 and a selection of the Variant H3 that does not correlate with the H3 or H3.3. The P. vivax CenH3 was predictes with a search of the genome with the P. falciparum CenH3 as the query and a TblastN alogithm the resulting gene coordinate was on the Pv_6871 contig at 209705-210193. T. gondii proteins (CenH3 (on TGG_995361 contig) and Bub3 (TGG_995291 contig) were seached for in toxodb.org and confirmed with experimental products and sequences. The Bub3 used are as follows: Human Bub3 (Accession # O43684), Mouse Bub3 (Accession # AAD38038), Zebrafish Bub3 (Accession # AAH83205), budding yeast (Accession # CAA99216). E. telleria Bub3 on the contig00020804 from the Sanger Instutute was theoretically arranged from BLAST data.
Transfection

TgCenH3 was inserted into the tubYFP-YFP/sagCAT expression plasmid (Gubbels et al., 2003) in place of the first YFP gene and a tubLDH1-myc/sagCAT created by Mazumdar, J based on the previous plasmid (University of Georgia, Dept Cell Biology, unpublished). TgBub3 was inserted into the tub-myc/sagCAT expression plasmid.

50µg (in 100µl TE) of plasmid DNA is prepared for transfection first by adding 11µl 3M Sodium Acetate (NaOAc), and 250µl 100% Ethanol. The mixture was incubated at –20°C for 2 minutes and centrifuged for 10 minutes at 14,000rpm to pellet the DNA. The pellet was washed with 70% ethanol and centrifuged again for 2 minutes at 14,000 rpm. The ethanol is decanted in a sterile environment and the pellet is dried for 10-15 minutes. The plasmid DNA was then dissolved in 100µl of cytomix (120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/KH₂PO₄ pH 7.6, 25 mM HEPES pH 7.6, EDTA 2 mM, MgCl₂ 5 mM, 2 mM adenosine triphosphate (added fresh), and 5 mM glutathione (added fresh)).

A freshly lysed tachyzoite T. gondii culture was needle passed, filtered through a sterile 3µm polycarbonate filter, centrifuged (1500g for 20 min at 4°C). The parasites were resuspended at 3.3x10⁷/ml in cytomix. The 100µl of plasmid DNA (50µg) in cytomix is added to 300 µl (10⁷) of parasites and placed in a 2 mm gap electroporation cuvette. The parasites were electroporated with a single 1.5kV pulse, a resistance setting of 25Ω, and a capacitor setting of 25µF generated by a BTX ECM630 apparatus (Genetronix). The cuvette was left undisturbed for 15 minutes. The parasite solution was used to infect 6 well plates of HFF cells with 50-100µl per well. The infection was incubated for 48 hours and viewed either with direct GFP fluorescence or with Immunofluorescence.
Phagemid Library Screening

Primers were designed for conserved 3’ regions of the probable *T. gondii* CenH3 as compared to the histone H3 and other CenH3 in the loop1 region (Malik and Henikoff, 2003). The sequences were ggtacgcctcaacctgcgcactgcaatgcc and gcaggagtcgacagatctcttgattccc. The PCR product of these primers was used as a $^{32}$P labeled probe in a Phagmid cDNA library. The 25 ng of the PCR product in 23µl dH$_2$O was denatured in a boiling water bath, followed by the addition of 15µl of Random primers buffer mixture, 2µl dCTP, 2µl dGTP, 2µl dTTP, 5µl α-p32 dATP, and 1u Klenow Fragment on ice to the PCR product to complete the 50µl Random primer labeling solution (Abersham). The mixture is then incubated at room temperature for 1 hour, inactivated and denatured at 100°C and quickly chilled.

BB4 strain E.coli were infected with the lamda phagemids containing pBluescript phagemids (Stratagene: Lamda ZAP premade library) *T. gondii* RH cDNA library (a gift from Michael White, Montana State Univeristy) and plated on large petri dishes in NZY top agarose (5g of NaCl, 2g of MgSO$_4$.7H2O, 5 g of yeast extract,10 g of NZ amine (casein hydrolysate), 0.7% agarose,1 dH$_2$O, pH 7.5) on a base agarose (5g of NaCl, 2g of MgSO$_4$.7H2O, 5 g of yeast extract, 10 g of NZ amine, 1.5% agar in 1L dH$_2$O, pH 7.5) and allowed to grow and lyse the *E. coli* leaving phagmids. The phagmids were lifted off the agarose with charged nylon membranes and UV cross linked per manufacture protocol (Stratagene).

The nylon membranes were hybridized with the 3’ DNA probe. Phagemids displaying the radioactive $^{32}$P probe exhibited exposed points once the film was developed. These colonies were excised from the agarose and grown in NZY media and plated on large petri dishes as previously described. The preceding was repeated with the same probe. 22 separate colonies were isolated following the second round of phagmid screening.
To recover the plasmid clones the Rapid Excision Kit (Stratagene) was used. The cDNA clones were evaluated by restriction digest and PCR. Two clones were found to contain the DNA which hybridized (E1 and C5). These two were sequenced. Neither one was complete. Both still contained introns, but C5 looked the most complete. Primers were constructed from this sequence.
3: Results

Microtubular Spindles Persist throughout Endopolygeny

The intracellular replication of *Sarcocystis neurona* exhibits a nucleus that gains size without dividing until the final round of DNA replication when nuclear division and cytokinesis occur in concurrence with DNA replication. This nucleus is continuously surrounded by the apicoplast and it divides in the final stage with the nucleus (Figure 2 E-H). An interesting microtubule morphology was demonstrated which consisted of either small intensely stained mini-spindle or typical mitotic spindle (Figure 2A and D). The number of mini-spindles or extended spindles increased as the size of the nucleus increased (Vaishnava et al., 2005). This leads us to hypothesize that the schizont nucleus is undergoing alternating phases of mitosis and DNA synthesis during this phase and that the microtubules might hold on to the DNA throughout the entire process (Figure 3). We did not know the position of the microtubule mini-spindle with respect to the nuclear envelope. The hypothesis that the DNA is attached to the microtubules throughout schizont development would require an intracellular localization. The resolution of immunofluorescence imaging was not high enough to distinguish if the microtubule mini-spindle were within the nuclear envelope or outside of the nuclear envelope. In order to elucidate the position of microtubule mini-spindle, we employed transmission electron microscopy (TEM).

TEM samples were prepared from *S. neurona* infected host cells grown in petri dishes in order to produce sections of flattened host cells and growing parasite parallel to the petri dish base. We performed this to produce TEM images that were consistent with the immunofluorescent images.
The mitotic spindle stage (Figure 4) and microtubule dot stage (figure 5) were both imaged. Both display nuclear material that is not visually condensed into chromosomes in the manner of yeast or animal mitotic cells. The nuclear membrane is continuous in both stages and did not appear broken down in any images collected. Centrosomes are displayed with a centriole like structure outside of the nucleus and with the plastid in close proximity.

The mitotic spindle phase has microtubules that extend through the schizont nucleus forming monopolar spindles extending from the centrosomes as previously observed. Microtubules (Figure 4C small white arrows) extend from areas around centrosomes with in the nuclear envelope (Figure 4C large white arrows) through the schizont nucleus in similar fashion as microtubules in the immunofluorescence images. The nuclear membrane (figure 4B black arrows) is not broken down even in areas adjacent to the centrosomes. The centrosome is attached to the nuclear envelope via microtubules (Figure 4B large black arrow). The plastid surrounds the centrosomes and has microtubules running between them (Figure 4B large white arrow). The plastid is one continuous structure (Figure 4A). The plastid encircles the nuclear envelope and surrounds the centrosomes.

In the microtubule dot stage, a tight bundle of microtubules is observed within the nuclear membrane in a tight cone shaped basket (Figure 5 B and C black arrow), the apex of which is adjacent to a paired centriole centrosomes (Figure 5A-C single white arrow). There is no indication that microtubules cross the nuclear membrane or that the nuclear membrane is broken down around the centriole. There are electron dense regions at the end of the spindle cone (Figure 5 B and C at the white arrows). We hypothesize that this is the kinetochore of chromosomes that remain attached to the microtubules, but more data is needed to establish this. In the serial sections (Figure 3 B and C), the electron density is mostly concentrated at the end of
the microtubule cone, but there is also a dense region behind this region. This could possibly be a DNA rich area as the chromosomes fall away from the kinetochore or possibly an area where the DNA is condensed.

The microtubule spindle is intranuclear for both morphological phases the mitotic like spindle stage and the dot stage. The spindles MTOC are the centrosomes outside of the nuclear membrane. The centrosomes are also tightly associated with a long organelle thought to be the apicoplast.

**Is the organelle encircling the nucleus actually the plastid?**

The apicoplast has been shown to be essential for the life cycle and associated with the mitotic spindle of *T. gondii*. A large filamentous organelle has been seen in close proximity of the developing schizont nucleus in immunofluorescence images of *S. neurona* with antibodies to plastid protein acyl carrier protein (Waller et al., 1998). In our TEM images, a large organelle encircling the endopolygeny nucleus was observed. It has four membranes, as characteristic for the plastid (Kohler et al., 1997). In order to confirm that this organelle was in fact the plastid, immunolabeling electron microscopy using the ACP antibody was performed.

Infected host cells were prepared for Immuno-EM as described in the materials and methods and embedded in LR White. Sections were cut and exposed to the ACP antibody and a corresponding Gold conjugated secondary antibody before staining. The antibody labeled the lumen of a long organelle with multiple membranes that is closely associated with the nucleus (Figure 6 white arrows). This is consistent with organelles labeled with the same antibody in *T. gondii* from which the protein was purified (Striepen et al., 2000; Waller et al., 1998) and stains this organelle in both the TEM and immunofluorescence images of *S. neurona* (Vaishnava et al., 2005). This confirms the interesting position of the plastid in the developing schizont lending
validity to the previous images of the plastid’s development and its position in other species of Apicomplexa.

**Do the Apicomplexan histones have a stage specific conformation?**

DNA staining using DAPI suggested that some condensation of DNA coincides with the presence of the monopolar spindles (Figure 10B and C). The TEM images showed areas of high electron density within the nucleus suspected of being condensed DNA. Histone modifications have been used in eukaryotic cells as indicators for gene expression patterns and DNA condensation. Four highly conserved histones (H2A, H2B, H3, and H4) make up the nucleosome, a multi-protein structure (Luger et al., 1997). Histone modifications include acetylation, methylation, and phosphorylation of lysine, arginine and serine amino acid residues (Felsenfeld and Groudine, 2003). Histone modifications were proposed to be a code for the expression and repression of genes (Strahl and Allis, 2000) and many pieces of the code has been deciphered. Histone H3 and H4 have many of the most important modification decoded so far. Various modifications in H3 and H4 allow or indicate gene transcription or silencing (Richards and Elgin, 2002). Acetylation of the histones is present when genes are being expressed and the DNA is in an euchromatic conformation (Turner, 2000). Methylation of amino acid residues have different effect so the condensation state of the DNA depending on which amino acid is methylated (McManus et al., 2005; Richards and Elgin, 2002; Strahl et al., 1999) and these modification are reversible (Bannister et al., 2002). Phosphorylation is on the other hand has one major indication in the eukaryotic nucleus. Phosphorylation of Serine 10 is highly correlated with chromosome condensation and is thought to signal for this condensation. It has been observed as a mitosis marker in many organisms from Human to *Tetrahymena* (Wei et al., 1998).
Antibodies against a peptide section of H3 with the phosphorylated serine 10 modification have been used in many species to display mitotic chromosomes and show that the nucleus is in a mitotic phase (Adams et al., 2001; Crosio et al., 2002; Wei et al., 1998). Since H3 and other histones are highly conserved proteins (Figure 13) the same antibodies can be used across species and even kingdoms. All have shown the modification’s presence only during late interphase and mitosis (Hendzel et al., 1997; Wei et al., 1998; Wei et al., 1999). The presence of the modification increases as the cell enters prophase as the nuclear material condenses into chromosomes and the nuclear envelope breaks down. The modification persists until after telophase when the nuclear envelope reforms and the chromosomes decondense. This has been exhibited from Tetrahymena micronuclei division to human cells mitosis (Wei et al., 1998; Wei et al., 1999).

We hypothesized that there should be a difference in the histone staining between the mitotic spindle phase and the microtubule dot phase. The phosphorylated serine 10 seemed to be the best candidate because it is established during mitosis. If it was turned on during one of the microtubule stages (the mitotic spindle phase or dot phase) and not in the other, it would support our working model. It could be used to establish which phases corresponded to the phases of synthesis or mitosis.

In order to establish a difference between the long spindle phase and the short spindle phase of *Sarcocystis neurona* in terms of DNA condensation we employed peptide antibodies to the H3 phosphorylated serine-10. We exposed fixed *S. neurona* and *T. gondii* infected host cells to this antibody. The resulting images displayed a lightly stained section surrounding the nucleus in *S. neurona* (Figure 7A-E) and a distinct mark near the nucleus in *T. gondii* (Figure 7 F-H). The level of signal was approximately 100 fold less in the parasites as compared to mitotic host
cells. The staining inside the nucleus was no different from the background in both cases. The image of the histone modification seemed peculiarly like images of the plastid (Vaishnava et al., 2005) (Figure 2E). Since both of these antibodies were raised in rabbits a colocalization experiment was not possible. Instead the antibody was exposed to free merozites of *T. gondii* (Figure 7I-N). The antibody signal showed areas of the parasite that also contained the plastid DNA.

The plastid genome is a 35 kilobase circular DNA strand and does not contain any histone proteins (Kohler et al., 1997; Wilson et al., 1996), but a western blot was performed in order to distinguish if the protein that was being marked was a histone or another protein that shared a similar epitope (Figure 7 O and P). On a western blot of *T. gondii* protein extract, the antibodies marked proteins much larger than histone H3 (17KDa). This indicates that the nuclear material is either not condensed during mitosis or does not use the H3 phosphorylated serine 10 as the other eukaryotic cells. Thus, we could not establish a stage specific histone conformation with the phosphorylated serine 10.

**Do the Apicomplexan have a heterochromatic histone modification?**

Since we could not establish a stage specific histone modification with the phosphorylated serine-10, a highly conserved histone modification, we decided to consider other established histone modifications. Since we thought that the schizont’s mitotic DNA would probably not be undergoing transcription and possibly be silenced, heterochromatin markers were utilized. The histone modification methylated lysine 9 is a good indicator of heterochromatin (Hwang et al., 2001; Jacobs et al., 2001; Noma et al., 2001). The modification is founding regions of chromatin which are not actively transcribed, but also requires other factors to repress transcription (Stewart et al., 2005). The residues can be methylated with a one (mono-), two (di-), or three (tri-) methyl
groups (Paik and Kim, 1967) and these have been shown to be expressed at different locations and time periods (Wu et al., 2005). Recently, an increase in tri-methylations of H3K9 was correlated to mitosis in human cells (McManus et al., 2005). If an expression pattern of any of the H3-K9 methylations could be established, it would help correlate the microtubule spindle morphology to the cell cycle state.

Immunofluorescence was performed with antibodies to all three different methylation types on *T. gondii* and *S. neurona* (figure 8). In all three cases, there was not a significant amount of signal displayed within the nucleus. The H3 monomethylated K9 antibody showed a low amount of signal within the nucleus when performed with a high concentration of antibody and a long exposure time. Both H3 dimethylated and trimethylate K9 display no signal within the nucleus, but they marked the conoid in the developing merozoites in *T. gondii* (Figure 8 M and P at white arrow), in merozoites that have recently entered the host cell in *S. neurona*, newly divided merozoites in *S. neurona*, and the remnants of the conoid in large *S. neurona* schizonts displayed as a dot at the apical end of the cell (figure 7 G at white arrow). This staining could indicate the presence of dimethylated and trimethylated tubulin in the subpellicular and in the microtubules of the conoid, though these antibodies were generated against a histone peptide (Ohta et al., 1987; Szasz et al., 1993).

Again, we found no stage specific histone modification, but we also found little indication that there is any heterochromatin contained within the *T. gondii* or *S. neurona* nucleus.

**Do Apicomplexan have a euchromatic histone modification?**

With no stage specific histone modification much less a H3 histone modification exhibited in either the phosphorylated serine 10 or methylated lysine 9 serine 10, we decided perhaps the opposing histone modification, H3 methylated lysine 4, would help to elucidate the
situation. This histone H3 modification has been shown to indicate euchromatic DNA, nuclear material that in actively being transcribed, and thus not condensed (Bernstein et al., 2002; Strahl et al., 1999). This histone modification is an antagonist for the methylation of H3K9 (Wang et al., 2001) and promotes the acetylation of the histone molecules (Bernstein et al., 2002; Noma and Grewal, 2002). We hypothesized that the schizont might be transcribing mRNA, but that it would not be at a high level and mostly centered around the nucleoli. Three antibodies were tested on fixed infected host cells: monomethylated K4, dimethylated K4 and trimethylated K4 Histone H3.

Contrary to the expectation, these antibodies produced intense labeling within the nucleus (figure 9). The monomethylated H3K4 gave the lowest signal of the three even when concentrated and with a longer exposure time, but it still produced more signal than the methylate H3K9 antibodies (figure 9 A-C). Monomethylated H3K4 has been indicated as a gene silencer in Chlamydomonas, a green algae (van Dijk et al., 2005). The H3 dimethylated and trimethylated K4 antibodies both displayed similar signal within the nucleus (figure 9 D,G and M, P). The signal label was evenly distributed all over the nucleus; there were no obvious areas where the signal was excluded at this resolution. The signal was present in T. gondii and S. neurona throughout development (Figure 10). Both the mitotic spindle stage and the mini-spindle stage in S. neurona were intensely stained. The monopolar spindle stage (Figure 10 A-C) has a slight increase in intensity over the mini-spindle stage (Figure D-F) which might indicate a condensation of the chromosomes. A western blot of the antibody indicated that the protein was approximately the size of histone H3 (Figure 10G). This indicates that the DNA is actively being transcribed and may not be condensed in the same way as other eukaryotic organisms or not condensed at all.
Since *S. neurona* did not exhibit condensation H3 modifications, but did display euchromatic H3 modifications, we decide to look at other H3 euchromatic modifications. This was performed to give a different perspective on the state of the histone modifications of the DNA. With the current understanding of histone modifications, we expected other euchromatin histone modifications to be present. Methylation of H3K4 promotes acetylation of the H3 and the H4 histones which promotes transcription of the DNA (Bernstein et al., 2002; Noma and Grewal, 2002).

Acetylations of residues in the histone H3 (as well as histone H4) are a major histone modification in euchromatic DNA (Wu and Grunstein, 2000). Acetylation of the lysine 14 is promoted by the phosphorylation of H3 serine 10. The H3K14 specific acetyltransferase, Gcn5, binds tighter to phosphorylated H3 than nonphosphorylated creating a correlation between the two modifications (Cheung et al., 2000; Lo et al., 2000). Acetylation of H3K9 along with all H3 acetylation produces a higher transcription level of its DNA. Methylation of the lysine 4 site may be a precursor to the acetylation of the lysine 9 (Bernstein et al., 2002; Noma and Grewal, 2002).

We used antibodies to these different modifications in immunofluorescence microscopy on fixed infected host cells. The H3 acetylated K9 antibody gave a strong signal from the nucleus in much like the even coverage displayed in the methylated K4 images (Figure 11A and E). This is to be expected since the acetylation of the K9 residue is thought to be dependent on the methylation of the K4. This supports the hypothesis that the majority of the genetic material is in a euchromatic state.

However, the signal for H3 acetylated lysine 14 antibody is much lower though still present (figure 9I and M). The signal is present in the nucleus, but required higher concentration
of antibody and longer exposure time compare to the acetylated K9. The signal does not densely cover the nucleus, though it is equally distributed amongst the DNA (not spotty as many images of the histone antibodies typically express). The morphology of the signal does change, however, from the growing *S. neurona* schizont (Figure 9I crossed arrow) to the 64N schizont (figure 9I the white arrow). The signal only encompasses the DNA in the growing schizont, but in the schizont undergoing nuclear separation the signal covers an area outside of the nucleus. The morphology does not change in the *T. gondii*. It remains at the lower level throughout the intracellular cycle. A signal is present in an area outside of the nucleus which looks similar to the plastid sequence displayed in phosphorylated H3S10 and the plastid staining. Though acetylated H3K14 is not a distinct spindle phase morphology marker, it does have a different morphology during a different time period of the cell cycle. This suggests that not all the DNA is actively being transcribed or the K14 acetylation may be involved in some way with nuclear condensation or mitotic signaling. The acetylation of K14 has been indicated as being synergistically linked to the phosphorylation of H3S10 (Cheung et al., 2000; Lo et al., 2000). Since there is no P-H3S10 within the cell, it could be that the acetylated K14 acts as similar marker.

For all the antibodies used, the entire nucleus seems to exhibit the histone modification. We probed for unmodified histones to see if there was any DNA incorporated with histones that did not categorize into either category of either euchromatin or heterochromatin. The label gave a low signal through out the cell that compared to the level of signal in the series of methylated lysine 9 experiments. This indicates that the signal displayed in the methylated K4 and acetylated K9 and K14 because the modifications are present. This also gives evidence that the absence of signal in methylated K9 indicates that there is none of this modification present.
A microtubule morphology specific H3 histone modification was not found. The DNA was found to constantly be modified in what has been shown to be the euchromatin conformation. This would indicate that the DNA is either always in an active transcription state even during mitosis or that the H3 histone modification system is differs from the system in other organisms.

**How are the multiple chromosomes organized within the polyploid schizont?**

As *S. neurona* grows within the host cell from one parasite to 64 parasites, the Schizont nucleus increases in size. We have seen that there is a scaffold for the organization of the nucleus in the persistent microtubules lead by the centrosomes, the MTOC. We have also observed how the centrosomes multiply spreading around the nucleus as the amount of DNA within the nucleus and the size of the nucleus increase. The schizont is polyploid and its nucleus contains up to 32-64 sets of chromosomes which do not appear to be separated by the nuclear membrane. We assume that the DNA must be organized within the nucleus in some manner. We hypothesized that individual sets of chromosomes are restricted to specific areas of the nucleus through constant interaction with the mitotic spindle microtubules.

Since we were interested in visualizing individual chromosomes and DNA loci, we employed fluorescent in situ hybridization (FISH). FISH allows to identify specific DNA within a fixed cell. Two DNA probes specifically hybridized to the *S. neurona* genes SnSAG1 and SnSAG2, genes that code for surface proteins indicated in possible involvement with host cells attachment (Howe et al., 2005) were employed.

In late stage schizonts, the SAG1 hybridized 1:1 to the daughter nuclei (Figure 12A). The SAG1 cosmid hybridized with DNA evenly distributed over the schizont (figure 12B small white arrows). Pairs of signal that correspond to sister genes are also seen in other schizonts (Figure B
large white arrows). All DNA did not localize in one single point or a few randomly scattered points within the schizont. Rather it localized to ordered positions within the nucleus. To confirm that the DNA was hybridizing with actual DNA and not an artifact, the SAG1 cosmid was hybridized along with SAG2 cosmid (Figure 12D). They stained separate loci that produced similar pattern. The SAG2 cosmid stained more than one place per dot of SAG1, suggesting that it may contain a repeat. This hybridization pattern suggests that the DNA for each individual developing parasite is collected into an area around the polyploidy nucleus.

If the microtubules were the driving force of this organization destroying them would change the pattern of staining causing it to become random. Oryzaline has been used previously to destroy microtubules in *S. neurona* which caused a breakdown of the organization of the plastid (Vaishnava et al., 2005). Oryzaline was added to developing schizonts and the DNA was hybridized to the sample. The SAG1 DNA probe hybridized areas scattered through the treated schizonts (Figure 12 E) the organization observed in the untreated schizonts was lost. This suggests that the spindle structures do support the arrangement of the DNA in the developing schizont as well as the plastid.

The individual daughter cell’s DNA is arranged within the schizont and the microtubules are involved in the construction of the organization, but it is unknown how the microtubules perform this action. The cycling of the mitotic spindle phase and the dot phase could have an effect on this organization.

**Where is the DNA attached to the Spindle?**

The *S. neurona* schizont nucleus has been shown to have cycling microtubule morphology during its development. The DNA is organized within the polyploid nucleus and the DNA organization is dependent on the microtubule scaffold, but how or where is the DNA
attached to the spindle? In eukaryotic organisms, the kinetochore is the place of attachment of chromosomes and spindle microtubules during mitosis (McAinsh et al., 2003). They help move and recruit the chromosomes to daughter cells. Centrosomes have been established in Plasmodium and Theleria (Waters and Janse, 2004). We hypothesize that *S. neurona* also has kinetochores that connect the DNA to the spindle and that this connection persist throughout both the mitotic spindle phase and the dot phase. We expect the kinetochore in the mini-spindle phase to be closely associated with the microtubules to keep the arrangement of the DNA in the polyploid nucleus. In order to elucidate this process we tried to produce antibodies to Apicomplexan kinetochore proteins.

Centromeric histone H3 (CenH3) replaces the Histone H3 proteins at the centromere/kinetochore (Palmer et al., 1991; Yoda et al., 2000). CenH3 is conserved throughout eukaryotic organisms (Blower et al., 2002; Cervantes et al., 2006; Henikoff et al., 2000; Jin et al., 2004; Shelby et al., 1997; Stoler et al., 1995; Talbert et al., 2002). It has been established that the CenH3 is present throughout mitosis only at the centromere and is required for proper chromosome segregation (Cervantes et al., 2006; Stoler et al., 1995; Sugimoto et al., 2000; Sullivan et al., 2001). The CenH3 has an extended N-terminus over the normal H3 which is highly variable between species and the amount of conservation between different lineages is not consistent (Malik and Henikoff, 2003; Malik et al., 2002). While highly divergent, the CenH3 from different species show certain characteristics. The loop 1 region (the first turn after the first conserved Alpha helix) of the of CenH3’s is longer than the conserved H3 and they commonly lose a conserved glutamine residue in the alpha 1 helix (Malik and Henikoff, 2003).

Since the genomic data is limited in *S. neurona* we used more complete genomic data sets to construct the protein. Plasmodium histone H3 (a highly conserved protein) was used as a
query in a search of the *Toxoplasma gondii* genomes with the BLAST algorithm, TblastN (Figure 14A). The results identified the H3 proteins (normally two copies H3 and H3.3 a slight variant) and one copy that had conserved regions but varied from the histone H3s (Figure 14A). We generated primers in the regions that were out of the conserved regions, but close enough to likely be in the mRNA. The RT-PCR product of these primers was then used as a probe to screen a phagemid cDNA library. Two cDNAs were discovered which contained mature 3’ends comparative to the TgH3 and TgH3.3 genes, however these did not contain a fully processed 5’ end. Primers were constructed for a 5’RACE that elucidate the full cDNA (Figure 14B). Other CenH3 from related species including *Plasmodium falciparum* (and other plasmodium species) and *Cryptosporidium parvum* were uncovered with the TBLASTN algorithm and protein text searches. Alignments of these show a large divergence in the 5’ end of the proteins (figure 13) as compared to alignments between mammal and plant cenH3 alignments, but the longer 5’ ends of *Saccharomyces cerevisiae, Caenorhabditis elegans, Drosophila melanogaster* CenH3 gives evidence to the TgCenH3 longer 5’end (Malik and Henikoff, 2003). Primers for the gene were made and the gene’s cDNA was produced (Figure 14C) by PCR and inserted into a *T. gondii* expression vector. The plasmid was transfected into *T. gondii* parasite, but with no expression could be detected. A peptide polyclonal antibody was produced to both the TgCenH3 (Figure 13B in yellow region) and PfCenH3 (Figure 13B in the gray region). The peptide was selected by the Biosource epitope selector computer program from the complete protein sequences. The specificity of the polyclonal antibodies to the initial peptide was confirmed by Biosource. Both antibodies gave no immunofluorescence signal within the nucleus of their respective organism.
We also cloned the *T. gondii* BUB3 gene to explore a second independent potential kinetochore marker. BUB3 protein is a kinetochore protein that blocks the microtubule attachment site until the microtubule attaches, and it is replaced by mad1 (Guenette et al., 1995; Martinez-Exposito et al., 1999). They are present at the kinetochore during pre-prophase and prophase when the chromosomes are condensed, but before the microtubules attach (Basu et al., 1998; Campbell and Hardwick, 2003). The protein in *Toxoplasma gondii* and *Cryptosporidium parvum* was discovered by text searches of data sets created from protein used as queries in BLAST algorithm, while the *Eimeria telleria* was found with a blast search using the TgBUB3 genomic sequence. The TgBUB3 and the EtBUB3 were then TblastX (translated DNA versus translated DNA) against only each other. The exons hit each other significantly and gave a good map for a construction of the cDNAs of the genes and proteins. The TgBub3 aligned well with other BUB3 proteins across phylums (figure 15). Primers were made for a cDNA PCR which produced a fragment with the predicted length (Figure 16J). The cDNA was then inserted into an expression vector and transfected into *T. gondii* parasites. This produced an over expression of the protein which caused a disruption of the cell cycle and an aneuploidy phenotype (figure 16A and D). This suggests that a correct level or timing of Bub3 is required for a normal cell cycle.
4: Discussion:

The Persistent Spindle and the Centrosomes

The mitotic spindle persists throughout endopolygeny. It cycles between a mini-spindle stage spindle and a mono-polar long stage spindle. Both stages are contained within the nuclear envelope which remains intact even in close proximity to the centrosomes. The centrosome seems to be the organelle controlling the organization of the cycling mitosis with microtubules running to the plastid and to the nuclear envelope.

The mitotic spindle system is similar to fission yeast that is established early in the replication phase and persists to the end of mitosis. Besides the increase in number of daughter cells upon cytokinesis, other differences are displayed between the cell cycle of fission yeast and *Sarcocystis neurona* that could help elucidate the system of mitosis in Apicomplexa.

The centrosomes of *S. neurona* remain outside of the nucleus throughout the cell cycle. It remains close to the nucleus throughout G1 and G2 but with the onset of mitosis it moves closer to the nuclear envelope in an invagination which does not break down, but begins coordination of a mitotic spindle. It is unclear whether it moves close to the nucleus before or after the first DNA replication period. From this point, it divides and replicates with the DNA moving the DNA around the nucleus, resulting in a loose organization of the DNA with in the polyploid nucleus. In contrast, the spindle pole body of fission yeast spends most of its time close to the nucleus through most of interphase and replicates in the cytosol. With the onset of DNA synthesis, the bridge linked SPBs moves into an invagination of the nuclear envelope. The nuclear envelope breaks down in the invagination. The SPBs become continuous with the
nuclear envelope. A mitotic spindle is constructed and maintained from the SPB until the end of mitotis. After mitosis, the SPB moves out of the nuclear envelope and reside in the cytosol until the next round of replication, though it has been suggested that it may be continuously connected to the DNA through out interphase.

The centrosome is the organizing structure of the spindles, but where are the microtubules nucleated within the nucleus or at the centrosome? The SPB of fission yeast controls the mitotic spindle through direct contact. The *S. neurona* centrosome does not rely on this direct contact with the nuclear environment. It is separated from the nuclear environment by the nuclear envelope. It must recruit spindle nucleating molecules such as γ-tubulin and Spc98p. The distribution of these molecules through the cell cycle could help elucidate the microtubule nucleation process. Whether they lie within the nucleus, through the cytosol or in the PCM would show where the nucleation of the spindle is performed. It is possible that the *S. neurona* could control the spindle in a similar manner to plants with their scattered γ-tubulin and apex Spc98p and no centrosome.

Recently, a membrane occupation recognition nexus (MORN) protein, MORN1, was discovered in *T. gondii* that tightly co-localized next to the centrosome. MORN proteins allow for interaction of proteins with phospholipids of membranes. The MORN1 marks the centrocone an apicomplexa structure associated with the intranuclear spindle. The MORN1 labels the inner membrane of the nuclear envelope lined open cone and the inner surface below the membrane of the floor which suggests it is porforated. The centrocone contains the mini-spindle. It is suggested that since the MORN1 is continuously expressed, the centrocone is persistent throughout its life cycle. This protein also co-localizes with the mini-spindles in *S. neurona* (Gubbels MJ, 2006). This helps to better understand the microtubule morphology of *S. neurona*. 
The centrocone contains the mini-spindles, though the floor of the centrocone and the open apex are not observed probably due to cross section. Though, no centrocone like structures were observed in the extended mitotic spindle stage only a broader invagination of the nuclear envelope. This could be a difference between the two apicomplexan species.

MORN1 maybe the involved in an interaction and/or connection between the centrosome and the nuclear envelope connecting the controlling structure to the internal scaffold of the mitotic spindle. Further understanding of the interactions of MORN1 could help to elucidate the problem nucleation of the intranuclear mitotic spindle and mini-spindle especially in relation to the $\gamma$-tubulin and Spe98p.

**One Continuous Plastid Encircles the Nucleus and the Centrosome**

The plastid is one continuous structure that encircles the nucleus. It encircles the nucleus and surrounds the centrosomes. It is directly adjacent to the nuclear envelope, but does not cross it. The plastid appears enveloped by the centrosomes perhaps to stabilize their placement surrounding the nucleus. The exact opposite of this could be possible. Thus, the centrosome could use its organelle organizing capabilities to recruit the plastid to the area around the nuclear envelope. This would help to divide the plastid and assure that it will be in each daughter merozoite. The plastid has been shown to be an essential organelle and coordinated with the centrosome and mitotic spindle during division in *T. gondii*. Within *S. neurona*, the plastid’s development directly reflects the development of the nucleus increasing in size as the schizont nucleus increases. It would be interesting to elucidate the plastid DNA development in endopolygeny and establish the system that controls the distribution of the DNA. The centrosomes’ microtubules might play a role in this or similar mechanisms may be involved in both the mitotic spindle and the plastid DNA distribution.
**Phosphorylation of H3 Serine 10 is not present in Apicomplexa**

Neither *T. gondii* nor *S. neurona* displayed a significant amount of the histone modification phosphorylated H3 serine 10. This is a highly conserved histone modification linked to condensation of DNA during mitosis. The lack of this modification in these species draws questions about the system of histone modification within the Apicomplexa. Other histone modifications have been seen to not follow a strict conservation from model organism to model organism, but the P-H3S10 has been displayed from another alveolate Tetrahymena to yeasts to animals. The implications of not having this histone modification are not known.

*S. neurona* DNA displays a higher concentration of DNA DAPI signal as in the long spindle stage which looks like the DNA is condensed, though the chromosomes are not visually condensed. The use of FISH with full chromosomes would help to better understand the condensation of the DNA within the mitosis cycle. A similar study has been performed in budding yeast which also does not have visually condensed chromosomes, but the condensation was elucidated (Guacci et al., 1994).

**Acetylated H3 Lysine 14 could be used as indicator for mitotic cycling**

With the absence of P-H3S10 modification, it is quite interesting to find a signal for Acetylated H3 lysine 14 even in a lower proportion to the other euchromatin modification. The H3 acetylated lysine-14 is controlled by a different pathway than the Acetylated H3K9. It acts in concert with the Phosphorylation of H3 Serine 10. The phosphorylation helps to recruit the HAT (Histone Acetyl Transferase) specific for H3K14, Gnc5. It binds much better to a phosphorylated histone H3 than non- phosphorylated. The phosphorylation is thought to arrange for the acetylation event. It is possible that limiting the action of the Gnc5 for the H3 K14 acetylation by not phosphorylating the H3S10 could be use to limit the aspects of DNA condensation or
allow mitosis to proceed while the parasite can still use its DNA (to produce proteins). It is also possible that there is another pressure that forced the loss of this histone modification.

Gcn5 has been established in apicomplexa species an the acetylation activity has been described (Fan et al., 2004; Hettmann and Soldati, 1999; Sullivan and Smith, 2000). Quite recently a study described two separate Gcn5 proteins, TgGcn5-A and TgGcn5-B, with distinctive acetylation targets. TgCen5-B specifically targets the H3 lysine 18 and the TgGcn5-A targets the H3 tail (which would acetylate the H3K14) (Bhatti et al., 2006). This adds to the complexity of the situation. Neither Gnc5 acetylation activity has been related to the the lack of phosphorylation of the histone H3 has been described.

Another recently published study on acetylation of *P. falciparum* histones found widespread acetylation in the histone H3 lysine and the H4 lysine residues. They exhibited acetylation activity by the recombinant *P. falciparum* Gcn5 on both the H3 K9 and K14 residues. They also described a lower level of H3K14 acetylation *in vivo* compare to other acetylation sites, though they attributed this to a modification of the H3 sequence at site 9 in *P. falciprum* H3 (Figure 13) (Miao et al., 2006). We suggest that it is the lack of phosphorylation at the Serine 10 residue. The affinity of the Gnc5 is probably lower than in other systems. A comparison between the activity level of Gnc5 in apicomplexa and another organism with a P-H3S10 (turned on and off) could help establish this affinity. Perhaps the Gnc5 has a higher affinity without the H3 phosphorylation or perhaps the loss of the modification attenuates the signal for a physiological reason.

**Histone modification in Typical Euchromatic Modifications and no Heterochromatin (Is There any Heterochromatin?)**
In all the experiments, the nucleus contained modifications that indicated euchromatin and no heterochromatin. Could this be possible or could the histone modification be a signal for something else? The amount of DNA contained within the nucleus of *T. gondii* is much smaller than human host cells seen in the images (Consortium, 2004; Kissinger et al., 2003). There are fewer non-coding regions in *T. gondii* than human genomes. This could account for heterochromatin histone modification signals not being observed at the resolution of this study.

It’s is also possible that the monomethylated H3K4 acts as a heterochromatin marker. It was displayed at a lower level than other methylated H3K4s and has been indicated as a gene silencing signal in other organisms (van Dijk et al., 2005).

The euchromatin modifications were more intense in the parasites than in the host cells indicating that they might be involved in some other signal process. Perhaps, they even indicate the exact opposite signal to the cell one of heterochromatin and silencing.

It would be beneficial to resolve the histone modifications further. Chromatin immunoprecipitation (ChIP) experiments using genes with known expression patterns could be employed to perform this task. The modification pattern could be compared to the expression pattern of the genes. Perhaps a novel modification pattern would be resolved that could be exploited as a drug target.

Distribution of H3.3 could also be used in some way in the system of DNA expression. H3.3 has been shown to displace histone H3 in active chromatin in other organisms (Choi et al., 2005). Perhaps, members of Apicomplexa could rely on this system more heavily to indicate active genes rather than caniconal histone markers. Expression patterns of H3.3 would also help to better our understanding of gene expression in Apicomplexans. The expression of H3.3 has
been established in *P. falciparum* life cycle (Miao et al., 2006), but its effect on gene expression is not established.

The Nucleus is organized during Endopolygeny

We observed that the developing *S. neurona* polyploid nucleus is organized. All the sets of chromosomes in the polyploid schizont nucleus do not split up de novo in the last stage of endopolygeny. The DNA copies moved with the centrosome around the nucleus as it increases in ploidy. The understanding of when the DNA replicates and when it divides is not understood. A correlation of the cycling spindle morphology with the replication pattern of the DNA could help to illuminate the time points of replication and separation.

CenH3 might not be used in Apicomplexa as in Other Phyla

CenH3 is conserved throughout the plant, animal and fungi kingdoms. We were able to identify a gene with a histone H3 like P-terminus and an extended N-terminus in *T. gondii*. It also had characteristic conserved amino acids and extended loop regions. This genetic sequence was amplified in cDNA, confirming its transcription. However peptide antibodies to this, TgCenH3, and a potential *Plasmodium falciparum* CenH3 both gave no signal. TgCenH3 also gave no signal in transfection experiments with GFP or myc plasmid vectors. It is also possible that our clone contained a mutation or that we miss annotated the gene resulting in a faulty antibody epitopes.

This suggests that the CenH3 might not be used as a marker for the centromere. The cDNA data contradicts this, but not all stages of the life cycle of *T. gondii* and *P. falciparum* were examined. PfCenH3 was also established with RT-PCR recently, but was unable to be confirmed by mass spectrometry during the tachyzoite stage (Miao et al., 2006). Perhaps the CenH3 is used only in the sexual replication and another variant histone indicates the centromere.
during mitosis. It is also possible that both peptides we used were not good epitopes for antibody because of folding or DNA interaction. Though the antibodies were specific for the peptide sequence they might not have been displayed well enough in the cellular environment.

There is much evidence that centromere and kinetochore exists in Apicomplexa. The genomic data in *Plasmodium* and *Theileria* is in support of this idea. The over expression of bub3, a kinetochore checkpoint protein, causing aneuploidy and halting proper replication in *T. gondii* also lends evidence to establish this. This would suggest that the apicomplexan members have a kinetochore that has components that have homology to other eukaryotes or that the Bub3 works without a kinetochore. A further study of kinetochore proteins would help to elucidate the construction of the kinetochore system and its effect on the parasitic mitosis.
5: Figures

Figure 1
Figure 1

Schemes for nuclear division in Apicomplexa

Endodyogeny: Cytokinesis directly follows a single DNA replication and nuclear division event.

Schizogony: The chromosomes replicate and the nuclei divide multiple times within the mother parasite before cytokinesis proceeds. Endopolygeny: The DNA replicates multiple times, but the daughter nuclei do not divide until the final mitotic event and subsequent cytokinesis.
Figure 2

Two Microtubule morphologies and their Relation to the Plastid

A-C exhibits a *S. neurona* schizont with a typical mitotic spindle morphology stained with anti-tubulin antibodies. The spindles have a close relationship to the centrosomes stained with anti-centrin antibodies. D-F show the other tubulin morphology of a dot formation surrounding the nucleus. These tubulin dots are also closely related to the anti-centrin stained centrosomes. In the close up, The dots (black arrow) are flanked by the centrosomes (white arrows). E-F displays the relationship between the anti-ACP stained plastid, the microtubule morphology and the nucleus. The plastid encircles the nucleus and is tightly coordinated to the microtubule dots. (adapted from (Vaishnava et al., 2005))
Figure 3
Figure 3

**Working model for Nuclear Division of Sarcocystis neurona**

1) a Merozoite infects a host cell and (2) begins replication by entering mitosis. It cycles between two tubulin morphologies a typical mitotic spindle during a mitotic phase (M) and (3) shorter tight spindles in the synthesis phase (S). It does this for 2-3 days and 5 rounds of DNA replication (up to 32N). (4) There is an onset of nuclear division with the final round of mitosis (M) (64 N) and (5) a subsequent cytokinetic event budding (B).
Figure 4
Figure 4

Electron micrographs of *S. neurona* schizont in the mitotic spindle pole phase.

A) The plastid (P) is shown to be a continuous structure surrounding the nucleus (N) and electron dense regions, centrosomes, are exhibited between the two. B) A magnification of the two centrosomes. The nuclear envelope is not broken down even under the centrosome (little black arrows). The plastids four membranes are indicated by the little white arrows. The centrosome is interacting with both the plastid (large white arrow) and the nucleus (large black arrow) via microtubules coordinating the actions of both. C) At the white arrows are microtubules that are running through the nucleus (N) in an astra pattern from the centrosomes (at the large white arrows).
Figure 5
Figure 5

Electron Micrograph of *S. neurona* with the microtubule dot morphology

A) An early Schizont with a plastid (P) in close proximity to the nucleus (N). An invagination of the nuclear envelope is at the white arrow and an electron dense area with in the nucleus is at the black arrow. B and C are serial sections. At the black arrow in B a tight basket of electron dense microtubules are exhibited while in C the microtubules are are less dense. The image in B displays a dense microtubule outer section and C shows a cross section of the cone like structure. This microtubule basket is the microtubule dot. There are electron dense areas at the white arrows in both sections at the end of the microtubule basket. We suspect these are the kinetochore of chromosomes in continuous connection with the spindle throughout mitosis. The highlighted white arrow outside of the nucleus displays a paired centrosome containing centrioles. The nuclear envelope is not broken down.
Figure 6

**Immunolabeled Electron Micrograph of Plastid (anti-ACP) antibody on *S. neurona*.**

Plastid antibodies and Gold Conjugate antibodies to the plastid antibodies were exposed to a section of paraformaldehyde and glutaraldehyde fixed *S. neurona* in host cells. A) A cross section of Sn displays the anti-ACP antibodies with gold in a long continuous organelle that is associated with the nucleus (N), the plastid (P). B) A magnification of the plastid showing the gold (at the white arrows) present mostly in the plastid membranes, though the membrane are not easily distinguished with this fixation.
Figure 7
Figure 7

Anti-H3 phosphorylated serine-10 antibodies in Apicomplexan parasites.

A-E are images of *S. neurona* anti-Phosphorylated H3 Serine 10 (A), anti-α tubulin (B), anti-H3pS10 and anti-α tubulin (C), DAPI (D) and anti-H3pS10 and DAPI (E). This exhibits that the major staining is external of the nucleus and the microtubule mini-spindle. The morphology resembles plastid morphology (see figure 2E). [Anti-H3pS10 Antibody was used at 1:1000 and a 1:200 secondary with a 1 second exposure in immunofluorescence.] F-H is anti-H3pS10 (F), DAPI (G), and a merge of the two (H) in *T. gondii*. The majority of staining in *T. gondii* was to a single point near the nucleus much like the plastid. I-K and L-N are anti-H3pS10 (I and L), DAPI (J and M) and a merge (K and N) on *T. gondii* merozoites. The majority of the anti-H3pS10 signal is localized to the plastid DNA and thus to the plastid. O and P are western blots of anti-H3pS10 on *T. gondii* merozoite protein lysate. The majority of the signal is to proteins much larger than the 17KDa histone H3. These are probably the plastid proteins it is staining.
Figure 8

*Sarcocystis neurona*

H3
Mono-methylated
Lysine 9

H3
Di-methylated
Lysine 9

H3
Tri-methylated
Lysine 9

*Toxoplasma gondii*

H3
Mono-methylated
Lysine 9

H3
Di-methylated
Lysine 9

H3
Tri-methylated
Lysine 9

Figure 8
**Figure 8**

**H3 methylated lysine-9 is not present on a distinguishable level in Apicomplexan.**

Images of the Antibody’s signal in the first column (A,D,G,J,M,P), DAPI signal image in the second column (B,E,H,K,N,Q) and a merge in third column (C,F,I,L,O,R). Anti-H3-methylated-K9 antibodies (mono-, di-, tri) were used at 1:100 and a secondary concentration of 1:200. Camera exposures in *S. neurona* were 3 seconds. Camera exposures in *T. gondii* were 1 second for di methylated K9 and Trimethylated K9 and 3 seconds for monomethylated K9. No significant signal is displayed within the nucleus in any of the parasites. A significant signal was imaged in the conoid region out side of the nucleus in *T. gondii* with the di-methylated K9 (M at the arrow) and Tri-methylated K9 (P at the arrow) antibodies and in *S. neurona* in dots outside of the nucleus thought to be the remnant of the parasite’s conoid (G at the arrow). In G the host cell displays regions of significant signal not seen in any of the parasites.
Sarcocystis neurona

H3
Mono-methylated
Lysine 4

H3
Di-methylated
Lysine 4

H3
Tri-methylated
Lysine 4

Toxoplasma gondii

H3
Mono-methylated
Lysine 4

H3
Di-methylated
Lysine 4

H3
Tri-methylated
Lysine 4

Figure 9
Figure 9

**Apicomplexan parasites display H3 Lysine 4 methylation.**

Images of the Antibody’s signal in the first column (A,D,G,J,M,P), DAPI signal image in the second column (B,E,H,K,N,Q) and a merge in third column (C,F,I,L,O,R). Anti-H3-dimethylated K4 and Anti-H3-trimethylated K4 antibodies were used at 1:1000 and a 1:200 secondary with a 0.7 second exposure in immunofluorescence and the anti-H3-monomethylated K4 antibody was diluted at 1:250 and exposed for 3 seconds. A significant amount of signal was imaged with all three antibodies in both *S. neurona* and *T. gondii*, but a much larger amount with anti-H3-dimethylated K4 (D and M) and anti-H3-trimethylated K4 (G and P). The signal in anti-H3-dimethylated K4 and anti-H3-trimethylated K4 was significantly more than the host cells. The host cells have more DAPI signal, but less stain with the antibody. All of the DNA is stained; there are no significant portions unstained at this resolution. The apical end of the parasite was marked with the anti-H3-trimethylated K4 (P) similar to the anti-H3-trimethylated K9 antibody.
Figure 10

Apicomplexan parasites display H3 Lysine 4 methylation throughout the cell cycle.

A and D are stained with Anti-H3-dimethylated K4 antibodies, B and E are DAPI signal, C and F are stained with anti-α tubulin. Both the mitotic spindle morphology (A-C) and the microtubule dot morphology (D-F) contain the H3-dimethylated K4 modification. The signal is slightly stronger in the spindle poles of (A) suggesting that the DNA is more concentrated and perhaps condensed during the mitotic event. G) A Western blot of Anti-H3-dimethylated K4 antibodies on T. gondii merozoite protein lysate. The majority of the signal is to proteins approximately 17KDa, the size of histone H3.
Figure 11
Apicomplexan parasites display different amount of H3 Acetylation.

Images of the antibody’s signal in the first column (A,E,I,M,Q,U), DAPI signal image in the second column (B,F,J,N,R,V), both the antibody and DAPI in the third column (C,G,K,O,S,W) and phase contrast images in the fourth column (D,H,L,P,T,X). The anti-H3-acetylated K9 antibody was diluted 1:1000 and exposed for 0.7 seconds. It displayed a significantly large amount of signal comparable to the Anti-H3-dimethylated K4 and Anti-H3-trimethylated K4 in coverage of the nucleus and signal strength in both *T. gondii* (E) and *S. neurona* (A). The anti-H3-acetylated K14 antibody was diluted 1:250 and exposed for 3 second. It displayed significantly less signal than the anti-H3-acetylated K9, but signal is present within the nucleus (I and M). *T. gondii*’s signal does not change (M). There is a change in the signal pattern of *S. neurona*. The signal is higher in the final stage of endopolygeny (at the white arrow in I) compared to early stage schizonts (crossed arrow in I). The antibody also marks the plastid *T. gondii* (at the arrow in M). (Q-T and U-X) The anti-unmodified-H3 antibody was used to distinguish if any of the H3 histones were in an unmodified form. It was diluted 1:100 and exposed for 5 seconds. Very little signal was displayed but the major amount was in the plastid region in *T. gondii* (at the white arrow in U) lending evidence to a histone like epitope in the plastid.
Figure 12
Figure 12

The genetic material is organized around the *S. nucleus*.

SAG1 was labeled with digoxigenin and SAG2 to biotin. These genetic probes were allowed to hybridize with developing schizonts. A) The SAG1 was displayed as 1:1 with developed *S. neurona* merozoites. B) SAG1 exhibits a non-random organization around the polyploid schizont nucleus (at the small white arrows). It is also displayed as sister genes in the schizont on the left (at the large white arrows); these are also spread around the nucleus and not congregated together. C) SAG2 is also spread around the nucleus, but has more location than the SAG1. It is specific to the *S. neurona* as compared to the host cell nucleus in the same frame. D) The SAG1 and SAG2 locate to different DNA within the nucleus. SAG2 has more than one location per SAG1 location suggesting that the probe contains repeats. E) The SAG1’s organization is destroyed when treated with Oryzalin as plastid organization is destroyed.
A) Histone H3 Homology

B) Apicomplexan CenH3s

Figure 13
The TgCenH3 does not show the conserved homology of the TgH3.

A) The histone H3 is highly conserved the *Toxoplasma gondii* H3 is here show aligned with *Plasmodium falciprum* (Pf), the human (Hs) and the mouse (Mm) histone H3. B) The TgCenH3 is well conserved in the P-terminus is conserved, but the N-terminus is divergent even in closely related apicomplexan species. *Plasmodium vivax* (Pv) variant H3, *Thileria aunulata* (Ta) variant H3 (potential CenH3). Peptide Antibodies were created to the highlighted peptides in PfCenH3 (in Gray) and TgCenH3 (in Yellow).
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### B

- **5' Nested Race**
  - 1.4KB product

### C

- **CenH3 cDNA**
  - 1.4KB product

**Figure 14**
Figure 14

**Evaluation of the TgCenH3**

A) A list of proteins that share significant homology to the P.falicprum H3 from Toxodb.org. The First row is the confirmed H3, the second in the confirmed H3.3 and the third is the probable CenH3 that was evaluated. B) A nested 5’RACE preformed with the TgCenH3 specific 3’ end primers stated in the materials and methods. C) The cDNA product of the *T. gondii* CenH3 primers.
Figure 15
Genomic data mined TgBUB3 is similar to other BUB3s.

TgBub3 was created from a text search at Toxodb.org for bub3. The punative sequence was used as a TblastX query into the Eimeria genome to extract the genomic sequence of the *Eimeria* gene. The *T. gondii* BUB3 genomic sequence was used as a TblastX query against the *Eimeria* genomic sequence. From the conserved regions the exons for TgBUB3 were extrapolated. Alignment of the TgBUB3 with Human (Hs), Mouse (Mm), Zebrafish (Dr), Eimeria T. (Et) budding yeast (Sc) shows the Eimeria and *T. gondii* genes to be significantly similar to other experimentally defined BUB3s.
Figure 16

Bub3 Tranfection

TgBub3 cDNA PCR 1KB Fragment
Figure 16

**BUB3 elucidation in *Toxoplasma gondii*.**

J) shows the cDNA amplified in *T. gondii* with primers created with the hypothetical cDNA extrapolated from genomic data. A-F display the Bub3 transient tranfection in a tubulin expression plasmid linked to myc marker and probed with Antibodies to myc. This displays a disrupted cell cycle resulting in aneuploidy of the *T. gondii* parasites. G-I exhibit the untranfected *T. gondii* probed with the myc antibody.
6: Conclusion

The mitotic spindle persists throughout the Schizont development located within the nuclear envelope. It is organized by the centrosome which also recruits the plastid. Further investigation into this mechanism could elucidate further differences between the parasites and their host cells.

The lack of the highly conserved histone modification H3 phosphorylated serine 10 is interesting. This along with the distinctive histone modification of the H3 methylation could be exploited, if there is a evident difference between the controls of the histone modifying proteins in the parasites and the host cell. More investigation is needed to decipher the system.

The organization of the endopolygeny nucleus is interesting, but more knowledge about the relationship to the microtubule morphology and the centrosome cycles is needed to relate the data to the body of evidence.

The lack of CenH3 evidence is stirring, but incomplete. A broader scope of the protein in other stages life cycle could elucidate its function in Apicomplexa. If CenH3 is down regulated or not used through the schizont stage, the difference of systems may allow for a novel manipulation of the parasite.
7: References


I contributed work to two articles which are included in the following pages:

**Analysis of the Sarcocystis neurona microneme protein SnMIC10: protein characteristics and expression during intracellular development**

I provided the immuno-electron micrograph displaying the SnMic10 antibodies within the apical end an intracellular Schizont (Figure 4).

**Plastid segregation and cell division in the apicomplexan parasite Sarcocystis neurona**

I provided all the electron microscopy images (in Figure 1 and 5) which were discussed in detail in this thesis.
Analysis of the *Sarcocystis neurona* microneme protein SnMIC10: protein characteristics and expression during intracellular development

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**Abstract**

*Sarcocystis neurona*, an apicomplexan parasite, is the primary causative agent of equine protozoal myeloencephalitis. Like other members of the Apicomplexa, *S. neurona* zoites possess secretory organelles that contain proteins necessary for host cell invasion and intracellular survival. From a collection of *S. neurona* expressed sequence tags, we identified a sequence encoding a putative microneme protein based on similarity to *Toxoplasma gondii* MIC10 (TgMIC10). Pairwise sequence alignments of SnMIC10 to TgMIC10 and NcMIC10 from *Neospora caninum* revealed approximately 33% identity to both orthologues. The open reading frame of the *S. neurona* gene encodes a 255 amino acid protein with a predicted 39-residue signal peptide. Like TgMIC10 and NcMIC10, SnMIC10 is predicted to be hydrophilic, highly alpha-helical in structure, and devoid of identifiable adhesive domains. Antibodies raised against recombinant SnMIC10 recognised a protein band with an apparent molecular weight of 24 kDa in Western blots of *S. neurona* merozoites, consistent with the size predicted for SnMIC10. In vitro secretion assays demonstrated that this protein is secreted by extracellular merozoites in a temperature-dependent manner. Indirect immunofluorescence analysis of SnMIC10 showed a polar labelling pattern, which is consistent with the apical position of the micronemes, and immunoelectron microscopy provided definitive localisation of the protein to these secretory organelles. Further analysis of SnMIC10 in intracellular parasites revealed that expression of this protein is temporally regulated during endopolygeny, supporting the view that micronemes are only needed during host cell invasion. Collectively, the data indicate that SnMIC10 is a microneme protein that is part of the excreted/secreted antigen fraction of *S. neurona*. Identification and characterisation of additional *S. neurona* microneme antigens and comparisons to orthologues in other Apicomplexa could provide further insight into the functions that these proteins serve during invasion of host cells.

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**Keywords:** Sarcocystis; Microneme; Apicomplexa; Equine protozoal myeloencephalitis; Invasion

1. Introduction

The apicomplexan parasite *Sarcocystis neurona* is the primary causative agent of equine protozoal myeloencephalitis, the most commonly diagnosed neurologic disease of horses in the United States (Dubey et al., 1991, 2001a; Hamir et al., 1992; MacKay, 1997). The natural life cycle of *S. neurona* utilises the opossum as a definitive host (Fenger et al., 1997; Dubey and Lindsay, 1998) and a variety of small mammal intermediate hosts, including raccoons, nine-banded armadillos, skunks, and cats (Dubey et al., 2000, 2001b; Cheadle et al., 2001a,b; Tanhauser et al., 2001). Horses are infected by ingesting feed and water sources contaminated with *S. neurona* sporocysts (Fenger et al., 1997; Dubey and Lindsay, 1998). In the infected animal, parasites ultimately invade cells within the central nervous system, occasionally causing focal inflammation and neurological damage. Seroprevalence of *S. neurona* in horses is substantial (MacKay et al., 2000), and the incidence of equine protozoal myeloencephalitis is considerably lower (MacKay et al., 2000), and the specific factors and events

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contributing to the pathogenesis of this disease are poorly understood.

Apicomplexan parasites are highly polarised cells that are characterised by the presence of an apical complex. This complex is formed by specialised cytoskeletal elements, including the conoid, and three distinct populations of secretory organelles called micronemes, dense granules, and rhoptries. Collectively, the secretory organelles of the apical complex aid in the organisms’ ability to invade host cells and to establish an intracellular environment that is suitable for survival and propagation. Based on protein characteristics and secretion kinetics during cell invasion, microneme proteins appear to be involved in attachment and entry into host cells (reviewed in Dubremetz et al., 1998). Multiple microneme proteins have been identified thus far (reviewed in Tomley and Soldati, 2001), and it is likely that these proteins work in concert to accomplish the critical function of attachment and invasion.

Like other members of the phylum, *S. neurona* merozoites possess the secretory organelles micronemes and dense granules; however, little is known about the protein contents of these organelles in this species. In an effort to obtain information regarding the molecular composition of *S. neurona*, an expressed sequence tag (EST) sequencing project was conducted to generate a database of gene sequences from this parasite (Howe, 2001). Herein, we describe a microneme protein from *S. neurona*, SnMIC10, which was identified from the collection of *S. neurona* EST glycerol stocks, and the cDNA inserts were sequenced using the T3 primer to confirm their identity. To obtain a full-length SnMIC10 cDNA clone, the 5’-end of the *SnMIC10* mRNA transcript was amplified from the cSn1 *S. neurona* cDNA library (Howe, 2001) using a reverse-orientation primer SnMIC10.R (5’-ACCTGACCATACTGTGC-3’) and the T3 primer of the pBlueScript cloning vector. The resulting PCR product was ligated into the pCR®2.1 vector (Invitrogen) and sequenced. A BLASTN search of dbEST with the obtained sequence identified an apparent full-length cDNA, SnES-T4a13c09, which was recovered for further study. The sequence of *SnMIC10* is available through GenBank accession #AF532594.

2.3. Sequence analysis


2.4. Recombinant SnMIC10 expression and polyclonal antisera production

The SnMIC10 open reading frame (ORF), without the predicted signal peptide, was PCR amplified using the primers SnMIC10.exp.f (5’-GATCCATACTGTGC-3’) and SnMIC10.exp.r (5’-GATCAAGCTTCCGGTGACCTTTC-3’), which were designed to incorporate a *Nde*I and a *Hind*III restriction site into the 5’- and 3’-end, respectively. The amplified fragment was digested with *Nde*I and *Hind*III and ligated into *Nde*I/*Hind*III-digested pET-22b expression vector (Novagen), creating the plasmid pPrSnMIC10. *Escherichia coli* strain BL21-CodonPlus (Stratagene) cells were transformed with pPrSnMIC10, and a clone that expressed a high level of recombinant SnMIC10 (rSnMIC10) was selected for use. After induction of expression with isopropyl-β-D-thiogalactopyranoside, rSnMIC10 was purified by nickel-column chromatography using the B-PER™ 6xHis Spin purification Kit (Pierce). Purified rSnMIC10 was run in a 12% SDS-PAGE minigel, excised from the gel, and used to immunise one rabbit and one rat for polyclonal antisera production (Cocalico Biologicals, Inc.).
2.5. SDS-PAGE and Western blot analysis

Proteins and whole parasite lysates were suspended in SDS sample buffer, with or without 2-mercaptoethanol, and supplemented with protease inhibitor cocktail composed of 4-(2-aminoethyl)benzenesulfonyl fluoride, E-64, bestatin, leupeptin, aprotinin, and sodium EDTA (Sigma). Proteins were separated in 12% polyacrylamide gels (Laemmli, 1970). For Western blot analysis, proteins were transferred to nitrocellulose membranes by semi-dry electrophoretic transfer in Tris-glycine buffer (pH 8.3). Membranes were blocked with PBS containing non-fat dry milk, 0.1% TWEEN 20, and 5% normal goat serum (NGS), and then incubated for 1 h in primary antibody solution. After multiple washes with PBS containing 0.1% NGS and non-fat dry milk, the membranes were incubated with goat anti-rabbit IgG (1:10,000) or goat anti-rat IgG (1:20,000) secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Labs, Inc.). After further washing, the membranes were processed for chemiluminescent detection using SuperSignal® substrate (Pierce) or ChemiGlow® substrate (Alpha Innotech, Corp.) and exposed to radiograph film or documented with an imaging system (Alpha Innotech, Corp.).

2.6. Secretion assays

The excretory/secretory antigen fraction from S. neurona was assayed as previously described for T. gondii (Carruthers and Sibley, 1999). Freshly egressed S. neurona merozoites were harvested as described above and washed once with secretion medium (RPMI with 10 mM HEPES + / –3% FBS). Parasites were resuspended in secretion medium and placed in microfuge tubes for incubation. Merozoites were removed from the secretion medium by centrifugation (two times at 1,000 × g, 5 min, 4°C), and supernatants were either stored at −20°C or immediately processed for Western blot analysis, as described above. To assess protein quantities, spot densitometry was performed on the FluorChem 8800 using AlphaEase FC processing and analysis software.

2.7. Indirect immunofluorescence assay of extracellular and intracellular merozoites

For analysis of extracellular parasites, freshly harvested merozoites were washed with PBS and resuspended in culture medium. Approximately 1.25–2.5 × 10⁶ parasites were spotted on a poly-l-lysine-coated slide and allowed to air-dry. For analysis of intracellular parasites, bovine turbinate host cells were grown on Lab-Tek® II Chamber Slides™ (Nalgene Nunc International) and inoculated with 0.8–1.0 × 10⁶ freshly harvested merozoites. Slides were fixed for 15 min at 4°C in 2.5% formalin-PBS containing 0.25% glutaraldehyde. For permeabilisation of slides, slides were incubated in 0.2% TX-100/PBS for 30 min. Slides were blocked for 30 min with 10% NGS/PBS, followed by incubation for 1 h at room temperature with primary antibody. Slides were rinsed and incubated with goat anti-rat IgG or goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) or Texas Red (Jackson ImmunoResearch Laboratories) at a 1:200 dilution. The slides were mounted in Vectashield® Mounting Medium with DAPI (Vector Laboratories, Inc.) and examined with a Zeiss axioscope (Carl Zeiss) equipped for phase contrast and epifluorescence microscopy.

2.8. Immunoelectron microscopic (Immuno-EM) examination of intracellular merozoites

For immunoelectron microscopy, infected cells were fixed with 2% paraformaldehyde and 0.1% glutaraldehyde in 0.5 × PBS for 20–30 min on ice. Fixed cells were washed, scraped and centrifuged. The pellet was progressively dehydrated with ethanol and gradually infiltrated with LR-White (London Resin Company) on ice. Finally the sample was transferred to a gelatin capsule and polymerised at 50°C. Ultrathin sections cut from trimmed blocks and recovered on formvar coated grids were placed on 50-µl drops of the polyclonal anti-rSnMIC10 antibody (400:1 dilution) in PBS/2% BSA, followed by incubation with 1:50 goat anti-rabbit IgG conjugated with 10 nm gold (Bibinternational) and a post-labelling wash of PBS/2% BSA containing 0.125 M NaCl. After immunolabelling, sections were stained and analysed with 4% uranyl acetate and 4% lead citrate and observed using a JEO JEM-100 CX II microscope.

3. Results

3.1. Identification of a S. neurona MIC10 homologue (SnMIC10)

To identify potential microneme proteins from S. neurona, the collection of S. neurona ESTs was searched with the sequences of previously identified T. gondii MIC proteins. BLAST searches of dbEST using TgMIC10 as the query identified six contiguous S. neurona ESTs that exhibited moderate similarity to TgMIC10 from T. gondii and NcMIC10 from N. caninum (Expect values <10⁻⁴). Alignment of the S. neurona sequence contig with the TgMIC10 and NcMIC10 sequences confirmed the sequence similarity (30% sequence identity, 48% similarity) observed in the initial BLAST searches, and it further suggested that the longest cDNA from the contig (SnEST4a16d01) was truncated at the 5’ end (data not shown).

To obtain a full-length cDNA of SnMIC10, the 5’ end of the gene was amplified from the cSn1 cDNA library using the T3 primer of the pBlueScript cloning vector and a reverse orientation primer (SnMIC10.R) based on the sequence of SnEST4a16d01. The resulting 700-bp ampli-
fication product was cloned into the pCR®-2.1 vector and designated pSnMIC10.5. Sequence analysis of pSnMIC10.5 revealed the expected sequence overlap with SnEST4a16d01, thus demonstrating that these are valid contiguous sequences. A BLASTN search of dbEST with the pSnMIC10.5 sequence identified two additional S. neurona ESTs that appeared to be full-length cDNAs of the putative SnMIC10 gene. The longer of these two ESTs, SnEST4a13c09 (GenBank accession # BE635951), was recovered and re-designated pcSnMIC10.

Complete double-strand sequence analysis of pcSnMIC10 revealed a 2,040 bp cDNA insert with a predicted ORF of 765 bp that encodes a protein of 255 amino acids. The 5′ untranslated region (UTR) of pcSnMIC10 was 235 bases long, while the 3′ UTR was greater than 1 kb in length. Analysis of other SnMIC10 cDNAs revealed several alternative poly-adenylation sites. Amplification of the SnMIC10 genomic locus indicated that the gene lacks introns (data not shown). A hydrophilicity plot of the SnMIC10 amino acid sequence suggested an amino-terminal hydrophobic stretch that corresponds to a predicted signal peptide of 39 amino acids (cleavage between Gly39 and Thr40). Removal of the signal peptide yielded a mature protein of 216 amino acids that has an expected molecular weight of 24.4 kDa. The SnMIC10 primary sequence does not contain any predicted N-glycosylation and O-glycosylation sites.

To determine the level of sequence conservation in the MIC10 orthologues, a multiple sequence alignment was generated using the presumptive mature forms (i.e. less the predicted N-terminal signal peptides) of SnMIC10, TgMIC10 and NcMIC10. This analysis revealed 33% sequence identity between SnMIC10 and TgMIC10 and 27% identity between SnMIC10 and NcMIC10 (Fig. 1). Relative to TgMIC10 and NcMIC10, the mature SnMIC10 contains a 68 amino acid extension on the amino-terminal end of the protein and an additional seven residues on the carboxyl-terminus. Like its homologues, SnMIC10 is devoid of cysteine residues or predicted adhesive domains, is expected to be predominantly alpha-helical in nature, and is rich in charged amino acid residues thereby resulting in an overall hydrophilic protein. In contrast to the nine diglutamic acid repeats observed in TgMIC10 (Hoff et al., 2001), only two of these repeated elements are present in SnMIC10.

3.2. Characterisation of SnMIC10

To produce reagents for the study of native SnMIC10, a recombinant form of the protein (rSnMIC10) was expressed in E. coli and used to immunise animals for polyclonal antiserum production. Western blot analysis using the resulting anti-rSnMIC10 sera revealed a single protein in S. neurona merozoites that migrated at approximately 24 kDa (Fig. 2), consistent with a calculated Mr of 24.4 kDa for the mature SnMIC10. Additionally, SnMIC10 was found in the aqueous phase of a TX-114 partitioning assay (Fig. 2), as expected from the predicted hydrophilic nature of the protein.

The clear homology between SnMIC10 and the MIC10 proteins from T. gondii and N. caninum suggested that SnMIC10 is a microneme protein of S. neurona. Immunofluorescent labelling with the anti-SnMIC10 serum showed a polar staining pattern in extracellular merozoites, consistent with the apical location of micronemes (Fig. 3). To obtain definitive localisation of SnMIC10, S. neurona merozoites were examined by immuno-EM. As shown in Fig. 4, the majority of the immunogold label decorated the micronemes of intracellular merozoites. A small amount of

![Fig. 1. Multiple sequence alignment of the mature proteins (i.e. less the predicted N-terminal signal peptides) showed 31 and 27% identity between SnMIC10 and the TgMIC10 and NcMIC10, orthologues, respectively. Numbers indicate the residue position. Capital letters in the consensus line indicate absolute amino acid conservation. Symbols indicate agreement in two of the three sequences and high consensus value (similar amino acid) in the third sequence substitution. Lower case letters indicate that the third sequence substitution is low consensus (i.e. dissimilar residue).](image-url)
immunogold label was observed in small vesicles anterior to the nucleus, and this likely represents SnMIC10 in the Golgi apparatus. Collectively, these data demonstrated that SnMIC10 is a microneme protein of *S. neurona* merozoites.

### 3.3. Secretion of SnMIC10

To assess whether SnMIC10 is a component of the *S. neurona* excreted/secreted antigen fraction, freshly egressed merozoites were incubated in medium, and the supernatants of these incubations were examined by Western blot for the presence of the protein. As shown in Fig. 5, SnMIC10 was present in the supernatant, consistent with the active secretion of the protein by extracellular parasites. Secretion was dependent on temperature (Fig. 5B), with increased amounts of SnMIC10 detected in supernatants from parasites incubated at 37°C. To determine the quantity of SnMIC10 present in the supernatants, the digital Western blot images captured with the CCD camera-equipped FluorChem 8800 were analysed by spot densitometry using AlphaEase FC processing and analysis software. These analyses indicated that approximately 15–18% of the total cellular SnMIC10 was present in the supernatants (Figs. 5A,B). The secreted SnMIC10 co-migrated in SDS-PAGE with the non-secreted form in lysed merozoites, thus indicating that SnMIC10 did not undergo proteolytic processing during secretion. To monitor for cell lysis, the quantity of surface antigen SnSAG4 or parasite actin in the supernatants was similarly determined. Approximately 6% of the total SnSAG4 (Fig. 5A) or 9% of total parasite actin (Fig. 5B) was detected in the supernatants, demonstrating that some inadvertent cell lysis occurred during the incubation. However, the amount of cell lysis was insufficient to account for the quantity of SnMIC10 present in the supernatants.
3.4. SnMIC10 expression during intracellular growth

To assess whether SnMIC10 is constitutively expressed during endopolygeny and to determine the location of the protein during parasite propagation, immunofluorescent labelling of SnMIC10 was performed on intracellular parasites. This analysis revealed SnMIC10 label at one pole of intracellular parasites at 16 h post-inoculation (Fig. 6A). At approximately 4 days post-inoculation when multiple different developmental stages were present, early- to mid-stage schizonts showed minimal SnMIC10 labelling with only a small, condensed spot observed on one or both poles (Fig. 6B). An increase in SnMIC10 staining was seen in the mid- to late-stage schizonts, with the label dispersed in a punctate pattern throughout the body of the schizont (Fig. 6C). In the final stages of schizont maturation when cytokinesis occurred, the SnMIC10 label became organised to the anterior pole of each individual merozoite (Fig. 6D).

4. Discussion

Micronemes are secretory organelles of the Apicomplexa that are important for attachment and invasion of host cells by these intracellular parasites (Dubremetz et al., 1998). Since the process of host cell invasion appears to be fairly conserved, members of the Apicomplexa are expected to share numerous microneme protein homologues. However, the various species of Apicomplexa occupy a broad range of parasitological niches (e.g. hosts and cell types), and this undoubtedly has necessitated some divergence in the microneme contents of the different members of this phylum. Although there is significant information regarding the composition of micronemes in several members of the Apicomplexa (e.g. T. gondii), only two microneme proteins have been identified thus far in the genus Sarcocystis (Tomley and Soldati, 2001). In the present study, we have identified and characterised a microneme protein of S. neurona merozoites, which has been designated SnMIC10.

Identification of the SnMIC10 gene was accomplished by searching the S. neurona EST database with the sequence of TgMIC10 from T. gondii. The SnMIC10 protein shares only about 30% sequence identity with TgMIC10 and the MIC10 homologue from N. caninum, NcMIC10. However, the general protein characteristics of the MIC10 orthologues are predicted to be similar, suggesting that this protein has a relatively conserved function in all three parasite species.

The homology of SnMIC10 with TgMIC10 and NcMIC10 suggested that SnMIC10 is a microneme protein of S. neurona merozoites, which was confirmed by immunofluorescence and immuno-EM analyses. As expected for a microneme protein, SnMIC10 was found to be part of the S. neurona excretory/secretory antigen fraction, as seen previously for the microneme contents of other Apicomplexa (Carruthers et al., 1999; Brydges et al., 2000; Bumstead and Tomley, 2000). Similar to what was observed for TgMIC10 (Hoff et al., 2001), the secreted SnMIC10 co-migrated in Western blots with SnMIC10 from whole merozoite lysate, indicating that the protein is not processed during secretion. This contrasts with numerous other microneme proteins, which have been shown to undergo proteolytic processing upon release from the parasite (Achbarou et al., 1991; Brydges et al., 2000; Lovett et al., 2000; Brecht et al., 2001). Similar to what has been seen for microneme proteins of other Apicomplexa (Bumstead and Tomley, 2000; Lovett et al., 2000; Carruthers et al., 1999; Brydges et al., 2000), the secretion of SnMIC10 was shown to be temperature sensitive, with a marked increased in SnMIC10 release observed at 37°C when compared to both 0 and 25°C.

Microsequencing of TgMIC10 demonstrated that Asp59 was the amino-terminal residue of the mature protein, thus indicating that the signal peptide is 19 amino acids longer than predicted since this protein does not undergo post-translational processing (Hoff et al., 2001). The sequence alignment of SnMIC10 with TgMIC10 and NcMIC10
revealed that a 68-residue amino-terminal extension exists on the *S. neurona* orthologue. The amino-terminus of mature SnMIC10 was not verified by microsequencing, so we cannot rule out post-translational processing of SnMIC10 that would yield a protein equal in length to TgMIC10 and NcMIC10. However, migration of SnMIC10 in SDS-PAGE was consistent with the expected Mr for the described primary sequence, suggesting that the 68-amino acid extension is part of the mature protein. It is unknown whether there is any functional significance to the amino-terminal extension.

*Sarcocystis neurona* propagates by endopolygeny, a developmental process similar to schizogony in which a single parasite gives rise to multiple daughter merozoites. During endopolygeny and schizogony, DNA synthesis is dissociated from cell division, thereby producing a large polyploidy cell. In the final stages of development, cytokinesis occurs to form the individual merozoites. In the final stages of development, cytokinesis occurs to form the individual merozoites. Immunofluorescent analysis of intracellular *S. neurona* revealed that expression of SnMIC10 is temporally regulated during endopolygeny. The SnMIC10 label was maintained at one pole of intracellular parasites for up to 16 h post infection. As the parasites progressed through endopolygeny, the SnMIC10 label decreased, thus suggesting that SnMIC10 and possibly other microneme proteins are not expressed during this phase of the cell cycle. Interestingly, a small spot of labelling was always seen on one or both poles of the schizont, and this most likely represents the remnant micronemes from the primary merozoite that invaded the host cell. As the schizonts matured, SnMIC10 production was apparently up-regulated, and the protein label appeared in a dispersed, punctate pattern throughout the body of the developing schizont. These spots are presumably the nascent micronemes of the newly-forming daughter cells. At the completion of cytokinesis, the SnMIC10 label had become located at the apical end of each merozoite. The down-regulation of SnMIC10 observed during endopolygeny is concordant with the loss of micronemes seen by ultrastructure analysis of *Sarcocystis* spp. schizonts (Dubey et al., 1989), and it is predictable for proteins that are not needed during the prolonged and complex intracellular development of these...
parasites. Somewhat similar to what was observed for SnMIC10, the microneme protein EtMIC2 of *Eimeria tenella* can be weakly detected in the cytoplasm of immature schizonts, but staining becomes more intense and concentrated to the apical end of the newly-formed merozoites as the schizonts mature (Tomley et al., 1996). In contrast to the aforementioned organisms that undergo endopolygeny/schizogony, the microneme proteins MIC1, MIC2, and MIC3 are present throughout intracellular development of *T. gondii* (Achbarou et al., 1991), which is consistent with the continued existence of micronemes in the developing daughter cells during endodyogeny (Hu et al., 2002).

The contents of the micronemes are known to be heterogeneous, and each of the assorted microneme proteins likely serves a different function to help accomplish host cell invasion. A number of microneme proteins have been shown to possess various adhesive domains, including integrin I domains, apple domains, EGF-like domains, and thrombospondin type I regions (Tomley and Soldati, 2001). These domains likely provide the parasites with an array of host cell-attachment capabilities. The absence of adhesive domains in the MIC10 orthologues and the lack of host cell binding by TgMIC10 (Hoff et al., 2001) suggest that this microneme protein is not involved in a receptor-ligand interaction with a host cell surface molecule during the invasion process. Unfortunately, the primary sequences of the three MIC10 orthologues provide little indication of this protein’s function, so the specific role served by MIC10 during infection remains unknown. However, it can be assumed that regions of sequence conservation are likely important for a function(s) that is shared by all three MIC10 orthologues. The nine diglutamic acid repeats and the RK(R/Y)HEEL repeat sequences seen in TgMIC10 are not well conserved in the *S. neurona* homologue nor are they prevalent in the NcMIC10 orthologue (Hoff et al., 2001). Consequently, these repeat elements may be necessary only for a task that is specific to TgMIC10. Both *T. gondii* and *N. caninum* are amenable to molecular genetic manipulations, and the generation of MIC10 deficient mutants may provide some insight into how this protein serves as a virulence factor for this group of important pathogens.

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Plastid segregation and cell division in the apicomplexan parasite *Sarcocystis neurona*

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**Summary**

Apicomplexan parasites harbor a secondary plastid that is essential to their survival. Several metabolic pathways confined to this organelle have emerged as promising parasite-specific drug targets. The maintenance of the organelle and its genome is an equally valuable target. We have studied the replication and segregation of this important organelle using the parasite *Sarcocystis neurona* as a cell biological model. This model system makes it possible to differentiate and dissect organelar growth, fission and segregation over time, because of the parasite’s peculiar mode of cell division. *S. neurona* undergoes five cycles of chromosomal replication without nuclear division, thus yielding a cell with a 32N nucleus. This nucleus undergoes a sixth replication cycle concurrent with nuclear division and cell budding to give rise to 64 haploid daughter cells. Interestingly, intranuclear spindles persist throughout the cell cycle, thereby providing a potential mechanism to organize chromosomes and organelles in an organism that undergoes dramatic changes in ploidy. The development of the plastid mirrors that of the nucleus, a continuous organelle, which grows throughout the parasite’s development and shows association with all centrosomes. Pharmacological ablation of the parasite’s multiple spindles demonstrates their essential role in the organization and faithful segregation of the plastid. By using several molecular markers we have timed organelle fission to the last replication cycle and tied it to daughter cell budding. Finally, plastids were labeled by fluorescent protein expression using a newly developed *S. neurona* transfection system. With these transgenic parasites we have tested our model in living cells employing laser bleaching experiments.

**Introduction**

The phylum Apicomplexa represents a large and diverse group of protozoan parasites. Among these are the human pathogens that cause malaria, AIDS-related encephalitis (*Toxoplasma*) and severe enteritis (*Cryptosporidium* and *Cyclospora*). The phylum also contains many parasites of substantial veterinary importance such as *Theileria*, *Babesia*, *Eimeria* and *Sarcocystis*. Aside from their obvious medical and economic importance protozoan parasites have long fascinated cell biologists as model organisms. Recent cell biological work on Apicomplexa has been focused on the cellular structures and molecules involved in their ability to invade mammalian cells, their peculiar cell cycle and mechanisms of cell division, and the presence of several unique organelles. One of these organelles, which has received particular attention, is the apicomplexan plastid or apicoplast.

Given that the cells of the mammalian host lack plastids, the discovery of the apicoplast has provided an entire organelle of potential parasite-specific drug targets. A number of these targets have been validated including several metabolic pathways confined to the plastid, as well as the maintenance of the organelle and its genome (McFadden and Roos, 1999; Ralph et al., 2004). Our interest is focused on apicoplast division and segregation during parasite development. Plastid division is best understood in chloroplasts. Interestingly, chloroplasts still use many elements of their ancestral (cyanobacterial) bacterial division machinery. All plant and algal genomes studied so far harbor two gene families homologous to the bacterial *FtsZ* gene (Miyagishima et al., 2004; Osteryoung et al., 1998; Stokes and Osteryoung, 2003). *FtsZ* is a prokaryotic homolog of tubulin and the main structural molecule in the bacterial division ring (Lowe and Amos, 1998; Lutkenhaus and Addinall, 1997). Plant *FtsZ* is targeted to the lumen of the chloroplast where it forms rings at the site of constriction, very much like its bacterial homolog (Kuroiwa et al., 2002; McAndrew et al., 2001; Vitha et al., 2001). Assembly and positioning of this ring is controlled and aided by the homologs of the bacterial cell division proteins MinD and MinE (Colletti et al., 2000; Dinkins et al., 2001; Itoh et al., 2001). In addition to these proteins found in all bacteria, higher plant chloroplasts also employ homologs of Ftn2 and Artemis, which are essential...
cell division proteins that are phylogenetically restricted to cyanobacteria (Fulgosi et al., 2002; Vitha et al., 2003). Recently, an eukaryotic element of the division machinery has been identified, a dynamin-like protein that forms a ring on the cytoplasmatic side and is required for proper organellar fission (Gao et al., 2003; Miyagishima et al., 2003). Surprisingly, our extensive comparative genomic analysis of the completed or nearly completed genomes of several plastid-harbor ing apicomplexan parasites (P. falciparum, P. yoelii, T. gondii, Eimeria tenella and Theileria annulata) did not identify a single homolog for any member of this conserved machinery. This is despite the fact that several proteins such as FtsZ are highly conserved and readily identified by BLAST search in the genomes of a diverse set of plants and algae (V.S. and B.S., unpublished data).

How is plastid division accomplished in apicomplexan parasites in the absence of the conserved machinery? Previous work on Toxoplasma has shown these organisms to employ a genuinely eukaryotic mechanism. Specifically, the dividing plastid associates with the centrosomes of the mitotic spindle (Striepen et al., 2000). As a result of this association the ends of the plastid are inserted into the budding daughter cells and segregated. Based on the observation that all Apicomplexa have lost the bacterial machinery, we hypothesized that these apicomplexan parasites have a plastid division mechanism that is genuinely eukaryotic. Our work on Toxoplasma has shown these organisms to employ a genuinely eukaryotic mechanism. Specifically, the dividing plastid associates with the centrosomes of the mitotic spindle (Striepen et al., 2000). As a result of this association the ends of the plastid are inserted into the budding daughter cells and segregated. Based on the observation that all Apicomplexa have lost the bacterial machinery, we hypothesized that these apicomplexan parasites have a plastid division mechanism that is genuinely eukaryotic.

Materials and Methods

Host cells and parasites

Sarcocystis neurona strain SN3 was propagated in primary bovine tubinate (BT) cells. BT cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, HyClone) supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 2 mM L-glutamine, 5 U/ml penicillin, 10 µg/ml streptomycin, and 1 ml/l fungizone (Invitrogen). A stable transgenic Toxoplasma gondii strain expressing the reporter ferredoxin NADH reductase-red fluorescent protein (FNR-RFP) was established and cultivated in human foreskin fibroblast (HFF) cells as previously described (Striepen et al., 2000).

S. neurona transfection

Plasmids for S. neurona transfection experiments were initially derived from the T. gondii expression vector pTB1YFP-YFP/SagCAT (Gubbels et al., 2003; Striepen et al., 2000) by replacement of the Toxoplasma tubulin promoter and the selectable marker cassette with the S′ flanking region of the S. neurona SAG1 locus (Howe et al., 2004; R.Y.G., V.S., B.S. and D.K.H., unpublished data). To engineer the plastid reporter construct pSnsagFNR-RFP used in this study, the coding region of FNR-RFP along with the 3′ UTR derived from the T. gondii DHFR-TS gene was excised from plasmid pTB1FNR-RFP (Striepen et al., 2000) with NotI and BglII and ligated into pSnsagYFP-YFP digested with NotI and BglII, placing the transgene under the control of the S. neurona promoter. The coding sequence of the T. gondii acyl carrier protein was excised for plasmid pTBACPGFPsagCAT (Waller et al., 1998) using BglII and AvrII and was ligated into pSnsagYFP-YFP equally restricted to replace the first YFP cassette to yield pSnsagACP-YFP.

Fluorescence microscopy

For immunofluorescence, filter-purified S. neurona merozoites were purified by filtration through 3 µm polycarbonate filters and resuspended in cytomi x (120 mM KC1, 0.15 mM CaCl2, 10 mM K2HPO4/KH2PO4, pH 7.6, 25 mM Hepes pH 7.6, 2 mM EDTA, 5 mM MgCl2, 2 mM ATP, 5 mM glutathione) to a density of 3x107 parasites per ml. 50 µg plasmid DNA were ethanol precipitated, resuspended in 100 µl cytomi x, mixed with 300 µl parasites in a 2 mm electroporation cuvette, and exposed to a 1800 V, 25 Ω and 25 µF pulse generated by a BTX ECM 630 apparatus (Genetronix).

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times post-infection for 10 minutes using 4% paraformaldehyde in PBS, followed by permeabilization with 0.25% (w/v) Triton X-100 in PBS. The following primary antibodies were used at the indicated dilutions: affinity purified polyclonal rabbit antiserum against T. gondii acyl carrier protein [ACP; 1:400; kindly provided by G. I. McFadden, University of Melbourne (Waller et al., 1998)]; monoclonal rabbit 12G10 raised against α-tubulin [1:25; kindly provided by J. Frankel, University of Iowa (Jerka-Dziadosz and Frankel, 1995; Jerka-Dziadosz et al., 1995)]; monoclonal mouse and polyclonal rabbit anti-centrin [1:100; kindly provided by J. L. Salisbury, Mayo Clinic (Pauletti et al., 1996)]; rat polyclonal anti-SnMic10 (Hoane et al., 2003) and rabbit polyclonal anti-IMC3 (Gubbels et al., 2004). All antibodies were diluted in PBS plus 1% BSA and detected using Alexa Fluor 488- or Alexa Fluor 546-conjugated goat anti-mouse, anti-rabbit or anti-rat antibodies (Molecular Probes). DNA was stained by incubation with 2 μg/ml 4′,6′-diamino-2-phenylindole (DAPI; Molecular Probes) for 10 minutes, excess dye was removed by washing, and coverslips were mounted with Gel/Mount (Biomeda Corporation). Fluorescence images were captured with a CCD camera (C4742-95; Hamamatsu) on a Leica DMIRBE microscope. Image acquisition, contrast adjustment and channel merging were performed using openlab software (Improvision).

To estimate the DNA content of individual schizont nuclei, the intensity of DAPI staining was measured in situ by image analysis. Fixed cultures were stained for tubulin and DNA as described above. Images were collected in the red and DAPI channel with constant exposure time and within the linear range of the CCD camera (the contrast was not adjusted). Nuclei were defined as objects by generating binary image masks for each image using a thresholding function. The mean (background-subtracted) pixel intensity was multiplied by the area of each nucleus to obtain a cumulative intensity measurement in arbitrary units. Host cell nuclei were measured as internal controls and provided results consistent with a confluent monolayer mostly arrested in G1 (the bulk of the measurements clustered around a mean of 7.5 × 10⁶ units Std 1.2 × 10⁶, n=20), and two cells with 12 and 16.5 × 10⁶ units, probably representing G2 and S phase, respectively.

Laser bleaching experiments
For deconvolution microscopy and laser bleaching experiments confluent BT and HFF coverslip cultures were infected with transgenic FNR-RFP-expressing S. neurona and T. gondii, respectively. Cultures were observed on a Delta Vision Spectris deconvolution workstation (Applied Precision) with an Olympus 60× NA 1.2 water immersion lens 48 hours after transfection. Coverslips were mounted in medium (Secure-Seal, Molecular Probes) and maintained at 37°C during the observation. A 25 mW argon laser was used to bleach the sample, by exposing it to five 488 nm pulses for 300 mseconds each at 1.5 mW laser power. The size of the laser spot was nominally diffraction limited. Stacks of optical sections were taken before and after the bleach with z-increments of 0.3 μm and xy spacing of 0.1 μm. The pre- and the post-bleach images were set to identical contrast setting using the image-scaling function in the Softworx software (Applied Precision). Total projection images of sequential z-stacks were rendered using Velocity software (Improvision).

Drug treatment
The dinitroaniline herbicide oryzalin was obtained from Riedel-deHaen (Seelze, Germany), and a 10 mM stock solution was prepared.
in DMSO. Confluent BT cell coverslip cultures were infected with *S. neurona*, allowed to develop for 36 hours, and then treated with 2.5 μg/ml oryzalin for either 24 hours or 48 hours prior to processing for immunofluorescence analysis.

**Electron and immunoelectron microscopy**

BT cells were grown to confluency in polystyrene Petri dishes and infected with *S. neurona* merozoites. Cells were fixed in situ, 72 hours post infection, with 1% glutaraldehyde and 1% OsO₄ in 50 mM sodium phosphate buffer pH 7.2 for 1 hour on ice, followed by overnight staining with 0.5% aqueous uranyl acetate. Samples were dehydrated using a progressive ethanol series, ethanol was gradually exchanged for resin and samples were embedded in EPON (Electron Microscopy Sciences) directly in the dish. Blocks of polymer were cut from the dishes and ultrathin sections were taken parallel to the monolayer using a diamond knife. Sections were placed on Formvar-coated grids, stained with 4% uranyl acetate and 4% lead citrate and examined using a JEO JEM-100 CX II microscope.

For immunoelectron microscopy, infected cells were fixed with 2% paraformaldehyde and 0.1% glutaraldehyde in 0.5× PBS for 20-30 minutes on ice. Fixed cells were washed, scraped and centrifuged. The pellet was progressively dehydrated with ethanol and gradually infiltrated with LR-White on ice. Finally the sample was transferred to a gelatin capsule and polymerized at 50°C. Ultrathin sections cut from trimmed blocks and recovered on Formvar-coated grids were placed on 50 μl drops of the polyclonal anti-ACP antibody (1:400 in PBS 2% BSA) followed by 1:50 goat anti-rabbit IgG conjugated with 10 nm gold (BBinternational). After immunolabeling, sections were stained and analyzed as described above.

**Results**

**Successive rounds of synchronous mitosis give rise to a polyploid parasite**

*Sarcocystis* is an obligate intracellular parasite. After invasion of the host cell, the parasite develops over 2-3 days during which the nucleus seems to grow continuously until it fragments into multiple nuclei, which are packaged into daughter cells (Speer and Dubey, 1999; Speer and Dubey, 2001). We have followed these events by fluorescence microscopy using *S. neurona*-infected tissue cultures as a model. The schizonts are lemon shaped and lie directly within the host cell cytoplasm. Their central large nuclei were intensely stained by the DNA intercalating dye DAPI (Fig. 1). Using a monoclonal antibody raised against α-tubulin from *Tetrahymena thermophila* (Jerka-Dziadosz and Frankel, 1995), we observed only moderate and diffuse staining in the parasite cytoplasm. This is in agreement with the previous reports that schizonts lack subpellicular microtubules, which are characteristic of the invasive stages of Apicomplexa (Morrissette and Sibley, 2002a). Within the nucleus however, two consistent and mutually exclusive staining patterns were observed: multiple intranuclear bundles of microtubules, or intensely stained dots right under the nuclear envelope (Fig. 1A,D). The microtubule bundles seemed to emanate from points close to the surface of the nucleus and coincided with apparent condensation of the DNA staining. This suggests that they represent mitotic spindles, segregating chromosomes (Speer and Dubey, 1999). To confirm this finding, infected cultures were double labeled with the anti-tubulin antibody and an anti-centrin serum. Centrin is a cytoplasmatic calcium binding protein that is concentrated in centrosomes surrounding the centrioles (Salisbury, 1995). This serum has previously been shown to label the centrosomes in the related parasite *T. gondii* (Striepen et al., 2000). As shown in Fig. 1C-F, the poles of these spindles clearly coincided with the centrin label. Note that the multiple centrosomes are linked by individual microtubules that span across the nucleus.

**Short intranuclear spindles are maintained throughout the cell cycle**

The staining patterns observed for the mitotic spindles were often too complex to count their numbers unambiguously. The dots in the second staining pattern however, could be easily counted and their number increased in geometric progression (2, 4, 8, up to 64; Fig. 2A-F; note that the dot often consists of two very close and poorly resolved structures). The increase in

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**Fig. 2.** *S. neurona* forges nuclear division and cytokinesis for five cell cycles prior to the budding of 64 daughter cells. Cell cultures were infected with *S. neurona* merozoites and fixed and processed for immunofluorescence 24-72 hours after infection. Cells were incubated with a monoclonal antibody against α-tubulin (red) and DAPI to stain DNA (blue). Developing schizonts present two mutually exclusive staining patterns: multiple spindles throughout the nucleus coinciding with DNA condensation or dots in proximity to the nuclear envelope (see Fig. 1). The number of these dots increased with the size of the schizont in a geometric progression (2-64, A-F). (G) Plot of the intensity of DNA staining of each nucleus (in arbitrary units, as shown for a parasite and host cell nucleus in inset) against the number of tubulin dots per nucleus (100 random fields were analyzed, individual measurements are shown as red dots, black lines and error bars indicate the mean and standard deviation, respectively). Progression from 32 to 64 (E,F) produces 64 daughter nuclei. Note that the dots often appeared as doublets (D).
the number of these structures seemed to be directly correlated with the size of the nucleus. To quantify this relationship, the DNA content of individual nuclei was measured by image analysis. Schizont nuclei were defined using a binary mask (see Material and Methods for detail) and the cumulative intensity of their DAPI label was recorded for each mask. Fig. 2G shows such measurements for nuclei analyzed from 100 fields plotted against the number of tubulin dots recorded for each respective nucleus. The DNA content of schizont nuclei is clearly correlated with the number of dots and as might be expected doubles synchronously with the former indicating that these dots might be associated with or represent minute spindles. Upon double labeling with centrin antiserum, each red tubulin dot was flanked by two green centrosomes (Fig. 1D-F, see inset in F). To further investigate the nature of these structures, infected cultures were fixed and embedded for electron microscopy. Fig. 1G shows a section through a young schizont (the large central nucleolus and the homogenous chromatin structure suggests that this cell is in interphase). Consistent with immunofluorescence images, the electron micrographs demonstrate short baskets of microtubules within the nucleus (Fig. 2H,I, black arrowheads). These microtubules emanate from centrosomes (white arrowheads with black outline), which lie outside of the nuclear envelope in the cytoplasm. As many as 32 of these short spindles were observed associated with a single polyploid nucleus. In even later developmental stages, the nucleus is segregated into 64 daughter nuclei following the last chromosomal duplication event (Fig. 2F). Our analysis concludes that all stages in the developing schizont present either typical spindles (associated with mitosis and DNA condensation), or short interphase spindles. We failed to observe stages devoid of microtubular structures in the nucleus.

The plastid is maintained as a single organelle throughout the intracellular development

To identify the plastid in \textit{S. neurona} and to follow its fate through development, parasites were analyzed by immunofluorescence using a polyclonal antibody raised against \textit{T. gondii} acyl carrier protein (ACP). ACP is a nuclear-encoded protein that is posttranslationally targeted to the lumen. ACP has been previously used as a plastid marker against \textit{T. gondii} immunofluorescence using a polyclonal antibody raised against the \textit{T. gondii} plastid protein ACP (A) and DAPI (B). A single round organelle was detected close to the nucleus colocalizing with the extranuclear plastid DNA (arrowhead). \textit{S. neurona}-infected cultures were fixed 48 hours post-infection (E-H), and simultaneously incubated with antibodies against ACP (green, E), α-tubulin (red, F) and DAPI (G). The plastid in developing schizonts appeared to be a single tubule wrapped around the nucleus (H). (I-L) Transfection plasmids were constructed that place the genes of two plastid targeted proteins from \textit{T. gondii} (FNR and ACP, fused to RFP (red) or YFP (green), respectively) under control of a \textit{S. neurona} promoter element. Plasmids were introduced into \textit{S. neurona} merozoites by electroporation prior to infection. Transformed cultures were observed by fluorescence microscopy in living cells 36 hours after transfection.

\textbf{Fig. 3.} The plastid in \textit{S. neurona} schizonts is a single tubular structure in close association with the nucleus and its spindles. Extracellular \textit{S. neurona} merozoites (A-D) were fixed and incubated with an affinity-purified rabbit antiserum raised against the \textit{T. gondii} plastid protein ACP (A) and DAPI (B). A single round organelle was detected close to the nucleus colocalizing with the extranuclear plastid DNA (arrowhead). \textit{S. neurona}-infected cultures were fixed 48 hours post-infection (E-H), and simultaneously incubated with antibodies against ACP (green, E), α-tubulin (red, F) and DAPI (G). The plastid in developing schizonts appeared to be a single tubule wrapped around the nucleus (H). (I-L) Transfection plasmids were constructed that place the genes of two plastid targeted proteins from \textit{T. gondii} (FNR and ACP, fused to RFP (red) or YFP (green), respectively) under control of a \textit{S. neurona} promoter element. Plasmids were introduced into \textit{S. neurona} merozoites by electroporation prior to infection. Transformed cultures were observed by fluorescence microscopy in living cells 36 hours after transfection.

\textbf{In vivo laser bleaching studies confirm a continuous plastid}

The presence of a large continuous plastid is highly interesting as it mirrors the development of the nucleus in \textit{S. neurona} and could lend additional support to the model that nucleus and plastid are organized and segregated by the same molecular machinery. Whether the plastid is indeed continuous could not be concluded with certainty based on immunofluorescence analysis, since some samples seemed to show small breaks in the plastid tubule depending on fixation conditions. To avoid fixation artifacts, we developed a transfection system for \textit{S. neurona} and no differences were observed between parasites undergoing mitosis and those in interphase (data not shown).
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*neurona* to establish fluorescent-protein-expressing parasites for in vivo analysis (R.Y.G., V.S., B.S. and D.K.H., unpublished). Transfection vectors were constructed with the promoter region of the *S. neurona* SAG1 gene, driving the expression of a reporter gene. The gene for two well-characterized *T. gondii* plastid-targeted proteins (ACP and FNR) were translationally fused to YFP or RFP, respectively. Parasites transfected with these plasmids by electroporation were used to infect coverslip cultures. Images were taken 24-36 hours after transfection without fixation. Transfection with both of these independent reporters resulted in fluorescent labeling of a tubular organelle in close association with the nucleus (Fig. 3I-L).

*S. neurona* plastids, imaged in vivo, appeared continuous and showed no obvious breaks (see three-dimensional reconstruction derived from deconvolved serial optical z sections in Fig. 4E and Movie 1 in supplementary material). To directly test for organellar continuity, laser bleaching experiments were performed. Given the high rates of diffusion over the small cellular distances, exposure of the distal end of the organelle to a series of laser pulses should bleach fluorescence in the entire organelle. If the organelle is discontinuous, bleaching should remain partial (see Fig. 4A,B for a schematic outline of the experiment). A series of short exposures to the 488 nm laser line at modest power settings was applied to one end of a plastid labeled in vivo with FNR-RFP (a soluble luminal marker). Prior to and after bleaching, z-stacks of fluorescent images were collected. Sustained bleaching of a small portion of the *S. neurona* plastid resulted in bleaching of the entire organelle consistent with free marker diffusion through the organelle (Fig. 4C-H; E and H are total projections of the z-stacks, a rendered version of this data is available as a movie: see Movie 1 in supplementary material). To ensure that the imaging process itself did not cause excessive bleaching and that the laser provides sufficient spatial precision, *T. gondii* was used as a well-suited control. A *T. gondii* plastid labeled with FNR-RFP was bleached in a vacuole containing multiple tachyzoites. Bleaching was restricted to the single organelle targeted (Fig. 4I-N). Recovery of the fluorescent signal was observed in both *S. neurona* and *T. gondii* plastids over a period of 1 hour (data not shown). Signal recovery probably occurred by import and is consistent with the kinetics of apicoplast targeting in *P. falciparum* (van Dooren et al., 2002) and *T. gondii* (Derocher et al., 2005).

Centrosomes and spindles are essential for plastid organization and segregation.

In the related parasite *T. gondii*, plastids are associated with the centrosomes during cell division (Striepen et al., 2000). To establish if and when such association occurs in the single tubular plastid in *S. neurona*, infected cultures were triple-labeled with anti-ACP (plastid, green), anti-α-tubulin (spindle, red) and DAPI (DNA, blue). The plastid was found to be in close contact with the nucleus throughout its development. The area of contact between nucleus and plastid coincided with the poles of spindles observed during mitosis and short spindles maintained through interphase (Fig. 3). Double labeling with antibodies against ACP and centrin

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**Fig. 4.** In vivo laser bleaching experiments show that the *S. neurona* plastid is a continuous organelle. In vivo laser bleaching experiments were performed to test if a fluorescent marker freely diffuses along the entire length of the tubular plastid. The extent of bleaching predicted for organelles with continuous (A) and discontinuous (B) lumen is depicted schematically. FNR-RFP-expressing *S. neurona* (C-H) and *T. gondii* (I-K) parasites were imaged in vivo. Plastids were exposed to a series of short laser pulses at the position indicated by the laser symbol (see Materials and Methods for detail). Cells were imaged before (pre) and after (post) bleaching. (C,F,I,L) Single fluorescence images at the focal plane; (D,G,J,M) merged images of C,F,I and L with the respective DIC image; (E,H,K,N) rendered 3D projection of the entire z-stack. (A quicktime movie of these data is available in supplementary material.) Upon exposing the distal end of the *S. neurona* plastid to multiple laser pulses the entire organelle is bleached (F-H). (I-K) Experiments to control for the spatial precision of the laser were performed with FNR-RFP expressing *T. gondii* parasites. (L-N) A single plastid was exposed to the laser resulting in bleaching of only the targeted organelle (white arrowhead) without affecting its nearest neighbors.
revealed an equally tight association between plastid and centrosomes (Fig. 5A, arrowheads indicate individual centrosomes). To validate this association at higher magnification, *S. neurona*-infected cells were fixed and embedded directly in the tissue culture dish. Blocks were cut and trimmed to preserve the original orientation and sections were then taken parallel to the cell monolayer. Fig. 5B-D shows electron microscope images of serial sections taken through a developing *S. neurona* schizont. A tubular multi-membranous organelle was observed that wrapped around the upper right portion of the central nucleus. At higher magnifications, the four distinct membranes surrounding the plastid were apparent (Fig. 5D). Four centrioles can be identified in Fig. 5C (white arrowheads) that lie in intimate proximity to the plastid [also see Speer and Dubey (Speer and Dubey, 1999)]. To confirm the identification of the organelle as a plastid, *S. neurona*-infected cultures were also fixed and embedded for immunogold labeling. Again a tubular organelle was observed in close proximity to the nucleus. This organelle shows strong and specific labeling for the plastid protein ACP (Fig. 5E,F).

**Pharmacological ablation of spindles disrupts plastid organization**

If spindles play an important role in plastid organization their disruption should affect plastid morphology. To test this, parasite microtubules were disrupted by treatment with oryzalin. Oryzalin is a dinitroaniline that has been shown to affect both subpellicular and spindle microtubules in Apicomplexa (Morrisette and Sibley, 2002a; Morrisette and Sibley, 2002b). Cultures infected with *S. neurona* were allowed to develop for 36 hours and then treated with 2.5 µg/ml...
oryzalin for 24 hours prior to processing for immunofluorescence. A profound loss of plastid organization was observed (Fig. 6C,D). In treated cells, the organelle coiled to the middle of the cell. Even though the organization of the plastid around the nucleus was lost in the drug-treated parasites (compare to untreated control in Fig. 6A,B), centrosome association was maintained. Upon double labeling of drug-treated parasites with anti-centrin and anti-ACP antibody multiple centrosomes were observed associated with the plastid (white arrowheads indicate individual centrosomes in Fig. 6E).

Plastid fission concurs with the last mitosis and daughter cell budding
To endow each daughter cell with a plastid the tubular plastid has to undergo fission. In order to time this event precisely, a molecular marker was used. SnMIC10 is a secretory protein targeted to the micronemes, which probably plays a role in host cell invasion. Importantly, its expression shows tight developmental regulation (Hoane et al., 2003). Fig. 7A shows three consecutive developmental stages of *S. neurona*: a developing schizont (1), a late schizont during budding (2) and a terminal stage schizont showing completed daughter cell formation (3). Expression of SnMic10 is undetectable in the developing schizont (Fig. 5B), but initiates towards the end of the last replication cycle with staining first seen as a dot at mid-cell most probably representing the Golgi or a premicroneme compartment. After completion of daughter cell formation, SnMic10 shows a typical micronemal pattern underlying the apical surface of the merozoite. Plastids were always tubular in the absence of SnMIC10 staining (Fig. 7C,D1), and fission concurred with the beginning of Mic10 expression. This concurrence of plastid fission with the last cell cycle was also evident by triple labeling for plastid, microtubules and DNA (Fig. 7E-H).

To investigate the role of spindles in plastid fission, infected cultures were allowed to develop for 36 hours and then treated with oryzalin for 48 hours prior to processing for immunofluorescence. Plastid fission was evident in the absence of spindles in treated cultures (Fig. 6G). However, these plastids were of unequal size and had lost the nuclear association (Fig. 6H) observed in untreated parasites. We were interested to learn if the observed fission could be a consequence of daughter cell formation in the absence of spindles. For this purpose, infected cultures were treated with oryzalin as described above and labeled with an antibody to *T. gondii* IMC3. IMC3 is a component of the inner membrane complex underlying the parasites plasma membrane, and antibodies to this complex reveal newly forming daughter cells (Fig. 6J) (Gubbels et al., 2004). We observed that drug treatment did not prevent the formation of daughter cells (Fig. 6K,L), however, nuclear segregation and packaging was severely impaired, yielding daughter cells devoid of a nucleus.

Discussion
Plant chloroplasts divide by binary fission and several constrictive rings have been described (Kuroiwa et al., 2002; McAndrew et al., 2001; Vitha et al., 2001). Many of the proteins that make up these rings, and the elements that regulate ring position, are conserved between plastids and their cyanobacterial ancestor (Osteryoung and Nunnari, 2003). By contrast, our previous work in *T. gondii* has indicated that the replication of parasite secondary plastids occurs in association with the centrosomes of the mitotic spindle (Striepen et al.,...
The current study shows this to be a conserved mechanism among Coccidia and it might provide a model to understand plastid division in the malaria parasite *Plasmodium*. In *Sarcocystis*, we have found that there is tight association of the plastid with the mitotic spindle and its centrosomes using several molecular markers examined at the light and electron microscopic level. If the mechanism is conserved, why do plastids show such diverse morphology? An obvious difference between *T. gondii* and *S. neurona* are their divergent cell cycle models. In *T. gondii*, the segregation of chromosomes is immediately followed by nuclear division and cytokinesis, and parasites maintain a haploid genome (Radke et al., 2001; Sheffield and Melton, 1968). In *S. neurona*, however, the number of spindles and centrosomes that we observed in growing schizonts suggests that this organism completes six replication cycles prior to daughter cell formation (Fig. 2). The first five cycles involve a succession of chromosomal duplication and segregation accomplished by multiple synchronous spindles throughout the nucleus, but nuclear division and cytokinesis are postponed, resulting in a 32 N nucleus (see Fig. 8 for a schematic outline of this process). The sixth cycle concurs with nuclear segregation and cytokinesis. The surprising persistence of spindles throughout these six replication cycles (with a peculiar ‘mini spindle’ during interphase) could provide a mechanism organizing chromosomes and organelles in later highly polyploid stages.
Indeed, pharmacological ablation of spindles results not only in a disorganized nucleus unable to properly segregate during budding, but also a disorganized plastid (Fig. 6). Molecular probes for the identification of centromeres and kinetochores are needed to fully test this model for chromosomes.

It is important to note how closely the development of the plastid mirrors the nuclear events. In *T. gondii*, an ovoid organelle is divided and segregated at the same time as the nucleus in every replication cycle. By contrast, in *S. neurona* we observed a continuous tubular plastid that grows alongside the nucleus (Figs 3, 4 and 5). Organelle continuity was experimentally confirmed in vivo using transgenic parasites (Fig. 5). Plastid fission into 64 individual daughter plastids occurs synchronously with the final nuclear division and segregation thus indicating that plastid fission is timed and regulated in a cell cycle-dependent way. This observation is consistent with a model in which the growing daughter cell pellicle, in conjunction with spindle attachment, could provide the constriction needed for fission (Striepen et al., 2000). An alternative model suggests the action of constrictive rings, similar to those seen in bacteria and plant chloroplasts (Matsuzaki et al., 2001). We have seen no evidence for plastid division rings in *T. gondii* or *S. neurona*, and we have not detected in apicomplexan genomes any of the genes for the conserved proteins associated with these structures (S.V. and B.S., unpublished data). We cannot exclude the possibility that such rings exist and are missed because they are extremely short lived, but they would have to consist of unique molecular components lacking homology to the cyanobacterial division apparatus. If constrictive rings are involved in plastid fission in *S. neurona*, their formation must be controlled in a cell cycle-dependent way and they have to form at multiple defined points along the tubular organelle in a spindle pole dependent-manner.

Spindles are clearly critical for the spatial organization of the plastid during parasite development, but are they required for fission? Ablation of spindles through pharmacological treatment did not prevent fission but broke the coordination of plastid and nuclear division (Fig. 6). Importantly, this treatment leaves intact plastid-centrosome association as well as daughter cell budding. This suggests that these two processes are sufficient for plastid fission and packaging, but that spindles are essential to coordinate this process with nuclear division. This model is in agreement with the observation that spindle ablation in *T. gondii* can lead to the formation of anucleate daughter cells, which in some cases still carry a plastid (Morrissette and Sibley, 2002b). The observation of sustained centrosome association under oryzalin treatment also suggests that this association is not mediated by microtubular structures and mechanisms.

The clarity of the subcellular structure in *S. neurona* when viewed by light microscopy is exceptional among apicomplexan species. This clarity combined with the parasite’s peculiar cell cycle makes it a unique model for the cell biological analysis of cell division in Apicomplexa. Transfection technology, as demonstrated in this report, now opens the door to mechanistic studies through genetic manipulation and in vivo analysis.

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