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Review

Fatty acid metabolism in the *Plasmodium* apicoplast: Drugs, doubts and knockouts

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ABSTRACT

The malaria parasite *Plasmodium* possesses a relict, non-photosynthetic plastid known as the apicoplast. The apicoplast is essential for parasite survival, and harbors several plant-like metabolic pathways including a type II fatty acid synthesis (FASII) pathway. The FASII pathway was discovered in 1998, and much of the early research in the field pursued it as a therapeutic drug target. These studies identified a range of compounds with activity against bloodstage parasites and led to the localization and characterization of most enzymes in the pathway. However, when genetic studies revealed FASII was dispensable in bloodstage parasites, it effectively discounted the pathway as a therapeutic drug target, and suggested these compounds instead interfered with other processes. Interest in FASII then shifted toward its disruption for malaria prophylaxis and vaccine development, with experiments in rodent malaria models identifying a crucial role for the pathway in the parasite's transition from the liver to the blood. Unexpectedly however, the human malaria parasite *P. falciparum* was recently found to differ from rodent models and require FASII for mosquito stage development. This requirement blocked the production of the FASII-deficient forms that might be used as a genetically attenuated parasite vaccine, suggesting the pathway was also unsuitable as a vaccine target. This review discusses how perception of FASII has changed over time, and presents key findings about each enzyme in the pathway to identify remaining questions and opportunities for malaria control.

Contents

Abbreviations: ACC, acetyl-coenzyme A carboxylase; ACP, acyl carrier protein; ACPS, acyl carrier protein synthase; ACS, acyl-coenzyme A synthetase; CoA, coenzyme A; DHAP, dihydroxyacetone phosphate; dim, cyclohexanedione; DOXP pathway, 1-deoxy-D-xylulose-5-phosphate/non-mevalonate isoprenoid precursor synthesis pathway; ER, endoplasmic reticulum; G3PAT, glycerol-3-phosphate acyltransferase; G3PDH, glycerol-3-phosphate dehydrogenase; GAP, genetically attenuated parasite; GFP, green fluorescent protein; FASI, type I fatty acid synthesis; FASII, type II fatty acid synthesis; fop, aryloxphenoxypropionate; PDH, pyruvate dehydrogenase; PEP, phosphoenolpyruvate; pPT, plastidic phosphate transporter.

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1. Introduction

Malaria is the most significant parasitic disease of humans, with over a third of the world's population considered at risk, and approximately 200 million cases reported each year [1]. Malaria is caused by *Plasmodium*, a single-celled protist in the phylum Apicomplexa. The *Plasmodium* life cycle is fascinating and complex, and several reviews provide detailed descriptions of its stages and their potential to be targeted for malaria control [2–5]. Briefly, infection begins when a small number of *Plasmodium* sporozoites are injected into the skin by the bite of a female *Anopheles* mosquito. The sporozoites travel to the liver and invade hepatocytes, where they proliferate asymptomatically to produce tens of thousands of liver stage merozoites. The merozoites are then released into the bloodstream, where they infect red blood cells to begin the replication cycle responsible for the symptoms of malaria. As parasite numbers increase, sexual forms of the parasite called gametocytes also start to appear in the blood. If ingested by a mosquito, these differentiate into gametes and fuse to produce zygotes in the lumen of the insect midgut. Zygotes then develop into motile ookinetes, which traverse the midgut epithelium and transform into oocysts on the outside of the gut wall. Finally, these oocysts divide to produce thousands of sporozoites, which migrate to the salivary glands ready for transmission to the next human host.

Malaria researchers utilize a range of tools to study the biology of *Plasmodium* across its life cycle. The bloodstages of the human malaria parasite *P. falciparum* can be maintained indefinitely *in vitro* [6], and gametocytes can be readily generated through manipulation of the culture conditions [7,8]. Gametocyte cultures can also be used to infect mosquitoes, and the resulting sporozoites can be isolated for infection of human hepatocytes *in vitro* [9]. More commonly, however, studies of the *Plasmodium* mosquito and liver stages rely on rodent malaria models such as *P. berghei* and *P. yoelii*. These models enable the entire *Plasmodium* life cycle to be safely perpetuated *in vivo*, and also allow for blood and liver stage parasites to be analyzed *in vitro* [10]. Complementing these experimental systems are technologies for the genetic modification of each species [11–15] and resources such as the *Plasmodium* genome database PlasmoDB [16]. Malaria research has also benefited enormously from studies in the related apicomplexan parasite *Toxoplasma gondii*. The *T. gondii* life cycle has multiple developmental stages, but the majority of research is focused on the tachyzoite stage, which is easily maintained in nucleated human cells *in vitro*. *T. gondii* is far more genetically tractable than *Plasmodium* and offers a wider range of tools for its modification [17,18]. These features have made the parasites invaluable as a surrogate for *Plasmodium*, and numerous discoveries about shared aspects of biology have emerged from studies in *T. gondii*.

Both *Plasmodium* and *T. gondii* possess an apicoplast, a relict non-photosynthetic plastid homologous to the chloroplasts of plants and algae [19]. The apicoplast was acquired by secondary endosymbiosis, a process by which the ancestor of the parasite engulfed and retained a eukaryotic alga and its plastid [19–22]. This intriguing evolutionary history has resulted in the apicoplast being surrounded by four membranes, and has shaped numerous other aspects of the organelle's biology. Like other plastids, the apicoplast has its own genome and machinery for transcription and translation [23]. However, the vast majority of apicoplast proteins are encoded by the parasite nucleus and must be imported into the organelle, in most cases courtesy of a distinctive two-part targeting sequence at their N-terminus [24,25]. There are approximately 500 proteins putatively targeted to the apicoplast in *P. falciparum*, and a detailed map of the organelle's metabolism has been assembled [26]. The apicoplast harbors pathways with similarities to those in plants plastids and cyanobacteria, including a FASII pathway, a non-mevalonate (DOXP) isoprenoid precursor synthesis pathway, an iron–sulfur cluster assembly pathway, and part of a heme synthesis pathway. These pathways are fed by precursors imported from the cytoplasm and mitochondrion, and produce metabolites and cofactors required for a range of cellular processes [19,20]. These metabolic activities undoubtedly account for why the apicoplast is essential in both *Plasmodium* and *T. gondii* [27–30], although not every pathway appears to be required at each stage of the life cycle.

Since its identification in 1998, the FASII pathway of the apicoplast has received considerable attention as a potential drug target [24,31–33] (Fig. 1). Fatty acids are required for membrane lipid synthesis and other essential cellular processes, and their production represents a central aspect of parasite lipid metabolism. As *Plasmodium* was previously assumed to rely entirely on the host for fatty acids [34,35], the discovery of FASII offered an exciting new opportunity for drug design. Indeed, the pathway appeared to be the ideal target, with no homologue in humans, and a range of existing compounds already established as FASII inhibitors in other organisms [36–38]. When these compounds showed activity against bloodstage parasites [24,39,40], it indicated FASII was essential, and seemingly validated the pathway as a therapeutic drug target. In efforts to develop more potent inhibitors, a myriad of studies were then undertaken in both *Plasmodium* and *T. gondii*. Numerous compounds were tested for inhibition of parasite growth [41–61], and many FASII enzymes were characterized [49,52,55,62–79], allowing further optimization of lead compounds against their putative targets. These studies identified several promising anti-malarial drug candidates, some of which displayed activity against bloodstage parasites at nanomolar concentrations [54,59].

Alongside these drug studies, researchers also began to investigate the fate of fatty acids produced by FASII. Two pathways for

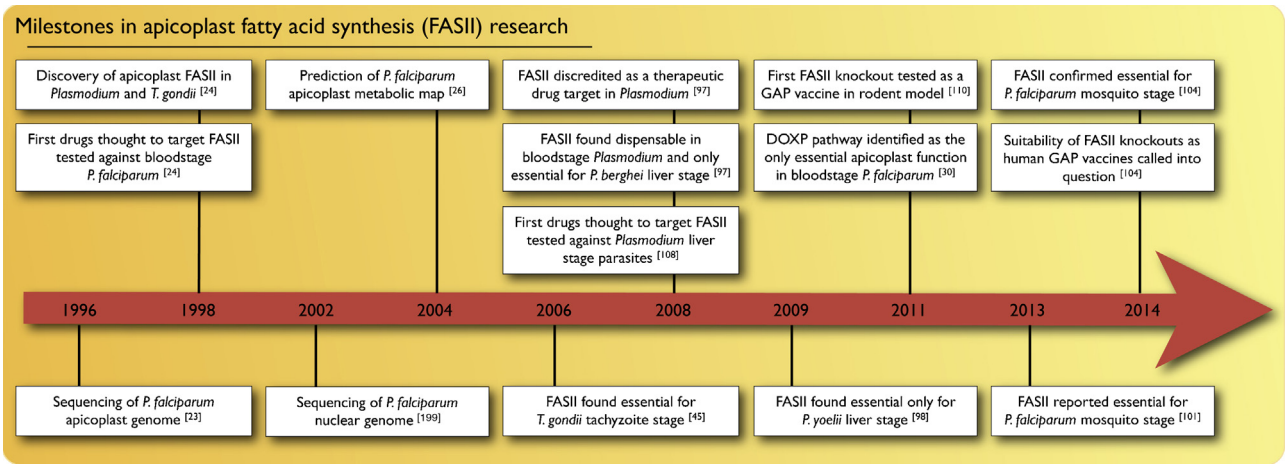

Fig. 1. Timeline of milestones in apicoplast fatty acid synthesis research in *Plasmodium* and *T. gondii*. *Abbreviations*: DOXP pathway, 1-deoxy-D-xylulose-5-phosphate/non-mevalonate isoprenoid precursor synthesis pathway; FASII, type II fatty acid synthesis pathway; GAP, genetically attenuated parasite.

the utilization of FASII products were identified in the apicoplast, one for the synthesis of the enzyme cofactor lipoic acid [80], and another for incorporation of fatty acids into precursors for membrane lipid synthesis [26,81]. The lipoic acid synthesis pathway was extensively studied in *Plasmodium* and *T. gondii*, and the localization and activity of its enzymes have been confirmed [80,82–84]. These and other studies established the lipoic acid synthesis pathway as the source of the lipoate cofactor of the apicoplast pyruvate dehydrogenase complex [45,85], which is an integral part of FASII [63], revealing a curious inter-dependence between the two pathways. The lipid precursor synthesis pathway received comparably less attention, but enzymes putatively able to attach fatty acids to a glycerol backbone were identified in both *Plasmodium* and *T. gondii* [26,81], suggesting the apicoplast could generate and export substrates for the synthesis of membrane lipids. In addition, a putative mechanism for fatty acid export from the apicoplast was identified [26], providing a further avenue for FASII products to contribute to lipid synthesis in other cellular compartments.

Beyond the apicoplast, the fate of FASII-derived fatty acids was more difficult to predict [26,86], and the function of the pathway was called into question as other aspects of parasite lipid metabolism were examined. Several studies revisited the ability of parasites to scavenge from the host, and found bloodstage *P. falciparum* could readily take up, modify, and incorporate exogenously supplied fatty acids into a range of parasite lipids [87,88]. Indeed, fatty acid scavenging was found to be essential for bloodstage *P. falciparum*, and appeared to be the major factor governing the fatty acid composition of parasite lipids [88–92]. *T. gondii* was similarly shown to take up and incorporate fatty acids from host cells [81,93], and a suite of proteins putatively involved in fatty acid scavenging and remodeling were correspondingly identified in the *Plasmodium* and *T. gondii* genomes [86–88,94]. These proteins included acyl-coenzyme A synthetases, fatty acid binding proteins, fatty acid desaturases, and a distinct set of fatty acid elongases predicted to be involved in further extending both scavenged and newly-synthesized fatty acids. In addition, *T. gondii* was found to possess a putatively cytosolic type I fatty acid synthase (FASI) [95,96], adding yet another layer of complexity to fatty acid metabolism in that organism. With each of these discoveries, the role of FASII became increasingly difficult to discern, and researchers could only speculate why it might be essential given its potential redundancy with these other pathways [81,87].

As research into FASII continued, suspicions also began to emerge about the specificity of several inhibitors, with compounds showing a lack of correlation between activity against parasites and recombinant FASII enzymes [44,47,56,57,78,79]. These doubts prompted researchers to investigate FASII using genetic approaches, and core components of pathway were targeted for deletion in both *Plasmodium* and *T. gondii* (Fig. 1). Although FASII was indeed essential for *T. gondii* tachyzoites [45], the pathway was found to be completely dispensable in bloodstage *Plasmodium*, and only required for liver stage development in the rodent models [97,98]. These findings were further supported by additional genetic [99–104] and biochemical experiments [30], and the DOXP pathway emerged as the only essential metabolic function of the apicoplast in bloodstage parasites *in vitro*. These studies discredited FASII as therapeutic anti-malarial drug target, and confirmed that each of its supposed inhibitors likely also interfered with other processes. At the same time, these data explained the requirement for fatty acid scavenging and remodeling in bloodstage *Plasmodium*, and several enzymes involved in these processes were identified as alternate possible targets for the so-called "FASII" inhibitors [105–107].

Following the discovery that FASII was required for liver stage development in the rodent models, interest in the pathway then shifted toward its disruption for malaria prophylaxis (Fig. 1). Several compounds thought to target FASII were tested against *Plasmodium* liver stages, and showed promising activities *in vitro* [53,108,109]. FASII knockouts were similarly identified as possible candidates for genetically attenuated parasite (GAP) vaccines, and selected lines were evaluated for their safety and ability to provoke protective immune responses in mice. Although attenuation was incomplete in some FASII knockouts [100,103], others could provide safe and long-lasting protection [110], and hopes were high that similar strategies might be used to generate a human GAP vaccine. However, when enzymes of FASII were recently targeted for deletion in *P. falciparum*, it revealed a previously unknown difference between the human and rodent parasites, with loss of the pathway instead resulting in a block in sporozoite development [101,104] (Fig. 1). This effectively discounted the *P. falciparum* FASII knockouts for use as a human GAP vaccines, and forced researchers to once again reconsider the role of pathway and its potential to be targeted for malaria control.

This review will summarize the extensive body of research on FASII in *Plasmodium* and consider how key studies have shaped our view of the pathway over time. The data for each FASII enzyme will be presented separately, allowing any gaps in knowledge about their individual roles to be highlighted. Studies in *T. gondii* will be included wherever relevant, and data from drug and genetic experiments in each organism will be integrated to identify alternate

possible targets for the so-called "FASII" inhibitors. Research on the utilization and export of fatty acids from the apicoplast will also be examined, and the remaining questions and opportunities for FASII research in *Plasmodium* will be discussed.

2. The FASII pathway

The FASII pathway of the apicoplast begins with the import of substrates from the cytoplasm, and through a series of reactions involving nine separate enzymes and the acyl carrier protein (ACP), results in the production of saturated fatty acids eight or more carbons in length [66,92,111] (Fig. 2). The majority of FASII enzymes have been characterized or targeted for deletion in *Plasmodium* and *T. gondii*, providing a detailed understanding of the pathway and its requirement in both organisms (Table 1). FASII can be divided into three phases termed 'preparation', 'initiation', and 'elongation'. The preparation phase describes the import of the glycolytic intermediate phosphoenolpyruvate (PEP) from the cytoplasm and its conversion into acetyl-CoA, ATP and reducing equivalents for the following steps in the pathway [112]. The initiation phase involves the conversion of acetyl-CoA into malonyl-ACP and acetoacetyl-ACP, which serve as the carbon donor and first substrate for the fatty acid elongation cycle, respectively [33]. Finally, the elongation phase describes the reaction cycle that extends the growing fatty acid chain by two carbon units per turn, ultimately resulting in the production of mature length acyl-ACPs [33,66].

2.1. Plastidic phosphate transporters (pPTs)

The first step in the preparation phase of FASII involves the transport of PEP into the apicoplast by the pPTs (Fig. 2). The pPTs are antiporters that move phosphorylated carbon compounds across membranes in exchange for inorganic phosphate. *Plasmodium* species possess two pPTs, and in *P. falciparum* these have been localized to the innermost and outermost apicoplast membranes by epitope tagging and protease protection assays [113]. The apicoplast localization the *P. berghei* homologue of the inner membrane pPTs has also been demonstrated by epitope tagging [114]. Assay of the *P. falciparum* pPTs *in vitro* has revealed the two transporters possess very similar substrate preferences, recognizing PEP with high affinity, and dihydroxyacetone phosphate (DHAP) and 3-phosphoglycerate with lesser but considerable affinity [112]. The *Plasmodium* pPTs are therefore predicted to act in tandem to transport these compounds into the apicoplast, providing PEP for use in FASII, plus substrates for both the DOXP and lipid precursor synthesis pathways [112,113] (see Section 4). Although the *T. gondii* genome encodes only one pPT [115], it appears to perform an equivalent function to the *Plasmodium* transporters. The apicoplast localization of the *T. gondii* pPT has been confirmed by epitope tagging [115,116], and its ability to transport PEP, DHAP and 3-phosphoglycerate have been demonstrated *in vitro* [117]. In addition, immuno-electron microscopy has indicated the *T. gondii* pPT may be targeted to multiple apicoplast membranes [118], although the proximity of the four bilayers prevents its precise localization from being determined.

The pPTs have been targeted for deletion in *P. berghei* and *T. gondii* [114,117], allowing the requirement for the transporters in each organism to be identified (Table 1). However, as they provide substrates for multiple apicoplast pathways [112,113,117], the phenotype of the pPT knockouts must be interpreted within this context. In *P. berghei*, loss of the inner membrane pPT appeared lethal for bloodstage parasites, whereas loss of the outer membrane pPT resulted in defects in both mosquito and liver stage development [114]. For the inner membrane pPT, the phenotype of the knockout cannot be attributed to loss of FASII given the pathway is dispensable in bloodstage parasites [97–104]. Instead, it likely reflects the role of the transporter in supporting the DOXP pathway, which is essential in bloodstage *P. falciparum* [30] and presumably also *P. berghei*. Conversely, as loss of the outer membrane pPT was tolerated by bloodstage parasites [114], it indicates apicoplast metabolism was not completely abolished in the knockout. This suggests substrates such as PEP can enter the organelle independently of the outer membrane pPT, and identifies a crucial difference in the permeability of the innermost and outermost apicoplast membranes. However, as defects were observed in the knockout at other life stages [114], it indicates the outer membrane pPT is likely the most efficient route for substrates to enter the apicoplast. For *T. gondii*, loss of the pPT was lethal for tachyzoites [117], consistent with the requirement for FASII at that stage [45]. However, as the phenotype of the pPT knockout was more acute than for the FASII knockout (see Section 2.6), it suggests the loss of other apicoplast pathways may result in more immediate consequences for parasite growth [117].

2.2. Pyruvate kinase (PKII)

The second step in the preparation phase of FASII is the conversion of PEP into pyruvate by pyruvate kinase (Fig. 2). Pyruvate kinases catalyze the transfer of a phosphate group from PEP to ADP, forming pyruvate and ATP. *Plasmodium* and *T. gondii* possess two pyruvate kinases, a type I isoform (PKI) involved in glycolysis in the cytoplasm, and the type II isoform (PKII) of the apicoplast [115,119,120]. Like the pPTs, PKII is expected to support multiple aspects of apicoplast metabolism, providing pyruvate for both the FASII and DOXP pathways and ATP for a range of enzymatic reactions [112,117]. The apicoplast localization of the *P. falciparum* and *T. gondii* PKII have been confirmed by antibody labeling and epitope tagging [115,120], and the activity of *T. gondii* enzyme has been demonstrated *in vitro* [62]. Interestingly, the *Tg*PKII displayed an exclusive preference for GDP over ADP as a phosphate acceptor [62], distinguishing it from most other pyruvate kinases. However, as *Tg*PKII was additionally targeted to the mitochondrion [62], it is not clear how closely this substrate preference will be shared with the *Plasmodium* enzyme.

As neither the *Plasmodium* nor *T. gondii* PKII have been targeted for deletion, the importance of the enzyme for parasite survival is yet to be established (Table 1). However, there is considerable evidence to suggest it is likely to be essential for both organisms. Firstly, PKII is predicted to generate pyruvate for both the FASII and DOXP pathways [112,117], which are required in *T. gondii* tachyzoites and blood stage *P. falciparum*, respectively [30,45]. Secondly, PKII is predicted to be a major source of energy in the apicoplast, with only one other pathway for ATP generation identified in the organelle in *T. gondii* [117], and no other sources known in *Plasmodium* [112]. Thus, PKII likely represents a viable drug target in both *Plasmodium* and *T. gondii*, and further research into the enzyme and its role in apicoplast metabolism is clearly needed.

2.3. Pyruvate dehydrogenase complex (PDH)

The last step in the preparation phase of FASII is catalyzed by PDH, which converts pyruvate into carbon dioxide and acetyl-CoA for subsequent reactions in the pathway (Fig. 2). PDH belongs to the α-keto dehydrogenase complex family, and is composed of four polypeptides (E1α, E1β, E2 and E3) that assemble with thiamine pyrophosphate and NAD+ to form the three functional domains of the enzyme. PDH also requires a lipoic acid cofactor, which must be synthesized and attached to the E2 subunit by enzymes of the lipoic acid synthesis pathway (see Section 3). The *Plasmodium* and *T. gondii* genomes encode the subunits of a single PDH complex (E1α,

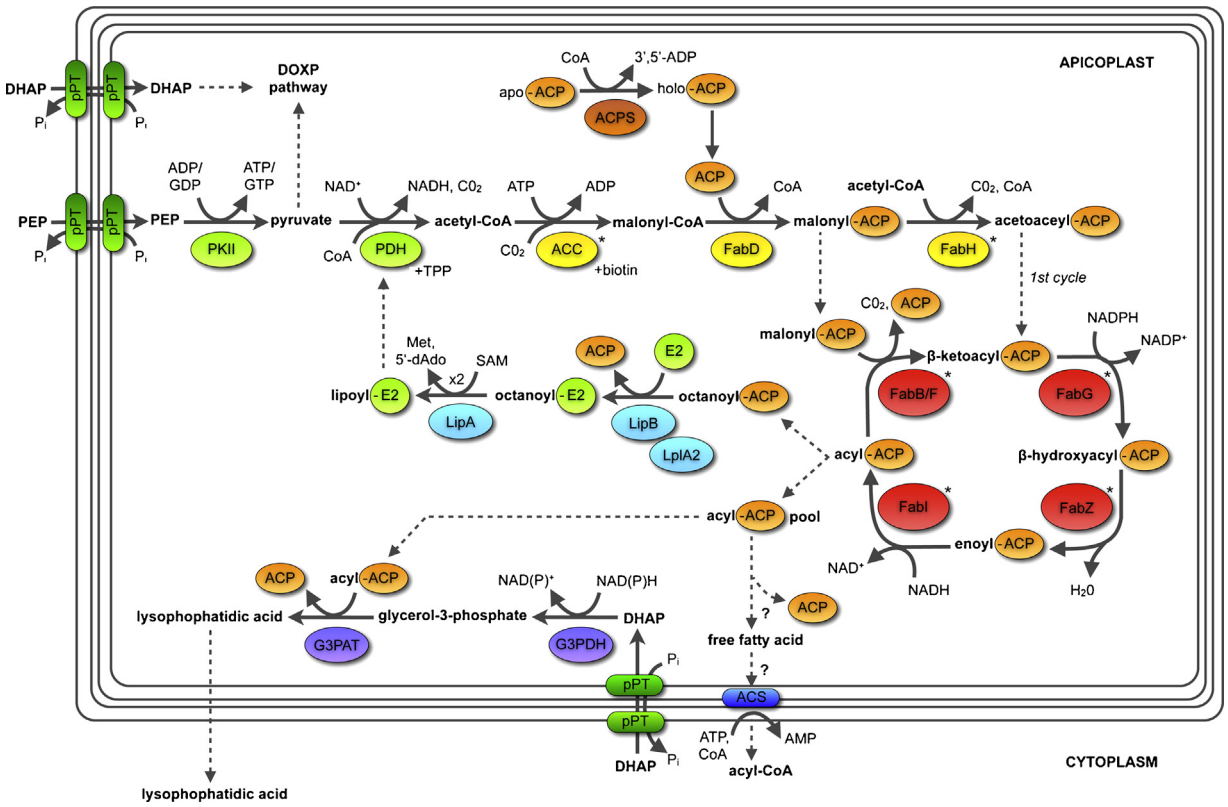

Fig. 2. Fatty acid metabolism in the *Plasmodium* apicoplast. The plastidic phosphate transporters (pPTs, dark green) transport phosphoenolpyruvate (PEP) and dihydroxyacetone phosphate (DHAP) into the apicoplast for the DOXP pathway and the synthesis and utilization of FASII fatty acids. Enzymes of the 'preparation' phase of FASII (light green) generate ATP and reducing power for the apicoplast, and produce acetyl-CoA for subsequent steps in the pathway. Enzymes of the 'initiation' phase of FASII (yellow) convert acetyl-CoA into the acetoacetyl-ACP precursor required for the very first cycle of chain elongation, and also the malonyl-ACP used as the carbon donor for subsequent elongation cycles. The acyl carrier protein (ACP, orange) is involved in both the 'initiation' and 'elongation' phases of FASII, but must first be converted into its holo-form by ACP synthase (dark orange). Enzymes of the 'elongation' phase of FASII (red) extend the growing fatty acid by two carbons per cycle, producing the eight carbon octanoyl-ACP and longer saturated acyl-ACPs. Enzymes of the lipoic acid synthesis pathway (blue) convert octanoyl-ACP into the lipoic acid cofactor required by the E2 subunit of the PDH complex, although the exact role of LplA2 is unclear (see text). Longer acyl-ACPs are used by enzymes of the lipid precursor synthesis pathway (purple) to generate lysophosphatidic acid that is putatively exported and then used for membrane lipid synthesis in other cellular compartments. Long chain acyl-ACPs can also be exported from the apicoplast directly *via* an acyl-CoA synthetase (ACS, dark blue). However, as no apicoplast thioesterase has been identified to act upstream of the ACS, and the localization of the ACS enzymes are yet to be confirmed (both shown by '?'), questions still remain about the mechanism of apicoplast fatty acid transport (see text). Enzymes investigated as potential drug targets indicated by asterisks. Enzymatic reactions shown by solid arrows. *Abbreviations*: 3′,5′-ADP, 3′,5′-adenosine diphosphate; 5′dAdo, 5′-deoxyadenosine; CoA, coenzyme A; DOXP pathway, 1-deoxy-D-xylulose-5-phosphate/non-mevalonate isoprenoid precursor synthesis pathway; FASII, type II fatty acid synthesis pathway; Met, methionine; Pi, inorganic phosphate; SAM, S-adenosylmethionine; TPP, thiamine pyrophosphate.

E1β, E2 and E3 I), and a second E3 II subunit that forms part of the α-keto dehydrogenase complexes of the mitochondrion [115,121]. Although PDH complexes are commonly found in both plastids and mitochondria, the localizations of the E1α, E1β, E2 and E3 I subunits in *Plasmodium* and *T. gondii* indicate the PDH complex is uniquely targeted to the apicoplast [63,85,99,115,121,122]. This finding initially caused some speculation about the acetyl-CoA source for the mitochondrion, but this was recently resolved with the discovery of a related α-ketoacid dehydrogenase with PDH-like activity in that organelle [123]. Further support for the apicoplast localization of the PDH complex come from detection of the enzyme with antibodies against lipoylated proteins in *P. falciparum* and *T. gondii* [83,85], and the activity of the *P. falciparum* PDH E2 subunit has also been demonstrated *in vitro* [63].

PDH is the first apicoplast enzyme to uniquely serve FASII, and genetic studies of the enzyme have proven particularly valuable for understanding the role of the pathway across the *Plasmodium* life cycle. Furthermore, as subunits of the complex have been deleted in *P. falciparum*, *P. berghei* and *P. yoelii*, it is just one of two FASII enzymes to be the subject of genetic studies in all three species (Table 1). In each case, loss of PDH function had no effect on the growth of bloodstage parasites [99–101], consistent with the dispensability of FASII at this stage [97,98,102–104]. Beyond the blood stage, loss of the PDH complex resulted in similar outcomes for *P. berghei* and *P. yoelii*, with knockouts displaying defects only in late liver stage development [99,100]. The *P. berghei* PDH knockout was evaluated as a GAP vaccine, and although the line was less attenuated than its *P. yoelii* counterpart [99], promising results were achieved by combining it with a second genetic modification [100]. However, when the *P. falciparum* PDH knockout was found to arrest prior to sporozoite development [101], it demonstrated for the first time that human and rodent malaria parasites must differ in their requirement for FASII across the parasite life cycle. This key finding effectively discounted the use of such knockouts as human GAP vaccines, and revealed a critical and previously unknown role for FASII in the *P. falciparum* mosquito stages.

2.4. Acetyl-CoA carboxylase (ACC)

The first step in the initiation phase of FASII involves ACC, which catalyzes the synthesis of malonly-CoA from acetyl-CoA and bicarbonate using ATP and a biotin cofactor (Fig. 2). ACC enzymes are composed of three domains, and are classified as multi-functional or dissociative enzymes depending on whether these domains are encoded by one gene or several genes. *Plasmodium* species possess a single multi-functional ACC, and in *P. falciparum* the enzyme has

Table 1
Summary of experimental data for enzymes involved in fatty acid synthesis and utilization in the apicoplast of *Plasmodium* and *T. gondii*.

		Localization				Activity				Knockout			
	Enzyme	*Pf*	*Pb*	*Py*	*Tg*	*Pf*	*Pb*	*Py*	*Tg*	*Pf*	*Pb*	*Py*	*Tg*
Fatty acid synthesis (FASII)	Phosphate transporter (pPT) of outer membrane[#] PF3D7_0508300[^]	✓			✓	✓			✓		✓ *ML*		✓
	Phosphate transporter (pPT) of inner membrane PF3D7_0530200	✓	✓			✓					✓ *B*		
	Pyruvate kinase (PKII) PF3D7_1037100	✓			✓				✓				
	Pyruvate dehydrogenase E1α subunit (PDH E1α) PF3D7_1124500	✓		✓	✓					✓ *M*	✓ *L*	✓ *L*	
	Pyruvate dehydrogenase E1β subunit (PDH E1β) PF3D7_1446400				✓								
	Pyruvate dehydrogenase E2 subunit (PDH E2) PF3D7_1020800	✓			✓	✓							
	Pyruvate dehydrogenase E3 subunit (PDH E3) PF3D7_0815900	✓		✓	✓							✓ *L*	
	Acetyl-CoA carboxylase (ACC) PF3D7_1469600	✓			✓				✓	✓			
	Malonyl-CoA:ACP transacylase (FabD) PF3D7_1312000					✓							
	Acyl carrier protein (ACP) PF3D7_0208500	✓	✓	✓	✓	✓							✓
	Acyl carrier protein synthase (ACPS) PF3D7_0420200												
	β-ketoacyl-ACP synthase III (FabH) PF3D7_0211400	✓				✓							
	β-ketoacyl-ACP synthase I/II (FabB/F) PF3D7_0626300					✓				✓ *M*	✓ *L*	✓ *L*	
	β-ketoacyl-ACP reductase (FabG) PF3D7_0922900			✓		✓							
	β-hydroxyacyl-ACP dehydratase (FabZ) PF3D7_1323000			✓	✓	✓						✓ *L*	
	Enoyl-ACP reductase (FabI) PF3D7_0615100	✓	✓	✓		✓			✓	✓ *M*	✓ *L*		
Utilization and export	Octanoyl-ACP:protein transferase (LipB) PF3D7_0823600	✓				✓				✓	✓ *L*		
	Lipoic acid synthase (LipA) PF3D7_1344600	✓			✓	✓			✓	✓ *			
	Lipoate protein ligase (LplA2) PF3D7_0923600	✓				✓					✓ *M*		
	Glycerol-3-phosphate dehydrogenase (G3PDH) PF3D7_1114800			✓								✓ *L*	
	Glycerol-3-phosphate acyltransferase (G3PAT) PF3D7_1318200			✓				✓ *L*				✓ *L*	
	Acetyl-CoA synthase (ACS) PF3D7_0215000, PF3D7_0215300												

[^], PlasmoDB gene IDs for *P. falciparum*
[#], studies of the sole pPT in *T. gondii* presented here
B, defect in blood stage; *M*, defect in mosquito stage; *L*, defect in liver stage
*, attempts to delete in bloodstage parasites reported unsuccessful

been localized to the apicoplast by epitope tagging [102]. Consistent with the presence of both FASI and FASII pathways in *Toxoplasma* [106], the *T. gondii* genome encodes two multi-functional ACC enzymes named ACC1 and ACC2. The *T. gondii* ACC1 is homologous to the *Plasmodium* ACC, and both its apicoplast localization and activity have been confirmed [64,124,125]. The *T. gondii* ACC2 was correspondingly localized to the cytosol [124], and has been identified as the likely source of malonyl-CoA for FASI [106].

There has been considerable interest in blocking the *Plasmodium* ACC with drugs, and for several years the enzyme thought to be the primary target of the aryloxyphenoxypropionate (fop) and cyclohexanedione (dim) herbicides. Fops and dims inhibit the multi-functional ACCs of grasses but not the dissociative ACCs of other plants. As both fops and dims showed activity against bloodstage *Plasmodium*, the compounds were assumed to target the multi-functional apicoplast ACC [40,41,42]. Similarly, although

only fops showed activity against *T. gondii*, their ability to inhibit the recombinant *Tg*ACC1 but not *Tg*ACC2 suggested they were specific for the apicoplast enzyme [43,64]. The ability of fops to block the incorporation acetate into fatty acids by *T. gondii* was similarly interpreted as evidence for specific inhibition of FASII [81], although more recent insight suggests acetate labeling instead reflects other processes (see Section 2.6). When FASII was shown to be dispensable in bloodstage *Plasmodium* [97–101,103,104], it indicated both classes of herbicide likely also had other targets. Indeed, recent genetic studies in *P. falciparum* have revealed loss of ACC has no effect on parasite growth or susceptibility to dims, indicating no part of the herbicide's activity can be attributed to inhibition of the enzyme [102] (Table 1). For *T. gondii*, the requirement for FASII in tachyzoites [45] means some activity of the fops against *Tg*ACC1 is possible. However, as acetate labeling in *T. gondii* has now been shown to reflect fatty acid extension by the distinct elongase pathway [111] (see Section 2.6), the ability of the fops to inhibit incorporation of the substrate into fatty acids suggests the compounds might instead target this aspect of parasite lipid metabolism.

2.5. Malonyl-CoA:ACP transacylase (FabD)

The next step in the initiation phase of FASII involves FabD, which catalyzes the transfer of a malonyl group from malonyl-CoA to ACP, producing malonyl-ACP for subsequent steps in the pathway (Fig. 2). The *Plasmodium* and *T. gondii* FabD have sequence features consistent with apicoplast targeting, and the activity of *Pf*FabD has been demonstrated by *in vitro* assay [65]. However, aside from structural modeling of the *Pf*FabD [126], little is known about the enzyme (Table 1). The only other research on FabD comes from the related apicomplexan parasite *Eimeria tenella*, where the apicoplast localization and activity of the enzyme have been confirmed [127]. FabD is therefore one of the least well characterized components of FASII, and considerable scope exists for further research on the enzyme.

2.6. Acyl-carrier protein (ACP)

ACP plays a central role in the initiation and elongation phases of FASII, with every step of the pathway from FabD onwards reliant on the protein (Fig. 2). ACP binds to fatty acids and their precursors *via* its phosphopantetheine prosthetic group, and is essential to shuttle substrates between separate enzymes of the pathway. The apicoplast localization of the *P. falciparum* and *T. gondii* ACP have been confirmed by multiple methods [24,25], and both the *P. berghei* and *P. yoelii* proteins have been detected using antibodies [97,128]. The function of *Pf*ACP has been demonstrated by complementation [129] and recombinant expression in *E. coli* [65,67], and also through its ability to support FASII enzymes *in vitro* [65,66]. There have been several structural studies of *Pf*ACP [130–133] and a considerable amount is known about the carrier's conformation, stability and redox state in bloodstage parasites. Intriguingly, *Pf*ACP has also been found to possess enzymatic activity, catalyzing both self-acylation and malonyl-transferase reactions *in vitro* [68,69]. ACP therefore appears to be more than just a 'carrier', and likely has an even more significant role in FASII and apicoplast metabolism than currently appreciated.

There are no reported attempts to target the *Plasmodium* ACP for deletion (Table 1), but genetic studies of the protein in *T. gondii* have proven highly informative. As deletion of *Tg*ACP resulted in defects in apicoplast development and the ultimate death of tachyzoites, it demonstrated FASII was essential for organelle maintenance and parasite survival [45]. To this day, this remains the only direct genetic evidence for the indispensability of FASII at this stage of the *T. gondii* life cycle (Fig. 1), and the only study to provide a concrete link between the pathway and apicoplast development. The *Tg*ACP knockout also provided the first genetic tool to test if exogenously supplied acetate could serve as a carbon source for FASII. Despite the absence of a putative transporter to import the substrate into the apicoplast, acetate incorporation into fatty acids had been assumed to reflect FASII activity [46,81]. However, as acetate labeling was unaffected in the *Tg*ACP knockout, it argued acetate incorporation must occur *via* another pathway [45], and the fatty acid elongases of the parasite endoplasmic reticulum (ER) were subsequently shown to be responsible [111]. Further experiments with the *Tg*ACP knockout then established glucose labeling as a more accurate measure of FASII activity, and revealed the major products of the pathway in *T. gondii* were 14–16 carbons in length [111]. This important research therefore affirmed PEP derived from glucose as the ultimate carbon source for FASII (Fig. 2), and provided the first experimental evidence for the length of the pathway's products in apicomplexan parasites.

2.7. Acyl-carrier protein synthase (ACPS)

ACP is initially synthesized in its apo-form, lacking the phosphopantetheine prosthetic group required to form a covalent attachment with fatty acids and their precursors. Before participating in FASII, ACP must be converted into its holo-form by ACPS, which catalyzes the transfer of a phosphopantetheine group from CoA to the carrier (Fig. 2). Therefore, although ACPS does not itself participate in FASII, its activity is essential for the function of the pathway. The *P. falciparum* and *T. gondii* genomes encode an ACPS with a putative apicoplast targeting sequence [26], and a second phosphopantetheinyl transferase associated with FASI is also present in *Toxoplasma* [134]. Curiously, the apicoplast ACPS appears to be bi-functional, with both the *P. falciparum* and *T. gondii* enzymes possessing a putative metal-dependent hydrolase domain in addition to their phosphopantetheinyl transferase domain [134]. However, as the enzyme has not been characterized or targeted for deletion in either parasite (Table 1), the function of this second domain and its relevance for FASII remain unknown.

2.8. β-Ketoacyl-ACP synthase III (FabH)

The final step in the initiation of phase of FASII involves the condensation of malonyl-ACP with acetyl-CoA by FabH (Fig. 2). This reaction generates CoA, carbon dioxide, and the four-carbon acetoacetyl-ACP used in the very first cycle of chain elongation. The apicoplast localization of the *P. falciparum* FabH has been confirmed using a green fluorescent protein (GFP) reporter [25], and its activity has been demonstrated by complementation [135] and *in vitro* enzyme assay[65,67]. Like other β-ketoacyl-ACP synthases, *Pf*FabH also possesses acetyl-CoA:ACP transacylase activity, catalyzing the conversion of acetyl-CoA and ACP into acetyl-ACP and free CoA with lesser efficiency [65,67]. However, as neither the *Plasmodium* nor *T. gondii* FabH have been targeted for deletion (Table 1), its requirement in parasites is yet to be formally established.

The *P. falciparum* FabH has been the subject of several inhibitor studies, and was initially believed to be the target of thiolactomycin analogs and certain sulfur-containing compounds. Thiolactomycin is a naturally occurring antibiotic that inhibits the β-ketoacyl-ACP synthases of bacteria [37]. As both thiolactomycin and its analogs showed activity against *P. falciparum* and *T. gondii*, they were assumed to target either FabH or FabB/F [24,40,44–46] (see Section 2.9). When tested for inhibition of the enzymes *in vitro*, thiolactomycin displayed activity only against FabB/F, but its analogs appeared to be specific for FabH [65,78]. A range of sulfides, sulfonamides and sulfonyls were also identified as potential FabH inhibitors, and similarly displayed activity against *P. falciparum* and *Pf*FabH *in vitro* [47,48]. However, as both the thiolactomycin

analogs and sulfur-containing compounds showed inconsistencies in their activity against parasites and the recombinant *Pf*FabH [44,47,78], their proposed mode of action was called into question. When FASII was shown to be dispensable in bloodstage *Plasmodium* [97–104], it confirmed these compounds must indeed also target other processes. For the sulfonamides, the activity of similar compounds against the *Plasmodium* carbonic anhydrase flags that enzyme as an alternate possible target [136], However, it is difficult to speculate if this is also the case for the sulfides and sulfonyls, leaving the question of their mode of action open. For the thiolactomycin analogs, disruption of neutral lipid synthesis appears to be the likely cause of their paraciticidal activity in *T. gondii* [46], and the same might possibly also be true in *Plasmodium*.

2.9. β-Ketoacyl-ACP synthase I/II (FabB/F)

The initial step in the elongation phase of FASII involves FabB/F, which catalyzes the condensation of malonyl-ACP with the growing acyl-ACP, forming carbon dioxide and a β-ketoacyl-ACP product that has been extended by two carbon units (Fig. 2). This step is however bypassed in the very first elongation cycle, where FabH instead performs the equivalent condensation reaction. The *P. falciparum* and *T. gondii* FabB/F possess sequence features consistent with apicoplast targeting, and the activity of *Pf*FabB/F has been demonstrated by complementation [137] and *in vitro* assay[66,70]. Although *Pf*FabB/F shares sequence similarity with both FabB and FabF β-ketoacyl-ACP synthases, the enzyme is most similar to FabF in activity [137], and its structure has correspondingly been modeled on the FabF of cyanobacteria [138].

The *P. falciparum* FabB/F was previously thought to be the target of thiolactomycin (see Section 2.8) and the fungal metabolite cerulenin, which inhibits the β-ketoacyl-ACP synthase steps of both FASI and FASII in other organisms [139]. Cerulenin was able to inhibit both *P. falciparum* [39–41] and the recombinant *Pf*FabB/F [70], and in the absence of a plasmodial FASI pathway, was assumed to specifically target that enzyme. This hypothesis was seemingly also supported by the ability of cerulenin to block the incorporation of malonyl-CoA into fatty acids in parasite extracts [39]. However, as for the other compounds thought to target FASII, it is now evident both thiolactomycin and cerulenin must also target other processes. For thiolactomycin, the structural similarity to its analogs suggest a common mode of action, and by extension, that thiolactomycin may also target neutral lipid synthesis in *Plasmodium* (see Section 2.8). For cerulenin, the β-ketoacyl-ACP synthase of the *Plasmodium* fatty acid elongase pathway has been proposed an alternate possible target, with the enzyme putatively inhibited by the compound in trypanosomes, and also thought to use malonyl-CoA as a carbon donor [106].

Genetic studies of FabB/F have been performed in *P. berghei*, *P. yoelii* and *P. falciparum*, making it only the second FASII enzyme to be targeted for deletion in all three species (Table 1). In each case, loss of FabB/F produced no effect on bloodstage parasites [98,103,104], consistent with dispensability of FASII at that stage [97,99–102]. For *P. berghei* and *P. yoelii*, loss of FabB/F resulted in similar outcomes beyond the bloodstage, with knockouts displaying defects only in late liver stage development [98,103]. Both knockouts were evaluated as GAP vaccines, and although the *P. yoelii* FabB/F knockout gave promising results [110], its *P. berghei* counterpart was markedly less attenuated and frequently produced breakthough bloodstage infections [103]. For *P. falciparum*, deletion of FabB/F instead led to a block in sporozoite development [104], mirroring the phenotype of the PDH knockout in the same species [101]. This key finding further dismissed the possibility of using such knockouts as human GAP vaccines, and provided valuable independent confirmation that the role of FASII must differ between *P. falciparum* and the rodent models (Fig. 1).

2.10. β-Ketoacyl-ACP reductase (FabG)

The second step in the elongation phase of FASII is performed by FabG, which catalyzes the reduction of β-ketoacyl-ACP to β-hydroxyacyl-ACP using NADPH as an electron donor (Fig. 2). The *Plasmodium* and *T. gondii* FabG have N-terminal sequences consistent with apicoplast targeting, and the localization of the *P. yoelii* enzyme has been confirmed by epitope tagging and visualization in liver stage parasites [98]. The activity of *Pf*FabG has been demonstrated *in vitro*, and its preference for NADPH over NADH as an electron donor has been established [49,66,71–73]. The crystal structure of *Pf*FabG has been solved [49], and key residues involved in ACP binding have been also identified by site-directed mutagenesis [74]. However, FabG is yet to be targeted for deletion in either *Plasmodium* or *T. gondii* (Table 1), making it the only enzyme of the FASII elongation cycle yet to be the subject of genetic studies.

The *Plasmodium* FabG was initially thought to be the target of the antimicrobial and antihelminthic agent hexachlorophene and a number of flavanoids. As hexachlorophene and its analogs showed activity against bloodstage *P. falciparum* [49], liver stage *P. yoelii* [108] and the recombinant *Pf*FabG [49], the compounds were assumed to specifically target the enzyme. Several flavanoids were similarly shown to inhibit *P. falciparum* growth [50] and *Pf*FabG *in vitro* [51,66], although in many cases they showed equivalent or greater activity against FabZ or FabI (see Sections 2.11 and 2.12). However, when FASII was found to be dispensable in bloodstage *Plasmodium* [97–104], it became clear both classes of compound must also target other processes. Hexachlorophene has since been shown to inhibit the glutamate dehydrogenases of *P. falciparum*, and in particular the enzyme of the apicoplast [140], suggesting it may instead act by disrupting redox balance in the organelle. However, as hexachlorophene inhibits the growth of liver stage *P. yoelii* [108], it remains possible some part of its activity results from inhibition of FabG at this stage. For the flavanoids, the range of biological activities documented for the compounds suggest they may have multiple different targets in parasites. Indeed, flavanoids have been linked to inhibition of carbon metabolism [141], polyamine biosynthesis [142], and DNA replication [143] in other parasitic protists, suggesting one or all of these pathways may likewise be targeted in *Plasmodium*.

2.11. β-Hydroxyacyl-ACP dehydratase (FabZ)

The third step in the elongation phase of FASII is catalyzed by FabZ, which removes water from β-hydroxyacyl-ACP to yield enoyl-ACP (Fig. 2). The apicoplast localization of the *P. yoelii* and *T. gondii* FabZ have been confirmed by epitope tagging and detection with antibodies [45,75,98], and the activity of the *P. falciparum* and *T. gondii* enzyme have been demonstrated *in vitro* [52,66,75]. Like other dehydratases, *Pf*FabZ could catalyze both the forward (dehydration) and reverse (hydration) reactions, and favored the latter when assayed alone [52]. However, in the presence of other components of the elongation cycle, the equilibrium was shifted toward the dehydration reaction as is expected to occur *in vivo* [66]. The crystal structure of *Pf*FabZ has also been solved, providing details of the enzyme's conformation in two states [144,145].

The *P. falciparum* FabZ was previously thought to be a target of the synthetic 'NAS' compounds and a range of both flavanoids and acetylenic fatty acids. NAS-21 and NAS-91 inhibit the β-hydroxyacyl-ACP dehydratase of mycobacteria [106], and as they were effective against *P. falciparum* and *Pf*FabZ *in vitro* [41,52], were assumed to target the apicoplast enzyme. This hypothesis was seemingly further supported the ability of the NAS compounds

to inhibit incorporation of acetyl-CoA into fatty acids in parasites [52], and by co-crystalization of the inhibitors with the recombinant *Pf*FabZ [146]. Flavanoids (see Section 2.10) and acetylenic fatty acids [53] were similarly effective against *P. falciparum* and *Pf*FabZ *in vitro*, although they often also inhibited FabG or FabI, suggesting they acted by targeting multiple FASII enzymes. However, when both FabZ [98] and FASII [97,99–104] were found to be dispensable in bloodstage *Plasmodium*, it once again called the activity of these compounds into question. For the NAS compounds, the stearoyl-CoA desaturase of *P. falciparum* has been suggested as an alternate possible target [106], although this cannot easily account for their inhibition of acetyl-CoA and malonyl-CoA incorporation, indicating yet other aspects of lipid metabolism may also be affected (see Section 2.12). For the flavanoids (see Section 2.10) and acetylenic fatty acids, it appears multiple processes may be inhibited by the compounds, with the structural similarity of the latter to typical fatty acids suggesting they act as toxic analogs and therefore potentially interfere with numerous aspects of lipid metabolism.

The requirement for FabZ across the parasite life cycle has been investigated in *P. yoelii*, and knockout parasites were unaffected in the blood or mosquito stages [98], consistent with other FASII enzymes in rodent models [97,99–104] (Table 1). The liver stage development of the knockout was not analyzed directly, but sporozoites lacking *Py*FabZ were unable to establish a bloodstage infection in mice, indicating the enzyme was required for the parasite's transition from the liver to the blood [98]. As this replicated the phenotype of other FASII knockouts in rodent models [97,99,100,103] and the *Py*FabB/F knockout analyzed in the same study [98], it strongly suggested *Py*FabZ is required for late liver stage development.

2.12. Enoyl-ACP reductase (FabI)

The final step in the elongation phase of FASII involves FabI, which catalyzes the reduction of enoyl-ACP to acyl-ACP using NADH as an electron donor (Fig. 2). The apicoplast localization of the *P. falciparum* FabI has been confirmed by epitope tagging [97] and detection with antibodies in oocyst and liver stage parasites [104], and both the *P. berghei* and *P. yoelii* enzymes have also been visualized [98,99,114]. The activity of the *P. falciparum and T. gondii* FabI have been demonstrated *in vitro*, and both appear to have a strict requirement for NADH over NADPH, contrasting with the more relaxed specificity of the bacteria homologue [39,55,66,76,77]. The crystal structure of the *P. falciparum*, *P. berghei* and *T. gondii* FabI have also been solved [55,56,79,97,147–150], providing considerable insight into the structural biology of the enzyme.

The *P. falciparum* FabI has been the subject of more inhibitor studies than any other FASII enzyme, and for brevity not all will be discussed here. The majority of studies have focused on the antibacterial triclosan, which inhibits the enoyl-ACP reductase of bacteria [139]. As triclosan was active against bloodstage *Plasmodium* and inhibited incorporation of acetate and malonyl-CoA into fatty acids, it was presumed to target FabI and block the activity of the FASII pathway [39]. This finding prompted a suite of further studies, which confirmed triclosan and analogs could bind, inhibit, and be crystalized in complexes with the recombinant *Pf*FabI [54–57,76,79,97,147,151]. Although some compounds showed discrepancies in their activity against parasites and the recombinant enzyme [56,57,79], complementary studies in *T. gondii* [58,77] gave further support for triclosan's proposed mode of action. Interest in FabI steadily increased, and a variety of other compounds were also tested for their inhibition of the enzyme including pyrazoles [152], flavanoids [51,66], rhodanines [59,153], sesquiterpenes [60,61] and acetylenic fatty acids [53]. Indeed, for a time, it appeared as though FabI was the most promising drug target of all the FASII enzymes.

Despite this massive research effort, however, the discovery that FabI [104] and thus FASII [98–104] were dispensable in bloodstage *Plasmodium* asserted all of these compounds must also target other processes. For triclosan, the equivalent enzyme of the *Plasmodium* fatty acid elongase pathway has been identified as an alternate possible target [105,107], but for many of the other compounds their mechanism of action remains unknown. Research into triclosan continued after this key finding, and both the parent compound [109] and several analogs showed activity against *P. berghei* liver stages *in vitro* [107], suggesting they may be useful as prophylactics or multistage anti-malarials. However, as triclosan displayed considerable variation in its efficacy against in rodent malaria models *in vivo* [97,154], the compounds seem unsuitable for either therapeutic or prophylactic use in their current state. For *T. gondii*, evidence suggests triclosan may indeed act at least partially through inhibition of FabI, with the compound able to abolish FASII-dependent lipoylation of the PDH complex (see Section 3), and over-expression of the enzyme able to decrease the parasite's susceptibility to the drug [85]. Further efforts have therefore been made to optimize these analogs for use against *T. gondii*, but few have so far proven sufficiently effective against parasites *in vivo* [149,155–157].

Genetic studies of FabI in *Plasmodium* have arguably made a more significant contribution to our understanding of FASII than any other enzyme. The landmark study by Yu et al. demonstrated for the first time that the pathway was dispensable in bloodstage *Plasmodium*, and paving the way for genetic studies of other FASII enzymes [98–104] (Fig. 1). The study was similarly the first to demonstrate FASII was required for liver stage development in rodent models [97], prompting greater interest in the biology of this stage and suggesting novel avenues for malaria prophylaxis [100,103,110]. Furthermore, by showing acetate incorporation into fatty acids could continue in the knockouts, the work invalidated acetate labeling as a measure of FASII activity in *Plasmodium* [97], just as it had been in *T. gondii* (see Section 2.6). This finding had massive ramifications for the interpretation of drug studies relying on acetate labeling for target validation, and would give valuable clues for many compounds about their genuine mode of action [105]. Later analysis of the *Pf*FabI knockout during mosquito stage development then gave important confirmation for a differing role of FASII in human and rodent malaria parasites, with loss of the enzyme blocking sporozoite development as in the *P. falciparum* FabB/F [104] and PDH knockouts [101]. These studies therefore represent decisive events in the FASII timeline (Fig. 1), and mark two of the most significant turning points in our evolving understanding of the pathway.

3. The lipoic acid synthesis pathway

The production of the octanoyl-ACP by FASII marks an important branch point in apicoplast fatty acid metabolism. From here, the short fatty acid can either be retained by FASII and further elongated, or diverted from the pathway for use in lipoic acid synthesis (Fig. 2). Lipoic acid is a sulfur-containing derivative of octanoic acid that serves as an essential cofactor for certain multisubunit enzyme complexes. In *Plasmodium* and *T. gondii*, lipoic acid is required for the activity of the apicoplast PDH complex (see Section 2.3) and three additional enzyme complexes located in the mitochondrion [80,83,85]. The apicoplast harbors three enzymes involved in lipoic acid metabolism named LipB, LipA and LplA2 (Fig. 2 and Table 1). The canonical apicoplast lipoic acid synthesis pathway is composed of LipB and LipA, which catalyze the conversion of octanoyl-ACP into lipoic acid using S-adenosylmethionine [82]. This pathway has been established as the primary source of the cofactor for the PDH complex, with a separate scavenging pathway identified as the principal source of lipoic acid for the

mitochondrial enzyme complexes [158–160]. LplA2 is partially redundant with LipB, and represents an alternate first step in the apicoplast lipoic acid synthesis pathway [84]. However, as LplA2 is also targeted to the mitochondrion [84] and required for the lipoylation of two enzyme complexes there [161], the enzyme appears to have multiple roles in lipoic acid metabolism.

3.1. Octanoyl-ACP:protein transferase (LipB)

The first step in the lipoic acid synthesis pathway involves LipB, which catalyzes the transfer of octanoyl groups from octanoyl-ACP to the E2 subunit of the PDH complex (Fig. 2). The *Plasmodium* and *T. gondii* LipB genes have been identified [80] and the localization of *P. falciparum* enzyme has been confirmed by GFP reporter assay [82]. The activity of *Pf*LipB has been demonstrated by complementation in *E. coli* [82], and like its bacterial homologue [162], is believed to catalyze the transfer either of octanoate or lipoate to the PDH complex. However, as LipA enzymes typically prefer protein-bound octanoate over octanoyl-ACP [162,163] (see Section 3.2), LipB is expected to principally catalyze the octanoyl transferase reaction *in vivo* [84].

Genetic studies of LipB have been performed in both *P. falciparum* and *P. berghei*, providing insight into the enzyme's requirement in each species (Table 1). In *P. falciparum*, loss of LipB resulted in a moderate acceleration in bloodstage growth rate, suggesting the enzyme was involved in cell cycle control, but was ultimately dispensable at this stage [84]. Deletion of *Pf*LipB also resulted in a marked decrease in PDH lipoylation, supporting its identification as the primary octanoyl transferase of the apicoplast [84]. However, as PDH lipoylation was not completely abolished in the *Pf*LipB knockout, it suggested another enzyme could partially complement for its loss, and LplA2 was subsequently identified as a second but less efficient source of apicoplast octanoyl transferase activity [84] (see Section 3.3). In *P. berghei*, disruption of LipB had no effect on bloodstage growth, but again led to a substantial decrease in PDH lipoylation [164]. Beyond the bloodstage, loss of *Pb*LipB resulted in defects only in late liver stage development, with knockout sporozoites typically failing to establish a patent infection in mice [164]. As this phenotype mirrored that of the PDH and FASII knockouts in rodent models [97–100], it indicated the defect most likely resulted from disruption of FASII [164]. However, as the *Pb*LipB knockout was less attenuated than the FASII knockouts, it suggested FASII activity was not completely abolished, and indicated the reduced level of PDH lipoylation maintained in the line was sufficient to allow some parasites to successfully complete liver stage development.

Genetic studies of LipB have also proven useful in identifying a link between apicoplast lipoic acid synthesis and the lipoylation of mitochondrial enzyme complexes. The previous consensus was that lipoic acid metabolism in the apicoplast and mitochondrion were strictly compartmentalized, with the apicoplast lipoic acid synthesis pathway entirely responsible for lipoylation of the PDH complex, and the mitochondrial scavenging pathway wholly responsible for lipoylation of enzyme complexes in that organelle [80,82,83,85,159,160]. This view was supported by genetic and biochemical studies in *Plasmodium* and *T. gondii*, which demonstrated loss of FASII impacted only PDH lipoylation [45,165], and lipoic acid scavenged from the medium contributed uniquely to lipoylation of mitochondrial enzyme complexes [83,85]. However, in addition to decreasing PDH lipoylation, both the *P. falciparum* and *P. berghei* LipB knockouts displayed a lesser but significant reduction in the lipoylation of mitochondrial enzymes [84,164]. These findings challenge the view that apicoplast and mitochondrial lipoic acid metabolism act in strict isolation, and indicate further research is required to fully understand the role of LipB and the lipoic acid synthesis pathway beyond the apicoplast.

3.2. Lipoic acid synthase (LipA)

The second step in the lipoic acid synthesis pathway is catalyzed by LipA, which introduces two sulfur atoms into the PDH-bound octanoate to convert it to lipoate (Fig. 2). LipA is an iron–sulfur cluster containing enzyme that relies on 5′-deoxyadenosine radicals generated by the reductive cleavage of S-adenosylmethionine for activity [166]. The apicoplast localization of the *P. falciparum* and *T. gondii* LipA have been confirmed by GFP reporter assay [80,82], and the activity of the enzymes have been demonstrated by complementation in *E. coli* [80,82]. The requirement for LipA in *Plasmodium* is yet to be formally demonstrated, with attempts to generate a knockout reported as unsuccessful [167] (Table 1). However, as both LipB [84,164] and the PDH complex [99–101] are dispensable in bloodstage *Plasmodium*, our current understanding of lipoic acid metabolism cannot easily explain why LipA might be refractory to deletion.

3.3. Lipoate protein ligase (LplA2)

The first step in the apicoplast lipoic acid synthesis pathway can alternatively be performed by LplA2, an atypical lipoate attachment enzyme that appears to possess some octanoyl transferase activity (Fig. 2). Lipoate protein ligases catalyze the ATP-dependent transfer of free lipoic acid to target proteins and are typically associated with the scavenging of free lipoate [167]. *Plasmodium* and *T. gondii* possess two lipoate protein ligases, a canonical enzyme named LplA1 involved in mitochondrial lipoic acid scavenging, and the more divergent LplA2.[159]. *Pf*LplA2 has been localized to the apicoplast and mitochondrion using antibodies and fusion of the full length protein to GFP, providing convincing evidence it is targeted to both organelles [84]. The activity of *Pf*LplA2 has been investigated using several approaches, and its function as a lipoate protein ligase is supported its ability to lipoylate the bacterial PDH when recombinantly expressed [83,161]. However, conflicting results for complementation and *in vitro* activity assays [83,84,161] suggest *Pf*LplA2 is at best a poor lipoate protein ligase, and indicate its primary function is instead as an accessory to LplA1 in the mitochondrion [161].

The requirement for LplA2 has been investigated to some extent in *P. berghei*, with loss of the enzyme reported to cause defects in mosquito stage development [158] (Table 1). Regrettably, no further details about the *Pb*LplA2 knockout have been published, making it difficult to speculate about why the enzyme may be essential at this stage. However, as PDH and FASII are dispensable for the mosquito stages in rodent parasites [97–100,103], the phenotype of the *Pb*LplA2 knockout cannot be attributed to a decrease in PDH lipoylation, and must instead reflect an impact on mitochondrial lipoic acid metabolism. Further research is therefore required to determine why LplA2 is essential at this stage, and to identify its precise function in the apicoplast given its apparent redundancy with LipB.

4. The lipid precursor synthesis pathway

One fate for full length fatty acid products of FASII is incorporation into lipid precursors in the apicoplast (Fig. 2). The majority of parasite lipids are phosphoglycerolipids and acylglycerols that possess a common glycerol and fatty acid backbone ultimately derived from a precursor known as phosphatidic acid [168,169]. Based on genomic data, the *Plasmodium* apicoplast was initially predicted to possess a three step pathway for phosphatidic acid synthesis using acyl-ACP from FASII and DHAP imported by the

pPTs [26] (see Section 2.1). Recent investigation of the pathway in *P. yoelii* has confirmed the apicoplast localization of its first two enzymes, and demonstrated the activity of the second by complementation [128] (Table 1). However, the enzyme predicted to catalyze the third step in the pathway was instead localized to the ER in *P. yoelii*, and no alternative capable of fulfilling its function in the apicoplast could be identified [128]. These findings suggest the *Plasmodium* apicoplast lipid precursor synthesis pathway may be incomplete, with the two known enzymes only sufficient to catalyze the conversion of acyl-ACPs and DHAP into the intermediate lysophosphatidic acid [128] (Fig. 2). A putative apicoplast lipid precursor synthesis pathway has also been identified in *T. gondii* [81], but neither their localization nor activity of its enzymes have been investigated.

4.1. Glycerol-3-phosphate dehydrogenase (G3PDH)

The first step in the apicoplast lipid precursor synthesis pathway involves G3PDH, which catalyzes the conversion of DHAP into glycerol-3-phosphate using NAD(P)H as an electron donor (Fig. 2). Glycerol-3-phosphate dehydrogenases catalyze the reversible reduction of DHAP to glycerol-3-phosphate using NADH, NADPH or $FADH_2$ cofactors. *Plasmodium* parasites possess three G3PDH isoforms, two putatively involved in a glycerol-3-phosphate shuttle in the mitochondrion [170–172], and another targeted to the apicoplast [128]. The localization of the *P. yoelii* apicoplast G3PDH has been confirmed by epitope tagging and visualization in liver stage parasites [128]. The activity of apicoplast G3PDH is yet to be demonstrated, but like plant chloroplast enzymes [173,174], is expected to favor the reduction of DHAP to glycerol-3-phosphate *in vivo*.

The requirement for the apicoplast G3PDH has been investigated in *P. yoelii*, and loss of the enzyme had no effect on blood or mosquito stage parasites, but resulted severe defects in late liver stage development [128] (Table 1). As this phenotype replicated that of the FASII knockouts in rodent models [97–100,103], it suggested apicoplast lipid precursor synthesis may be a major route for newly-synthesized fatty acids to be incorporated into parasite lipids. The phenotype of the *P. yoelii* G3PDH knockout similarly identified it as a potential GAP vaccine, and immunization with the line was able to give excellent protection in mice [128]. However, given the requirement for FASII differs between human and rodent malaria parasites [97–104], the same may equally be true for the apicoplast lipid precursor synthesis pathway, casting doubt on its potential to be targeted for human GAP vaccine development.

4.2. Glycerol-3-phosphate acyltransferase (G3PAT)

The second step in the apicoplast lipid precursor synthesis pathway involves G3PAT, which catalyzes the transfer of a fatty acid from acyl-ACP to glycerol-3-phosphate to produce lysophosphatidic acid (Fig. 2). Glycerol-3-phosphate acyltransferases use acyl-ACP, acyl-CoA or acyl-phosphate as acyl donors, and catalyze the formation an ester linkage between the transferred fatty acid and the first carbon of glycerol-3-phosphate [169]. *Plasmodium* parasites possess two G3PAT enzymes, one involved in lipid precursor synthesis in the ER [175], and a second targeted to the apicoplast [128]. The localization of the *P. yoelii* apicoplast G3PAT has been confirmed by epitope tagging and visualization in liver stage parasites, and the activity of the enzyme has been demonstrated by complementation, confirming both its function and preference for acyl-ACP as an acyl donor [128].

The requirement for the apicoplast G3PAT has been investigated in *P. yoelii*, and deletion of the enzyme had no effect on blood or mosquito stage parasites, but resulted in severe consequences for late liver stage development [128] (Table 1). The *P. yoelii* G3PAT knockout was therefore evaluated as a GAP vaccine, and proved to be a powerful immunogen in the rodent model [128]. However, as for the apicoplast G3PDH knockout analyzed in the same study, it is unclear whether the same strategy can be used to generate a human GAP vaccine. The similarity in phenotype between the *P. yoelii* apicoplast G3PAT knockout and FASII knockouts in rodent models [97–100,103] again suggests the apicoplast lipid precursor synthesis pathway is a major avenue for newly-synthesized fatty acids to be incorporated into parasite lipids. The pathway is therefore the only fate of full length FASII products to be characterized in any detail, with comparatively little known about the mechanism of fatty acid export from the organelle (see Section 5).

The apparent absence of an enzyme to follow the G3PAT in the *Plasmodium* apicoplast is quite puzzling. The missing enzyme is a lysophosphatidic acid acyltransferase, and it was predicted to catalyze the transfer of a fatty acid from acyl-ACP to lysophosphatidic acid to form phosphatidic acid [26]. The candidate enzyme was investigated in *P. yoelii*, but was instead localized to the ER, and no alternative apicoplast enzyme could be identified [128]. This suggested the *Plasmodium* apicoplast lipid precursor synthesis pathway was incomplete, and indicated lysophosphatidic acid was likely exported from the organelle for conversion into phosphatidic acid in the ER [128]. Lysophosphatidic acid is more easily transported across membranes than phosphatidic acid, and this could perhaps explain why production of the intermediate in the apicoplast might be advantageous. However, given the *Plasmodium* ER also harbors a G3PAT isoform [175], a source of lysophosphatidic acid is already present in the organelle. No precedent exists for an incomplete phosphatidic acid synthesis pathway in other plastid-bearing organisms [128], making it difficult to speculate about how the two pathways might interact. Indeed, although the phenotype of the G3PDH and G3PAT knockouts [128] demonstrates the two pathways are non-redundant, it is currently not clear why this may be the case. One explanation could be the two G3PAT isoforms incorporate fatty acid species of differing lengths or degrees of saturation, producing precursors that give rise to lipid species with different physiochemical properties and functions. Further research into apicoplast lipid precursor synthesis may therefore give considerable insight into how FASII fatty acids are utilized by parasites, and investigation of the pathway in additional *Plasmodium* species is highly desirable.

5. Fatty acid export from the apicoplast

A second fate for mature length products of FASII is export from the apicoplast for utilization and incorporation into lipids at other cellular locations. Apicoplast fatty acid export is not well characterized, but is believed to involve membrane-bound acyl-CoA synthetase (ACS) enzymes [26] (Fig. 2). ACS enzymes are thought to be involved in fatty acid transport across various parasite membranes, and a number of ACS isoforms exist in both the *Plasmodium* and *T. gondii* genomes [26,35,86,176]. Two *P. falciparum* ACS isoforms are predicted to localize to the apicoplast [26], and *in silico* modeling of the enzymes has given some support for their proposed activity [176]. However, neither the localization nor activity of the putative apicoplast ACS enzymes have been confirmed (Table 1), and additional questions about apicoplast fatty acid export also remain to be addressed.

5.1. Acyl-CoA synthetases (ACS)

ACS enzymes catalyze the ATP-dependent transfer of free fatty acids to CoA, producing AMP and pyrophosphate while simultaneously facilitating the movement of the fatty acids across a membrane (Fig. 2). The *Plasmodium* and *T. gondii* genomes encode

multiple ACS isoforms that are predicted to participate in the intracellular transport and scavenging of fatty acids [86,177]. The number of ACS isoforms varies between species, with four present in most *Plasmodium* species and six found in *T. gondii* [86,176]. In *P. falciparum* however, the ACS gene family has undergone considerable expansion, and comprises 13 isoforms as a result of recent gene duplication events [176]. The *P. falciparum* ACS8 and ACS9 possess predicted apicoplast targeting sequences [26], and conserved regions of the proteins have been modeled on the bacterial ACS, identifying their putative substrate binding motifs [176]. Homologues of *Pf*ACS9 also exist in other *Plasmodium* species [176], and may likewise be targeted to the apicoplast. However, neither the localization nor activity of the putative apicoplast ACS isoforms have been investigated in *Plasmodium* (Table 1), and no prediction has been made for the targeting of the *T. gondii* enzymes.

Further questions about apicoplast fatty acid export arise when the proposed mechanism is compared to that in plant chloroplasts. In plants, ACS enzymes are located on the outer chloroplast membrane [178] and are believed to mediate the transfer of fatty acids to the ER at sites where the organelles are in close contact [179]. As ACS enzymes catalyze the transfer of free fatty acids to CoA, this process is reliant on an acyl-ACP thioesterase, which releases fatty acids from ACP to permit their diffusion across the inner chloroplast membrane [178,179]. The discovery of similar membrane contact sites between the apicoplast and ER in *Plasmodium* and *T. gondii* [180,181] support the presence of an equivalent pathway in parasites, and metabolic labeling has given direct evidence for FASII fatty acid export in *Toxoplasma* [111]. However, as no candidate apicoplast acyl-ACP thioesterase can be identified in either *P. falciparum* or *T. gondii* [26,31], it is not clear how free fatty acids might be generated for the apicoplast ACS enzymes (Fig. 2). Furthermore, as apicoplasts are bound by two additional membranes in comparison to plant chloroplasts, the mechanism of fatty acid export across the outer organelle membranes remains a mystery. The export of FASII products is therefore one of the least well characterized aspects of apicoplast fatty acid metabolism, and the area would benefit greatly from additional research attention.

6. Discussion

The story of FASII is a fascinating example of the twists and turns a research trajectory can take. Initially hailed as the quintessential drug target, later relegated to possible prophylactic target and GAP vaccine strategy, only then to be found intractable for human vaccine production (Fig. 1). Certainly, FASII has experienced highs and lows unlike any other malaria parasite metabolic pathway. Where will FASII research take us in the future? And how can we best benefit from the substantial research investment that has already been made? Although the initial rationale behind the FASII drug studies was often flawed, their data nonetheless provide a wealth valuable information about the pathway. The search for better inhibitors prompted researchers to localize, characterize and crystalize many FASII enzymes, contributing detailed information about their mechanism of action and structural biology. Later genetic studies then built upon these foundations, revealing when FASII was required in the parasite life cycle, and identifying intriguing differences between *P. falciparum* and the rodent parasite species in their fatty acid acquisition strategies at certain life stages. Together, these efforts have made FASII one of the most well characterized pathways of the apicoplast, and contributed significantly toward our understanding of organelle function. At the same time, these studies have uncovered new questions about inhibitors and the FASII pathway, offering a host of opportunities for further drug development and basic biological research.

The discovery that FASII was dispensable in blood stage *Plasmodium* raised an obvious question: if inhibitors are not acting through ablation of the pathway, how are they killing parasites? For several of the supposed "FASII" inhibitors, studies in other parasites and re-evaluation of the experimental data have allowed for alternate possible targets to be identified [46,105–107,136,140–143]. In many cases, these represent other aspects of lipid metabolism [46,105–107], consistent with an essential role for fatty acid scavenging or remodeling in bloodstage parasites [97,108]. These alternate possible targets remain to be validated, and we should be cautious about making assumptions without genetic evidence for their indispensability – let us not make the same mistake twice. However, as some of the inhibitors showed activity against bloodstage parasites at sub-micromolar concentrations [54,59], it may be advantageous to pursue these compounds and their targets further. Equally, even without validation of their bloodstage targets, the "FASII" inhibitors may still have potential for further development as therapeutic antimalarials. Although knowledge of their mode of action is clearly desirable, it is by no means a prerequisite for clinical use [182], and it would seem foolish to discount these leads given the urgent need for new anti-malarial drugs [183,184].

Beyond their potential as stand-alone therapeutic antimalarials, there is still considerable promise for "FASII" inhibitors as prophylactics or partner drugs. Although only a fraction of the inhibitors have been tested against liver stage *Plasmodium* [107–109], their activity *in vitro* suggests they may equally be suited to malaria prevention. As in the bloodstage, these compounds presumably target other pathways in liver stage parasites. However, as so many of the compounds bind and inhibit FASII enzymes *in vitro* [41,47–49,51–55,57,59–61,65,66,70,76,78,146,151,152], it strongly suggests at least part of their activity may be specific. If FASII is essential in liver stage *P. falciparum* as might be predicted, these compounds would be ideal candidates for future development [98,183], with the pathway remaining an attractive drug target for the same reasons described initially [185]. Conversely, if FASII is not critical for liver stage development in *P. falciparum*, these inhibitors could be optimized for activity against their other putative targets. In either case, there is a strong argument for testing more "FASII" inhibitors against liver stage *Plasmodium*, particularly given the very limited number of drugs presently available for use against this stage [2]. Finally, with the recent discovery that "FASII" inhibitors can decrease re-activation of 'dormant' bloodstage *P. falciparum* generated by artemisinin treatment *in vitro* [186], it appears the compounds may also have potential as partner drugs for this important front-line therapeutic agent. Further research in to the "FASII" inhibitors and their suitability for use in artemisinin combination therapy is therefore also a high priority.

With its early focus on drug development, much of the research on FASII in *Plasmodium* has centered on characterizing the 'druggable' enzymes of FASII. While these studies have given us invaluable insight into the structure and function of the enzymes pursued as drug targets, other aspects of the pathway received comparably little attention. There is a notable lack of research on FabD and ACPS, presumably due to a paucity of inhibitors known to target the enzymes in other systems [126,134]. Similarly, aside from lipoic acid, there are several gaps in knowledge concerning the origin of the reducing equivalents and cofactors required by FASII enzymes. Both FabG [49,66,71,72,73] and FabI [39,55,66,76,77] require reducing equivalents for their activity, indicating two units of NAD(P)H are required for each elongation cycle. The PDH complex is able to generate one unit of NADH per molecule of acetyl-CoA [20], but as no other potential source has been identified in the *Plasmodium* apicoplast [185], it is not clear how the remaining reducing equivalents are generated for FASII or other apicoplast pathways. Although a triose phosphate shuttle has been identified as a second source of reducing equivalents in the *T. gondii* apicoplast [117], there is no evidence for such a pathway in *Plasmodium* [21,26,112,185], leaving the balance of reducing power in the

parasite organelle in apparent deficit. The source of the biotin, thiamine pyrophosphate and CoA required by ACC, PDH and ACPS are also somewhat unclear, with enzymes involved in the synthesis or scavenging of these cofactors identified in *Plasmodium* [26,187,188], but little research to date addressing their provision to the apicoplast [189–193]. To gain a more wholistic understanding of FASII, characterization of its remaining enzymes and elucidation of these underlying pathways is a necessity.

Perhaps one of the greatest gaps in knowledge about FASII concerns the fate of its fatty acid products. Research into lipoic acid synthesis has defined the destiny of the octanoyl-ACP produced by FASII [80,82,84], but comparatively little is known about how mature length fatty acids are utilized in *Plasmodium*. The *P. falciparum* FASII pathway appears to predominantly produce fatty acids 14 carbon units in length [92], and the same may also be true in other *Plasmodium* species. These mature length fatty acids are assumed to be used primarily for lipid synthesis [97,98,194], but the nature of the lipid species they are incorporated into and the biological functions they perform remain poorly defined. The presence of an apicoplast lipid precursor synthesis pathway [128] indicates some FASII fatty acids are used for lipid precursor synthesis without modification, with enzymes such as fatty acid desaturases and elongases found only in other cellular compartments [86,195]. Equally, the identification of a putative mechanism for apicoplast fatty acid export [26] suggests some FASII products may be desaturated or elongated prior to incorporation into lipid precursors and lipids [86,195]. This apparently complex network of pathways presumably allows parasites to channel FASII products into a wide range of lipid species, and likely offers some degree of flexibility for integrating the apicoplast pathway with fatty acid and lipid scavenging. However, it has also so far prevented the fate of FASII fatty acids from being determined, with genetic experiments providing valuable information about when the pathway is required, but limited in their ability to address the question of *why*. To unambiguously identify the lipid species reliant on FASII fatty acids for their synthesis, and to uncover why the pathway is essential at some life stages but not others, additional methodologies such as metabolic labeling will need to be employed.

In the absence of direct evidence for the fate of apicoplast synthesized fatty acids, there has been considerable speculation about their likely role in parasite lipid metabolism based on examination of FASII knockout phenotypes. In rodent models, loss of FASII ultimately leads to defects in late liver stage development and merozoite formation [97–100,103], but also consistently reduces parasite size [97–100], nuclear content [97–99], and expression of the key merozoite surface protein MSP1 [97–100,103]. Similarly, although loss of FASII in *P. falciparum* ultimately causes defects in oocyst maturation and sporozoite formation [101,104], it also impacts oocyst size [104]. Based on these observations, two main hypotheses have been presented to describe why FASII may be required for these processes. The first hypothesis proposes FASII is required to supply bulk lipid synthesis at these stages [97–99,104,106,194,196], providing fatty acids for incorporation into a range of lipid species to complement scavenging from the host. This view is supported by the developmental stages at which FASII knockouts arrest [98,99,104], as although *P. falciparum* and the rodent models differ in when the pathway is required, both oocyst division (sporogony) and liver stage division (schizogony) represent times of immense lipid demand and membrane biogenesis. This hypothesis is further supported by the reduced size of knockouts [194], the viability of parasites completing liver stage development in lines where attenuation is incomplete [97], and the apparent ability of parasites to up-regulate FASII in response to decreased lipid uptake from the host [92,197]. The second hypothesis proposes FASII is required to provide a specific fatty acid or lipid species that cannot be scavenged at these times [97–99,104,106,196]. As expression of the merozoite protein MSP1 is consistently affected by loss of FASII in the rodent models, it has been suggested the pathway may be required to provide fatty acids for the synthesis of the protein's glycophosphatidylinositol anchor [97,106,194,198]. However, this might equally reflect a more general failure to complete late liver stage development, as suggested by the decreased size and nuclear content of FASII knockouts. Furthermore, as knowledge of fatty acid scavenging and modification in both the liver and mosquito stages is limited [196,198], it is difficult to identify which fatty acid species may be limiting at these times. Finally, as some *Plasmodium* FASII knockouts also displayed reduced apicoplast development [99,104], it has been suggested the pathway may provide fatty acids for apicoplast membrane synthesis [104,198] as apparently occurs in *T. gondii* [45]. Although it is certainly possible that FASII fatty acids are utilized for the synthesis of apicoplast membrane lipids, the normal development of the organelle at other life stages in the knockouts [97–104] suggests this again likely reflects a more general delay in parasite development rather than loss of specific fatty acid or membrane lipid species.

Knowledge of the fate of FASII fatty acids will equally aid in understanding why *Plasmodium* species differ in their requirement for the pathway. Although loss of FASII invariably affects liver stage development in rodent models, the severity of the phenotype differs between species, with *P. berghei* knockouts consistently displaying less attenuation [97,100,103] than their *P. yoelii* counterparts [98–110]. This disparity has been attributed to differences in the virulence of parasite strains [97,194], but could also represent true differences in liver stage metabolism between species [97]. More pressingly, it will be important to establish why *P. falciparum* and the rodent models differ in their requirement for FASII across their life cycle [97–104]. The unique reliance on FASII for mosquito stage development in *P. falciparum* suggests key aspects of fatty acid metabolism must vary between human and rodent malaria parasites at this stage [104]. However, whether this reflects differences in the rate of sporozoite production, response of parasites to growth in a laboratory setting, or species-specific variation in the biological function of certain fatty acid and lipid species is yet to be determined [104]. Finally, it will be imperative to establish if the pathway is also required for the *P. falciparum* liver stage. This will necessitate the use of conditional knockout strategies or clever genetic experiments to circumvent the block at the preceding developmental stage [104], but it will be essential to determine if FASII is valid as a prophylactic drug target and complete our understanding of the pathway in human malaria parasites.

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