THE FUNCTIONAL CHARACTERIZATION OF APICOMPLEXAN TYPE II FATTY ACID SYNTHESIS IN *TOXOPLASMA GONDII*

by

JOLLY MAZUMDAR

(Under the Direction of Boris Striepen)

ABSTRACT

Apicomplexan parasites cause important human diseases including malaria and AIDS associated opportunistic infections. Effectiveness of current drug treatments are challenged by side effects and wide spread resistance. The discovery of the apicoplast, an organelle derived from a prokaryote, and the metabolic pathways within, presents novel drug targets unique to the parasite. Apicoplast localized Type II fatty acid synthesis (FASII) is one such pathway. The remarkable divergence of apicoplast FASII from human FASI makes it a potential drug target. But the biological functions of this pathway are currently unknown. Moreover, some apicomplexans including *Toxoplasma gondii*, encodes an additional FAS I pathway. In the presence of potentially redundant mechanisms, the functional significance of apicoplast FASII remains elusive.

The research presented here focuses on the elucidation of apicoplast FASII functions in the apicomplexan, *T. gondii*. Using a novel two marker approach, we engineered a TgFASII mutant, by the conditional knock-out of acyl carrier protein (ACP), a central FASII component. FASII knock down significantly reduced the growth and viability of parasites in cultured cells.

FASII mutants formed smaller plaques, and were unable to establish disease in a mouse model, indicating an essential requirement of FASII for the growth and pathogenesis of *T. gondii*. Biochemical functions, extensively characterized by protein analysis, immunofluorescence assays, metabolic labeling and fluorescent transgene expression, indicate a role of FASII in the production of lipoic acid, an essential cofactor for the parasite's sole pyruvate dehydrogenase complex (PDH). We also show a role of FASII in maintenance of the apicoplast. FASII knock down produces drastic effects on apicoplast morphology, resulting in organelle loss. Consistent with previous reports suggesting robust scavenge of fatty acids from the host cell by *T.gondii*, loss of FASII did not affect bulk fatty acid biosynthesis.

In conclusion, we have generated a genetic model for the rigorous analysis of TgFASII functions. We show an essential requirement of apicoplast FASII for the maintenance of the apicoplast and enzymes within, including PDH. Most importantly, the critical nature of apicoplast FASII for the growth and pathogenesis of parasites validate this pathway as a viable drug target.

INDEX WORDS: Apicoplast; plastid; fatty acid synthesis (FAS); Type I and Type II FAS; conditional gene knock-out

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DEDICATION

To my parents. Thank you for your ever lasting love and support. Also to my grandparents (Dadu and Arektama) and to the loving memories of Dadu and Thamma.

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CHAPTER 1

INTRODUCTION

The phylum Apicomplexa constitutes more than 4500 species of obligate intracellular protozoan parasites (Levine, 1988) which includes clinically significant pathogens such as *Plasmodium falciparum* and *Toxoplasma gondii*. While *P. falciparum* is infamous for causing malaria that claims at least two million human lives annually (Gardiner et al., 2005), *T. gondii*, another apicomplexan is fast gaining attention as the leading cause of encephalitis in AIDS patients (Luft and Remington, 1992). A globally prevalent opportunistic pathogen, it severely affects congenitally infected infants and immunocompromised patients (Swisher et al., 1994). Significant challenges persist in the treatment of these parasitic infections. Multiple drug resistant strains of *P. falciparum* are being isolated at an increased frequency (Vogel, 2005), and pyrimethamine, the most common treatment for *T. gondii* is frequently associated with adverse side effects and poor patient tolerance (Katlama et al., 1996; Lisenfeld et al., 1999), creating an urgent need for novel drugs.

P. falciparum and *T. gondii*, like most other apicomplexans harbor an apicoplast, a relict chloroplast organelle of cyanobacterial origin (Cavalier-Smith, 1982). The apicoplast is essential for parasite survival (Fichera and Roos, 1997; He et al., 2001; McConkey et al., 1997) and preliminary genomic and biochemical evidence indicates a role of this organelle in the parasite's overall metabolism. At least three metabolic pathways, including the biosynthesis of isoprenoids (Jomaa et al., 1999), heme (Ralph et al., 2004; Varadharajan et al., 2002) and fatty acids (McLeod et al., 2001; Waller et al., 1998) have been identified within the apicoplast.

The apicoplast localized biosynthetic pathways are prokaryotic in nature and distinctly different from their host counterparts. This divergence of the apicoplast metabolic pathways and their sensitivity to inhibitors specific to prokaryotic enzymes, presents an ideal platform for therapeutic exploitation. One such pathway with immense therapeutic potential is the apicoplast localized Type II Fatty acid synthesis. Fatty acids are essential cellular requirements, and the Type II FAS pathway has been identified in both *T. gondii* and *P. falciparum* (Waller et al., 1998). Apicoplast FASII is highly divergent from human FASI and can be specifically targeted by certain prokaryotic FASII inhibitors resulting in the inhibition of parasite growth (McLeod et al., 2001; Surolia and Surolia, 2001; Waller et al., 2003; Zuther et al., 1999). However, the specificity of some of the inhibitors has been questioned (Jones et al., 2005; Paul et al., 2004), and biological validation of the therapeutic potential of this pathway is yet to be provided. The functions of apicoplast FASII are currently unknown. FASII lipids are speculated to play a role in essential functions, including the formation of parasitophorous vacuole (Fichera and Roos, 1997), and fulfillment of parasite fatty acid needs (Ralph, et al., 2004) however, experimental evidence of these hypothesis is still lacking.

This research is aimed at understanding the biological contributions of apicomplexan FASII pathway towards parasite survival. In the work presented here, we engineered a mutant FASII strain by the conditional knock-out of its central component acyl carrier protein (ACP) in the model apicomplexan, *T. gondii*. For this, we took advantage of the conditional gene expression system recently developed by Meissner and colleagues (Meissner et al., 2002). We developed a novel YFP based negative selection screen to successfully target the locus of native ACP gene by double homologous recombination. Phenotypic characterization of our conditional mutant suggests the essential requirement of FASII pathway for the growth and viability of

parasites both *in vitro* and *in vivo*. Furthermore, infection with TgFASII mutant strain followed with down regulation of ACP by tetracycline, conferred protection in the mouse model to subsequent *T .gondii* infections, and thus demonstrates potential as a live attenuated vaccine. In addition, we and others have recently identified an additional mitochondrial localized fatty acid biosynthetic pathway in *T. .gondii*, similar to the Type I FAS of *Cryptosporidium parvum* (Mazumdar et al., 2006; Zhu et al., 2000). The presence of two fatty acid biosynthetic pathways in *T. gondii* offers an excellent opportunity for the functional dissection of apicoplast fatty acid metabolism, and the therapeutic validation of the apicoplast localized FAS II pathway. This study is a valuable contribution to the ongoing research efforts focused on the therapeutic exploitation of the FAS II pathway for the treatment of malaria and toxoplasmosis.

The dissertation is organized into five chapters. Chapter 2 is a review of the literature available on the biology of the apicoplast and the multiple drug targets it presents. Chapter 3 presents a novel strategy for gene targeting, exemplified in the construction of a conditional TgFASII mutant. We present a positive-negative selection employing drug treatment and cell sorting, which offers significant improvements in the frequency of successful allelic replacements. Chapter 4 investigates the effect of FASII knock down on parasite growth and pathogenesis and examines the overall contribution of *T. gondii* FASII pathway in the biology of the parasite. Chapter 5 summarizes the significant findings of our experiments and presents future direction of our research.

The work presented in this dissertation, sheds new light on the complexity of apicomplexan fatty acid metabolism, characterizes the functions of the one of the pathway, the apicoplast localized Type II fatty acid biosynthesis, and validates it as a viable drug target.

CHAPTER 2

THE APICOPLAST; A RELICT CHLOROPLAST IN APICOMPLEXAN PARASITES (REVIEW OF THE LITERATURE)

The apicoplast is an organelle found within most apicomplexan parasites and bears remarkable similarity to secondary plastids. Plastids per se are remnants of cyanobacteria, a common photosynthetic eubacteria that were engulfed by plants and algae by the process of endosymbiosis (Gray, 1992; Gray and Spencer, 1996). Examples of plastid include the actively photosynthesizing green "chloroplast" in plants, bright red "rhodoplasts" in red algae and the golden brown "chromoplasts" present inside algae like diatoms and dinoflagellates. Non-photosynthetic versions of the plastid also exists and includes organelles such as "amyloplasts" and "leucoplasts" found in certain parasitic plants and underground plant tissues. The identification of a plastid homologue in members of the phylum Apicomplexa, an organelle typically found in plants and algae, (Kohler et al., 1997; McFadden et al., 1996; Wilson et al., 1994) was therefore quite a surprise. The apicomplexan plastid, commonly called the "apicoplast" (Kohler et al., 1997), harbors several metabolic pathways unique to the parasite and has received considerable attention in recent years as a depot for novel drug targets.

This chapter presents an overview of the apicoplast, its origin and maintenance. It discusses the various modifications that occurred in its evolutionary path from being a free living cyanobacterium to an intracellular organelle, including its novel division and protein import mechanisms. Lastly this chapter presents the various metabolic functions of the apicoplast and their potential as future drug targets.

2.1 Endosymbiotic Origin of Plastids

Plastids are endosymbiotic organelles predominant in plants and algae. Commonly associated with photosynthesis these organelles are metabolically diverse. Apart from photosynthesis, plastids have been shown to function in controlling oxidative stress as well as biosynthesis of fatty acids, isoprenoids, heme, amino acids and other small compounds (Weeden, 1981). Initiated by the engulfment of a free living photosynthetic eubacteria (*cyanobacterium*) by a eukaryotic host cell (Gray, 1992), the endosymbiotic relationship of the plastid is characterized by the retention and integration of the captured endosymbiont into the host cell (McFadden, 1995). Endosymbiosis provides an aggressive strategy for the host cell to acquire multiple new functions at once and is best illustrated in plants and algae that acquired photosynthesis by the endosymbiotic relationship with a *cyanobacterium* (Gray, 1992).

The steps leading to the domestication of the once free-living endosymbiont into an organelle typically include: 1) the loss and transfer of genes from the endosymbiont genome into the host nuclei; 2) the concomitant establishment of a mechanism for the import of the nuclear encoded proteins back into the organelle; and 3) the establishment of a mechanism for the division and transmission of the organelle.

2.1.1 Primary Plastids

Plastids descending directly from the engulfment of a *cyanobacterium* by a eukaryotic host are called “primary plastids”. These organelles are bound by two membranes which are homologous to the inner and outer membranes of the cyanobacterial endosymbiont (Jarvis and Soll, 2001). Primary plastids are found in glaucophytes, red algae, green algae and their closest relatives, the land plants.

The presence of plastids across diverse lineages poses an obvious question. Did primary plastids arise from one endosymbiotic event or are the descendants of several independent events? Though this debate continues (Stiller et al., 2003), analyses of plastid morphology, biochemistry, gene content/ organization, and molecular phylogenies of numerous plastid RNAs and proteins, suggests that primary plastids are monophyletic, i.e the product of a single endosymbiotic event (Besendahl et al., 2000; Delwiche et al., 1995; Helmchen et al., 1995).

2.1.2 The Apicoplast is a Secondary Plastid and Probably Monophyletic

Plastids in apicomplexans, heterokonts, cryptophytes, haptophytes, dinoflagellates, euglenoids and chlorarachinophytes on the other hand have a secondary origin and are called "secondary plastids". These plastids evolved via secondary endosymbiosis, a process in which one of the primary-event algae is itself eaten by another eukaryote and degenerated into an organelle (Archibald and Keeling, 2002; Cavalier-Smith, 2000; Moreira and Philippe, 2001), (Fig. 2.1).

The theory of secondary endosymbiosis was first proposed for *Euglena* chloroplasts (Gibbs, 1978) and was later supported by work in *Cryptomonads* (Douglas et al., 1991). Like primary endosymbiosis this process too involved transfer and losses of a large number of genes from the plastid genome, development of a new protein targeting system and the establishment of a mechanism for its transmission. Secondary plastids are typically surrounded by three or four membranes derived from the endosymbiont plasma membrane and the secondary host endomembrane, respectively (Gibbs, 1978; Hopkins et al., 1999; Striepen et al., 2000). Unlike primary plastids which are believed to have a single origin, phylogenetic evidence suggests, secondary plastids have originated more than once and originate from both green algal and red algal lineages (Keeling et al., 2004; Palmer, 2003; Yoon et al., 2004).

The apicoplast, a non-photosynthetic organelle is a secondary plastid, and harbors its own genome. The apicoplast genome, a 35kb circle was first observed ultrastructurally by Kilejian in the avian parasite *P. lophurae* (Gutteridge et al., 1971), followed by similar observations in *P. berghei* (Dore et al., 1983) and *Toxoplasma gondii* (Borst et al., 1984). Today the apicoplast has been identified in members of all four apicomplexan lineages (*Coccidian, Haemosporidia, Gregarinia* and *Piroplasmida*) (Gleeson, 2000; Lang-Unnasch et al., 1998; McFadden and Waller, 1997). The only apicomplexans currently thought to lack a plastid are *Colpodella,* a phagotrophic flagellate with an apical complex (McFadden, 1997b) and the more clinically significant *Cryptosporidium parvum* (Zhu et al., 2000a). Repeated efforts to identify the plastid at the molecular and ultrastructural level and genomic level have yielded negative results (McFadden, 1997a; Zhu et al., 2000a).

At least two lines of study indicate a monophyletic origin for the apicoplasts in apicomplexans. Firstly, comparison of apicoplast genomes from two coccidians (*Eimeria Tenella* and *T. gondii*), one haemosporidian (*P. falciparum*) and one piroplasmid (*Theileria annulata*) show remarkable similarity in their genomes with regard to gene content and gene organization (Denny et al., 1998). Secondly, tree topologies derived from phylogenetic analyses of plastid- and nuclear-encoded ribosomal RNA genes are strikingly similar, which not only suggests a common origin for the apicomplexan plastids but also the co-evolution of the apicoplast and the nucleus in apicomplexan parasites (Lang-Unnasch et al., 1998; Obornik et al., 2002).

2.1.3 Red Algal Affiliation of the Apicoplast

Since secondary plastids could arise from both the red alga (rhodophytes) and the green alga (chlorophytes), the evolutionary affiliation of the apicoplast has been frequently debated. Apicomplexans are sister groups to dinoflagellates, a diverse group of algae harboring a

peridinin containing secondary plastid of the red algal lineage. Based on its close association to the dinoflagellates, the apicoplast was initially considered a descendant of the red algal lineage (McFadden and Waller, 1997; Palmer, 1992; Wilson et al., 1994). But since many secondary plastids have a green algal lineage, a green algal origin of the apicoplast was also proposed**.** In the absence of classical plastid markers such as photosynthetic pigments (chlorophyll) and enzymes (rubisco), initial studies of the plastid evolution largely relied on comparative molecular approaches such as phylogenetic analysis. Contrary to its red algal affiliation, initial phylogenetic trees constructed from plastid-encoded genes of ribosomal RNAs and ribosomal proteins indicated a weak affiliation of the apicoplast with the euglenoid plastids (Egea and Lang-Unnasch, 1995; Gardner et al., 1994; Howe, 1992), which belong to the green plastid lineage (Delwiche, 1999). Weak support for a green algal ancestry of the apicoplast was furthered by sequence analysis of *tufA* gene coding for the protein synthesis factor Tu, located on the plastid genome (Kohler et al., 1997). On the other hand, the presence of ORF470/*ycf*24/*suf*B genes, characteristic of the red algal genome, in the apicoplast genomes of *P. falicparum* and *P. berghei* provided strong evidence for the red algal affiliation of the apicoplast (Williamson et al., 1994; Wilson, 1993; Yap et al., 1997). At about the same time while the green algal ancestry was being proposed, comparison of gene loss patterns and gene rearrangements between the apicoplast genomes and several other plastids (two rhodophytes, one cryptophyte and one diatom) suggested an evolutionary affiliation of the apicoplast outside the green algal lineage (Blanchard and Hicks, 1999; McFadden, 1997a). Finally, the strongest proof for the red algal affiliation of the apicoplast was provided by Zhang and colleagues, who sequenced and introduced dinoflagellate plastid sequence for phylogenetic analysis and demonstrated a clear

relation between dinoflagellate chloroplasts and apicomplexan plastids (Zhang et al., 1999; Zhang et al., 2000).

The red algal affiliation of the apicoplast witnessed an interesting turn when Cavalier-Smith put forth the “Chromalveolate” hypothesis (Cavalier-Smith, 1999). This theory not only advocated a shared origin for the apicoplast and the dinoflagellate plastids, but further suggested that all secondary plastids that derive from the red algal lineage are monophyletic deriving from the same secondary endosymbiotic event. The chromalveolate hypothesis presumes a common origin for plastids among heterokonts, cryptophytes and haptophytes (the Chromista) as well as apicomplexans, dinoflagellates and ciliates (the Alveolata), uniting both chromistans and alveolates into one big monophyletic clade, the “Chromalveolatea” (Cavalier-Smith, 1999). According to the chromalveolate hypothesis, the chromistan and alveolata lines diverged after acquisition of the secondary plastid. It further suggests that all chromalveolates harboring a non-photosynthetic derivative of the plastid including all ciliates, the various apicomplexans, members of the Chromista that lack a plastid and all dinoflagellates that contain a non-photosynthetic plastid (Cavalier-Smith, 1986; Cavalier-Smith, 1999; Cavalier-Smith, 2000; Cavalier-Smith, 2002) subsequently lost the capability to photosynthesize. Fig.2.2. depicts the schematic representation of the chromalveolate tree of life.

Experimental support for the “chromalveolate” hypothesis comes from the phylogenetic analysis of GAPDH (glyceraldehyde-3-phosphate dehydrogenase), a central metabolic enzyme of glycolysis and the Calvin cycle (Fast et al., 2001). Plastid-bearing organisms have two versions of GAPDH, a cytosolic and a plastid isoform. The plastidic GAPDH of plants, green algae and red algae are cyanobacterial-like supporting their cyanobacterial origin. On the other hand, the genes for plastid GAPDH of dinoflagellates and cryptophytes are closer to eukaryotic

cytosolic genes. It is thought that the cytosolic GAPDH in dinoflagellates and cryptophytes underwent a duplication eventually replacing the plastid isoform. Comparison of the cytosolic and plastidic GAPDH genes of the apicomplexan *T. gondii* with other GAPDH sequences, clusters the apicomplexan plastidic and cytosolic GAPDH sequences with those genes from dinoflagellates, heterokonts and cryptophytes, and not with the sequences from plants, green algae and red alga (Fast et al., 2001), offering strong support for a common origin of all secondary plastids of the red algal lineage. However, phylogenetic analysis of cytosolic GAPDH does not support the proposition that cryptophytes, haptophytes, heterokonts and alveolates (including dinoflagellates) recently divereged from a common ancestor (Falkowski et al., 2004; Fast et al., 2001; Harper and Keeling, 2003; Takishita et al., 2004), and thus the chromalveolate hypothesis awaits further testing.

2.2 Apicoplast Division: A Novel Mechanism

The apicoplast is a semi-autonomous organelle capable of vertical self- transmission to daughters. It harbors its own genome, a 35 kb circle and encodes genes functional in their own transcription and translation (Cai et al., 2003; Roos et al., 1999; Wilson et al., 1996). Sensitivity to inhibitors of prokaryotic DNA replication, protein translation and transcription (Fichera and Roos, 1997; Williamson et al., 1996), made apparent the parasite's dependence on the apicoplast. The recent identification of a *T. gondii* mutant with an apicoplast segregation defect further corroborates initial pharmacological observations (He et al., 2001a). Mutant parasites lacking an apicoplast were incapable of continued growth in culture and eventually died (He et al., 2001a; Striepen et al., 2000). Apicoplast division, an organellar house keeping function has thus gained importance as a potential drug target. Until recently the molecular mechanism of apicoplast

division and segregation were poorly understood. Since the apicoplast derives from a *Cyanobacterium*, it was initially considered to follow the bacteria cell division model.

2.2.1 Plastid Division Apparatus: A Prokaryotic Blueprint

Bacterial cell division is characterized by the formation of a division ring also called the "Z ring" initiated by the self assembly of protein FtsZ at the middle of a dividing bacterium (Bi and Lutkenhaus, 1991). FtsZ, the most conserved element of bacterial cell division machinery is a tubulin-like molecule with GTPase activity (Lowe and Amos, 1998; Nogales et al., 1998). It is hypothesized to function in bacterial cell division by either directly participating in cytokinesis or by generating the contractile force required to complete cytokinesis. In bacteria the positioning of FtsZ is regulated by several other proteins such as MinC, MinD and MinE (Errington et al., 2003; Weiss, 2004).

Homologues for FtsZ and helper protein MinD have been identified in plants (Colletti et al., 2000; Kanamaru et al., 2000; Osteryoung et al., 1998; Strepp et al., 1998) , photosynthetic protists, red algae and a cryptophyte (Beech and Gilson, 2000; Beech et al., 2000; Takahara et al., 2000) and their importance in chloroplast division is unambiguously demonstrated by gene knockouts and RNA interference experiments in plants (Osteryoung et al., 1998; Strepp et al., 1998). In addition, a tripartite plastid –dividing ring that contains two different homologues of FtsZ has been located at the midpoint of dividing chloroplasts (Vitha et al., 2001). Other bacterial cell division proteins such as ARC6 (Pyke, 1999), descendant of the cyanobacterial division protein Ftn2 (Vitha et al., 2003), FtsH protein (Itoh et al., 1999) are also present in a variety of plastid bearing organisms (higher plants, mosses, red algae, cryptophytes). Recently, a eukaryotic GTPase like dynamin, ARC 5 and other dynamin-like proteins of eukaryotic origin has been shown to participate in chloroplast division (Gao et al., 2003).

The plastid division machinery is thus a chimera which still retains several prokaryotic cell division components and at the same time utilizes new components derived from the host cell (Osteryoung, 2000).

2.2.2 The Apicoplast Displays Divergent Division Mechanism

Apicomplexans, on the other hand lack FtsZ homologues or any other conserved bacterial cell division proteins (Striepen et al., 2000; Vaishnava et al., 2005). This is quite a surprise since FtsZ homologues have been isolated in certain phylogenetic groups that harbor secondary plastids of the red algal origin (Miyagishima et al., 2004), including the cryptophyte *Guillardia theta* (Fraunholz et al., 1998), the heterokont alga *Mallomonas splendens* (Beech et al., 2000) and the diatom *Thalassiosira pseudomona* (Armbrust et al., 2004). Moreover, unlike plant chloroplast division, apicoplast division proceeds in intimate synchrony with the nuclear division of the host cell. This is indicated by the precise inheritance of a single apicoplast by the daughter cells of both *P. falciparum* and *T. gondii* (Waller and McFadden, 2000), irrespective of their diverse cell division modes resulting in a variable number of daughter cells produced. *P. falciparum* follows schizogony, a process characterized by multiple nuclear divisions prior to cytokinesis while *T. gondii* replicates by endodyogeny where each nuclear division is followed by cytokinesis (Cai et al., 2003; Morrissette and Sibley, 2002).

In the absence of conserved division components, apicoplast division was explored in live cells of both *T. gondii* and *P. falciparum* by visualization of an apicoplast targeted fluorescent reporter protein, GFP (Green fluorescent protein) (Van Dooren et al., 2005, He et al., 2001a; Striepen et al., 2000; Waller and McFadden, 2000). Pioneering study using fluorescent apicoplasts by Striepen and colleagues (Striepen et al., 2000) revealed a division model unique to the apicoplast.

Employing elegant microscopy their study traced the structural changes of the fluorescent apicoplast during the life cycle of *T. gondii* and made the following observations (Striepen et al., 2000). In its non-dividing state the apicoplast localizes as a round structure in *T. gondii*. Once apicoplast division commences, the organelle elongates and the two ends appear to associate with a cellular structure. The apicoplast genome which exists as a particulate structure "nucleoid" associates with the end of this elongating organelle as well (Striepen et al., 2000). Antibody staining with Centrin, a marker for centriole association and alpha-tubulin, a marker for microtubule showed a close association of the dividing plastids with the centrosomes and the ends of the intranuclear mitotic spindle (Striepen et al., 2000). A close association of the centrosomes with the (posterior) end of plastids can be seen even in non-dividing apicoplasts during interphase. Finally, the study showed that apicoplast divides concurrently with the nucleus (Striepen et al., 2000).

Treatment with dinitroaniline herbicides like oryzalin and ethafluralin that disrupt microtubule formation and blocks nuclear division, resulted in cells with multiple centrosomes and spindles, resembling an artificial schizont (Morrissette and Roos, 1998; Shaw et al., 2000). Furthermore, the apicoplast in these artificial schizonts, which are either present as multiple distinct nucleoids or one reticulate structure maintain close association with multiple centrosomes, and suggests an association independent of the mitotic spindle in *T. gondii* (Striepen et al., 2000). A centrosome dependent model for apicoplast segregation has been recently validated in *Sarcocystis neurona*, another apicomplexan parasite with a divergent cell division model (Vaishnava et al., 2005).

In conclusion, the apicoplast division model presents two fundamental differences with the bacterial division model, schematically represented in Fig.2.3. Firstly, unlike plant

chloroplasts, apicoplast division proceeds in the absence of FtsZ. Secondly, the apicoplast divides in close association with the host mitotic apparatus and replication machinery.

2.2.3 Apicoplast Genome Replication

The apicoplast harbors its own genome, a 35kb circular DNA. The apicoplast genome is highly reduced and encodes genes functional in their own transcription and translation (Cai et al., 2003; Roos et al., 1999; Wilson et al., 1996). The apicoplast harbors multiple copies of the 35 kb circular DNA which are physically linked into concatamers. The copy number varies between 1 and 15 in *P.falciparum*, and between 6 and 25 in *T.gondii* (Fichera and Roos, 1997; Kohler et al., 1997; Matsuzaki et al., 2001). More than 90% of the apicoplast genome is present as covalently closed circular molecules in *Plasmodium* species (Wilson and Williamson, 1997). In contrast the *T.gondii* apicoplast genome has been shown to consist mostly of linear tandem arrays of the 35Kb circle (Williamson et al., 2001). The apicoplast DNA stained with intercalating dyes like DAPI, appear as an extra-nuclear spot smaller than the organelle itself, suggesting the maintenance of the apicoplast genome as intra-organellar DNA (Matsuzaki et al., 2001; Striepen et al., 2000).

Information on apicoplast genome replication and segregation is scarce. Based on observed frequency distribution of linear oligomers of different size, Williamson and colleagues proposed a rolling circle model for the replication of apicoplast genome in *T.gondii* (Williamson et al., 2001). The apicoplast genome of *P.falciparum* has been suggested to replicate bi-directionally with the center of the large inverted repeat serving as the origin of replication (Singh et al., 2005; Williamson et al., 2002). Linear apicoplast DNA molecules have also been reported from *Eimeria tenella* (Dunn et al., 1998) and *Neospora caninum* (Gleeson and Johnson, 1999).

2.2.4 Apicoplast Segregation Defect

The biological validation of the essential nature of the apicoplast comes from a mutant with a plastid segregation defect, engineered by He and colleagues (He et al., 2001a). *T. gondii* RH cell line, transiently transfected with a "poison construct" consisting of the green fluorescent protein (GFP) with an N-terminal plastid-targeting leader sequence and a C-terminal signal for organellar targeting to the rhoptries (a parasite specific secretory organelle), generate abnormal apicoplast morphology. Interestingly, of all the daughter cells within a parasitophorous vacuole, representative of the clonal expansion of a single invasion event, only one expressed the GFP fluorescence. This was in striking contrast to wild type parasites where each daughter cell within a parasitophorous vacuole expresses the apicoplast signal. The mutant apicoplast appeared to replicate and grow but never divide, resulting in unequal segregation and thus generating daughter cells lacking the apicoplast. Despite the apicoplast's inability to divide and segregate into the daughter cells, cell division (endodyogeny) and even replication of the plastid genome seem to proceed unaffected (He et al., 2001a).

The plastid-deficient mutants displayed delayed-death phenotype similar to previous observations made with certain inhibitors (Fichera et al., 1995). The mutants were able to grow and divide normally within the initial infected host cell, but were unable to sustain growth and died soon after reinvasion of another host cell (He et al., 2001a). The mutants revealed membranous inclusions containing cytoplasmic material inside the apicoplast. GFP localized only to the periphery of the plastid as opposed to normal GFP localization in the apicoplast lumen. The large membranous inclusions were speculated to result from the entrapment of the fusion proteins across the apicoplast membranes probably due to its inability to translocate completely across the four bounding membranes (He et al., 2001b). Interestingly, replacement of

the C-terminal rhoptry targeting signal in the “poison construct” with a conventional alpha-helical transmembrane domain or a GPI anchor abolished its ability to target efficiently to the apicoplast or to disrupt apicoplast division (He et al., 2001b).

2.3 Apicoplast Protein Targeting; a Novel Machinery

2.3.1 Protein Import in Primary Plastids

Plastid genomes are heavily reduced. Plastids in green plants encode for approximately 150 genes (Bruce, 2001) as compared to almost 3200 genes encoded by the genome of a modern day *Cyanobacterium* (Kaneko et al., 1996). While some genes have been permanently lost, many others have been transferred to the nuclear genome of the eukaryotic host (Baldauf and Palmer, 1990; Martin et al., 1998; McFadden, 2001; Rujan and Martin, 2001). In spite of the small genome size, the plastid serves as the hub for various metabolic activities, a feat it accomplishes by importing nuclear-encoded proteins back into the plastid.

In primary plastids such as plant and algal chloroplast the trafficking of nuclear encoded proteins from the cytoplasm to the plastid is mediated with the help of the “transit peptide”, an N-terminal extension thought to be acquired by some unknown evolutionary process during endosymbiosis (Bruce, 2001). The chloroplast which is bound by two membranes harbors components of the protein translocation machinery that span across both the membranes. Transit peptides are hypothesized to partition protein from the cytoplasm on to the chloroplast surface by specifically interacting with chloroplast lipids Monogalactosyldiacylglycerol (MDGD), Sphingolipids (SL) and Phosphoglycerides (PG) (Horniak et al., 1993; Kerber and Soll, 1992; van't Hof and de Kruijff, 1995), an interaction suggested to be aided by a 14-3-3/Hsp70 chaperone complex (May and Soll, 2000). The second step in the process of organellar

trafficking is the receptor- mediated interaction of the transit peptides with the outer and inner chloroplast translocon components, Toc (translocon of the outer chloroplast membrane) and Tic (translocon of the inner chloroplast membrane) (Hirsch et al., 1994; Jarvis and Soll, 2001; Kessler et al., 1994; Kovacheva et al., 2005).

Following translocation of the chloroplast proteins across the two membranes, a stromal processing peptidase such as CPE cleaves the transit peptide generating the mature chloroplast protein. The "free" transit peptide is subsequently degraded by some unknown peptidase (Richter and Lamppa, 1998). Transit peptides therefore play a key role in the trafficking of nuclear-encoded proteins into the plastid. Bioinformatic analysis of the *Arabidopsis* genome identified at least 3500 proteins that harbor a transit peptide (Bruce, 2001).

Plant and algal transit peptides are typically 25-125 amino acids in length. They are basic and enriched in serine and threonine but lack a consensus sequence or secondary structure (von Heijne and Nishikawa, 1991).

2.3.2 Protein Import in Secondary Plastids

Protein import into secondary plastids is more complex than primary plastids. Firstly secondary endosymbionts underwent a second round of gene transfer (both organelle- and nucleus-encoded genes were again transferred, this time to the nucleus of the second host cell). Secondly they are bound by one or two additional membranes.

In apicomplexans, the apicoplast is surrounded by four membranes. Of the two additional membranes, the outermost membrane is thought to derive from the phagosome and the membrane beneath also called the "periplastid membrane" from the plasma membrane of the secondary endosymbiont (McFadden, 1999). Import of proteins across the multi-membranes of the apicoplast is mediated by a bi-partite N-terminal extension which comprises of a classical

"signal peptide" followed by a plant-like transit peptide (Waller et al., 1998; Waller et al., 2000) (Fig. 2.4.).

2.3.3 Proteins Target to the Apicoplast Via the Secretory Pathway

Several studies confirm the trafficking of apicoplast proteins via the secretory pathway. The signal peptide routes apicoplast proteins into the ER (DeRocher et al., 2000; Waller et al., 1998; Waller et al., 2000; Yung and Lang-Unnasch, 1999) and the plant-like transit peptide effects the translocation of the proteins across the inner organellar membranes (McFadden, 1999; Schwartzbach et al., 1998; van Dooren et al., 2001).

Molecular analysis of the apicomplexan leader sequences indeed reveals a bipartite nature. The extreme N-terminal region is 16-34 amino acids in length and contains a hydrophobic domain followed by a Von Hiejne cleavage site, similar to the classical secretory signal peptide that target proteins to the endomembrane system. The signal peptide is followed by an extension functionally equivalent to the plastid transit peptide (Waller et al., 1998; Waller et al., 2000). GFP fusions of the N-terminal bipartite extensions of the apicomplexan leader peptide indicate that the leader sequences are both necessary and sufficient to direct import of the reporter protein into the plastid in *P. falciparum* and *T. gondii*. Deletion of just the transit peptide caused proteins that now contained only an N-terminal signal peptide fused to GFP, to be secreted from the cell into the parasitophorous vacuole (DeRocher et al., 2000; Waller et al., 2000). While removal of the signal peptide alone led to the accumulation of the fluorescence in the cytosol (Waller et al., 2000).

2.3.4 Apicoplast Transit Peptides

The apicoplast transit peptides, an extension downstream of the N-terminal signal peptide is considered functionally equivalent to the plant and algal transit peptide. Like primary transit

peptides they bear a net positive charge and are enriched in serine and threonine. Apicoplast transit peptides are variable and range from 57-107 amino acids in *T. gondii,* to the relatively shorter 30-42 amino acids in *P. falciparum* (Waller et al., 1998). Additionally, the net positive charge of *P. falciparum* transit peptides derives from an enrichment of arginine and lysine instead of serine and threonine, as observed in *T. gondii* (Waller et al., 1998).

Transit peptides possess distinct subdomains (Bruce, 2000; von Heijne et al., 1989), and some of their functional characteristics include their ability to form helices with galactolipids of the plastid membranes (van't Hof et al., 1993; Wienk et al., 2000), and the capacity to interact with chaperones and peptidases (Rial et al., 2000; Richter and Lamppa, 1998). Whether apicomplexan transit peptides also display these features is yet to be established, but domain swapping experiments clearly demonstrates redundancy between the apicomplexan and transit peptides from plants. For example, apicoplast transit peptides for the ribosomal protein S9 could effect targeting of green fluorescent protein into isolated pea chloroplasts (DeRocher et al., 2000). Substantial deletions of the N-terminal 42 amino acid S9 transit peptide further suggests that targeting information resides mostly in the N-terminus of the transit peptide (DeRocher et al., 2000; Yung et al., 2001).

Transit peptides lack a consensus sequence or a regular secondary or tertiary structure (von Heijne and Nishikawa, 1991). However, despite the lack of a predicted structural motif, the primary sequence of the majority of plant transit peptides can be identified using computational programs such as ChloroP (Emmanuelsson et al., 1999; Peltier et al., 2000). Since enrichment for Ser and Thr and the net positive charge are also characteristic of transit peptide-like domains of *T. gondii*, some *Toxoplasma* transit peptides are correctly recognized by ChloroP (DeRocher et al., 2000).

2.3.5 Processing of N-terminal Leaders

Following its targeting to the ER, the signal peptide undergoes cleavage by a signal peptidase during translocation across the ER. The processing can be detected by Western analysis of apicoplast proteins in *P. falciparum* and *T. gondii* (He et al., 2001b; Mazumdar et al., 2006; Waller et al., 1998). In *Euglena gracilis* which harbors a secondary plastid and routes protein via the secretory pathway, pulse chase labeling of SSP, a chloroplast targeted protein specifically shows removal of the signal peptide in the ER (Sulli and Schwartzbach, 1996). The apicoplast transit peptide domain on the other hand, is most likely removed once the protein has been translocated across the plastid's membrane, as occurs with transit peptides in plant chloroplasts (He et al., 2001b; van Dooren et al., 2002; Waller et al., 1998; Waller et al., 2000). Not much is known about the primary transit peptide cleavage site and only one loosely defined motif has been identified thus far (Bruce, 2000; Emmanuelsson et al., 1999)**.** Similarly, no consensus cleavage motif could be deduced from the apicoplast proteins sequenced thus far including ACP, FabI, FabZ and FNR (Harb et al., 2004; Surolia and Surolia, 2001; van Dooren et al., 2001; Waller et al., 1998). Furthermore, analysis of the cleavage pattern of these proteins reveals interesting variations. For example the mature *P. falciparum* ACP harbors a 16 aa N-terminal stretch that is different from cyanobacterial ACP and enriched in Lys and Asn, seeming to suggest inaccurate processing16 residues upstream of the predicted cleavage motif (van Dooren et al., 2002). On the other hand, analysis of the transit peptide of Tg FNR displays at least two independent transit peptide domains (Harb et al., 2004).

2.3.6 Protein Trafficking from ER to the Apicoplast, an UnknownMechanism

While we know that nuclear-encoded proteins are directed to the ER by the signal peptide, events leading to the transport of these proteins from the ER to the apicoplast are yet to

be elucidated. The mechanism is relatively lucid in the secondary plastids of chromistan algae (cryptophytes, heterokonts and haptophytes) which harbor ribosomes on their outer most plastid membrane (Gibbs, 1981). Proteins synthesized by these ribosomes are able to reach the inner plastid membranes without further trafficking through the endomembrane system. The situation is complex in apicoplasts whose outer membranes are completely devoid of ribosomes. Two models have been proposed to explain protein trafficking from the ER to the outer membranes of the apicoplast. Bodyl, in 1999 hypothesized that proteins are trafficked from the ER to the apicoplast with the help of shuttling vesicles (Bodyl, 1999). Experimental support for the shuttling vesicle theory comes from the apicoplast-deficient cells of *T. gondii*, in which plastid-targeted GFP has been observed in vesicles located in the apical region of the cell (He et al., 2001b). Though attractive, the model does not however explain how transit peptides induce packaging of apicoplast-bound proteins into the appropriate vesicles. The second model, proposed by van Dooren and colleagues, suggest all secreted proteins 'wash- past' the plastid by default, and those bearing transit peptides are sieved out by receptors on the apicoplast membranes (van Dooren et al., 2000). Both models are attractive, but lack experimental support.

Another aspect which remains unresolved is the involvement of the Golgi apparatus in protein-trafficking to the apicoplast. The golgi-disrupting agent Brefeldin A (BFA) does not ablate targeting or processing of apicoplast proteins. Moreover, the attachment of the C-terminal ER-retrieval signal HDEL to recombinant apicoplast-targeted proteins does not inhibit apicoplast targeting (Roos et al., 2002). However, recent analysis by DeRocher and colleagues, offer evidence that protein trafficking to the apicoplast may depend on some BFA-sensitive GTP exchange factors (DeRocher et al., 2005). Fig. 2.4 presents a schematic summarization of protein targeting to the apicoplast.

2.3.7 Transport Across the Periplastid Membrane; an Open Question

The apicoplast, a secondary plastid is surrounded by four membranes. The product of two subsequent engulfment processes, the apicoplast derives its outermost membrane from the phagosome (part of the endomembrane system), the membrane beneath also called the "periplastid" membrane from the algal cell wall, and the two innermost membranes (the outer and inner plastid envelope membranes) from the primary plastid (Fig.5A). Protein import across these four membranes is mediated by a bipartite leader sequence. The signal peptide can traffic proteins across the outer membrane of the apicoplast and the transit peptide mediates traffic across the two inner membranes. Interestingly, the bipartite sequence does not explain protein trafficking across the periplastid membrane, and this open question has been reviewed in great detail by several authors (Cavalier-Smith, 1999; Kroth and Strotmann, 1999; van Dooren et al., 2001) .

Four models that account for protein trafficking across the periplastid membrane have been proposed, which are detailed in Fig. 2.5. Gibbs in 1981 suggested that proteins, that cross the outer membrane, may be packaged into vesicles which shuttle across the periplastid space (the space between the periplastid membrane and the outer plastid envelope membrane) and fuse with the periplastid membrane (Gibbs, 1981). This hypothesis was supported by the presence of vesicles and tubules (termed the 'periplastidial reticulum) within the periplastid space in various algae (Gibbs, 1981). In its counter argument, proteins from the periplastid vesicles would be released into the space between the two innermost membranes. This would prevent the transit peptides from interacting with the Toc complex/transit peptide receptors that are believed to be located on the cytosolic side of the outer plastid envelope membrane (Cavalier-Smith, 1999).

The second model simplifies the situation by considering the existence of large pores

within the periplastid membrane which would allow proteins to pass through freely (Cavalier-Smith, 1999; Kroth and Strotmann, 1999). This model eliminates the need for a special target to translocate proteins across the periplastid membrane. Like primary plastids which import proteins via the transit peptide, the transit peptides inherent in these proteins would effect translocation across the two plastid envelope membranes. One obvious drawback of the large pore theory is its failure to account for the leakage of proteins out from the periplastid space. In addition, it fails to answer why the apicoplast would retain an apparently non-functional membrane and not simply lose it in the course of evolution.

Cavalier-Smith in 1999 proposed a two way vesicle shuttling between the two middle membranes (Cavalier-Smith, 1999). The highlight of this theory is the insertion of the Toc complex/transit peptide receptors from the outer plastid envelope membrane onto the periplastid membranes. Such an insertion would allow the proteins to be translocated across the two middle membranes employing the same transit peptide. Furthermore, the two way shuttling would also integrate galactolipids present on the outer plastid envelope membrane onto the periplastid membrane. In plants, galactolipids present on the outer membrane of the chloroplast are thought to be necessary for the transit peptide-receptor interaction (Douce and Joyard, 1990) and thus play an important role in protein trafficking. In apicomplexans, the presence of galactolipids in any of the plastid membranes is so far preliminary (Marechal et al., 2002). In addition, this hypothesis fails to account for the insertion of the Toc components and transit peptide receptors and galactolipids in the wrong orientation, which would make them inaccessible to the apicoplast transit peptides.

The most recent model proposed by van Dooren and colleagues, suggest the dual insertion of Toc complex components onto the outer plastid envelope membrane as well as the

periplastid membrane (van Dooren et al., 2000). Though this model does not specify how a secondary plastid would achieve this dual targeting, it does eliminate the involvement of vesicles for protein trafficking. According to this model transit peptides alone would be sufficient to allow proteins to be translocated across the inner three membranes. None of the above models are complete and offer opportunities for further research.

2.4 Apicoplast Functions

The plastid harbors a diverse metabolism. In addition to photosynthesis, plastids are the site for production of fatty acids, isoprenoids, heme, starch, aromatic amino acids and other metabolic products. The apicoplast lacks photosynthetic functions, however similar to non-photosynthetic plastids; the apicoplast is believed have metabolic functions and at least three metabolic pathways have been identified in this organelle. A comprehensive view of *Plasmodium* apicoplast metabolism has been recently presented by Ralph and colleagues (Ralph et al., 2004)

2.4.1 Heme Biosynthesis

One of the functions initially suggested for the apicoplast was synthesis of heme for mitochondrial respiration (Wilson et al., 1991). Heme is an essential component needed for the synthesis of cytochromes, chlorophyll, phycobilins and the corrin nucleus of vitamin B_{12} (Obornik and Green, 2005). Both prokaryotes and eukaryotes employ heme biosynthesis but differ in the initial part of the pathway, the synthesis of 5-aminolevulinate (ALA). In photosynthetic eukaryotes and all prokaryotes outside the α-proteobacterial group, ALA is synthesized in the plastid by the C_5 pathway starting with the 5C precursor glutamate. While in α-proteobacteria and non-photosynthetic eukaryotes such as animals, fungi and apicomplexans,

ALA is synthesized in the mitochondria by the condensation of succinyl-CoA with glycine by δ-aminolevulinate synthase (ALAS), also known as the Shemin pathway (Roberts et al., 2002). The mitochondrial localization of ALAS clearly indicates the mitochondria as the site for the initial step of heme biosynthesis. However, some of the subsequent enzymes have been predicted to be apicoplast targeted (Varadharajan et al., 2002). For example, an important downstream step in the heme biosynthesis pathway is the conversion of ALA to phorphobilinogen by δ-aminolevulinate dehydratase (ALAD or HemB). *P. falciparum* reveals a plastid localized HemB similar to the plants but distinct from the cytosolic Hem B of animals and fungi (Gardner et al., 2002; Sato et al., 2000). In addition, an orthologue of uroporphyrinogen III synthase enzyme (HemD), acting at a later stage of the heme biosynthesis pathway is predicted to be targeted to the apicoplast in *T. gondii*, while other enzymes such as, uroporphyrinogen decarboxylase (HemE) and ferrocheletase (HemH) are identified as apicoplast targeted in *P.falciparum* (Ralph et al., 2004), indeed suggesting the mosaic distribution of this pathway between the mitochondria and the apicopast in apicomplexans. We have identified homologs of Hem B (TgTigrScan_1739), HemE (TgTwinScan_0195) and Hem H (TgGLEAN_5447) genes in *T. gondii* genome database (ToxoDB). Our analysis, however, fails to identify an N-terminal hydrophobic signal, and may need further bioinformatics characterization.

2.4.2 Isoprenoid Biosynthesis

The first indication of the apicoplast's possible involvement in isoprenoid biosynthesis came from the identification of an apicoplast localized 1-deoxy-xylulose-5-phosphate (DOXP) in *P. falciparum* (Jomaa et al., 1999) and later in *T. gondii* (Seeber, 2003). Isoprenoids are diverse compounds made up of repeated units of Isopentenyl phosphate (IPP). The function of isoprene units ranges from serving as the prosthetic group of several enzymes such as thiamine

pyrophosphate (TPP) and DOXP synthase (DXS) to being the structural backbone of ubiquinones and dolichols, compounds that are involved in electron transport and formation of glycoproteins. Animal and fungal cells synthesize isoprenoids via the acetate/mevalonate pathway and use mevalonate, a 5 carbon molecule as the precursor (Lichtenthaler et al., 1997). Alternatively, bacteria and plant chloroplasts synthesize isoprenoids employing a second biosynthetic pathway which is dependent on 1-deoxy-xylulose-5-phosphate (DOXP) instead (Arigoni et al., 1997; Disch et al., 1998; Lange et al., 1998; Lichtenthaler et al., 1997; Schwender et al., 1996). This alternative pathway also known as the non-mevalonate pathway has been recently identified in apicomplexan parasites, as mentioned above.

Several extra-plastidic roles of isoprene units have been identified in apicomplexans, ranging from the isoprenylation of dolichols (Couto et al., 2004) and tRNAs (Ralph et al., 2004) to the prenylation of ubiquinones (Vial, 2000). Plants satisfy many of these demands for isoprenes through a cytosolic mevalonate pathway in addition to the plastidic DOXP pathway. Genome mining, however, fails to identify a cytosolic mevalonate pathway in apicomplexans. Furthermore *P. falciparum* exhibit low sensitivity to mevastatin, a mevalonate pathway inhibitor (Couto et al., 2004). These findings suggest that the cytosolic and mitochondrial demands for isoprene subunits are probably met by the apicoplast localized isoprenoid biosynthetic pathway. Surprisingly, recent pharmacological analysis show *T. gondii* is resistant to fosmidomycin, the phosphonate inhibitor of the non-mevalonate pathway, which inhibits the enzyme 1-deoxy-xylulose 5 phosphate reductoisomerase. Moreover, *T. gondii* is sensitive to bisphosphonates, which typically target farnesyl pyrophosphate synthase (FPPS) of the mevalonate pathway (Ling et al., 2005). Genome analysis, however, does not support the presence of a mevalonate pathway for isoprenoid biosynthesis in *T. gondii*.

2.4.3 Fatty Acid Synthesis

Fatty acids are critical for membrane biogenesis and cell homeostatis and are essential cellular requirements. Both eukaryotes and prokaryotes synthesize fatty acids *de novo* employing a multi-enzyme fatty acid synthase system (FAS) (Smith, 1994). Contrary to previous speculations suggesting the complete absence of *de novo* fatty acid synthesis in apicomplexan parasites (Holz, 1977), Waller and colleagues in 1998, reported the identification of at least two apicoplast targeted FAS components in both *P. falciparum* and *T. gondii* (Waller et al., 1998). Subsequently diverse FAS pathways have been identified in all three clinically significant apicomplexan parasites of the humans, *T. gondii, P. falciparum* (Ralph et al., 2004; Surolia and Surolia, 2001; Waller et al., 1998) and *C. parvum* (Zhu et al., 2000b). The apicoplast FAS enzymes of *T. gondii* and *P. falciparum* are homologous to bacterial and plant chloroplast enzymes, which typically harbor the type II FAS pathway (FASII), characterized by the presence of enzymes as distinct units. The FASII pathway is remarkably divergent from the eukaryotic FASI pathway, which harbors the complete set of FAS enzymes on a single polypeptide (Smith, 1994). Similar to *P. falciparum*, the genome of *T. gondii* is now known to encode all the components of the FASII pathway, (Table 2.1.) (Mazumdar, Unpublished observation; Ralph et al., 2004), and three enzymes characterized thus far including acyl carrier protein (ACP), fatty acyl dehydratase (FabZ) and enoyl reductase (FabI) have been shown to localize to the apicoplast (Ferguson et al., 2005; Waller et al., 1998).

One of the primary functions of the chloroplast FAS II pathway is the generation of fatty acids for cellular purposes. Active incorporation of acetate, a 2C precursor in both *T. gondii* and *P. falciparum* provides strong indication for the parasite's capability for fatty acid biosynthesis, which some researchers suggest to be derived from the apicoplast FASII pathway (Bisanz et al.,

2006; Surolia and Surolia, 2001). Consistent with observations made previously suggesting robust scavenging of lipids by *T. gondii*, disruption of FASII functions did not affect bulk fatty acid synthesis as detected by radio labeled acetate incorporation (Charron and Sibley, 2004 and chapter 4). Interestingly, unlike in plant chloroplasts, our analysis (presented in Chapter 4) did not find an apicoplast targeted acetyl-CoA synthetase (ACoS), an enzyme critical for the activation of acetate to acetyl-CoA, the 2C precursor for fatty acid biosynthesis. In plants ACoS is also required for the export of plastid lipids to the ER for glycerolipid synthesis (Schnurr et al., 2000; Schnurr et al., 2002). While *P. falciparum* seems to encode at least one apicoplast isoform of acetyl-CoA synthetase enzyme (NP702246), the ACoS isoform in *T. gondii* (TgTwinScan_3199) (ToxoDB) does not bear apicoplast targeting signal. However, the inhibition of apicoplast FASII did drastically affects parasite growth and viability, suggesting the role of FASII in essential functions (chapter 4). The exact functions of apicoplast fatty acids are yet to be ascertained. Ralph and colleagues, speculate the role of apicoplast fatty acids in the production of phosphatic acids by the acylation of G3P by the apicoplast localized glycerol-3-phosphate acyltransferase (ACT1) and 1-acyl-glycerol-3-phosphate acyltransferase (ACT2), and the biosynthesis of either oleic and/ or palmitoleic acids by the action of an apicoplast localized stearoyl-CoA-desaturase (Ralph et al., 2004). We did find a fatty acyl desaturase with putative apicoplast targeting leader (TgTwinScan_6030). However, we did not find isomers of ACT1 or ACT2 in *T. gondii*. Also, both *T. gondii* and *P. falciparum* seems to lack thioesterase, an enzyme necessary to cleave acyl chain from acyl-acyl carrier protein, before export out of the apicoplast (Mazumdar, Unpublished observation; Ralph et al., 2004), and as mentioned above, our analysis indicates the absence of the enzyme ACoS in *T. gondii*, which is necessary to export the lipids out into the ER. These observations indicate that the role of apicoplast fatty acids might not lie in

the synthesis of bulk fatty acids for the parasite, but for their utilization primarily within the apicoplast. With the help of a genetic model, we have explored parts of this aspect in *T. gondii*, and our findings are presented in chapter 4.

2.4.4 *Toxoplasma gondii* Presents Unusual Fatty Acid Metabolism

During this study we and others have identified a second fatty acid synthesis pathway in *T. gondii* (TgTwinScan_0460) similar to the FAS I pathway of *C. parvum* (Mazumdar, unpublished observation; Zhu et al., 2000b). The FAS I pathway is present in mammals and fungi and characterized by the presence of a multi-functional polypeptide. TgFASI is a large polypeptide (9940 aa) with 41 predicted enzyme domains (ToxoDB). Immunofluorescence assay with a polyclonal rabbit serum against *C. parvum* FASI indicates a mitochondrial localization of TgFASI (M.J., Crawford, personal communication; J. Mazumdar, unpublished observation). Currently the biological functions of *T. gondii* FAS I are unknown.

2.4.5 Carbon and Energy Sources for Apicoplast Metabolism

Chloroplasts of non green plant tissues such as root cells, meet their carbon and energy requirement by the import of hexose and triose phosphates from the cytoplasm (Ralph et al., 2004). Results emerging from the analysis of apicomplexan genomes indicate a striking resemblance in the mechanisms that the apicoplast and non-green plastids employ to fulfill their anabolic requirements. Non-green plastids import C6 sugars including hexose phosphate and phosphoenol pyruvate (PEP) via a plastidic phosphoenolpyruvate/ phosphate translocator (PPT). We and others have identified a putative PPT, with a hydrophobic N-terminal extension, characteristic of a nuclear-encoded plastid targeted protein, in the genomes of both *T. gondii* (ToxoDB, Tg Twinscan_6854) and *P.falciparum* (Ralph et al., 2004), suggesting PEP import by the apicoplast. Following import, PEP is converted to pyruvate by a pyruvate kinase, yielding

ATP (Fischer et al., 1997). Both the *T. gondii* and *P. falciparum* genomes encode a plastidic pyruvate kinase, TgTwinScan_6522 and GBAAN35560, respectively. Furthermore, both *P.falciparum* and *T.gondii* harbor an apicoplast-localized Pyruvate Dehydrogase Complex (PDH) (Foth et al., 2005), which decarboxylates pyruvate to generate acetyl-CoA and NADH, the carbon precursor and reducing energy for fatty acid biosynthesis.

The 3 C intermediate, Dihydroxyacetone phosphate (DHAP) is another important source of cytosolic carbon in non-photsynthetic plastids. Plants import DHAP by the triose phosphate transporter (TPT) (Flugge, 1999), which is either converted to glyceraldehyde-3-phosphate (GA3P) by the chloroplast triose phosphate isomerase (TPI), or to glycerol-3-phosphate (G3P) by glycerol phosphate dehydrogenase (GpdA). GA3P is a precursor for isoprenoid biosynthesis while G3P is primarily used for the biosynthesis of phospholipids. We have identified isoforms of TPT (TgTwinScan_3278), TPI (TgTwinScan_5952) and GpdA (TgGLEAN_1006) in the genome of *T. gondii*, each with a putative apicoplast targeting signal, suggesting the import and modification of DHAP by *T. gondii* apicoplast. Similar observations have also been made for *P. falciparum* apicoplast (Ralph et al., 2004). A list of enzymes involved in apicoplast localized carbon and energy generation identified in ToxoDB is presented in Table 2.2.

Surprisingly, unlike non-photosynthetic plastids which import glucose to obtain ATP and reducing power, genome analysis indicates the absence of a glycolytic pathway or the pentose phosphate pathway in the apicoplast (Ralph et al., 2004). Moreover, *T. gondii* and *P. falciparum* lack an ADP/ATP translocator that is present in both chloroplasts and non-green plastids (Kampfenkel et al., 1995; Neuhaus et al., 1997). It is thus not clear how the apicoplast obtains ATP or reducing power. Ralph and colleagues, predict the generation of NAD(P)H during the conversion of GA3P to 1,3 diphosphoglycerate (1,3-DPGA) by the glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) as a possible source of reducing power for the apicoplast (Ralph et al., 2004). In addition to a cytosolic GAPDH, *T. gondii* harbors a putative apicoplast- targeted GAPDH (Fast et al., 2001). Moreover, the apicoplast has also been shown to harbor the terminal components of the light dependent electron transport chain, ferredoxin (Fd) and ferredoxin dependent NADP reductase (FNR) (Pandini et al., 2002; Vollmer et al., 2001). Plant Fd accepts electrons from photosystem I (PS I) and FNR transfers these electrons to $NADP^+$, generating NADPH. In the absence of light the flow of electrons is reversed, FNR oxidizes NADPH to generate reduced Fd (Fd_{red}). Pandini and colleagues, have identified a similar FNR-dependent reduction of Fd in *T. gondii* (Pandini et al., 2002).

Fd_{red} is essential for the activity of several ferredoxin-dependent enzymes. One potential apicoplast enzyme that may depend on Fd_{red} is stearoyl-CoA desaturase (Vollmer et al., 2001). In addition, Fd_{red} is predicted to participate in the biogenesis of [Fe-S] clusters. The [Fe-S] cluster assembly pathway previously thought to be located exclusively in the mitochondria of eukaryotes have now been identified in the plastids of plants and apicomplexans as well (Initiative, 2000; Seeber, 2002). Fd_{red} is an essential requirement for mitochondrial Fe-S cluster biogenesis and is a critical requirement in the desulfuration of cysteine, which yields sulfur for the Fe-S cluster (Lange et al., 2000). The *P. falciparum* apicoplast harbors a cysteine desulphurase enzyme (SufS), and similar to mitochondrial SufS, is hypothesized to depend on apicoplast Fd_{red} for activity (Ralph et al., 2004). The genome of *T. gondii* encodes a putative apicoplast targeted SufS (TgTwinScan_0723) (Table 2). Apicoplast proteins which are likely to depend on apicoplast [Fe-S] cluster include lipA (involved in lipoic acid biosynthesis) and the enzymes of the isoprenoid biosynthetic pathway IspG and IspH, and MiaB (tRNA methylthiotransferase).

2.5 The Apicoplast Presents Novel Drug Targets

2.5.1 DNA Replication, Transcription and Protein Translation

Interestingly, even before the complete molecular characterization of the apicoplast, *P. falciparum* and *T. gondii* were shown to be sensitive to rifampicin (Wilson et al., 1996), and its derivative rifabutin (Olliaro et al., 1994). The apicoplast is now known to harbor a RNA polymerase homologous to the α_2,β,β' DNA-dependent RNA polymerase of cyanobacteria and other prokaryotes (Gray and Lang, 1998), which is highly sensitive to rifampicin. The apicomplexans, *P. falciparum* and *T. gondii* are also sensitive to fluroquinolone ciprofloxacin, a drug which specifically inhibits prokaryotic but not eukaryotic DNA replication topoisomerases (Fichera and Roos, 1997).

In addition to drugs affecting apicoplast DNA replication and transcription, apicomplexan parasites are also inhibited by prokaryotic protein translation inhibtors including doxycycline, clindamycin and spiramycin (McFadden and Roos, 1999; Ralph et al., 2001). Furthermore, apicomplexans parsites are sensitive to lincosamides (lincomycin and clindamycin) and macrolides (erythromycin and azithromycin) (Fichera et al., 1995; Gleeson, 2000), which block protein synthesis by interacting with peptidyl transferase domain of bacterial 23SrRNA (Steigbigel, 1990), as well as certain thiopeptide antibacterials (thiostrepton and micrococcin) (McConkey et al., 1997; Rogers et al., 1997; Rogers et al., 1998) (Sullivan et al., 2000), which are predicted to act by binding to the guanosine triphosphatase (GTPase) binding domain of the large subunit rRNA blocking apicoplast translation (Clough et al., 1997; McConkey et al., 1997; Rogers et al., 1997; Rogers et al., 1998). None of the above targets have been pursued further for therapeutic purposes.

2.5.2 Isoprenoid Synthesis: a Viable Drug Target in *Plasmodium falciparum*

In addition to the house keeping functions, two apicoplast metabolic pathways have also emerged as potential drug targets, isoprenoid biosynthesis and fatty acid biosynthesis. For example, fosmidomycin, an antibiotic specifically targeting non-mevalonate isoprenoid biosynthesis is highly active against recombinant *P. falciparum* DOXP reductoisomerase and inhibits growth of *P. falciparum* both *in vitro* and *in vivo* (Jomaa et al., 1999). Clinical trials with fosmidomycin as an anti-malarial are underway (Lell et al., 2003). However, *T. gondii* is not sensitive to fosmidomycin (Ling et al., 2005).

2.5.3 Fatty acid synthesis, a Potential Drug Target for *T. gondii* and *P. falciparum*

Parasite dependency on FAS II pathway is demonstrated by significant inhibition of both *P. falciparum* and *T. gondii* by inhibitors of FAS II biosynthesis. The antibiotic thiolactomycin, which is specific for FAS II enzyme β-ketoacyl-ACP synthase (FabH) significantly inhibits *T.gondii* and *P.falciparum in vitro* (R.G. Donald, personal communication; (Waller et al., 1998). Parasite growth in cultured cells is also inhibited by triclosan (McLeod et al., 2001; Surolia and Surolia, 2001), a broad spectrum antibiotic (Levy et al., 1999), active against enoyl-ACP reductase (FabI), which catalyzes the NADH-dependent reduction of enoyl-ACP into a saturated acyl chain (Heath and Rock, 1995). Triclosan binds to and inhibits purified *P. falciparum* enoyl-ACP reductase (Perozzo et al., 2002) and is equally effective against blood stage malarial parasites in the animal model ((Surolia and Surolia, 2001).

Parasite dependency on FAS II is also demonstrated by sensitivity of apicomplexan acetyl-CoA carboxylase (ACC), (catalyzes the first step of the FAS II reactions) to aryloxyphenoxypropionate class of anti-graminicidal herbicides (‘fops’ and ‘dims’) (Konishi and Sasaki, 1994). Although the chloroplast ACC is a single large polypeptide in the grasses, in other

plants it is made up of four subunits that are insensitive to aryloxyphenoxypropionates. The apicoplast targeted ACC carboxylase in *T. gondii* is similar to the multi-domain chloroplast enzyme found in diatom algae and grasses (Jelenska et al., 2001), and is likewise sensitive to aryloxyphenoxypropionate herbicides *in vitro* (Zuther et al., 1999).

Sensitivity of apicomplexan parasites to FAS II inhibitors makes apicoplast FAS II an excellent drug target. However, recent reports have raised concerns about the specificity of triclosan and thiolactomycin (Jones et al., 2005; Paul et al., 2004), creates a pressing need for the biological validation of apicoplast FAS II functions. The presence of apicoplast FAS II in *T. gondii*, offers the unique opportunity for the functional analysis of FAS II in this model organism.

2.5.4 *Toxoplasma gondii*; a Model for the Analysis of Apicoplast FAS II Functions

Beyond its own clinical significance, *T. gondii* is an excellent model for studying other apicomplexans (Donald and Roos, 1994; Kim and Weiss, 2004). *T. gondii* is easy to cultivate in cultured cells compared to *P. falciparum* and *C. parvum*. It is relatively larger in size and bears distinct organelles, making *T. gondii* tachyzoites a good sample for microscopic and ultrastructural analysis (Kim and Weiss, 2004). Most importantly, *T. gondii* has an established repertoire of genetic tools including stable molecular transformation (Donald and Roos, 1993; Kim et al., 1993), genetic selection (Donald and Roos, 1994; Donald and Roos, 1995; Kim et al., 1993; Mazumdar et al., 2006), genetic complementation (Striepen et al., 2002) and conditional gene expression system (Meissner et al., 2002). Employing a conditional genetic knock-out model, we have made substantial progress in the understanding of apicomplexan TgFAS II functions. Our findings and future goals are presented in subsequent chapters.

2.6 Conclusions

Apicomplexan parasites are a class of infectious protozoans that causes malaria, severe enteritis and AIDS associated opportunistic infections in humans. Most of these apicomplexans harbor a plastid like organelle, called the apicoplast, a secondary endosymbiotic organelle of cyanobacterial origin. The apicoplast is essential for parasite survival. Every apicomplexan parasite harbors exactly one apicoplast. The apicoplast is a semi-autonomous organelle; it bears its own genome and maintains a semi independent mode of replication and transmission, intimately linked to parasite cell division. Interestingly, unlike its cyanobacterial ancestor, the apicoplast harbors a highly reduced genome. Barring a few house keeping genes, most of the apicoplast proteins are encoded on the nuclear genome and imported back to the apicoplast. Here, they are involved in a variety of metabolic functions including the biosynthesis of heme, isoprenoids and fatty acids. Apicoplast metabolic pathways are parasite specific and remarkably divergent from the human counterparts, thus presenting drug targets unique to the parasite. Apicoplast localized Type II fatty acid biosynthesis is one such potential drug target. Both *P. falciparum* and *T. gondii* is sensitive to certain FAS II inhibitors. Though the therapeutic potential of several FAS II enzymes are actively being investigated, the biological functions of apicomplexan FAS II are yet to be characterized. To determine the validity of FAS II pathway as a potential drug target, we engineered a genetic model of Tg FAS II pathway and characterized its functions in *T .gondii*. Our findings presented in chapter 4 are of significance to the development of anti-parasitic drugs based on FAS II pathway, and offers hope for the exploitation of this pathway for the development of future therapeutics against both *Plasmodium falciparum* and *Toxoplasma gondii*.

Figure 2.1: The Secondary endosymbiotic origin of the apicoplast. Secondary endosymbiosis is a two step engulfment process. First, a cyanobacterium was endocytosed by a eukaryote, generating primary plastids. Genes were transferred from the bacterial genome to the host nucleus. A subsequent endocytosis, involving the engulfment of the autotrophic eukaryote harboring the primary endosymbiont by the ancestors of present day apicomplexan parasite, marks the origin of the apicoplast. A second round of gene transfer occurred from the endosymbiont's chloroplast and nuclear genome to the host nuclear genome. Unlike the double membranes of primary plastids, the apicoplast is surrounded by four membranes. The inner two membranes represent membranes acquired from the *cyanobacterium*. The third membrane from inside (periplastid membrane) derives from the endosymbiont plasma membrane. The outermost membrane results from the host phagosome/ endomembrane system.

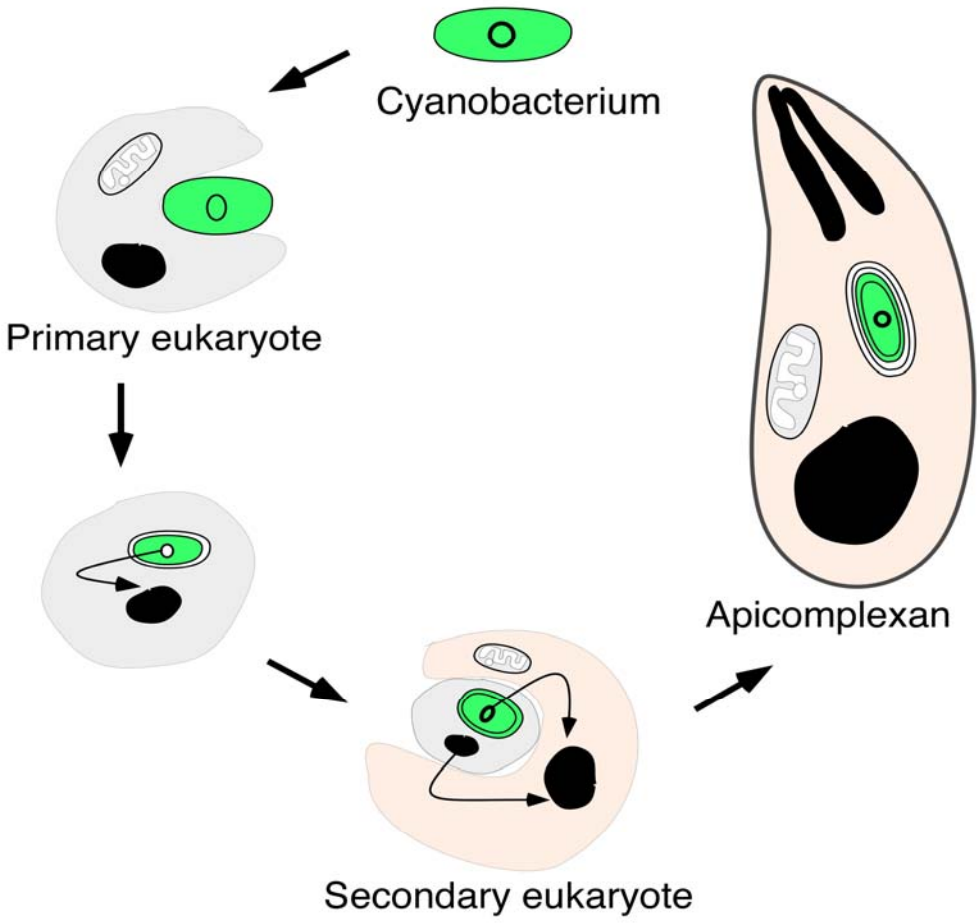

Figure 2.1. The secondary endosymbiotic origin of the apicoplast

Figure 2.2: The Chromalveolate tree of life. Chromalveolate hypothesis proposes a common origin for plastids among chromista and alveolata. It suggests all secondary plastids with a red algal affiliation are monophyletic. Chromista (heterokonts, cryptophytes and haptophytes) and Alveolata (apicomplexan, dinoflagellates and ciliates) diverge after acquisition of the secondary plastid. This hypothesis predicts that members of chromista that lack a plastid and apicomplexans , ciliates and dinoflagellates that lack a photosynthetic plastid, lost the capability to photosynthesize subsequent to their divergence. In contrast, secondary plastids of green algal origin results from several independent endosymbiotic events.

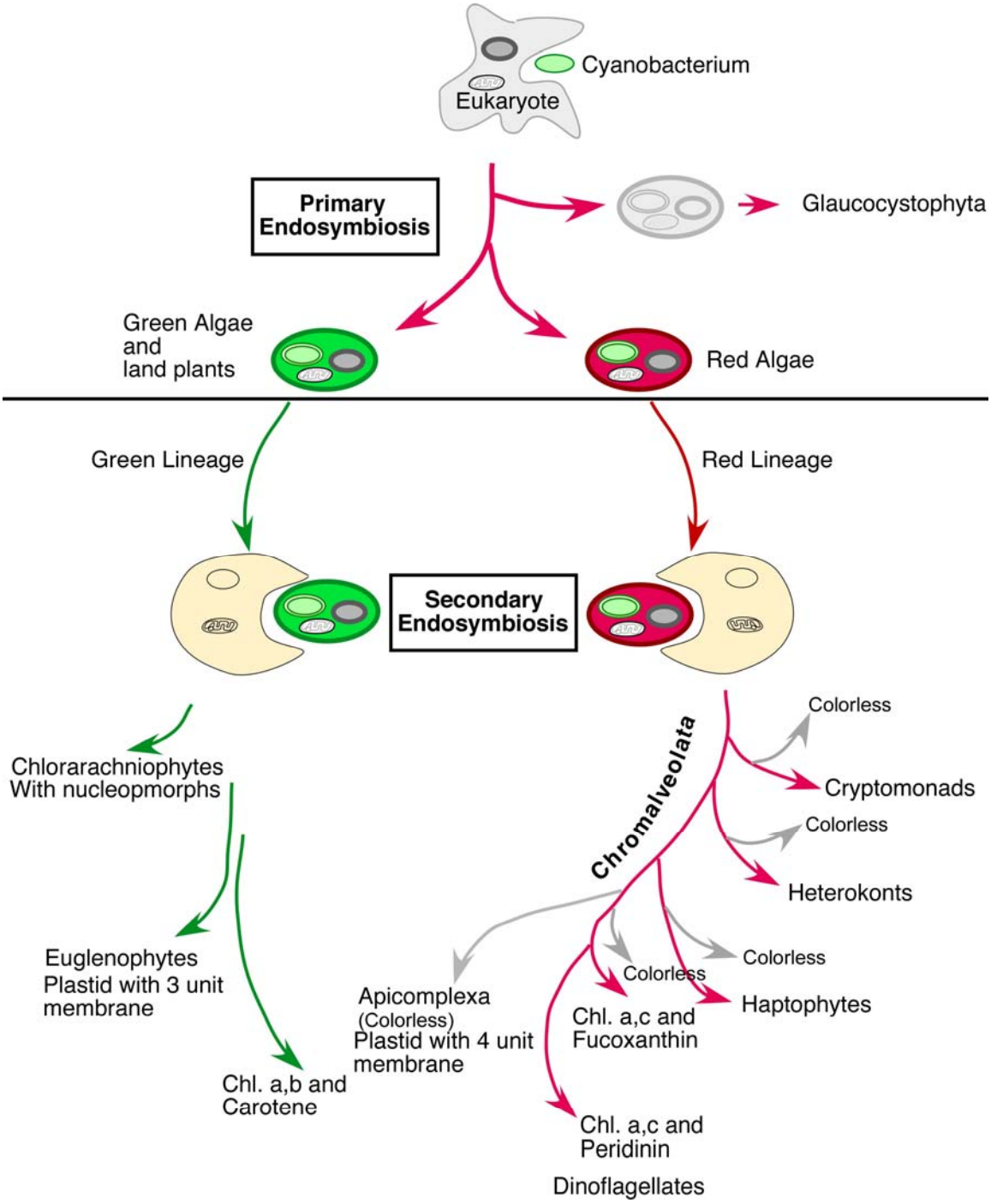

Figure 2.2. Schematic representation of the "Chromalveolate" tree of life (Adapted from Delwiche, 1999).

Figure 2.3: Hallmarks of bacterial and apicoplast division models.

(A) Bacterial division is characterized by the appearance of the FtsZ ring at the septum of the dividing bacteria. The localization of the Z ring is facilitated by molecular chaperones minD, minE and Arc6. **(B)** Apicoplast division proceeds in the absence of Z ring and is intimately linked to parasite division (Striepen et al., 2000). 1) In non-dividing parasites, the apicoplast is located apical to the nucleus and the apicoplast genome is associated with a single centriole. 2) Following initiation of parasite division, the plastid genome is replicated and divided into two equal portions, each associating with the newly divided centriole. 3) The intranuclear spindle forms and the dumb-bell shaped plastid migrate to lie on top of the dividing nucleus, with the centrioles attached to the spindle. 4) As the spindle extends, the plastid is pulled into a U shape and ultimately cut by the growing daughter cells. 5) Each half of the plastid remains associated with the centriole and migrates into the budding daughter parasites with dividing nucleus. 6) Post nuclear fission each daughter nucleus relocates to the center of the dividing daughter parasites. Parasite division is completed when the fully divided and assembled daughters bud out of the mother cell.

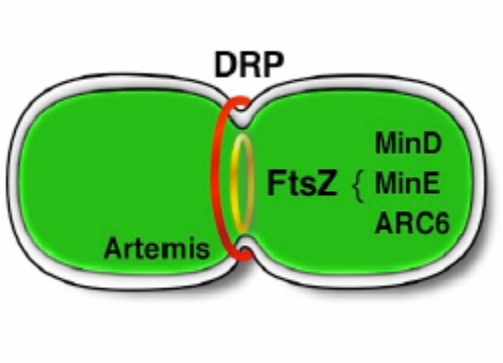

Figure 2.3 A) The FtsZ ring is the hallmark of bacterial cell division

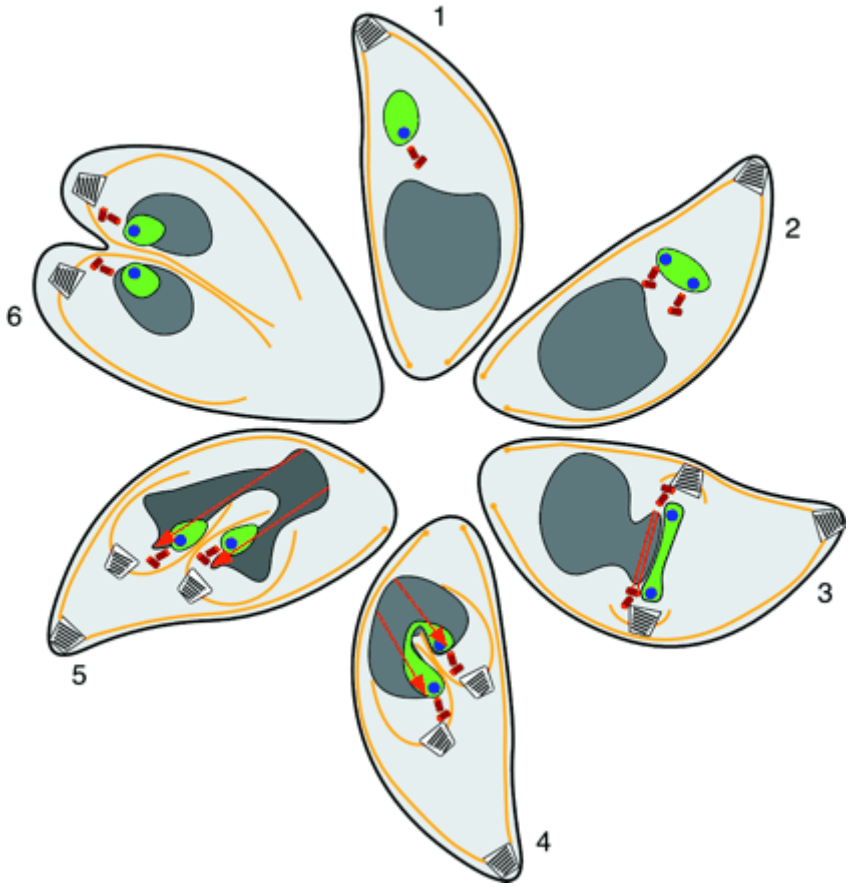

Figure 2.3 B) Model illustrating the molecular link between apicoplast and parasite cell division (Striepen et al., 2000).

Figure 2.4: Apicoplast protein import. Nuclear encoded apicoplast proteins are targeted via a bipartite N-terminal signal sequence. The extreme N-terminal signal sequence targets the protein to the ER. The transit peptide subsequently targets the protein to the apicoplast. Following translocation of the protein across the apicoplast inner membranes into the lumen, the transit peptide is cleaved by a resident peptidase, such as plpC. Western blot analysis of apicoplast proteins correctly detects both precursor and mature protein bands. Molecular mechanisms directing the protein from the ER to the apicoplast, as well as signals mediating transport across the periplastid membrane are currently unknown.

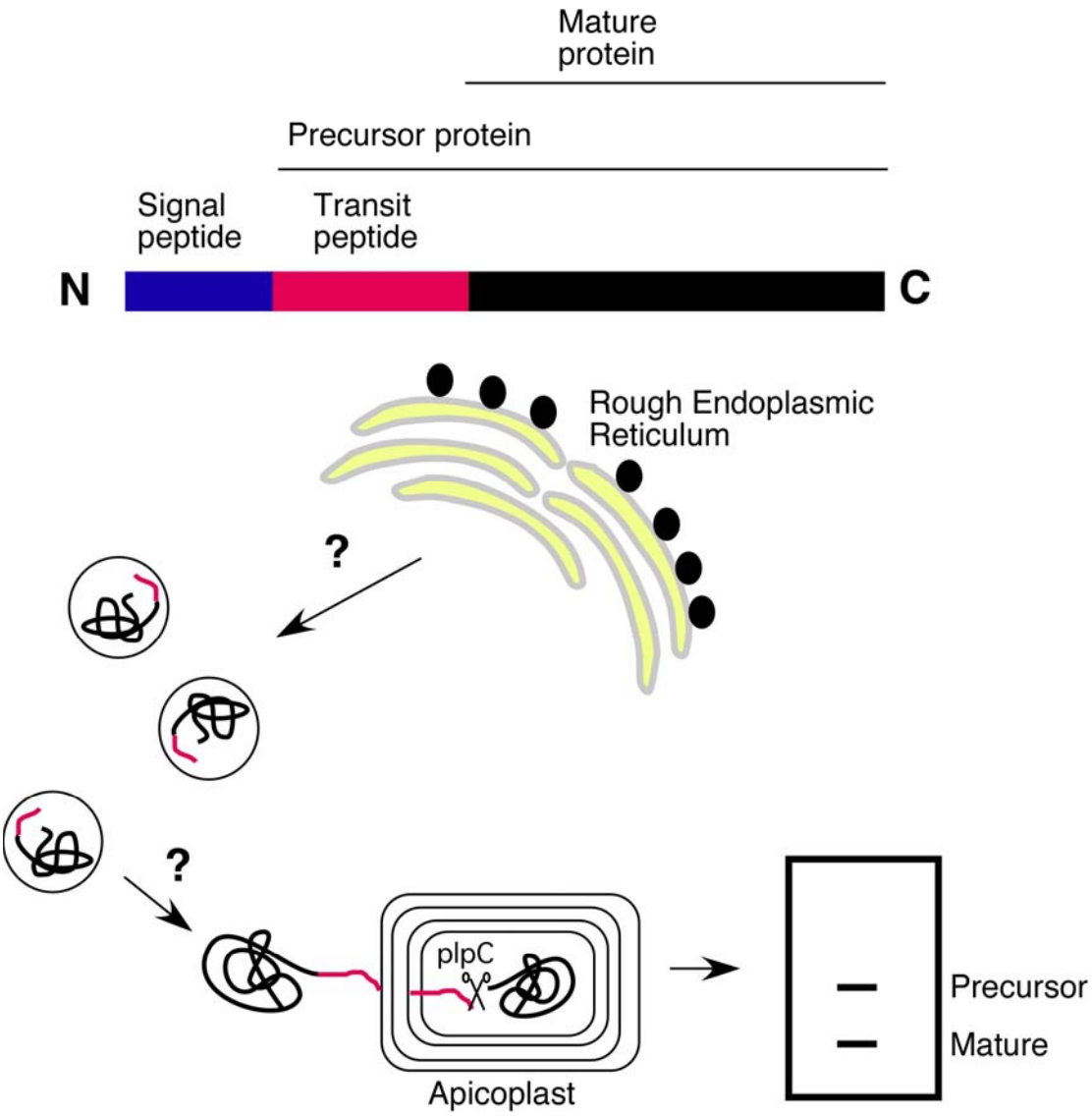

Figure 2.4. Apicoplast protein import

Figure 2.5: Models for protein trafficking across the periplastid membrane. A) The vesicle shuttling theory. Apicoplast proteins shuttle across the periplastid membrane bound in vesicles. The proteins are released in the space between the outer and inner plastid membranes, eliminating interaction between Toc components and the proteins. It fails to explain the presence of Toc components present on the cytosolic side of the outer plastid membrane. **B)** The membrane pore model. Large pores in the periplastid membrane facilitate protein trafficking. This model eliminates the need for special targeting signal, but does not account for reverse flow of proteins into the periplastid space. **C)** The two way vesicle shuttling model. Toc components from the outerplastid membrane are integrated in the periplastid membrane via vesicle shuttling. However, such a shuttling will lead to integration of components in the wrong orientation preventing the interaction of Toc components with transit peptides. **D)** Dual targeting hypothesis. Toc components are targeted in the right orientation to both the periplastid membrane and According to this theory apicoplast proteins could traffick across the periplastid and the outerplastid membrane. No mechanism capable of dual targeting the Toc components, to the cytosolic side of the periplastid membrane and the outer plastid membrane, has been elucidated.

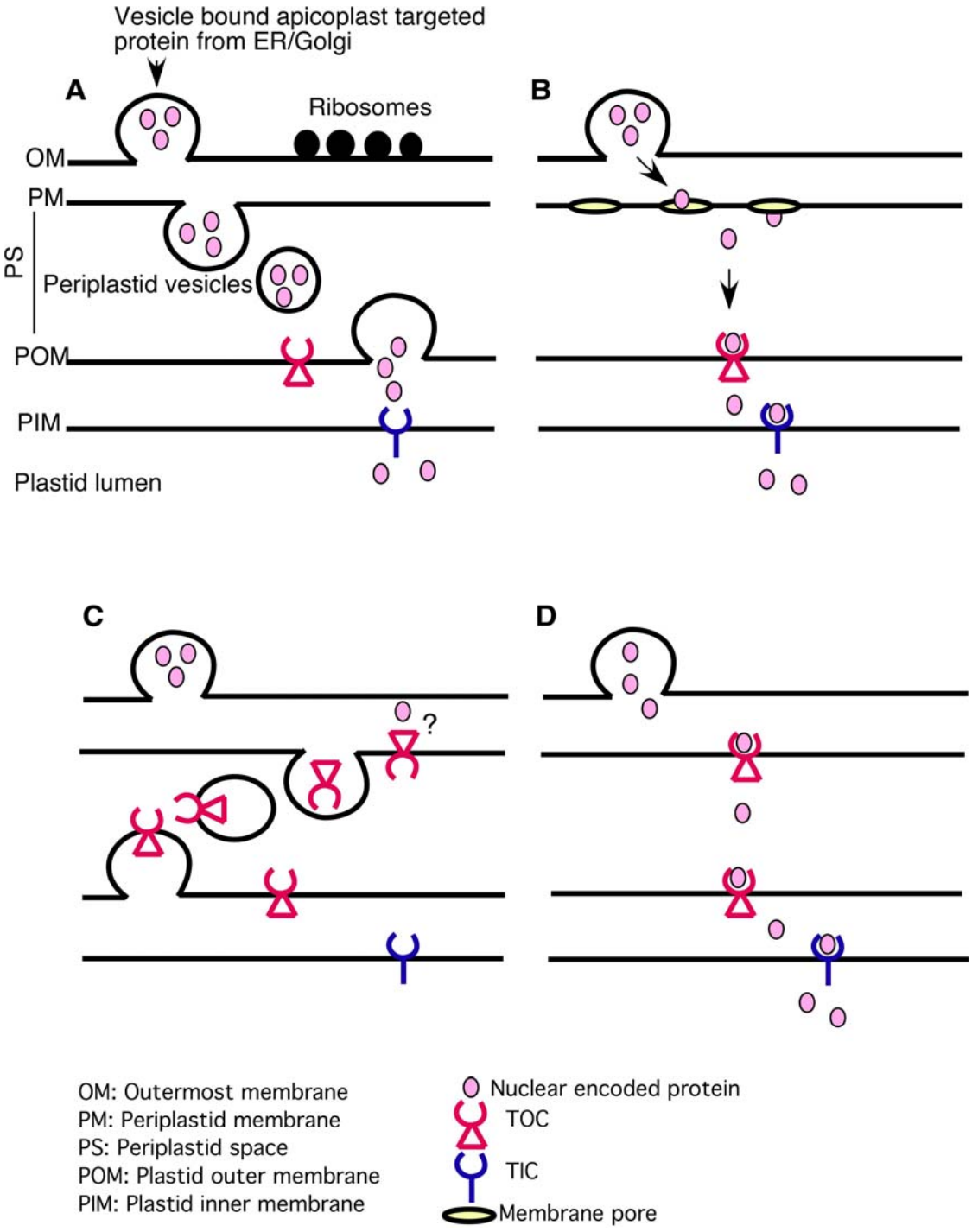

Fig 2.5. Models for protein trafficking across the periplastid membrane

Table 2.1: *T. gondii* genome encodes the complete set of FAS II enzymes. The structural arrangement of FAS II is characterized by the distinct presence of the enzyme components as individual genes. All seven components (six enzymes and one carrier protein) have been identified in the *T. gondii* genome (Fig A1 provides general information on FAS II enzymes, reactions catalyzed and immediate downstream functions in the apicoplast). Table 1 represents the gene ID of the individual FAS II proteins as identified in ToxoDB release 3.1. Sequence analysis placed these proteins nearest to corresponding proteins from other apicomplexans and plants, systems known to harbor FAS II enzymes.

Table 2.1: ToxoDB identification of *T. gondii* FAS II enzymes

Gene	*T. gondii* (Query)	Gene Bank Nearest neighbour	p-score	Chromosome/ Scaffold ID
Malonyl-CoA-[acyl-carrier-protein] transacylase (*fabD*)	TgGLEAN_6300	*Brassica napus* CAB45522	$3e^{-43}$	X 995361
3-oxoacyl-[acyl-carrier-protein]III (*fabH*)	TgTwinScan_5818	*Nostoc spp.* BAB77763	$6e^{-95}$	VIII 995362
3-oxoacyl-[acyl-carrier-protein] reductase (*fabG*)	TgTwinScan_1472	*Plasmodium falciparum* CAD51191	$3e^{-51}$	XII 995286
Beta-hydroxyacyl-[acyl-carrier-protein] dehydratase (*fabZ*)	TgGLEAN_2150	*Toxoplasma gondii* AAC72191	$8e^{-129}$	1b 995334
		Plasmodium falciparum AAM75408	$2e^{-43}$	1b 995334
Enoyl-[acyl-carrier-protein] reductas (*fabI*)	TgGLEAN_8302	*Toxoplasma gondii* AAQ74987	0.0	XII 995291
		Brassica napus CAC41369	$3e^{-104}$	XII 995291
3-oxoacyl-[acyl-carrier-protein] synthase I/II (*fabB*)	TgTwinScan_0386	*Plasmodium falciparum* CAG25076	$6e^{-126}$	1a 994723
Acyl carrier protein (*acp*)	TgTwinScan_7096	*Toxoplasma gondii* AAC63956	$3e^{-95}$	VIIb 995636
		Fusobacterium nucleatum AAC94356	$2e^{-20}$	VIIb 995636

Table 2.2: A list of newly identified apicoplast enzymes with putative roles in carbon and energy generation in the *T. gondii* genome. Presence of several apicoplast localized metabolic pathways is confirmed in *T. gondii*. Our search for enzymes, with putative functions in the generation of precursor for fatty acids, isoprenoids and cellular energy has yielded promising candidates. Some of these enzyme isomers harbor the N-terminal hydrophobic signal, a characteristic of apicoplast targeted proteins. Further bioinformatic analysis needs to be done to confirm the organellar affiliation of some of the other proteins lacking the obvious apicoplast targeting signal. (TwS: Tg TwinScan), (TgS: Tg GlimmerScan), (GLN: Tg GLEAN)

Table 2.2: Identification of apicoplast genes involved in carbon and energy generation

Gene	Query	*T.gondii*	p-score	SP	GBNN	p-score
Phosphate/ Phosphoenol pyruvate translocator (PPT)	*Arabidopsis thaliana* AAB40646	TwS_7513	9.5 e-43	+	*Toxoplasma gondii* AAZ04384	0.0
					Chromobacterium violaceum NP_901854	2 e-55
Pyruvate Kinase (PK)	*Plasmodium falciparum* NP700836	TgS_6611	5.1 e-69	+	*Nitrobacter hamburgensis* ZP00626051	1 e-81
Phosphate/ Triose phosphate transporter (TPT)	*Arabidopsis thaliana* AAC83815	TwS_3278	1.1 e-47	+	*Cryptosporidium parvum* XP_627044	1 e-37
		TwS_6854	8.1 e-0.5	-	*Plasmodium falciparum* CAD51448	6 e-75
Triose phosphate isomerase (TPI)	*Arabidopsis thaliana* 2009415A	TwS_5952	1.4 e-53	+	*Secale cereale* CAA81487	3 e-52
		TwS_5404	2.1 e-62	-	*Tetrahymena thermophila* EAR88549	1 e-70
Glycerol phosphate dehydrogenase (Gpd A)	*Arabidopsis thaliana* NP187426	GLN_1006	9.1 e-07	+	*Theileria annulata* CAI73748	9 e-92
		GLN_1825	4.6 e-07	-	*Theileria annulata* CAI73748	7 e-102
Cysteine desulfurase (SufS)	*Arabidopsis thaliana* AAK00758	TwS_0723	2.7 e-06	+	*Cryptosporidium parvum* XP_625374	2e -104
		TwS_1029	4.3 e-131	- short	*Danio rerio* XP_691182	2 e-136
δ-aminolevulinate dehydratase (ALAD/Hem B)	*Arabidopsis thaliana* CAB 65342	TgS_2021	1.6 e-50	?	*Odontella sinensis* CAC36186	3 e-88
Uroporphyrinogen synthase III (Hem D)	*Arabidopsis thaliana* CAC85827	TgS_1739	2.1 e-05	+ short	No hit	
Uroporphyrinogen decarboxylase (Hem E)	*Arabidopsis thaliana* NP 974316	GLN_1257	5.7 e-35	?	*Salinibacter rubber* YP_445821	5 e-85
Ferrochelatase (Hem H)	*Arabidopsis thaliana* CAA51819	GLN_5447	7.1 e-18	?	*Thiobacillus denitrificans* YP_315791	6 e-65

CHAPTER 3

A NOVEL SELECTION FOR THE EFFICIENT DETECTION OF ALLELIC REPLACEMENTS IN *TOXOPLASMA GONDII* [1]

[1]Jolly Mazumdar and Boris Striepen. Manuscript to be submitted to Cytometry A.

Abstract

Targeted gene deletion mediated by homologous recombination is a powerful tool for functional gene analysis. But the high frequency of non-homologous recombination in *Toxoplasma gondii* often hampers the efficiency of the process. Exploiting the parasite's ability to fluoresce following genomic integration of yellow fluorescent protein (YFP), we present here a simple selection strategy aimed to increase the frequency of double homologous recombinants in *T. gondii*. In addition to the incorporation of the chlorampheicol resistant CAT gene between flanking sequences homologous to the target gene (in this case acyl carrier protein, ACP) for positive selection of transformants, we incorporated a second marker, YFP outside the homologous sequence, to obtain counter selection against the fluorescent non-homologous recombinants. Of all stable transformants isolated by chloramphenicol selection, only one percent of the population was non-fluorescent, of which ~10% were positive for allelic replacement. Absence of native ACP locus was confirmed by PCR and Southern analysis. The enhanced enrichment of double homologous recombinants offered by CAT-YFP selection, over CAT (chloramphenicol) selection alone demonstrates the effectiveness of YFP negative selection. YFP can be used in all parasite background and expressed independently as a transgene, offering distinct advantages over existing negative selection strategies.

Abbreviations: ACP (acyl carrier protein); CAT (chloramphenicol acetyl transferase); YFP (yellow fluorescent protein) and FACS (fluorescence activated cell sorting)

Keywords: *Toxoplasma gondii*, homologous recombination; allelic replacement; positive- negative selection

3.1: Introduction:

Apicomplexan parasites cause severe human diseases and urgently needs novel drug targets. Hope for new anti-parasitic drugs rely on the recently discovered apicoplast (McFadden et al., 1996), an essential organelle which harbors parasite specific pathways and processes. The recent sequencing of both *P. falciparum* and *T. gondii* genomes (Gardner et al., 2002; Kissinger et al., 2002; Kissinger et al., 2003) have unveiled a plethora of encoded proteins, many of which are apicoplast localized. However, most of these proteins lack functional characterization. One such class of proteins includes the components of apicoplast localized Type II fatty acid synthase system (FAS II), whose therapeutic potentials are now actively being investigated (McLeod et al., 2001; Sharma et al., 2003; Surolia and Surolia, 2001; Waller et al., 1998; Waller et al., 2003). FAS II pathway is essential in bacteria; but the critical nature of this pathway in apicomplexans is yet to be tested. To elucidate the biological functions of apicomplexan FAS II, we attempted to engineer a FAS II mutant by the conditional knock-out of its central component, ACP in the model apicomplexa, *T. gondii*.

Gene knock-outs offer a powerful tool for the functional analysis of genes. However, direct gene knock-outs are limited to the characterization of non-essential gene functions, since strains carrying null mutations in essential genes are not recoverable for further analysis (Kim et al., 1993; Mital et al., 2005).“Conditional gene knock-out”, an alternative approach which combines gene inactivation with controlled gene expression, permits the analysis of essential genes. It allows a selected gene to be regulated, and thus offer strategic depletion of the gene under study. Recently, a tetracycline controlled gene expression system (*Tg* Tet-R) has been developed for *T. gondii* (Meissner et al., 2002).

The construction of a conditional gene knock-out is a two step process, and involves the stable expression of a regulatable transgene, followed by the deletion of the native locus mediated by double homologous recombination (Donald and Roos, 1994; Thomas and Capecchi, 1987). The technique of gene deletion or allelic replacement relies on the homologous exchange of an endogenous gene portion with exogenous DNA carried on a targeting vector, introduced into the cells. A targeting vector is typically characterized by a linear array of wild type sequence flanking on both sides of an altered gene sequence, or a drug marker (Thomas and Capecchi, 1987). Once within the cell, double recombination between the homologous pair present on the targeting vector and the genome, specifically replaces the native gene with the selectable marker, offering a phenotype for positive selection (Folger et al., 1984; Kim et al., 1993; Thomas and Capecchi, 1987) (Fig 3.2a).

Though positive selection has been employed to generate mutants in *T. gondii* (Donald and Roos, 1994; Kim et al., 1993; Meissner et al., 2002; Mital et al., 2005; Soldati et al., 1995), they are often time consuming and inefficient. Mutant screens based solely on positive selection are frequently hampered by significant false positive background, generated as a result of the parasite's natural tendency for non-homologous recombination (Brecht et al., 1999; Donald and Roos, 1994).

An alternative approach, that significantly enriches rare events of homologous recombination in a population, employs combined "positive-negative" selection of the transformants (J. Stougaard, 1993; Terada et al., 2002; Thykjaer et al., 1997). In addition to a positive marker provided between the flanking wild type sequences in the gene replacement cassette, positive-negative selection incorporates a second marker outside the homologous region in the cassette. Integration dictated by non-homologous recombination incorporates the negative

marker in the genome, and offers a phenotype for counter selection against non-homologous recombinants (Thykjaer et al., 1997) (Fig 3.3a). Present strategies for negative selection in *T.gondii* include the use of parasite genetic markers uracil phosphoribosyl transferase (UPRT) (Donald and Roos, 1995) and hypoxanthine-xanthine guanine phosphoribosyl transferase (HXGPRT) (Donald et al., 1996), bacterial cytosine deaminase (CD) (Fox et al., 1999) and the Herpes simplex virus thymidine kinase (HSTK) (Fox et al., 2001; Radke and White, 1998).

To its disadvantage, the use of UPRT and HXGPRT negative selection is restricted to parasite strains lacking these genes or the corresponding metabolic pathways (i.e UPRT$^-$ and HXGPRT $^-$ parasite genetic background) (Donald et al., 1996; Donald and Roos, 1995). HSTK and CD, which confers sensitivity to ganciclovir (Iltzsch, 1993) and 5-fluorouracil (Donald and Roos, 1995; Pfefferkorn, 1978), respectively, can be used in any genetic background, but it cannot be expressed by an endogenous promoters and works when fused to the bi-functional Dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene or CAT gene (Fox et al., 1999; Fox et al., 2001; Radke and White, 1998). The designing of the conditional gene expression system around *T.gondii* Rh strain (Meissner et al., 2002), a cell line positive for both HXGPRT and UPRT, and the technical necessity for the counter selection gene to be expressed independently of DHFR-TS or CAT genes in the targeting vector, calls for the development of alternative selection strategies.

Naturally fluorescent proteins (green/yellow/red) have recently become available for stable expression in *T.gondii* (Kim and Boothroyd, 1995; Striepen et al., 1998). Typically used for the observation of cellular processes (Gubbels et al., 2003; Gubbels and Striepen, 2004; Gubbels et al., 2004; Striepen et al., 2000), the fluorescent phenotype generated by genomic integration of fluorescent transgenes, offers convenient detection by fluorescence- activated cell

sorter (FACS) (He et al., 2001; Striepen et al., 1998). We exploited this property of YFP, a variant of green fluorescent protein (Cubitt et al., 1999), to develop a screen for the counter selection of homologous recombinants. We incorporated YFP outside of the 3' homologous sequence in the ACP targeting construct harboring CAT, between the flanking sequences. FACS analysis of stable transformants post chloramphenicol selection produced two distinct peaks, corresponding to fluorescent parasites generated by the ectopic integration of YFP and non-fluorescent parasites, representing homologous recombinants. In confirmation with previous observations of high frequency of non-homologous recombination in *T. gondii* (Brecht et al., 1999; Donald and Roos, 1994), fluorescent parasites constituted almost 99% of the entire chloramphenicol resistant population.

CAT-YFP selection offered a 1000 fold enrichment of homologous recombinants. In striking contrast to our earlier futile attempts to isolate ACP knock-outs in a large screen of parasites (> 400 parasite clones) in the absence of negative selection, we isolated 2 mutants lacking ACP gene in 25 clones, when we specifically screened the non-fluorescent population.

3.2: Materials and methods

Parasite cell lines and selection

RH strain *T. gondii* tachyzoites (and all cell lines derived from the strain) were serially passaged in primary human foreskin fibroblast (HFF) cells as described previously (Roos et al, 1994). HFF cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated bovine serum in the presence of 2 mM glutamine, penicillin, streptomycin and gentamycin. Host medium was replaced with DMEM containing 1% dialyzed fetal calf serum prior to infection and supplemented with 1 μg/ml anhydrotetracycline (ATc) (IBA, St.

Louis, MO) where indicated. Freshly released extracellular tachyzoites were filtered through 0.3μM nucleopore polycarbonate membrane filter, centrifuged at 1500 x g for 20 minutes, re-suspended in 1X Phosphate Buffered Saline (pH 7.4) and counted for various experiments. To generate stable transformants $3x10^7$ freshly lyzed tachyzoites were transfected with 50μg DNA and selected with 1μM/ml pyrimethamine (Donald and Roos, 1993) or 10 μM chloramphenicol (Kim et al., 1993), according to the selectable marker provided in the transformation vector.

Plasmid construction and generation of cell lines

To generate ptet07sag4-ACPmyc/sagCAT, the parent vector ptubYFP^1-YFP^2/sagCAT (Gubbels et al., 2003) was sequentially modified (Fig. 3.1A). First, YFP^1 coding sequence downstream of promoter tub was exchanged with ACP coding region with *Bgl* II/ *Avr* II to generate ptub-ACPYFP/sagCAT. The 549 bp ACP fragment was amplified from Tg cDNA (Waller et al., 1998) with oligonucleotides ACP-1 5' TGAAGATCTATGGAGATGCATCCCCGCAAC 3' and ACP-2 5'ATGCCTAGGCTTGGCTTTCTCGATATAG 3'. Next tub promoter region was replaced with tetO7sag4 using PpuM I/Bgl II. The tetO7sag4 fragment was amplified from p7tetOS4LacZCAT (Meissner et al., 2001) with oligonucleotides tetO7-1 5'CCAAGGACCCGGTACCGAGCTCGACTTTCAC 3' and tetO7-2 5'GCAGATCTGGTTGAAGACAGACGAAAGCAGTTGC 3'. The resulting plasmid ptetO7sag4-ACPYFP/sagCAT was modified to replace YFP-dhfr with epitope tag myc-dhfr3' retrieved from pgra1-PCNA-cmyc-dhfr/HXGPRT with *Avr* II/*Not* I digestion. Lastly, sagCAT was replaced with DHFR-TS drug resistance cassette retrieved from TSc3ABP, described previously (Striepen et al., 2002) with PpuMI – XbaI/ApaI end filling, to generate ptet07sag4-ACPmyc/DHFR-TS. To generate ACP/ACPi cell line, RH Tati tachyzoites (Meissner et al., 2002) were transformed with ptetO7sag4-ACPmyc/DHFR-TS and selected with pyrimethamine.

The strain RH-YFP was generated by transformation with ptub-YFPYFP/sagCAT and selection with chloramphenicol.

Targeting of the native ACP locus

pACP-KO-CAT/YFP was engineered on the backbone of a CAT vector, a kind gift of Martin Gasten (University of Dusseldorf, Germany) and flanked on both sides with ~1.0 and 3.1 kb of 5' and 3' flanking sequences of *acp* gene. 5' ACP flanking sequence was amplified from Tg gDNA using olignucleotides ACP-KO-1 5'GCTGGTACCTGCCTCATCGCCAGAATG-3' and ACP KO-2 5' TCCAGATCTTAGTTCTGGTGTGAGAGGTGTGCA-3 and cloned with *Kpn*I/*Bcl*I. ACP 3' flanking sequence was amplified from gDNA using oligonucleotides ACP-KO-3 5'ATGGATTCTATTTTCCTCGTGTCGGCGTTGTG-3' and ACP-KO-4 5' TCTGCGGCCGCTTACTTGTACAGCTCGTCCATGCCG-3' with *Pac*I/*Not*I. tubYFP fragment was amplified from ptubYFPYFP/sagCAT with oligonucleotidesYFP-1 5'ATGCATATGATGCATGTCCCGCGTTCGTGA-3' and YFP-2 5' TCTGCGGCCGCTTACTTGTACAGCTCGTCCATGCCG-3' and incorporated downstream of ACP 3'flanking sequences with *Nde*I/*Not*I. Prior to transformation, the vector was linearized with *Kpn*I/*Not*I, and gel purified to remove the bacterial backbone of the vector to raise the frequency of double homologous recombination. ACP/ACPi cells were transformed with pACP-KO/CAT and selected with chloramphenicol.

pTgACP-KO/DHFR-TS was a kind gift from Michael Crawford (University of Pennsylvania, Philadelphia, PA). Remaining pKO-ACP-CAT vectors were generated by PCR amplification of specific sequence lengths from gDNA with elongase reaction mix (Invitrogen, Carlsbad, CA) and incorporation into CAT vector (Fig.3.2B).

Counter selection of heterologous recombinants by FACS

For flow-cytometric analysis of parasites, 1 x 10^6 freshly released parasites were suspended in 1ml 1X PBS. Three hundred thousand cells were analyzed on a MO-FLO instrument (Cytomation, Fort Collins, CO) using a 488 nm argon laser for excitation and an emission filter with a band pass of 530/540nm. The data were managed with Summit version 3.1 (Cytomation) and prepared for publication with Flowjo version 4.0.2 (Treestar, San Carlos, CA). Non-fluorescent parasites were sorted into the wells of a 384-well tissue culture plate (Falcon-Becton Dickinson, Franklin lanes, NJ) containing 50 µl ED1 media and clonally expanded.

Molecular analysis of ACP mutants

PCR screening: Genomic DNA was isolated from *T. gondii* ACP/ACPi and RH strains by phenol extraction, as described previously (Sambrook et al., 1982). Clones were screened for presence or absence of native *acp* by PCR amplification with oligonuleotides ACP-KO1 5' TTCCGACTGGACTGCACACCTCTCACACCAGAACT3' and ACP-KO2 5'CTCCAGTGATCGTATGTCTCCTTGAGACTCTTA-3'. Mutants lacking the ACP locus were detected by the amplification of a single 1.3kb product. Wild type RH amplified a 1.5kb product and ACP/ACPi heterologous recombinants amplified both 1.5kb and 1.3kb products.

Southern blot analysis: Loss of native ACP and gene copy number of CAT was tested by Southern blot analysis. Genomic DNA (5µg) was digested overnight with *Bgl*II, *Bam*HI and *Xho*I, restriction endonucleases, the fragments were separated on a 0.8% agarose gel and transferred to nitrocellulose membrane. Probes were prepared as follows. P1, a 500 bp amplification product of ACP intron sequence amplified from RH gDNA with oligonucleotides ACP intron-1 5' ACAACGGTGTCTCGGACGGAGTCGTTGCAAGTC-3' and ACP intron-2 5'-TCTGGTTAACCAACTGGGTTGTTCAACGAGGCA-3'. P2 (CAT coding region) and P3

(3’ flanking sequence of the genomic locus) was retrieved from ptub-CAT-GFP and pKO-ACPYFP/sagCAT vectors with BglII/AvrII and XhoI/BglII restriction digestion respectively. All probes were gel purified and labeled by random priming (Gibco, Gaithersberg, MD). Membrane was hybridized at 42°C in a 50% formamide solution, nitrocellulose blots were washed three times in 2× SSC (0.30 M NaCl plus 0.030M sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS) at room temperature (10 min each) and then twice in 0.1× SSC containing 0.1% SDS at 42°C (30 min each). Signals were detected by autoradiography. Determination of loss of native ACP gene and presence of CAT coding region was based on comparison of hybridization signals between ΔACP/ACPi strain and wild type RH control.

Expression and regulation of ACPi

Immunofluorescence microscopy: Confluent monolayers of HFF cells were grown on coverslips in six-well plates, and infected with freshly lysed tachyzoites of the strains. Cells were fixed, usually 36 hours post infection for 10 minutes with 3% paraformaldehyde in PBS and subsequently permeabilized for 10 minutes with 0.25% Triton X (w/v) in PBS. Endogenous ACP was detected with α-ACP affinity purified polyclonal rabbit antiserum (1:1000) (Waller et al., 1998), kindly provided by G.I. McFadden (University of Melbourne, Australia). To detect ACPi carrying c-Myc epitope tag, parasites were immunostained with α-cmyc antibody (monoclonal 9E10) (1:1000). Primary antibodies were diluted in 1%BSA in PBS and detected using Alexa Fluor-488 or 546 goat anti rabbit or anti mouse antibodies (Molecular Probes now Invitrogen). DNA was stained by incubation with 2μg/ml 4’6’-diamino-2-phenylindole (DAPI), (Molecular Probes) for 10 minutes. Excess stain was removed by washing, and coverslips were mounted by Gel/ Mount (Biomeda Corporation, CA). Fluorescent images were captured with a CCD camera

(C474295-Hamamatsu) on a Leica DMIRBE microscope. Image acquisition, contrast adjustment and channel merging were performed using openlab software (Improvision).

Western blot analysis: Lysate from freshly released $5x10^6$ parasites were separated on NuPAGE-Novex 12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred on Protran nitrocellulose membranes, 0.45μm (Whatman Schleicher and Schuell BioScience, Keene, NH) for western analysis. The membrane was immunoblotted with primary antibodies 9E10 (1:2000), α–ACP (1:10,000) and α-Mic2 (1:10,000) and detected with anti-mouse and anti-rabbit HRP labeled secondary antibodies, (1:3000) (Sigma, St. Louis, MO). Signal was developed with the ECL plus chemiluminescence kit (Amersham Pharmacia, Piscataway, NJ) on Kodak BioMax MS autoradiographic film (Fischer Scientific, Pittsburg, PA).

3.3: Results

Isolation of a stable cell line expressing tetracycline regulatable ACP

Acyl carrier protein (ACP), a central component of type II fatty acid biosynthesis pathway (FAS II) is essential for shuttling growing fatty acyl chains between the FAS II enzymes (Wong et al., 2002). To analyze the biological functions of Tg FAS II pathway, we conditionally targeted Tg ACP. The genome of *T.gondii* encodes a single copy of ACP gene (TgTwinScan_7096) (ToxoDB). Therefore, to maintain continued activity of the *Tg* FAS II pathway in the absence of wild type ACP, we first expressed an inducible copy of ACP (ACPi) by ectopic integration into the genome. For this, *T. gondii* RH TAti strain, stably expressing a tetracycline (Tc) responsive transactivator (TAti strain) (Meissner et al., 2002) and endogenous ACP, were transfected with myc-tagged ACP (ACPi) and selected with pyrimethamine (Fig.3.1A). The resulting cell line expressed both endogenous and inducible ACP and was

designated ACP/ACPi. Expression and localization of ACPi was detected by immunofluorescence assay (Fig.3.1B). ACPi fused to myc epitope was detected with α-myc 9E10 monoclonal antibody (Fig.3.1B). Apicoplast targeting of ACPi was confirmed by co-localization with endogenous ACP signal detected with α-ACP polyclonal rabbit serum (Fig.3.1B, right panel). Parasite nucleus was stained with DAPI (Fig.3.1B, left panel). Previously, the addition of ATc has been shown to down regulate the activity of the tetO7sag4 conditional promoter (Meissner et al., 2002). ATc regulation of ACPi was monitored by immunofluorescence assay (Fig.3.1C) and Western blot analysis (Fig.3.1D). ATc treatment specifically ablated the expression of ACPi, detected by the loss of signal with α-myc antibody in both assays (Fig 3.1C, right panel and 3.1D). Mic2 was used as loading control (Fig.3.1D). Expression of endogenous ACP was not affected by ATc treatment (Fig.3.1C, middle panel). A clone demonstrating maximum ACP downregulation with Atc treatment was selected and in this background targeted for native ACP deletion. Absence of endogenous ACP would place the parasites under the direct control Tg ACPi, thus offering a strategy for the conditional determination of the phenotype of Tg ACP deficient parasites.

Tg ACP/ACPi strain demonstrates a high frequency of non-homologous incorporation of pKO-ACP vector

Allelic replacement aims for targeted gene deletion mediated by double homologous recombination, (Fig.3.2A). Detection of recombinants relies on the incorporation of a marker gene, offering a phenotype for selection. Incorporation of the chloramphenicol acetyl transferase (CAT) gene, offering resistance to the drug chloramphenicol, has been frequently employed for isolation of double homologous recombinants in *T.gondii* (Kim et al., 1993; Meissner et al., 2002; Mital et al., 2005). To target ACP, we engineered a knock-out vector, pKO-ACP/sagCAT carrying CAT gene between linear arrays of 5’ and 3’ wild type sequences, flanking the native

ACP locus, and used this vector to transform ACP/ACPi (DHFR-TS/pyrimethamine resistant) strain. Previously, alleles in *T.gondii* have been targeted with varying lengths of wild type sequences ranging from < 1kb to >10 kb (Donald and Roos, 1994; Kim et al., 1993; Mital et al., 2005). We therefore targeted the native ACP locus with several vectors carrying different lengths of wild type sequences (1.0kb to 4.0kb) (Fig 3.2B). To increase the frequency of double recombination, the vectors were linearized prior to transformation and subsequently selected with chloramphenicol. Stable transformants were cloned by limiting dilution and screened for the absence of native ACP by genomic PCR (explained in detail in the next section and Fig 3.3 C and D). However, most clones screened amplified a 1.5 Kb native ACP locus and the 1.3 Kb SagCAT replacement sequences, indicative of heterologous recombination (data not shown). We have also tried replacing native ACP locus with DHFR-TS gene. ACP/ACPi (chloramphenicol resistant) strains were transformed with pKO-ACP/DHFR-TS and similar to previous transformations, revealed high frequency of heterologous recombination (data not shown). The simultaneous amplification of the native ACP locus and the CAT replacement sequence by majority of the clones, confirmed the high frequency of random integration of exogenous DNA in *T. gondii,* as previously reported (Brecht et al., 1999; Donald and Roos, 1994)..

In conclusion, we were unable to isolate double recombinants for ACP locus in a screen of more than 500 parasite clones (400 for pKO-ACP/sagCAT and 100 for pKO-ACP/DHFR-TS), a screen comparatively (>4 fold) larger than conducted for other *T. gondii* genes (Jeffery Mital, personal communication, Michael Crawford, personal communication). To enrich double homologous recombinants in a population we tested alternative strategy. Genomic integration of YFP offers a fluorescent phenotype (Fig 3.2C). Exploiting this property we developed a screen for the counter-selection of homologous recombinants.

Efficient detection of Tg ΔACP/ACPi mutants using double marker strategy

Combined "positive negative" selection offers selective enrichment of homologous recombinants (Fig 3.3A) and has been employed to isolate double recombinants in direct gene knock-out experiments in several systems including the apicomplexan parasite *Plasmodium falciparum* (Duraisingh et al., 2002), fungus (Takahashi et al., 2004), yeast (Hartzog et al., 2005) and plants (Miao and Lam, 1995). However, positive- negative selection for the generation of conditional knock-out mutants is yet to be reported in apicomplexan parasites. To gain counter-selection against non-homologous recombinants in *T. gondii*, we engineered a new KO vector with a tub-YFP expression unit incorporated outside the 3' region of homology (Fig.3.3A). As shown in Fig 3.2C, incorporation of YFP generates fluorescent parasites (Fig. 3.3B green dash) which can be easily distinguished from non-fluorescent parasites (Fig. 3.3B black dash) in a fluorescence-activated cell sorter (FACS) (Fig 3.3B). According to the experimental design, integration of pKO-ACP-YFP by non-homologous recombination would incorporate YFP, generating fluorescent parasites, while homologous integration will maintain the parasites in their non-fluorescent state (Fig.3.3A). Following stable selection of transformants with chloramphenicol, we subjected more than 300,000 transformation events to negative selection. FACS analysis indicated the presence of two distinct chloramphenicol resistant populations (Fig 3.3B). While fluorescent transformants representing heterologous recombinants constituted almost 99% of the population, approximately one percent (3000 parasites) of the population was non-fluorescent (dark phenotype) (Fig.3.3B.). Dark parasites were sorted and clones were screened for the absence of native ACP locus by genomic PCR (Fig.3.3 C and D). Sequence

specific primers were designed to generate only a 1.3 kb product in the mutant locus (Fig 3.3C). Mutants carrying a deletion in the native ACP locus amplified a single 1.3kb product (Fig 3.3D). ACP/ACPi heterologous recombinants amplified both native ACP (1.5kb) and sagCAT sequence (1.3kb) (Fig 3.3D). Wild type RH and pKO DNA served as control for endogenous ACP and sagCAT amplification product, respectively (Fig.3.3D). Double homologous mutants lacking native ACP locus was detected at a high frequency of 1 in 12 non-fluorescent parasites. Loss of native *acp* locus was also confirmed by Southern analysis (Fig.3.3E). Compared to wild type RH control, when probed with P1 (*acp* intron sequence), P2 (CAT coding region) and P3 (3'flanking genomic sequence), the mutant DNA hybridized only with P2 and P3 (Fig 3.3E), confirming the replacement of native ACP by site specific integration of a single copy of CAT gene. The mutant lacking native ACP locus was designated ΔACP/ACPi.

3.4: Discussion

We attempted to engineer a mutant of Tg FAS II by the conditional knockout of its central component, ACP, for the functional analysis of apicoplast localized *T.gondii* FAS II pathway. For this, we took advantage of a conditional gene expression system recently developed for *T.gondii* (Meissner et al., 2002). A prerequisite of this technique, we first generated a cell line with stable expression of an inducible copy of ACP (ACPi). Simultaneous expression of both inducible and native copies of ACP, allowed us to make the parasites dependent on ACPi, and offered a strategy to down regulate ACP for phenotypic analysis of ACP deficient parasites. In the background of ACPi expression, we targeted the native ACP locus with different replacement vectors. We relied on positive selection with the drug chloramphenicol for detection of stable transformants. We next screened stable clones for

mutants lacking the native ACP locus. To our disappointment, our screen indicated a very high frequency of heterologous recombination and we were unable to isolate the desired mutants.

To enrich for homologous recombinants, we next considered combined positive-negative selection of transformants, a strategy which counter selects hemologous recombinants. Available *T.gondii* markers are technically limited for use as negative markers in the conditional KO strategy. HXGPRT and UPRT gene markers can be employed only in HXGPRT $^{-}$ and UPRT $^{-}$ parasite background, and bacterial CD and viral TK is functional only when fused to DHFR-TS or CAT genes. To develop a novel platform allowing counter selection of heterologous recombinants in *T.gondii*, we incorporated YFP in the ACP targeting vector. YFP is a naturally fluorescent protein, which among other purposes are now routinely being employed in our lab as a marker for stable selection of transformants. Incorporation of YFP outside the homologous sequence in the ACP KO vector allowed us to isolate at least 3000 putative homologous transformants from more than 300,000 chloramphenicol resistant stable transformants. Specific analysis of homologous recombinants, not only makes the genetic screen time efficient, but also significantly improves the frequency of mutant isolation. In our efforts to target ACP, combined CAT-YFP selection offered a 1000 fold enrichment of homologous recombinants over chloramphenicol selection alone, and we were able to isolate two mutants in the initial 24 clonal strains screened.

YFP offers several advantages over existing negative selection strategies for *T. gondii*. YFP is a non-parasite protein (Cubitt et al., 1999) and can be expressed in any genetic background. More importantly, unlike CD and HSTK, this study, and others show that YFP can be expressed independently by a tubulin promoter and thus can be incorporated at any desired site on a vector (Gubbels et al., 2003; Gubbels et al., 2004).

Conditional gene knock-out is a robust tool for the analysis of gene function and especially significant in systems such as *P. falciparum*, the causative agent of malaria and *T. gondii*, a prominent HIV associated pathogen where high throughout genome wide gene inactivation approaches (siRNA' induced inactivation) are yet to translate to regular applications. Recently, the conditional gene expression system, has been shown to function in *P. falciparum* (Meissner et al., 2005). The efficient expression of GFP, variant of YFP in *P. falciparum* (Gubbels and Striepen, 2004; VanWye and Haldar, 1997; Waller et al., 1998; Waller et al., 2000), along with the demonstrated efficiency of YFP negative selection, thus offers a promising platform for generation of conditional knock-outs in both *P. falciparum* and *T. gondii*.

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Figure 3.1: Expression and regulation of inducible ACP (ACPi). (A) Sequential modification of ptub-YFPYFP/SagCAT to generate ptetO7sag4-ACPmyc/DHFR-TS. 1) ACP coding region was introduced with *Bgl*II/*Avr*II cloning. 2) tubulin promoter sequence was exchanged with regulatable promoter tetO7sag4 with *PpuM*I/*Bgl*II. 3) Second YFP cassette was replaced with myc epitope tag cassette with *Avr*II/*Not*I. 4) DHFR-TS cassette was cloned in to replace sagCAT by *PpuM*I/*Apa*I-*Xba*I fill in cloning (resulting in loss of *Apa*I site in the final vector product). **(B)** Expression and localization of ACPi. RH Tati cells stably transformed with ptetO7Sag4-ACPcmyc/DHFR-TS were fixed and stained with antibodies to ACP and c-myc epitope. While α-ACP antibody detects both ACP and ACPi, α-myc antibody specifically detects ACPi. Detection and colocalization of ACP and myc signal indicates expression and apicoplast targeting of ACPi. Parasite nucleus is detected with DAPI. **(C and D)** ATc regulation of ACPi. Parasites were treated with ATc for 5 days prior to immunostaining. ATc treatment specifically ablates ACPi expression (C, right panel). All micrographs were exposed for the same time. Parasite protein immunoblotted with α-myc antibody fails to generate signal in the presence of ATc. Mic2 is used for loading control (D).

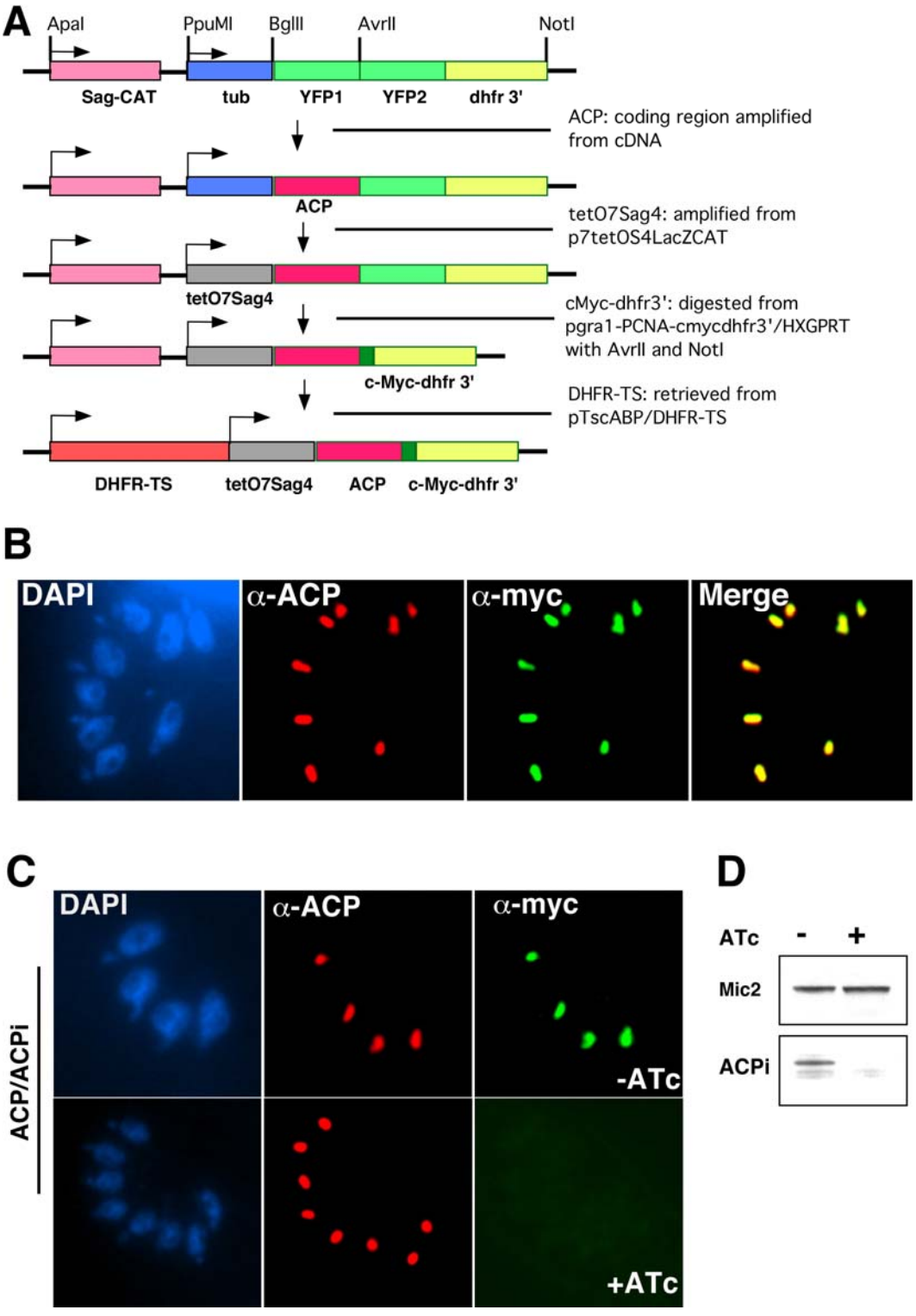

Fig 3.1 Expression and regulation of ACPi

Figure 3.2: Conventional single marker strategy for endogenous ACP replacement. (A) Schematic representation of Allelic replacement mediated by double homologous recombination. Targeting sequence carries wild type sequences flanking a marker offering phenotype for selection, in this case chloramphenicol resistance gene, CAT. Homology between genomic and vector sequence allows pair wise alignment which promotes homologous recombination. 1) Double homologous recombination between the two homologous pairs replaces the targeted gene with the CAT. Transformants are CAT + and resistant to chloramphenicol. 2) The risk of false positive: The CAT gene can also be incorporated by random integration and single homologous recombination. Transformants are CAT + but not the desired mutant (false positive). **(B)** Various pKO-ACP vectors engineered to target native ACP locus. To maximize allelic replacement efficiency, these vectors have varying lengths of flanking sequences and different selection markers. **(C)** Expression of YFP generates fluorescent phenotype and thus offers a marker for selection.

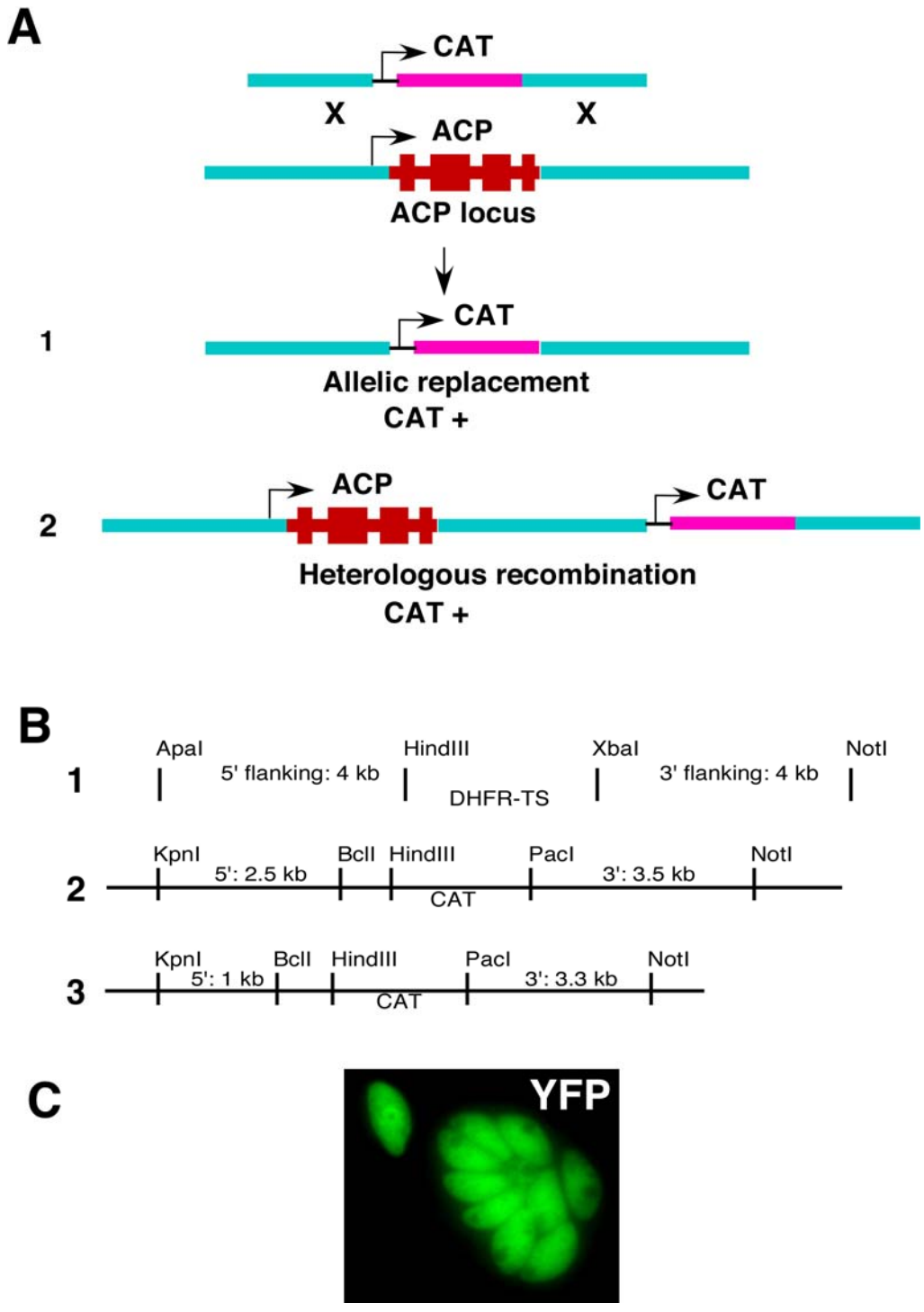

Fig 3.2. A single marker strategy for allelic replacement of endogenous ACP

Figure 3.3: Gene targeting of the ACP locus using a positive/negative selection scheme to enrich homologous recombinants. (A) Schematic outline of positive negative selection for homologous recombination at the ACP locus. (**B**) FACS profiles of parent strain (black), a YFP expressing clone (green) and a population of stable drug resistant parasites after transfection with the double marker KO construct (grey, dimmest 1% of parasites were sorted and cloned). One in twelve of these clones showed successful targeting of the locus (compared to >400 clones unsuccessfully screened using a single marker approach). (**C**) Replacement of native ACP gene by chloramphenicol acetyl transferase (CAT) gene yields altered restriction profile after digestion with restriction enzymes *BamH*I, *Bgl*II and *Xho*I. (**D)** PCR detection of endogenous (1.5 kb) and inducible (1.3 kb) ACP genes. The pKO targeting plasmid and RH genomic DNA serve as control for KO insert and native ACP respectively. (**E**) Southern analysis of *BamH*I /*Bgl*II and *BamH*I/*Xho*I digests of RH ΔACP/ACPi genomic DNA with probes P1 (ACP intron, hybridizes to native ACP but not to the ectopic minigene copy), P2 (CAT) and P3 (3' non coding region present in both loci). These results demonstrate a successful knock-out of the native ACP locus.

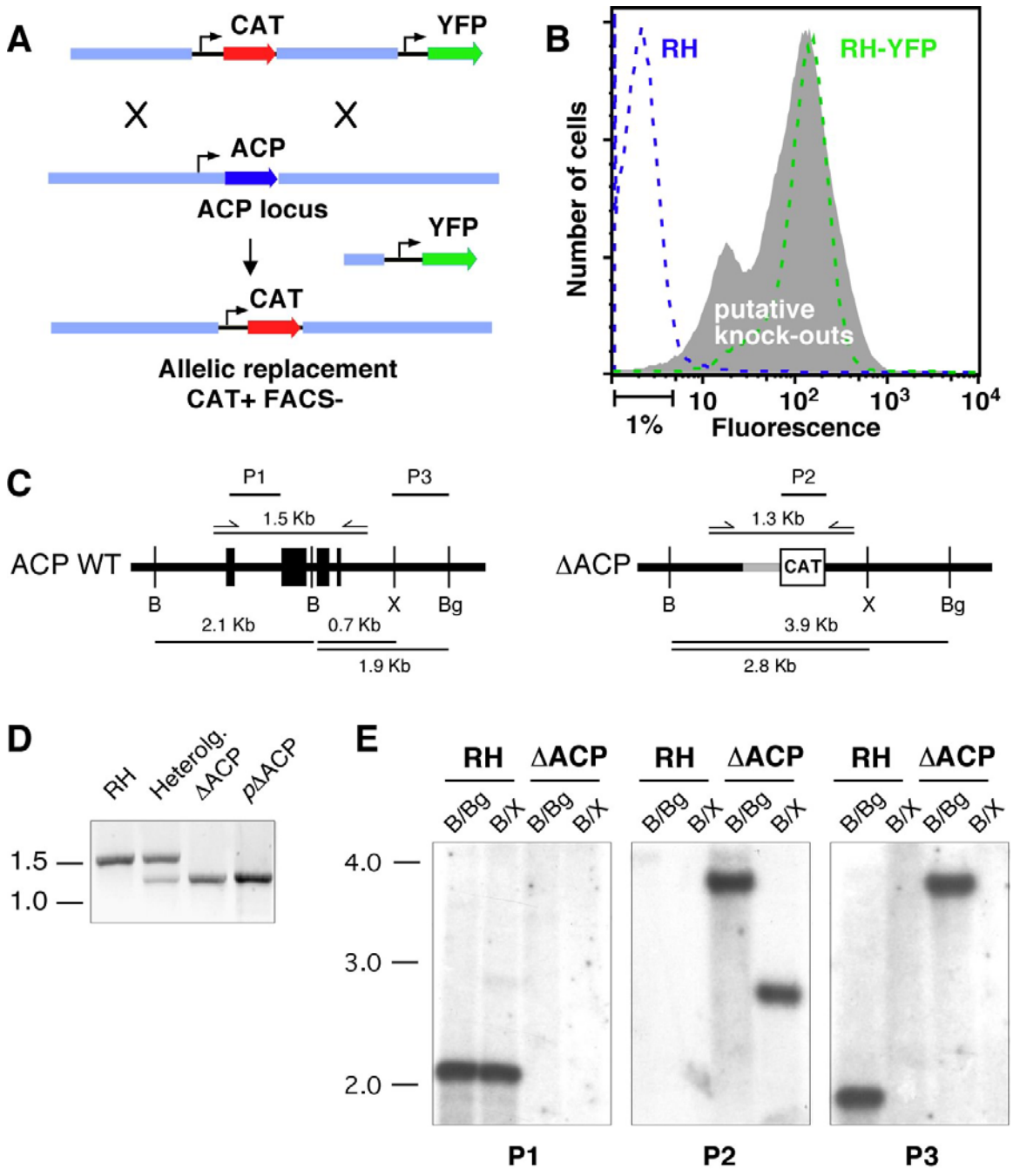

Fig 3.3. A two marker strategy for detection of gene replacement.

CHAPTER 4

APICOPLAST FATTY ACID SYNTHESIS IS ESSENTIAL FOR ORGANELLE BIOGENESIS AND PARASITE SURVIVAL IN *TOXOPLASMA GONDII* [2]

[2]Jolly Mazumdar, Emma Wilson, Christopher Hunter and Boris Striepen. Manuscript submitted to Proceedings of the National Academy of Sciences.

Abstract

Apicomplexans are a class of infectious protozoan parasites which cause important human diseases including malaria and AIDS associated opportunistic infections. Drug treatment for these diseases are not satisfactory and challenged by the lack of efficacy, side effects and most importantly resistance. The recent discovery of the apicoplast, a chloroplast like organelle of cyanobacterial origin within apicomplexans presents multiple drug targets unique to the parasite. To test the biological contributions and the therapeutic potential of one such pathway, the apicoplast localized Type II fatty acid biosynthesis (FAS II) we have engineered a FAS II gene knock-out model in *Toxoplasma gondii*. For this, we conditionally targeted acyl carrier protein (ACP), an essential FAS II enzyme. Disruption of FAS II leads to severe growth defects in cultured cells. Moreover, mutant strains are incapable of causing disease not only in the immunocompetent mice but also in mice that are immunocompromised (lack interferon-γ). Surprisingly, we find that apicoplast FAS II contributes minimally to bulk fatty acids of the parasite. Instead, we show that this pathway provides lipoic acid, a necessary cofactor of the parasite's sole pyruvate dehydrogenase enzyme. Furthermore, knock down of apicoplast fatty acid biosynthesis also produces dramatic changes in apicoplast morphology resulting in organelle loss. In conclusion, with the help of a genetic model we demonstrate the essential role of Tg FAS II pathway in parasite viability and pathogenesis, and validate its potential as a future drug target.

Abbreviations: ACP, acyl carrier protein, DAPI, 4', 6-diamidino-2-phenylindole; FAS, fatty acyl synthase; FNR, ferredoxin NADH reductase; YFP, yellow fluorescent protein.

Keywords: *Toxoplasma,* Apicomplexa, fatty acid biosynthesis, apicoplast, chloroplast

4.1: Introduction

Apicomplexan parasites are one of the leading causes of infectious diseases in humans. *Plasmodium falciparum*, the causative agent of malaria claims an estimated 2 million lives annually (Gardiner et al., 2005), and *Toxoplasma gondii*, an opportunistic pathogen is a prominent cause of encephalitis in AIDS patients (Lisenfeld et al., 1999; Luft and Remington, 1992). Significant therapeutic challenges such as lack of efficacy, side effects and emergence of wide spread drug resistance creates a pressing need for the development of novel drugs (Katlama et al., 1996; Vogel, 2005), a challenge, whose solution may lie in the recent discovery of a chloroplast like organelle, the apicoplast (Fichera and Roos, 1997; McFadden and Roos, 1999) . The apicoplast ultimately derives from a *cyanobacterium* (Kohler et al., 1997; McFadden et al., 1996; McFadden and Waller, 1997). It is critical for parasite survival (Fichera et al., 1995; He et al., 2001a; McConkey et al., 1997) and harbors multiple prokaryotic metabolic pathways which are fundamentally divergent from their analogous counterparts in humans. This divergence between the parasite and host metabolic pathways presents an ideal platform for the development of novel parasite specific therapeutics. The apicoplast localized type II fatty acid biosynthesis is one such pathway.

Among other functions, fatty acids play a key role in membrane biogenesis, and are essential cellular requirements. Eukaryotes and prokaryotes synthesize fatty acids *de novo* employing two divergent forms of the multi-enzyme Fatty Acid Synthase (FAS) system respectively. While the eukaryotic FAS I is a multifunctional polypeptide (Smith, 1994), the prokaryotic FAS II, prevalent in bacteria and plant chloroplasts, is characterized by the discrete arrangement of these enzymes (Harwood, 1996; Smith, 1994). Whether the structural difference between FAS I and FAS II pathways offers therapeutic potential, has been extensively

investigated in *Escherichia coli* and other bacteria (Cronan and Rock, 1996; Schroeder et al., 2002), and indeed several inhibitors specific for bacterial FAS II including triclosan, thiolactomycin, cerulenin, isoniazid and diazaborines, have been characterized (Heath et al., 2001).

The identification of the FAS II pathway, in clinically important apicomplexan parasites (Waller et al., 1998), has received attention as a drug target. Both *P. falciparum* and *T. gondii* encode the complete set of FAS II enzymes ((Ralph et al., 2004) and our analyses), and the therapeutic potential of some components, notably enoyl-ACP reductase (FabI), ketoacyl-ACP synthases (FabH) and fatty acyl dehydratases (FabZ) are being actively investigated (Sharma et al., 2003; Surolia and Surolia, 2001; Waller et al., 2003). FAS II inhibitors triclosan and thiolactomycin have been shown to affect parasite growth (McLeod et al., 2001; Sharma et al., 2003; Surolia and Surolia, 2001; Waller et al., 2003). However, the specificity of these drugs has recently been questioned (Jones et al., 2005; Paul et al., 2004). Furthermore, it appears that in addition to FAS II, apicomplexans also harbor fatty acyl elongases and in the case of *Toxoplasma*, a FAS I pathway (Zhu, 2004 and our analyses).

Inhibition of the FAS II pathway in plants and bacteria results in abnormal cell growth, membrane deformation and ultimately death (Mou et al., 2000; Turnowsky et al., 1989; Vilcheze et al., 2000). However, any essential roles for apicomplexan FAS II are yet to be demonstrated. Apicoplast localized fatty acid biosynthetic pathway has been speculated to play a role in important functions including production of specialized lipids for parasite utilization and the formation of parasitophorous vacuole, a membranous structure formed around the parasite at the time of host cell invasion (Fichera et al., 1995), but await characterization.

To rigorously evaluate the biological contributions of apicomplexan FAS II pathway towards parasite survival, we engineered a gene model permitting the functional analysis of apicoplast FAS II pathway in *T. gondii*. We targeted FAS II by the conditional knock-out of its central component acyl carrier protein (discussed in chapter 3). Using plaque assays and quantification of transgene expression, we show that FAS II knock down severely restricts the growth of parasites in cell culture and leads to cell death. The inability of mutant parasites to cause infection in the mouse model, clearly establishes the pathogenic nature of FAS II. Moreover, the mutant strain effectively vaccinates mice, offering protection against a new infection by wild type strain. Contrary to the general presumption linking FAS II pathway to the *de novo* synthesis of parasite fatty acids, the production of fatty acids as measured by the uptake of radio labeled acetate was unaffected by the loss of Tg FAS II. However, knock down of FAS II did impair lipoylation of the parasite's sole pyruvate dehydrogenase enzyme complex (PDH), which is critical for the generation of acetyl-CoA, a 2C metabolic precursor. Furthermore, FAS II knock down also affects apicoplast morphology, resulting in organelle loss. In conclusion, our findings clearly demonstrate the essential role of apicoplast FAS II for parasite survival and pathogenesis and validate this pathway as a novel drug target.

4.2: Materials and Methods

Parasite cell lines and selections: ACP/ACPi, ΔACP/ACPi and all other transgenic lines derived from these strains were serially passaged in primary human foreskin fibroblasts (HFF) as described in chapter 3 (materials and methods). Unless specified otherwise, cells were pre-incubated in 1μg/ml ATc for six days. HFF cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated bovine serum in the presence

of 2 mM glutamine, penicillin, streptomycin and gentamycin, as mentioned in chapter 3. To generate stable transformants 3 x 10^7 freshly released recipient cells were transfected and selected by FACS. FACS sorting of YFP was achieved using excitation of 488nm and emission filter with a band pass of 530/540 nm respectively.

Plasmid construction and transgene expression: Parasite lines expressing YFP-YFP transgene were transfected with plasmid ptubYFP-YFP/sagCAT, described previously (Gubbels et al., 2003) pTubFabZYFP/sagCAT was generated by exchanging promoter tetO7sag4 in ptetO7sag4FabZYFP/sagCAT, (Mazumdar, unpublished data) with promoter tub, retrieved from ptubYFPYFP/sagCAT with *PpuM*I/*Bgl*II. All cells stably expressing YFP-YFP transgene were selected by FACS. Cells expressing, cytosolic, apicoplast and dense granule markers were transiently transfected with ptub-YFPYFP/sagCAT, ptub-FNRRFP/sagCAT (Striepen et al., 2000) and ptub-P30GFP/sagCAT (Striepen et al., 2001). Cells expressing ectopic superoxide dismutase (SOD2) were transiently transfected with pT8-SOD2-ty/sagCAT, a kind gift from Dr. Dominique Soldati (University of Geneva, Geneva, Switzerland).

***In vitro* growth assay:** Growth of parasites in cultured cells was observed ten days after infection by plaque assay, as previously described (Pfefferkorn and Pfefferkorn, 1976). Briefly, 10 days after infection, HFF monolayers were fixed, stained and scored for number. 50 plaques from triplicate flasks were measured for area (short axis x long axis x Pi/4).

***In vitro* fluorescence assay**: Growth of parasites was monitored as described previously (Gubbels et al., 2003). Briefly, Costar, 96 well sterile tissue culture well plates with optical bottoms (Falcon/Becton-Dickinson, Franklin Lanes, N.J) were seeded with host cells. Monolayers were infected with 2500 freshly lysed parasites resuspended in parasite medium without phenol red (Gibco, BRL Life technologies, Rockville, Md) and supplemented with ATc

and drugs for specific experiments. Plates were read daily in a BMG fluorescent plate reader (Offenburg, Germany) and both excitation (510nm) and emissions (540nm) were read from the bottom. YFP fluorescence was excited with a single flash from a Xenon lamp and emission signals were recorded with a standard gain of 60. Final data from quadrupole recording was expressed as percentage positivity (PP) to correct for cell to cell fluorescence variation, as decribed previously (Gubbels et al., 2003). A PP of 0% represents the background level and 100% PP represents maximum fluorescent signal within a particular experiment.

Virulence assay in mice: For the virulence studies in mice, tachyzoites were harvested from freshly lysed HFF cells, washed in PBS and counted microscopically. At the indicated days, 1000 tachyzoites were injected intraperitoneally (i.p) in 0.2ml PBS into 6-8 week old C57B1/6 mice. Groups of 5 mice received sterile drinking water supplemented with 0.1% sucrose. In indicated groups, the drinking water was further supplemented with 0.2mg ATc /ml. The animal experiments were conducted with ACP/ACPi and ΔACP/ACPi strain in parallel. For interferon gamma assay, the mice were injected with 500μg α-IFNγ antibody on days 0 and 5 of the experiment. Mice groups that survived infection were re-challenged with i.p injection of 10,000 RH tachyzoites.

Metabolic labeling: For analysis of fatty acyl methyl esters, 1 $x10^8$ parasites were incubated with 10μCi ^{14}C- labeled Na-acetate in 1 ml DMEM for 4h at 37^0C and 5% CO_2. Antibiotics thiolactomycin (a kind gift from John Onydeka, Merck, NJ), cerulenin (Sigma, St louis, MO) or ethanol (control) was added as indicated. Total lipids were extracted with chloroform/methanol (2:1), and subjected to Folch's washing, as previously described (Folch et al., 1957). The extract was dried under liquid nitrogen and taken up in anhydrous methanol containing 0.2% sulfuric acid and 0.1% benzene, heated at 70^0C for 4 h and extracted with hexanes (Fosbrooke and

Tamir, 1968). Fatty acyl methyl esters (FAME's) were analyzed on RP-18 HPTLC (Analtech Inc., Newark, DE). Preheated plates were spotted with 10µl of samples and developed in methanol/chloroform/water (75:25:5). Signals were amplified with EN^{3}HANCE autoradiography enhancer spray (PerkinElmer, Boston, MA).

Immunostaining of parasites and proteins: Immunofluorescence assay and Western blot analysis were performed as described in Chapter 3 (materials and methods). All primary and secondary antibodies for immunofluorescence assay were used at 1:1000 and 1:300 dilutions respectively. α-ACP polyclonal rabbit serum, a kind gift of Dr. Geoff McFadden (University of Melbourne, Parkville, Australia) and α-cMyc 9E10 monoclonal antibodies (Roche Molecular Biochemcials, Mannheim, Germany) were used to detect endogenous and inducible ACP. α-lipoylated E2 monoclonal antibody, 2H-4C8 (Migliaccio et al., 1998), kindly gifted by Dr. Eric. Gershwin (University of California SanDiego, San Diego, CA) and α-LA polyclonal antibodies (Calbiochem, San Diego, CA) was used to detect lipoylation of apicoplast and mitochondrial proteins. Apicoplast and mitochondrial expression of SOD2 protein was detected with α-ty monoclonal antibody, a kind gift of Dr. Dominique Soldati (University of Geneva, Geneva, Switzerland). For Western blot analysis of proteins, primary antibodies were used at the following dilutions, polyclonal α-ACP and monoclonal α-Mic2 antibodies, a kind gift from Jean Francois Dubremetz, (Universite de Montpellier 2, Montpellier, France) at (1:10,000), α-2H-4C8 and α-LA at (1:5000) and all HRP conjugated secondary antibodies (Sigma, St. Louis, MO) at 1:3000 dilution. Parasites expressing transgenes YFPYFP, FNR-RFP, P30-GFP and FabZ-YFP were directly visualized under fluorescence microscope.

4.3: Results

ATc treatment generates ACP null condition in ΔACP/ACPi strain

Phenotypic analysis in a conditional gene knock-out relies on the stringent regulation of the inducible copy in the absence of the native gene. To ensure ACP null condition, ATc treated ΔACP/ACPi were tested for loss of ACP expression by immunofluorescence assay and Western blot analysis (Fig.4.1 A and B). Parasites were immunostained with an α-myc antibody, which specifically reacts with myc-tagged ACPi as shown earlier (Chapter 3). ATc treatment ablates ACPi expression in both ACP/ACPi and ΔACP/ACPi cells (Fig.4.1 A panels F and L). In contrast α-ACP antibody which recognizes both ACP and ACPi, can be detected in parental line (panel E), but is lost in mutant cell line, indicating the absence of endogenous ACP (panel K). Western blot analysis of parasite proteins immunoblotted with α-ACP antibodies confirmed the above observations (Fig 4.1B). Nuclear encoded apicoplast proteins are synthesiszed as precursor proteins (p) which are proteolytically processed to the mature form (m) in the apicoplast (DeRocher et al., 2000; Waller et al., 1998), also detailed in section 2.3.5. Note the presence of both precursor and mature proteins for ACP and ACPi on the blot and the slow mobility of ACPi due to the epitope tag. ATc treatment ablates all protein signals in mutant cells, but specifically down regulates ACPi signal in parent cells (Fig. 4.1B). Mic2 serves as loading control (Fig 4.1B). We further analyzed the kinetics of ATc-mediated ACP knock-down (Fig. 4.1C). Protein levels of ACPi in cells treated with ATc for different time periods were analyzed by Western blot. The ACPi precursor protein shows a dramatic reduction within 24 hours suggesting a fast effect of ATc on transcription. Mature ACPi appears to be a stable protein and is diluted by growth; after 5 days ACPi is no longer detectable. The test was performed in ACP/ACPi cells to use native ACP as an internal control. To ensure full ACP knockdown all

biochemical experiments described below were conducted with cells treated with ATc for at least six days.

Mutants are severely impaired for growth in cultured cells and animal model

We next examined the effect of ACP depletion on parasite growth. *T.gondii* is an obligate intracellular parasite and grows within the host cells by clonal expansion. This process can be easily visualized in cultured cells by assaying for plaques generated by host cell lysis as a result of the clonal growth of parasites (Pfefferkorn and Pfefferkorn, 1976). Ten days after inoculation of fibroblast monolayers, both ATc treated and untreated parasite strains formed comparable numbers of plaques. However, ΔACP/ACPi parasites under ATc treatment generated distinctly smaller plaques (Fig 4.2A and B lower panel). Mutants formed plaques at a number comparable to the parent line (Fig 4.1B upper panel), but displayed a more than 70 percent reduction in plaque size (area was measured for 50 plaques per flask, n=3, $p<0.0001$ students t test). To quantify the growth rate dynamically, we stably expressed a YFP-YFP transgene in the parent and mutant strains, and assayed for growth over an extended period of time by the measurement of fluorescence, as described previously (Gubbels et al., 2003). Initially ATc treated ΔACP/ACPi parasites grow at a rate indistinguishable from untreated controls but slow sharply after 5 days (Fig. 4.2D, red squares). Parasites pre-incubated with ATc for 5 days (2nd passage, red triangles) show only minimal growth (no viable parasites could be detected by plaque assay after a third passage under ATc, data not shown). No significant growth effects of ATc treatment were observed in the ACPi parental strain (Fig 4.2C). These findings indicate that apicoplast FAS II is required for the normal growth of parasites in cultured cells.

FAS II is essential for pathogenesis in a mouse model

To investigate the consequence of slow growth on parasite pathogenesis, we took advantage of a mouse model. To establish if ACP and thus FASII knock down affects pathogenesis, mice were infected with 1000 ΔACP/ACPi or ACPi tachyzoites (10 mice per group). ATc was provided in the drinking water for five mice while the other mice received a placebo. The parental strain produced high parasitemia and killed mice within 17 days of intraperitoneal inoculation irrespective of ATc treatment (Fig. 4.3A). In contrast, mice infected with the ΔACP/ACPi receiving ATc were completely protected and at no point showed parasites or signs of disease; control mice receiving a placebo all died within 17-24 days of infection (Fig. 4.3B). Infection with the mutant combined with ATc treatment effectively vaccinated mice, these mice were refractory to a subsequent lethal challenge with 10,000 wild type RH parasites (Fig. 4.3C). Life-threatening toxoplasmosis is often associated with AIDS or other severe immunosuppression. Interferon γ (IFN γ) is the key mediator for the control of *T. gondii*. Mice lacking IFN γ are exquisitely susceptible (Suzuki et al., 1988) and infection in these mice progresses markedly faster (Hunter et al., 1993). To test the effect of FAS II depletion on parasite pathogenesis in an immunocompromised model, mice ablated for IFN γ with α-IFN γ antibody were infected with ΔACP/ACPi parasites. In the absence of ATc all animals died in half of the time observed in the initial experiment. Importantly, mice receiving ATc were again fully protected (Fig 4.3D). Comparison of peritoneal fluid from the infected mice indicates high parasitemia in the absence of ATc (Fig 4.3E). Taken together, these data suggest that apicoplast fatty acid synthesis is essential for robust growth and pathogenesis regardless of the host's immune-status.

Bulk fatty acid synthesis is unaffected in ΔACP/ACPi mutants

De novo fatty acid (FA) synthesis proceeds with the sequential incorporation and reduction of 2:C carbon moieties into growing FA chains. The incorporation of ^{14}C- acetate, a 2:C labeled precursor is routinely used to measure FA biosynthesis (Bisanz et al., 2006; Charron and Sibley, 2002). A recent study used [^{14}C]- acetate labeling of extracellular tachyzoites and interpreted radiolabeled lipids as apicoplast FASII derived (Bisanz et al., 2006). To investigate the role of the apicoplast FAS II pathway in fatty acid biosynthesis, we monitored the incorporation of ^{14}C-acetate in the mutant and parent cells. To our surprise, ATc treatment did not affect the incorporation of ^{14}C-acetate in either the parent or the mutant strain (Fig 4.4A). The fatty acid profile generated by both strains comprised mostly of C:18 (Stearic acid), very long chain FA and some C:16. The labeled products were parasite specific and not host FAS I products as direct labeling of host cells generate a different profile of C:16 and shorter FA's (Fig 4.4B). Earlier, in the course of this study we had identified an additional FAS I pathway (TgTwinScan_0460) (ToxoDB) in *T. gondii*. This suggested that ^{14}C-acetate incorporation might be due to FA elongation activity of FASI (Zhu, 2004) and/or FA elongases (three genes are present in the *T. gondii* genome, TgTwinScan_3930, 2967 and 6237) (ToxoDB), rather than *de novo* synthesis by the apicoplast- localized FASII pathway.

To test this hypothesis we monitored acetate incorporation in the presence of FAS inhibitors thiolactomycin and cerulenin. Thiolactomycin specifically inhibits the condensation reaction of the FAS II pathway. Cerulenin on the other hand inhibits the condensation reaction in both the eukaryotic FAS I and prokaryotic FAS II pathway (Magnuson et al., 1993). Consistent with our hypothesis acetate incorporation was sensitive to cerulenin but resistant to

thiolactomycin (Fig. 4.4C and D). In control experiments both drugs blocked parasite growth demonstrating their principal activity (Fig 4.4 E and F). These findings suggest that the principle role of FAS II pathway does not lie in the biosynthesis of bulk fatty acids as measured by labeled acetate incorporation.

ACP down regulation inhibits lipoylation of the Pyruvate Dehydrogenase enzyme complex

In plant chroplasts, fatty acid biosynthesis relies on the 2C precursor acetyl-CoA for fatty acid biosynthesis (Mooney et al., 2002). In a cell, acetyl-CoA can be either mobilized from acetate by the action of acetyl-CoA synthase (ACoS) or generated from pyruvate by the action of pyruvate dehydrogenase complex. Inspection of the *T. gondii* genome reveals that the apicoplast lacks an acetyl-CoA synthase and relies on pyruvate dehydrogenase (PDH) for acetyl-CoA production. The putative ACoS in the genome (TgTwinScan_3199) (ToxoDB) appears to lack apicoplast targeting motifs.

The activation of PDH complex depends on lipoylation, characterized by the addition of lipoic acid moiety (LA) on to the E2 subunit of PDH multi-enzyme complex. Lipoic acid is an essential cofactor for oxidative decarboxylases and involved in the response to oxidative stress. *T. gondii* harbors several LA-modified proteins in its mitochondrion and the apicoplast (Thomsen-Zieger et al., 2003). In prokaryotes, LA can be synthesized *de novo* from octanoyl-ACP, (a FAS intermediate) by LipA. Analysis of the *T.gondii* genome reveals the presence of a single LipA gene localized within the apicoplast. A second gene, LplA which acts on scavenged LA is exclusively targeted to the mitochondrion (Thomsen-Zieger et al., 2003). To assess if apicoplast FAS II can also provide precursors for lipoic acid biosynthesis we examined protein lipoylation in parent and mutant cell line by immunofluorecsence and western analysis (Fig 4.5 A and B). Cells were immunoblotted with 2H-4C8, an anti-lipoylated peptide monoclonal

antibody that preferentially recognize E2 subunit of the apicoplast Pyruvate Dehydrogenase enzyme complex (PDC). Lipoylation of PDC-E2 is specifically down regulated in ΔACP/ACPi cells treated with ATc (Fig. 4.5 A and B). We next investigated if the mitochondrial LA is scavenged from the plastid LA pool. Cells were stained and immunoblotted with α-LA polyclonal antibody which recognizes both mitochondrial and apicoplast lipoylated proteins (Fig 4.5 C and D). ATc treatment did not affect mitochondrial lipoylation in ΔACP/ACPi cells. Consistent with observations in *P. falciparum* (Wrenger and Muller, 2004), these findings confirm the presence of a FAS dependent LA pathway in the apicoplast and a LA salvage pathway in the mitochondrion in *T. gondii*. Interestingly, based on our observation, the mitochondrial LA pathway is not dependent on apicoplast FAS II.

FAS II is essential for apicoplast biogenesis

Disruption of FAS II functions result in defective chloroplast biogenesis in plants (Mou et al., 2000). Moreover, FAS II lipids are implicated to play an important role in chloroplast protein import, previously discussed in section 2.3.6 and 7. To evaluate protein trafficking and apicoplast biogenesis under FASII knock down conditions, we transiently transfected ATc treated parasites with the apicoplast marker ferredoxin NADPH reductase-RFP (FNR-RFP). While the untreated mutant showed typical plastid labeling in 30% of the cells only very few fluorescent apicoplasts could be detected in treated mutants (Fig. 4.6A). No difference in plastid labeling was observed in the parent strain under drug. Control experiments with markers for the cytoplasm (YFP-YFP) or the secretory pathway (P30-RFP) showed no significant difference between ATc treated and untreated parasites indicating that this effect is specific to the apicoplast (Fig. 4.6A). To further investigate this phenotype, we constructed a stable parasite line expressing the apicoplast marker FabZ-YFP in the mutant background. Examination of cultures

treated with ATc for 0, 2 and 6 days revealed three distinct vacuole phenotypes: all parasites in a vacuole showing a fluorescent apicoplast (A), a portion of them show fluorescent apicoplast (P), or none (N) (Fig 4.6D, right panel). The phenotypes were scored. ATc treatment lead to a progressive loss of plastid signal (Fig. 4.6 B and C). After 6 days typical plastid labeling was lost in 80% of the vacuoles (Fig 4.6D, right panel). We also made similar observation with an endogenous apicoplast marker, HU protein (Fig 4.7A) (quantification data not shown). To see if the morphological defects were specific to the apicoplast, mutant cells were transfected with Superoxide dismutase 2 (SOD2), a marker protein which is simultaneously targeted to the apicoplast and the mitochondrion. Presence of intact mitochondrion indicated an apicoplast specific effect of FAS II knock down (Fig 4.7B). Our frequent observation of a single large apicoplast per parasite vacuole was reminiscent of a previously described apicoplast division mutant (Fig. 4.6 B and C) (He et al., 2001a), also discussed in section 2.2.4.

4.4: Discussion

De novo synthesis of fatty acids is mediated by a multi-enzyme fatty acid synthase system (FAS). Interestingly, the primary functions of eukaryotic FAS I and prokaryotic FAS II are conserved, but the structural difference between the two offers scope for therapeutic exploitation. Genomic and biochemical analysis provide strong support for the presence of a prokaryotic FAS II pathway in apicomplexan parasites, *P. falciparum* and *T. gondii*. While the inhibition of parasite growth by FAS II inhibitors make FAS II an attractive drug target, the specificity of these drugs have recently come under scrutiny, and evidence directly linking biological functions of FAS II to parasite survival are missing. To better understand the biological role of FAS II pathway and to validate its therapeutic potential, we have engineered

the first genetic model permitting analysis of FAS II functions. Using a mutant strain lacking acyl carrier protein, a critical component of the FAS II pathway, we have studied the functions and effects of FAS II depletion in *T. gondii*.

Previous studies have suggested *de novo* fatty acid biosynthesis as the primary function of apicoplast FAS II (Bisanz et al., 2006; Surolia and Surolia, 2001). To our surprise, the FAS II mutant strain efficiently synthesizes C16 and C18 fatty acyl chains, usual FAS products. Moreover, the synthesis is resistant to FAS II inhibitor thiolactomycin, but arrested by cerulenin, a FAS I inhibitor, suggesting the involvement of some pathway other than FAS II for the bulk synthesis of fatty acids. Indeed, a second pathway similar to the eukaryotic type I FAS has been recently identified in *T. gondii*. Interestingly, the depletion of FAS II inhibits the biosynthesis of specialized apicoplast FAS II products, such as lipoic acid, suggesting that the primary role of apicoplast FAS II might not be synthesis of bulk fatty acids, but of specialized products for use within the apicoplast, including lipoic acid. Lipoic acid is critical for the activation of several enzyme complexes, including the sole Pyruvate dehydrogenase complex (PDH) of the parasite. Therefore, depletion of apicoplast FAS II should render apicoplast PDH inactive. PDH is essential for generation of acetyl-CoA, a common metabolic precursor. As this is the only PDH found in apicomplexans (Foth et al., 2005), FAS II disruption therefore might impair acetyl-CoA dependent pathways in the apicoplast and beyond. If the apicoplast exchanges acetyl-CoA with other compartments (especially with the mitochondrion, the site for TCA cycle and ATP production) remains to be elucidated. Our mutant analysis also demonstrated organellar biogenesis defects suggesting that FASII supplies the apicoplast with essential lipids. These lipids could be important for the growth and division of plastid membranes (Mou et al., 2000; Turnowsky et al., 1989) or be required for organellar protein import (Chen and Li, 1998).

Most importantly, we demonstrate that the apicoplast FAS II pathway is essential for parasite survival and pathogenesis. FAS II mutant strains grow slowly and are unable to cause infection in the mouse model. Furthermore, infection with mutant strains vaccinates mice against subsequent infections. In the absence of effective vaccination available for malaria or toxoplasmosis, the attenuated strain demonstrates potential as a vaccine candidate. In conclusion, our findings clearly establish the essential requirement of apicoplast FAS II pathway for the growth and pathogenesis of parasites, and validate its potential as a novel drug target.

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Figure 4.1: ATc induced ACP null condition. **(A)** Parasites were treated with ATc and fixed and stained with antibodies to ACP (middle panel) and to Myc (lower panel). Micrographs were taken under identical exposure conditions. ATc treatment ablates myc signal in both parent and mutant cell lines (panel F and L). Lack of signal with α-ACP antibody in ATc treated ΔACP/ACPi cells, indicates the absence of endogenous ACP (panel K). ACP signal in untreated ΔACP/ACPi is due to the presence of ACPi (panel H). **(B)** Western blot analysis confirms above observation. Parent cells express the precursor (p) and mature (m) ACP and ACPi. p and m ACPi migrate slower due to appended myc tag. ΔACP/ACPi parasites exclusively express the precursor (pACPi) and mature (mACPi) protein. ATc treatment of ΔACP/ACPi parasites ablates ACPi expression generating the conditional mutant condition. Mic2 is used for loading control. **(C)** Kinetics of ATc regulation tested by western analysis in ACP/ACPi strain. Suppression of transcription is quick and the precursor signal ablates within 24 hours of ATc treatment. The mature protein is stable, can be detected for 5 days and is diluted by growth. Native ACP serves as internal control.

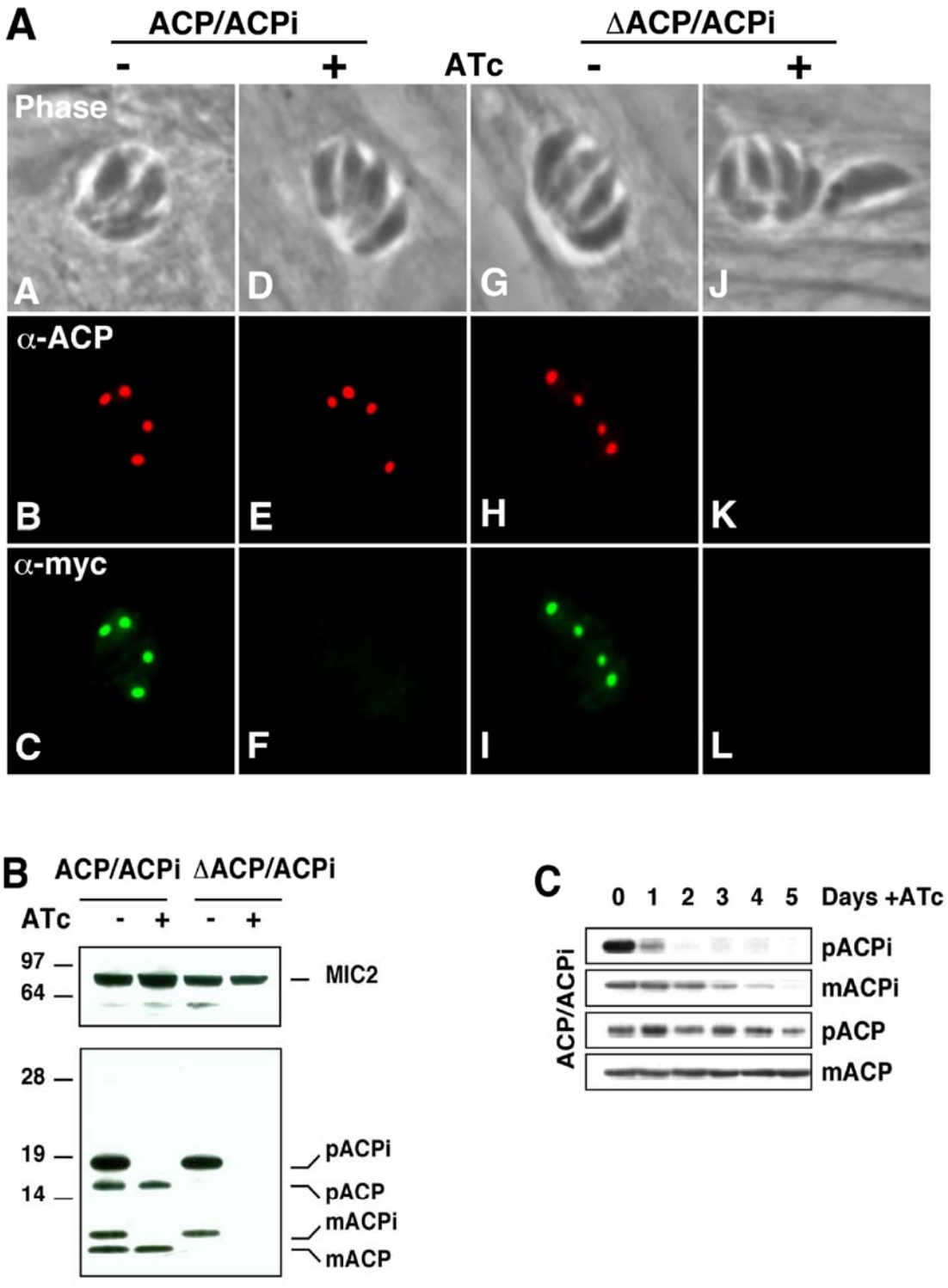


Fig. 4.1 ATc induced ACP null condition

Figure 4.2: FAS II knock down reduces parasite growth in cultured cells (A) ATc treated ΔACP/ACPi parasites form smaller plaques compared to parent cells. **(B)** Quantification of plaque area of 50 plaques from three assays indicate a >70 fold reduction in ATc treated ΔACP/ACPi plaque size compared to untreated parent and mutant cells (2B lower panel). Mutants under ATc maintain normal invasion efficiency as indicated by plaque counts (2B upper panel). The percentage of 100% indicate successful invasion in the absence of ATc for ΔACP/ACPi parasites. **(C and D)** The rate of intracellular growth monitored over 10 days after infection. Fluorescence of YFP-YFP expressing lines were measured in a fluorescent plate reader **(C)** ACP/ACPi cells maintained comparable growth rate in the presence and absence of ATc. **(D)** Growth of mutant cells reduced sharply after 5 days of ATc treatment and was severely restricted when pre-incubated with ATc for more than 6 days.

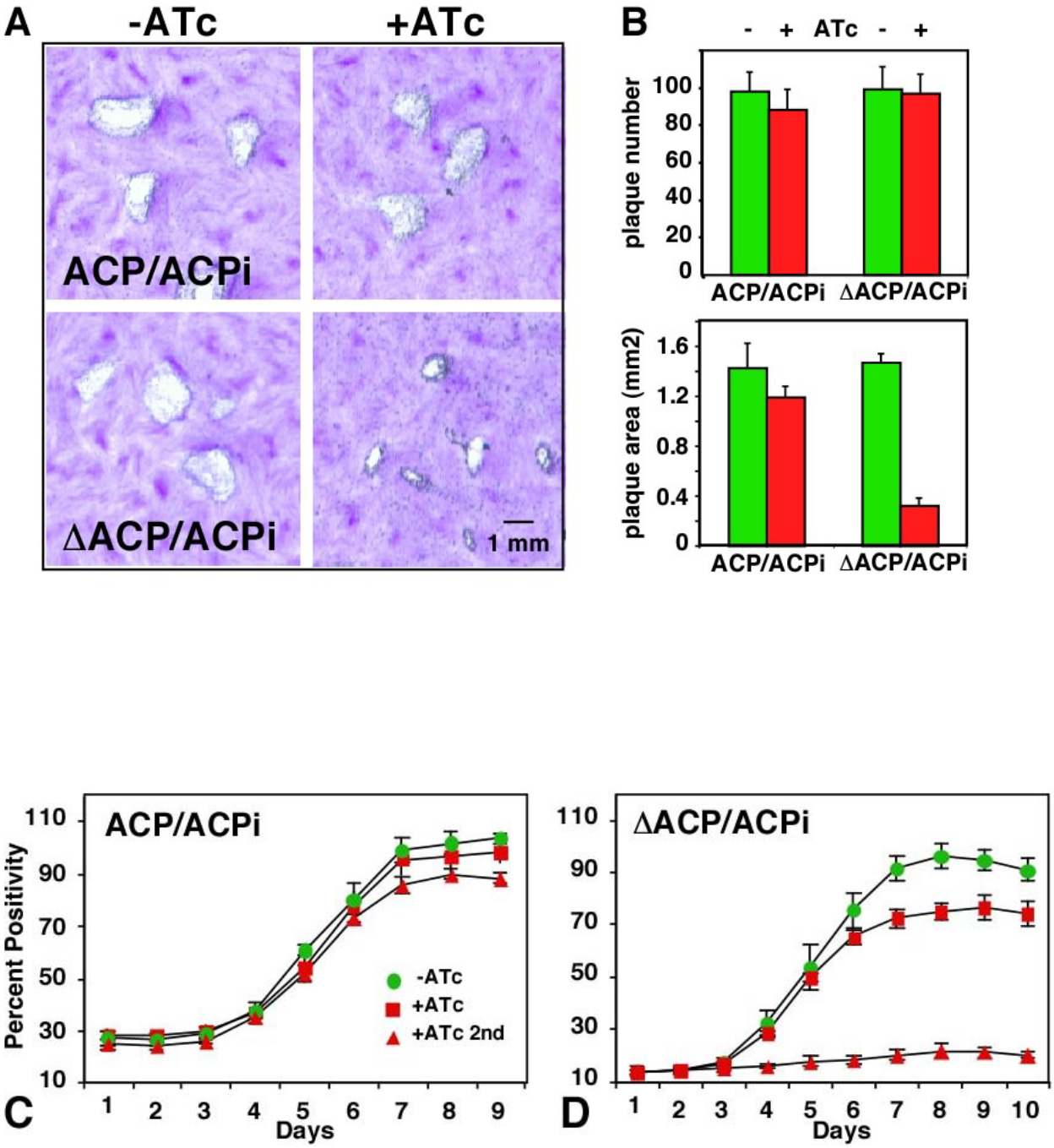


Fig 4.2: Parasite growth under FAS II knock down condition

Figure 4.3: ΔACP/ACPi mutant parasites do not cause disease in the mouse model. Groups of 10 C57B1/6 mice were infected with **(A)** ACP/ACPi and **(B)** ΔACP/ACPi tachyzoites by intraperitoneal injection. Mice received 0.2mg/ml ATc (red) or placebo (green). **(A)** All animals in groups infected with ACP/ACPi tachyzoites succumbed to infection within 17 days of infection irrespective of the absence or presence of ATc. **(B)** After 24 days only the group that were infected with ΔACP/ACPi and received ATc treatment survived. Mice cured of infection were observed for 100 days and no pathology was detected. **(C)** Subsequent re-challenge of these mice with 10,000 RH wild type tachyzoites, did not cause infection. All naïve mice were killed within 13 days of infection. **(D)** Mice were immunosupressed with antibodies to IFN-γ (arrow indicates IFN-γ antibodies, 500 μg/mice administered i.p on days 0 and 5) and infected and treated as described for B. All mice on placebo but not ATc died. **(E)** Peritoneal fluid from infected mice visualized under the microscope shows a higher parasite count in the absence of ATc treatment.

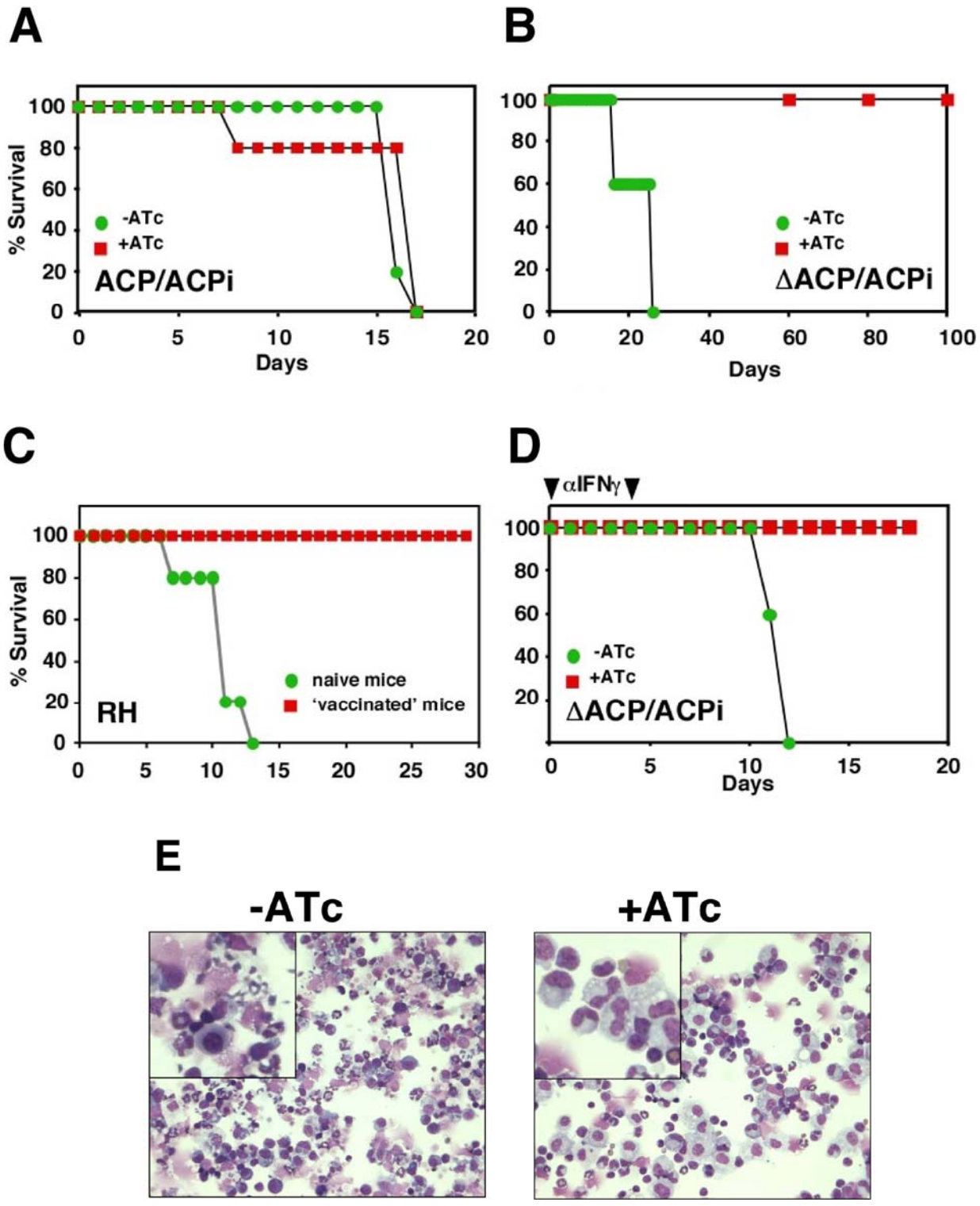
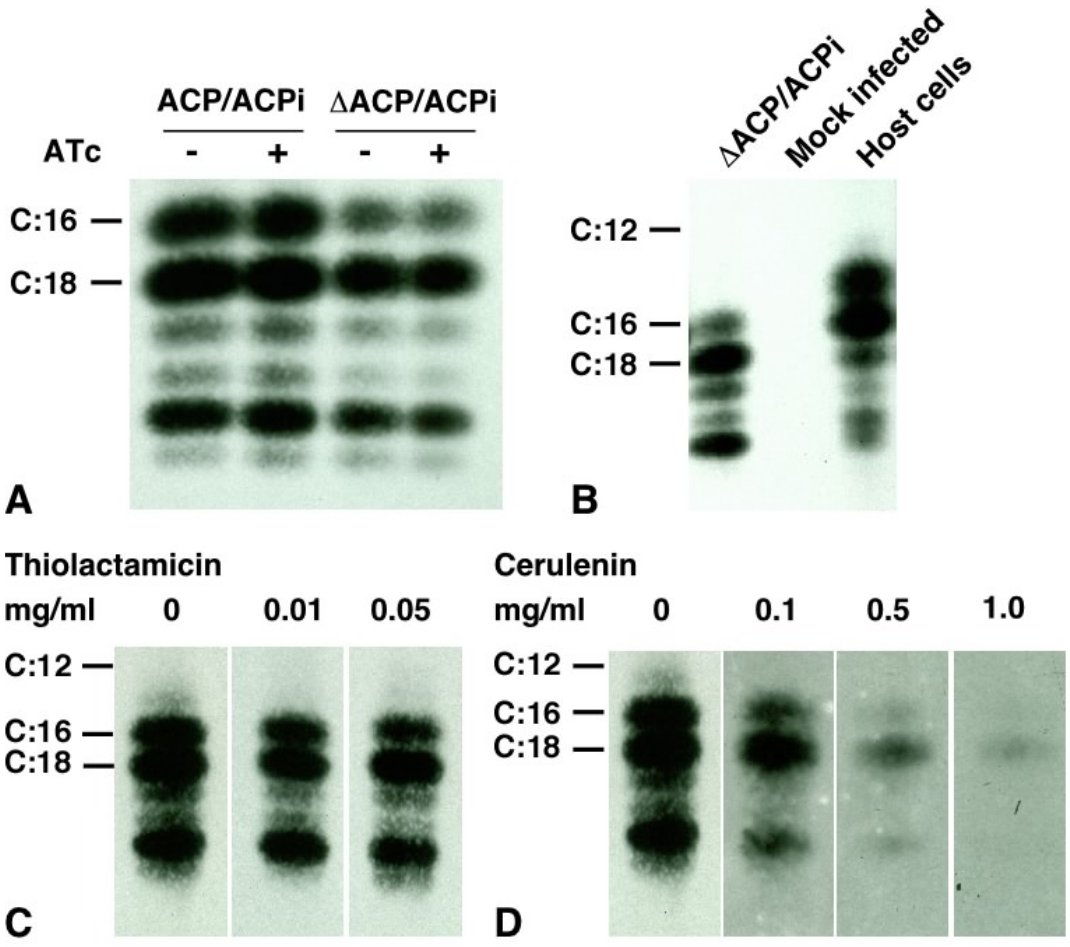


Fig 4.3: Pathology of mutant strain infection in the mouse model

Figure 4.4: Synthesis of bulk fatty acids is unaffected by ACP knock down. **(A)** Parents and mutants were treated with ATc for 6 days and extracellular tachyzoites were incubated with ^{14}C-acetate. Radiolabeled fatty acids were analyzed by RP-TLC. Major products detected comprised of C:18, long chain FA and some C:16 (methylated radiolabeled fatty acid of known chain length were run in parallel). **(B)** Mock infection or direct labeling of host cell cultures served as control for potential host cell contamination. **(C and D)** Acetate incorporation into FA is resistant to the FASII inhibitor thiolactomycin and sensitive to cerulenin, FASI and FAS II inhibitor. **(E)** Parasite growth measured by fluorescence assay is sensitive to thiolactomycin.



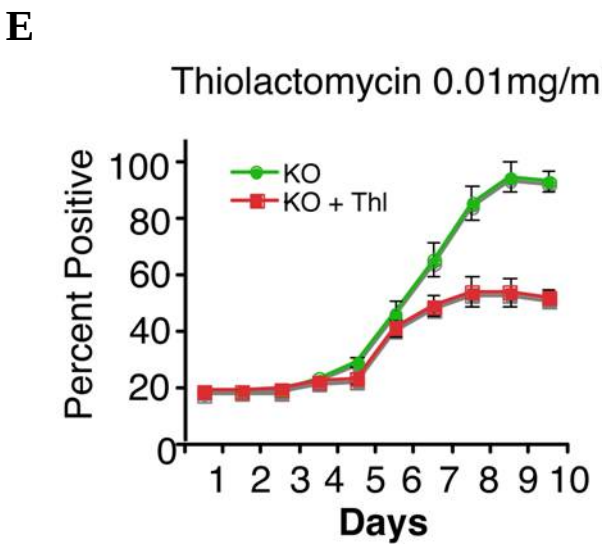


Fig. 4.4. Biochemical and pharmacological analysis of FAS II mutants

Figure 4.5: FAS II knock down displays organelle specific lipoylation impairment.

(A and B) Immunofluorescence and western analysis of parent and mutant cells with 2H-4C8 anti-lipoylated peptide monoclonal antibody that preferentially recognizes lipoylated apicoplast Pyruvate Dehydrogenase complex (PDC). ATc treatment completely abolishes lipoylation of apicoplast PDH. Arrows indicate signal from antibodies 2H-4C8 and HU, a *T.gondii* protein associated with the apicoplast genome and used here as an independent apicoplast control. Mic2 is used for loading control. **(C and D)** A polyclonal serum raised against LA-KLH recognizes both apicoplast (P) and mitochondrial (M) lipoylated proteins. Note mitochondrial lipoylation is unaffected by ATc treatment.

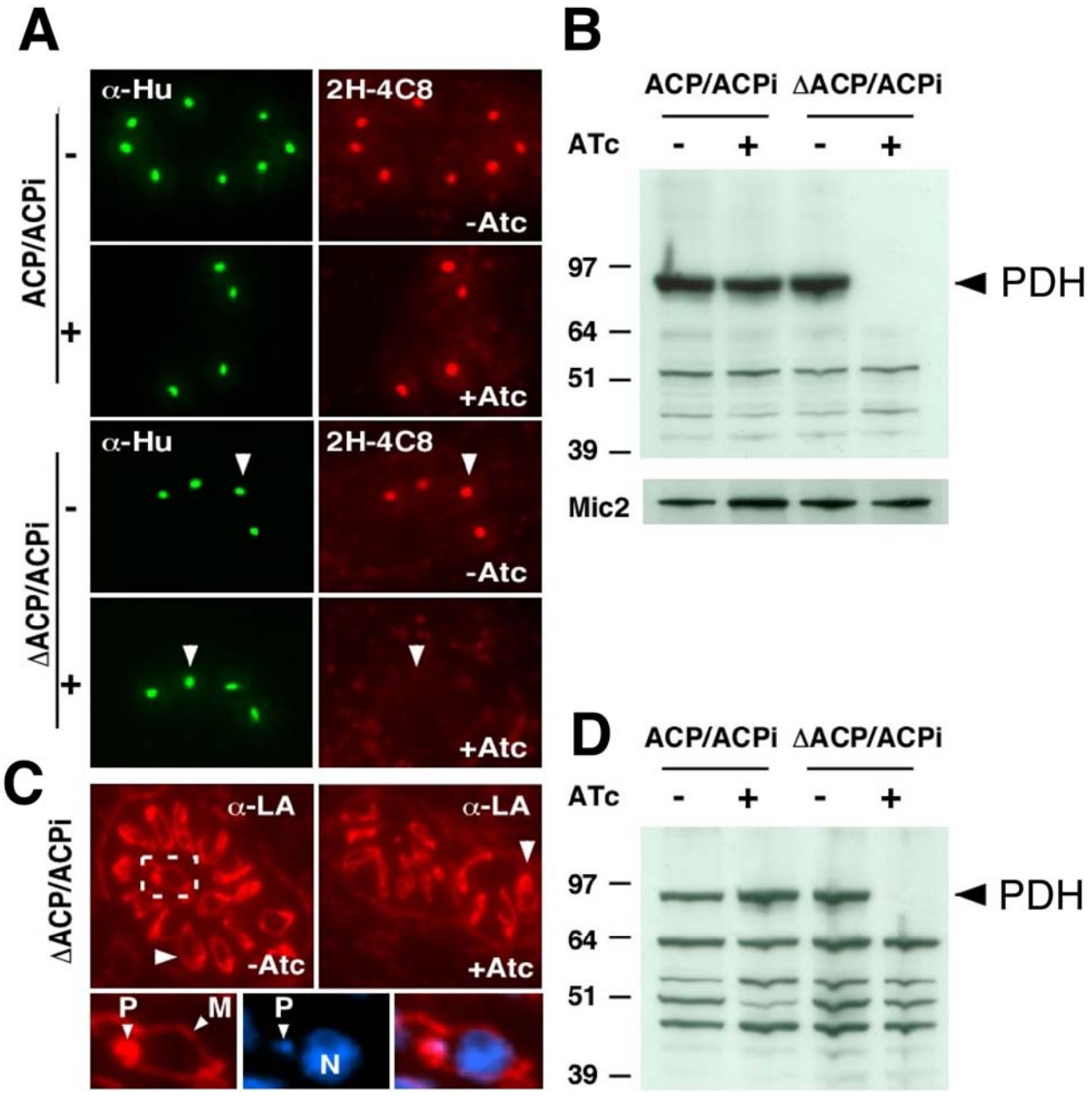


Fig. 4.5. Organelle specific impairment of enzyme lipoylation

Figure 4.6: FAS II knock down leads to defects in apicoplast morphology and biogenesis. **(A)** ACP/ACPi and ΔACP/ACPi cells were grown for 6 days in the presence or absence of ATc and then transfected with plasmids resulting in the expression of FNR-RFP (apicoplast), P30-RFP (dense granules and parasitophorous vacuole) and YFP-YFP (cytoplasm) and seeded onto coverslip cultures. After 24 h parasites were scored for fluorescent protein expression. **(B, C)** A ΔACP/ACPi line stably expressing the apicoplast marker FabZ-YFP was treated with ATc for 3 days and imaged. Vacuole with a single or few large apicoplasts are frequently observed (arrow, parasitophorous vacuoles is indicated by dotted line). **(D)** The same line was treated with ATc for different times and plastid morphology was scored using the three categories indicated. Percentage of parasite vacuoles displaying apicoplast morphology defects increase over time.

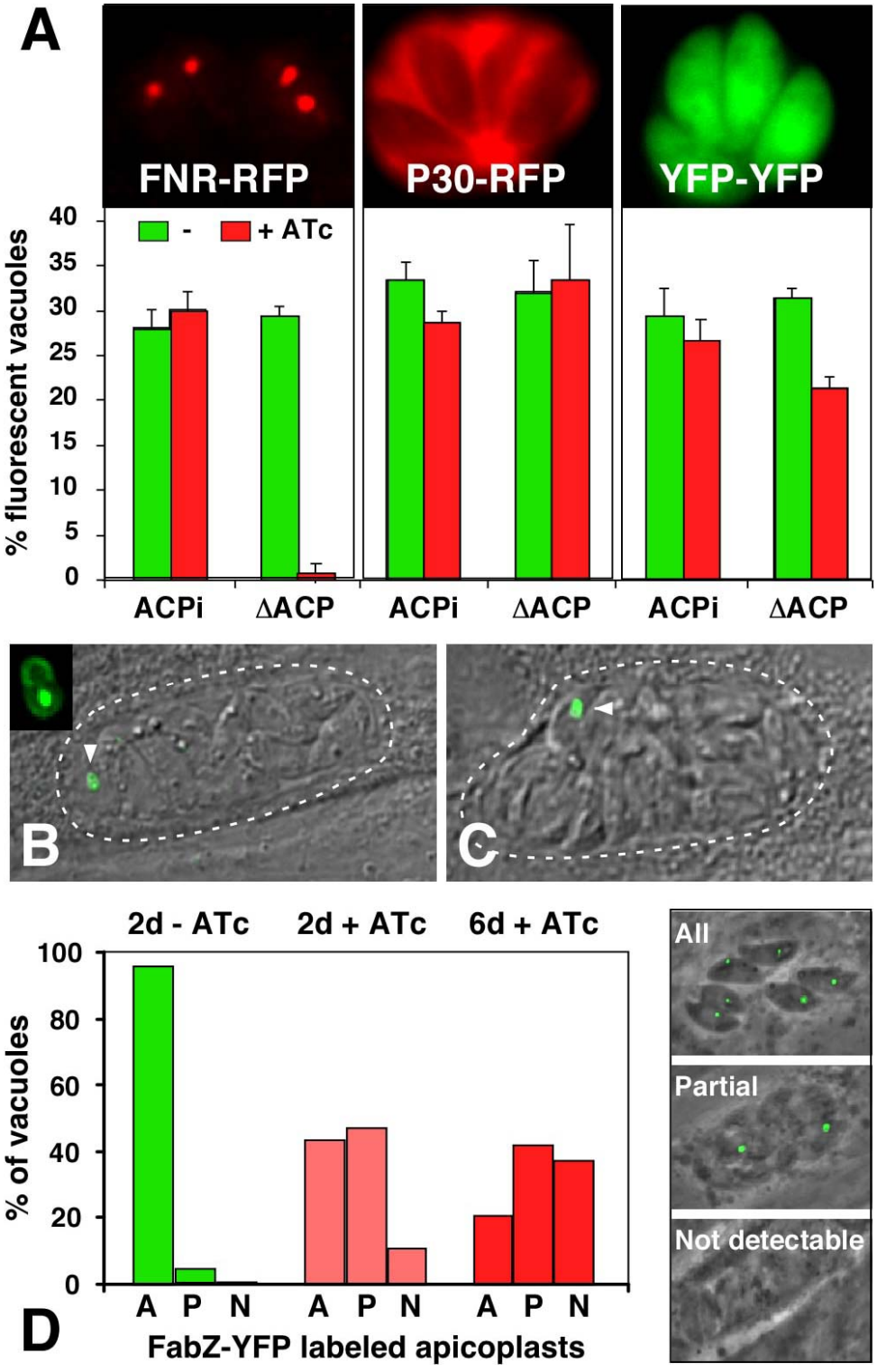


Fig.4.6: FAS II depletion results in apicoplast morphology defects

Figure 4.7: Morphological defects are organelle specific. (A) Apicoplast morphological defects (A, P and N) were also observed with endogenous apicoplast marker protein HU. Mutant cells treated with ATc for six days and immunostained with polyclonal anti-Hu antibody (generated by Shipra Vaishnava), University of Georgia, GA. **(B)** Parasite line ΔACP/ACPi/FabZ-YFP was transiently transfected with a plasmid encoding a Ty-tagged superoxide dismutase 2 (this protein targets to both apicoplast and mitochondrion, D. Soldati, unpublished). Transfectants were incubated in the presence (A-C) or absence of ATc (D-F). After two days cells were fixed and stained using antibodies to GFP (FabZ, red) or TY (SOD2, green). Note that while as shown in Fig. 4.6, FabZ-YFP staining is abolished by FASII knock-down, mitochondrial morphology appears unaffected.

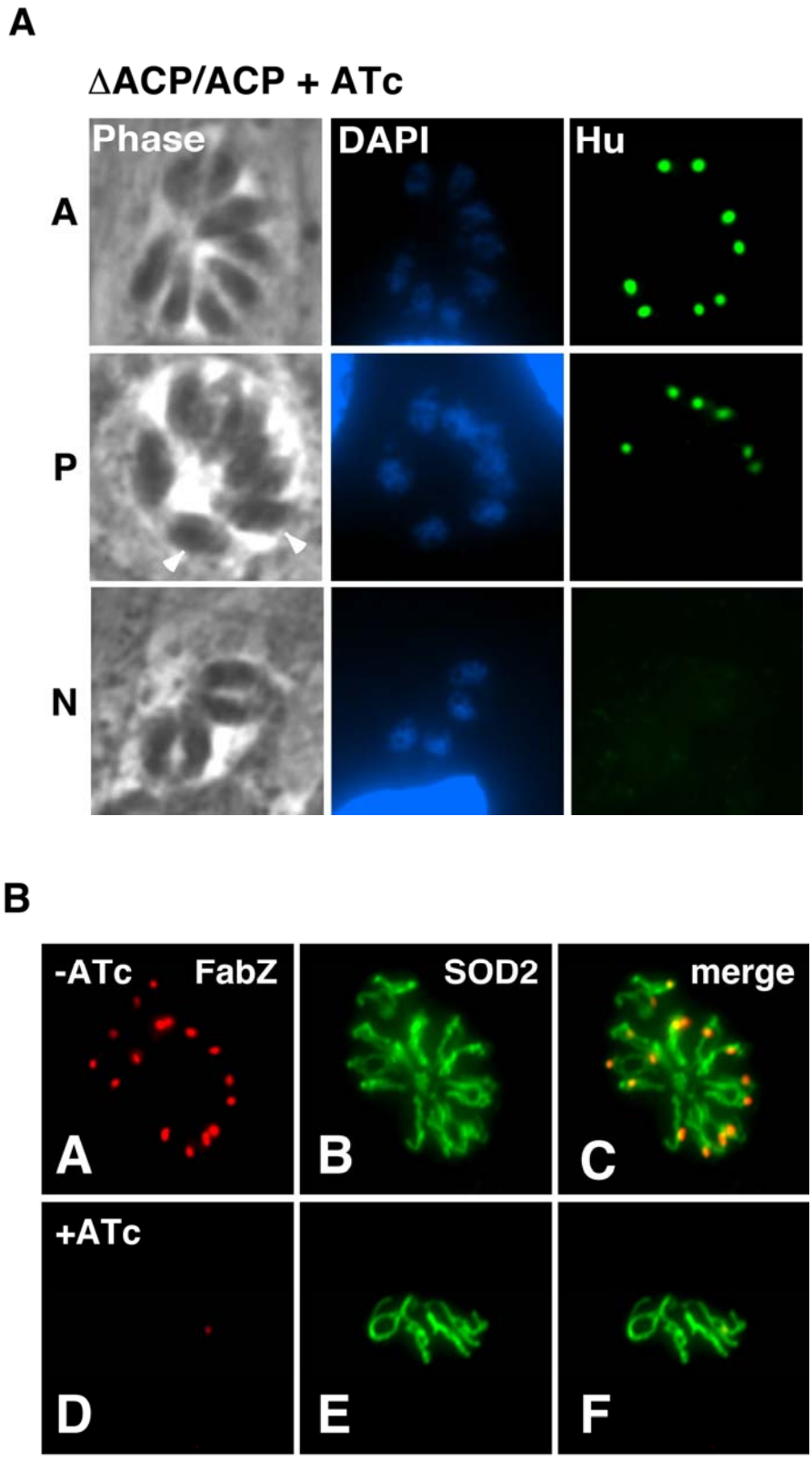


Fig 4.7. Morphological defects are organelle specific

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

An urgent need for novel anti-apicomplexan drug targets

Malaria caused by *Plasmodium* spp is estimated to kill at least two million people every year. *T. gondii*, a prominent opportunistic pathogen, is a major cause of mortality in HIV and other immuno-suppressed patients (Brindle et al., 1991; Slavin et al., 1994). *C. parvum*, an apicomplexan capable of water borne outbreaks, causes severe diarrhea and currently lacks effective treatment (Mead, 2002). Together, apicomplexan diseases remain a leading cause of global morbidity and mortality, creating an urgent need for the development of novel drugs.

Earlier we discussed the identification of a parasite specific organelle, the apicoplast. Found within most apicomplexan parasites, the apicoplast is derived from a prokaryote (*cyanobacterium*) by the process of engulfment. It is essential for parasite viability, and among other functions, it harbors several central metabolic pathways within its confines. The prokaryotic nature of metabolism and the absence of these pathways in the human host present the ideal platform for the development of drugs that are parasite specific, and least toxic to the host cells. Apicoplast localized fatty acid biosynthesis (FAS II) is one such parasite specific pathway. Both *P. falciparum* and *T. gondii* harbor a FAS II pathway. Preliminary pharmacological analysis indicates sensitivity of these parasites to certain FAS II inhibitors. But prior to this study the biological validation for the essential nature of apicoplast FAS II pathway was lacking.

5.1: Conclusions

5.1.1: Our research thus far: Apicoplast localized FAS II pathway is essential for parasite survival and pathogenesis

In the face of persistent therapeutic challenges, the hope for future treatment largely relies on the validation of potential drug targets. To evaluate the biological contributions of apicoplast FAS II pathway, we engineered a conditional knock-out mutant of FAS II pathway in, *T. gondii*. Beyond its own clinical significance, the ease of cultivation and genetic manipulation makes *T. gondii*, a model apicomplexan. To generate the mutant, we targeted the central component of FAS II pathway, acyl carrier protein for deletion. The success of our genetic approach largely relied on the development of a novel CAT-YFP "drug-FACS" combination selection for the rapid detection of allelic replacement events. Described in chapter 3, we foresee a wider application of our novel selection strategy for gene deletion experiments in both *T. gondii* and *P. falciparum*, otherwise a challenging task in apicomplexan parasites. Once generated, we analyzed FAS II functions in the mutant line devoid of ACP. The findings of our study are detailed in chapter 4 and can be summarized as follows:

1) Apicoplast FAS II is essential for parasite survival and pathogenesis;
2) Apicoplast fatty acids are important for the maintenance of apicoplast morphology and organelle biogenesis;
3) The major biochemical role of apicoplast FAS II lies in the production of specialized metabolites such as lipoic acid and not necessarily in the *de novo* synthesis of bulk lipids for the parasite;

4) Apicoplast supplies factors critical for parasite survival. Acetyl-CoA, a central metabolic precursor may be one such candidate. It remains to be seen if apicoplast acetyl-CoA serves the parasite biochemical pathways beyond the apicoplast.

Based on our analyses, the primary function of apicoplast FAS II pathway might lie in the maintenance of the apicoplast and processes within. Thus, consistent with previous observations (Charron and Sibley, 2002), *T. gondii* might indeed scavenge the bulk of the lipids from host cells. Apicomplexan parasites are known to be robust lipid scavengers. The role of *T. gondii* FAS I and fatty acyl elongases are yet to be elucidated. However, based on the detection of very long chain fatty acids in our analysis, it is reasonable to speculate the possible involvement of Tg FAS I and fatty acyl elongases in the modification and elongation of synthesized lipids (Fig 5.1). If confirmed, such a function for *T. gondii* FAS I will be consistent with observations made in *Cryptosporidium parvum* (Zhu, 2004), another clinically significant apicomplexan. In addition to furthering the understanding of apicoplast FAS II functions, our research thus creates scope for the future analyses of other apicomplexan pathways and apicoplast functions.

5.2: Future direction

5.2.1 Analysis of apicomplexan FAS I, using *T. gondii* as the model organism

Initially thought to lack mechanisms for *de novo* biosynthesis of fatty acids, data emerging from several apicomplexan genome sequence projects now reveal a highly diverse apicomplexan fatty acid metabolism (Table 5.2A). While some apicomplexans indeed lack a fatty acid biosynthetic pathway (Gardner et al., 2005), the clinically significant *P. falciparum* and *T. gondii* has confirmed presence of a FAS II pathway. Recently, the apicoplast localized

FAS II pathway has been identified in the agriculturally significant apicomplexan parasite, *Eimeria.tenella*, as well (Cai et al., 2003; Zhu, 2004). Interestingly, *C. parvum*, which is presumed to lack an apicoplast, lacks the FAS II pathway. Instead, *C. parvum* harbors a cytosolic fatty acid synthase system which is closer to the eukaryotic Type I FAS (Zhu et al., 2000).

C. parvum lacks drug treatment, which is often attributed to the lack of knowledge of basic metabolic pathways in this organism. The discovery of Cp FAS I pathway and the sensitivity of *C. parvum* to FAS I inhibitor cerulenin, offers one potential drug target (Zhu et al., 2000). However the specificity of cerulenin to CpFAS I over human FAS I is not known. Moreover, the contribution of CpFAS I towards parasite survival is yet to be elucidated. As mentioned before, during the course of this study, we and others have identified a FAS I pathway in *T. gondii* as well (Mazumdar et al., 2006; Zhu et al., 2000). Sequence analysis of the two pathways indicates remarkable similarity. This presence of FAS I pathway in the model organism *T. gondii*, presents an excellent opportunity for the future analysis of apicomplexan FAS I functions.

5.2.2 A conditional approach to analyzing Tg FASI^{-} phenotype

Tg FAS I (TgTwinScan_0460) is a large polypeptide ~9000 aa, encoded over a region of > 35,000 bp and has 19 exons (ToxoDB). It shares remarkable sequence similarity with *C. parvum* FAS I (Fig 5.2B). Immunofluorescence assay with a polyclonal rabbit serum reactive against *Cp*FASI, indicates a mitochondrial localization of Tg FASI (our analysis) (Fig 5.2C). To test for FAS I functions and to evaluate its functions towards parasite survival, we applied several strategies to engineer a mutant of FAS I pathway in *T.gondii*. The very large size of Tg FAS I gene makes it impractical for it to be expressed as a conditional transgene using standard molecular biology techniques. We therefore first tried direct gene knock out of Tg FAS I. For

this, we targeted the first three exons for allelic replacement, the knock-out vectors were designed to remove the start codon and thus to inhibit translation of the protein. We were unable to disrupt FAS I gene, presumably because Tg FAS I has essential functions. Next, we tried to swap the FAS I endogenous promoter with a tet regulatable promoter, to put the parasites under the direct control of a regulatable FAS I protein. Knocking –in a regulatable promoter directly upstream of the protein of interest, would allow the convenient down-regulation of the protein and subsequent phenotypic characterization. We targeted a 3kb region immediately upstream of the putative start codon (*T. gondii* promoter activity is usually characterized within ~1.5kb upstream region, (Kibe et al., 2005; Mercier et al., 1996)) for exchange with the tetO7sag4 promoter. However, in control experiments we were unable to drive expression of YFP protein with the putative FAS I promoter region, suggesting either a weak promoter activity or the absence of a functional promoter in the selected region. In the absence of an accurate gene model for Tg FASI, we postponed the approach.

5.2.3 Targeting FAS I by the conditional knock-out of FAS I Phospho-pantetheinyl transferase (Type I PPTase)

Attachment of a phosphopantetheinyl moiety to the newly synthesized holo-ACP, mediated by the enzyme Phosphopatetheinyl transferase (PPT), is critical for the activation of fatty acid synthases (Joshi et al., 2003; Lambalot et al., 1996; Mootz et al., 2001). Recently two different classes of PPT have been identified in apicomplexan parasites. Biochemical analysis indicates that the activation of *C. parvum* FAS I depends on a surfactin production element (SFP) type PPT (CpSFP-PPT), and on a holo-acp synthase type PPT in *P. falciparum* (PfACPS-PPT) (Cai et al., 2005). While PfACPS-PPT appears phylogenetically related to proteobacterial type PPT, CpSFP-PPT is closer to the mammalian counterpart. Both SFP type and ACPS type PPT

(TgSFP-PPT and TgACPS-PPT) implicated in the specific activation of FAS I and FAS II enzymes, has been identified in *T. gondii* (Cai et al., 2005) (Table 5.1). Visualization of Tg SFP-PPT tagged to fluorescent reporter YFP, indicates a mitochondrial localization similar to FAS I (Fig 5.2C).

To investigate the functions of Tg FAS I, we now aim to disrupt Tg FAS I functions by the conditional knock-out of its activation enzyme, TgSFP-PPT. Unlike FAS I polypeptide, SFP-PPT is a 1329 bp long gene. *T. gondii*, encodes a single copy of SFP-PPT gene (TgTwinScan_1208) (ToxoDB). We are thus first aim to establish a cell line expressing a regulatable copy of SFP-PPT (SFP-PPTi). We have engineered a plasmid ptetO7sag4-SFP-PPTcmyc/DHFR-TS for transfection into Tati cells. The plasmid was constructed by exchanging ACP with SFP-PPT coding region (retrieved from plasmid tub-SFP-PPTYFP/sagCAT, a kind gift of Dr. David Roos, University of Pennsylvania, PA) in the vector ptetO7sag4-ACPmyc/DHFR-TS with BglII/AvrII, previously described in chapter 3. The fusion of SFP-PPT to myc epitope tag will permit easy detection of SFP-PPT transgene. The cells are currently under selection for stable incorporation of ectopic SFP-PPTmyc. Once established, the transgene will be tested for expression, localization and ATc regulation. In the background of inducible SFP-PPT expression, we will target endogenous SFP-PPT for gene deletion, an approach similar to discussed in chapter 3. Functional deletion of the endogenous gene in the presence of regulatable ectopic copy will place the parasites under the direct control of endogenous SFP-PPT, allowing for convenient phenotypic characterization. We are currently engineering targeting constructs. We have successfully PCR amplified 2.5 kb 3’ and 5’ flanking sequences from Tg genomic DNA, and is in the process of cloning it into the KO vector. Once generated, the Tg FAS I mutants will be tested for their biochemical role, sensitivity to various FAS

inhibitors and most importantly for their contribution in the growth and pathogenesis of parasites. Such a study will permit the complete understanding of fatty acid biosynthesis in *T. gondii*, and might further research on FAS I based drug development for *C.parvum*.

5.2.4 Analysis of the role of apicoplast acetyl-CoA

One of the primary functions of the pyruvate dehydrogenase complex (PDH) involves the decarboxylation of pyruvate to yield acetyl-CoA and NADH. In plants, which maintain organelle specific PDH, acetyl-CoA and NADH generated in the chloroplast feeds into essential metabolic pathways such as fatty acid biosynthesis, whereas the acetyl-CoA generated in the mitochondrion is oxidized to CO_2 and H_2O by the TCA cycle to yield ATP (Mooney et al., 2002). Interestingly, apicomplexan parasites *P. falciparum* and *T. gondii*, maintain a single pyruvate dehydrogenase complex (PDH) which is exclusively located in the apicoplast. As demonstrated in chapter 4, FAS II knock down which affects parasite growth and viability, also inhibits lipoylation (activation) of this PDH complex. However, even though it lacks a PDH, several TCA cycle enzymes have been identified within the apicomplexan mitochondrion (Gunther et al., 2005; McMillan et al., 2005). The source of mitochondrial acetyl-CoA is currently unknown and if apicoplast acetyl-CoA serves as the link for mitochondrial pathways for energy generation, remains to be elucidated. Based on our findings linking FAS II down regulation to inhibition of PDH lipoylation, it could be well speculated that the apicoplast FAS II is retained to supply precursors for the maintenance of the apicoplast and organelles beyond. In this respect, it will be interesting to test if mitochondrial functions are linked to apicoplast acetyl-CoA (Fig 5.3). To test this, we plan to regenerate apicoplast lipoic acid biosynthesis in the FAS II mutant background by targeting the mitochondrial lipoic acid scavenge enzyme lplA, to the

apicoplast. Giel van Dooren, in our lab has annotated mitochondrial lplA, an enzyme active in the lipoic acid scavenge pathway. He next aims to target the mitochondrial lplA enzyme (TgTwinScan_3273) to the apicoplast of the ΔACP/ACPi cells, by appending an apicoplast targeting sequence to the N-terminus of the protein. It will be interesting to see if introduction of the LA scavenge pathway can activate the apicoplast PDH complex (can be detected by immunological reagents). If tested positive, parasite growth will be observed. A reversion of the growth phenotype will help determine if PDH generated acetyl-CoA and NADH are factors responsible for normal growth and viability of the parasite. Currently, information on the presence of lipoic acid transporters in the apicoplast is unknown, and if apicoplast localized lplA will have access to exogenous lipoic acid, remains to be seen.

Figure 5.1: A model for lipid biosynthesis in *Toxoplasma gondii.* *T.gondii* harbors 3 sources of fatty acids. It can scavenge lipids from the host cell. It harbors an apicoplast localized prokaryotic Type FAS II pathway, and recently an additional FAS I pathway has been identified as well. The main role of the apicoplast-localized FAS II pathway appears to lie in the biosynthesis of specialized lipids including lipoic acid for use by the apicoplast. *T.gondii* also harbors a eukaryotic FAS I pathway and modifying enzymes elongases which functions in the elongation of typical FAS product C:16 (palmitic acid). Our biochemical assay offers support that the parasites probably derive bulk lipids, (C:16) from the host cells which are further modified by FAS I and elongases to produce long chain fatty acids. Modification of FAS II lipids by FAS I remains a possibility, but is yet to be determined.

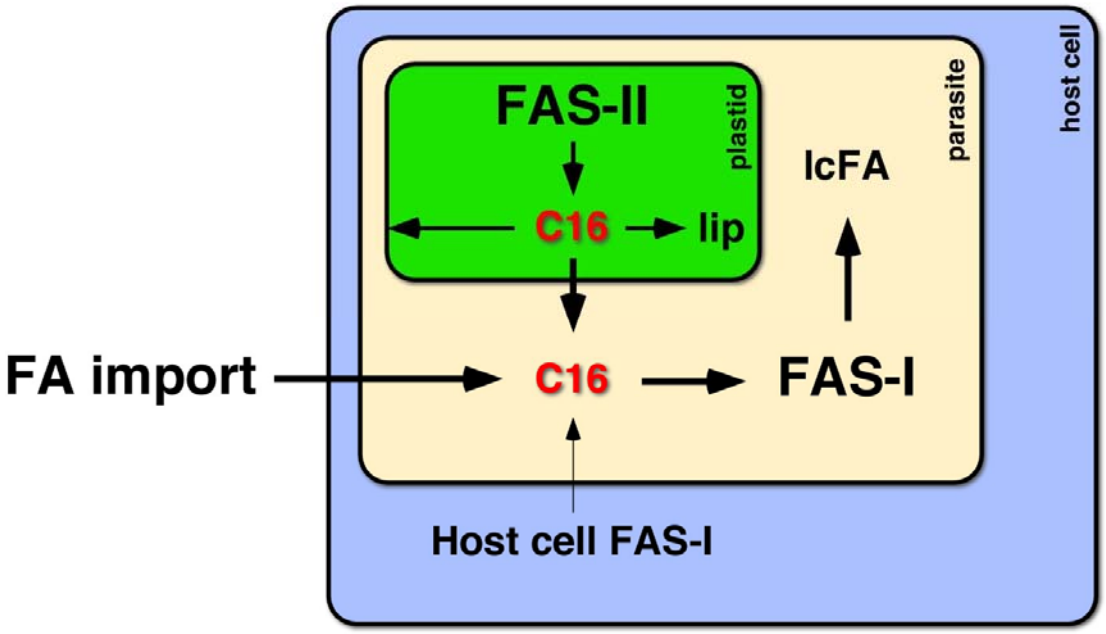


Fig. 5.1: **A model for lipid biosynthesis in *Toxoplasma gondii***

Table 5.1: Apicomplexans display diversity in lipid biosynthesis. Both prokaryotic and eukaryotic type fatty acid biosynthesis pathways and fatty acid modifying enzymes are identified in apicomplexans. Accessory enzymes supporting the presence or absence of these FAS pathways have also been identified accordingly. The presence of both FAS I and FAS II, makes *T. gondii* an excellent model for studying apicomplexan lipid biosynthesis.

Figure 5.2: Preliminary characterization of Tg FAS I pathway. (A) An alignment of a portion of Cp FAS I and Tg FAS I. CpACP phosphopantetheinyl binding domain (PBD) and Tg PBD show many conserved regions, indicating high sequence similarity. **(B)** Mitochondrial localization of Tg FAS I. Immunostaining of parasites with a polyclonal antibody against CpFAS I generate mitochondrial signal. **(C)** Expression of Tg SFP-PPT transgene indicates mitochondrial localization as detected with YFP fluorescence.

Table 5.1: Diversity of apicomplexan lipid biosynthesis

Apicomplexan	FAS II	FAS I	ACPS-PPT	SFP-PPT	Elongase
P. falciparum	+	-	+	-	+
C. parvum	-	+	-	+	+
T. gondii	+	+	+	+	+

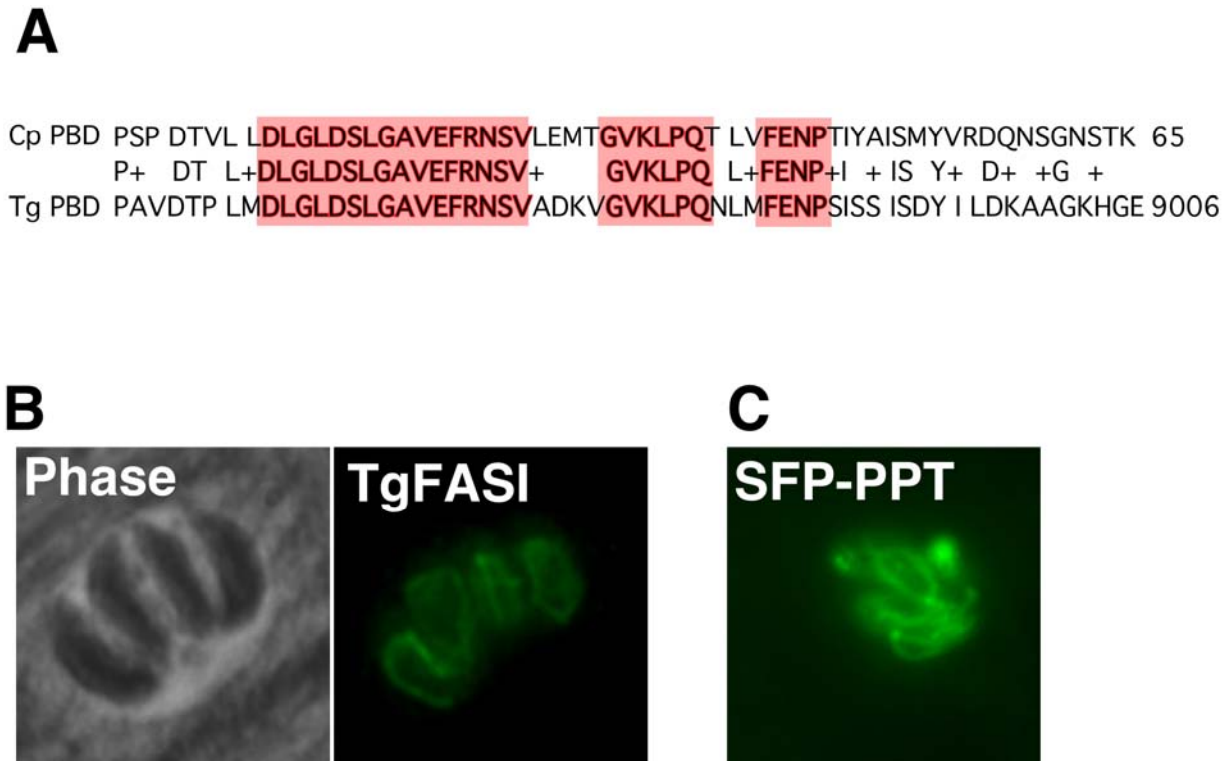


Fig 5.2. Preliminary characterization of *T. gondii* FAS I pathway

Figure 5.3: A model for the analysis of apicoplast-mitochondrial biochemical interaction. Activation of PDH depends on *de novo* synthesis of cofactor lipoic acid by lipA and lipB and FAS II intermediate octanoyl ACP. Inactivation of PDH should shut down acetyl-CoA generation. If acetyl-CoA feeds into mitochondrial and cytosolic pathways is unknown. Potential candidate pathway could be TCA cycle for the generation of energy. The apicoplast and mitochondrion maintains a close physical contact. This has been hypothesized to favor sharing of metabolic enzymes, as observed for heme biosynthesis (section 2.5.2).
Mitochondria harbors lplA which can scavenge lipoic acid from host cell, for lipoylation of TCA cycle enzymes. Targeting of lplA to the apicoplast in the FAS II mutant background should restore lipoylation of apicoplast PDH (if apicoplast harbors lipoic acid transporters). A functional PDH in the absence of apicoplast FAS II provides a good model for the testing of importance of apicoplast acetyl-CoA in parasite metabolism.

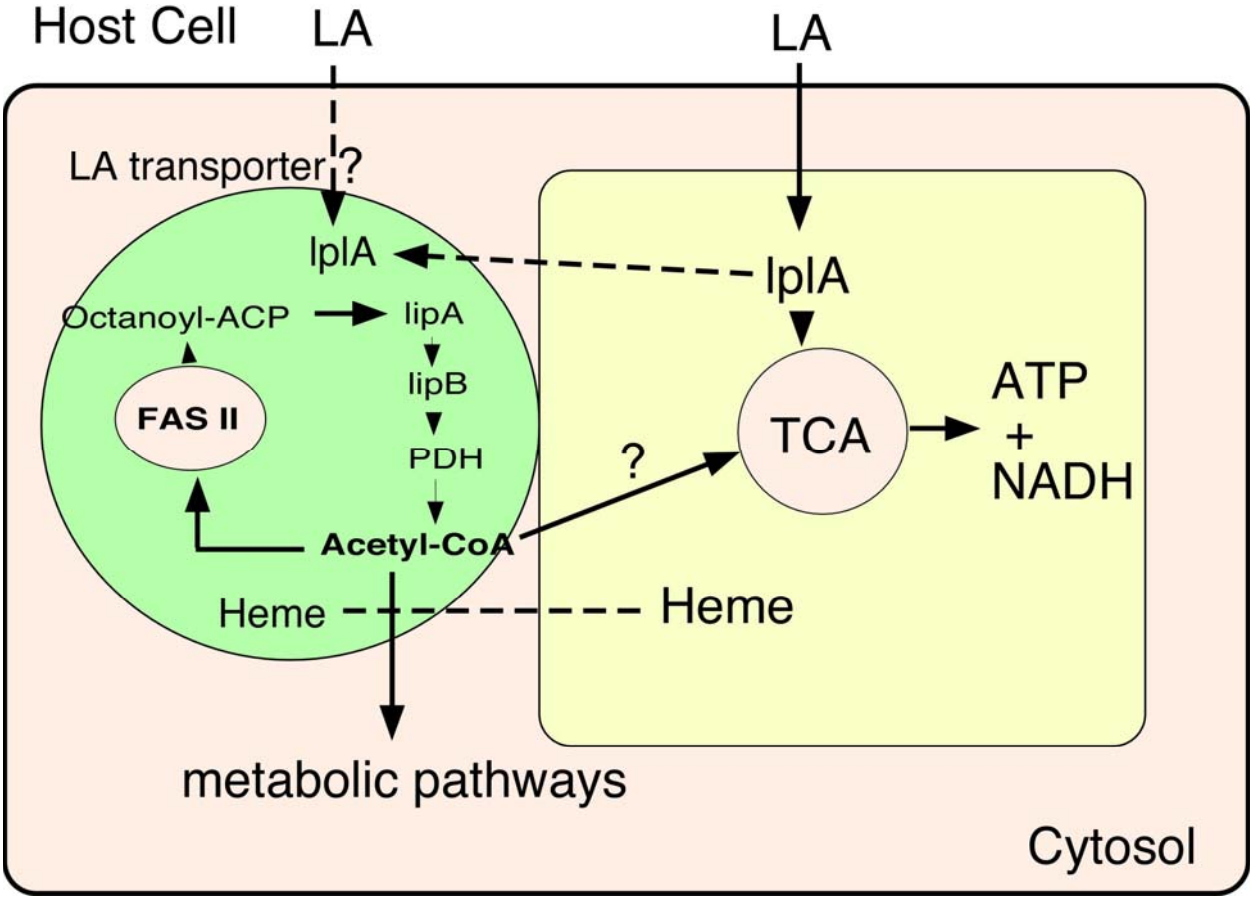


Fig 5.3: Possible points of biochemical interaction between the apicoplast and the mitochondrion

APPENDIX A

Figure A1: **Reactions catalyzed by FAS II enzymes**. Fatty acids are synthesized by sequential incorporation of acetyl-CoA (2:C precursor).

1: **ACCase**: carboxylation of acetyl-CoA to malonyl-CoA. This is the commited step of fatty acid synthesis reactions and requires input of energy, ATP.

2: **Fab D and AT**: catalyzes conversion of malonyl-CoA and acetyl-CoA to malonyl-ACP and acetyl-ACP respectively.

3. **Fab H:** Condensation of malonyl-ACP + acetyl-ACP to generate acetoacetyl-ACP

4. **Fab G:** Reduction of acetoacetyl-ACP to D-3 hydroxybutyryl-ACP

5. **Fab Z:** D-3 hydroxybutyryl-ACP is dehydrated to crotnoyl-ACP (trans Δ^2 enoyl-ACP)

6. **Fab I:** Crotnoyl-ACP is reduced to Butyryl-ACP (4:C intermediate)

7. **Fab B/F:** Condensation of butyryl-ACP and malonyl-ACP, to initiate a new round of elongation

8. **ACP:** Carrier protein carrying growing acyl-chains.

FAS II reactions usually terminates at C:16 (Palmitic acid). Thioesterase which cleaves fatty acyl chain from fatty acyl-ACP is absent in apicomplexans. FAS II intermediate Octanoyl-ACP (C:8) is precursor for lipoic acid (LA) biosynthesis. LA is required for activation of PDH, which generates acetyl-CoA from pyruvate.

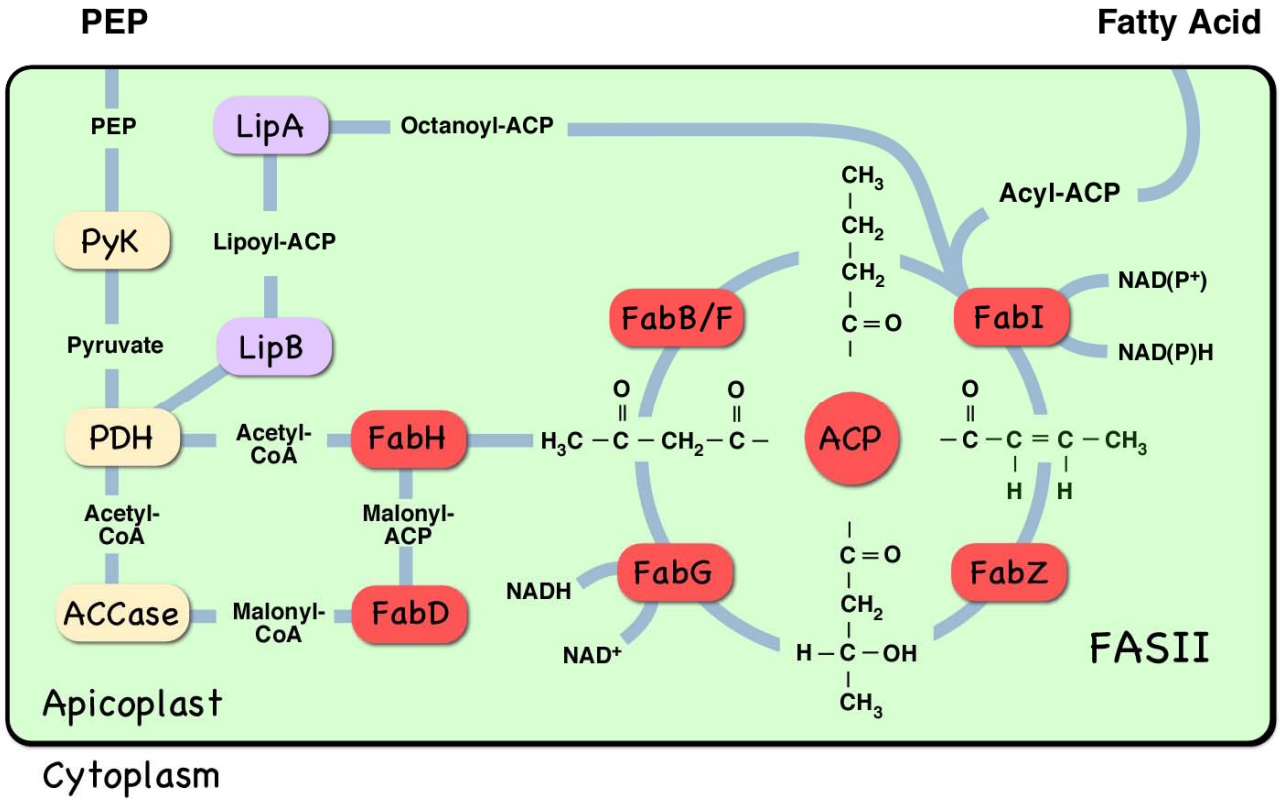


Fig. A1: Reactions catalyzed by FAS II enzymes and immediate downstream function in the apicoplast.

APPENDIX B

Figure B1: FAS II inhibitor thiolactomycin generates apicoplast morphology defects. Non-mutant Rh strain expressing a fluorescent plastid marker, FNR-RFP (red) and fluorescent dense granule marker, P30-GFP were treated with 0.05mg/ml thiolactomycin. Secretion of dense granule proteins into the parasitophorous vacuole (PV) stains the PV green. Cells were imaged on different days. Three day after Thl treatment several vacuoles displaying defective apicoplast morphology were captured, detected by loss of red apicoplast signal (middle and lower panel). Untreated cells maintained normal apicoplast morphology at all times (Upper panel). These results were consistent with our previous observations made in FAS II knock-out mutants.

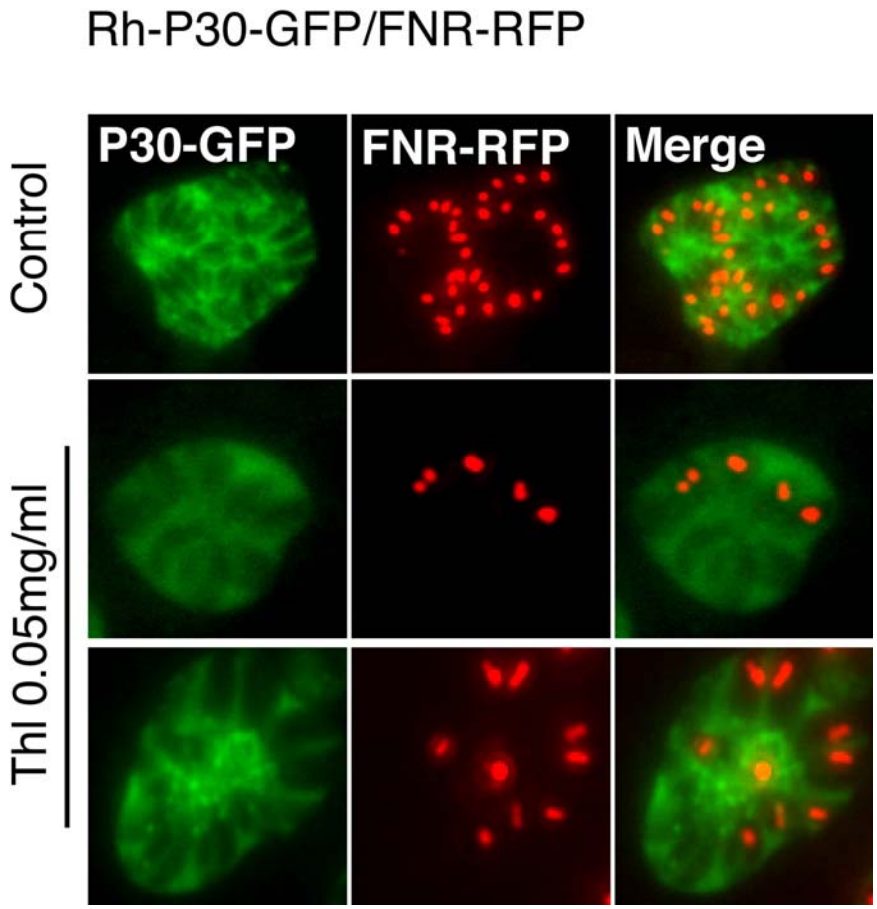


Fig. B1: Thiolactomycin treatment generates apicoplast morphology defect

APPENDIX C

Figure C1: Vectors for targeting Tg FAS I pathway. Tg FAS I is a large gene which comprises of 19 exons. To knock-out Tg FAS I function, we applied two different approaches for gene deletion. **(A)** Deletion of the start codon to inhibit translation of the protein. Tg FAS I currently lacks a fully annotated gene model. We therefore targeted the first three exons to ensure removal of initiation codon. **(B)** Construction of a targeting cassette pKI-FAS I to replace endogenous FAS I promoter with tetO7Sag4 regulatable promoter. The targeting vector was sequentially constructed to incorporate a 5' and 3' homologous sequence flanking a regulatable promoter and gene marker for selection. Tub-YFP was incorporated outside 3' homologous sequence. The vector was linearized prior to transformation. The strategy was to knock-in tetO7Sag4 regulatable promoter in front of FAS I exons, to gain conditional expression of the protein. Note, FAS I promoter region and initiation site is yet to be confirmed.

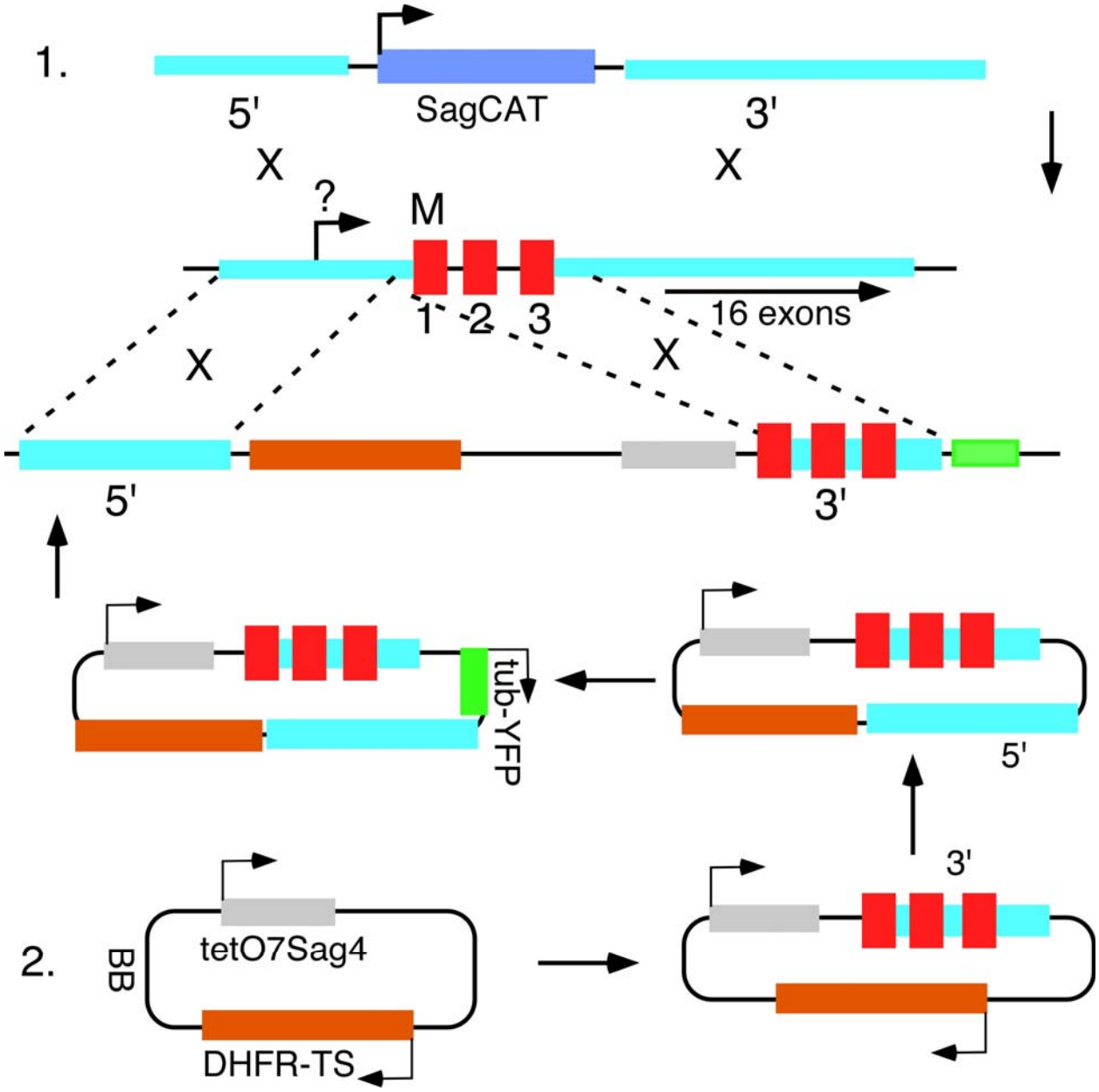


Fig. C1: Vectors for targeting of *T. gondii* FAS I pathway

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