Characterization of the Plasmodium falciparum and P. berghei glycerol 3-phosphate acyltransferase involved in FASII fatty acid utilization in the malaria parasite apicoplast

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Summary

Malaria parasites can synthesize fatty acids via a type II fatty acid synthesis (FASII) pathway located in their apicoplast. The FASII pathway has been pursued as an anti-malarial drug target, but surprisingly little is known about its role in lipid metabolism. Here we characterize the apicoplast glycerol 3-phosphate acyltransferase that acts immediately downstream of FASII in human (Plasmodium falciparum) and rodent (Plasmodium berghei) malaria parasites and investigate how this enzyme contributes to incorporating FASII fatty acids into precursors for membrane lipid synthesis. Apicoplast targeting of the P. falciparum and P. berghei enzymes are confirmed by fusion of the N-terminal targeting sequence to GFP and 3′ tagging of the full length protein. Activity of the P. falciparum enzyme is demonstrated by complementation in mutant bacteria, and critical residues in the putative active site identified by site-directed mutagenesis. Genetic disruption of the P. falciparum enzyme demonstrates it is dispensable in blood stage parasites, even in conditions known to induce FASII activity. Disruption of the P. berghei enzyme demonstrates it is dispensable in blood and mosquito stage parasites, and only essential for development in the late liver stage, consistent with the requirement for FASII in rodent malaria models. However, the P. berghei mutant liver stage phenotype is found to only partially phenocopy loss of FASII, suggesting newly made fatty acids can take multiple pathways out of the apicoplast and so giving new insight into the role of FASII and apicoplast glycerol 3-phosphate acyltransferase in malaria parasites.

Introduction

Malaria is caused by Plasmodium parasites, which have a complex life cycle involving multiple stages in the human and mosquito hosts (Greenwood et al., 2008; Aly et al., 2009). Plasmodium parasites require fatty acids for membrane lipid synthesis and other essential activities, and possess both an endogenous fatty acid synthesis pathway and mechanisms for scavenging fatty acids from the host (Déchamps et al., 2010). The parasite fatty acid synthesis pathway is located in the apicoplast, a reduced plastid organelle homologous to chloroplasts of plants and algae (van Dooren and Striepen, 2013). As in chloroplasts and bacteria, the apicoplast harbours a dissociative, or type II fatty acid synthesis (FASII) pathway, which is intrinsically different to the FASI pathway of humans (Waller et al., 1998). This characteristic, and the assumption that FASII was essential in blood stage parasites, led to its promotion as a target for anti-malarial drugs, and considerable research was directed toward identifying FASII inhibitors.
falciparum may be reliant on FASII in the mosquito stages (Schaijk et al., 2009). This in turn sparked the hypothesis that P. falciparum may be reliant on FASII in the mosquito stages for synthesis of the GPI anchor of the circumsporozoite protein (CSP) or other essential surface proteins (van Schaijk et al., 2014), but so far there is no definitive experimental evidence to support or distinguish these hypotheses.

As in other organisms, malaria parasites are predicted to synthesize phospholipids and the lipid moieties of GPI anchors from a precursor known as phosphatidic acid (Déchamps et al., 2010). Phosphatidic acid is produced via a two-step pathway involving a glycerol 3-phosphate acyltransferase (G3PAT) and lysophosphatidic acid acyltransferase (LPAAT), which rely on conserved histidine and aspartate residues in a ‘HX₄D’ motif (Heath and Rock, 1998) to catalyse the attachment of fatty acids to the first and second positions of glycerol 3-phosphate, respectively. Plasmodium parasites have been predicted to have two sets of these enzymes, one set in the apicoplast and another set in the endoplasmic reticulum (ER) (Santiago et al., 2004; Lindner et al., 2014). The identification of a putative mechanism for apicoplast fatty acid export (Ralph et al., 2004) indicates FASII can potentially contribute fatty acids to both of these pathways. To date, however, there have been relatively few studies to provide evidence for FASII fatty acids being used in phosphatidic acid synthesis in either organelle. The P. falciparum ER G3PAT has been characterized and uses a range of fatty acids including myristate (C14:0) (Santiago et al., 2004), providing suggestive data that FASII may provide substrates to this enzyme. The P. yoelii apicoplast G3PAT has also been characterized and its deletion found to phenocopy FASII null mutants in the late liver stage (Lindner et al., 2014), providing a more tangible link between the two pathways in this model. Surprisingly, however, the same study also found the predicted P. yoelii apicoplast LPAAT was localized to the ER, indicating the apicoplast pathway may differ from that in other plastid-bearing organisms and instead only produce the intermediate lysophosphatidic acid (Lindner et al., 2014). Therefore, although the apicoplast and ER acyltransferases are predicted to be integral for linking FASII with membrane lipid synthesis, their precise role in FASII fatty acid metabolism is yet to be elucidated.

In this study, we characterize the apicoplast G3PAT of P. falciparum (Pf apiG3PAT) and P. berghei (Pb apiG3PAT) to further explore how this enzyme contributes to FASII fatty acid metabolism. We confirm the apicoplast targeting and activity of Pf apiG3PAT and investigate residues in its predicted active site by targeted mutagenesis. We establish the dispensability of Pf apiG3PAT in the blood stage, and compare the phenotype of mutant and wild type parasites in both standard media and conditions that induce FASII activity to make additional inferences about fatty acid metabolism at this stage. Performing complementary studies in P. berghei, we verify the apicoplast localization of Pb apiG3PAT and demonstrate the enzyme is critical for parasite development in the late liver stage, consistent with findings for its P. yoelii homolog. We further show that the...
Pb apiG3PAT deletion mutant phenocopies the P. berghei FASII null mutant phenotype in many respects, but not in the severity of the MSP1 expression defect, revealing novel insight into the role of the enzyme in lipid metabolism and the complex interplay between pathways for fatty acid metabolism in the apicoplast and ER.

Results and discussion

Pf apiG3PAT is targeted to the apicoplast and functions as a typical G3PAT

Pf apiG3PAT was initially implicated as a putative apicoplast protein on the basis of a predicted apicoplast targeting sequence at its N-terminus (Ralph et al., 2004). Further in silico analysis of Pf apiG3PAT confirmed the presence of a putative apicoplast targeting sequence of 70 amino acids that contained a predicted signal peptide and predicted transit peptide as expected (Zuegge et al., 2001; Foth et al., 2003; Petersen et al., 2011) (Supplementary Fig. 1).

To test if this putative apicoplast targeting sequence was functional, we generated the transgenic Pf apiG3PAT<sub>1-70</sub> gfp parasite line, which expressed the predicted Pf apiG3PAT targeting sequence fused to a C-terminal green fluorescent protein (GFP) reporter. Live fluorescence microscopy of blood stage Pf apiG3PAT<sub>1-70</sub> gfp parasites revealed that GFP was directed to a discrete cellular compartment characteristic of the apicoplast (van Dooren et al., 2005) in ring, trophozoite and schizont stage parasites (Fig. 1A). Immunofluorescence microscopy of Pf apiG3PAT<sub>1-70</sub> gfp parasites confirmed GFP was targeted to the apicoplast by co-localization with the apicoplast marker, acyl carrier protein (ACP; Waller et al., 2000) (Fig. 1B). Western blot of bacterial extracts confirms expression of the tagged Pf apiG3PAT (expected mass of 47 kDa). D–E. Mutation of the conserved histidine or aspartate in the ‘HX<sub>4</sub>D’ motif of Pf apiG3PAT abolishes its ability to restore growth in the E. coli mutant. Bacteria were transformed with vectors encoding His-tagged versions of the histidine to alanine mutant (Pf apiG3PAT<sub>H195A</sub>) or aspartate to alanine mutant (Pf apiG3PAT<sub>D200A</sub>), empty vector or E. coli G3PAT positive control. Western blot of bacterial extracts confirms expression of the tagged Pf apiG3PAT<sub>1-70</sub> gfp parasites confirmed GFP was targeted to the apicoplast by co-localization with the apicoplast marker, acyl carrier protein (ACP; Waller et al., 2000) (Fig.
et al. next sought to test whether, as in other characterized G3PAT-deficient bacterial mutant, confirming that \( Pf apiG3PAT \) was active as a G3PAT. This demonstrated that \( Pf apiG3PAT \) vector grew markedly transformed bacteria using anti-His antibodies also verified \( Pf apiG3PAT \) (Fig. 1C), consistent with findings for \( Py apiG3PAT \) (Lindner et al., 2014). Western blot analysis of extracts from transformed bacteria using anti-His antibodies also verified that the tagged \( Pf apiG3PAT \) was expressed and soluble as expected. This demonstrated that \( Pf apiG3PAT \) expression was sufficient to partially restore growth in the PfG3PAT-deficient bacterial mutant, confirming that \( Pf apiG3PAT \) was active as a G3PAT. Having demonstrated that \( Pf apiG3PAT \) was active, we next sought to test whether, as in other characterized G3PATs (Heath and Rock, 1998; Lewin et al., 1999; Turnbull et al., 2001; Tamada et al., 2004), the conserved histidine and aspartate residues of the ‘HX₄D’ motif were important for catalysis. To investigate this, we individually mutated the conserved histidine and aspartate residues of \( Pf apiG3PAT \) to alanine, then tested the resultant proteins for their ability to rescue growth in the bacterial complementation assay. Bacteria transformed with the vector encoding the \( Pf apiG3PAT_{HX4D} \) histidine to alanine mutant had the same residual growth rate as bacteria transfected with the empty vector control, suggesting the conserved histidine was required for \( Pf apiG3PAT \) activity (Fig. 1D). However, we were unable to detect this protein by Western blot analysis despite numerous attempts, and so could not exclude the alternate hypothesis that alteration of the residue had affected the expression, folding or stability of the protein. Bacteria transformed with the vector encoding the \( Pf apiG3PAT_{D200A} \) aspartate to alanine mutant were similarly indistinguishable from the empty vector control, and in this case weak expression of the protein could be detected by Western blot analysis (Fig. 1E). These observations indicated that \( Pf apiG3PAT \) likely resembled characterized G3PATs in its reliance on the conserved histidine and aspartate of the ‘HX₄D’ motif for activity or folding, indicating the enzymes putatively share a common mechanism of catalysis. \( Pf apiG3PAT \) is dispensable in asexual blood stage parasites Having confirmed the apicoplast localization and activity of \( Pf apiG3PAT \), we next sought to determine the requirement for the enzyme in blood stage parasites. Because we expected \( Pf apiG3PAT \) would primarily or exclusively use FASII fatty acids, we predicted that it would be dispensable in asexual blood stage parasites. To test this we used a double cross over homologous recombination strategy to disrupt the \( Pf apiG3PAT \) locus, inserting the human dihydrofolate reductase (hDHFR) drug resistance cassette into coding sequence to truncate the enzyme and eliminate the ‘HX₄D’ active site motif (Supplementary Fig. 2). Drug-resistant parasites were obtained from two independent transfections in the D10 wild type background, and the successful integration of the hDHFR cassette into the \( Pf apiG3PAT \) locus was confirmed by PCR for each line (Supplementary Fig. 2). We also attempted to transfected into the mosquito-transmissible NF54 wild type background on three separate occasions without success (data not shown). Proceeding with the two lines in the D10 background, we observed both \( Pf apiG3PAT \) (−) lines were viable and grew normally as determined by frequency of subculturing, suggesting \( Pf apiG3PAT \) was indeed dispensable for blood stage growth.

As we had employed a transfection strategy that relied on negative selection to eliminate wild type parasites rather than cloning by limiting dilution (Maier et al., 2006), we also sought to verify the disruption of the \( Pf apiG3PAT \) locus by whole genome sequencing of one line. This analysis again confirmed the successful integration of the hDHFR cassette into the \( Pf apiG3PAT \) locus, and although three single base changes were identified between the mutant line and the wild type parent across the entire genome, targeted PCR-based sequencing of these regions in the second \( Pf apiG3PAT \) (−) line revealed that they were not conserved (sequencing data deposited in NCBI Short Read Archive under accession number SRP071808, PCR data not shown). Therefore, we reasoned the ability to recover \( Pf apiG3PAT \) (−) parasites was not because of compensatory mutations or insertion of the selectable marker at other loci, and hence that \( Pf apiG3PAT \) activity was truly dispensable in blood stage parasites.

To investigate whether disruption of \( Pf apiG3PAT \) produced any subtle phenotype in the growth or development of blood stage parasites, one \( Pf apiG3PAT \) (−) line was selected for further characterization. First, to monitor
apicoplast development in the asexual blood stage cycle, we performed immunofluorescence microscopy using antibodies against the apicoplast marker ACP (Waller et al., 2000). No difference was observed between the Pf apiG3PAT (−) line and wild type parasites in apicoplast appearance in ring, trophozoite or schizont stages, indicating disruption of the enzyme did not impact apicoplast development in the blood stage (Fig. 2A). Transmission electron microscopy further confirmed this finding, with typical apicoplast ultrastructure and membrane organization observed in trophozoite stage parasites of both lines, and whorls of membrane seen in the apicoplast lumen in the Pf apiG3PAT (−) parasite as previously reported for wild type (Lemgruber et al., 2013) (Fig. 2B). Disruption of Pf apiG3PAT therefore had no appreciable impact on apicoplast development in blood stage parasites grown in standard culture conditions, indicating the enzyme was not required for apicoplast membrane synthesis and was either non-active or functionally redundant in this stage.

To investigate whether disruption of Pf apiG3PAT had any effect on parasite growth or cell cycle progression, the replication rate of synchronized parasites from the Pf apiG3PAT (−/C0) and wild type line were compared using an established four day growth assay (Mitamura et al., 2000; Mi-Ichi et al., 2006). No difference was observed between Pf apiG3PAT (−) and wild type parasites in replication rate in standard culture conditions, with both lines found to increase in parasitemia between five and six fold per cycle (Fig. 3A). Similarly, no appreciable difference was detected between Pf apiG3PAT (−) and wild type parasites in the proportion of rings or trophozoites present each day of the assay (Supplementary Fig. 3), providing preliminary evidence that disruption of Pf apiG3PAT had no effect on either parasite growth or cell progression in the blood stage. This lack of growth phenotype was further supported by GC-MS analysis of magnetically isolated Pf apiG3PAT (−) and wild type infected red blood cells, which showed disruption of the enzyme had no significant affect on the overall fatty acid profile of parasite lipids (Fig. 3B and C, Supplementary Table 2). Together, these findings demonstrated that Pf apiG3PAT was dispensable in blood stage parasites in standard culture conditions, and suggested the enzyme did not normally contribute to bulk membrane lipid synthesis in this stage.

Last, as it has previously been reported that Pf apiG3PAT and FASII expression is up-regulated in P. falciparum ex vivo isolates displaying a ‘starvation’ transcriptional response (Daily et al., 2007), we hypothesized that Pf apiG3PAT may be important in blood stage parasites in environments where exogenous fatty acids are limiting. To test this, we repeated the growth assay using lipid-depleted media containing only palmitate (C16:0) and oleate (C18:1), because these are the minimum fatty acid set required to support parasite growth, and the conditions were previously shown to also induce FASII activity (Botté et al., 2013). Again, no difference was observed between the Pf apiG3PAT (−) line and wild type in their fold replication per cycle in lipid-depleted conditions, with both lines failing to
increase in parasitemia after the first cycle (Fig. 3D). Furthermore, although the growth of Pf apiG3PAT (-) and wild type parasites was clearly reduced in lipid-depleted media, both displayed normal cell cycle progression, indicating a low number of successful replication events had indeed taken place (Supplementary Fig. 3). Thus disruption of Pf apiG3PAT caused no further reduction in growth in lipid-depleted media beyond that observed for wild type. This suggested that incorporation of FASII fatty acids into membrane lipid precursors in the apicoplast was not essential at this stage, and by extension, that these fatty acids were instead likely exported and utilized by other pathways.

**Pb apiG3PAT is targeted to the apicoplast and expressed in liver stage parasites**

Pb apiG3PAT shares strong sequence similarity with Pf apiG3PAT and is likewise predicted to possess an apicoplast targeting sequence at its N-terminus (Supplementary Fig. 1). To verify that Pb apiG3PAT is targeted to the apicoplast, we used a 3’ gene replacement strategy to generate the Pb apiG3PAT ha gfp transgenic line, which expressed the full-length protein fused to C-terminal hemagglutinin (HA) and GFP tags (Supplementary Fig. 4). We chose this approach as it resulted in the tagged protein being expressed from the endogenous promoter, and could therefore also provide information about when in the life cycle Pb apiG3PAT was expressed. No GFP fluorescence was observed in blood stage parasites, oocysts or salivary gland sporozoites by live fluorescence microscopy, indicating Pb apiG3PAT was not expressed at detectable levels in these stages (data not shown). GFP fluorescence was however observed in liver stage parasites in a structure resembling the apicoplast (Stanway *et al.*, 2009), indicating that Pb apiG3PAT is normally expressed at this stage (Supplementary Fig. 5). Apicoplast targeting of Pb apiG3PAT was then confirmed by immunofluorescence microscopy and colocalization of the HA and GFP tags with the FASII enzyme FabI (Yu *et al.*, 2008) (Fig. 4). This demonstrated that Pb apiG3PAT is localized to the apicoplast and expressed in liver stage parasites, consistent with the observed up-regulation of FASII enzymes at this stage in rodent malaria models (Tarun *et al.*, 2008).

**Pb apiG3PAT is dispensable in blood and mosquito stage parasites**

Having shown that Pb apiG3PAT was targeted to the apicoplast and expressed in liver stage parasites, we next sought to test whether it was essential for late liver stage development like Py apiG3PAT (Lindner *et al.*, 2014) and FASII (Yu *et al.*, 2008; Vaughan *et al.*, 2009) in rodent malaria models. For this, a double cross over homologous recombination strategy was used to disrupt Pb apiG3PAT.
by inserting the hDHFR cassette into its coding sequence, mirroring the approach we used to disrupt PfapiG3PAT (Supplementary Fig. 6). Drug-resistant parasites were obtained from two independent transfections and cloned by limiting dilution, and successful disruption of the PbapiG3PAT locus in each clone was confirmed by PCR (Supplementary Fig. 6). The ability to recover PbapiG3PAT (−) parasites indicated the enzyme was not essential in asexual blood stage parasites, consistent with the dispensability of PyapiG3PAT (Lindner et al., 2014) and FASII at this stage (Yu et al., 2008; Vaughan et al., 2009). Furthermore, comparison of the PbapiG3PAT (−) clone 1 to wild type parasites in an in vivo blood stage growth assay revealed no apparent difference between the lines (Fig. 5A), confirming that as for PfapiG3PAT, disruption of the P. berghei enzyme had no appreciable impact on growth at this stage.

Next, to determine whether disruption of PbapiG3PAT altered parasite transmission or development in the mosquito, we compared the numbers of male gametocytes, ookinetes, oocysts and salivary gland sporozoites produced by PbapiG3PAT (−) and wild type parasites. Preliminary experiments indicated that neither of the PbapiG3PAT (−) clones displayed any defects in mosquito stage development, consistent with our inability to detect expression of PbapiG3PAT by fluorescence microscopy at these stages.

More detailed analysis of PbapiG3PAT (−) clone 1 further confirmed that disruption of the enzyme had no appreciable impact on these stages, with the mean numbers of male gametocytes, ookinetes, oocysts or salivary gland sporozoites from five experiments closely approximating those observed for wild type (Fig. 5B). Disruption of PbapiG3PAT therefore had no apparent impact on parasite transmission or development in the mosquito, consistent with the finding that neither PyapiG3PAT (Lindner et al., 2014) or FASII (Yu et al., 2008; Vaughan et al., 2009) is required in these life stages in rodent malaria models.

PbapiG3PAT is required for normal sporozoite infectivity in vivo

After establishing that disruption of PbapiG3PAT did not affect mosquito stage development, we sought to test whether PbapiG3PAT (−) sporozoites differed from wild type in their ability to produce patent blood stage infections in vivo. To compare the infectivity of PbapiG3PAT (−) and wild type, we intravenously injected sporozoites into outbred Swiss Webster mice, and time to blood stage patency was monitored. Preliminary experiments with the PbapiG3PAT (−) clones indicated they were both markedly attenuated in infectivity relative to wild type (data not shown), consistent with the observed phenotype of the P. berghei FASII null mutants (Yu et al., 2008; Annoura et al., 2012; Nagel et al., 2012).

Fig. 4. PbapiG3PAT is expressed in liver stage parasites and localizes to the apicoplast. Immunofluorescence microscopy of PbapiG3PAT ha gfp liver stage parasites 48 h post-infection confirms the GFP and HA signals co-localize with the apicoplast marker FabI. Parasite and host cell nuclei stained with DAPI. Scale 10 μm.

Fig. 5. PbapiG3PAT (−) parasites show normal development in the blood and mosquito stages. A. Blood stage growth of PbapiG3PAT (−) and wild type parasites is indistinguishable in a four-day in vivo growth assay. Error bars show mean of six mice ± standard error. B. Numbers of exflagellating male gametocytes and mosquito stage parasites in the PbapiG3PAT (−) and wild type lines are comparable. Exflagellation rate determined per 10^7 red blood cells (RBCs) immediately prior to mosquito infection, and ookinetes, oocysts and salivary gland sporozoites counted 20-22 h, 14-15 days and 21-22 days post-infection, respectively. Results from five biological replicates, with mean values indicated by bars.

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2013). Selecting \textit{Pb apiG3PAT} (−) clone 1 for further analysis, we then compared time to patency with wild type after injection of 1000 or 10,000 sporozoites in six independent experiments. Similar to previous experiments with outbred mice (Jaffe \textit{et al.}, 1990; Scheller \textit{et al.}, 1994), injection of 10,000 wild type sporozoites produced infections in all six mice after an average of 4.2 days, while injection of 1000 wild type sporozoites produced infections in four mice after an average of 5.8 days (Table 1). By comparison, injection of either dose of \textit{Pb apiG3PAT} (−) sporozoites produced infections in a smaller fraction of mice and with substantial delays (4–5 days) relative to wild type (Table 1). To test whether the attenuation of \textit{Pb apiG3PAT} (−) blood stage patency was influenced by the route of sporozoite infection, we also assessed the time to patency after infection by biting with 10 mosquitoes. Once again, \textit{Pb apiG3PAT} (−) sporozoites produced infections in fewer mice than wild type, with the one mouse that did become infected doing so with an approximate 6 day delay relative to the control (Table 1). This demonstrated that \textit{Pb apiG3PAT} (−) sporozoites were markedly less infective than wild type regardless of the infection route, with disruption of the enzyme resulting in comparable attenuation to that reported for the \textit{P. berghei} FASII null mutants (Yu \textit{et al.}, 2008; Annoura \textit{et al.}, 2012; Nagel \textit{et al.}, 2013).

\textit{Pb apiG3PAT} is required for the normal growth and maturation of late liver stage parasites in vitro

The reduction in \textit{Pb apiG3PAT} (−) sporozoite infectivity hinted that as for FASII (Yu \textit{et al.}, 2008; Vaughan \textit{et al.}, 2009), the enzyme may be required for parasite development in the late liver stage. To investigate this, we compared the ability of \textit{Pb apiG3PAT} (−) and wild type sporozoites to invade and develop within HepG2 hepatocytes in vitro. \textit{Pb apiG3PAT} (−) and wild type sporozoites did not differ in their ability to invade hepatocytes, indicating the enzyme was not required for the initial establishment of liver stage infections (data not shown). Focusing on \textit{Pb apiG3PAT} (−) clone 1, we then investigated whether the enzyme was required for the growth or development of liver stage parasites. First, to investigate if disruption of \textit{Pb apiG3PAT} affected liver stage parasite growth, we performed immunofluorescence microscopy of \textit{Pb apiG3PAT} (−) clone 1 and wild type at various times post-infection using antibodies against the cytosolic marker HSP70 and measured parasite cross-sectional area in vivo. We then compared time to patency with wild type after injection of 1000 or 10,000 sporozoites in six independent experiments. No difference in mean parasite size was observed between \textit{Pb apiG3PAT} (−) and wild type parasites at 24 h post-infection, demonstrating the enzyme was not required for parasite growth in the early liver stage (Fig. 6A). However, at 48 and 66 h post-infection, \textit{Pb apiG3PAT} (−) parasites were significantly smaller than wild type as determined by a two-tailed t-test, with mean areas 22% and 26% less than the control, respectively (Fig. 6B and C, Supplementary Fig. 7). Disruption of \textit{Pb apiG3PAT} therefore negatively impacted the growth of parasites in the late liver stage, consistent with the requirement for both \textit{Py apiG3PAT} (Lindner \textit{et al.}, 2014) and FASII for normal growth at this stage in rodent malaria models (Yu \textit{et al.}, 2008; Vaughan \textit{et al.}, 2009).

Next, to investigate whether disruption of \textit{Pb apiG3PAT} affected the ability of late liver stage parasites to complete development, we assessed their capacity to produce detached cells and merosomes, which are the final liver stage forms normally observed \textit{in vitro} (Sturm \textit{et al.}, 2006). To examine the formation of detached cells and merosomes, we collected culture supernatants from hepatocytes at 66 and 72 h post-infection, stained briefly with Hoechst nuclear stain, then counted these exoerythrocytic forms by live fluorescence microscopy on three separate occasions. Consistent with previous reports (Nagel \textit{et al.}, 2013), wild type parasites produced the majority of detached cells and merosomes at 66 h post-infection, with smaller numbers of these forms detected at the 72 h (Fig. 6D). By contrast, \textit{Pb apiG3PAT} (−) clone 1 was never seen to produce detached cells or merosomes at either 66 or 72 h. Disruption of \textit{Pb apiG3PAT} therefore severely compromised the ability of parasites to complete liver stage development \textit{in vitro}. This in turn suggested the reduced infectivity of \textit{Pb apiG3PAT} (−) sporozoites resulted from defects in liver stage merozoite production, although evidently a small

### Table 1. \textit{Pb apiG3PAT} (−) parasites show reduced sporozoite infectivity in vivo.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Route</th>
<th>Sporozoites</th>
<th>Fraction patent</th>
<th>Days to patency</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Pb apiG3PAT} (−)</td>
<td>Intravenous</td>
<td>10 000</td>
<td>5/6</td>
<td>8.8</td>
</tr>
<tr>
<td>Wild type</td>
<td>Intravenous</td>
<td>10 000</td>
<td>6/6</td>
<td>4.2</td>
</tr>
<tr>
<td>\textit{Pb apiG3PAT} (−)</td>
<td>Intravenous</td>
<td>1000</td>
<td>1/6</td>
<td>10</td>
</tr>
<tr>
<td>\textit{Pb ANKA WT}</td>
<td>Intravenous</td>
<td>1000</td>
<td>4/6</td>
<td>5.8</td>
</tr>
<tr>
<td>\textit{Pb apiG3PAT} (−)</td>
<td>Mosquito bite</td>
<td>10 mosquitoes</td>
<td>1/6</td>
<td>11</td>
</tr>
<tr>
<td>\textit{Pb ANKA WT}</td>
<td>Mosquito bite</td>
<td>10 mosquitoes</td>
<td>6/6</td>
<td>4.5</td>
</tr>
</tbody>
</table>

* Mean days to patency calculated from mice that developed a patent infection.
Fig. 6. P. berghei Pb apiG3PAT (−) parasites are smaller in the late liver stage and fail to produce merosomes in vitro.

A. Cross-sectional area of Pb apiG3PAT (−) and wild type liver stage parasites was determined by immunofluorescent labelling with antibodies against the cytosolic protein HSP70. Pb apiG3PAT (−) and wild type parasites do not significantly differ in size at 24 h post-infection as determined by a two tailed t-test (n.s., non-significant).

B and C. Pb apiG3PAT (−) parasites are significantly smaller than wild type at 48 and 66 h post-infection, indicating the enzyme is required for normal parasite growth in the late liver stage (**, p-value <0.05, *** p-value <0.001). Error bars show mean ± standard error.

D. Numbers of detached cells and merozoites produced by Pb apiG3PAT (−) and wild type parasites at 66 and 72 h post-infection. Pb apiG3PAT (−) parasites were not observed to produce detached cells or merosomes at either time point, indicating disruption of the enzyme severely compromised completion of liver stage development in vitro.

number of merozoites were still produced in vivo, consistent with reports for the P. berghei FASII null mutants (Yu et al., 2008; Nagel et al., 2013).

Pb apiG3PAT is required for normal apicoplast and nuclear development in the late liver stage, but is not critical for expression of merozoite surface protein 1

Having established that Pb apiG3PAT (−) parasites showed similar defects in late liver stage growth and maturation to FASII null mutants in rodent malaria models (Yu et al., 2008; Vaughan et al., 2009), we next tested whether disruption of the enzyme reproduced any of the other defects reported for these mutants (Pei et al., 2010; Annoura et al., 2012; Nagel et al., 2013). To explore whether disruption of Pb apiG3PAT similarly affected apicoplast and nuclear development in the late liver stage, we performed immunofluorescence microscopy of Pb apiG3PAT (−) clone 1 and wild type parasites with antibodies against ACP and DAPI nuclear stain at various time points post-infection. No difference was observed between Pb apiG3PAT (−) and wild type parasites at 24 h post-infection, demonstrating the enzyme was not required for apicoplast or nuclear development in the early liver stage (Fig. 7A). By contrast, Pb apiG3PAT (−) parasites had noticeably smaller apicoplasts and fewer nuclei than wild type at 48 and 66 h (Fig. 7B and C). Moreover, whereas wild type parasites had typically undergone schizogony and the apicoplast and nuclei had divided into daughter merozoites by 66 h, Pb apiG3PAT (−) parasites were never seen to progress to this stage. This further supported our finding that disruption of Pb apiG3PAT severely impacted liver stage merozoite production, and suggested that like FASII (Yu et al., 2008; Vaughan et al., 2009; Pei et al., 2010), the enzyme may be directly or indirectly required for apicoplast and nuclear development in the late liver stage.

Next, we investigated whether as in FASII null mutants in rodent models (Yu et al., 2008; Vaughan et al., 2009; Pei et al., 2010; Annoura et al., 2012; Nagel et al., 2013), disruption of Pb apiG3PAT affected the expression of MSP1 or its localization to the plasma membrane in late liver stage parasites. For this, we used immunofluorescence microscopy to localize MSP1 in Pb apiG3PAT (−) clone 1 and wild type parasites at 48 and 66 h post-infection. To enable the plasma membrane of early liver stage parasites to be examined, we also performed immunofluorescence microscopy of parasites at 24 h post-infection using antibodies against CSP as previously described (Lindner et al., 2014). No difference was observed between Pb apiG3PAT (−) and wild type in CSP staining at 24 h, confirming disruption of the enzyme did not impact parasite growth or plasma membrane morphology in the early liver stage (Fig. 8A). Surprisingly, we also failed to detect any perceivable difference in MSP1 staining at 48 h, with Pb apiG3PAT (−) parasites again appearing smaller than wild type, but nonetheless showing a diffuse pattern of MSP1 staining similar to the control (Fig. 8B). This indicated that...
although late liver stage growth was reduced in *Pb apiG3PAT* (−/C0) parasites, the expression and localization of MSP1 were not noticeably altered, contrasting with the phenotype reported for FASII null mutants (Yu et al., 2008; Vaughan et al., 2009; Pei et al., 2010; Anouora et al., 2012; Nagel et al., 2013). Consistent with our finding that loss of *Pb apiG3PAT* severely impaired merozoite production, we did observe differences between the lines at 66 h, with MSP1 staining typically detected around individual merozoites in the wild type, but remaining patchy or indicative of only limited plasma membrane invagination in *Pb apiG3PAT* (−) parasites (Fig. 8C). This suggested that as for FASII in the rodent malaria models, *Pb apiG3PAT* was likely involved in synthesizing lipids for the developing merozoite membranes. However, as MSP1 staining was still readily detected in *Pb apiG3PAT* (−) parasites, it again indicated that MSP1 expression was not impacted to the same extent as for the *P. berghei* FASII null mutants (Yu et al., 2008; Anouora et al., 2012).

To more quantitatively compare the proportions of MSP1-expressing parasites in the *Pb apiG3PAT* (−) clone 1 and wild type lines, we performed immunofluorescence microscopy of *Pb apiG3PAT* (−) and wild type parasites using anti-MSP1 antibodies at 48 h post-infection. Similar patterns of MSP1 expression are observed in *Pb apiG3PAT* (−) and wild type parasites, indicating disruption of the enzyme does not markedly affect plasma membrane morphology or MSP1 expression at this stage.

**Fig. 7.** *Pb apiG3PAT* (−) parasites have impaired apicoplast and nuclear development in the late liver stage. A. Immunofluorescence microscopy of *Pb apiG3PAT* (−) and wild type liver stage parasites using antibodies against the apicoplast marker ACP and DAPI nuclear stain at 24 h post-infection. *Pb apiG3PAT* (−) and wild type parasites show similar patterns of ACP and DAPI staining at this time point, indicating apicoplast and nuclear development are not affected by disruption of the enzyme in the early liver stage.

B and C. Immunofluorescence microscopy of *Pb apiG3PAT* (−) and wild type liver stage parasites using anti-ACP antibodies and DAPI nuclear stain at 48 and 66 h post-infection. *Pb apiG3PAT* (−) parasites have noticeably smaller apicoplasts and fewer nuclei than wild type at both time points, indicating apicoplast and nuclear development are severely impaired by disruption of the enzyme in the late liver stage. Scale bar 10 μm.

**Fig. 8.** *Pb apiG3PAT* (−) parasites show altered plasma membrane morphology in the late liver stage but still express merozoite surface protein 1.

A. Immunofluorescence microscopy of *Pb apiG3PAT* (−) and wild type parasites using antibodies against the CSP at 24 h post-infection. *Pb apiG3PAT* (−) and wild type parasites show similar patterns of CSP staining at this time point, indicating plasma membrane morphology is not affected by disruption of the enzyme in the early liver stage.

B. Immunofluorescence microscopy of *Pb apiG3PAT* (−) and wild type parasites using antibodies against merozoite surface protein 1 (MSP1) at 48 h post-infection. Similar patterns of MSP1 expression are observed in *Pb apiG3PAT* (−) and wild type parasites, indicating disruption of the enzyme does not markedly affect plasma membrane morphology or MSP1 expression at this stage.

C. Immunofluorescence microscopy of *Pb apiG3PAT* (−) and wild type parasites using anti-MSP1 antibodies at 66 h post-infection. *Pb apiG3PAT* (−) and wild type parasites differ noticeably in their pattern of MSP1 staining at this time point, suggesting disruption of the enzyme affects formation of merozoite membranes in the late liver stage. Importantly, although there was only one instance where evidence of plasma membrane invagination was observed for *Pb apiG3PAT* (−) parasites (middle panel), MSP1 expression was still readily detected in the line. DNA with DAPI. Scale 10 μm.
microscopy of late liver stage parasites using antibodies against both MSP1 and the parasitophorous vacuole marker UIS4 (Mueller et al., 2005). Using UIS4 staining to identify parasites, we scored the proportion that was MSP1-positive at 48 and 66 h in three independent experiments. Consistent with our observation using MSP1 antibodies alone, no significant difference was observed between \( \text{Pb} \) apiG3PAT (−) and wild type at 48 h, with over 75% of parasites scored as MSP1 positive in each line (Fig. 9, Supplementary Fig. 8). A modest but significant decrease was observed between \( \text{Pb} \) apiG3PAT (−) and wild type at 66 h, with the mean proportion of MSP1 positive parasites found to be 64% and 78%, respectively (Fig. 9, Supplementary Fig. 8). However, this equated to only an 18% reduction relative to wild type, the MSP1 phenotype observed for \( \text{Pb} \) apiG3PAT (−) parasites was still far more mild than reported for the \( \text{Pb} \) apiG3PAT mutant in blood stage parasites, but severe defects observed for the \( \text{Pb} \) apiG3PAT mutant in growth and merozoite formation in the late liver stage that ultimately result in markedly decreased parasite infectivity.

Together, these findings extend upon previous research on the \( \text{P. yoelii} \) apicoplast G3PAT, and reveal new information about how pathways in both the apicoplast and ER contribute to incorporating FASII fatty acids into precursors for membrane lipids such as the MSP1 GPI anchor. We provide important confirmation that the requirement for FASII for late liver stage development in rodent malaria models likely reflects a need for fatty acids for membrane lipid production, and demonstrate that fatty acid synthesis and scavenging pathways can differ in their contribution to certain lipid species. As human and rodent malaria parasites differ in their requirement for FASII across the parasite life cycle, it is not yet clear how closely these findings will be echoed in \( \text{P. falciparum} \). However, should future experiments reveal that FASII and \( \text{Pf} \) apiG3PAT are similarly

**Conclusions**

In this study, we have characterized the \( \text{P. falciparum} \) and \( \text{P. berghei} \) apicoplast G3PAT and assessed the phenotype of deletion mutants to investigate how the enzyme contributes to linking FASII with membrane lipid production in each \( \text{Plasmodium} \) species. We confirm apicoplast targeting of the \( \text{P. falciparum} \) and \( \text{P. berghei} \) enzyme and demonstrate the activity of \( \text{Pf} \) apiG3PAT by complementation, providing evidence that FASII fatty acids can contribute to the initial step in phosphatidic acid synthesis in the apicoplast of both human and rodent malaria parasites. We find that disruption of the enzyme largely mirrors the phenotype of the FASII null mutants in each species, with no apparent defect observed for the \( \text{Pf} \) apiG3PAT mutant in blood stage parasites, but severe defects observed for the \( \text{Pb} \) apiG3PAT mutant in growth and merozoite formation in the late liver stage that ultimately result in markedly decreased parasite infectivity.

Additionally, our findings extend upon previous research on the \( \text{P. yoelii} \) apicoplast G3PAT, and reveal new information about how pathways in both the apicoplast and ER contribute to incorporating FASII fatty acids into precursors for membrane lipids such as the MSP1 GPI anchor. We provide important confirmation that the requirement for FASII for late liver stage development in rodent malaria models likely reflects a need for fatty acids for membrane lipid production, and demonstrate that fatty acid synthesis and scavenging pathways can differ in their contribution to certain lipid species. As human and rodent malaria parasites differ in their requirement for FASII across the parasite life cycle, it is not yet clear how closely these findings will be echoed in \( \text{P. falciparum} \). However, should future experiments reveal that FASII and \( \text{Pf} \) apiG3PAT are similarly

**MSP1 expression**

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Assuming the \( \text{P. berghei} \) MSP1 GPI anchor is composed of the same fatty acids, the greater reliance on FASII would be consistent with the pathway contributing the myristate (C14:0) and potentially also fatty acids for its diacylglycerol moiety, whereas \( \text{Pb} \) apiG3PAT may only be required for production of the latter or not necessary for synthesis of the anchor at all. Our findings are therefore consistent with FASII fatty acids taking multiple pathways out of the apicoplast into lipids, with some fatty acids reliant on \( \text{Pb} \) apiG3PAT for incorporation into precursors for membrane lipid synthesis, while others are putatively exported prior to being incorporated into lipids such as the MSP1 GPI anchor.
required for the P. falciparum liver stage, we anticipate this information will help guide further research and assist in identifying the most strategic aspects of lipid metabolism to target for malaria prophylaxis.

**Experimental Procedures**

**Routine maintenance of P. falciparum**

*P. falciparum* D10 wild type parasites and transgenic lines were maintained as previously described (Trager and Jensen, 1976) at 2% haematocrit in RPMI-HEPES supplemented with AlbuMAX II (Gibco) for all experiments unless otherwise stated.

**Generation and analysis of Pf apiG3PAT₁₋₇₀ gfp transgenic parasites**

The region encoding the predicted *Pf apiG3PAT* apicoplast targeting sequence was amplified from *P. falciparum* genomic DNA using primers P1 and P2 (Supplementary Table 1) and introduced into the pHBlR vector between the *Pf*HSP86 promoter and GFP coding sequence as previously described (van Dooren et al., 2005). The resulting *Pf apiG3PAT₁₋₇₀ gfp* vector was transfected into ring stage parasites using the standard electroporation protocol (Wu et al., 1995; Crabb and Cowman, 1996). Parasites were then cultured in the presence of 2.5 μg ml⁻¹ blasticidin S (Invitrogen) to select for episomal maintenance of the vector.

Live microscopy of *Pf apiG3PAT₁₋₇₀ gfp* parasites was performed by staining cultures briefly with 1 μg ml⁻¹ 4’,6-diamidino-2-phenylindole (DAPI), before immobilizing cells in media on glass coverslips pre-treated with 0.1% polyethyleneimine. Images were acquired using a Leica SP2 inverted confocal microscope at ambient temperature, and merged and contrast adjusted using ImageJ software (NCBI).

**Generation and analysis of Pf apiG3PAT (~) parasites**

Two regions of the *Pf apiG3PAT* coding sequence were amplified from *P. falciparum* genomic DNA using primers P7, P8, P9 and P10 (Supplementary Table 1) and introduced into the pCC-1 transfection vector (Maier et al., 2006). The resulting vector was transfected into ring stage parasites using the standard electroporation protocol (Wu et al., 1995; Crabb and Cowman, 1996), then selection was performed with 5 nM WR99210 (Jacobus Pharmaceuticals) and 1 μM 5-fluorocytosine (Sigma) as previously described (Maier et al., 2006). Disruption of the *Pf apiG3PAT* locus in the two independently derived parasite populations was confirmed by PCR using primers P11, P12 and P13, with primers T1 and T2 used independently of each other. Identically derived parasite populations was confirmed by PCR using primers P11, P12 and P13, with primers T1 and T2 used as controls (Supplementary Table 1).

Modification of the *Pf apiG3PAT* locus in the principal line examined in this study was additionally confirmed by whole genome sequencing as previously described (Straimer et al., 2012). Briefly, PCR-free libraries were prepared using NEBNext DNA Library reagents (NEB) and NEXTFlex DNA Barcodes (Bio Scientific). Eight libraries were multiplexed with 8% PhiX control and run across two lanes on the Illumina HiSeq 2500 system using single-end sequencing. Sequencing data was analysed using Galaxy (Giardine et al., 2005; Blankenberg et al., 2010; Goekcs et al., 2010). Reads were mapped to the *P. falciparum* 3D7v.10.0
was performed as previously described (MacRae et al., 2009). Variants were called using Freebayes (Garrison and Marth, 2012), filtered for quality and read depth with GATK tools (Auwera et al., 2013) (Quality > 50.0, Read Depth > 20), and annotated using Snpeff (Cingolani et al., 2012) (PF3D7v9.1 genome).

Immunofluorescence microscopy of parasites was performed using anti-ACP, Alexa Fluor goat anti-rabbit secondary antibodies and DAPI as above. Transmission electron microscopy was performed for quality and read depth with GATK tools (Auwera et al., 2013) (Quality > 50.0, Read Depth > 20), and annotated using Snpeff (Cingolani et al., 2012) (PF3D7v9.1 genome).

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Experimental animals and routine maintenance of P. berghei Swiss Webster mice of four to six weeks of age were used for all experiments, and were sourced from the University of Melbourne Zoology Animal Facility or Monash University Animal Research Platform. All experiments were conducted in accordance with the local Prevention of Cruelty to Animals legislation and the University of Melbourne Animal Ethics Committee guidelines under ethics permits 1112043.1 and 1413078.

Mice were infected with P. berghei ANKA wild type or transgenic parasite lines by intraperitoneal injection, and parasitemia was monitored by thin Giemsa-stained smears. Numbers of male gametocytes were assessed by monitoring exflagellation as described (Sturm et al., 2015), and mice were deemed suitable for mosquito infection when >3 exflagellation events per 1000 RBCs were observed. Adult female Anopheles stephensi mosquitoes aged 3 to 7 days were infected by feeding on parasitized mice until engorged. Mosquitoes were maintained on 10% sucrose at 27°C and 80% humidity, and naive mice were infected after 21 days by biting or intravenous injection of sporozoites into the tail vein.

Infection of HepG2 cells with P. berghei sporozoites HepG2 cells were grown at 37°C and 5% carbon dioxide in Advanced MEM medium (Gibco) containing 10% heat-inactivated fetal bovine serum, 2 mM GlutaMAX (Gibco), 1% penicillin/streptomycin (HyClone) and a variable concentration of amphotericin B (Hyclone). Cells were seeded onto glass coverslips in 24-well plates or glass-bottomed culture dishes pre-treated with rat tail collagen type I (Sigma), and allowed to grow overnight before infection with sporozoites. Media was then changed 1–2 h after infection and twice daily thereafter for all experiments.

Generation and analysis of Pb apiG3PAT ha gfp parasites The Pb apiG3PAT coding sequence (minus stop codon) was amplified from P. berghei genomic DNA using primers P14 and P15 and introduced into the pREP3 transfection vector, which was derived from the pL0006 vector (Malaria...
Research and Reference Reagent Resource Center) by addition of the GFP coding sequence downstream of the multiple cloning site. The resulting vector was linearized by cutting with BstEII, then transfected into schizont stage parasites using the Nucleofector device (Lonzza) as previously described (Janse et al., 2006). Pyrimethamine-resistant parasites were recovered and clonal lines obtained by limiting dilution in 10 mice. The correct integration of the vector into the \( P_b \) apiG3PAT locus was confirmed by PCR using primers P16, P17 and P18, with primers T1 and T2 used as controls (Supplementary Table 1).

Live imaging of liver stage parasites was performed 48 h post-infection. Cells were stained briefly with 5 \( \mu \)g/ml Hoehst 33342 (Life Technologies), then imaged using a Leica SP5 inverted laser scanning confocal microscope in a 37 C temperature controlled chamber. Images were taken sequentially for the two channels and processed using ImageJ software (NCBI).

Immunofluorescence microscopy of liver stage parasites was performed 48 post-infection. Cells were washed in PBS, fixed in 4% paraformaldehyde, washed again and permeabilized in 0.15% Triton X-100 before blocking in 3% bovine serum albumin. Labelling was performed with mouse anti-GFP (Roche), rabbit anti-Fabl (gift from David Fidock; used as anti-ACP antibodies were temporarily unavailable), Alexa Fluor goat anti-mouse-488 and anti-rabbit-546 secondary antibodies (Molecular Probes). Samples were then stained with 1 \( \mu \)g/ml DAPI, mounted in Fluorescence Mounting Medium (DAKO), and imaged using a Leica SP5 at ambient temperature as above.

**Generation and analysis of \( P_b \) apiG3PAT (-) parasites**

Regions of the \( P_b \) apiG3PAT coding sequence and 3' UTR were amplified from \( P. \) berghei genomic DNA using primers P19, P20, P21 and P22 (Supplementary Table 1) and introduced into the pL0006 vector (Malaria Research and Reference Reagent Resource Center). The vector was linearized with SacII and ApaI, then transfected into parasites and used as controls (Supplementary Table 1).

Blood stage parasite growth assays were performed as previously described (Sturm et al., 2015) by intravenously injecting three sets of two mice with 1.0 \( \times \) 10\(^5\) infected RBCs, then monitoring parasitemia daily by thin Giemsa-stained smears.

Male gametocyte and mosquito stage development of parasites were assessed by determining the mean exflagellation rate and mean number of ookinetes, oocysts and salivary gland sporozoites for five separate infections. Exflagellation rate was determined as previously described (Sturm et al., 2015), then mosquito cages were infected and 10 individuals sacrificed at each time point. For ookinetes, blood boluses were isolated from mosquitoes 20–22 h post-infection, pooled and stained with mouse anti-p28 (gift from Robert Sinden) and goat anti-mouse Alexa Fluor 488 (Molecular Probes), then parasites were counted as described (Sturm et al., 2015) on a haemocytometer using an Olympus IX73 epifluorescence microscope. For oocysts, midguts were isolated from mosquitoes 14–15 days post-infection, stained with 2% Mercurochrome (Sigma), then viewed using an Olympus BH-2 light microscope. For sporozoites, salivary glands were isolated 21–22 days post-infection, disrupted in PBS, and then parasites were counted on a haemocytometer using an Olympus CK2 microscope.

Sporozoite infectivity in vivo was assessed by measuring the time to patency following intravenous injection of 1000 or 10 000 sporozoites or exposure to 10 infected mosquitoes for ten minutes. Parasitemia was monitored on days 3–14 post-infection by thin Giemsa-stained smears as described (Nagel et al., 2013). Smears were viewed using an Olympus BH-2 light microscope with 40× oil objective, and patency was judged by scanning a minimum of 20 adjacent fields of view as previously reported (Lindner et al., 2014).

Liver stage parasite size was measured by performing immunofluorescence microscopy of parasites at 24, 48 and 66 h post-infection. Cells were fixed as above and labelled with mouse anti-HSP70 (gift from Moriya Tsuji), goat anti-mouse Alexa Fluor 488 (Molecular Probes) and DAPI, before mounting in Fluorescence Mounting Medium (DAKO). Imaging was performed using an OMX V4 Blaze in wide field deconvolution mode, with z-slices acquired across the entire depth of the parasites. Image deconvolution and maximum projection were performed using softWORx software (Applied Precision), and parasite cross-sectional areas calculated using ImageJ (NCBI). Experiments were repeated three times with >50 parasites imaged per replicate, and statistical analysis was performed using Prism version 6.0 (GraphPad).

Detached cell and merozoite formation by parasites was assessed by collecting culture supernatants 66 and 72 h post-infection. Cells were stained briefly with 5 \( \mu \)g/ml Hoehst 33342 (Life Technologies), the counted using the Leica SP2 inverted microscope in epifluorescence mode. Experiments were repeated three times, with a minimum of four wells counted on each occasion.

Immunofluorescence microscopy of liver stage parasites to examine apicoplast and plasma membrane morphology was performed by fixing and labelling with rabbit anti-ACP, mouse anti-CSP (gift from Louis Schofield) or mouse anti-MSP1 (gift from Paul Gilson), then with Alexa Fluor secondary antibodies and DAPI before mounting as above. Images were acquired with a Leica SP2 inverted microscope in epifluorescence mode and processed using ImageJ (NCBI).

Immunofluorescent microscopy of liver stage parasites to quantify MSP1 staining was performed by fixing and staining with mouse anti-UIS4 (gift from Photini Sinnis) and anti-MSP1 antibodies, then with secondary antibodies and DAPI before mounting and imaging as above. Experiments were
repeated three times with >100 parasites counted per replicate, and statistical analysis was performed using Prism version 6.0 (GraphPad).

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References


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Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s web-site:

Table S1. Primers used in this study.

Table S2. Mean relative fatty acid abundance in total lipid extracts from infected RBCs isolated from cultures of Pf apiG3PAT (–) or wild type parasites.

Fig. S1. Alignment of Pf apiG3PAT and Pb apiG3PAT showing the predicted apicoplast targeting sequence and ‘HX4D’ motif characteristic of glycerol 3-phosphate acyltransferases.

Fig. S2. Generation of Pf apiG3PAT (–) parasites.

Fig. S3. Disruption of Pf apiG3PAT does not affect blood stage cell cycle progression on standard or lipid-depleted media.

Fig. S4. Pb apiG3PAT tagging strategy and confirmation by PCR.

Fig. S5. Live fluorescence microscopy of Pb apiG3PAT ha gfp liver stage parasites.

Fig. S6. Pb apiG3PAT knockout strategy and confirmation by PCR.

Fig. S7. Representative immunofluorescence images of Pb apiG3PAT (–) and wild type parasites as used for measuring liver stage cell size.

Fig. S8. Representative immunofluorescence images of Pb apiG3PAT (–) and wild type parasites as stained for scoring expression of merozoite surface protein 1 (MSP1) in the late liver stage.
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