FATTY ACID SYNTHESIS IN THE APICOMPLEXAN PARASITE

TOXOPLASMA GONDII

by

SRINIVASAN RAMAKRISHNAN

(Under the Direction of Boris Striepen)

ABSTRACT

Apicomplexan parasites are obligate intracellular pathogens infecting humans and many animals. Members of this group are causative agents of severe diseases like malaria, cryptosporidiosis, toxoplasmosis and babesiosis. Similar to other intracellular pathogens, apicomplexan parasites rely on both synthetic and import mechanisms to obtain necessary nutrients for growth. Consequently, these metabolic pathways have been the focus of drug development studies.

Components of the metabolism of fatty acids are validated drug targets against intracellular bacterial and viral pathogens. Likewise, this process has been considered as a suitable target against apicomplexan infections. However, fatty acid synthesis in apicomplexan parasites seems to be a very complex phenomenon due to presence of multiple mechanisms for lipid synthesis and acquisition. Understanding the details of these mechanisms is key to identifying the strongest drug target.

Toxoplasma gondii is an apicomplexan parasite, which habors three mechanisms of fatty acid synthesis. The parasite harbors both prokaryotic type II (FASII) and eukaryotic type I (FASI) pathways of fatty acid synthesis. Additionally, the parasite appears to contain a fatty acid

elongation machinery. In this research, we attempt to determine the role and significance of these pathways in *T. gondii* tachyzoites, the parasite life cycle stage responsible for acute toxoplasmosis.

While the FASII pathway is essential for parasite growth, its precise contribution towards overall fatty acid synthesis has not been determined yet. Little is known about the function and significance of FASI and FAE pathways in *T. gondii*. Characterizing these pathways has been especially challenging due to expected similarity in their products and substrates. Here, we have developed a novel strategy by coupling genetics with metabolomics to individually dissect the precise role of each synthesis mechanism.

We show that fatty acid synthesis is a crucial determinant for *T. gondii* infection. We also show that the parasite selectively uses these pathways to generate specific products that are necessary for continuous growth and thereby for establishing an acute infection.

INDEX WORDS: Apicomplexa, fatty acid, *T. gondii*.

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DEDICATION

This work is dedicated

to my brother Sharavanan for being so supportive, inspiring, and encouraging

and to my parents Lata and Ramakrishnan for their countless sacrifices.

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CHAPTER 1

INTRODUCTION

Apicomplexa are obligate intracellular protozoan parasites, which cause devastating diseases in humans and animals. Toxoplasmosis is a disease caused by the apicomplexan parasite *Toxoplasma gondii*. Humans become infected by consumption of food or water contaminated with *T. gondii* oocysts or tissue cysts. The disease presents mild symptoms in most immunocompetent individuals but can be life-threatening in those with a weak immune system.

Based on its degree of pathogenicity in humans, the disease has been classified into two stages – an acute stage and a latent stage. Acute stage refers to the severe form of the disease where actively dividing parasites cause tissue and organ damage, debilitating the infected individual. Immunocompetent individuals present minor symptoms like muscle aches, fever, and rarely require treatment. But, in immunocompromised individuals, the disease can cause major symptoms like encephalitis, myocarditis or pneumonitis (1-3) and can only be controlled by drug treatment (4). Immune or drug pressure (5) limits the damage, but fails to clear parasites that have entered the latent state. Latent stage caused by dormant parasites is asymptomatic. However, in absence of immune pressure, the dormant parasites are often reactivated and cause recurring episodes of acute infection with severe symptoms (6).

Acute and latent infections are caused by two different stages of parasite life cycle within the human host, the tachyzoite and bradyzoite stage respectively. *T. gondii* tachyzoites are motile, asexual and fast multiplying parasite forms. These tachyzoites actively invade and

multiply in almost all nucleated cells. The majority of tachyzoites multiply within the host cell giving rise to more tachyzoites, which are released by the lysis of the infected cell. However, some tachyzoites may convert to bradyzoites after invasion. Bradyzoites are slow replicating forms, which remain dormant by forming a tissue cyst (7). These slow-diving bradyzoites evade the immune system, resist drug treatment and are responsible for the latent form of the disease. Unlike bradyzoite development, the active multiplication of tachyzoites is responsible for organ damage and clinical symptoms associated with toxoplasmosis. Hence, it is critical to inhibit the growth of *T. gondii* tachyzoites to reduce the severity of the disease.

T. gondii tachyzoites can be controlled with a combination of pyrimethamine and sulfdiazine, drugs that target parasite folic acid synthesis (8). However, resistance against these agents is always a risk (9, 10). Atovaquone is another drug of choice, which seems to be active against both acute and latent stages. However, atovaquone resistance in *T. gondii* has emerged recently and is a growing concern (11, 12). The budding resistance along with the toxicity associated with these drugs suggests an immediate need for better therapeutic agents and newer drug targets. Hence, more thorough understanding of parasite metabolism is needed for the identification of new targets.

Numerous aspects of the *T. gondii* metabolism have been studied in detail (13). The recent development of molecular and biochemical tools has elevated our understanding of several metabolic pathways in the parasite (14). However, some of these pathways have only been characterized partially and further research is required to determine their accurate role and contribution towards parasite biology. Fatty acid synthesis in *T. gondii,* is one such process; the details of which are poorly understood (15). *T. gondii,* similar to other eukaryotes, uses fatty acids for protein modification (16) and phospholipid synthesis (17). Moreover, the presence of

specialized membranous organelles like micronemes, rhoptries and a parasitophorous vacuole suggests that the parasite could have a much higher demand for fatty acids as compared to other eukaryotes. Consequently, this parasite has developed multiple routes to acquire fatty acids. *T. gondii* can not only import fatty acids but also harbors three mechanisms to synthesize its own fatty acids.

In this thesis we investigate the fatty acid synthesis pathways in *T. gondii* tachyzoites, the stage responsible for acute infection. The parasite harbors three mechanisms for synthesis – a fatty acid synthesis type I (FASI) pathway, a fatty acid synthesis type II (FASII) pathway and a fatty acid elongation (FAE) pathway (18). It has been shown that the FASII pathway is required for the growth of *T. gondii* tachyzoites. However, precise products of the FASII pathway were not identified (15). Moreover, the FASI pathway and FAE pathway remain uncharacterized. In this research we make an attempt to determine the precise role and significance of each fatty acid synthesis pathway in tachyzoites. The second chapter in this thesis reviews the current knowledge about fatty acid synthesis pathways in protozoan parasites with a special focus on apicomplexan and kinetoplastid parasites. Chapter 3 describes how we develop a new strategy by combining genetics with metabolomics to identify the precise products of FASII pathway and fatty acid elongases in *T. gondii*. Chapter 4 and Chapter 5 describe the use of this strategy to identify the role and significance of FAE and FASI pathway respectively. The last chapter provides an overall conclusion for this research on *T. gondii* fatty acid synthesis pathways.

All together, this dissertation unravels the role and significance of individual pathways contributing towards fatty acid synthesis in *T. gondii*.

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CHAPTER 2

REVIEW OF LITERATURE

LIPID SYNTHESIS IN PROTOZOAN PARASITES[1]

[1] Ramakrishnan, S., Serricchio, M., Striepen, B. and Bütikofer, P. Submitted to *Progress in lipid research,* 01/28/2013.

2.1 ABSTRACT

Lipid metabolism is of crucial importance for pathogens. Lipids serve as cellular building blocks, signalling molecules, energy stores, posttranslational modifiers, and pathogenesis factors. Here we discuss the tremendous advances that have been made in the understanding of the synthesis and uptake pathways for fatty acids in apicomplexan and kinetoplastid parasites. Many of the most important human parasites belong to these two phyla including *Plasmodium*, *Toxoplasma*, *Cryptosporidium*, *Trypanosoma* and *Leishmania*. Parasite lipid synthesis differs in significant ways from its counterpart in the human host in both phyla. Parasites have acquired novel pathways through endosymbiosis, as in the case of the apicoplast, they have dramatically reshaped substrate and product profiles, and they have evolved specialized lipids that are used to interact with or to manipulate the host. These differences potentially provide opportunities for drug development. However, a number of recent studies demonstrate that targets have to be carefully evaluated. The parasites rely on a complex system of uptake and synthesis to satisfy the needs. The parameters of this system change dramatically as the parasite transits through the various stages of its lifecycle. We outline the lipid pathways for key species in detail as they progress through the developmental cycle and highlight those that are of particular importance to the biology of the pathogens and/or are the most promising targets for parasite specific treatment.

2.2. INTRODUCTION

Diseases caused by protozoan parasites are among the most pressing concerns on the global health agenda. Within their ranks are not only some of the most widespread and important infectious diseases, but also many of the most sorely neglected [1]. In this review we will focus on two protozoan phyla in particular, Apicomplexa and Kinetoplastida, which stand out in their overall public health impact with respect to incidence and severity of the diseases they cause [2]. Not only are these the most important groups of parasites, they also are the best studied. Facile experimental model systems have been established for several members of both phyla. Driven by powerful genetics these models have yielded considerable insight into parasite metabolism. There are numerous significant metabolic differences between these parasites and their human host, and we will highlight these differences, as they afford opportunities for drug development. We will provide a brief introduction to the parasite models that are the focus of this article for readers unfamiliar with the organisms. We will describe the particular importance of lipid metabolism for pathogens to introduce the subsequent systematic and comprehensive discussion of the biosynthesis pathways of major lipid classes for both phyla.

2.2.1 Apicomplexan Parasites

Apicomplexans are eukaryotic pathogens named after the complex of secretory and cytoskeletal organelles located at the apical end of the parasite cell. Apicomplexa are intracellular parasites and the organelles of the apical complex are required for host cell invasion. Three genera represent a particular threat for human health and therefore will be the main focus

of this review: *Plasmodium*, *Toxoplasma* and *Cryptosporidium*. Note, however, that there are numerous additional apicomplexans that are important veterinary pathogens.

Five species of *Plasmodium* collectively are the causative agents of malaria, a disease that claims the lives of about a million individuals every year. Most of these deaths are due to *Plasmodium falciparum* and occur in small children in sub-Saharan Africa. *Plasmodium* has a complex life cycle, and as we will see, profound metabolic and biochemical changes are associated with each life cycle transition. Infection of a human occurs through the bite of a mosquito, whereby a small number of sporozoites are injected with the saliva. The sporozoites travel to the liver and invade hepatocytes, where they massively proliferate. Merozoites are then released into the bloodstream, where they invade and replicate in red blood cells. It is this intraerythrocytic phase that is responsible for the disease. Control of malaria rests on the prevention of transmission (e.g. through treated bed nets) and drug treatment. The genetic malleability of the parasite and its propensity to evolve drug resistance [3] has long haunted malaria control. This famously includes the loss of chloroquine as an effective therapeutic. There is grave concern about the future of the current artemisinin-based treatment regimen with first signs of treatment failure emerging in South East Asia [4, 5]. A constantly evolving portfolio of new anti-malarials is required to keep up with the ever-changing parasites. Understanding parasite metabolism is an important cornerstone of this agenda.

Toxoplasma and *Cryptosporidium* were initially recognized as opportunistic infections in immune-compromised individuals and received particular attention as late stage manifestations of AIDS [6, 7]. The sexual phase of the *Toxoplasma* life cycle occurs in the intestinal tract of cats and results in the shedding of spore-like oocysts, which then become highly infectious to other animals and humans upon consumption of contaminated food or water. Within

intermediate hosts, tachyzoites cause rapid systemic infection. The onset of immune control eliminates tachyzoites, but bradyzoites persist within tissue cysts for the life of the host. In immune-compromised adults, chronic infection is reactivated leading to *Toxoplasma* encephalitis. In addition, congenital toxoplasmosis is a significant concern during pregnancy. While anti-folate treatment is effective in controlling tachyzoites and acute disease, there are no drugs available to eliminate chronic infection, which is problematic in a variety of clinical settings [8].

Cryptosporidiosis is an acute enteric disease that typically is self-limiting. However, recent studies show that in particular in malnourished children the disease can be severe, protracted, and life threatening [9]. *Cryptosporidium* has a single host life cycle restricted to the intestinal epithelium, and oocysts are the only mode of transmission. Nitazoxanide was approved by the FDA for treatment but only shows moderate efficiency in immune-competent children and produces no benefit in immune-compromised patients. Finding a reliable drug remains an important goal [10].

2.2.2. Kinetoplastid parasites

Kinetoplastids are flagellated protists that share a mitochondrial genome of unique organization and localization called the kinetoplast. There are numerous human pathogens among these early-branching eukaryotes, African and American trypanosomes and many different species of *Leishmania*. All of these pathogens have insect and mammalian hosts, and the stages associated with the various stages of the life cycle can be morphologically distinguished based on the position and length of the flagellum. More importantly, they also

show remarkable metabolic differences. Stages from insect and mammalian hosts can be cultured axenically, which has greatly facilitated biochemical and genetic studies.

Trypanosoma cruzi is the causative agent of Chagas disease, which is endemic in large parts of South America (note that although both the parasite and the vector are present in North America, transmission is rare). *T. cruzi* is transmitted by reduvid, or kissing bugs. Promastigote stages replicate within the bug's midgut and infective metacyclic trypomastigotes are deposited with the feces onto the skin of the mammalian host. In the mammalian host, the parasite cycles between replicative intracellular amastigotes and trypomastigotes. Importantly, replicating amastigotes are free in the host cell cytoplasm providing intimate access to host metabolites. Chagas disease is characterized by chronic and progressive inflammatory tissue damage, in particular of the heart muscle. Treatment is available but is not consistently effective and suffers from significant adverse effects [11].

Trypanosoma vivax, *T. congolense* and *T. brucei brucei* cause Nagana, an important cattle disease that severely limits ranching and dairy production in many parts of Africa. *T. brucei gambiense and rhodesiense* are morphologically indistinguishable from the bovine subspecies and are the causative agents of Human African Trypanosomiasis, also known as sleeping sickness. All African trypanosomes are vectored by tsetse flies, where they are present as procyclic trypomastigotes, epimastigotes and metacyclic trypomastigotes. In the mammalian host, trypomastigotes replicate extracellulary in the bloodstream causing anemia and cachexia. In humans, the blood phase is ultimately followed by invasion of the central nervous system. Untreated infection is invariably fatal. Several drugs are available for treatment of Human African Trypanosomiasis and Nagana, but there are severe limitations. Some of the drugs have grave and even life threatening adverse effects, are limited in their potency once the parasites

have entered the brain, or are only active against certain species or subspecies. Lastly, there is treatment failure and overt resistance to several drugs, in particular in veterinary practice [12-14].

Parasites of the genus *Leishmania* are transmitted by a variety of sandflies, where promastigotes replicate in the midgut and are mechanically introduced into the bite site. In the mammalian host, *Leishmania* parasites proliferate as intracellular amastigote in macrophages, and they do so within a fully matured and acidified phagolysosome [15].

A large number of different *Leishmania* species are responsible for a broad spectrum of disease manifestations, with currently 12 million people being infected worldwide. In its most benign form, leishmaniasis presents as a self-limiting skin ulcer at the bite site (cutaneous leishmaniasis). In contrast, progressive non-healing erosion of mucosal tissue in the vicinity of the bite site is characteristic for mucocutaneous forms. In the most severe visceral disease, parasites spread systemically causing hepatosplenomegaly, cachexia and immunosuppression. The etiology of these various forms appears to be closely linked to an unbalanced immune response to the chronic infection resulting in too much or too little inflammation and parasite control. Visceral leishmaniasis is fatal if not treated. Several drugs are available [16], but treatment of leishmaniasis remains challenging due to adverse effects, the requirement of lengthy regimens, limited drug availability and expenditure [17].

2.2.3. Lipids and Pathogens

Lipids are critical to the biology of all cells and organisms. They are the main structural elements of all biological membranes, they anchor glycoconjugates and many proteins to membranes, they serve as signaling molecules within and between cells, and they represent a highly efficient store and source of energy and reduction power. Lipids are of particular importance for pathogens, and some pathogens deliberately seek out lipid-rich host niches [18], or enhance the availability of lipids by manipulating the host [19, 20]. Intracellular pathogens have evolved sophisticated mechanisms to manipulate and tap into the lipid metabolism of their host cells. These include interference with vesicular and non-vesicular cellular lipid trafficking in viral [21], bacterial [22] and protozoal [23] pathogens. Within host cells, intracellular pathogens often develop in specialized vacuoles and the flow of lipids between host and pathogen-controlled membranous compartments is key to the pathogen's ultimate success [15, 24-26].
Lipids are not only used by pathogens as food or structural building blocks, but are also important pathogenesis factors that allow the pathogen to evade immune responses, manipulate host processes, and cause disease. In many cases these are specialized lipids synthesized by the pathogen [27]. The best-characterized example in protozoan parasites is a class of specialized glycosylphosphatidylinositol (GPI) lipids. They include GPI-anchored lipophosphoglycans of *Leishmania* species, which are crucial for host specificity and survival of the parasite in the sand fly vector [28, 29], and are also thought to modulate the initial interaction with the mammalian host [30]. In African trypanosomes, GPI-anchored variant surface glycoproteins are at the heart of the antigenic variation mechanism used to enable chronic infection. In *Plasmodium* and

Toxoplasma, precursor GPI lipids are believed to play important roles as toxins and immune modulators [31-33].

Due to the importance of specialized lipids in mycobacteria, lipid synthesis has been a major target of drug development for tuberculosis [34]. Such examples are also present in protozoan parasites. As we will describe in detail, kinetoplastids and apicomplexans rely on a number of mechanisms for lipid synthesis that are not found, or different from those used in the mammalian host. These include fatty acid synthesis in the mitochondrion and the plastid, specialized elongation and desaturation pathways, and differences in downstream pathways of phospholipid synthesis. The success of miltefosine as an orally available *Leishmania* drug is one important validation of lipid metabolism as a drug target. Lipid turnover in pathogens is complex and involves numerous mechanisms of uptake and synthesis. In the following we will systematically review fatty acid and uptake pathways in both apicomplexans and kinetoplastids.

2.3 FATTY ACID SYNTHESIS IN PROTOZOAN PARASITES

The genomes of protozoan parasites encode the enzymes for three distinct biochemical pathways involved in fatty acid synthesis. While there are significant differences between these pathways, the underlying chemistry and sequence of enzymatic reactions is highly conserved (Fig. 2.1). All pathways synthesize fatty acids by successive addition of two carbon units to a growing carboxylic acid chain that is held via the pantetheinyl group of acyl carrier protein (ACP) or coenzyme A (CoA). The length of this starter chain may vary. Fatty acid synthesis type I and II (FASI and FASII) typically produce fatty acids *de novo*, while the fatty acid elongation (FAE) pathway adds two carbon units to a typically much longer starter molecule. As detailed

below, FASI and FASII differ in their architecture, FASI is expressed as a single very large polypeptide whereas FASII has multiple individual components (Fig. 2.1B,C). The source of carbon to be added is malonyl-CoA, the activated form of a three carbon dicarboxylic acid. Malonyl-CoA is generated by acetyl-CoA carboxylase (ACC) from two molecules of acetyl-CoA (this step typically regulates the flux of fatty acid synthesis). Decarboxylative condensation of the malonyl substrate with the starter yields a chain elongated by two carbon units. The carbons are then fully reduced by the successive action of ketoacyl reductase, dehydratase and enoyl reductase. At this point the fatty acid can be elongated through condensation with another malonyl-CoA, or released by cleaving the thioester bond holding the carboxyl end.

Not all fatty acid synthesis pathways are present in all apicomplexans. The FASII pathway has been thoroughly characterized in *Toxoplasma* and *Plasmodium,* but is absent in piroplasms and *Cryptosporidum*. A subgroup of Apicomplexa (the Coccidia and *Cryptosporidium*) also harbor a FAS I megasynthase.

The genomes of kinetoplastid parasites encode the enzymes of two pathways to synthesize fatty acids, a FASII pathway and a FAE pathway. Both pathways have been characterized in *T. brucei* using genetic and biochemical approaches. The genomes of other kinetoplastid parasites, namely *T. cruzi* and *Leishmania major*, encode clear homologs of the enzymes of these pathways, suggesting that fatty acid metabolism of these parasites may be similar to that of *T. brucei*. However, their roles have not been studied experimentally. Table 2.1 provides an overview of the distribution of the three pathways among well-characterized parasite species discussed in this review.

2.3.1 The FASII pathway in apicomplexans

The FASII pathway is the stereotypical prokaryotic mechanism of fatty acid synthesis. Much of what we know about the pathway comes from studies in *E. coli* [35], however, it is widely distributed among bacteria and typically represents their main and often only source of fatty acids. All enzymes as well as the ACPs are expressed as individual polypeptides. In addition, the FASII pathway is found in some eukaryotes, where it is localized within organelles derived through endosymbiosis involving bacteria. Plants contain this pathway within the chloroplast, which is believed to be derived from a cyanobacterium [36] and represents the sole site of fatty acid *de novo* synthesis in plants. Recent work has also described a FASII pathway in mitochondria of certain eukaryotes, where its role is still unclear but likely includes the synthesis of lipoic acid [37]. In apicomplexan parasites the FASII pathway is found in the apicoplast, a plastid-like organelle that is derived from a red alga through secondary endosymbiosis [38]. Similar to plastids of plants and algae, the apicoplast is not only home to a FASII pathway but also to a non-mevalonate isoprenoid synthesis pathway and a portion of the heme pathway [38, 39]. The enzymes of the FASII pathway have been characterized in several apicomplexan species, with particular attention on *P. falciparum*, from which β-ketoacyl-ACP reductase [40], β-ketoacyl-ACP synthase [41] and β-ketoacyl-ACP dehydratase [42] have been expressed as recombinant enzymes and subjected to kinetic analyses. In addition, there is structural information available for *P. falciparum* 3-oxoacyl-ACP reductase [43] and β-hydroxyacyl-ACP dehydratase [44].

Since the FASII pathway is not a major source of fatty acids for the mammalian host, it has been suggested that this pathway might represent a parasite-specific drug target against

apicomplexan parasites. Consistent with this hypothesis, *Plasmodium* parasites were found to be sensitive to triclosan inhibition *in vitro* and *in vivo* [45]. Triclosan targets the enoyl-ACP reductase of the bacterial FASII pathway [46] and is a common ingredient of anti-bacterial soaps. Similarly, *Toxoplasma* and *Babesia* were reported to be susceptible to triclosan [47, 48], suggesting that enoyl-ACP reductase may represent a general target to inhibit growth of apicomplexan parasites and prompting further studies to characterize the *Plasmodium* enzyme and to find more potent inhibitors [49-51].

At around the same time, the genomes of several apicomplexans were sequenced, revealing that not all Apicomplexa have a FASII pathway. These include genera that apparently lost the apicoplast, such as *Cryptosporidium*, but also genera that still harbor an apicoplast such as *Plasmodium* [52-54]. Importantly, the FASII pathway and the presumptive triclosan target, enoyl-ACP reductase, are absent from *Babesia* and *Theileria* parasites, both of which are susceptible to triclosan at a dose comparable to the malaria parasite [53-56]. This, together with the observation that some bacteria lacking enoyl-ACP reductase are susceptible to triclosan [57] and the lack of inhibition of enoyl-ACP reductase by triclosan in *T. brucei* [58], raised doubts about the specificity of the compound for the FASII pathway. These concerns were further heightened when new triclosan derivatives were tested. Careful structure-activity relationship studies revealed that the activity of triclosan against enoyl-ACP reductase did not correlate with the activity against parasites [59].

With the pharmacological support for an essential role for FASII weakened, genetic studies were conducted in *Toxoplasma* and *Plasmodium*. A conditional *T. gondii* mutant was constructed in the gene for FASII ACP. When apicoplast ACP expression was blocked in this mutant by exposure to anhydrous tetracycline, parasite growth in culture was significantly reduced, while

continued suppression resulted in parasite death [60]. Furthermore, mice challenged with this mutant parasite were cured from a lethal infection by tetracycline treatment. When analyzed biochemically, these mutants showed a pronounced loss of lipoic acid production in the apicoplast. This was consistent with a loss of FASII activity, which is thought to provide octanoic acid-ACP, i.e. the precursor of apicoplast lipoic acid *de novo* synthesis [60-62]. In conclusion, *Toxoplasma* FASII is required for parasite development and pathogenesis.

The importance of FASII was revisited using genetic approaches in *P. falciparum* and two rodent malaria species [59, 63]. The studies demonstrated that the FASII enzymes are not expressed in the blood stages, which are the cause of clinical malaria. Furthermore the genes for several FASII enzymes including the presumptive triclosan target, enoyl-ACP reductase, could be deleted from the genome without impairing parasite growth in the blood stage or their development in the mosquito. Lastly, *P. berghei* enoyl-ACP reductase null mutants were as susceptible to triclosan as wild type parasites and the same was true for parasites expressing enoyl-ACP reductase carrying point mutations that commonly confer robust resistance to triclosan [59]. In conclusion, the anti-parasitic effect of triclosan is not due to inhibition of FASII, and FASII is not a valuable target for anti-malaria therapy to cure blood stage infection. Importantly however, the pathway was found to be essential for the parasite in the liver phase of the infection [59, 63]. Taken together, the studies in *Toxoplasma* and *Plasmodium* demonstrate that the importance of the FASII pathway is highly stage- and host cell-dependent. While *Toxoplasma* and the *Plasmodium* liver stage require FASII for survival, other stages and parasite species can apparently satisfy their needs by salvage of fatty acids from the host. This is consistent with findings in bacteria, where the importance of the FASII pathway is governed by the availability of exogenous fatty acids [64, 65].

While the requirement of the apicoplast FASII pathway is now well understood for life cycle progression of certain apicomplexans, its role in fatty acid metabolism of parasites has remained unclear. Is it a true engine of *de novo* synthesis of fatty acids, as in chloroplasts of plants, or does it have a more specialized and local role [66]? As pointed out above, biochemical analysis of FASII mutants in *T. gondii* suggested a link between FASII and lipoic acid synthesis [60, 61]. Lipoic acid in the apicoplast is required for the activity of the E2 subunit of pyruvate dehydrogenase, which in turn is required to supply the substrate for fatty acid synthesis, acetyl-CoA [67, 68]. In addition, lipoic acid is a cofactor for mitochondrial enzymes and, therefore, may be exported from the apicoplast to mitochondria. However, several studies have shown that mitochondrial lipoylation is independent of *de novo* synthesis of lipoic acid in the apicoplast but instead relies on lipoate salvage from the host [61, 69-71].

In summary, the role the FASII pathway in apicomplexan parasites is much more complex and diverse than initially anticipated. Its value as a drug target depends on the parasite species and the life cycle stage under consideration. Parasites harboring this pathway utilize it for *de novo* synthesis of fatty acids as well as to supply the substrate for lipoic acid synthesis (Fig. 2.2).

2.3.2 The FASII pathway in kinetoplastids

The FASII pathway in kinetoplastid parasites appears similar to the FASII pathway from other organisms. An ACP holds the growing acyl chain while the chain is modified by a synthase, dehydratase and two reductases. The major difference between the apicomplexan and kinetoplastid FASII is their organellar localization. While the FASII pathway in apicomplexan

parasites localizes to the apicoplast, the FASII pathway in kinetoplastids is found in the mitochondrion [72]. Mitochondrial localization of FASII has been confirmed in *T. brucei* by epitope tagging of the individual TbFASII enzymes. Three of the proteins, ACP, dehydratase and ketoacyl-ACP synthase, have been studied in detail. The presumptive dehydratase component of the TbFASII pathway complements the loss of the corresponding enzyme in yeast, i.e. it restores lipoic acid synthesis and respiration [73]. Genetic analysis in *T. brucei* shows that deletion or RNAi knockdown of ACP or ketoacyl-ACP synthase blocks growth of procyclic and bloodstream forms in culture [72], demonstrating that TbFASII is essential in both life cycle forms. Biochemical analyses using a cell free system and radioactive precursors demonstrate palmitate to be the final product of the TbFASII pathway. While capable of fatty acid production, the pathway shows moderate activity and contributes only about 10% of total parasite fatty acid synthesis activity [72]. This suggests that an alternate mechanism of synthesis may be the major contributor to the overall fatty acid pool in kinetoplastids. A second and maybe more important role for TbFASII appears to be the synthesis of octanoic acid ACP for lipoic acid production. It has been shown that ablation of the FASII pathway in *T. brucei* blocks lipoic acid synthesis [72], indicating that the TbFASII pathway is the main source of octanoic acid for lipoic acid synthesis.

Are there additional roles for FASII-synthesized fatty acids beyond lipoylation? Biochemical analyses indicate reduced phospholipid synthesis in TbFASII mutants [72], and loss of mitochondrial ACP decreases the levels of certain mitochondrial phospholipids. This change in phospholipid composition coincides with changes in morphology and membrane potential of the parasite mitochondrion. Loss of ACP also affects the respiratory complexes and significantly reduces cytochrome-mediated respiration [74]. A more recent report on TbFASII highlights the

importance of the pathway for kinetoplastid DNA segregation in *T. brucei* bloodstream forms. This segregation defect is believed to be due to altered phospholipid composition of the mitochondrial membrane, resulting in disturbed assembly and structure of the tripartite assembly complex that is required for kDNA segregation [75].

The importance of the FASII pathway in other kinetoplastids has not been addressed yet. However, it has been demonstrated that expression of *L. major* dehydratase and ketoacyl reductase complements the respiration defect of yeast mutants for the corresponding enzymes (43). In addition, genome mining indicates the presence of additional FASII components in both *L. major* and *T. cruzi* (acyl carrier protein: LmjF.27.0290 and Tc00.1047053511867.140; ketoacyl synthase: LmjF.33.2720 and Tc00.1047053504157.20; enoyl-ACP reductase: LmjF.04.0290 and Tc00.1047053506627.20). All candidate proteins are predicted to localize to the mitochondrion, based on the presence of an N-terminal leader sequence. Together, these data suggest that the FASII pathway is likely conserved among kinetoplastids.

2.3.3. The FASI pathway in apicomplexans

Several apicomplexan parasites encode a FASI megasynthase. The overall chemistry of FASI is similar to that of FASII. The reaction sequence is initiated by loading an acyl starter moiety to the phosphopantetheinyl arm of the ACP domain, followed by elongation cycles catalyzed by a synthase, a dehydratase and two distinct reductase domains [76, 77]. In contrast to FASII, all activities and domains of FASI are part of a single polypeptide chain. FASI is the fatty acid synthesis mechanism typically found in eukaryotes, including humans and yeast, however, it has also been described in certain bacteria [78, 79]. Among apicomplexan parasites, candidate

genes for the FASI pathway have been reported in *Cryptosporidium* [80], *Eimeria* [81], and *Toxoplasma* [66]. Bioinformatic and chemical analyses of apicomplexan FASI pathways suggest important differences compared to their mammalian counterparts. Structurally, mammalian FASI is a unimodular protein comprised of seven enzymatic domains. These enzymatic domains act sequentially for several cycles of elongation on an acyl moiety attached to ACP. Bioinformatic analysis of *C. parvum* FASI suggests that it is a multimodular protein where each module contains a set of multiple enzymatic domains [80]. The unusual architecture of apicomplexan FASI indicates that the mechanism of chain elongation is quite different from that of mammalian FASI. It is likely that the substrate is undergoing a single round of elongation in each module prior to being transferred to the ACP of the next module. The fate of the elongated product, in both mammals and apicomplexans, depends on the final domain of the enzyme. In mammals, a thioesterase domain releases the elongated product by hydrolytic cleavage [76], whereas in apicomplexan parasites, the thioesterase domain is replaced by a reductase domain, suggesting that the released product may be a fatty alcohol rather than a fatty acid [82].

The divergence of the apicomplexan FASI is not limited to its structural organization but may include its substrate specificity. Our current knowledge on the specificity of apicomplexan FASI is limited to data from the *C. parvum* enzyme, CpFASI. Biochemical experiments with recombinant CpFASI indicate that it may act as a fatty acid elongase rather than in *de novo* synthesis. Elongation versus *de novo* activity of FASI is defined by the substrate specificities of the starter and termination domains. The starter domain of CpFASI showed preference for long chain fatty acid substrates, with palmitoyl-CoA showing the highest activity in substrate competition assays [83], whereas the terminator domain showed preference for very long chain fatty acids, i.e. hexacosanoyl-CoA [82]. Expression of enzyme modules in *E. coli* is a remarkable

idea to make these giant enzymes tractable, but may have limitations. Heterologous expression and the reliance on CoA-bound model substrates may modulate the specificity of the domains. Also, interaction with other enzymes *in vivo* may not be fully mimicked in an *in vitro* system. Nonetheless, these results strongly support the idea that CpFASI is involved in the production of very long chain fatty alcohols and, thus, is functionally different from mammalian FASI. Instead, CpFASI may resemble bacterial FASI (phylogenetic analyses appear to support a potential direct relationship via horizontal gene transfer). For example, it has been shown that FASI from *Mycobacterium* is capable of generating very long chain fatty acids using palmitate as substrate [84], and pathogenic mycobacteria use their FASI enzyme to elongate fatty acids imported from the host cell [85]. The resulting very long chain fatty acids can then be utilized for mycolic acid biosynthesis. It is likely that *C. parvum* FASI has a similar function, i.e. to elongate palmitate imported from the host cell to generate very long chain fatty acids.

Since the overall architecture of CpFASI is similar to FASI in *Toxoplasma* and *Eimeria*, it is likely that these proteins work in a similar fashion and perform a similar role. It is interesting to note that apicomplexan parasites harboring this pathway also harbor polyketide synthases (PKS) and form oocysts that are shed into the environment. These oocysts are surrounded and protected by an oocyst wall that confers remarkable chemical resistance. Oocysts are impervious to bleach and water chlorination, and the wall has a yet to be characterized lipid component. In contrast, apicomplexan parasites lacking FASI and PKS, like hematozoans [53, 54, 66], do not form such environmentally stable oocysts. It is tempting to speculate that the products of the FASI enzyme could play an important role in oocyst formation, however, such a link has not been demonstrated experimentally.

PKS, like FASI, are multifunctional polypeptides and the apicomplexan enzymes appear to be type I PKS [86]. While the overall architecture of PKS enzymes resembles that of FASI enzymes, there are important differences. FASI fully reduces each added two-carbon unit. In contrast, PKS megasynthases typically lack certain domains, resulting in chains with multiple keto or hydroxyl groups [87]. These reactive groups can then be precursors for complex secondary products (this can but not always does involve additional enzymes). By varying the domain architecture, a myriad of compounds can be synthesized, including many molecules with importance in medical microbiology, such as toxins [88] and antibiotics [89]. PKS from *C. parvum* is the only characterized apicomplexan PKS so far, but its product is not known [90]. As for FASI, Zhu and colleagues used modular expression to study this enzyme. Interestingly, they found that the loading unit of PKS preferred very long fatty acids as substrates [91]. Dinoflagellates, a phylum of photosynthetic protists most closely related to apicomplexans, are well known to produce a variety of polyketide toxins (the typical cause of shellfish poisoning and red tide massive fish kills). These toxins appear to be the product of PKS-type enzymes and phylogenetic analysis has linked PKS from *Cryptosporidium* and some dinoflagellate PKS [92]. While initial studies in dinoflagellates have focused on toxins, it now appears that there may be additional roles for polyketides and related metabolites in the biology of these organisms [93]. Cysts are not restricted to apicomplexans but also found in dinoflagellates. It is tempting to speculate that FASI and PKS may collaborate to produce a special lipid of the oocyst wall and that such walls may predate apicomplexan parasitism. A similar collaboration between FASI and PKS has been reported for certain fungal [94] and bacterial [95] species.

2.3.4. The FAE pathway in apicomplexans

FAE represents the third mechanism of fatty acid synthesis in apicomplexan parasites. Similar to the FASII pathway, the FAE pathway operates using single monofunctional polypeptides to catalyze each elongation cycle. Every elongation cycle starts with a condensation reaction catalyzed by an elongase enzyme, however, in contrast to *de novo* synthesis systems, the FAE begins with a much longer starter (16:0 or longer). The product of this enzyme is modified sequentially by a ketoacyl reductase, a dehydratase and an enoyl reductase to produce a fatty acid that is elongated by a two carbon unit [96]. A major mechanistic difference that distinguishes the FAE pathway from the FASII pathway is the anchor molecule to which the growing acyl chain is attached. Unlike the FASII pathways that use ACPs, the FAE pathway employs CoA as an anchor molecule for fatty acid elongation [96, 97].

The FAE pathway is present in all eukaryotes. In plants [98], animals [99] and yeast [100], the pathway localizes to the ER and is important for proper growth and development [101, 102]. In apicomplexan parasites, elements of the FAE pathway were first characterized in *C. parvum*, which encodes a single fatty acid elongase localized in the parasitophorous vacuole membrane. This implies that the enzyme is secreted from the parasite and could act on substrates derived from the host cell. This may be part of a specialized uptake and modification process. It would be very interesting to localize additional components of the *Cryptosporidium* FAE machinery to further characterize this unusual compartmentalization. Biochemical studies on the recombinant enzyme showed that its preferred substrates are myristoyl-CoA and palmitoyl-CoA. Starting with myristoyl-CoA the enzyme can conduct up to two rounds of elongation generating stearic acid as the final product [103].

In contrast to *C. parvum,* in most organisms the FAE pathway employs multiple elongases with defined substrate and product length specificities that can act successively. While yeast contains three elongase genes [102], mammals contain six [96] and plants as many as twenty-one, highlighting the particular importance very long chain fatty acids, fatty alcohols and waxes play in the biology of plants [104]. Similar to *S. cerevisiae*, *T. gondii* and *P. falciparum* encode three fatty acid elongases [66]. Metabolic labeling experiments with radioactive myristate and palmitate suggest fatty acid elongation to be active in blood stage malaria parasites [105]. Similarly, labelling with radioactive acetate detects incorporation into long chain fatty acids, suggesting a role for FAE pathway in *T. gondii* tachyzoites [60, 66]. Fatty acid elongases exhibit chain length specificity for products and this has been previously reported for yeast and plant enzymes. However, how ELOs measure the actual chain lengths of their products was puzzling for some time. A seminal article by Denic and Weismann has elucidated this mechanism [100]. Fatty acid elongases in yeast are membrane proteins of the ER with their active sites exposed on the cytoplasmic surface. A lysine residue on the luminal side of a transmembrane helix is responsible for sensing the methyl end of the fully elongated fatty acid. Using mutational analyses, it was shown that the position of this lysine residue in the sixth transmembrane helix determines the length of the fatty acid released from a fatty acid elongase [100]. Specificity of elongase enzymes also influences the essentiality of FAE pathway. In yeast the loss of individual elongase enzymes is tolerated, whereas simultaneous disruption of two fatty acid elongases is lethal [102]. This has been attributed to overlapping specificity of the fatty acid elongases [102]. Besides yeast, the products of the FAE pathway are essential in mammals and plants as well.

While it can be suggested that apicomplexans might be using two different systems (FAE and FASI) to synthesize long chain fatty acids, it is not known what the products are and what are

their precise roles in the parasite. In plants [101] and yeast [106], loss of the FAE pathway affects phospholipid synthesis. Long chain fatty acids could be performing a similar role in apicomplexan parasites as well. However, such a link between phospholipid synthesis and fatty acid elongation in apicomplexan parasites has yet to be studied experimentally.

2.3.5. The FAE pathway in kinetoplastids

The kinetoplastid FAE pathway comprises of multiple elongases, dehydratases and reductases. The best-characterized components of this pathway are the four elongase enzymes of *T. brucei*. The *T. brucei* FAE resides in the membrane of the ER [107] (Fig. 2.3). A transgenic version of TbElo3 fused to GFP co-localized with blue-white DPX staining, a dye that preferentially accumulates in the ER [107]. While at first glance the enzymes and their localization in *T. brucei* appear to be quite similar to those in other eukaryotes, their functions are remarkably different. Unlike any other FAE pathway studied so far, the TbFAE pathway is capable of synthesizing fatty acids *de novo*. The key to this ability lies in the chain length specificity for the starter. While FAE pathways typically use a medium or long chain fatty acyl-CoA as primer, the TbFAE can use butyryl-CoA to prime the elongation process. The names of the elongase enzymes in the pathway (ELO1-4) refer to the order in which they act in the pathway. The specific role of each elongase enzyme was determined by analyzing ELO mutants in *T. brucei* bloodstream forms [107]. Fatty acid elongases are generally divided into two categories: elongases that act on saturated and monounsaturated fatty acids and elongases that act on polyunsaturated fatty acids (PUFAs). In the TbFAE pathway, ELO1-3 act on saturated fatty acids, whereas ELO4 is specific for PUFAs. Chain elongation for the TbFAE pathway starts with

the action of TbELO1, which uses butyryl-CoA to generate decanoyl-CoA, which is then extended by TbELO2 to myristoyl-CoA. The identification of this synthesis route for myristoyl-CoA solved a long-standing conundrum. The membrane of *T. brucei* bloodstream forms is covered with a dense coat of variant surface glycoproteins, which allows the parasite to evade host immunity through antigenic variation. These proteins are attached to the plasma membrane via a GPI anchor, which represents the first fully characterized GPI structure in eukaryotes [108]. Its composition is unique in that the fatty acyl chains are composed of myristic acid exclusively [109], requiring an elaborate fatty acid remodeling process to ensure this exclusivity [110]. Myristic acid is in low abundance in the serum of the mammalian host and it was puzzling for a long time as to how the parasite satisfies its enormous demand for this fatty acid. Myristoyl-CoA synthesized by the peculiar FAE *de novo* pathway solved this puzzle. Unexpectedly, however, *T. brucei* bloodstream forms lacking the FAE pathway showed no growth defect in culture and were not attenuated *in vivo* in the rat model [107].

Analysis of TbELO3 shows that this enzyme is capable of generating stearoyl-CoA from myristoyl-CoA. Importantly, this enzyme is only expressed in *T. brucei* procyclic forms, which synthesize surface proteins and GPI anchors that differ from bloodstream forms. The major acyl groups on procyclic GPI anchors are palmitate and stearate, and not myristate [111], suggesting that TbELO3 is the main provider of fatty acids for GPI anchors in procyclic trypanosomes. In contrast to the situation in *T. brucei* bloodstream forms, FAE appears to be essential for growth of procyclic parasites in culture. Altogether, these results show FAE in *T. brucei* to be the dominant source of fatty acids – even for shorter species like myristate. Thus, the importance of a fatty acid synthesis pathway for trypanosome survival depends on the life cycle stage and may be influenced by the availability of fatty acids in the host environment.

Components of the FAE pathway have also been detected in *T. cruzi* and *L. major*. A search of the respective genome sequences suggests the presence of candidate genes for ketoacyl-CoA reductases, dehydratases, and enoyl-CoA reductases in all kinetoplastid parasites. While *T. brucei* harbors four elongase genes, *T. cruzi* seems to contain five and *L. major* as many as fourteen. None of the encoded candidate enzymes has been studied experimentally. However, the elongases detected in *T. cruzi* and *L. major* are believed to be orthologs of TbELO1-3, or TbELO4, based on phylogenetic analysis [112]. The presence of additional elongase enzymes in *L. major* and *T. cruzi* may be related to the fact that the GPI anchors in these parasites have longer fatty acids [113] compared to those in *T. brucei*. In addition, the lipid portion of *Leishmania* lipophosphoglycan is composed of very long (24 and 26 carbon) alkyl chains that are likely derived from very long fatty acids [113].

2.3.6. Synthesis of unsaturated fatty acids

Unsaturation of fatty acids alters their physical and chemical properties. Incorporation of unsaturated fatty acids into membrane lipids can increase the overall flexibility and fluidity of membranes. The degree to which unsaturated fatty acids are used varies considerably between organisms. Unsaturated fatty acids are synthesized from saturated fatty acids by the action of desaturases [114]. Unsaturated fatty acids are classified into two main categories, monounsaturated fatty acids (MUFAs) and PUFAs. The synthesis of MUFAs occurs by introduction of a single double bond and is universally catalyzed by the enzyme stearoyl-CoA desaturase (SCD) [115-117]. SCD in the apicomplexan parasite *P. falciparum* was identified only recently [118]. The enzyme seems to be active in blood stage parasites [105] and is required

for growth [118]. Data from other systems suggest that expression of SCD can be influenced by certain PUFAs [119]. Whether such an effect on SCD is responsible for PUFA-mediated inhibition of *Plasmodium* growth [120] has yet to be determined. Genome searches in apicomplexan parasites reveal the presence of SCD in *Toxoplasma, Eimeria* and *Neospora*, but not in *Theileria, Babesia* or *Cryptosporidium*.

The action of desaturases is not limited to the formation of MUFAs. Additional desaturases distinct from SCD are responsible for the synthesis of PUFAs. These types of desaturases have not been reported in apicomplexan parasites. The presence of polyunsaturated fatty acids in *T. gondii* [119] and the demonstration of linoleic acid in *C. parvum* [119] and *Eimeria* oocysts [119, 121] suggests that these parasites could have evolved a mechanism to import the required polyunsaturated fatty acids from the host.

Kinetoplastids are able to synthesize PUFAs and they harbor multiple desaturases that can act sequentially. The pathway begins with SCD, which has been characterized experimentally in *T. brucei* and is essential for both bloodstream and procyclic forms [122]. Genome analysis of *Trypanosoma* and *Leishmania* further reveals the presence of delta-12, delta-4 and omega-3 desaturases (the numbers indicate the position of desaturation with respect to the carboxy- (delta) or methyl- (omega) end of the chain respectively). In contrast, delta-6 and delta-5 desaturases are only found in *Leishmania* [123-125], indicating that *Leishmania* but not *Trypanosoma* have a complete pathway to synthesize C22 PUFAs. In trypanosomes, a PUFA-specific elongase (TbELO4) and a delta-4 desaturase may generate C22 PUFAs from host-derived C20 substrates. In addition to high amounts of C22 PUFAs, trypanosomes also contain high amounts of linoleic acid [126, 127]. The omega-3 desaturase present in both *Leishmania* and *Trypanosoma* is responsible for the synthesis of linoeleic acid and has received particular

attention. The absence of this enzyme in humans makes omega-3 desaturase a potential candidate for parasite-specific intervention [123, 128].

Taken together, these studies show that both apicomplexan and trypanosomatid parasites follow a common paradigm for generating MUFAs using SCD. In both parasite groups this enzyme is important for growth. However, the processes of obtaining PUFAs appear quite different. Apicomplexans parasites rely on fatty acid import whereas trypanosomatids are capable of synthesizing PUFAs.

2.4 SUBSTRATES AND ACTIVATION OF FA SYNTHESIS PATHWAYS

The difference in the evolutionary origin and cellular localization of fatty acid synthesis pathways in apicomplexans is also reflected in more peripheral aspects. A first example is the provision of malonyl-CoA, the main substrate for fatty acid synthesis, a second is the activation of ACPs that are critical in both FAS systems. Apicomplexan parasites that harbor both the FASI and FASII mechanism seem to have evolved two independent systems for substrate provision and enzyme activation. The malonyl-CoA substrate is generated by the action of ACC. Two genes encoding ACC have been identified in *T. gondii*. Localization studies suggest that one of these enzymes is recruited to the apicoplast while the other is cytosolic [129, 130]. The apicoplast-localized ACC is probably required to generate malonyl-CoA for the apicoplast-based FASII pathway, whereas cytosolic ACC may supply malonyl-CoA for the FASI pathway. *Plasmodium,* which lacks the FASI pathway, and *Cryptosporidium*, which lacks the FASII pathway, harbor only single genes for ACC [66]. The idea that the apicoplast and the cytoplasm rely on independent pools of malonyl-CoA is also consistent with our recent stable isotope

labeling experiments. We found that ^{13}C-acetate is readily incorporated into the products of the cytoplasm- and ER-based FAE pathways (see below). In contract, carbon derived from ^{13}C-glucose but not ^{13}C-acetate is incorporated into fatty acids produced by the FASII pathway [131]. The apicoplast pathway is solely fed through the import of phosphoenolpyruvate [68] from the cytoplasm.

Unlike apicomplexans, kinetoplastids harbor only a single ACC. In *T. brucei*, the enzyme is cytosolic and its main role is to supply the FAE pathway. RNAi experiments demonstrate that TbACC is required in procyclic forms but dispensible in bloodstream forms, thus mirroring the findings for TbELO mutants [132, 133]. It is likely, yet has not been demonstrated experimentally, that malonyl-CoA generated by cytoplasmic TbACC also serves as substrate for the mitochondrial FASII pathway.

Two independent ACP activating enzymes are present in *T. gondii* [66, 134]. In both the FASI and FASII pathway, apo-ACP is activated to the holo-ACP form by addition of a phosphopantetheinyl moiety. This reaction is catalyzed by phosphopantetheinyl transferase [135]. The two phosphopantetheinyl transferases in *T. gondii* have not been characterized in detail yet. However, one of them carries a bipartite leader and is likely localized to the apicoplast and activates the FASII ACP. The second protein is related to the cytoplasmic enzyme previously characterized in *Cryptosproidium* [134] and is probably involved in FASI (and PKS) activation. How *T. brucei* ACP is activated remains unknown, a candidate phosphopantetheinyl tranferase gene is not immediately evident in the genome.

2.5 FATTY ACID SYNTHESIS PATHWAYS AS DRUG TARGETS

Because lipid biosynthetic pathways have long been considered of little importance for parasite survival, their potential as drug targets has not been systematically addressed. After a brief discussion of selected compounds known to affect lipid metabolism in Kinetoplastida and Apicomplexa, we highlight potentially interesting lipid pathways unique to certain members of these groups of parasites, or alternatively, are common to several parasite species but differ from the corresponding pathways in mammals.

2.5.1 Drugs targeting FASII

Triclosan [46] and thiolactomycin [136, 137] are well studied inhibitors of type II FAS. Soon after the discovery of a FASII pathway in apicomplexan parasites, these drugs were tested for their efficacy against *Plasmodium*. Both triclosan [45, 47] and thiolactomycin [138] inhibit the growth of blood stage *Plasmodium* parasites. Additional drugs known to target bacterial fatty acid synthesis include epigallocatechin gallate [139], NAS-91 [140], and flavonoids [141], all of which were evaluated as anti-malarials. Green tea extracts containing epigallocatechin gallate, NAS-91 [42] or flavonoids [142] inhibit the growth of *P. falciparum* parasites *in vitro*. However, as detailed above, molecular analysis has shown the FASII pathway to be dispensable for growth of blood stage *Plasmodium* parasites. Therefore the anti-malarial activities of these drugs are likely due to mechanisms independent of FASII. This conclusion is further supported by the activity of epigallocatechin gallate against *Babesia,* an apicomplexan parasite lacking FASII [48, 143]. Similarly, NAS-91, which inhibits the dehydratase of mycobacterial FASII, was initially

thought to kill *Plasmodium* by the same mechanism. However, a more recent study demonstrates that it may also inhibit mycobacterial SCD [144]. In contrast to FAS II, *Plasmodium* SCD is required for intraerythrocytic development of the parasite [118]. Thus, SCD rather than FASII dehydratase appears to be the likely target of NAS-91.

Thiolactomycin, which is thought to be a more specific FAS II inhibitor, as well as several compounds derived from thiolactomycin were found to inhibit growth of *T. gondii* [145], *T. cruzi, T. brucei* [146, 147], and *L. donovani* [147]. In *T. gondii* and *T. brucei*, where this has been studied in more detail, the activity of thiolactomycin is consistent with molecular data indicating that the FASII pathway is essential for growth [72, 131]. Similar to their activity in *Plasmodium*, green tea extracts containing catechins also inhibit *T. cruzi* [148]. Whether this effect was due to the activity of catechins against *T. cruzi* FASII pathway is not known.

2.5.2 Drugs targeting FASI and FAE

Cerulenin is a more general fatty acid synthesis inhibitor, whose activity is not limited to a particular type of FAS pathway. It is effective against human cancer cells by inhibiting mammalian FASI [149, 150]. Cerulenin has also been tested and proven effective in inhibiting *C. parvum* FASI [83]. In addition, in kinetoplastids lacking FASI but containing the FAE pathway, cerulenin has been suggested to be effective by inhibiting ELO-2, but not ELO-1 [107, 151]. However, cerulenin shows little activity against *C. parvum* fatty acid elongase [103]. A small number of specific inhibitors of fatty acid elongation has been identified. However, the activity of these inhibitors appears limited to a specific subtype of plant fatty acid elongases [104].

2.5.3 Drugs targeting FA desaturation

The identification of an active metabolism for unsaturated fatty acids in protozoan parasites has stimulated interest in desaturase inhibitors as anti-parasitic compounds. Isoxyl, which has been used for a long time in the treatment of tuberculosis, is known to inhibit bacterial SCD, and also inhibits growth of *T. cruzi* epimastigotes with an EC_{50} of 2 μM [152]. However, this drug typically has to be activated by a bacterial monoxygenase to be effective [153]. A homolog of this enzyme is not evident when searching the genomes of apicomplexans and kinetoplastids. Further studies are needed to establish the target and mode of activation of isoxyl in parasites.

Methylsterculate, a methylester of sterculic acid, inhibits the intraerythrocytic growth of *P. falciparum*. The anti-malarial activity of methylsterculate can be reversed by oleic acid supplementation, strongly implicating *P. falciparum* SCD as the likely target of this drug [118]. Thiastearates (TS) are another class of compounds that target the synthesis of unsaturated fatty acids. 10-TS has been shown to inhibit *T. cruzi* SCD [152], whereas 12-TS and 13-TS prevent the conversion of oleate to linoleate and inhibit the growth of *T. cruzi* [152] and *T. brucei* [154]. The enzyme responsible for conversion of oleate to linoleate is delta-12 oleate desaturase. This enzyme is present in kinetoplastid parasites but absent in the human host, making it a strong candidate for a parasite-specific target. Note that based on bioinformatic analyses, this enzyme is absent in apicomplexan parasites, suggesting that 12-TS and 13-TS are likely not effective against Apicomplexa.

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Figures and Tables

Table 2.1: Comparision of fatty acid synthesis and modification pathways in apicomplexan and kinetoplastid parasites. The presence (+) or absence (-) of specific pathways is shown for well studied model species.

Species	**FASII**		**FASI**	**PKS**	**FAE**	
	Apicoplast	Mitochondrion			*De novo*	Elongation
Trypanosoma brucei	-	+	-	-	+	-
Trypanosoma cruzi	-	+	-	-	+	-
Leishmania major	-	+	-	-	+	-
Theileria annulata	-	-	-	-	-	-
Babesia bovis	-	-	-	-	-	-
Plasmodium falciparum	+	-	-	-	-	+
Toxoplasma gondii	+	-	+	+	-	+
Eimeria tenella	+	-	+	+	-	+
Cryptosporidium parvum	-	-	+	+	-	+

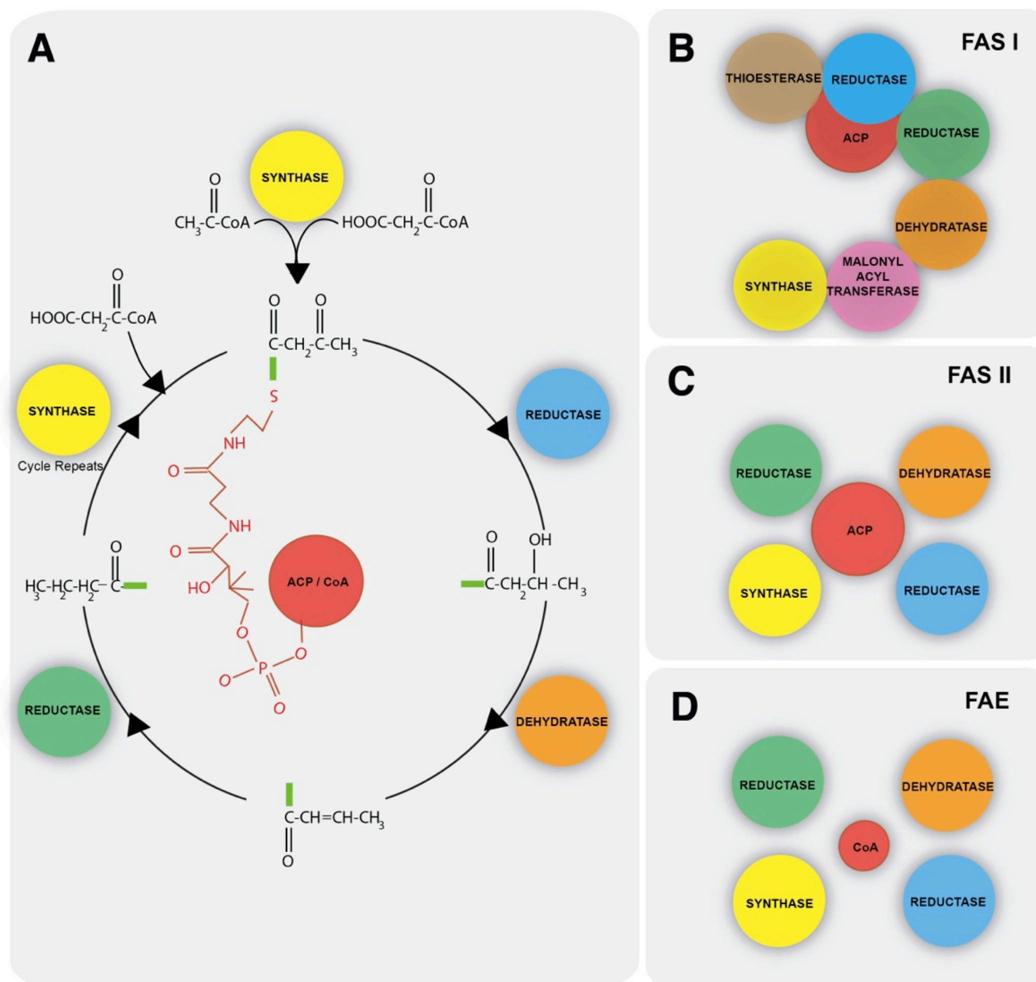

Figure 2.1: Three mechanisms of fatty acid synthesis. A, All fatty acid synthesis mechanisms follow a similar sequence of enzymatic reactions. A starter molecule is transferred to the phosphopantetheinyl group on ACP or CoA. The starter is then elongated by two carbon atoms and involving a four-reaction mechanism: decarboxylative condensation with a malonyl group by a synthase, reduction by a ketoreductase, dehydration by a dehydratase and reduction by an enoyl reductase. The product is released, or condensation with another malonyl group initiates

the next round of elongation. **B**, Fatty acid synthase type I. All enzymes are domains of a single polypeptide (note that the apicomplexan FASI has a more complex multimodular architecture). **C**, Fatty acid synthase type II. All enzymes are encoded as individual proteins. **D**, Fatty acid elongation pathway. The system consists of enzymes encoded as individual proteins, acting on a CoA-bound starter molecule – typically a longer fatty acid (16 carbon or longer). ACP, acyl carrier protein; CoA, Coenzyme-A; FASI, fatty acid synthase type I pathway; FASII, fatty acid synthase type II pathway; FAE, fatty acid elongation pathway.

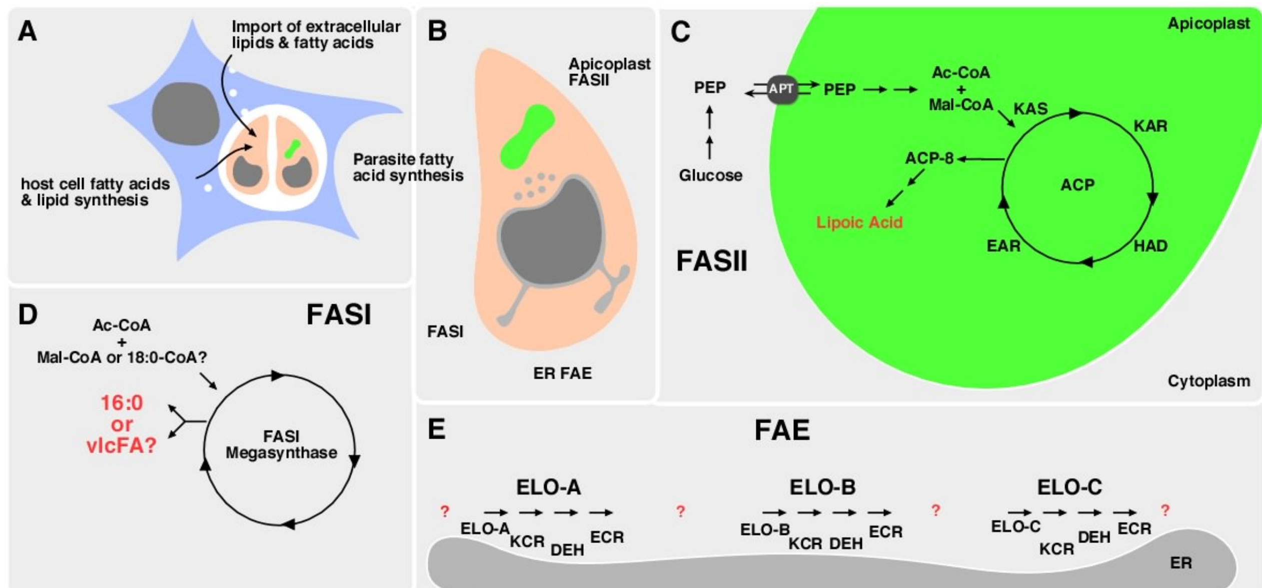

Figure 2.2: Apicomplexans can acquire fatty acids through a complex network of synthesis and uptake. **A**, *T. gondii* is shown as a representative apicomplexan parasite (pink) and intracellular pathogen capable of fatty acid and lipid salvage from the host cell (blue). This process can intersect host cell import as well as synthesis routes. **B**, In addition, the parasite harbors three fatty acid synthesis pathways that are localized to different cellular compartments **C**, Apicoplast (green)-localized FASII pathway produces lipoic acid **E**, Apicomplexan parasites

also maintain an ER-associated elongase system **D**, Apicomplexan FASI remains largely uncharacterized. Its stage-specific expression pattern and localization are not established. It is also unclear whether this megasynthase synthesizes fatty acids *de novo*, like the FASI of humans, or acts as an elongase for saturated fatty acids, as demonstrated for FASI of *C. parvum*.

PEP, phosphoenolpyruvate; Mal, malonate; Ac, acetate; vlcFA, very long chain fatty acids; Ac-CoA, acetyl-CoA; Mal-CoA, malonyl-CoA; ER, endoplasmic reticulum; KAS, ketoacyl-ACP synthase; KAR, ketoacyl-ACP reductase; HAD, hydroxylacyl-ACP dehydratase; EAR, enoyl-ACP reductase; ELO, elongase; KCR, ketoacyl-CoA reductase; DEH, acyl-CoA dehydratase; ECR, enoyl-CoA reductase. Other abbreviations are as in the legend of Fig. 2.1.

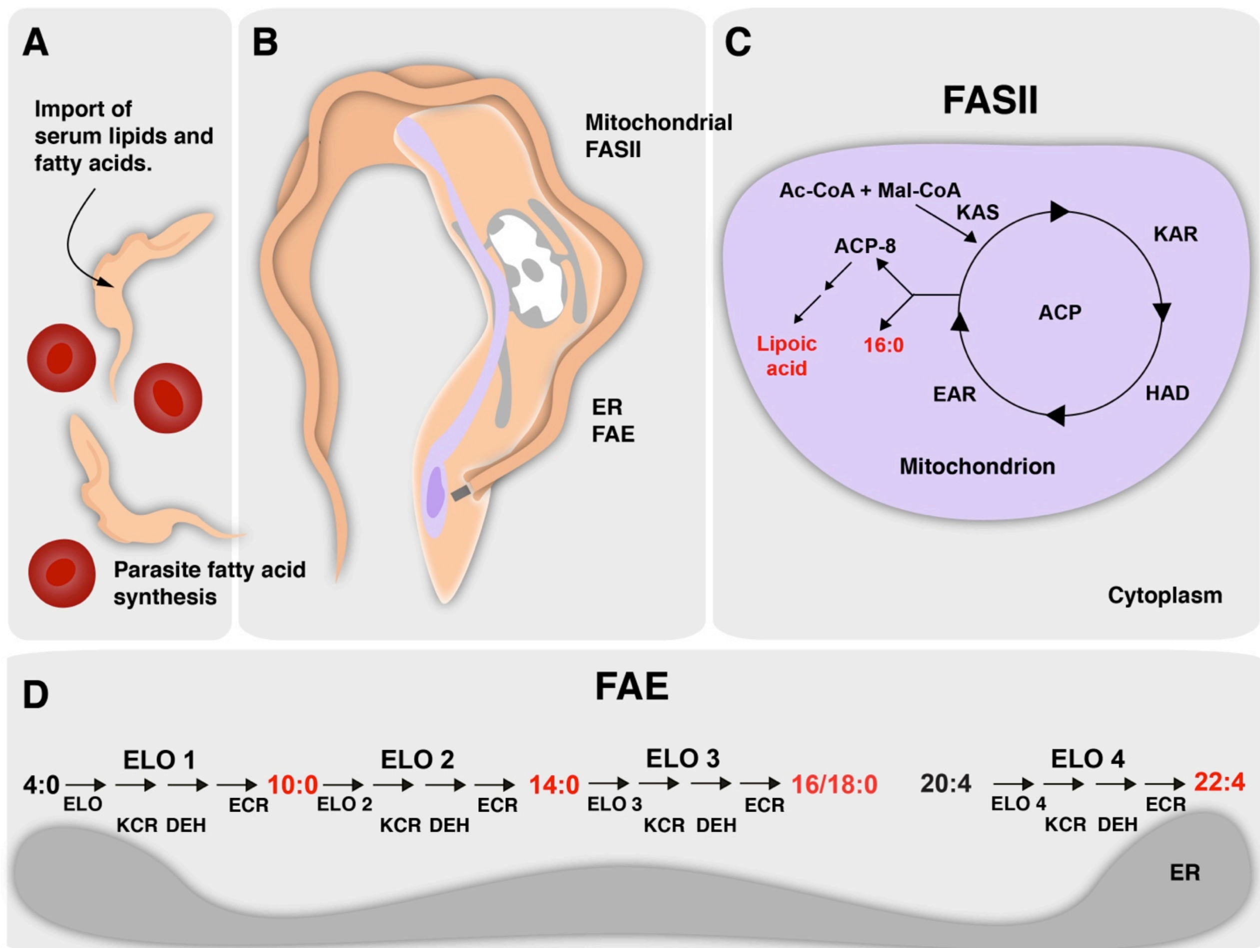

Figure 2.3: Kinetoplastid fatty acid synthesis occurs in the mitochondrion and the ER. Kinetoplastids obtain fatty acids using synthesis and import mechanisms. **A**, Kinetoplastid parasites replicate extracellularly in the bloodstream of the mammalian host, red blood cells are also shown (red). **B**, Additionally, the parasite harbors two mechanisms of fatty acid synthesis that are localized in two different organelles (redrawn in part after Vickerman K: Protozoology. Vol. 3 London School of Hygiene and Tropical Medicine, London, 1977). **C**, A FASII pathway localizes to the mitochondrion (light violet), where it is required for the synthesis of lipoic acid and palmitic acid. **D**, Kinetoplastid parasites also harbor an ER-localized fatty acid elongase system. Unlike all other organisms, kinetoplastid FAE is used for *de novo* synthesis of fatty acids. The kinetoplastid FAE uses butyrate and malonate as substrates to generate myristate/stearate and adrenate as products. Major products are highlighted in red. Mal, malonate; Ac, acetate. Other abbreviations are as in the legend of Fig. 2.2.

CHAPTER 3

THE APICOPLAST AND ENDOPLASMIC RETICULUM COOPERATE IN FATTY ACID BIOSYNTHESIS IN THE APICOMPLEXAN PARASITE *TOXOPLASMA GONDII* [1]

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3.1 ABSTRACT

Apicomplexan parasites are responsible for high impact human diseases such as malaria, toxoplasmosis and cryptosporidiosis. These obligate intracellular pathogens are dependent on both *de novo* lipid biosynthesis as well as the uptake of host lipids for biogenesis of parasite membranes. Genome annotations and biochemical studies indicate that apicomplexan parasites can synthesize fatty acids via a number of different biosynthetic pathways that are differentially compartmentalized. However, the relative contribution of each of these biosynthetic pathways to total fatty acid composition of intracellular parasite stages remains poorly defined. Here we use a combination of genetic, biochemical and metabolomic approaches to delineate the contribution of fatty acid biosynthetic pathways in *Toxoplasma gondii*. Metabolic labeling studies with ^{13}C-glucose showed that intracellular tachyzoites synthesized a range of long and very long chain fatty acids (C14:0-26:1). Genetic disruption of the apicoplast localized type II fatty acid synthase (FASII) resulted in greatly reduced synthesis of saturated fatty acids up to eighteen carbons long. Ablation of FASII activity resulted in reduced intracellular growth that was partially restored by addition of long chain fatty acids. In contrast, synthesis of very long chain fatty acids was primarily dependent on a fatty acid elongation system comprising three elongases, two reductases and a dehydratase that were localized to the endoplasmic reticulum. The function of these enzymes was confirmed by heterologous expression in yeast. This elongase pathway appears to have a unique role in generating very long unsaturated fatty acids (C26:1) that cannot be salvaged from the host.

3.2 INTRODUCTION

Apicomplexans are a phylum of single-celled eukaryotic parasites that includes the causative agents for important human diseases such as malaria, toxoplasmosis and cryptosporidiosis as well as numerous veterinary pathogens. Control of these diseases is challenging, in no small part due to rapid emergence of drug resistance which requires a continuous pipeline of new drugs with novel targets and/or modes of action. Apicomplexans are obligate intracellular pathogens and deploy complex mechanisms of synthesis and salvage to meet their essential nutritional requirements. The acquisition of lipids and fatty acids has emerged as a particularly important aspect of the metabolism of intracellular pathogens (1-3). Fatty acids are the main building blocks of membranes and also play a significant role in the post-translational modification of numerous proteins.

Apicomplexans were believed to be incapable of synthesizing their own fatty acids and to rely completely on host derived lipids. However, the discovery of the apicoplast, a plastid-like organelle challenged this view. As found for the chloroplasts of plants and algae, the apicoplast contains a type II fatty acid synthesis (FASII) pathway (4). Humans lack plastids and the associated FASII, and instead rely on type I fatty acid synthesis for the production of bulk long chain fatty acids. A FASII type synthase is present in human mitochondria but there are structural differences between its components and those of bacterial or apicomplexan FASII (5). Furthermore, apicoplast FASII also differs from the mammalian FASI pathway, thereby making it a potential target for the treatment of apicomplexan infections (6). Initial pharmacological studies on *Plasmodium falciparum* and *Toxoplasma gondii* supported this idea (7, 8). Subsequent genetic studies found essentiality of this pathway to be more complicated. We constructed a

conditional mutant for the apicoplast acyl carrier protein (ACP) in *T. gondii*. ACP is a key molecule of the FASII pathway: fatty-acyl intermediates are covalently bound to ACP as the enzymes of the pathway act on them. Loss of ACP in this conditional mutant affects apicoplast biogenesis and results in the death of the parasite. Moreover, mice infected with a lethal dose of *T. gondii* can be cured by suppression of the FASII pathway, suggesting its importance in the growth and survival of the parasite (9). In contrast, this pathway is not essential for the bloodstream and mosquito stages of the malarial parasite *Plasmodium*, although it is essential for the liver stage that is required for establishment of infection in the mammalian host (10, 11). Other apicomplexan parasites, such as *Cryptosporidium*, *Babesia* and *Theileria* appear to completely lack the FASII biosynthetic machinery suggesting that they are dependent on fatty acid salvage from the host (1).

While both *T. gondii* and *P. falciparum* have been shown to take up radiolabelled fatty acids from the culture medium (12, 13), these parasites are predicted to contain a number of other fatty acid biosynthetic pathways that could partly compensate for the loss of FASII (1, 14). These alternate mechanisms include a FASI pathway (in *T. gondii*, *Eimeria* and *Cryptosporidium*) and a putative fatty acid elongation pathway (in most apicomplexans with the exception of *Theileria* and *Babesia*). FASI in apicomplexans so far has only been studied in *Cryptosporidium parvum* (14, 15).

Overall these studies suggest that apicomplexan fatty acid metabolism is complex, and that multiple, potentially redundant biosynthetic pathways could contribute to the total cellular fatty acid pool. Identifying the specific biochemical activities of individual components, delineating their sequence and interaction, and relative importance under different growth conditions are critical for understanding host-parasite interaction and identifying potential drug

targets. Here we develop a combined genetic and metabolomic strategy to identify the products of FASII and the elongation pathways for *T. gondii*. *De novo* fatty acid synthesis was monitored in wild type parasites and in mutant parasite lines in which specific fatty acid biosynthetic enzymes were selectively ablated, by labeling parasite-infected host cells with ^{13}C-U-glucose and analysis of parasite lipids by gas chromatography-mass spectrometry (GC-MS). The activity of specific fatty acid elongases was further examined by heterologous expression and biochemical analyses. Our analyses suggest that the FASII and elongase pathways have non-redundant functions in intracellular tachyzoite stages in maintaining the fatty acid composition of bulk lipids and that these *de novo* pathways cannot be by-passed by fatty acid salvage from the host cell.

3.3 EXPERIMENTAL PROCEDURES

3.3.1 Gene identification and gene tagging

The protein sequences of the yeast fatty acid elongation machinery (ELO1, 2 and 3, keto acyl-CoA reductase, dehydratase and enoyl-CoA reductase; genbank IDs: NP_012339, NP_009963, NP_13476, AAS56194, NP_012438, NP_010269) were used as query sequences for BLAST searches against the *T. gondii* genome and genbank databases. In order to evaluate the computational predictions for these genes, we conducted RT-PCR experiments. The predictions for *T. gondii* ELO-A, ELO-B, ELO-C and DEH were confirmed. However, for ECR and KCR beginning and end of the coding sequence were different, and subsequently established by RT-PCR, subcloning and sequencing. The experimentally validated *T. gondii* genes described in this study are ELO-A, ELO-B, ELO-C, KCR , DEH and ECR (See Supplementary table

ST3.1 for genebank ID's). The full-length coding sequences for all genes were amplified from *T. gondii* cDNA using primers introducing flanking BglII and AvrII restriction sites, subcloned into plasmid pCR2.1 (Invitogen) and subsequently introduced into the equivalent sites of either plasmid pdt7s4H_3 or pdt7s4M_3 (16) placing them under the control of a tetracycline regulatable promoter and fusing a triple HA (pDT7S4H_3) or a triple c-Myc epitope tag (pDT7S4M_3), respectively, to the 3′-end. All primers used are listed in supplementary table ST3.1.

3.3.2 Parasite culture and construction of mutants.

Parasites were cultured and genetically manipulated as described (17). Conditional mutants were generated using the previously described two-step strategy (9, 18). Plasmids containing a second copy of the target gene under the control of a regulatable promoter (pdt7s4H_3) were stably introduced into a *T. gondii* tetracycline transactivator strain (19). Stable clones for ELO-A, B or C, respectively, were established in the presence of pyrimethamine. We next engineered a plasmid-based construct to delete the ELO-C locus and cosmid–based targeting vectors for ELO-A and B. For ELO-C sequences (~2kb) upstream and downstream of the ELO-C genomic locus were amplified by PCR and sub-cloned into the respective BamHI/KpnI and AatII/ SpeI restriction sites of the PTCY vector flanking a chloramphenicol resistance cassette (the vector also contains a YFP cassette for negative selection (9). This construct was linearized with SpeI and transfected into the pDT7S4-ELO-C-H_3 parasite line. Transgenic parasites which were chloramphenicol resistant and YFP negative were isolated. Clones were tested by PCR for integration and gene disruption was confirmed by Southern blot analyses. Cosmids (TOXP929/ELO-B or TOXO789/ELO-A) were modified to replace the coding sequence with a chloramphenicol resistance cassette by recombineering (18). Targeting

cosmids were transfected into the respective parasite and transgenic clones were selected in the presence of chloramphenicol. Mutants were identified as described for ELO-C. We conducted plaque assays in the absence or presence of 0.5 mM ATc to evaluate the growth of mutants (17). For chemical complementation assays myristic and palmitic acid were dissolved in equal molar amounts in ethanol, dried into a microfuge tube and complexed to fatty acid free BSA in water (Invitrogen, 1:3 molar ratio) with sonication prior to addition to normal growth medium as indicated.

3.3.3 Microscopy

Immunofluorescence microscopy was performed as previously described (20). Infected cover slip cultures were fixed with 3% paraformaldehyde in PBS, followed by permeabilization in 0.25% Triton-X 100 in PBS. After blocking rat anti-HA antibodies (Roche; Indianapolis,IN) were used at a dilution of 1:100, rabbit anti-ACP (a kind gift from Geoff McFadden, University of Melbourne (4)) at 1:1000, mouse anti-GFP (Torry Pines Bioloabs) at 1:400, and rabbit anti-myc (Roche Applied Science) at 1:100. Secondary antibodies were used at a dilution of 1:200 and were anti-rat Alexa Fluor 546, anti-rabbit Alexa Fluor 546, anti-mouse Alexa Fluor 488, and anti-rat Alexa Fluor 488 (all Invitrogen). Images were taken using an Applied Precision Delta Vision microscope, and images were deconvoluted and adjusted for contrast using Softworx. Protein localization annotations have been submitted to the ApiLoc database of protein subcellular localization in Apicomplexa (www.apiloc.biochem.unimelb.edu.au)

3.3.4 Western blotting

Western Blot analysis were performed as previously described (20) and used Rat anti-HA antibodies (Roche; Indianapolis, IN) at 1:100 and anti-GRA8 antibodies at 1:2000 (a kind gift from Gary Ward, University of Vermont (21)).

3.3.5 ^{14}C-acetate radiolabeling and thin layer chromatography

Parasites were grown in absence and presence ATc for 48 hours and free tachyzoites were metabolically labeled as previously described (9). Briefly, 10^8 tachyzoites were incubated with 10 μCi Na-[^{14}C]-acetate (Movarek) in 1 ml DMEM for 4h at 37°C and 5% CO_2. Total lipids were extracted with chloroform/methanol (2:1). The extract was dried and fatty acid methyl esters were subjected to acidic methanolysis. The resulting fatty acid methyl esters were extracted with hexane and analyzed on RP-18 HPTLC plates developed in methanol/chloroform/water (75:25:5) and exposed to film for one week.

3.3.6 Stable isotope labeling and metabolomic analyses

T. gondii-infected fibroblasts were grown in DMEM medium in T175 flask in the absence or presence of ATc for 48 hours. The medium was supplemented with 8 mM ^{13}C-U-glucose (final concentration of ^{13}C/^{12}C-glucose was 16 mM) 24 h prior to egress. Free parasites were separated from host cells by filtration through a membrane with 3 μm pore size. Parasites were quenched by rapid chilling of the cell suspension in a dry ice/ethanol bath and parasites

were recovered by centrifugation (4000 x g, 25 min, 0°C). Cell pellets were washed 3 times with ice cold PBS and cell aliquots (2×10^8 cells) transferred to microfuge tubes and centrifuged (10,000 × g, 30 sec, 0°C). Pellets were suspended in chloroform (50 μl) and vortex mixed thoroughly prior to the addition of 200 μl methanol:water (3:1). Samples were sonicated for one minute at RT and then incubated at 60°C for 20 min. Extracts were centrifuged at 10,000 × g for 5 min, and the supernatant subjected to biphasic partitioning by addition of 100 ml water. The organic phase was transferred to a partially-sealed capillary for methanolysis, dried with an internal standard of 1 nmol *scyllo*-inositol, and subsequently washed twice with methanol. Dried residues were dissolved in 0.5N methanolic-HCl (50 ml, Sigma-Aldrich) and the capillaries sealed under vacuum and incubated at 95°C overnight. Samples were neutralized with 10 ml pyridine (20 min) before being transferred to GC-MS vials, dried, and derivatized by addition of 25 μl of N-methyl-N-(trimethylsilyl)-trifluoroacetamide/trimethylchlorisane. Samples were analyzed on an Agilent 7890A-5975C GC-MS system. Splitless injection (injection temperature 280 °C) onto a 30 m + 10 m × 0.25 mm DB-5MS + DG column (J&W, Agilent Technologies) was used, using helium as the carrier gas. The initial oven temperature was 80 °C (2 min), followed by temperature gradients to 140 °C at 30 °C/min, from 140 °C to 250 °C at 5 °C/min, and from 250 °C to 265 °C at 15 °C/min. The final temperature was held for 10 min. Data analysis was performed using Chemstation software (MSD Chemstation D.01.02.16, Agilent Technologies) Abundance and label incorporation was calculated as previously described (22). Data shown are the average of three technical replicates and their standard deviation. They are representative of two biological experiments. Statistical significance was evaluated by Wilcoxon Rank Sum Testing with continuity corrections, using the R data analysis package (v2.14.0), where p-values of less that 0.05 were considered significant

3.3.7 Yeast media

Yeast strains were grown on YP (1% yeast extract, 2% peptone) or yeast synthetic complete (SC) media (yeast synthetic dropout medium supplement (SIGMA, St. Louis, USA), supplemented with 0.67% Difco yeast nitrogen base (Becton, Dickinson and Company, Sparks, USA), containing either 2% (wt/v) D-galactose and 0,05% (wt/v) D-glucose (GalGlu), 4% (wt/v) D-glucose (D) or 2% (wt/v) raffinose (R) as the carbon source(s). For solid media, 2% agar was added. Selections were carried out on plates lacking leucine (SCGalGlu -Leu). Counter-selections against plasmids containing a *URA3* marker were done on 5′-fluoroorotic acid plates (23) supplemented with GalGlu (5'FOAGalGlu). Liquid culture with 4% (wt/v) D-glucose was used to repress expression from the *GAL1*promoter. Strains subjected to spotting assay were grown over night in YPGalGlu, kept for 24h below an OD_{600}<1 then grown for 8h in YPRaf. The cell density was normalized to an OD_{600}=0.5 before being spotted on YPGalGlu, SCGalUra GalGlu and SC 5'FOA GalGlu.

3.3.8 Yeast strain construction

The yeast strain *S. cerevisiae* W1536 *Δelo2Δelo3* / YCp33 *GAL1 ScELO3* (*URA3*) (*Δade2, Δade3, his3-11, his-3-12, trp1-1, ura3-1, elo2::kanMX4, elo3::kanMX4*) has been described before (24-26). The yeast strain was transformed with either one of the following plasmids (all *LEU2*): YCp111 *GAL1-TgELO-B*, YCp111 *GAL1-TgELO-C* or YCpP111 *GAL1-TgELO-A* on SCGalD -Leu. Candidate transformants on SCGalD-Leu plates were picked and streaked on 5'-FOAGalD plates to select against the presence of the YCp33 *GAL1 ScELO3*

plasmid. Complementation of the *Δelo2Δelo3* mutations was indicated by the production of a large amount of FOA-resistant colonies. Presence of the plasmids carrying *T. gondii* constructs was verified by the ability of the FOA-resistant colonies to grow on SCGalD-Leu. These candidates were used in further experiments. Strain W1536-5B *Δtsc13*/ YCplac33 *GAL1 ScTSC13* (*MAT**a**; Δade2, Δade3, his3-11, his-3-12, trp1-1, ura3-1, tsc13::kanMX4*)was transformed with YCp111 *GAL1-TgTSC13* on SCGalD -Leu, and colonies complemented by *T. gondii TSC1* were identified and isolated in an analogous fashion as described above. Lastly, yeast mutants complemented by *T. gondii PHS1* were generated in a similar manner, using strain W1536-5B *Δphs1* / pYES2 *PHS1* (*MAT**a**; Δade2, Δade3, his3-11, his-3-12, trp1-1, ura3-1, phs1::kanMX4*) transformed with YCp111 *GAL1-TgPHS1*.

3.3.9 Preparation of ER extracts from yeast cells

Yeast cells were grown to exponential phase (2.10^6 cells.ml^{-1}) and then harvested by centrifuging for 15min at 1300g, 4°C. The pellet was washed with sterile water, and suspended in ice cold lysis buffer. The cells were disrupted with glass beads and centrifuged for 15min at 15000g at 4°C to remove the cell debris. The supernatants were centrifuged for 90min at 85000 *x g* in a Sorval Ti70 rotor at 4°C to generate pellet (P85) and supernatant (S85) fractions. The P85 was suspended in lysis buffer and used for the elongase assay. The protein concentrations were determined by the method of Lowry using bovine serum albumin as the standard. Equivalent amounts of total lysate, P85 and S85 were precipitated by adding trichloroacetic acid (TCA) to 10% final concentration and processed for immunoblotting.

3.3.10 Yeast fatty acid analysis

Yeast cultures were inoculated from an overnight starter culture to an OD_{600} of 0.05 in 100 ml YPGalGlu, allowed to grow to an OD_{600} of 0.5 and harvested by 5 minutes centrifugation at 4300g. Cells were washed in 50 ml water and harvested by centrifugation. The cell pellet flash frozen in liquid nitrogen and stored at -70ºC. Fatty acid analysis was performed following methods published earlier (27) with minor modifications. Briefly, the cell pellets were extracted with 1 ml chloroform/methanol/formic acid (10/10/1), followed by a second extraction with 1 ml chloroform/methanol/water (5/5/1 v/v). The combined organic phases were washed with an equal volume 1M KCl in 0.2M phosphoric acid and dried. Lipids were dissolved in 1 ml toluene and treated with 1 ml methanolic HCl (prepared by adding 0.5ml acetylchloride to 5ml cold, dry methanol) overnight at 50ºC. The solution was washed with 1 ml 4M KCl, the organic phase separated and the aqueous phase extracted with 1 ml hexane. The organic phases were pooled, washed with 0.5ml 0.5M ammonium bicarbonate, dried, redissolved in 100 μl hexane and subjected to gas chromatography on a HP6890 gas chromatograph with an HP5973 mass analyzer and equipped with a J&W scientific DB624 (30m x 0.32mm, stationary phase 1.8μm) column. 1μl was injected splitless (detector block 250ºC) and subjected to a temperature program from 60C to 250ºC (20ºC/min) and a hold period of 35min, flow 2ml/min. Masses were scanned from m/z 35 to 800, and a Wiley 275 spectral library was used for identification.

3.4 RESULTS

3.4.1 *Toxoplasma* is capable of *de novo* fatty acid synthesis

In apicomplexans, the enzymes of the FASII pathway are localized to the apicoplast (4). In plants, the homologous chloroplast pathway is the sole source of *de novo* synthesized fatty acids. We have previously constructed a *T. gondii* mutant with a conditional defect in this pathway. While the growth of this mutant was severely reduced upon inhibition of FASII, no difference was observed in the rate of fatty acid biosynthesis as measured using ^{14}C-acetate (9). We considered that FASII may not use acetyl-CoA generated from exogenous ^{14}C acetate. To test this we have established a new metabolic labeling procedure, in which the incorporation of ^{13}C- derived from ^{13}C-U-glucose into the entire cellular pool of fatty acids is detected by GC-MS analysis (Fig. 3.1A-C). When infected fibroblasts were labeled with ^{13}C-U-glucose, label was rapidly incorporated into tachyzoite lipids that were recovered from extracellular stages after host cell lysis and parasite egress. ^{13}C-U-glucose labeling of extracellular tachyzoites resulted in negligible labeling of parasite lipids indicating that *de novo* fatty acid synthesis occurs predominantly in intracellular stages and decreases dramatically following egress (data not shown). Host fibroblast cells are also capable of synthesizing fatty acids via a FASI system. In principal, ^{13}C derived from exogenous ^{13}C-glucose could be incorporated into host fatty acids and subsequently scavenged by intracellular tachyzoites. To investigate the extent to which fibroblasts synthesize fatty acids, uninfected fibroblasts were metabolically labeled with ^{13}C-U-glucose and incorporation of ^{13}C-into fatty acids was assessed by GC-MS. In contrast to the intracellularly labeled tachyzoites, negligible label was detected in the uninfected fibroblast

lipids (Table 3.1). These experiments, together with the data presented below, indicate that ^{13}C-labeling of tachyzoite fatty acids with ^{13}C-glucose represents *de novo* fatty acid biosynthesis within the parasite rather than uptake of labeled host lipids. In this context, it is notable that significant differences occur in the extent to which different fatty acids are labeled. While the long and very long chain fatty acids, C14:0, C16:0, C16:1, C18:1, C20:1 and C26:1 were strongly labeled (>60%) others, including C18:0, C22:0 and C24:1 were labeled to less than 20%. These differences could reflect differences in the turnover and achievement of isotopic equilibrium in different fatty acids pools, or more likely, differences in the rate of salvage of 'unlabeled' host fatty acids. Regardless, the results indicate that a number of major fatty acids in intracellular stages are primarily synthesized *de novo* rather than taken up from the host

3.4.2 Apicoplast FASII is required for the synthesis of long chain fatty acids

The detection of multiple mass isotopomers of the long chain fatty acids, differing by 2 atomic mass units (amu) in ^{13}C-U-glucose fed-parasites (Fig. 3.1B) indicated multiple rounds of incorporation of ^{13}C-acetyl-CoA and ^{12}C-acetyl-CoA into these chains, consistent with the operation of a FAS system. To investigate the contribution of the apicoplast-located FASII complex to cellular fatty acid synthesis, fibroblasts were infected with the *T. gondii* ΔACP/ACPi mutant which expresses components of the apicoplast FASII complex under the control of a tetracycline regulated promoter (9). When *T. gondii* ΔACP/ACPi-infected fibroblasts were incubated with ^{13}C-U-glucose in the absence of anhydrotetracycline (ATc), high levels of ^{13}C-incorporation were observed in tachyzoite lipids (Fig 3.1D-E). In contrast, when labeling was done in the presence of ATc, resulting in selective down-regulation of parasite FASII activity,

the labeling of tachyzoite fatty acids was strongly inhibited. In particular, labeling of the long chain saturated and unsaturated fatty acid C14:0 and C16:0 was reduced by >80% in the presence of ATc (Fig. 3.1E). In a control experiment, ATc was found to have no effect on the labeling of wild type tachyzoite fatty acids, indicating that ATc by itself does not inhibit ^{13}C-U-glucose uptake and catabolism to acetyl-CoA or fatty acid synthesis (Fig. 3.1C, Table 3.1). These experiments provide further direct support for the conclusion that labeling of parasite fatty acids with ^{13}C-U-glucose reflects parasite *de novo* synthesis, rather than salvage. They also suggest that the FASII pathway is directly responsible for the *de novo* synthesis of saturated long chain fatty acids (C14:0, C16:0) that are utilized, at least in part for the synthesis of unsaturated and very long chain fatty acids.

3.4.3 Growth defects due to loss of FASII pathway are only partially restored by chemical complementation with myristic and palmitic acid.

We have previously shown that loss of FASII activity leads to inhibition of parasite growth (9). As loss of FASII results in a deficiency in myristic and palmitic acid synthesis we investigated whether nutritional supplementation of FASII-deficient parasites with these fatty acids would restore growth. Parasite lines expressing red fluorescent protein were cultured in 96 well plates in medium supplemented with fatty acids complexed to BSA at concentrations ranging from 20 nM to 2 mM. These fatty acid concentrations have no effect on the growth of the wild-type parasites (data not shown). As expected, intracellular growth of the ΔACP/ACPi mutant was markedly reduced after six days in the presence of ATc (Fig. 3.2A) (9). Growth inhibition in the presence of ATc was delayed by 2 days in the presence of a combination of 1

mM myristic and 1 mM palmitic acid. However, fatty acid supplementation did not restore wild type growth or prevent death over longer periods, possibly reflecting inefficient transport to the parasite vacuole, increased fatty acid demand at later time points and/or the involvement of FASII in other lipid biosynthetic pathways, such as the synthesis of lipoic acid.

3.4.4 Parasites express an endoplasmic reticulum-associated fatty acid elongation system

Several very long chain fatty acids were labeled with ^{13}C-U-glucose in the absence of FASII suggested that additional fatty acid biosynthetic pathways are active during intracellular tachyzoite development (Fig 3.1). Bioinformatic analysis of the *T. gondii* genome indicates that these parasites may have a functional fatty acid elongase system. Using the sequences of the components of the yeast elongation pathway as query in similarity searches we found three candidate genes coding for fatty acid elongases, and a single candidate for each of the downstream reductases and the dehydratase in *T. gondii* (see table ST3.1).

We evaluated the coding sequence for all six genes by cDNA and RACE PCR followed by subcloning and sequencing. This analysis validated the computationally predicted gene models for four genes: ELO-A, B and C and the dehydratase, which is annotated as a hypothetical protein tyrosine phosphatase-like domain-containing protein in the *T. gondii* genome database. We established a diverging coding sequence for the presumptive enoyl-reductase and keto-acyl CoA reductase (see Experimental Procedures and supplementary Table ST3.1 for detail). The coding sequences were amplified and introduced into a parasite expression vector that appended a C-terminal epitope tag. These constructs were transfected into parasites and stable transgenic lines were established through drug selection and cloning (see Experimental Procedures for

details). The six epitope-tagged proteins localized to the same perinuclear structure in the parasite, as determined by immunofluorescence assays (IFA). For comparison we transfected these lines with an expression plasmid for Der1-GFP (20), a marker for the *T. gondii* endoplasmic reticulum. This marker co-localized with the epitope-tagged proteins indicating that these enzymes localize to the endoplasmic reticulum (Fig. 3.3) (note that ELO-C had a more restricted pattern at the apical face of the nuclear envelope reminiscent of the previously characterized ER exit site (28)). These observations are consistent with reports from other organisms where the components of the fatty acid elongation pathway have been found to form a multi-enzyme complex on the membrane of the endoplasmic reticulum (29, 30).

3.4.5 Candidate *T. gondii* genes rescue lethal yeast fatty acid elongation mutants.

We next tested the molecular function of each of the six enzymes by heterologous expression in well-defined yeast mutants lacking equivalent enzymes. For these phenotypic complementation experiments, we constructed yeast expression vectors under the control of the galactose activated/glucose repressed *GAL1* promoter. Constructs encoding the putative *T. gondii* fatty acid elongases were transformed into *S. cerevisiae* strain W1536 *Δelo2Δelo3*/ YCp33 *GAL1-ScELO3* (26). This mutant lacks the genes for both fatty acid elongase 2 and 3. This is a lethal double mutation (27) and the strain is only viable due to episomal expression of elongase 3 from a *GAL1* promoter. Following transformation with parasite test plasmids, loss of the *URA3*-containing yeast plasmid was achieved by counter-selection on media containing the *URA3* activated toxic substrate 5'-fluoroorotic acid. *T. gondii* ELO-B and ELO-C but not ELO-A rescued the growth deficiency of the Δ*elo2Δelo3* mutant strain (Fig. 3.4A). Functional

complementation was further validated by incubation of microsomal fractions prepared from the two respective complemented strains with radiolabelled malonyl-CoA and palmitoyl-CoA (31). As expected, this resulted in chain elongation of the substrate (data not shown). Similarly when expressed in yeast cells, the *T. gondii* candidates for dehydratase and enoyl-reductase were found to complement the growth of mutants in their respective yeast homologs (*S. cerevisiae* Phs1p and Tsc13, see Fig. 3.4B and Experimental Procedures for detail). Overall these experiments suggest that the *T. gondii* candidate genes indeed encode the enzymes of the fatty acid elongation system. We cannot make firm conclusions for TgELO-A and TgKCR that failed to complement. This may be due to lack of expression or poor interaction of the heterologous enzymes with the native yeast machinery.

3.4.6 Genetic ablation of fatty acid elongases in *T. gondii*.

To further investigate the functional significance of the fatty acid elongase system in *Toxoplasma,* we generated conditional parasite mutants for the condensing enzymes of the pathway. A parasite strain expressing epitope tagged ELO-B under a tetracycline-regulated promoter from an ectopic locus was constructed. In this background, we replaced the chromosomal copy of this gene with a chloramphenicol resistance cassette using homologous recombination. Chloramphenicol resistant parasites were screened by PCR to identify the clones with genetic disruption for ELO-B. Using a similar strategy we also engineered conditional mutants for ELO-C and ELO-A (see Experimental Procedures section for detail, note that some deletions were constructed using plasmids (9) and others with cosmids (18)). Deletion of the respective locus was confirmed for each mutant by Southern blot (Fig. 3.5 G-I). We evaluated

the ATc dependence of the expression of each of the genes by IFA and Western blot assays. For IFA, mutant parasites were grown in absence and presence of ATc for 24 hours prior to fixation and processing. For Western blots parasites were grown in ATc for 0-4 days. Both assays show rapid loss of the tagged proteins upon ATc addition. The proteins are essentially undetectable after 24 hours of treatment (Fig. 3.5). To determine the effect of the loss of these proteins on parasite growth we next performed plaque assays. Confluent host cell monolayers were infected with the mutant parasites in absence or presence of ATc. Plaques in the host monolayer were visualized by staining after 10 days. We detected no significant difference in plaque number or size for each of the three elongase mutants (Fig. 3.5L). The ACP mutant was used as a positive control and reduced plaque formation was readily observed as previously described (9). We conclude that individual loss of ELO-A, ELO-B or ELO-C does not impede parasite growth in tissue culture.

3.4.7 *T. gondii* ELO proteins are required for the synthesis of very long chain fatty acids.

We do not detect a growth phenotype in single elongase deficient parasites. This could reflect the possibility that fatty acids synthesized by the elongase system are not essential, the ELO enzymes are functionally redundant (27), and/or intracellular parasites can compensate for the loss of this pathways by salvaging necessary fatty acids from the host. To determine the specific biochemical role of individual elongases, the fatty acid biosynthesis capabilities of each mutant were initially analyzed using ^{14}C-acetate labeling. Parasites were grown in absence or presence of ATc, liberated from their host cells and incubated in fatty acid free medium supplemented with 10 μCi ^{14}C-acetate. Lipids were extracted after 4 hours and the corresponding

fatty acid methyl esters analyzed by thin layer chromatography (Fig. 3.6). The loss of ELO-B resulted in a moderate yet reproducible decrease in the labeling of all long chain fatty acids. Loss of ELO-C produced the complete loss of labeling in a single very long chain fatty acid. In addition we noted a marked increase in the abundance of a shorter fatty acid, suggesting that this may be a precursor that accumulates in the absence of the downstream enzyme (Fig. 3.6C). Ablation of ELO-A did not produce recognizable changes in the labeling pattern. While these analyses indicate clear changes in the biosynthetic capabilities of two of our mutants they are limited in their sensitivity and resolution. In particular they do not provide accurate measurement for the length and saturation of the affected fatty acids.

3.4.8 ELO-B and ELO-C produce monounsaturated long chain fatty acids in a two-step mechanism

To further investigate the substrate specificity of the different *T. gondii* fatty acid elongases, each of the ELO mutant parasites were metabolically labeled with ^{13}C-glucose in absence or presence of ATc and fatty acid mass isotopomers were analyzed by GC-MS. The labeling of saturated long chain fatty acids up to C16:0 was unaffected by the individual loss of activity of ELO-A, ELO-B and ELO-C (Fig 3.7), consistent with their synthesis being regulated by FASII. Loss of ELO-A activity was associated with a marked reduction in labeling of saturated and unsaturated fatty acids with chain lengths of 18 to 24 carbons. Ablation of ELO-A also resulted in a reduction in the cellular levels of these fatty acids. Significantly, both the abundance and level of labeling of the unsaturated fatty acid C16:1 increased in the absence of ELO-A. These results suggest that ELO-A is required for efficient elongation of C16:0 or C16:1

to longer species. In the absence of ELO-A, *de novo* synthesized C16:0 is apparently channeled into increased synthesis of C16:1.

In contrast, loss of expression of ELO-B or ELO-C resulted in enhanced labeling of long chain fatty acids, but reduced labeling of several very long chain fatty acids. Specifically, down-regulation of ELO-B activity severely ablated labeling of the mono-unsaturated very long chain fatty acids C20:1, C22:1, C24:1 and C26:1 (Fig 3.7A). On the other hand, down-regulation of ELO-C activity led to a highly selective loss of labeling of C26:1 and the accumulation of C22:1. These results suggest that ELO-B is largely responsible for the elongation of C18:1/C20:1 to C22:1, while the primary role of ELO-C is to elongate fatty acids from C22:1 to C26:1. This model is also supported by our measurements of overall fatty acid abundance (Fig. 3.7B). Loss of ELO-C results in a severe drop in the abundance of only C26:1, in contrast, loss of ELO-B affected several shorter monounsaturated fatty acids in addition to C26:1. This is reminiscent of the situation in *S. cerevisiae*, where ScElo2p (equivalent to TgELO-B) is capable of elongating fatty acids up to 24 carbons, preferring substrate of <22 carbon length. Likewise, ScElo3p and TgELO-C are alike in preferential production of very long chain fatty acids. Note that both *T. gondii* enzymes complement the yeast double mutant indicating that they can have overlapping and slightly broader specificity.

To distinguish between the potential activities of ELO-B we returned to the complemented yeast strains and analyzed their fatty acid contents. The wild type yeast, mutant *Δelo2Δelo3* complemented *S. cerevisiae ELO3,* as well as the mutant complemented by expression of TgELO-B, were grown on YPGalD medium. Total lipids were extracted and analyzed by GC-MS (see Experimental Procedures). As previously shown, when compared with wild type the *Δelo2Δelo3/ScELO3* mutant shows a much decreased level of fatty acids C20 and

higher. The double deletion strain carrying the TgELO-B construct, displayed some restoration of its longer chain fatty acids (C20-24), but not C26 or C26:1. Interestingly there seemed to be an overabundance of unsaturated fatty acids with no or only modest increase in saturated fatty acids (see GC-MS tracks in Fig. 3.8A and 3.8B). In particular, there was a striking accumulation of C22:1 compared to all other strains (see table 3.2 and table 3.3 and Fig. 3.8A).

3.5 DISCUSSION

Intracellular pathogens need to either scavenge or synthesize lipids in order to maintain the integrity of their own membranes and/or the membrane of specialized vacuoles within which they survive and proliferate. Lipid and fatty acid salvage mechanisms have been shown to be important for the intracellular growth of several intracellular bacteria. Similarly, apicomplexan parasites, have been shown to salvage lipids from the host endocytic pathway or cytoplasmic lipid bodies (12, 32-34). Nonetheless, recent bioinformatic, genetic and biochemical studies have suggested that intracellular *T. gondii* tachyzoite stages express multiple pathways of fatty acid biosynthesis and may be dependent on *de novo* synthesis of fatty acids for synthesis of bulk and specialized lipid classes (1). To dissect this complex network of uptake and synthesis, we have combined reverse genetics with metabolomic analyses. We define the function of individual fatty acid synthesis pathways and have developed a model that incorporates these findings (Fig. 3.9). The FASII pathway is localized to the apicoplast (shown in green) and is thought to utilize acetyl-CoA derived from glycolytic triosephosphates that are imported into the apicoplast via a phosphate translocator (18, 35). Our labeling studies provide strong support for this model. Specifically, while ^{14}C-acetate was not effectively incorporated into FASII products, ^{13}C derived from ^{13}C-U-glucose was strongly incorporated into this pathway. Ablation of FASII activity

resulted in a global reduction in fatty acid labeling. This was most apparent for the saturated long chain fatty acids C14:0 and C16:0, indicating that FASII is largely responsible for maintaining the cellular levels of these fatty acids. The pattern of ^{13}C-labeling of these fatty acids in FASII-competent parasites was also consistent with *de novo* synthesis from short chain (~C4) fatty acid precursors. Synthesis of this precursor is most likely dependent on apicoplast FabD (36) but apicoplast ACP has also been demonstrated to be capable of self-malonylation (37). Note that *T. gondii* also expresses two differentially localized acetyl-CoA carboxylases one in the apicoplast the other in the cytoplasm (38) that could provide malonyl-CoA for FASII and the elongation pathway, respectively. These findings demonstrate that the apicoplast, like the plant chloroplast is the main cellular site of fatty acid *de novo* synthesis. There are a number of important functions for fatty acid synthesis directly in the plastid including lipoylation, acylation of plastid proteins and the synthesis of plastid lipids. Indeed the loss of FASII in the apicoplast and the plant chloroplast perturbs organelle biogenesis and division leading to organelle loss (9, 39).

Does this suggest the significance for FASII to be entirely local? Following this idea the main function of FASII could be to maintain the apicoplast to support the organelle as the site of isoprenoid precursor synthesis (40, 41). In plant chloroplasts, FASII-derived fatty acids are rapidly exported from the plastid into the cytoplasm (42). Our experiments do not formally test this, and do not establish the localization of FASII derived lipids. We note however, that our measurements indicate that most (60-80%) of the parasite C14:0 and C16:0 are ^{13}C labeled, a finding that is inconsistent with FASII only supplying a small organellar pool of lipids. Moreover, our data suggest that C16:0 and C16:1 fatty acids synthesized by the apicoplast FASII complex are further elongated by the ER elongases, indicating that a significant fraction of the apicoplast-synthesized fatty acids are exported to the ER. We propose that the apicoplast is the

main source of C14:0 and C16:0 for intracellular stages of *T. gondii* and the liver stage of *Plasmodium*, but that in the *Plasmodium* erythrocyte and mosquito stages this need can be satisfied by salvage of fatty acids from the host. A greater dependency of *T. gondii* tachyzoites on *de novo* fatty acid synthesis is further supported by our finding that loss of FASII was only partially rescued by providing excess C14:0 and C16:0 in the culture medium. Overall these observations are consistent with important functions of FASII derived products within and beyond the apicoplast in *T. gondii*.

In addition to the FASII complex, we provide direct evidence for the activity of an elaborate fatty acid elongation pathway localized to the parasite endoplasmic reticulum. This fatty acid elongation pathway comprises four enzymatic steps. The extension of the carbon chain is initiated by a condensation reaction between acyl-CoA and malonyl-CoA catalysed by a fatty acid elongase. This is followed by reduction through keto-acyl CoA reductase, dehydration to enoyl-CoA, and final reduction to the elongated fatty acyl-CoA. We identified a full complement of six enzymes and support the sequence similarity based assignments experimentally by yeast complementation analysis for several candidates. Our ^{13}C-U-glucose labeling experiments in parasite mutants and heterologous expression in yeast mutants suggest that each of the elongases may act sequentially as outlined schematically in Fig. 3.9E. The *T. gondii* elongation system generates very long chain mono-unsaturated fatty acids. ELO-B elongates C18:1 to C22:1 in two carbon increments. Based on the yeast data its activity from C20:1 to C22:1 appears to be particularly robust. C22:1 is elongated in two final rounds by ELO-C to C26:1 (note that we do not fully resolve whether the synthesis of C24 is an overlapping function between the two elongases). The *T. gondii* ELO-B and ELO-C pathway is the sole source of C26:1 for the parasite and does not appear to elongate saturated fatty acids. The significance of this striking preference

for unsaturated fatty acids is unclear at this point. Unsaturated fatty acids have distinct physical properties resulting in differences in e.g. membrane fluidity and those may be more suited to the parasite's needs. It is notable that the levels of C26:1 in fibroblasts are very low (Table 3.1, Fig. 3.10), possibly accounting for the inability to generate multiple ELO genes.

What is the source of the ELO-B substrates? Monounsaturated fatty acids can be synthesized by the desaturation of a corresponding saturated fatty acid. A route used by many organisms is the conversion of C18:0 to C18:1 oleic acid by stearoyl-CoA desaturase (43). This enzyme is present in *T. gondii* (TGGT1_053030) and has been characterized in detail in *P. falciparum* (44). In *Plasmodium* it was found to be localized to the membrane of the endoplasmic reticulum and thus could work hand in hand with the ELO system. This system could potentially tap into the FASII generated fatty acid pool through the activity of ELO-A which appears to be required for elongation of C16:0 and C16:1 to C18:0 and C18:1, respectively. Specifically, ablation of ELO-A resulted in a marked decrease in synthesis of C18:0, which was associated with the concomitant increase in both the labeling and steady state concentration of C16:0/C16:1 (Fig 3.7). It is notable that only a minor pool of C18:0 is labeled with ^{13}C-U-glucose in wild type parasites (<10%) indicating that this fatty acid is primarily salvaged from the host. In contrast, C18:1 is labeled to a similar extent as C16:0 and C16:1 (>40%). These findings suggest that ELO-A primarily utilizes C16:0 synthesized in the apicoplast and C16:1 synthesized by the putative tachyzoite ER desaturase. The reduced cellular levels and rates of labeling of longer chain saturated/unsaturated fatty acids in ELO-A ablated parasites indicates that the C18:0 and C18:1 synthesized by ELO-A is selectively utilized by ELO-B and ELO-C, while the larger cellular pool of salvaged C18:0 appears to be poorly used. Collectively these observations indicate that synthesis of very long chain fatty acids in intracellular tachyzoites involves

extensive intraorganellar transport as well as some degree of substrate channeling. It should be noted that the *T. gondii* genome encodes a FASI pathway (Fig. 3.9D) but its role in the *T. gondii* fatty acid metabolism is not yet established.

While we demonstrate clear biochemical effects of suppressing individual ELO genes, we do not measure parasite growth impairment under tissue culture conditions. It is possible that the parasite can satisfy its needs through uptake. A recently described lipid uptake system in *T. gondii* may facilitate this process (45). Alternatively lack of growth defects may be due to redundancy within the ELO system itself. In yeast, the loss of a fatty acid elongase 2 or fatty acid elongase 3 can be tolerated. However, loss of both elongases results in synthetic lethality. It has been suggested that the enzyme partially complement each other and that the cells can tolerate the resulting slight differences in their fatty acid pools (27). Our yeast complementation results indicate that the *T. gondii* enzymes may also have overlapping substrate specificity. To establish whether a similar redundancy exists in *T. gondii* we sought to engineer a more complete ablation of the parasite ELO system. Our first approach was to attempt to generate a conditional double mutant in ELO-B and C. However, our attempts to generate such a mutant failed recurrently despite the fact that gene targeting was efficient for individual loci in previous experiments (data not shown). An alternative approach would be to delete a non-redundant downstream enzyme of the pathway, thereby affecting the activity of all three elongases. We attempted to generate conditional mutants for dehydratase and enoyl-CoA reductase. Again, we were unsuccessful in generating a mutant (data not shown). These negative results do not formally establish the essential nature of the *T. gondii* ELO pathway, but they are consistent with such a role.

Which aspect of parasite biology generates the heavy need for elongated fatty acids served by these elaborate and potentially redundant synthesis and uptake pathways? Very long

chain fatty acids are required for the synthesis of highly specialized lipids such as waxes in plants, but are also important components of phospholipids (27, 46). Loss of the elongation pathway affects the overall phospholipid pool in these organisms. *T. gondii* harbors a variety of phospholipids which not only differ in their head group but also in the length of the acyl-chains (47). The parasites can take up phospholipids but appear to depend on the synthesis of some species (32, 45, 48). Overall our results demonstrate that despite its substantial complexity the parasite fatty acid metabolism can be unraveled in molecular detail. Stable isotope labeling followed by GC-MS can track and quantify the synthesis of a large number of metabolites *in vivo* and was particularly useful for resolving the activities of different fatty acid biosynthetic enzymes. Furthermore, the fact that ^{13}C-labeled precursors can be added at normal physiological concentrations may increase the efficiency with which they can access intracellular compartments such as the *T. gondii* parasitophorous vacuole rather than being diverted into host cell metabolism. Conditional gene ablation provides focus and specificity to this broader metabolomic analysis. We provide functional annotation for two important parasite pathways here and suggest that this strategy could be used to great effect to understand the metabolic interaction between host and parasite in *T. gondii* and many other intracellular pathogens.

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Figures and tables

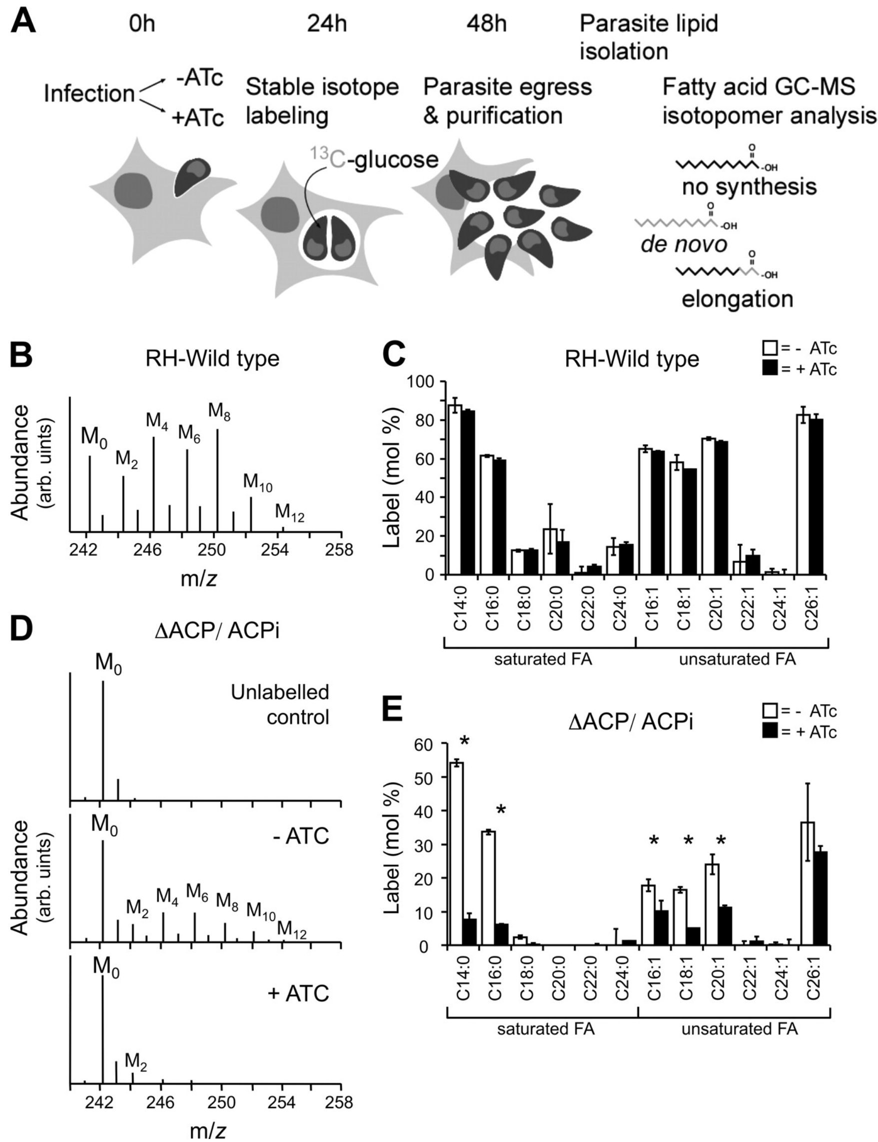

Figure 3.1: Metabolic labeling of intracellular tachyzoites with ^{13}C-U-glucose and analysis of *de novo* fatty acid biosynthesis. (A). *T. gondii*-infected fibroblasts were precultured in the presence or absence of ATc for 24 h and then labeled with ^{13}C-U-glucose 24 h prior to parasite egress. After host cell lysis, extracellular tachyzoites were metabolically quenched in a dry ice/ethanol bath and separated from host cell debris by filtration prior to metabolite extraction. Examples of different labeling patterns (^{13}C indicated in grey) and their interpretation are schematically shown for myristate (**B**) Lipids from ^{13}C-U-glucose-labeled tachyzoites were

subjected to methanolysis and TMS derivitization and analyzed by GC-MS. A portion of the mass spectrum containing the molecular ions of the methyl ester of myristate is shown. Unlabeled myristoyl methyl ester (M_0) has an m/z of 242 amu. Higher mass isotopomers (M_{1-12}) contain between 1 to 12 ^{13}C atoms, corresponding to incorporation of ^{13}C into the fatty acid biosynthetic pathways via ^{13}C-acetyl-CoA. (**C**) Incorporation of ^{13}C into the major fatty acids of intracellular wild type *T. gondii* tachyzoites (labeling is given as mol% of all labeled mass isotopomers (M_{1-12}) relative to M_0, after correction for natural abundance). The fatty acid notation Cn:m indicates the length of the fatty acid (n, carbon number) and degree of unsaturation (m, number of double bonds). Note that treatment of wild type parasites with ATc (black) does not result in any significant changes of the fatty acid labeling pattern when compared to untreated controls (white). (**D**). Intracellular tachyzoites of the *T. gondii* ΔACP/ACPi mutant carrying an inducible copy of FASII ACP were labeled with ^{13}C-U-glucose in the presence or absence of ATc. Total lipids were extracted from the purified tachyzoites and released fatty acid methyl esters (FAME) analyzed by GC-MS. The mass spectrum (molecular ion region from m/z 240-250) of the myristate FAME from unlabeled parasites, and from ^{13}C-glucose labeled parasites cultured in the absence or presence of ATc is shown. Incorporation of multiple $^{13}C_2H_4$ units into myristate is observed in the absence of ATc (ACP active) and largely inhibited in the presence of ATc (ACP repressed). The spectrum is representative of three individual experiments. (**E**) Total ^{13}C-incorporation into fatty acids in ΔACP/ACPi tachyzoites labeled in the presence (black) or absence (white) of ATc. Error bars represent standard deviation where $n = 3$. Fatty acids for which significant changes in labeling were observed in the presence and absence of ATc using the Wilcoxon Rank Sum Test (p-values less than 0.05) are indicated with an asterisk. The absolute amount of incorporation can vary from experiment to experiment depending on the

stage of the culture (no ATc in **C** and **E**). We therefore always directly compare ATc treated samples with an untreated control from the same culture batch.

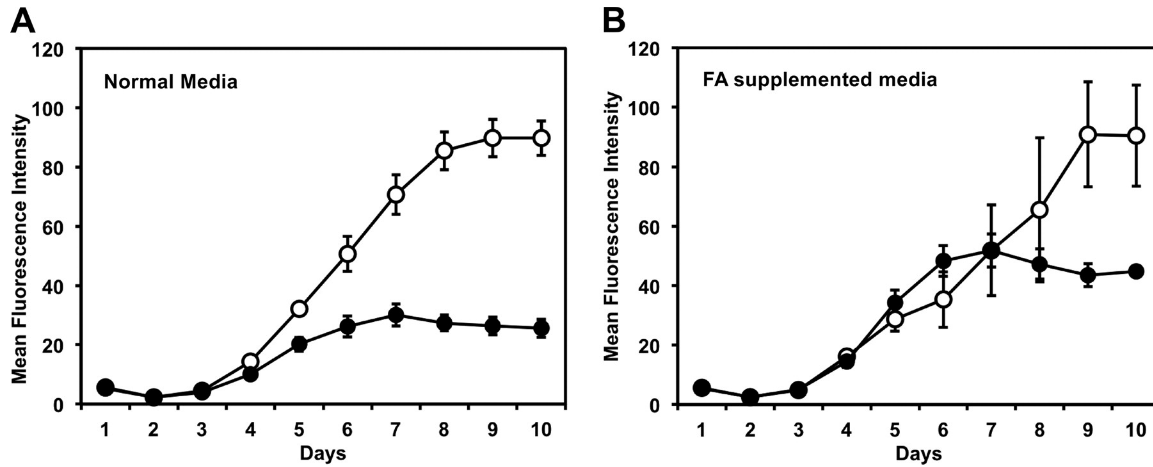

Figure 3.2: Growth of ΔACP/ACPi parasites in media supplemented with myristic and palmitic acid. Growth of ΔACP/ACPi parasites stably expressing a dTomatoRFP transgene was evaluated by measuring parasite fluorescence daily in a 96 well culture format (49). Parasites were grown in normal growth media supplemented with fatty acid free BSA (**A**) or media supplemented with 1 mM of each myristic and palmitic acid coupled to BSA (3:1 molar ration) (**B**). Parasites were grown in the presence (black) or absence (white) of ATc. The average of fluorescence intensity (arbitrary units) for three independent replicates is shown and error bars represent the standard deviation for each data point. Continuous culture in BSA conjugated fatty acids (or higher serum supplementation) did not lead to continuous growth of the ACP mutant in the presence of ATc.

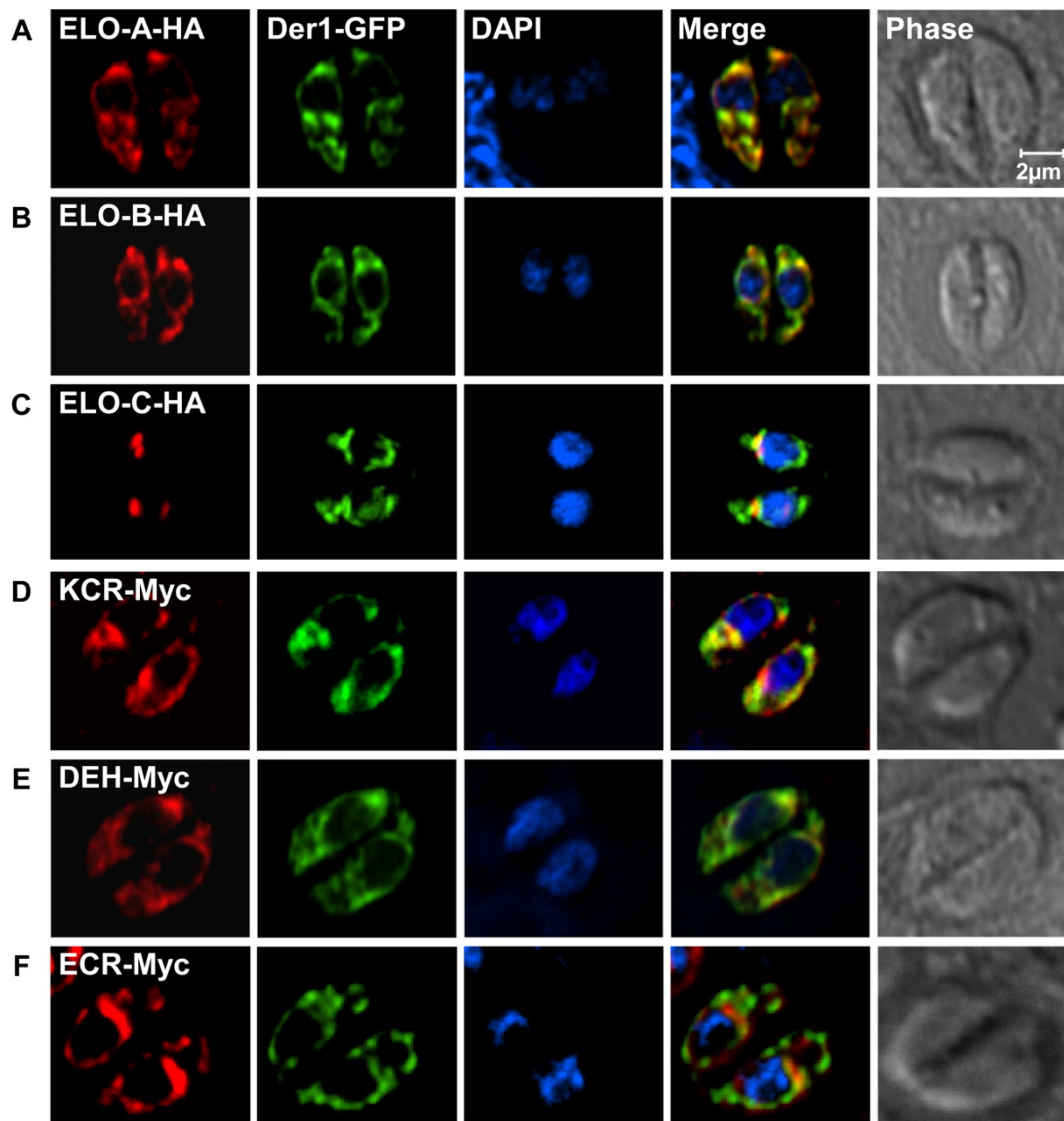

Figure 3.3: The *T. gondii* fatty acid elongation pathway is localized to the parasite endoplasmic reticulum. Transgenic parasite lines were allowed to infect cover slip cultures and were fixed and processed for immunofluorescence assay 24 hours later. Transgenic proteins were marked with an HA (**A**-**C**) or a Myc (**D**-**F**) epitope tag detected with suitable antibodies (red channel). All strains carried a Der1GFP marker (green) previously shown to localize to the endoplasmic reticulum (20). DAPI and phase contrast images are shown for comparison. See table ST3.1 for reference to *T. gondii* gene models and Experimental Procedures for detail on gene identification and cloning. Note that all six candidate fatty acid elongation enzymes are

found to be associated with the endoplasmic reticulum. Tagged ELO-C produces a more restricted pattern at the apical side of the nuclear envelope.

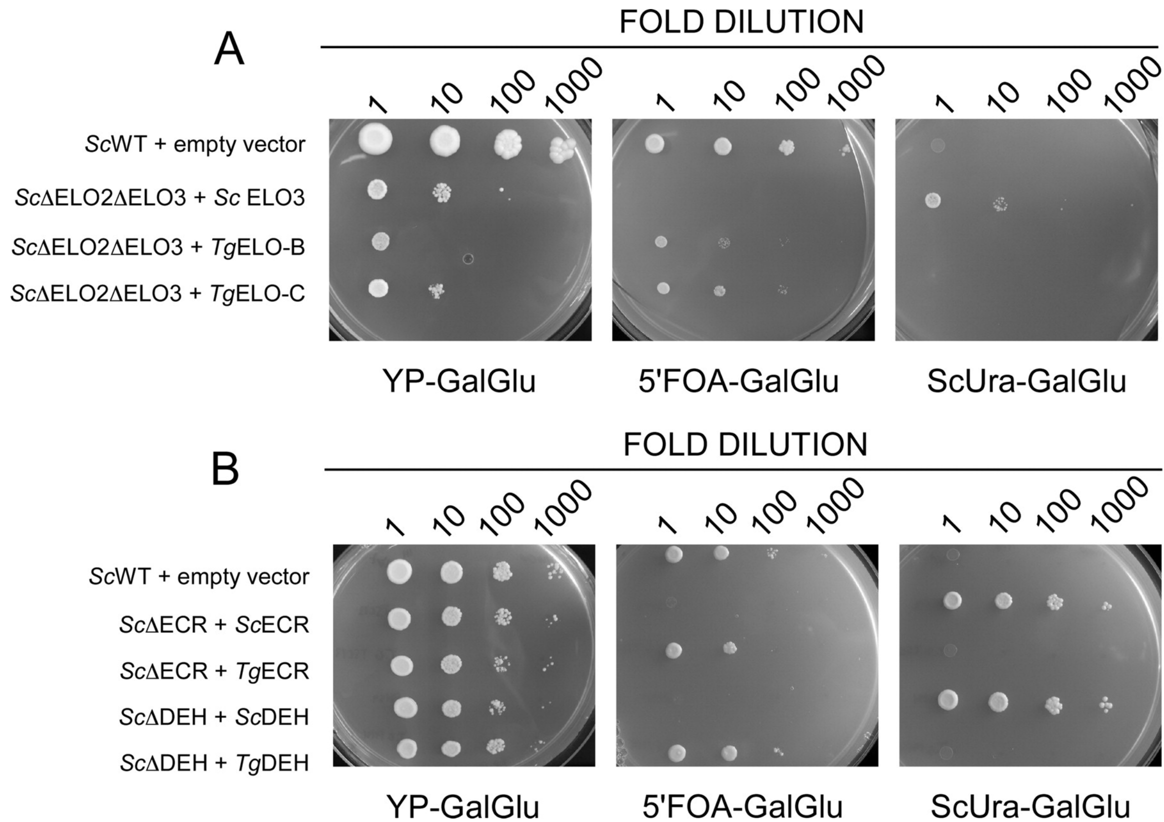

Figure 3.4: *T. gondii* genes complement yeast fatty acid elongation mutants. Yeast mutants with deletions in specific genes encoding fatty acid elongation enzymes were transformed with plasmids carrying *T. gondii* candidate genes as detailed in Experimental Procedures. The resulting strains were used to conduct spotting assays on YP-GalGlu media and demonstrate complementation (panels on the left). Control experiments using the same yeast cultures were done on media that selects against the yeast rescue plasmid and thus indicate that phenotypic complementation is due to presence of the *T. gondii* constructs only (5'FOA-GalGlu), or media that indicate presence of the yeast rescue plasmids only (ScUra-GalGlu) (center and right panels, respectively). Yeast cultures were progressively diluted prior to plating from left to right as indicated. (**A**) Complementation analyses for the yeast elongase *ScΔelo2Δelo3* double mutant

and the yeast (**B**) enoyl-CoA reductase (*Sc*DECR) and dehydratase (*Sc*DDEH) mutants respectively. Note that under restrictive conditions (5'FOA-GalGlu) growth depends on the presence of the *T. gondii* genes and that this growth can be fully suppressed using control media (ScUra-GalGlu). Only experiments that resulted in phenotypic complementation are shown here. See Table 3.2 for full detail on the genotype of all yeast strains.

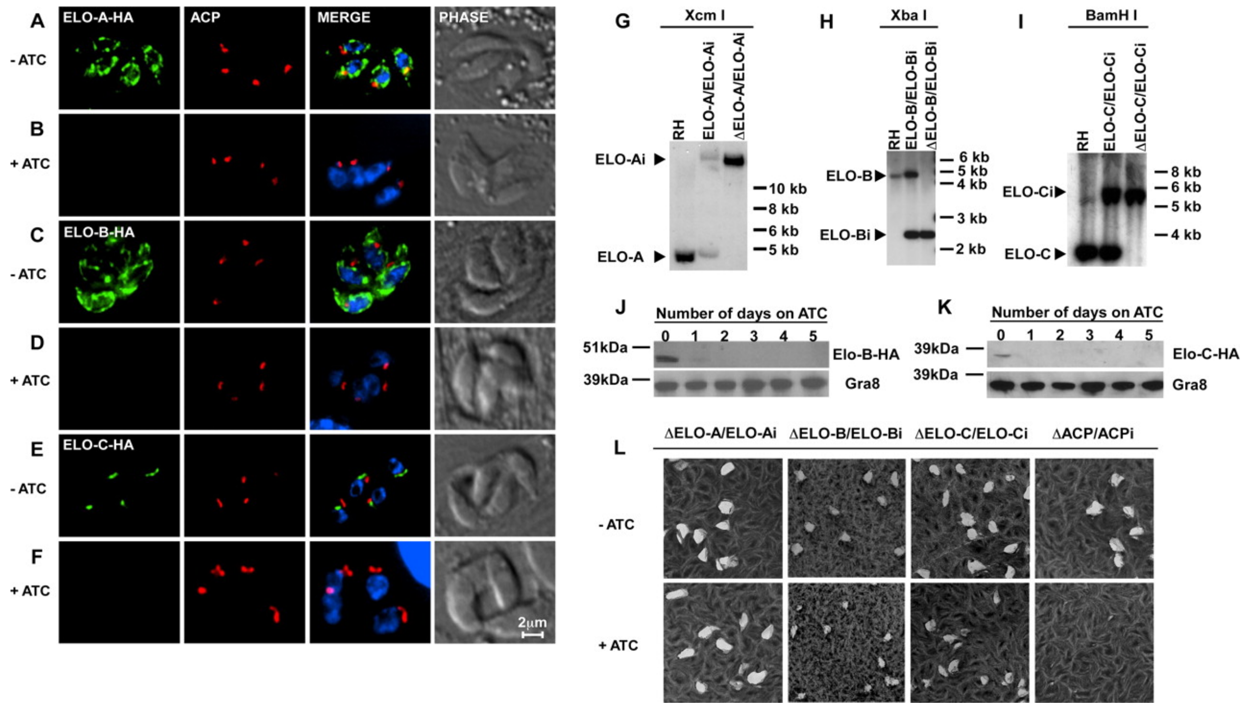

Figure 3.5: Conditional mutants lacking individual fatty acid elongases exhibit normal growth. We constructed conditional mutants for each of the three *T. gondii* ELO genes. (**A**-**F**) Immunofluorescence assay detecting the expression of the HA epitope tagged ectopic allele for the indicated ELO genes when grown in the absence (-ATc) or presence (+ATc) for 24 hours. Merge images also show DAPI staining of DNA. (**G**-**I**) Southern blot analysis probing genomic DNA from RH wild-type, a strain carrying both the native and the conditional allele, and the mutant carrying only the conditional allele (from left to right for each of the respective mutants as indicated). Probes detect the respective coding region of each gene and are detailed in

supplementary table ST3.2. In each blot the expected size of the restriction fragment for the native locus (e.g. ELO-B) and the conditional locus (e.g. ELO-Bi) is indicated with an arrowhead. Note loss of native loci. (**J** and **K**) Western blot analysis detecting ELO-Bi and ELO-Ci expression using an antibody against the HA tag. GRA8 is shown as a loading control. (**L**) The growth of the conditional ELO mutants was measured by plaque assay in absence and presence of ATc as indicated. ΔACP/ACPi serves as positive control.

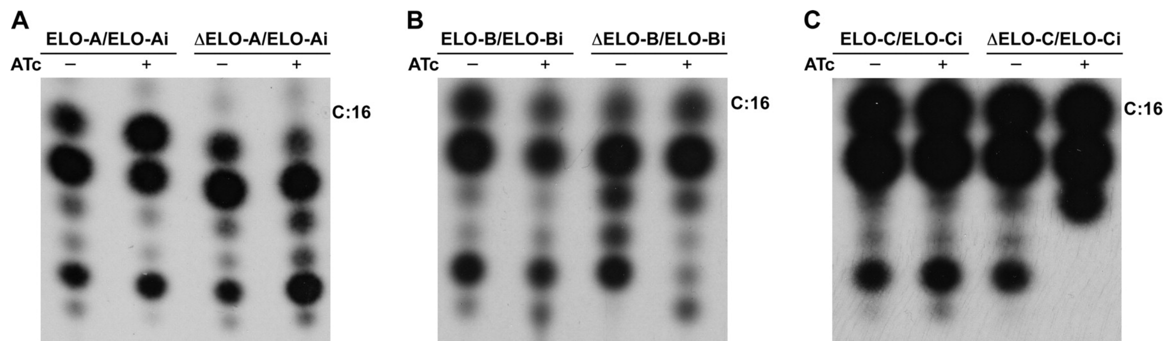

Figure 3.6: Radiolabeling analysis shows a reduction in long chain fatty acid synthesis in the *T. gondii* ELO-B and ELO-C mutant. *T. gondii* ELO mutants (**A**, ΔELO-A; **B**, ΔELO-B; and **C**, ΔELO-C) were metabolically labeled with ^{14}C-acetate, lipids were extracted, and fatty acid methyl esters were analyzed by reverse phase thin layer chromatography and representative autoradiographs for each mutant are shown. Each panel shows the parental strain carrying native and conditional locus (e.g. ELO-B/ELO-Bi) to left and the mutant (ΔELO-B/ELO-Bi) to the right grown in the absence (-) or presence (+) of ATc.

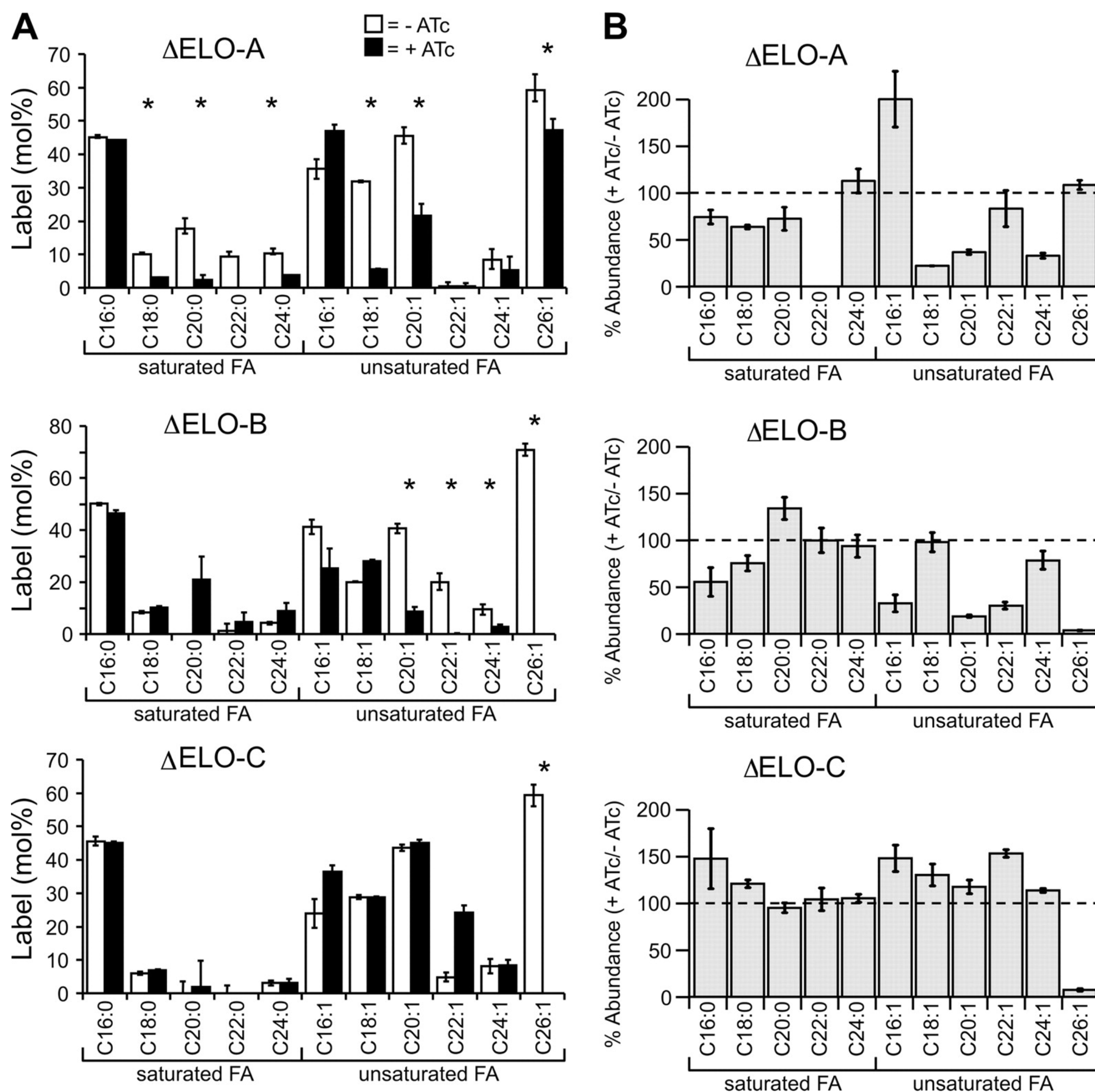

Figure 3.7: Loss of elongases results in selective reductions in the rate of synthesis and cellular levels of unsaturated fatty acids. Intracellular tachyzoites of the three ΔELO mutants were labeled with ^{13}C-U-glucose in the presence (black) or absence (white) of ATc as detailed in Fig. 3.1. (**A**) Level of ^{13}C-incorporation into the major fatty acids of the isolated tachyzoites. Error bars represent standard deviation where n=3-4 (**B**) Changes in the relative abundance of individual fatty acids in ΔELO tachyzoites in the presence of ATc as measured by GC-MS (values are given relative to the abundance of this fatty acid species in the no ATc control).

Results are indicative of >3 individual replicates. Individual values for all experiments are listed in Table 3.4. Fatty acids for which significant changes in labeling were observed in the presence and absence of ATc using the Wilcoxon Rank Sum Test (p-values less than 0.05) are indicated with an asterisk.

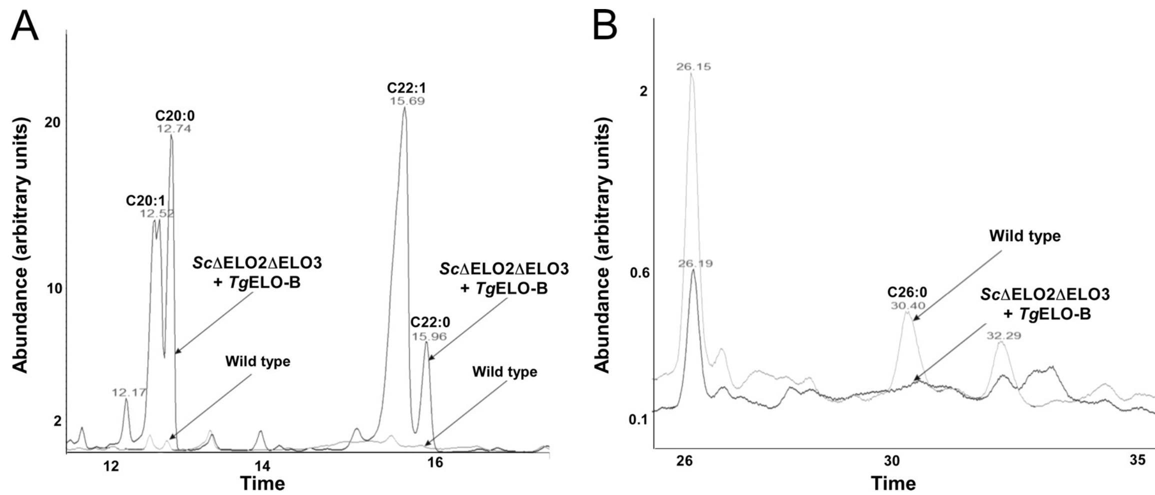

Figure 3.8: GC-MS analysis of fatty acid methyl esters from *S. cerevisiae* complemented with *T. gondii* ELO-B. Fatty acids from yeast Δ*elo2*/Δ*elo3* double deletion strain complemented by TgELO-B (See Fig 3.5) were extracted and subjected to GCMS analysis as described in Experimental Procedures. An overlay of the chromatogram of fatty acid methyl esters from this strain and the wild type is shown. (**A**) Enlargement of the region of the trace showing C22 and C24 methyl esters, (**B**) the region showing C26 methyl esters.

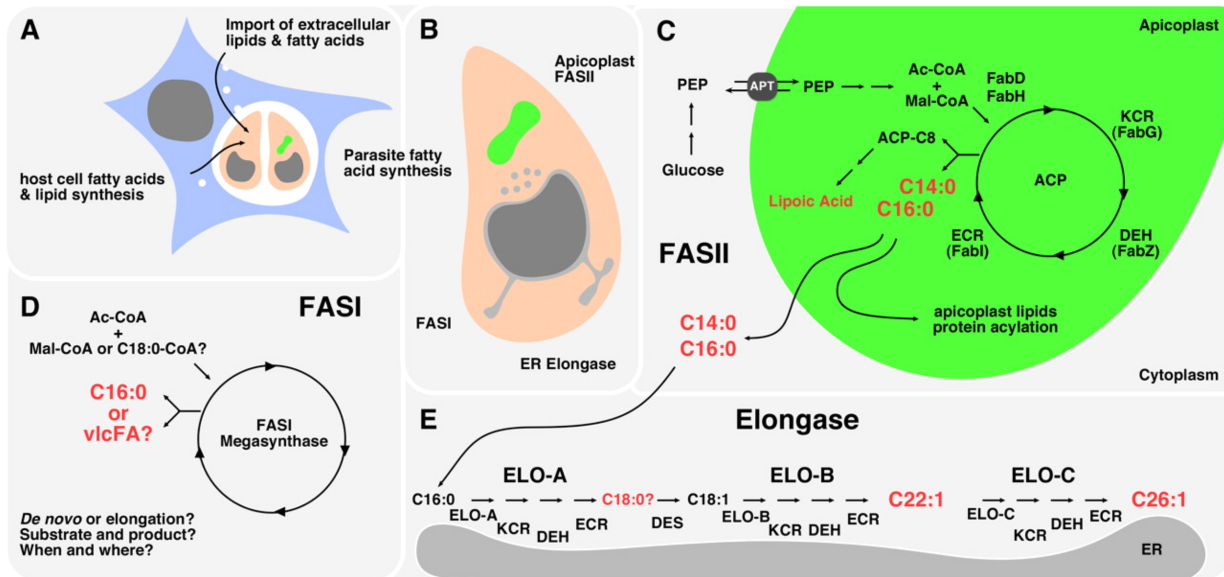

Figure 3.9: *Toxoplasma* acquires fatty acids through a complex network of synthesis and uptake. (**A**) *T. gondii* (pink) is an intracellular pathogen capable of fatty acid and lipid salvage from the host cell (blue). This process can intersect host cell import as well as synthesis routes. (**B**) In addition the parasite harbors three fatty acid synthesis pathways that are localized to different cellular compartments. (**C**) The apicoplast (green) localized FASII pathway produces significant amounts of myristic and palmitic acid in addition to lipoic acid relying on cytoplasmic glycolysis for precursors. (**E**) *Toxoplasma* also maintains an ER associated elongase system that synthesizes very long chain monounsaturated fatty acids, subsequently using the activity of ELO-B and ELO-C. (**D**) The *T. gondii* FASI remains largely uncharacterized. Its stage specific expression pattern and localization are not established. It is also unclear whether this megasynthase synthesizes fatty acids *de novo* like the FASI of humans or acts as an elongase for saturated fatty acids, as demonstrated for the FASI of *C. parvum*. Major products are highlighted in red. Des, desaturase; PEP, phosphoenolpyruvate; Mal, malonate; Ac, acetate; vlcFA, very long chain fatty acids.

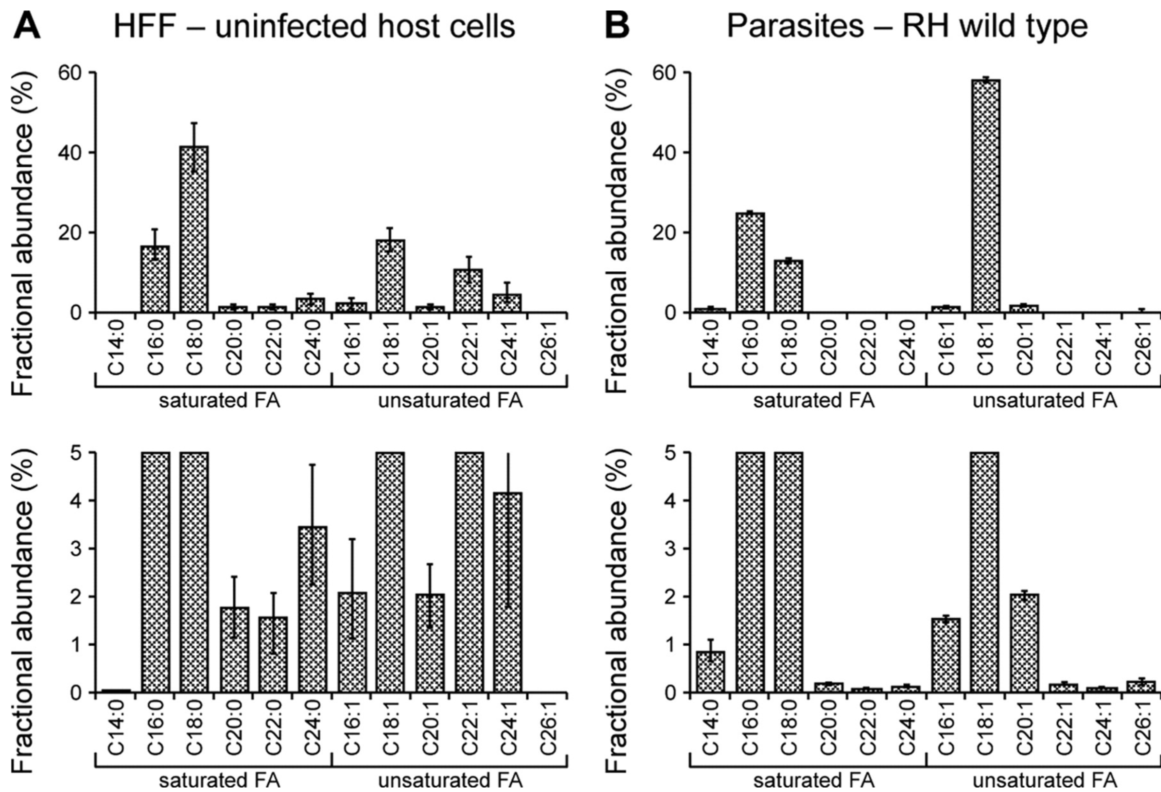

Figure 3.10. Total fatty acid composition of uninfected HFF host cells and RH tachyzoites. The total lipid extract of (**A**) HFF and (**B**) isolated purified tachyzoites (labeled in their host cells as detailed in the Experimental Procedures) was subjected to solvolysis in methanolic-HCl and derivitized in trimethylsylyl reagent, and fatty acid methylesters detected by GC-MS. The mole percent of the major fatty acids are shown (mean +/- SD). The lower panels show same data set scaled to a maximum of 5% fractional abundance to display low abundance species.

Table 3.1: Percent labeling of fatty acids derived from ^{13}C-U-glucose-fed tachyzoites and HFF. Wild type RH parasites were used to infect HFF and labeled in situ with ^{13}C-glucose. Uninfected HFF were labeled with ^{13}C-glucose under identical conditions. Percent labeling was determined by GC-MS analysis.

Cell type	**RH**	**RH**	**HFF**
+/- ATc	-	+	-
C14:0	87.73 (± 3.86)	84.68 (± 0.50)	nd
C16:0	61.56 (± 0.58)	59.01 (± 1.09)	0.00 (± 0.00)
C18:0	12.60 (± 0.55)	12.64 (± 0.73)	0.00 (± 0.00)
C20:0	23.64 (± 12.81)	16.84 (± 6.41)	0.00 (± 0.00)
C22:0	1.14 (± 2.95)	4.23 (± 1.05)	0.00 (± 0.00)
C24:0	14.57 (± 4.41)	15.38 (± 1.53)	0.00 (± 0.00)
C16:1	65.15 (± 1.62)	63.76 (± 0.47)	0.00 (± 0.00)
C18:1	57.97 (± 3.88)	54.59 (± 0.15)	1.76 (± 1.77)
C20:1	70.41 (± 0.82)	68.62 (± 0.71)	0.00 (± 0.00)
C22:1	6.81 (± 8.79)	9.76 (± 3.44)	0.00 (± 0.00)
C24:1	1.31 (± 1.87)	0.00 (± 2.58)	0.00 (± 0.00)
C26:1	82.83 (± 4.14)	80.37 (± 2.75)	nd

Table 3.2: Yeast strains constructed for complementation analysis.

Name	**Genotype of the yeast strain**
***Sc*ΔELO2ΔELO3+*Sc*ELO3**	W1536 elo2Δelo3Δ/ YCp33 *GAL1*-*Sc*ELO3
***Sc*ΔELO2ΔELO3+*Tg*ELO-B**	W1536 Δelo2Δelo3/YCP111 GAL *Tg*ELO-B
***Sc*ΔELO2ΔELO3+*Tg*ELO-C**	W1536 Δelo2Δelo3/YCP111 GAL *Tg*ELO-C
***Sc*ΔECR+*Sc*ECR**	W1536 Δtsc13/YCP33 GAL *Sc*Tsc13
***Sc*ΔECR+*Tg*ECR**	W1536 Δtsc13/YCP111 GAL *Tg*ECR
***Sc*ΔDEH+*Sc*DEH**	W1536 Δphs1/YCP33 GAL *Sc*Phs1
***Sc*ΔDEH+*Tg*DEH**	W1536 Δphs1/YCP111 GAL *Tg*DEH

Table 3.3: Fatty acid profiles of yeast wild type and complement *elo2*/*elo3* mutant strain. Values indicated are % of relative response units of total fatty acid methyl esters. P values were calculated using a two-tailed student's t-test, setting not detected fatty acid species to 0. *= $P < 0.05$ comparison to both *S. cerevisiae* WT and *elo2*/*elo3* double mutant complemented with *S. cerevisiae ELO3*. † $P < 0.05$ only in in comparison to the *S. cerevisiae elo2*/*elo3* double mutant complemented with *S. cerevisiae ELO3*.

The results of fatty acid extractions from two independent sets of cultures are shown. Fatty acyl species were identified by GC/MALDI mass spectroscopy as described in the Experimental Procedures.

	C14	C15	C16:1 /16	C18:1 /18	C20:1 /20	C22:1	C22	C24:1	C24	C26	C26-OH
TgELO-B	2.5 ± 1.2	4.4 ± 1.9	26.1† ± 3.8	36.3 ± 5.0	**12.4* ± 3.2**	**14.8* ± 1.3**	2.2 ± 1	**1.6* ± 0.2**	nd /0.1	nd	nd†
TgELO-C	2.1 ± 0.6	3.6 ± 2.3	47.2 ± 3.9	45.0† ± 0.4	2.2 ± 1.6	0.2 /nd	nd	nd	nd	nd	nd†
ScELO3	2.5 ± 0.1	2.4 ± 1.5	40.6 ± 0.8	51.9 ± 1.8	nd	nd	nd	nd	nd	1.1 ± 0.6	1 ± 0.1
WT	3.1 ± 1.4	1.6 ± 1.5	47.2 ± 6.5	46.1 ± 2.2	0.5 ± 0.1	0.3 ± 0.2	0.5 ± 0.5	nd	0.1 /nd	0.6 ± 0,4	0.7 /nd

nd: not detected

Table 3.4: Percent labeling of fatty acids derived from ^{13}C-U-glucose-fed tachyzoites. Mutant parasites were used to infect HFF and labeled in situ with ^{13}C-glucose in the presence or absence of ATc. Percent labeling was determined by GC-MS analysis. Fatty acids for which significant changes in labeling were observed in the presence and absence of ATc using the Wilcoxon Rank Sum Test (p-values less than 0.05) are indicated by asterisk.

Cell type	ACP		ELO-A	ELO-A	ELO-B	ELO-B	ELO-C	ELO-C
+/- ATc	-	+	-	+	-	+	-	+
C14:0	**54.08* (± 0.99)**	**7.66* (± 1.78)**	nd	nd	nd	nd	nd	nd
C16:0	**33.58* (± 0.68)**	**6.06* (± 0.22)**	45.17 (± 0.64)	44.24 (±0.19)	50.01 (± 0.45)	46.34 (± 1.47)	45.65 (± 1.28)	45.14 (± 0.35)
C18:0	**2.38* (± 0.51)**	**0.26* (± 0.28)**	**10.02* (± 0.50)**	**3.08* (± 0.03)**	8.40 (± 0.40)	10.18 (± 0.54)	5.95 (± 0.53)	7.02 (± 0.18)
C20:0	0.00 (±0.00)	0.00 (± 0.00)	**17.79* (± 3.14)**	**2.28* (± 1.60)**	0.00 (± 0.00)	20.99 (± 8.83)	0.00 (± 3.65)	1.97 (± 7.81)
C22:0	0.00 (± 0.00)	0.00 (± 0.39)	9.27 (± 1.61)	nd	1.26 (± 2.64)	4.62 (± 3.55)	0.00 (± 2.47)	0.00 (± 0.00)
C24:0	0.00 (± 4.82)	1.23 (± 0.08)	**10.21* (± 1.47)**	**3.75* (± 0.12)**	4.18 (± 0.42)	8.87 (± 3.04)	3.12 (± 0.81)	3.17 (± 1.14)
C16:1	**17.76* (± 1.76)**	**10.01* (± 3.17)**	35.63 (± 3.90)	47.04 (± 1.82)	41.27 (± 2.78)	25.38 (± 7.47)	23.94 (± 4.33)	36.37 (± 2.07)
C18:1	**16.37* (± 0.86)**	**4.96* (± 0.15)**	**31.95* (± 0.25)**	**5.42* (± 0.25)**	20.15 (± 0.08)	27.88 (± 0.64)	28.86 (± 0.53)	28.83 (± 0.19)
C20:1	**24.05* (± 2.99)**	**11.21* (± 0.61)**	**45.56* (± 1.48)**	**21.54* (± 3.51)**	**40.60* (± 1.72)**	**8.46* (± 1.93)**	43.64 (± 0.96)	45.09 (± 0.83)
C22:1	0.00 (± 1.82)	1.05 (± 1.50)	0.51 (± 1.26)	0.55 (± 0.82)	**20.12* (± 3.21)**	**0.00* (± 0.19)**	4.91 (± 1.38)	24.13 (2.22)
C24:1	0.31 (± 0.52)	0.00 (± 1.58)	8.36 (± 1.75)	5.29 (± 4.18)	**9.42* (± 2.07)**	**2.63* (± 1.14)**	8.10 (± 2.10)	8.39 (± 1.70)
C26:1	36.45 (± 11.48)	27.56 (± 1.86)	**59.32* (± 4.71)**	**47.30* (± 3.37)**	**70.80* (± 2.30)**	**0.00* (± 0.00)**	**59.41* (± 3.28)**	**0.00* (± 0.00)**

Supplementary Table ST1: Primers used for construction of tagged genes for subsequent localization of their protein products, gene deletions.

Name	Primers used for cloning with Restriction sites	Vector used for cloning	Genbank Acession numbers	Toxodb.org gene ID's
ELO-A	For_XmaI 5'-GTC ACC CGG GAT GTG GTC ACT CTG GCA GCT GTT TCA CT Rev_PstI 5'-GTC ACT GCA GTC AAT CTC TAC GAA GAC AAG CGC CGC CT	pDT7S4H_3	EEE21454	TGME49_053 880
ELO-B	For_BglII5'-GTC AAG ATC TAA AAT GGC GCC CAC AAT TGT TGA CG Rev_AvrII5'- GTC ACC TAG GAT CGG CTT TCC GAG CGC CTC C	pDT7S4H_3	EEE24212	TGME49_042 380
ELO-C	For_BglII5'-GTC AAG ATC TAA AAT GGA TAT CCG AGA AAC GAG T Rev_AvrII5'-GTC ACC TAG GCT GAG CCT TTT CCG CCT GGA TTT TC	pDT7S4H_3	EEE20374	TGME49_005 350
KCR	For_BclI 5'-GTACTGATCAGATCAATGGATCTCTTCAGT Rev_AvrII 5'-GTACCCTAGGCATGTCCTTCTTAGCC	pDT7S4M_3	JN544919	TGME49_071 890
DEH	For_BglII 5'- GTA CAG ATC TAA AAT GGC AGA AGC GGC Rev_AvrII 5'- CGC AGG CAG CGA AGA GAA GAA AAC ACA GCC TAG GGT AC	pDT7S4M_3	XP_002364371	TGME49_111 290
ECR	For_BglII 5'- GGA TCC AAA ATG AGG ATT TCT CTG AAG AAG CGC Rev_AvrII 5'- GTA CCC TAG GGA GAA TAA ACG GAA TGA TTG CGC	pDT7S4M_3	JN544918	TGME49_085 240
Primers used to recombineer gene targeting cosmids (50 bp recombination sequences are shown in upper case)				
ELO-A KO	For5'- CATACGTCATTTACCGAAATCCCAGTTGGCGTGGCAGAGAGATCACGAAAcctcgactacggcttccattggcaac Rev5'- GTGCCCCGTGCACGTTTCCACCCGTCTCTAAAATTGTTAAATGGCCTGAAatacgactcactatagggcgaattgg			
ELO-B KO	For5'- GTCTGTCCCCTTCTTTTGCCGCAGCTCTTCTCTCCTCCATCCCAGCCACGcctcgactacggcttccattggcaac Rev5'- TCATGAACTTACGCATCCCCTTTTGAATATGTGTCGGGGATTTTGCGTGTatacgactcactatagggcgaattgg			
Primers used to generate gene targeting plasmids				
ELO-C KO	Upstream for5' - ACT GGG TAC CGA GAG AAT CCA TCT GGC TAT TCC Upstream rev5' - ACT GAA GCT TTT TCC TGA ATC TAA AGA GGC TAG AAA Downstream for5' - ACT GGA CGT CAG GAC AGG GTG CAG AGT GAA GAT AAC Downstream rev5' - ACT GAC TAG TGG ATC ATT AGT CAA CAG GTC GTC			

Supplementary Table ST2: Primers used for Southern Blot probes

Name	Primers used for cloning with Restriction sites
ELO-A	For_5'- CTA CGC GTT CTC CGT CTT GCA GGC Rev_5'- AGA AGA CTC TTC GGG GTC ACT TGG C
ELO-B	For_5'- GTC AAG ATC TAA AAT GGC GCC CAC AAT TGT TGA CG Rev_5'- TCTACTTCGCTGTCGCTGCC
ELO-C	For_5'- GTC AAG ATC TAA AAT GGA TAT CCG AGA AAC GAG T Rev_5'- GTC ACC TAG GCT GAG CCT TTT CCG CCT GGA TTT TC

Supplementary Table ST3: Primers used for construction of tagged genes for cloning in Ycp111 GAL1 plasmid and expression in yeast.

Name	Primers used for cloning with Restriction sites	Epitope tag
ELO-A	For_PstI 5'- GTA CCT GCA GTA CCC CGG ACT ACG CT Rev_XbaI 5'- GTA CTC TAG ATC ATA CGA AGA CAA GC	HA_3
ELO-B	For_BglII5'-GTC AAG ATC TAA AAT GGC GCC CAC AAT TGT TGA CG Rev_SacI5'- GAG CTC CCC GGG TTA AGC GTA GTC CGG GAC GTC GTA CGG GTA	HA_3
ELO-C	For_BglII5'-GTC AAG ATC TAA AAT GGA TAT CCG AGA AAC GAG T Rev_SacI5'- GAG CTC CCC GGG TTA AGC GTA GTC CGG GAC GTC GTA CGG GTA	HA_3
KCR	For_BclI 5'- GTACTGATCAGATCAATGGATCTCTTCAGT Rev_KpnI/SacI 5'- GTA CGA GCT CGG TAC CCT CTC CTT ATT ACA GGT CCT	Myc_3
DEH	For_BglII 5'- GTA CAG ATC TAA AAT GGC AGA AGC GGC Rev_KpnI/SacI 5'- GTA CGA GCT CGG TAC CCT CTC CTT ATT ACA GGT CCT	Myc_3
ECR	For_BglII 5'- GGA TCC AAA ATG AGG ATT TCT CTG AAG AAG CGC Rev_KpnI/SacI 5'- GTA CGA GCT CGG TAC CCT CTC CTT ATT ACA GGT CCT	Myc_3

Supplementary Table ST4: Percent labeling of fatty acids derived from ^{13}C-U-glucose-fed tachyzoites and HFF. Wild type RH parasites were used to infect HFF and labeled in situ with ^{13}C-glucose. Uninfected HFF were labeled with ^{13}C-glucose under identical conditions. Percent labeling was determined by GC-MS analysis.

Cell type	RH	RH	HFF
+/- ATc	-	+	-
C14:0	87.73 (± 3.86)	84.68 (± 0.50)	nd
C16:0	61.56 (± 0.58)	59.01 (± 1.09)	0.00 (± 0.00)
C18:0	12.60 (± 0.55)	12.64 (± 0.73)	0.00 (± 0.00)
C20:0	23.64 (± 12.81)	16.84 (± 6.41)	0.00 (± 0.00)
C22:0	1.14 (± 2.95)	4.23 (± 1.05)	0.00 (± 0.00)
C24:0	14.57 (± 4.41)	15.38 (± 1.53)	0.00 (± 0.00)
C16:1	65.15 (± 1.62)	63.76 (± 0.47)	0.00 (± 0.00)
C18:1	57.97 (± 3.88)	54.59 (± 0.15)	1.76 (± 1.77)
C20:1	70.41 (± 0.82)	68.62 (± 0.71)	0.00 (± 0.00)
C22:1	6.81 (± 8.79)	9.76 (± 3.44)	0.00 (± 0.00)
C24:1	1.31 (± 1.87)	0.00 (± 2.58)	0.00 (± 0.00)
C26:1	82.83 (± 4.14)	80.37 (± 2.75)	nd

Supplementary Table ST5: Percent labeling of fatty acids derived from ^{13}C-U-glucose-fed tachyzoites. Mutant parasites were used to infect HFF and labeled in situ with ^{13}C-glucose in the presence or absence of ATc. Percent labeling was determined by GC-MS analysis. Fatty acids for which significant changes in labeling were observed in the presence and absence of ATc using the Wilcoxon Rank Sum Test (p-values less than 0.05) are indicated by grey coloring.

Cell type	ACP		ELO-A	ELO-A	ELO-B	ELO-B	ELO-C	ELO-C
+/- ATc	-	+	-	+	-	+	-	+
C14:0	54.08 (± 0.99)	7.66 (± 1.78)	nd	nd	nd	nd	nd	nd
C16:0	33.58 (± 0.68)	6.06 (± 0.22)	45.17 (± 0.64)	44.24 (±0.19)	50.01 (± 0.45)	46.34 (± 1.47)	45.65 (± 1.28)	45.14 (± 0.35)
C18:0	2.38 (± 0.51)	0.26 (± 0.28)	10.02 (± 0.50)	3.08 (± 0.03)	8.40 (± 0.40)	10.18 (± 0.54)	5.95 (± 0.53)	7.02 (± 0.18)
C20:0	0.00 (±0.00)	0.00 (± 0.00)	17.79 (± 3.14)	2.28 (± 1.60)	0.00 (± 0.00)	20.99 (± 8.83)	0.00 (± 3.65)	1.97 (± 7.81)
C22:0	0.00 (± 0.00)	0.00 (± 0.39)	9.27 (± 1.61)	nd	1.26 (± 2.64)	4.62 (± 3.55)	0.00 (± 2.47)	0.00 (± 0.00)
C24:0	0.00 (± 4.82)	1.23 (± 0.08)	10.21 (± 1.47)	3.75 (± 0.12)	4.18 (± 0.42)	8.87 (± 3.04)	3.12 (± 0.81)	3.17 (± 1.14)
C16:1	17.76 (± 1.76)	10.01 (± 3.17)	35.63 (± 3.90)	47.04 (± 1.82)	41.27 (± 2.78)	25.38 (± 7.47)	23.94 (± 4.33)	36.37 (± 2.07)
C18:1	16.37 (± 0.86)	4.96 (± 0.15)	31.95 (± 0.25)	5.42 (± 0.25)	20.15 (± 0.08)	27.88 (± 0.64)	28.86 (± 0.53)	28.83 (± 0.19)
C20:1	24.05 (± 2.99)	11.21 (± 0.61)	45.56 (± 1.48)	21.54 (± 3.51)	40.60 (± 1.72)	8.46 (± 1.93)	43.64 (± 0.96)	45.09 (± 0.83)
C22:1	**0.00 (± 1.82)**	**1.05 (± 1.50)**	**0.51 (± 1.26)**	**0.55 (± 0.82)**	**20.12 (± 3.21)**	**0.00 (± 0.19)**	**4.91 (± 1.38)**	**24.13 (2.22)**
C24:1	**0.31 (± 0.52)**	**0.00 (± 1.58)**	**8.36 (± 1.75)**	**5.29 (± 4.18)**	**9.42 (± 2.07)**	**2.63 (± 1.14)**	**8.10 (± 2.10)**	**8.39 (± 1.70)**
C26:1	**36.45 (± 11.48)**	**27.56 (± 1.86)**	**59.32 (± 4.71)**	**47.30 (± 3.37)**	**70.80 (± 2.30)**	**0.00 (± 0.00)**	**59.41 (± 3.28)**	**0.00 (± 0.00)**

CHAPTER 4

THE ESSENTIALITY OF FATTY ACID ELONGATION IN *TOXOPLASMA GONDII* DEPENDS ON ITS LIPID ENVIRONMENT.[1]

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4.1 ABSTRACT

Apicomplexan parasites are causative agents of important human diseases like malaria, cryptosporidiosis and toxoplasmosis. Fatty acid acquisition in these parasites is crucial for successful infection. The required fatty acids can either be synthesized or imported by the parasite. Following import, these fatty acids often undergo elongation to generate long chain fatty acids that could have specific roles in the parasite. Most apicomplexan parasites employ a fatty acid elongation (FAE) pathway for generating long chain fatty acids.

In the apicomplexan parasite *T. gondii*, the FAE pathway is comprised of three elongases, two reductases and a dehydratase. Conditional mutagenesis and metabolomic analysis of the fatty acid elongases suggested that this pathway is actively involved in generating long chain unsaturated fatty acids for the parasite. Whether the pathway is essential for parasite growth, remained unknown. With the goal of determining the essentiality of this pathway, we extended our approach to analyse the dehydratase enzyme of the FAE pathway. Dehydratase is involved in an intermediate step of every elongation cycle. Our results show that the loss of this protein affects the entire FAE pathway and results in reduced long chain fatty acid synthesis. The inability of the parasite to synthesize long chain fatty acid results in an immediate growth defect. This growth defect can be partially rescued by supplying long chain fatty acids in the parasite growth medium indicating that the products of the FAE pathway are essential for growth of *T. gondii*.

4.2 INTRODUCTION

Elongation is an important sector of fatty acid metabolism in all organisms. Fatty acids can either be synthesized or imported. Elongation and modification of these fatty acids is required before they can be utilized for specific cellular processes. Fatty acid elongation produces precursors for the formation of long chain lipids, which have been shown to perform important roles in protein trafficking (1), maintenance of cell membranes and maintenance of nuclear pore complex (2). Synthesis of long chain fatty acids is achieved by the activity of the fatty acid elongation pathway.

The fatty acid elongation (FAE) is a multistep process where each elongation cycle begins with a condensation reaction, that is catalyzed by a fatty acid elongase enzyme. The product of this reaction, is then reduced by a keto-acyl reductase (KCR), dehydrated by hydroxyl acyl-CoA dehydratase (HCD) and further reduced by the action of enoyl-CoA reductase (ECR). Most organisms harbor multiple fatty acid elongases, which are specific for the length of the fatty acid they utilize as substrate and the length of the fatty acid product that they generate. Consequently, a complete elongation pathway in any organism comprises several elongation cycles catalyzed by the same or different fatty acid elongases present in that organism. It has been speculated that these fatty acid elongases can have overlapping specificity in their choice of substrate and the length of products generated may vary. This is further supported by the fact that loss of two, but not one, fatty acid elongase in *S. cereviseae* causes lethality (3). Although the various elongation cycles may be initiated by different elongases, the reductases and dehydratase involved in every elongation cycle are believed to be the same. Therefore, loss of these enzymes ablates the entire elongation pathway. This has been demonstrated in *S. cereviseae* (4) and *Arabidopsis* (5) where the loss of dehydratase produces a concomitant reduction in long chain

fatty acid synthesis. Similarly, loss of the enoyl-CoA reductase in *Arabidopsis* results in the reduction of very long chain fatty acid content and affects plant morphogenesis (6). Additionally, data from *Arabidopsis* suggests that even though the plant genome encodes for two keto-acyl CoA reductases (KCR), only one of these two reductases is required for the FAE pathway (7). Whereas, loss of the other KCR has no effect on fatty acid elongation or plant morphogenesis.

Apicomplexan parasites are obligate intracellular pathogens. Therefore, access to required fatty acids may vary depending on the specific niche occupied by these organisms. Most apicomplexan parasites, however, harbor mechanisms to generate their own fatty acids. Among these parasites capable of lipid synthesis, *C. parvum* harbors a single fatty acid elongase capable of generating C16/ C18 fatty acid by using C14/C16 fatty acid substrate (8). However, it is not known if CpFAE is essential for parasite growth. In *Plasmodium* species, bioinformatic analyses suggest the presence of a fatty acid elongation pathway. The role and essentiality of this pathway remains undetermined. Based on radiolabelling experiments, it appears this pathway functions in blood stage parasites (9). In *T. gondii*, we have recently described the role of the TgFAE pathway in tachyzoites. By combining genetics with stable isotope labeling and isotopomer analysis we identified the specific products of each fatty acid elongase (10).

Here, we study whether the TgFAE pathway is required for the growth of parasite tachyzoites. We isolate conditional mutants for dehydratase and enoyl-CoA reductase enzymes of the TgFAE pathway. By measuring fatty acid synthesis using heavy isotope labelling, we show that the loss of dehydratase prevents the formation of all long chain fatty acids by the parasite. Growth analysis indicates that this pathway is crucial for parasite growth. Furthermore, we also show that, an excess of fatty acids supplied in the growth medium can partially restore the growth defect associated with the dehydratase mutant. Together these results highlight the

importance of TgFAE pathway and suggest that the essentiality of the apicomplexan fatty acid synthesis pathways is highly dependent on the fatty acids present in the surrounding environment of the parasite.

4.3 EXPERIMENTAL PROCEDURES

4.3.1 Parasite culture and construction of mutants.

T. gondii tachyzoites derived from strain RH were cultured and genetically manipulated as described previously (11). Conditional mutants for dehydratase and enoyl-CoA reductase were generated using a previously described promoter insertion strategy in a Ku80 mutant parasite line (12). For dehydratase, cosmid PSBMG05 was modified so that a tetracycline-regulated promoter was inserted prior to the start codon of the coding sequence. The cosmid was then transfected into the Ku80 mutant parasite line and transgenic parasites were selected in the presence of 1 μM pyrimethamine. Mutants were identified by PCR (detailed in Fig. 4.1B and 4.1C; See table ST4.1 for primer details). To manipulate enoyl-CoA reductase locus, a targeting plasmid was developed. This plasmid contained the tetracycline-regulated promoter and a pyrimethamine resistance cassette flanked by the ECR locus sequence (See table ST4.1 for primer details). Following transfection of Ku80 TATi (12) parasite line with this plasmid, transgenic parasites were selected in presence of pyrimethamine and mutants were identified via PCR (Fig 4.4B). Parasite growth was evaluated by plaque assay in absence or presence of 0.5 μM ATc (Fig 4.4C) (11).

4.3.2 ^{14}C Acetate radiolabelling and thin layer chromatography.

Parasites were grown in the absence or presence of 0.5 μM ATc for 48 hours, and free tachyzoites were metabolically labeled as previously described (13). Briefly, 10^8 tachyzoites were incubated with 10 μCi of sodium [^{14}C]acetate (Mavarek) in 1ml of Dulbecco's modified eagle medium for 4h at 37°C and 5% CO_2. Total lipids were extracted with chloroform/methanol (2:1). The extract was dried and fatty acid methyl esters were subjected to acidic methanolysis. The resulting fatty acid methyl esters were extracted with hexane and analyzed on the RP-18 HPTLC plates developed in methanol/chloroform/water (75:25:5) and exposed to film for 1week at -80°C.

4.3.3 Stable Isotope Labeling and Metabolomic Analyses

T. gondii infected fibroblasts were grown in DMEM in a T175 flask in the absence or presence of ATc for 48 h. Following these 48 hours, the medium was supplemented with 8 mM [U-^{13}C] glucose (final concentration of [^{13}C/^{12}C] glucose was 16 mM) and parasites were grown for another 24 hours. Upon complete host cell lysis, free parasites were separated from host cells by filtration through a 3-μm membrane. Parasite metabolism was quenched by rapid chilling of the cell suspension in a dry ice/ethanol bath and parasites were recovered by centrifugation (4000 X g, 25 min, 0 °C). Cell pellets were washed three times with ice-cold PBS, and cell aliquots (2 X 10^8 cells) were transferred to microcentrifuge tubes and centrifuged (10,000 X g, 30 s, 0 °C). Pellets were suspended in 300μl chloroform/methanol (2:1) and vortex-mixed thoroughly. Samples were sonicated for 5 mins at RT and then incubated at 60 °C for 20 min. Extracts were

centrifuged at 10,000 X g for 5 min, and the supernatant was dried and subsequently washed twice with methanol. Dried residues were dissolved in 25µL of methprep II (alltech). Samples were analyzed on an Agilent 7890A-5975C GC-MS system. Split (split ratio 10:1) injection (injection temperature 250 °C) onto a 30m + 10m X 0.25mm DB-5MS + DG column (J&W, Agilent Technologies) was used, using helium as the carrier gas. The initial oven temperature was 70 °C (1 min), followed by temperature gradients to 230 °C at 17 °C/2mins, from 230 to 325 °C at 25 °C/min. The final temperature was held for 10 min. Data analysis was performed using Chemstation software (MSD Chemstation D.01.02.16, Agilent Technologies). Abundance and label incorporation was calculated as described previously (14). Data is shown are the average of three technical replicates and their standard deviation. Statistical significance was evaluated by Wilcoxon Rank Sum Testing with continuity corrections, using the R data analysis package (version 2.14.0), where p values of <0.05 were considered significant.

4.3.4 Fatty acid complementation:

Preparation of fatty acids: 100mM of each fatty acid was measured and dissolved in 1mL of ethanol. 10ul (1mM) of each fatty acid was taken into a new 1.5mL tube. This fatty acid mixture was dried by nitrogen air/speed vacuum. 0.5mM fatty acid-free BSA (sigma-aldrich) in PBS was prepared and filter sterilized.This BSA was then added to the fatty acids to obtain 2:1 (FA:BSA) molar ratio. Fatty acids were solubilized by sonication in water bath for about 1 hour, or until fatty acids are dissolved completely. Mix 10-50ul (10-50uM) of each fatty acid into a tube and bring up to 1mL with BSA to prepare the final fatty acid mixture.

Preparing the parasites: Parasites were filtered through a 0.3 μm membrane to remove host cell debris and enumerated. For pre-incubation on ATc, parasites were grown for 72 hours on a T25 HFF flask in presence of 0.5 μM ATC. All parasites were washed twice with 10mL PBS before being used for the experiment. Growth assay measurement was performed in 96-well optical plates containing 5000 parasites in 200 μl of Delbecco's modified eagle media. Control wells did not contain any fatty acids wheras other wells contained fatty acids as listed in the respective figures. Growth of parasites in these wells were monitored using a plate reader for 9 days.

4.4 RESULTS

4.4.1 Generating conditional mutants for a constitutive component of the fatty acid elongation pathway.

We have previously shown that a fully functional fatty acid elongation pathway (FAE) is present in *Toxoplasma gondii* (10). All components of this pathway are expressed in the tachyzoite stage of the parasite life cycle. The primary enzymes of the pathway, fatty acid elongases are not essential for parasite growth under *in vitro* conditions (10). However, a possible overlapping activity of these elongases masks any defects associated with the loss of a single elongase enzyme.

Hence, to test the essentiality of the fatty acid elongation pathway, we decided to generate a mutant [for two fatty acid elongases simultaneously in the same parasite line. However, our attempts to generate such double mutants were not successful. As an alternate strategy we chose to generate conditional mutants for the dehydratase enzyme. This enzyme is required for

completing the elongation cycle started by each of the fatty acid elongases (4, 5). As a result, loss of this enzyme should completely ablate the overall fatty acid elongation pathway. To generate this mutant we initially used the parasite line expressing a tagged version of the dehydratase coding sequence under a regulatable promoter (10). Our attempts to replace the genomic copy of dehydratase with a drug selectable marker in this parasite line failed recurrently. We believed that the low recombination efficieny in *T. gondii* could be the reason behind our unsuccessful attempts to delete the genomic copy of the dehydratase gene. To resolve this problem we used the Ku80 mutant parasite line. Ku80 is a protein involved in non-homologous DNA end joining. This parasite line, therefore reduces the probability of non-homologous recombination thereby facilitating an increased probability to delete the genomic copy of a gene. We replaced the UPRT locus in this parasite line with a tagged and regulated copy of the dehydratase gene. The loss of UPRT locus allowed us to select for this line using FUDR. However, once again our attempts to delete the genomic copy of dehydratase were unsuccessful. We believed that the presence of the antigenic tag could affect the function of the regulatable dehydratase gene.

To avoid any interference from the tag we generated the dehydratase mutant using a promoter insertion strategy in the Ku80 Tati parasite line. This strategy has been previously established in our laboratory to generate conditional mutants of essential genes in *T. gondii* (12). We incorporated the regulatable promoter between the native promoter and the start codon of the dehydratase gene. After drug selection, we tested the resistant parasite clones via PCR screen. The result of this screen is presented in Figure 4.1B. Wild type parasites with an intact native promoter, produce a 3.5 kb band (Fig. 4.1B). The parasite clone where the native promoter has been displaced by the insertion of the regulatable promoter can be seen by the absence of a 3.5

kb band (Fig. 4.1B). Using control primers, which bind an gene unrelated to the FAE pathway, we can confirm the presence of genomic DNA in all samples in Fig. 4.1C.

Next, we verified that the expression of the dehydratase gene in this parasite line, can be regulated by addition or removal of tetracyline. We tested this using qRT-PCR (Fig. 4.1C), which allowed us to measure the copies of dehydratase m-RNA and therefore expression of the gene in the presence and absence of tetracycline. Our qRT-PCR showed that the expression of dehydratase was suppressed by addition of tetracycline. The control primer set amplifies ACCase, a gene whose promoter is unmodified in the dehydratase mutant. The expression of ACCase gene was not affected in the dehydratase mutant. This shows that the effect of tetracycline is specific to the dehydratase gene. Together these results show that we were able to generate a conditional mutant for the dehydratase enzyme of the fatty acid elongation pathway.

4.4.2 Loss of dehydratase affects the entire fatty acid elongation pathway and results in reduced synthesis of all the products.

Fatty acid elongases catalyse the starting step of every fatty acid elongation cycle. Dehydratase is required for the completion of the elongation cycle initiated by all elongases. Therefore, we expected that downregulation of dehydratase should affect the synthesis of all long chain fatty acids that are synthesized by these fatty acid elongases. Incubation of parasites with ^{14}C-acetate leads to the incorporation of radioactive carbon into newly synthesized fatty acids. These fatty acids were extracted, converted to methyl esters and identified using a reverse phase thin layer chromatography. To test is the overall long chain fatty acid synthesis was affected in the dehydratase mutant, we incubated extracellular parasites with ^{14}C-acetate in absence or

presence of ATc. Fig 4.2A shows the thin layer chromatogram of acetate labeling experiments with Ku80 tati parental parasites and the dehydratase mutant. The labeling profile of Ku80 Tati parental parasite line (Fig 4.2A, lanes 1 and 2) remains unchanged in the presence or absence of ATc indicating that ATc has no effect on these parasites. The fatty acid labeling pattern for the dehydratase mutant grown in absence of ATc matches that of the Ku80 Tati parasites (Fig 4.2A, lane 3). However, in presence of ATc, when the dehydratase protein is absent, a strong reduction in labeling of long chain fatty acids can be noticed. Label accumulates in the shorter fatty acid species, which could be the starting substrates for the fatty acid elongation pathway (Fig 4.2A, lane 4).

To further confirm the biochemical defect associated with the dehydratase mutant, we used a stable heavy isotope labelling and mass spectrometry approach. In this experiment, we add ^{13}C-glucose to the dehydratase mutant parasites growing in presence or absence of ATc. Following further growth for 24 hours, parasites are then separated from host cell debris and fatty acids are extracted and derivatizated, followed by GC-MS analysis. For parasites grown in absence of ATc, the ^{13}C is incorporated into the fatty acids, which are being actively synthesized by the parasites. However, for parasites grown in presence of ATc, where the dehydratase protein is downregulated, active incorporation of ^{13}C into the products of fatty acid elongation pathway should be markedly reduced. Figure 4.2B shows the percentage labeling of fatty acids with ^{13}C in the dehydratase mutant grown in absence or presence of ATc. It can be noted that the incorporation of label in the dehydratase mutant parasites grown in absence of ATc is very similar to the labelling profile of wild type parasites (10). For the dehydratase mutant grown in presence of ATc, a significant reduction in ^{13}C incorporation can be observed for lignoceric, oleic, gondoic, erucic and hexacosenoic fatty acids. These long chain fatty acids are the products

of fatty acid elongation pathway in *T. gondii*. Reduced labeling of these long chain fatty acids indicates an indispensable role for dehydratase in the fatty acid elongation pathway.

4.4.3 Dehydratase enzyme of the TgFAE pathway is required for the growth of *T. gondii* tachyzoites

Loss of dehydratase affects the overall synthesis of long chain fatty acids. These long chain fatty acids comprise a major portion (about 45%) of the total fatty acid pool in *T. gondii* (See table 4.1 for overall abundance of each fatty acid). Although the role of these fatty acids is unknown in *T. gondii*; in other organisms, long chain fatty acids are crucial for membrane maintainence, protein trafficking and are required for continuous cell growth. Owing to these important functions that can be performed by long chain fatty acids and their high percentage in *T. gondii* tachyzoites, we tested the growth of dehydratase mutant in this life cycle stage of the parasites. We approached this question in two ways. Firstly we carried out plaque assays, to measure growth of *T. gondii*. Parasites are added to a confluent monolayer of host cells. Parasite growth results in the lysis of host cells and formation of plaques, which appear as clearances in host monolayer crystal violet staining. Fig 4.3A shows that the plaque size and therefore the growth of the wild type parasites remain unaffected by the presence or absence of ATc. Dehydratase mutant parasites grow normally in absence of ATc. However, upon addition of ATc, the plaque size is significantly reduced. The smaller plaque size is indicative of a growth defect associated with the loss of dehydratase.

We transfected these parasites with the red fluorescent protein. Expression of the fluorescent protein in these parasites allows us to examine their growth rate by measuring

fluorescence increase over time in 96-well plates. Figure 4.3B shows the growth of parasites for a period of 10 days. Mutants grown in absence of ATc produce fluorescence growth curves indistinguishable from those obtained for wild type parasites. However, in presence of ATc parasite growth is reduced. This effect is even stronger in parasites that were pre-incubated for 3 days with ATc. In these pre-treated cultures, dehydratase is absent at the beginning of the growth assay experiments. Therefore, any defects due to the loss of this enzyme are detected early and easily. In our experiments, these pre-incubated parasites do not exhibit any growth. Together the plaque assay and growth assay experiments show that a marked growth defect is associated with the loss of dehydratase.

4.4.4 A second downstream enzyme of the FAE pathway shows growth phenotype similar to parasites lacking dehydratase

The final step in the FAE pathway is a reduction of the fatty acid to obtain a final product, which is two carbons longer. The enzyme responsible for this is enoyl-CoA reductase (ECR) that we have previously shown co-localizes to the endoplasmic reticulum and is able to complement its yeast homolog (10). Using a similar promoter insertion strategy as for dehydratase, we obtained knock down clones for ECR where a regulatable promoter could shut off expression of the gene by the addition of ATc (iECR). Targeting of the locus was confirmed by PCR screening as seen in figure 4.4B. Presence of a 2.5 kb band is diagnostic of a modified ECR locus. Growth of ECR mutants was tested by their ability to form plaques on a confluent host cell layer. Results in fig. 4.4C show that ECR mutants in presence of ATc produce smaller

plaques than in absence of ATc. Difference in plaque size indicates that ECR is required for parasite growth.

4.4.5 Loss of dehydratase can be restored by chemical complementation with monounsaturated fatty acids

Loss of FAE activity leads to inhibition of parasite growth. In the absence of the dehydratase enzyme, there is a reduction in certain long chain fatty acids. We investigated whether supplementing fatty acids in the normal growth medium could restore parasite growth. Mutant dehydratase parasites take 6 days to exhibit growth defects in presence of ATc (Fig. 4.3B). Therefore dehdyratase mutants expressing red fluorescent protein were grown in the absence or presence of ATc for 6 days and then used for fatty acid complementation analysis. Parasites were cultured in 96-well plates in medium supplemented with fatty acids complexed to FA-free BSA at a concentration of 250 μM. At these concentrations fatty acids have no effect on the growth of wild type parasites (data not shown). Combinations of saturated or monounsaturated fatty acids were tested, to see if loss of these fatty acids is the cause for parasite death in the absence of the FAE pathway.

When the parasites are grown in the absence of ATc, dehydratase is expressed and products of the FAE pathway are synthesized. Addition of monounsaturated fatty acids (Fig 4.5A) or saturated fatty acids (Fig 4.5B) does not affect parasite growth under this condition. However, when dehydratase expression is turned off by addition of ATc, only a mixture of monounsaturated fatty acids can rescue the observed growth defect (Fig. 4.5A). Interestingly,

saturated fatty acids do not rescue this growth defect (Fig 4.5B), indicating that the parasite has a specific need for monounsaturated fatty acids.

4.5 DISCUSSION

4.5.1 Fatty acid elongation in apicomplexan parasites is well conserved.

Components of the fatty acid elongation (FAE) pathway have been reported in all apicomplexan parasites that are capable of fatty acid synthesis. The presence of this pathway in *C. parvum* (8) and *T. gondii* (10) has been confirmed biochemically. In *Plasmodium* species, substantial evidence for the presence of a FAE pathway is lacking. However, it has been reported that blood stage *Plasmodium* parasites are capable of synthesizing long chain fatty acids (9), suggesting that the FAE pathway might be active during this life cycle stage of the parasites. Phylogenetic analyses of fatty acid elongases of different organisms place *T. gondii* and *Plasmodium* species together, suggesting that the enzymes of the FAE pathway in apicomplexan parasites are closely related (15). Characterization of *C. parvum* fatty acid elongase shows that this enzyme does not exhibit any activity towards polyunsaturated fatty acids (8). Our analyses of *T. gondii* elongases show that these enzymes are involved in synthesis of long chain monounsaturated fatty acids. Together, this indicates that *C. parvum* and *T. gondii* fatty acid elongases are similar in their activity and belong to a group of saturated/monounsaturated fatty acid elongases.

4.5.2 Fatty acid elongation pathway plays an important role in growth of all eukaryotes.

The fatty acid elongation pathway has been and characterized in yeast, plants and kinetoplastid parasites. In *S. cereviseae*, the pathway involves three fatty acid elongases. A double mutant for two of these fatty acid elongases affects growth, suggesting a direct relationship between FAE and cell multiplication (3). In plants, the essentiality of the FAE pathway is underscored in the mutants for the entire FAE pathway. *Arabidopsis* mutants for dehydratase and enoyl-CoA reductase enzymes of the FAE pathway exhibit reduced synthesis of long chain fatty acids and are defective in normal plant growth (5, 6). In the kinetoplastid parasite, *T. brucei*, the FAE pathway serves as a mechanism of *de novo* synthesis rather than fatty acid elongation. Inhibition of the *T. brucei* FAE pathway in fatty acid free media affects parasite growth, signifying that the products of this pathway are required for the multiplication of the parasite (16).

4.5.3 The importance of fatty acid elongation in apicomplexan parasites.

Although, among apicomplexan parasites, the FAE pathway was first described in *C. parvum*, the importance of the pathway towards parasite growth remained undetermined. Molecular and biochemical analysis of the *C. parvum* elongase clearly determines the functional specificity and expression profile of the enzyme. However the data does not provide any evidence for the role of this pathway in parasite growth. In *Plasmodium* the pathway remains uncharacterized and therefore its contribution toward parasite multiplication is yet uncertain. In *T. gondii*, analysis of fatty acid elongases clearly identifies the specific contribution of the

pathway towards the overall fatty acid pool of the parasite. However, no growth defect is associated with any of the *T. gondii* elongase mutants. It has been suggested that the fatty acid elongases and long chain fatty acids can exhibit a moderate redundancy in their functional activity. To avoid this redundancy, we generated conditional mutants for dehydratase and enoyl-CoA reductase enzymes of the TgFAE pathway. These enzymes are required for the completion of every elongation cycle in the FAE pathway. The dehydratase mutant clearly shows a marked decrease in the overall fatty acid elongation. This is reflected in both acetate labeling (Fig. 4.2A) as well as ^{13}C-glucose labeling experiments (Fig. 4.2B). Analysis of both dehydratase and enoyl-CoA reductase indicates that the loss of these enzymes affects parasite growth (Fig. 4.3A and Fig. 4.4C). This provides evidence that the products of the fatty acid elongation pathway are important for the survival of *T. gondii*. Considering that *T. gondii* is closely related to *C. parvum* and *Plasmodium*, it can be speculated that the FAE pathway is crucial in these parasites as well.

4.5.5 The essentiality of apicomplexan fatty acid synthesis is largely dependent on the parasite growth environment.

During their life cycle, apicomplexan parasites are exposed to a variety of environments that differ greatly in their metabolic composition. They cycle between the intracellular and extracellular environment, among tissues within a host, and among hosts. As a result, the metabolic pathways employed by these parasites change significantly from one life cycle stage to another. In the apicomplexan parasite *Plasmodium*, fatty acid synthase type II (FASII) pathway is believed to be the major source of *de novo* fatty acid synthesis. Genetic analysis suggests that the enzymes of the FASII pathway are not expressed in insect or blood stages but only in late

liver stage parasites. Moreover, this pathway only appears to be essential during the late liver stages of infection. The specific role of this pathway during this life cycle stage remains unclear. However, it can be proposed that the parasite growth environment could be responsible for such differences in the essentiality of the FASII pathway. The FASII pathway in apicomplexan parasites is believed to be similar to the plant FASII pathway. It has been demonstrated in plants that the addition of fatty acids to plant suspension cultures reduces fatty acid synthesis indicating that the activity of FASII pathway can be controlled by the presence of fatty acid in the environment(17). Similarly in parasites, it is highly likely that the lipids in the blood serum or in the insect gut prevent the expression and utilization of the parasite FASII pathway. However, restricted access to these lipids in the liver stages may cause the parasite to depend on its own synthetic mechanism during this part of the life cycle.

We have demonstrated that in *T. gondii*, the growth defects associated to the downregulation of the biosynthetic pathway can be rescued by exogenously providing fatty acids. These results highlight the importance of environmental lipid composition in parasite survival. Loss of the fatty acid elongation pathway prevents parasite growth. However, addition of fatty acids to the parasite growth medium rescues this growth defect to almost wild type levels (Fig. 4.5A-B). The ability to rescue this growth defect by addition of fatty acids to growth medium provides evidence that the essentiality of fatty acid elongation in *T. gondii* can be regulated by the presence of fatty acids in the external growth environment. Additionally, this suggests that in the presence of an alternative source such as the host fatty acids, the apicomplexan parasites would not have to be dependent on their own fatty acid synthesis mechanisms.

4.6.6 Why are long chain monounsaturated fatty acids so important in *T. gondii* ?

Fatty acids in general are important biomolecules in all organisms. They serve as substrates for oxidative phosphorylation in the mitochondrion. It is known that excessive long chain fatty acids can also cause uncoupling of oxidative phosphorylation in the mitochondria. This is believed to be due to the ability of these fatty acids to transport cations into the mitochondria thereby causing loss of the membrane potential (18). Therefore, it can be stipulated that at normal concentrations these long chain fatty acids could be involved in transporting and maintaining normal cationic concentrations in the mitochondria and that the loss of these fatty acids could affect the normal functioning of the mitochondria in *T. gondii*.

Long chain fatty acids are also required in phospholipid synthesis. In yeast, synthesis of certain phospholipids requires long chain fatty acids. In absence of these bases, phospholipid synthesis and cell growth is impaired (19). Even in plants, loss of fatty acid elongation affects the phospholipid pool and results in defective endomembrane trafficking (6). It is possible then that the growth defects we reported in *T. gondii* elongation mutants are due to compromised phospholipid synthesis.

Analysis of a yeast acetyl carboxylase mutant suggests a possible relation between long chain fatty acids and nuclear envelope structure (2). Additionally, long chain fatty acids are also required for targeting Pma1p (an ATPase protein) to the cell membrane surface. In absence of the C26-fatty acid, Pma1p ends up in the yeast vacuole instead of the cell membrane surface (20). Searches through the *T. gondii* genome revealed the presence of a Pma1p homolog which could be affected in the fatty acid elongation mutants. Additionally, similar to Pma1p, membrane trafficking of several other crucial proteins could be affected in the *T. gondii* mutants

leading to the observed growth defect. Our results show a clear requirement of long chain fatty acids in parasite growth. However, identifying the specific roles of these fatty acids in parasite growth requires further analysis. Characterizing their specific role will unravel important insights into the significance of fatty acid metabolism in apicomplexan parasites.

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Figures and tables

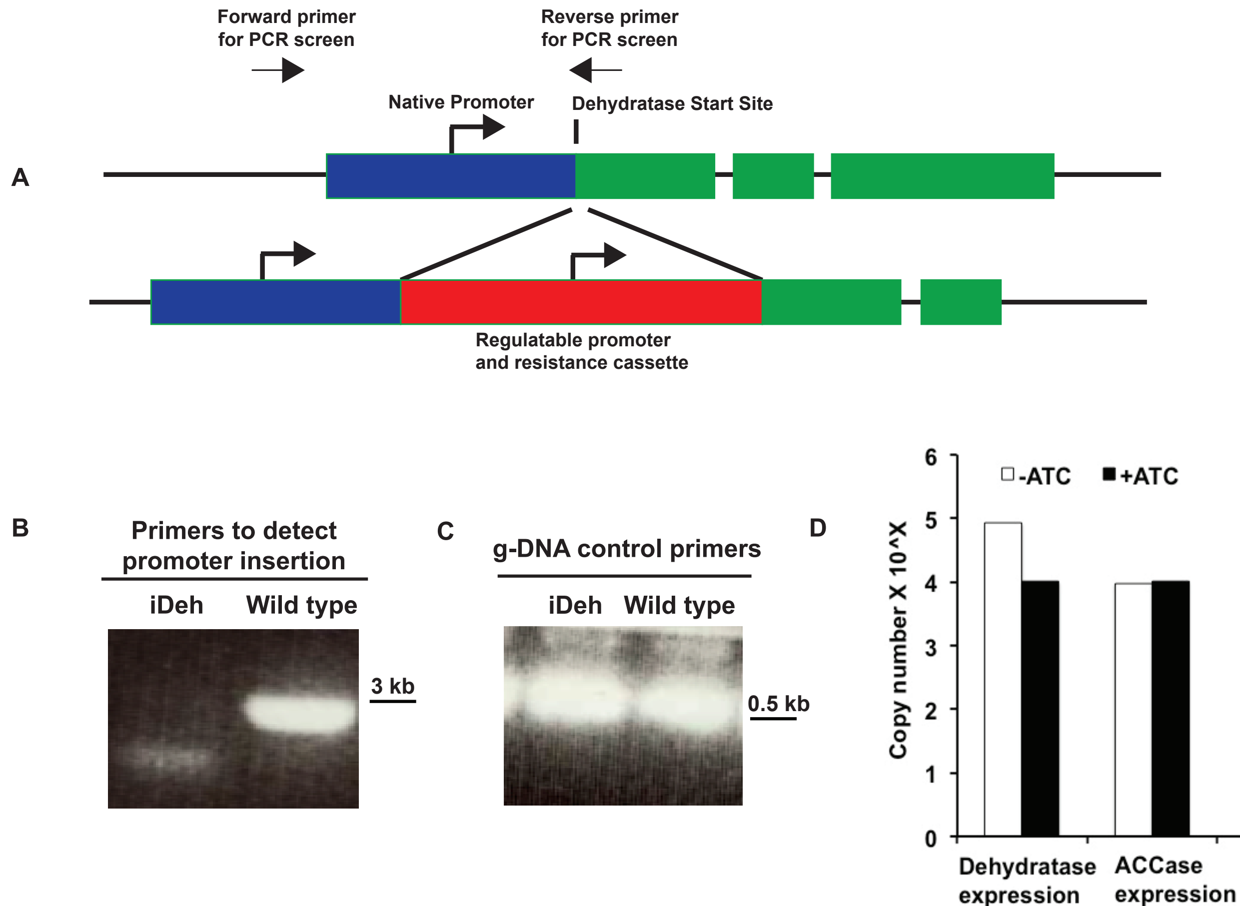


Figure 4.1: Generation of conditional mutant for dehydratase.

We generated conditional mutants for the dehydratase protein of the *T. gondii* fatty acid elongation pathway. **(A)** Depicts the promoter insertion strategy that was used to place the dehydratase gene under a regulatable promoter. **(B)** PCR analysis to identify parasite clones that carry regulatable promoter insertion. **(C)** PCR analysis showing the presence of genomic DNA in all samples. (D) RT-PCR results show that in dehydratase mutant, the expression of dehydratase gene is regulated by ATc, but the expression of ACCase gene remains unaffected.

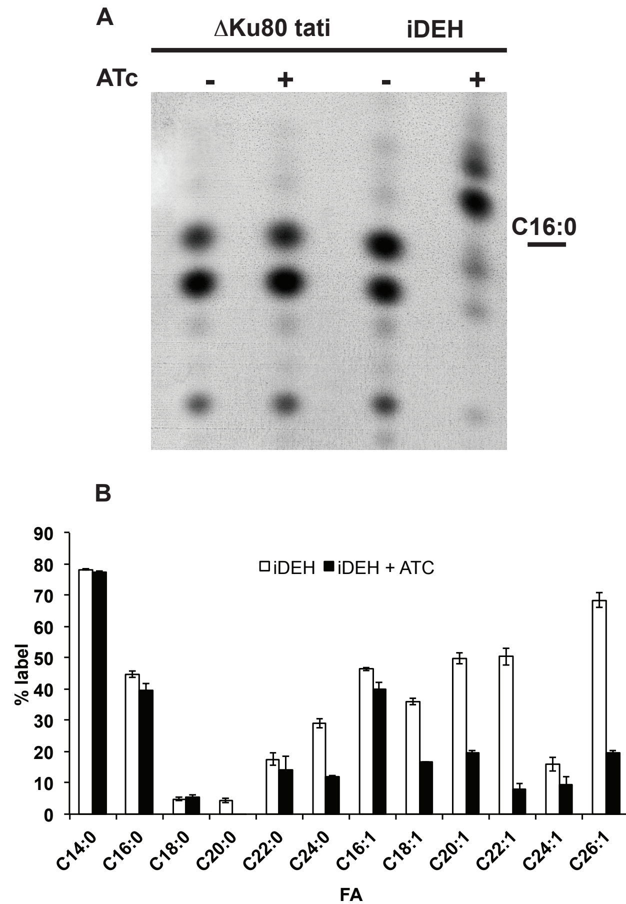
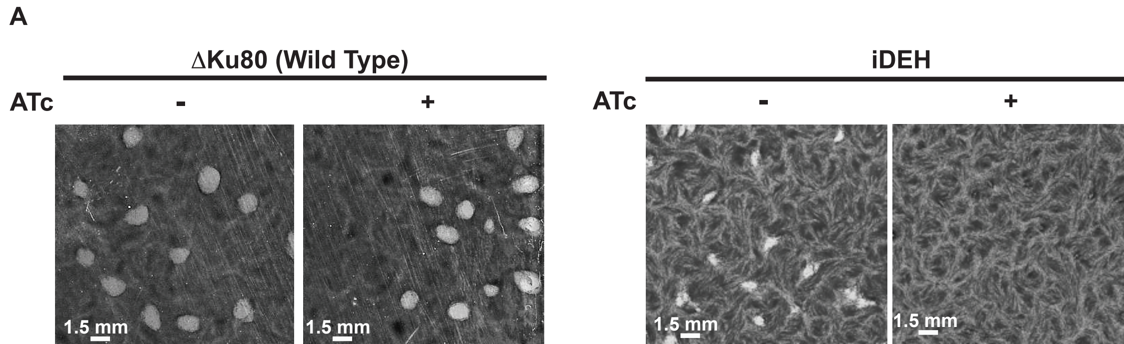


Figure 4.2: Dehydratase mutant exhibits reduced synthesis of long chain fatty acids.

(A) A *T. gondii* dehydratase mutant clone was metabolically labeled with [^{14}C]acetate; lipids were extracted, and fatty acid methyl esters were analyzed by reverse phase thin layer chromatography. The parental strain (ΔKu80 Tati) with native dehydratase promoter is shown on left and the mutant (iDEH) with the dehydratase gene under the control of a regulatable promoter is shown on right, grown in the absence (**-**) or presence (+) of ATc. **(B)** Intracellular tachyzoites of dehydratase mutant were labeled with [U-^{13}C]glucose in the presence (black) or absence (white) of ATc as detailed in (10). Level of ^{13}C incorporation into the major fatty acids (FA) of the isolated tachyzoites is shown. Error bars represent standard deviation where n = 3. Individual values for all experiments are listed in Table 4.1.

A



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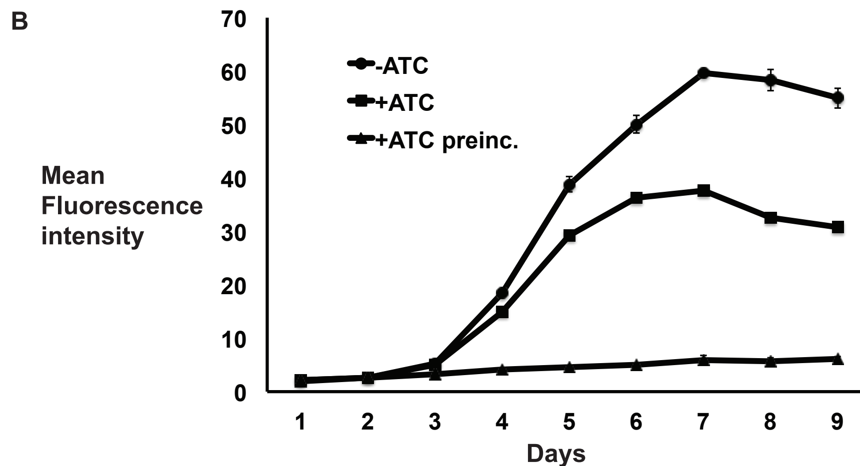

Figure 4.3: Loss of dehydratase affects parasite growth. (A) Growth of conditional dehydratase mutant was measured by plaque assay in absence or presence of ATc as indicated. **(B)** Fluorescence growth assays for the dehydratase conditional mutant growing in the presence or absence of ATc, or after preincubation with ATc (3 d +ATc) are indicated. Data points represent the mean of triplicate wells and error bars show standard deviation. Where no bar is shown the deviation was smaller than the symbol.

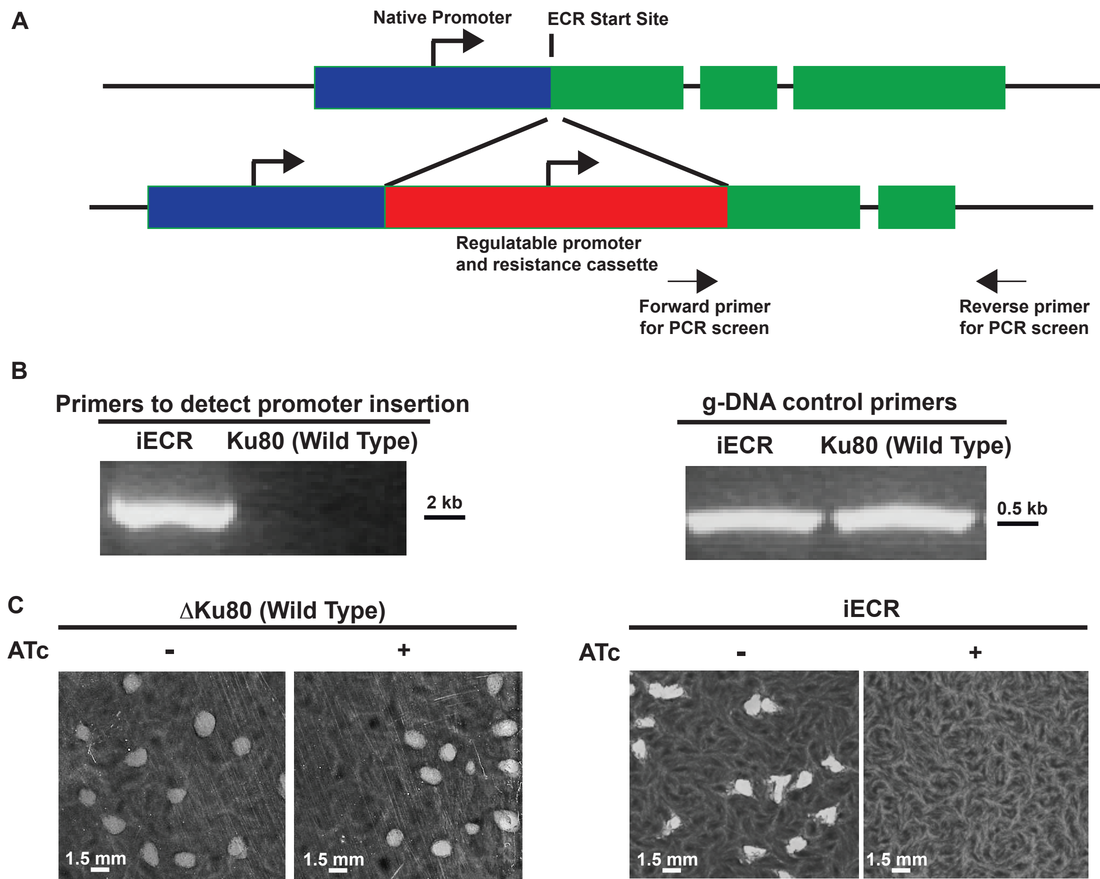

Figure 4.4: Conditional mutant for enoyl CoA reductase of the fatty acid elongation pathway affects parasite growth. We generated a conditional mutant for enoyl CoA reductase (ECR) of the fatty acid elongation pathway. **(A)** Depicts the promoter insertion strategy that was used to place the enoyl CoA reductase gene under a regulatable promoter. **(B)** The panel on left shows PCR analysis to identify parasite clones with the regulatable promoter insertion. Presence of band indicates that the regulatable promoter was inserted upstream of the ECR start codon. The panel on right displays PCR analysis showing the presence of genomic DNA in all samples. **(C)** Growth of iECR mutant was measured by plaque assay in absence or presence of ATc as indicated.

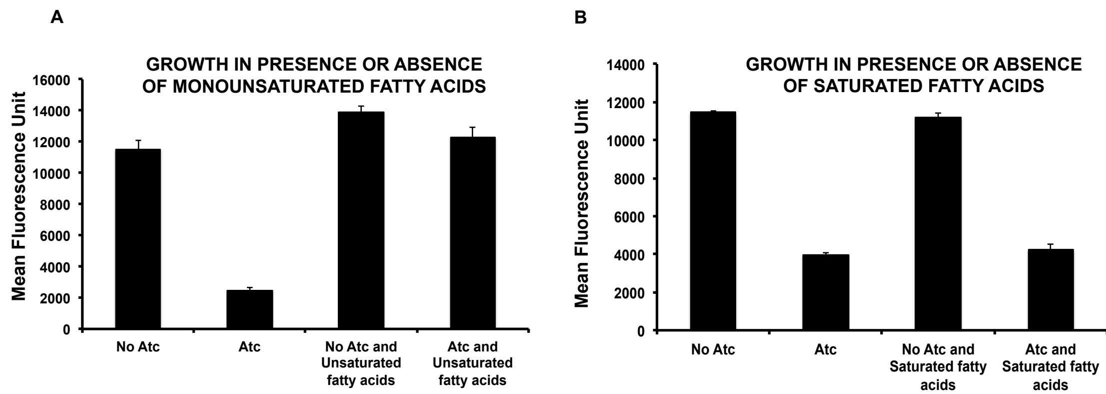

Figure 4.5: Monounsaturated but not saturated fatty acids rescue the growth defect associated with the dehydratase mutant. We attemped to restore the growth of dehydratase mutant by supplying various long chain (A) monounsaturated fatty acid mixture (C18, C20, C22, C24) or (B) saturated (C18, C20, C22, C24) fatty acid mixture at a concentration of 250μM for each fatty acid in a fluorescence growth assay experiment. For fatty acids 'Cn' where n refers to number of carbon atoms in each fatty acid used. Mean fluorescence intensity is displayed and each bar is a representative of triplicates. Error bars show standard deviation.

Table 4.1: Percent labeling of fatty acids derived from [U-^{13}C]glucose-fed tachyzoites. Dehydratase mutant parasites were used to infect HFF and labeled in situ with [^{13}C]glucose in the presence or absence of ATc. Percent labeling was determined by GC-MS analysis.

Cell type	Percent Labelling		Overall abundance (nM)	
+/- ATc	-	+	-	+
C14:0	76.28 (± 0.22)	77.48 (± 0.42)	18.69 (± 5.2)	18.02 (± 1.83)
C16:0	44.85 (± 1.09)	39.66 (± 2.04)	86.51 (± 7.78)	101.10 (± 22.14)
C18:0	4.85 (± 0.5)	39.84 (± 2.14)	65.8 (± 3.35)	76.62 (± 6.71)
C20:0	4.43 (±0.73)	nd	1.71 (± 0.12)	1.56 (± 0.12)
C22:0	17.56 (± 1.9)	14.26 (± 4.4)	0.54 (± 0.05)	0.6 (± 0.04)
C24:0	29.08 (± 1.46)	11.85 (± 0.5)	0.81 (± 0.12)	0.73 (± 0.12)
C16:1	46.44 (± 0.5)	39.66 (± 2.04)	10.19 (±1.23)	16.06 (±1.59)
C18:1	36.04 (± 1.02)	16.58 (± 0.29)	161.62 (± 20.25)	133.19 (± 18.59)
C20:1	49.93 (± 1.82)	19.62 (± 0.79)	9.7 (± 1.26)	4.46 (± 0.6)
C22:1	50.33 (± 2.62)	8.14 (± 1.65)	0.56 (± 0.07)	0.42 (± 0.03)
C24:1	15.86 (± 2.12)	9.30 (± 2.79)	0.45 (± 0.15)	0.56 (± 0.27)
C26:1	68.47 (± 2.3)	19.63 (± 2.3)	6.64 (± 1.8)	0.78 (± 0.04)

Supplementary table ST4.1: Primers used for construction and identification of mutants.

Name	Primers used for cloning with Restriction sites
Primers used to recombineer gene targeting cosmids (50 bp recombination sequences are shown in upper case)	
iDEH	For5'- GCCTTCTTCCTCTTCTTCCGCGTCTTCGACTTTGCTTCAGTCTCCTCACCGAATGGTAACCGACAAACGCGTTC Rev5'- CCAGGAACCTTTCCAGCGAGACTCGCTCGCGCAGGCGCCGCTTCTGCCATAGATCTGGTTGAAGACAGACGAAAGC
iDEH PCR screen primers	For5'- TCTTCTTCCGCGTCTTCGAC Rev5'- CGAGACAGGCGCAACTCCTT
Primers used to generate gene targeting plasmids	
iECR	Upstream for5' - GTACCATATGGTGTTGTCAGAATCTTTTCTGG Upstream rev5' - GTACGTATACGGTGACCTCGTGACAAGGACG Downstream for3' - GTACGGATCCATGAGGATTTCTCTGAAGAAGC Downstream rev3' - GTACGGATCCATGTCTAGACACTGCAGACACGC
iECR PCR screen primers	For 5'-CGCACGGCAGTCAGATAACAGGTGTA Rev 3'-GCATCAAGCCTGAAATCG

CHAPTER 5

FATTY ACID SYNTHASE TYPE I IS NOT REQUIRED FOR THE SYNTHESIS OF FATTY ACIDS IN *T. GONDII* TACHYZOITES.[1]

[1] Ramakrishnan, S., Docampo, M.D., MacRae, J.I., McConville, M.J. and Striepen, B. To be submitted to PLOS Pathogens.

5.1 ABSTRACT

Fatty acid synthesis type I (FASI) pathway is typically used by eukaryotes and has been extensively studied in mammals and yeast. The FASI megaenzyme in these organisms acts as a *de novo* synthase. Interestingly, in apicomplexan parasites, only a subgroup of parasites carries this gene. For *C. parvum* FASI, evidence points towards a role in fatty acid elongation rather than *de novo* synthesis. *T. gondii* seems to contain three systems FASI, FASII and FAE for fatty acid synthesis and elongation. Under this circumstance, it is unknown if FASI functions as a de novo synthase or a fatty acid elongase. In past few years, molecular analysis of apicomplexan FASI has been challenging due the novel architecture of the gene.

In this chapter, using modern techniques of genetic manipulation and biochemical characterization, we attempt to unravel the role of the FASI pathway. Our analysis shows that the pathway is not essential for growth of tachyzoite parasites in vitro. Moreover, loss of the FASI pathway does not affect lipid synthesis indicating that this mechanism of fatty acid synthesis is probably active in a different life cycle stage.

5.2 INTRODUCTION

The fatty acid synthase type I (FASI) pathway is comprised of a single polypeptide with multiple catalytic domains. This pathway is the major source of fatty acids in many animals and has been well characterized in yeast and mammals. In both systems, this enzyme acts as a *de novo* synthase with acetyl–CoA and malonyl Co-A as the starter substrates (1-3). An acyl transferase/malonyl-acyl tranferase domain is loads both substrates onto the ACP domain. The substrates are then condensed, reduced, dehydrated and further reduced to produce an acyl molecule elongated by two carbon units. This process is repeated several times to produce palmitic acid, the final product which is eventually cleaved off by the activity of a thioesterase (TE) domain of FASI (4). In contrast, in yeast, the final fatty acid is not cleaved but transferred onto Co-A with the help of a transferase domain (5).

The apicomplexan FASI seems both structurally and functionally distinct from other eukaryotic type I synthases studied so far. The enzyme is best characterized in the apicomplexan parasite *C. parvum*. Unlike the mammalian or fungal FASI, the CpFASI contains three enzymatic modules. Each module contains all the enzymatic domains sufficient for a complete round of elongation (6). Biochemical analysis of heterologously expressed modules of CpFASI enzymatic domains in *E. coli* suggests that this protein may act as a *de novo* synthase but rather a fatty acid elongase (7). Moreover, the final domain of CpFASI is neither a thioesterase nor a transferase, but a reductase domain (8). This suggests that apicomplexan FASI may function differently from other known FASI. While some insights were gained by studying the *C. parvum* enzyme, the biological role of FASI in apicomplexans has remained unknown. This is mainly because lack of genetic tools in *C. parvum* make it difficult to address this question. Here we study the role of

FASI using *T. gondii* as a model system. Analysis of *T. gondii* genome revealed the presence of a FASI gene candidate, whose architecture is similar to that of *C. parvum*. The availability of advanced molecular tools in *T. gondii* makes it an excellent system to study this pathway (9).

Another interesting aspect of the FASI pathway is its activation by the transfer of a phosphopantetheinyl group from CoA to the ACP domains on the enzymes. Yeast FASI self-activates itself with the help of a 4'-phosphopantetheinyl transferase (PPTase) domain present within the enzyme (10). In mammals, the PPTase enzyme that activates the ACP domains is encoded independent of the FASI enzyme. The activation of FASI in apicomplexans could resemble the mammalian system. A single PPTase enzyme has been identified and characterized in *C. pavum*. *T. gondii* contains two PPTases, presumably one for the activation of the FASII pathway in the apicoplast and the other for the activation of the FASI pathway. (11, 12).

Here, we evaluate the essentiality and role of the FASI pathway in *T. gondii*, by generating conditional mutants for both FASI and PPTase enzymes. By conducting lipid analyses of these mutants, we seek to shed light onto the role of this pathway in apicomplexan parasites.

5.3 EXPERIMENTAL PROCEDURES

5.3.1 Parasite culture and construction of mutants.

T. gondii tachyzoites strain RH were cultured and genetically manipulated as described previously (13). Conditional mutants for fatty acid synthase type I (FASI) and phosphopantetheinyl transferase (PPTase) were generated using a previously described promoter insertion strategy in Ku80 Tati parasite line (14). For FASI, a targeting plasmid was constructed. In this plasmid, a tetracycline-regulated promoter was flanked by upstream and downstream

sequences of FASI gene. The plasmid was then transfected in Ku80 mutant parasite line and transgenic parasites were selected in presence of pyrimethamine. Mutants were identified with the help of a PCR screen (described in Fig. 5.1D; See table ST5.1 for primer details). For phosphopantetheinyl transferase, a targeting plasmid contained the tetracycline promoter and pyrimethamine resistance cassette flanked by the PPTase gene sequence (See table ST5.1 for primer details). Following transfection of Ku80 tati parasite line with this plasmid, transgenic parasites were selected in presence of pyrimethamine and mutants were identified via a PCR screen (Fig 5.1F). The growth of these mutants were evaluated by conducting plaque assays in absence or presence of 0.5μM ATc (Fig 5.2B-C) (13).

5.3.3 Stable Isotope Labeling and Metabolomic Analyses

T. gondii infected fibroblasts were grown in DMEM in a T175 flask in the absence or presence of ATc for 48 h. The medium was supplemented with 8 mM [U-^{13}C] glucose (final concentration of [^{13}C/^{12}C] glucose was 16 mM) 24 h prior to egress. Free parasites were separated from host cells by filtration through a membrane with a 3-μm pore size. Parasites were quenched by rapid chilling of the cell suspension in a dry ice/ethanol bath and parasites were recovered by centrifugation (4000 X g, 25 min, 0 °C). Cell pellets were washed three times with ice-cold PBS, and cell aliquots (2 X 10^8 cells) were transferred to microcentrifuge tubes and centrifuged (10,000 X g, 30 s, 0 °C). Pellets were suspended in 300μl chloroform/methanol (2:1) and vortex-mixed thoroughly. Samples were sonicated for 5 mins at RT and then incubated at 60 °C for 20 min. Extracts were centrifuged at 10,000 X g for 5 min, and the supernatant was dried and subsequently washed twice with methanol. Dried residues were dissolved in 25μL of

methprep II (alltech). Samples were analyzed on an Agilent 7890A-5975C GC-MS system. Split (split ratio 10:1) injection (injection temperature 250 °C) onto a 30m + 10m X 0.25mm DB-5MS + DG column (J&W, Agilent Technologies) was used, using helium as the carrier gas. The initial oven temperature was 70 °C (1 min), followed by temperature gradients to 230 °C at 17 °C/2mins, from 230 to 325 °C at 25 °C/min. The final temperature was held for 10 min. Data analysis was performed using Chemstation software (MSD Chemstation D.01.02.16, Agilent Technologies). Abundance and label incorporation was calculated as described previously (15). Data is shown are the average of three technical replicates and their standard deviation.

5.3.4 Quantitative PCR

RNA was purified and c-DNA was synthesized from 2 X 10^8 tachyzoites using the Superscript III first strand synthesis kit from Invitrogen. Primers for qPCR were chosen so that the resulting amplicon is 300-350 bps in length. The amplicons were cloned into PCR2.1 using TOPO TA cloning kit from Invitrogen. The copy number of amplified taranscript was calculated using the insert size (300 bps) and vector size (3.9kb). A stock solution of 10^8 copies was used to prepare six dilutions ranging from 10^2 to 10^7. Final samples are prepared with 300ng of c-DNA extracted from mutant parasites. Running samples were prepared using the Bio-Rad SYBR green mix kit and PCR was carried out in Bio-Rad iQ5 multicolor PCR system. Data from the qPCR machine BIO-RAD iQ5 was exported to excel sheet and plotted to generate standard curve. The readings from unknown samples were extrapolated on the standard curve and the copy number in these samples was determined.

5.4 RESULTS

5.4.1 Generating conditional mutants for fatty acid synthase type I and 4'-phosphopantetheinyl transferase in *T. gondii* tachyzoites.

Fatty acid synthesis is an essential part of *T. gondii* metabolism. Bioinformatic analyses suggest the presence of a fatty acid synthase type I pathway in *T. gondii*. Three enzymes constitute the fatty acid synthase type I pathway; a fatty acid synthase type I (FASI) multienzyme, a 4'-phosphopantetheinyl transferase (PPTase) which activates the FASI enzyme and an acetyl CoA carboxylase which provides the substrate for the pathway. To determine the role of FASI pathway in parasite metabolism we decided to test the expression of these genes in parasite tachyzoites. m-RNA from freshly lysed tachzoites was extracted and reverse transcribed to generate a c-DNA pool. To detect FASI, PPTase and ACCase expression, primers binding the exons of these genes were engineered (See table ST5.1 for primer information). Using these primers, PCR reactions were conducted with either tachyzoite g-DNA or tachyzoite c-DNA as templates. Fig 5.1 shows that m-RNA for all enzymes of the FASI pathway was present in *T. gondii* tachyzoites. When g-DNA was used as template, FASI primers produced a 1.5 kb band whereas when c-DNA was used as template, the same FASI primers produced a 1 kb band (due to the splicing of a 500kb intron) (Fig. 5.1A). This suggested that FASI enzyme is expressed in parasite tachyzoites. Similarly expression of ACCase can also be detected (Fig. 5.1C). However, the low intensity of the PCR band from c-DNA template indicates that these enzymes are probably expressed at low levels. In contrast to FASI, PPtase seems to be expressed at high levels. With g-DNA as template, PPTase primers produce a band of 5 kb but with c-DNA as

template, a 3kb band can be observed due to splicing (Fig. 5.1B). Together these results suggest that the FASI enzyme transcript is expressed. The expression of PPTase suggests that the FASI could be activated and might be contributing towards fatty acid metabolism in *T. gondii* tachyzoites.

We next wanted to investigate function and importance of this pathway in *T. gondii* tachyzoites. We have previously characterized fatty acid synthesis from FASII and FAE in *T. gondii* using a conditional mutation strategy (16). Hence, we attempted to generate a conditional mutant for the FASI pathway. Owing to the long length of the FASI gene, we decided to replace the FASI promoter with a regulatable promoter. We engineered a plasmid in which the tetracycline-regulated promoter was flanked by FASI sequences (See table ST5.1 for primer details). Similarly a plasmid to replace the PPTase promoter with a tetracycline regulatable promoter was engineered (See table ST5.1 for primer details). Since PPTase is required for the activation of the FASI enzyme, regulation of PPTase expression would be an alternative way to regulate the FASI pathway. Next, we transfected the ku80 tati parasite line with either the FASI plasmid or PPTase plasmid and selected for pyrimethamine resistant parasites. Following selection, parasite clones were screened using FASI specific primers or PPTase specific primers (See table ST5.1 for primer details). The result of the screen is shown in figure 5.1D-5.1G. Parasite clones in which the FASI (Fig. 5.1D) or PPTase (Fig. 5.1F) promoter was not replaced produced a 3 kb band. But, parasite clones with promoters replaced lacked this 3kb band. These clones were identified as FASI (Fig 5.1D) and PPTase (Fig 5.1) promoter replaced clones and called iFASI and iPPTase respectively. The samples were further tested for presence of g-DNA using control primers (See table ST5.1 for primer details). A 0.5kb band showed that g-DNA was present in all samples (Fig. 5.1E and Fig 5.1G). Hence, we successfully replaced the gene

promoters with a regulated promoter. The placement of this promoter before the start codon of the FASI and PPTase gene would allow us to regulate the expression of these genes by addition or removal of tetracycline to the parasite growth medium.

5.4.2 Fatty acid synthase type I pathway is not required for growth of parasite tachyzoites in tissue culture.

In order to confirm that the expression of FASI and PPTase genes could be regulated in the promoter replaced parasite lines, we conducted quantitative RT-PCRs (qRT-PCR). iFASI and iPPTase parasite lines were grown either in absence or presence of anhydrous tetracycline (ATc) and subjected to m-RNA extraction. The extracted RNA was reverse transcribed to c-DNA and used as template for qPCR reactions. Our expectation was that in presence of ATc the expression of both FASI and PPTase should be lower than in absence of ATc. As expected the number of gene transcripts was at least 10-fold lower in presence of ATc (Fig. 5.2A), indicating that addition of ATc will allow us to reduce the transcription of FASI and PPTase genes in these parasite lines. The dehydratase mutant, which shows a growth defect in response to ATc was used as a positive control to demonstrate the change in numbers of the transcripts.

Next we monitored for changes in growth that may be associated with the loss of the FASI pathway. For this, we inoculated a confluent HFF monolayer with 500 parasites. These were grown for a period of 10 days in either absence or presence of ATc. During this period, the growth of parasites is indicated by the production of plaques in the host monolayer. The results of this experiment are shown in Fig 5.2B-5.2C. Both iFASI and iPPTase grew normally in absence or presence of ATc. Plaque size was indistinguishable between these samples. Plaque

size is a direct indicator of parasite growth. It can therefore be suggested that loss of the FASI pathway does not affect the growth of *T. gondii* tachyzoites under *in vitro* conditions.

5.4.3 Loss of FASI pathway does not affect the synthesis of fatty acids in *T. gondii* tachyzoites.

While the FASI pathway may not be required for parasite growth, it could still act in the synthesis of certain fatty acids in the parasite. We have previously used a ^{13}C labeling strategy to identify the precise roles the FASII and FAE pathways in *T. gondii* (16). In this strategy, we utilized the parasite's ability to incorporate ^{13}C into newly synthesized fatty acids, when ^{13}C-glucose was provided as a substrate in the growth medium. However, to determine the role of the FASI pathway, we decided to use ^{13}C-acetate as a labeling substrate. Acetate provided in the media is not imported into the parasite apicoplast and cannot be used by the FASII pathway (16). Hence, using ^{13}C-acetate we can specifically label the products of the FASI and FAE pathways. Additionally, using ATc on either the FASI or PPTase mutant, we can specifically ablate the FASI pathway. The extent of incorporation of ^{13}C into a fatty acid is a direct measure of the parasite's ability to synthesize that fatty acid. Therefore, using this strategy on iFASI and iPPTase, we can detect any fatty acids that are synthesized solely by the FASI pathway.

We grew both iFASI and iPPTase parasites in absence or presence of ATc for 48 hours to turn off the expression of the respective genes. After 48 hours, we added ^{13}C-acetate to the growth medium. Once the parasites had completely lysed out the host cells, they were filtered to remove host cell debris, subjected to fatty acid extraction and analysed using gas chromatography and mass spectrometry system (See section 5.3.3). Fatty acids in both iFASI and iPPTase parasite

lines grown in absence of ATc were labeled with ^{13}Carbon indicative of active fatty acid synthesis. However, for iFASI and iPPTase parasites grown in presence of ATc, the fatty acid labeling was indistinguishable from those grown in absence of ATc. The results were statistically analysed and are shown as percentage labeling in Fig 5.3. The percentage labeling of both saturated and unsaturated fatty acids in iFASI parasites remained unchanged in absence or presence of ATc (Fig 5.3A). Consistent with this result, iPPTase parasites, whether grown in the absence or presence of ATc, did not affect the labeling of any fatty acids (Fig 5.3B). This suggests that the FASI pathway is not a source of fatty acids in *T. gondii* tachyzoites.

5.5 DISCUSSION

Fatty acid synthase type I pathway (FASI) is present in many eukaryotes and also in certain prokaryotes (17). In all these organisms the FASI protein is synthesized as an inactive apoenzyme. The apo form is then activated by conversion to holoenzyme using either an internal or independent 4'-phosphopantetheinyl transferase (PPTase). Among apicomplexan parasites, the FASI enzyme and the FASI-activating PPTase enzyme has only been studied in *C. parvum*. These studies indicate that the pathway serves as a fatty acid elongase rather than a de novo synthase. We wanted to test whether the FASI pathway had a similar role in *T. gondii*. Our initial analysis of gene expression suggested that the FASI pathway is active in *T. gondii* tachyzoites (Fig. 5.1A), so we generated conditional mutants for the FASI pathway. We did this by placing the genomic copy of the FASI enzyme or PPTase enzyme under the control of a tetracycline regulated promoter. Surprisingly, loss of the pathway did not effect the parasite's growth. Furthermore, loss of the FASI pathway also did not affect lipid synthesis. Both medium and long chain fatty acids were being synthesized at the same levels in presence or absence of the FASI

pathway. This indicates that FASI pathway does not function as a de novo synthase or as a fatty acid elongase in *T. gondii* tachyzoites.

Interestingly, among apicomplexan parasites, the FASI pathway is only conserved in parasite species that can form cysts. These cysts are shed into the environment and have an extremely resistant cyst wall. It is possible that the products of the FASI pathway could be playing a role in cyst formation. Consistent with this hypothesis, apicomplexan oocysts are known to be acid fast positive, indicating that oocysts could be surrounded by a protective lipid layer {Bronsdon, 1984 #642}. However, further analysis of these gene candidates is necessary to confirm such a role.

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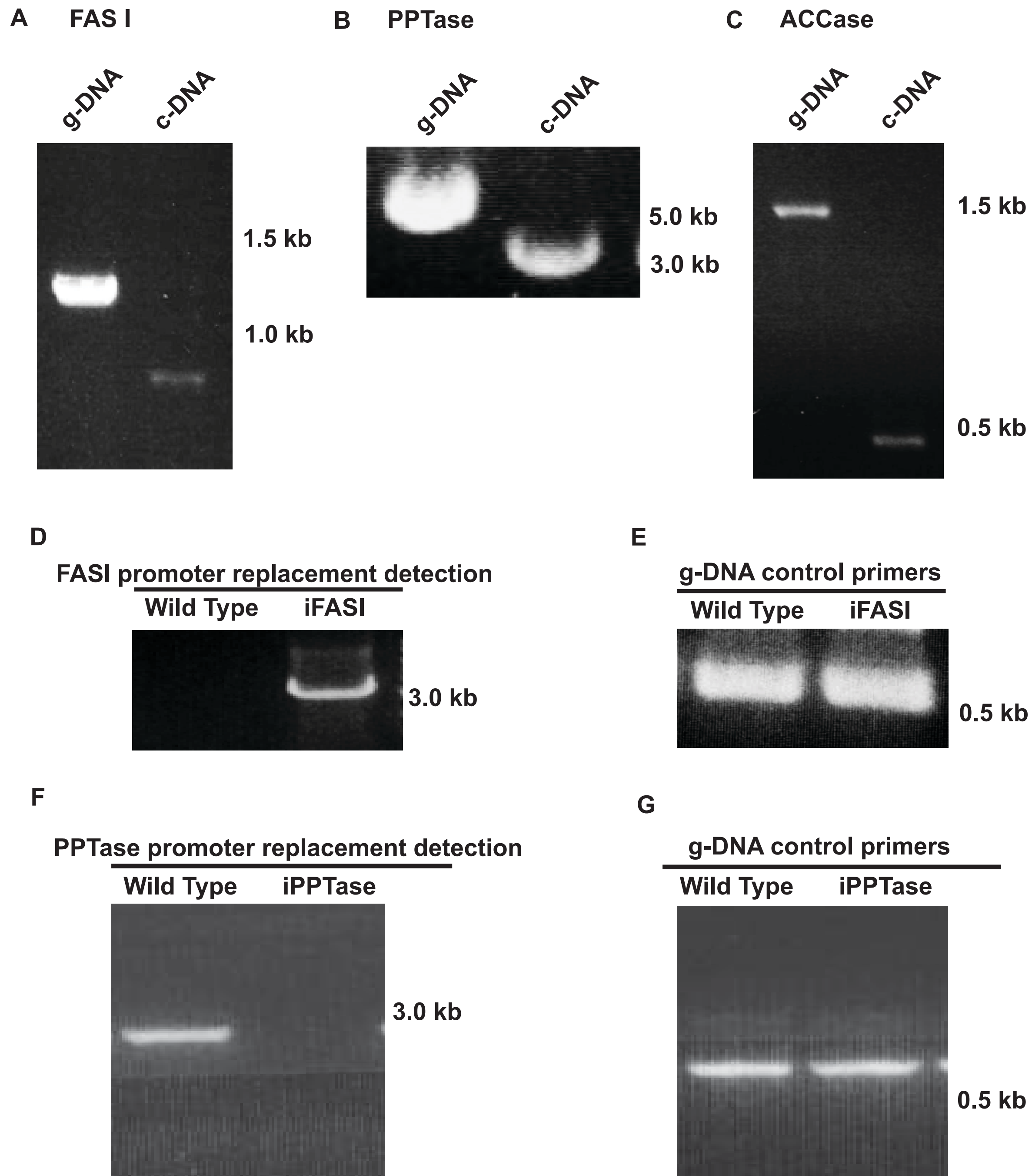
Figures and tables

Figure 5.1: Generation of conditional mutants for FASI pathway.

All components of Fatty acid synthase type I pathway are expressed in *T. gondii* tachyzoites. Primers that bind two different exons were engineered for fatty acid synthase type I (FASI), 4'-Phosphopantetheinyl transferase (PPTase), and acetyl CoA carboxylase (ACCase) genes. The two exons are separated by an intron and produce a shorter band when amplified from c-DNA. Such size difference between g-DNA and c-DNA amplicons can be detected for **(A)** FASI **(B)** PPTase and **(C)** ACCase indicating that these enzymes are expressed in parasite tachyzoites. To generate mutants for FASI pathway, native promoters for FASI and PPtase genes were replaced by tetracycline regulated promoters. Promoter replacement was detected by PCR analysis. (C) DNA from a parasite clone that lacks a 3.0 kb band indicates the replacement of the native promoter. Primer binding sites are detailed in Fig ST5.1. DNA from a wild type produces a 3.0 kb band indicating that the native promoter is intact. Both samples contain DNA indicated by the control bands in (D). (E) DNA from parasite clone produces a 3.0 kb band indicating the replacement of the native promoter. Primer binding sites are detailed in Fig ST5.1. But, DNA from wild type parasites lacks this 3.0 kb band indicating that the native promoter is intact. Both samples contain DNA which is indicated by the control bands in (F).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c-DNA
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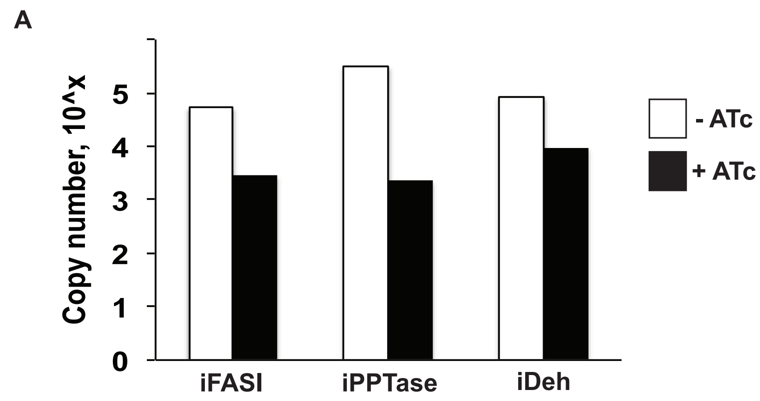
1.0 kb
B PPTase
g-DNA
c-DNA
5.0 kb
3.0 kb
C ACCase
g-DNA
c-DNA
1.5 kb
0.5 kb
D
FASI promoter replacement detection
Wild Type
iFASI
3.0 kb
E
g-DNA control primers
Wild Type
iFASI
0.5 kb
F
PPTase promoter replacement detection
Wild Type
iPPTase
3.0 kb
G
g-DNA control primers
Wild Type
iPPTase
0.5 kb

Figure 5.2: FASI pathway is not required for normal parasite growth under *in vitro* conditions. (A) To confirm that we could regulate the expression FASI and PPTase with tetracycline we performed qPCR. Addition of tetracycline reduces the copy number of FASI by 10 fold in FASI mutant (iFASI) and PPTase by 100 fold in PPTase mutant (iPPTase). Dehydratase regulation is shown as a control. Dehydratase mutant (iDeh) has a growth and biochemical phenotype indicating that a 10-fold reduction in copy number might be sufficient to detect an associated defect. The growth of these mutants was tested by their ability to form plaques on a confluent HFF layer. iFASI parasites(B) form plaques of identical size in absence or presence of ATc. Similarly size of plaques formed by iPPTase parasites (C) is unaffected by the addition of ATc indicating that FASI pathway is not required for the growth of *T. gondii* tachyzoites.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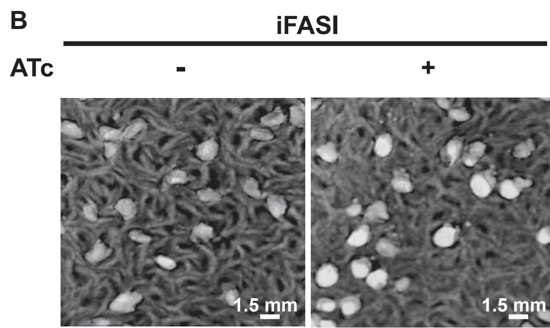
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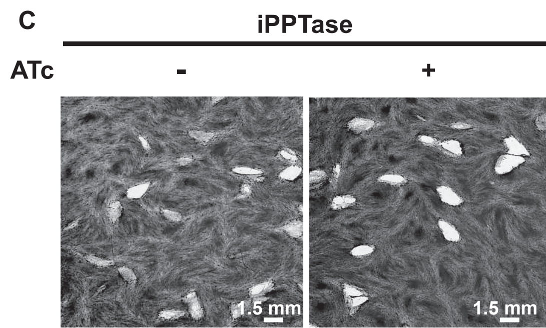
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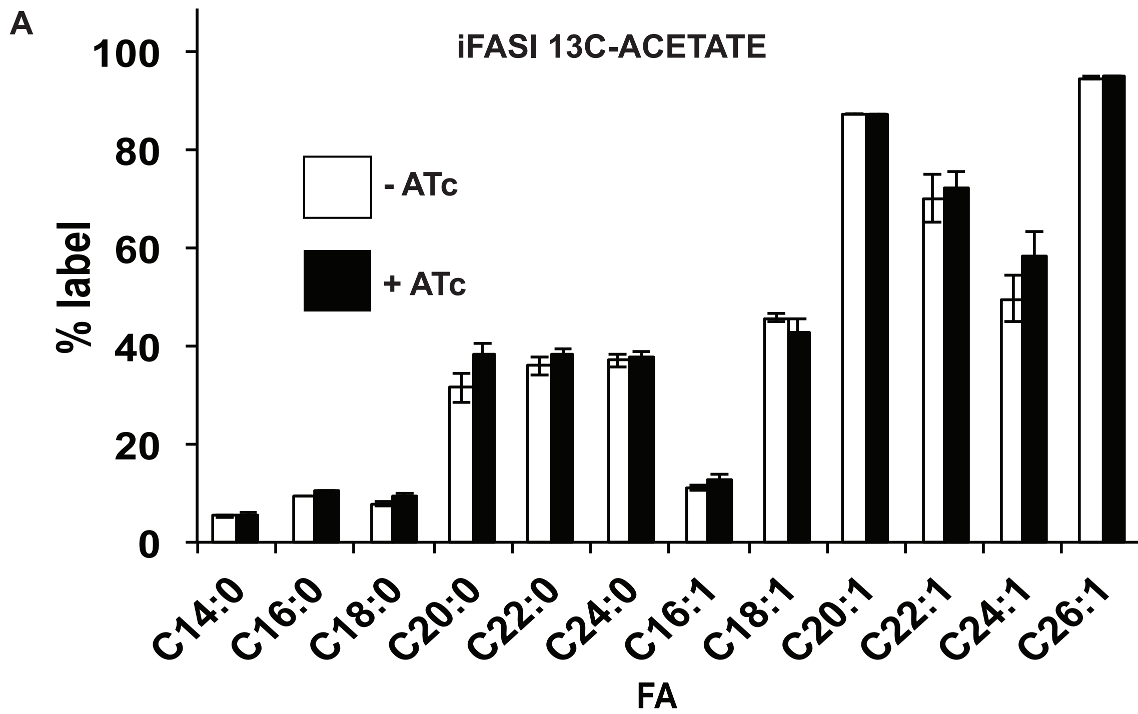
Figure 5.3: Synthesis of fatty acids remains unaffected upon the loss of FASI pathway.

(A) Intracellular tachyzoites of iFASI were labeled with [U-^{13}C]-acetate in the presence (black) or absence (white) of ATc as detailed in (16). Level of ^{13}C incorporation into the major fatty acids (FA) of the isolated tachyzoites is shown. Error bars represent standard deviation where n = 3. Individual values for all experiments are listed in table 5.1. **(B)** Intracellular tachyzoites of iPPTase were labeled with [U-^{13}C]-acetate in the presence (black) or absence (white) of ATc as detailed in (16). Level of ^{13}C incorporation into the major fatty acids (FA) of the isolated tachyzoites is shown. Error bars represent standard deviation where n = 3. Individual values for all experiments are listed in table 5.2.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C16:0
C18:0
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C24:0
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C20:1
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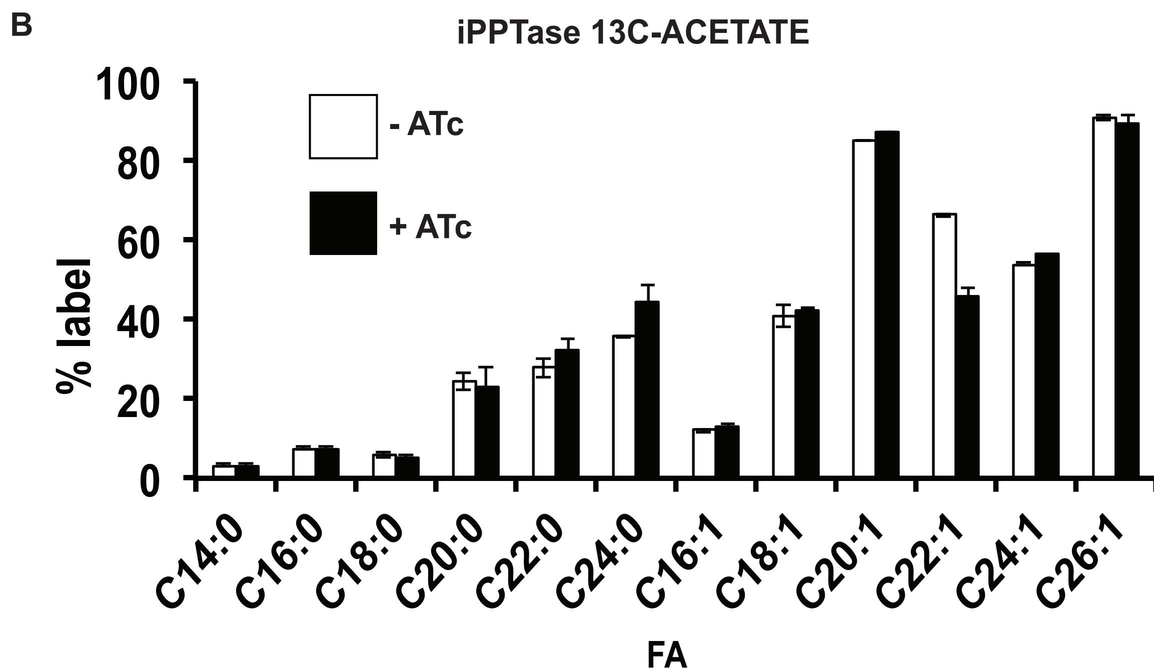
C24:1
C26:1
FA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C20:0
C22:0
C24:0
C16:1
C18:1
C20:1
C22:1
C24:1
C26:1
FA

Table 5.1: Percent labeling of fatty acids derived from [U-^{13}C] acetate fed FASI mutant (iFASI) tachyzoites.

iFASI parasites were used to infect HFF and labeled in situ with [U-^{13}C] acetate in the presence or absence of ATc. Percent labeling was determined by GC-MS analysis.

Fatty acid type	Percent Labelling		Overall abundance (nM)	
+/- ATc	-	+	-	+
C14:0	5.30 (± 0.23)	5.50 (± 0.49)	47.64 (± 6.0)	35.52 (± 0.62)
C16:0	9.26 (± 0.12)	10.44 (± 0.31)	226.76 (± 32.73)	157.41 (± 7.59)
C18:0	7.75 (± 0.32)	9.41 (± 0.65)	161.62 (± 28.58)	95.73 (± 11.08)
C20:0	31.59 (±3.08)	38.5 (±2.2)	4.36 (± 0.64)	2.61 (± 0.34)
C22:0	35.99 (± 1.9)	38.27 (± 1.2)	2.95 (± 0.51)	1.94 (± 0.22)
C24:0	37.02 (± 1.29)	37.63 (± 0.94)	5.36 (± 0.69)	3.65 (± 0.12)
C16:1	11.25 (± 0.65)	12.56 (± 1.19)	48.88 (± 7.61)	33.27 (± 1.25)
C18:1	45.71 (± 0.9)	42.70 (± 2.79)	518.99 (± 66.75)	376.52 (± 13.08)
C20:1	87.33 (± 0.03)	87.2 (± 0.1)	42.76 (± 6.4)	30.75 (± 0.52)
C22:1	70.15 (± 4.92)	71.96 (± 3.84)	4.62 (± 0.63)	3.43 (± 0.24)
C24:1	49.61 (± 4.63)	58.52 (± 1.84)	3.37 (± 0.65)	2.76 (± 0.16)
C26:1	94.63 (± 0.37)	94.97 (± 0.15)	37.38 (± 2.64)	24.39 (± 2.57)

Table 5.2: Percent labeling of fatty acids derived from [U-^{13}C] acetate fed PPTase mutant (iPPTase) tachyzoites. iPPTase parasites were used to infect HFF and labeled in situ with [U-^{13}C] acetate in the presence or absence of ATc. Percent labeling was determined by GC-MS analysis.

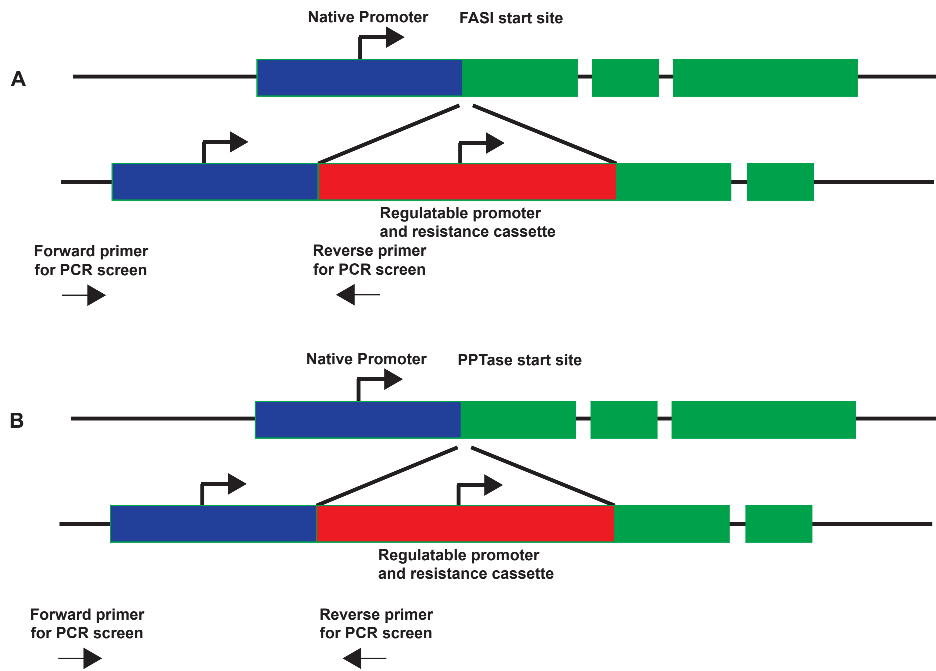
Fatty acid type	Percent Labelling		Overall abundance (nM)	
+/- ATc	-	+	-	+
C14:0	3.11 (± 0.39)	2.80 (± 0.99)	292.80 (± 37.2)	201.41 (± 69.77)
C16:0	7.64 (± 0.39)	7.28 (± 0.4)	1172 (± 208.37)	257.95 (± 257.95)
C18:0	5.94 (± 0.67)	5.10 (± 1.12)	740.48 (± 121.85)	485.05 (± 74.57)
C20:0	24.30 (±2.19)	23.02 (±4.86)	16.78 (± 2.03)	12.66 (± 2.13)
C22:0	27.69 (± 2.22)	32.20 (± 2.58)	7.62 (± 0.74)	4.88 (± 1.38)
C24:0	35.75 (± 0.26)	44.32 (± 4.02)	14.49 (± 2.01)	7.64 (± 2.08)
C16:1	12.00 (± 0.58)	12.87 (± 0.53)	201.18 (± 36.26)	129.68 (± 47.38)
C18:1	41.08 (± 2.92)	42.59 (± 0.21)	2332.03 (± 325.97)	1653.91 (± 553.31)
C20:1	85.16 (± 0.20)	86.86 (± 0.38)	188.02 (± 35.47)	124.58 (± 44.48)
C22:1	66.36 (± 0.45)	46.00 (± 2.19)	11.83 (± 1.36)	7.50 (± 2.49)
C24:1	53.93 (± 0.34)	56.24 (± 1.64)	7.95 (± 0.91)	5.91 (± 1.48)
C26:1	90.93 (± 0.71)	89.60 (± 2.00)	125.52 (± 24.53)	72.93 (± 11.75)

Supplementary table ST5.1: Primers used for construction and identification of mutants.

Name	Primers used for cloning with Restriction sites
Primers used to generate gene targeting plasmids	
iFASI	Upstream for5' - CCTTCTTCGGGTACCCTTTTTCGCTA Upstream rev5' - GTACAAGCTTTTGCAAAAATGCCCTCTC Downstream for3' - GCACGGGGACGTTTTGGGAGATCC Downstream rev3' - GTACACTAGTCGGCGTGCTGGGCGGCGC
iFASI PCR screen primers	For5'- CGACCGGCAGCTGTTAG Rev5'- ACCAGTAACTGTTCTGACTTCTTG
FASI qPCR primers	For5'- CAGAGGAGTGGATGGCAATTC Rev5'- GGCTTGCCCAACTTCCCA
iPPTase	Upstream for5' - GTACAGATCTATGAGGATTTCTCTGAAGAAGC Upstream rev5' - GTACCCCGTTATGTCTAGACACTGCAGACACGC Downstream for3' - GTACCATATGGTGTTGTCAGAATCTTTTCTGG Downstream rev3' - GTACGTATACGGTGACCTCGTGACAAGGACG
iPPTase PCR screen primers	For 5'- CGCCTTGGCGAATGTTCATGAC Rev 3'- CGACGTACACGCCTAGAACAA
PPTase qPCR primers	For5'- GAGAGCCGCGCACGC Rev5'- AGGCTTTCACGAACGCCTC
Primers to detect gene expression	
FASI	Forward (1048)- ATCCATCATTCTCGACCCCC Reverse (1049)- ATCCATCATTCTCGACCCCC
PPTase	Forward 1 (3142)- GAGAGCCGCGCACGC Reverse 1 (3143)- AGGCTTTCACGAACGCCTC

Figure ST 5.1: Modified promoter screening strategy.

PCR screen was used to detect successful promoter modification for FASI and PPTase genes. Binding site for (A) FASI screening primers and (B) PPTase screening primers are shown.



CHAPTER 6

CONCLUSION

Toxoplasmosis is a minor concern for individuals with a healthy immune system. However, the disease poses a significant health risk in organ transplant patients and in others with a weakened immune system. The pathology associated with the disease is due to activity of *T. gondii* tachyzoites. These tachyzoites destroy the cells and tissues at the site of their multiplication and subsequent lysis. The resulting damage incapacitates the infected individual and is lethal if untreated.

Obtaining essential nutrients is the key to a successful infection by any pathogen. Acquiring these nutrients is especially challenging for intracellular pathogens like *T. gondii*, because they have to rely heavily on their own synthetic machinery. As a result, these intracellular pathogens often evolve multiple routes to obtain their necessary nutrients. One such example is the necessity for fatty acids, and *T. gondii* harbors three mechanisms to generate these metabolites. A type II fatty acid synthesis mechanism (FASII) is localized to the apicoplast, a fatty acid elongation pathway localizes to the endoplasmic reticulum and a type I fatty acid synthase whose localization is still unknown. Using a genetic and metabolomics approach we dissected the overall contribution of the multiple fatty acid synthesis pathways in *T. gondii* tachyzoites. Based on our *in vitro* observations we draw the following conclusions.

1) *T. gondii* uses both the FASII and FAE pathways to synthesize fatty acids.

To analyse the role of the FASII pathway in *T. gondii*, we used the conditional mutant for the FASII acyl carrier protein. By heavy isotope labeling and GCMS analysis of fatty acids in this mutant, we identified myristate and palmitate as products of the FASII pathway. Using the same strategy, we were able to determine the products of the FAE pathway. We generated conditional mutants for all three fatty acid elongase proteins that are expressed in *T. gondii* tachyzoites. We then used these mutants and determined the elongation range for each elongase enzyme. Our results show that these elongases collaborate with the FASII pathway to generate long chain monounsaturated fatty acids as the final product. We established that the parasite tachyzoites use both FASII and FAE pathways to satisfy their requirement for fatty acids.

2) Fatty acid synthesis is an important determinant of parasite growth.

Previous results from our lab have shown that the FASII pathway is essential for the growth of *T. gondii* tachyzoites. Using a similar strategy, we have now evaluated the essentiality of the FAE pathway. We made a conditional mutant for the dehydratase enzyme of the pathway, therefore ablating the entire pathway. This is evident by the reduced synthesis of all long chain fatty acids. Growth of this mutant was analysed under *in vitro* conditions using two different strategies. A plaque assay experiment was used to measure the parasite's ability to grow, multiply and form plaques on a confluent host cell layer. Results from this experiment indicated that the dehydratase enzyme is essential for tachyzoite growth. We also directly measured the growth of this mutant using fluorescence analysis, and obtained the same result. Therefore, using

conditional mutagenesis we have shown that the FAE pathway is required for parasite growth. Considering that both the FASII and FAE pathways are essential for the growth of *T. gondii* tachyzoites, we propose that fatty acid synthesis plays an important role in acute toxoplasmosis.

3) *T. gondii* has a special requirement for long chain monounsaturated fatty acids.

Heavy isotope labeling and GCMS analysis of FAE mutants shows that the long chain monounsaturated fatty acids are the major products of the FAE pathway. Hence, we investigated if the growth defect observed in the dehydratase mutant is due to the loss of specific fatty acids. We supplied long chain saturated or long chain monounsaturated fatty acids in the parasite growth medium. Interestingly, the growth defect was only rescued by long chain monounsaturated fatty acids but not by saturated fatty acids. In the presence of these long chain unsaturated fatty acids, the growth of the dehydratase mutant was almost completely restored. Thus, our results show that *T. gondii* tachyzoites have a specific requirement for long chain monounsaturated fatty acids.

4) Fatty acid synthesis in *T. gondii* tachyzoites occurs independent of the FASI pathway.

Detection of FASI expression in *T. gondii* tachyzoites prompted us to investigate the role of this pathway in the parasite. In order to identify the role of the FASI pathway, we generated conditional mutants for the entire FASI pathway. The pathway is comprised of a single FASI enzyme, which is activated by a 4'-phosphopantetheinyl transferase (PPTase). We generated conditional mutants for both FASI and PPTase enzymes. Growth and biochemical analysis

showed that there were no defects associated with the loss of these proteins in *T. gondii* tachyzoites. Consequently, we conclude that the FASI pathway does not contribute towards fatty acid synthesis in tachyzoites.

Overall, our results show that, although the parasite harbors multiple routes for lipid synthesis, it selectively uses these synthetic mechanisms to obtain specific fatty acids in specific phases of it's life cycle. These differences may be governed by the need for particular lipids that function in these stages and reflect the availability of different lipids in different host cell niches.