Type II fatty acid synthesis is essential only for malaria parasite late liver stage development

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Summary

Intracellular malaria parasites require lipids for growth and replication. They possess a prokaryotic type II fatty acid synthesis (FAS II) pathway that localizes to the apicoplast plastid organelle and is assumed to be necessary for pathogenic blood stage replication. However, the importance of FAS II throughout the complex parasite life cycle remains unknown. We show in a rodent malaria model that FAS II enzymes localize to the sporozoite and liver stage apicoplast. Targeted deletion of FabB/F, a critical enzyme in fatty acid synthesis, did not affect parasite blood stage replication, mosquito stage development and initial infection in the liver. This was confirmed by knockout of FabZ, another critical FAS II enzyme. However, FAS II-deficient Plasmodium yoelii liver stages failed to form exo-erythroctic merozoites, the invasive stage that first initiates blood stage infection. Furthermore, deletion of FabI in the human malaria parasite Plasmodium falciparum did not show a reduction in asexual blood stage replication in vitro. Malaria parasites therefore depend on the intrinsic FAS II pathway only at one specific life cycle transition point, from liver to blood.

Introduction

Malaria parasites are protists belonging to the genus Plasmodium. They are obligate intracellular parasites that have two distinct replicating life cycle forms in the mammalian host. A massive one-time replication occurs in the liver after inoculation of sporozoite stages by the bite of an infected mosquito and results in the production and release of tens of thousands of exo-erythroctic merozoites (Prudencio et al., 2006). These merozoites infect red blood cells and initiate the cyclic replication that occurs within the blood stream. Blood stage infection leads to malaria disease with Plasmodium falciparum alone afflicting more than 500 million people annually (Snow et al., 2005). Plasmodium replication in red blood cells produces between 8 and 36 merozoites with each invasive cycle (Cowman and Crabb, 2006) whereas one-time replication in the infected hepatocyte produces up to 40 000 merozoites (Shortt et al., 1951) – an ~2000-fold difference. It is currently not well understood to what extent malaria parasites rely on parasitic scavenging of nutrients versus intrinsic synthesis for growth and replication.

Lipids are not only essential but are one of the most abundant components of all organisms and the malaria parasite needs a plentiful supply of lipids – specifically fatty acids for the membrane biogenesis necessary for invasive stage formation. Plasmodium parasites were initially assumed to lack the ability to synthesize their own fatty acids and thus rely on their hosts for lipid scavenging (Vial and Ancelin, 1992). However, this model came into question with the discovery of the apicoplast, a relict plastid organelle of Plasmodium (Kohler et al., 1997). Plant and algal plastids harbour several key biosynthetic pathways and the sequencing of the P. falciparum genome (Gardner et al., 2002) coupled with a detailed analysis of the proteins of known function that were targeted to the apicoplast (Foth et al., 2003) allowed the construction of an apicoplast-specific metabolic map (Ralph et al., 2004). The apicoplast is of cyanobacterial origin and one such apicoplast-targeted pathway is bacterial-like type II fatty acid synthesis (FAS II) (Waller et al., 1998), a de novo pathway by which Plasmodium can synthesize fatty acids from derivatives of acetate and malonate. The fatty acid chain extension step of FAS II is catalysed by four key enzymes – FabB/F, FabG, FabI and
FabZ and the substrate/product of each reaction is covalently bound to the acyl carrier protein (ACP) cofactor (Fig. 1A). Conversely, the mammalian FAS I pathway utilizes a single enzyme complex and is not present in Plasmodium based on genome sequence analysis (Bahl et al., 2003). This is not the case for all apicomplexan parasites – the genome of Toxoplasma gondii encodes both FAS I and FAS II enzymes, Cryptosporidium parvum has FAS I enzyme whereas Theileria annulata does not harbour either FAS I or FAS II pathways (Mazumdar and Striepen, 2007). Deletion of ACP from T. gondii has demonstrated that apicoplast fatty acid synthesis is essential for organelle biogenesis and parasite survival in this parasite (Mazumdar et al., 2006).

The four Plasmodium FAS II enzymes are promising drug targets because they are of bacterial origin. The P. falciparum enzymes have been expressed in vitro and used to reconstitute the elongation module of FAS II (Sharma et al., 2007). The in vitro system mimicked the in vivo machinery and known inhibitors of the enzymes of the elongation module caused the expected accumulation of intermediates. Thus, Plasmodium possesses a functional FAS II pathway. An early study identified a Plasmodium FabI and showed that the FabI inhibitor triclosan kills blood stage parasites (Surolia and Surolia, 2001) and subsequently a significant effort has been undertaken to develop blood stage FAS II inhibitors to treat malaria (Gornicki, 2003; Sato and Wilson, 2005; Wiesner and Seeber, 2005). Although the data suggested that FAS II is necessary for intra-erythrocytic replication, the expression of FAS II enzymes has not been studied throughout the complex infection cycle of the parasite and their importance in parasite progression throughout the life cycle remains unknown.

We recently carried out a liver stage transcriptome and proteome analysis in the model rodent malaria parasite Plasmodium yoelii and observed that (i) the transcription of FAS II genes was increased in liver stages when compared to salivary gland sporozoites and mixed blood stages. The expression profile for FabB/F, FabI, FabZ and FabG are shown. Note that for all four genes, expression is highly upregulated in liver stages.

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pared with blood stages; (ii) FAS II enzymes were present in the liver stage proteome; and (iii) hexachlorophene, an inhibitor of FabG, was able to inhibit liver stage development in vitro (Tarun et al., 2008). These results suggested that FAS II might be important for parasite liver infection. Here, we show that expression of FAS II only occurs during the pre-erythrocytic phases of parasite infection and this allowed unprecedented imaging of the sporozoite and liver stage apicoplast. Strikingly, gene knockouts of FabB/F and FabZ, two of the enzymes involved in fatty acid synthesis demonstrated that FAS II is critical for normal liver stage development but not for blood stage or mosquito stage development. Knockout parasites did not form the first generation of invasive merozoites, which are the critical parasite transition stages to move from the liver into the blood stream and initiate red blood cell infection. In addition, gene knockout of FabI from *P. falciparum*, a further enzyme involved in FAS II, demonstrated that FAS II is not critical for *P. falciparum* blood stage replication.

**Results**

*The transcript abundance of* *P. yoelii* FAS II genes is highly upregulated in late liver stage development*

We used quantitative RT-PCR (qPCR) to show upregulation of FAS II genes in liver stages because our previous microarray analysis had indicated preferential expression in liver stages of *P. yoelii* (Tarun et al., 2008). Transcript abundance was analysed in the different *P. yoelii* life cycle stages for the four FAS II genes encoding the enzymes involved in fatty acid extension (Fig. 1A). For all four genes, the level of transcription was highly increased in pre-erythrocytic stages but the most consistent and substantial expression was seen in late liver stages when compared with blood stages (Fig. 1B to E). The qPCR data suggest that FAS II is induced in liver stages and might thus play an important role for liver stage development.

To further investigate the expression of FAS II during the parasite life cycle we generated a transgenic *P. yoelii* line expressing a myc epitope-tagged FabI under the control of the endogenous FabI promoter (PyFabI-myc) (Fig. S1). The quadruple myc tag was fused to the carboxyl (C)-terminus of FabI and was followed by the 3′ UTR from *Plasmodium berghei* dihydrofolate reductase/thymidylate synthase (DHFR/TS). This expression cassette design should not alter the stage-specific expression of FabI as 3′UTRs are involved in mRNA stability, but not regulation of temporal expression. A similar strategy has previously been used to study the blood stage expression of *P. falciparum* subtilase 1 (Yeoh et al., 2007). PyFabI-myc allowed for the visualization of FabI expression throughout the parasite life cycle using indirect immunofluorescence assays (IFA) with anti-myc antibodies. FabI-myc expression was not detectable in the developing mosquito midgut oocysts and also not in oocyst sporozoites (Fig. S2). FabI-myc expression was first detected in salivary gland sporozoites and localized to a spherical structure close to the nucleus (Fig. 2A). All *P. yoelii* FAS II enzymes including *P. yoelii* FabI possess a bipartite leader sequence that predicts import into the apicoplast (data not shown). As FAS II enzymes localize to the apicoplast in the apicomplexan *T. gondii* (Waller et al., 1998), and FabI from the related apicomplexan *Eimeria tenella* was localized to the apicoplast (Ferguson et al., 2007), we assume that the FabI-myc expression we detected in salivary gland sporozoites is apicoplast specific (Fig. 2A). This is the first published data showing the *Plasmodium* sporozoite apicoplast. To analyse FabI expression during liver stage development, HepG2:CD81 hepatoma cells (Silvie et al., 2003) were infected with sporozoites from the PyFabI-myc line. At 7 h post infection (pi) when intra-cellular sporozoites initiate transformation to trophozoites, apicoplast morphology as determined by FabI-myc staining (Fig. 2B), was similar to that of salivary gland
sporozoites (Fig. 2A). At 14 h pi, parasite nuclear division commenced and the apicoplast initiated its division as indicated by the dumbbell shape (Fig. 2C). By 24 h pi, the apicoplast had formed a branched lariat-shaped structure (Fig. 2D), which became more elaborate with advanced liver stage development at 30 h pi (Fig. 2E). By 40 h pi (Fig. 2F) the apicoplast had differentiated into hundreds of intertwining structures that appeared to be segregating. These results show that FabI expression is initiated in salivary gland sporozoites and that there is robust apicoplast-specific FabI expression throughout liver stage development. Interestingly, the replication of the liver stage apicoplast bears striking similarities to that seen in the apicoplast of developing *P. falciparum* blood stages with reference to the changing appearance of the organelle during schizogony (van Dooren et al., 2005) but on a much more expansive scale. Strikingly, by 48 h pi (Fig. 2G), a time point when the *P. yoelii* liver stage schizont undergoes merozoite formation (Baer et al., 2007), FabI-myc expression was greatly reduced when compared with 40 h pi (Fig. 2F). This strongly suggests that FabI expression is downregulated shortly before or during exo-erythrocytic merozoite formation. Finally, we investigated whether FabI is expressed during asexual blood stage replication and found that Fabl-myc expression was not detected in blood stages of parasites.

To test the hypothesis that FAS II is not essential for blood stage development, we deleted *P. yoelii FabB/F*, the enzyme that catalyses the condensation of malonyl-ACP with the lengthening fatty acyl-ACP formed by FabI (Fig. 1A) from the parasite genome using a double cross-over recombination strategy (Menard and Janse, 1997; Tarun et al., 2007). We decided to delete *FabB/F* because it catalyses an essential step in fatty acid synthesis. PCR genotyping using specific primers pairs confirmed the recombination event and the deletion of the *FabB/F* gene (*fabb/f*−) in two cloned knockout parasite lines (*fabb/f*− clone 1) from two independent transfections. To assay the effect of *FabB/F* deletion on blood stage development, mice were intravenously (iv) injected with 1 ¥ 10^3 or 1 ¥ 10^6 *fabb/f*− clone 1 parasites and blood stage development was followed over time in comparison with wild-type (WT) parasites (Fig. 4A and B). There was no significant difference in the growth rate of *fabb/f*− clone 1 parasites and WT parasites. Normal clearance of the parasite that is observed for this non-lethal *P. yoelii* strain occurred for both WT and knockout approximately 20 days pi (Fig. 4A and B). The results demonstrate that the loss of *FabB/F* and therefore the loss of FAS II had no deleterious effect on *P. yoelii* asexual blood stage replication in vivo.
Furthermore, the blood stage fabb/f− parasites showed normal gametocyte development and male gamete exflagellation (data not shown). To confirm our observations with fabb/f− parasites we used similar methodologies to generate a parasite with a deletion in a second synthesis enzyme, FabZ. FabZ catalyses the dehydration of β-hydroxyacyl-ACP to form trans-2-enoyl-ACP (Fig. 1A). PCR genotyping confirmed the creation of fabz− parasites in two cloned knockout lines from independent transfections (Fig. S3). As for fabb/f− parasites, the deletion of FabZ had no deleterious effect on the growth of fabz− blood stage parasites (Fig. 4A and B). A similar blood stage phenotype from the deletion of a second enzyme in

FAS II adds strength to our hypothesis that FAS II is not necessary for blood stage replication.

P. yoelii FabB/F and FabZ are necessary for pre-erythrocytic stage infection

After transmission of either fabb/f− or fabz− to Anopheles stephensi mosquitoes, we observed normal development of mosquito midgut oocysts, formation of oocyst sporozoites and invasion of sporozoites into the salivary glands as indicated by the enumeration of salivary gland sporozoites in comparison with WT (Table S1: data for one clone for each deletion are shown). Therefore, FAS II is not necessary for parasite development in the mosquito. Next, salivary gland sporozoites were detected iv into BALB/c mice. Mice were injected with 10 000 and 50 000 (n = 8) knock-out sporozoites or with 10 000 WT sporozoites. Mice were assayed for blood stage parasitaemia every other day from day 3 post injection (pi) until day 15 by Giemsa-stained blood smears (Table 1). After 3 days, all mice injected with WT sporozoites exhibited patent blood stage parasitaemia. Strikingly, none of the mice injected with 10 000 and 50 000 fabb/f− sporozoites or fabz− sporozoites became blood stage patent and this was true for both clones of each of the knockouts. These results demonstrate that the lack of FAS II renders the pre-erythrocytic parasite unable to successfully infect the mammalian host.

P. yoelii fabb/f− parasites arrest late in liver stage development

To further investigate the phenotype of the knockout parasites we chose to follow the progression of fabb/f− clone 1 parasites in the liver. BALB/c mice were iv injected with 1 x 10⁶ sporozoites (WT or fabb/f−) and sacrificed at dif-
suggest that FabB/F is not essential for early liver stage development.

Salivary gland sporozoites were isolated from Anopheles stephensi mosquitoes and injected iv into BALB/c mice (one million for both WT and fabb/f−). The livers were removed at (A) 12 h pi and (B) 24 h pi, fixed and cut into 50 μm sections. Liver stage parasites were detected using an IFA utilizing antibodies to the circumsporozoite protein (CSP). Fluorescent staining was achieved with Alexa Fluor-conjugated secondary antibodies specific to rabbit (Alexa Fluor 488, green) and mouse (Alexa Fluor 594, red) IgG. Nuclear staining was achieved with DAPI. Differential interference contrast and fluorescent images were captured and processed using deconvolution microscopy and a merge of the captured images is presented on the far right pane (merge). The scale bars equal 5 μm. Note: As the liver stage progresses there is no difference in the size between the WT and fabb/f− parasite. The results suggest that FabB/F is not essential for early liver stage development.

Table 1. *P. yoelii* FAS II is critical for successful pre-erythrocytic stage infection.

| Parasite genotype | # sporozoites injected | # mice injected | # mice blood stage patent (day of patent) |
|-------------------|------------------------|----------------|------------------------------------------|
| Wild type         | 10 000                 | 10             | 10 (3)                                   |
| fabb/f− clone 1   | 10 000                 | 18             | 0 (--)                                   |
| fabb/f− clone 2   | 50 000                 | 18             | 0 (--)                                   |
| fabb/f− clone 3   | 10 000                 | 12             | 0 (--)                                   |
| fabb/f− clone 4   | 50 000                 | 12             | 0 (--)                                   |
| fabb− clone 1     | 10 000                 | 8              | 0 (--)                                   |
| fabb− clone 2     | 50 000                 | 8              | 0 (--)                                   |
| fabb− clone 3     | 10 000                 | 4              | 0 (--)                                   |
| fabb− clone 4     | 50 000                 | 4              | 0 (--)                                   |

a. Mice injected with 10 000 and 50 000 fabb/f− sporozoites were followed for 15 days post injection and never became blood stage patent.

b. Mice injected with 10 000 and 50 000 fabb− sporozoites were followed for 15 days post injection and never became blood stage patent.

Fig. 5. Development of *Plasmodium yoelii* WT and fabb/f− early liver stages in vivo. Salivary gland sporozoites were isolated from Anopheles stephensi mosquitoes and injected iv into BALB/c mice (one million for both WT and fabb/f−). The livers were removed at (A) 12 h pi and (B) 24 h pi, fixed and cut into 50 μm sections. Liver stage parasites were detected using an IFA utilizing antibodies to the circumsporozoite protein (CSP). Fluorescent staining was achieved with Alexa Fluor-conjugated secondary antibodies specific to rabbit (Alexa Fluor 488, green) and mouse (Alexa Fluor 594, red) IgG. Nuclear staining was achieved with DAPI. Differential interference contrast and fluorescent images were captured and processed using deconvolution microscopy and a merge of the captured images is presented on the far right pane (merge). The scale bars equal 5 μm. Note: As the liver stage progresses there is no difference in the size between the WT and fabb/f− parasite. The results suggest that FabB/F is not essential for early liver stage development.

P. falciparum FabI is not essential for blood stage growth

To test whether the FAS II pathway might be dispensable for a clinically relevant human malaria parasite, we decided to study FabI in the NF54 strain of *P. falciparum*. We were able to delete the *P. falciparum* FabI gene from blood stage parasites using double homologous cross-
over recombination in association with positive and negative selection. Deletion of the gene was confirmed by Southern blot analysis of the parental line (data not shown) and two clonal lines derived from the parental line (Fig. S4). We then compared the replication of the *P. falciparum fabi* blood stages with that of the WT NF54 strain. We saw no significant differences in replication efficiency between WT and *fabi*⁻ (Fig. 7 and data not shown).
Double homologous cross-over recombination was used to delete P. falciparum FabI and generate P. falciparum fabi− parasites. To assess blood stage growth, parasite cultures of both the WT NF54 and the fabi− clone E6 containing mainly ring stages were synchronized twice within 4 h using sorbitol (Lambros and Vanderberg, 1979). Parasite density was determined and the culture was diluted to 0.8% parasitaemia, 5% haematocrit. Cultures were maintained under an atmosphere of reduced oxygen at 37°C and medium was refreshed every 24 h. Growth, based on percentage parasitaemia, was monitored by Giemsa-stained thin blood smears every 24 h for 5 days. The experiment was carried out in triplicate and mean and standard deviations are shown. The results show that there are no significant differences in the growth of the two parasite lines.

shown) in the growth assay, which strongly suggests that, as for P. yoelii, the FAS II pathway is dispensable for P. falciparum blood stage growth.

Discussion

Although many metabolic functions have been assigned to the Plasmodium apicoplast (Ralph et al., 2004), including FAS II, it is not known if these pathways are essential for every life cycle stage of the malaria parasite. As the parasite inhabits both extracellular and intracellular niches and replicates in the mosquito vector and mammalian host, its ability to scavenge host nutrients to support replication presumably varies greatly depending on its environment. The study presented herein analyses for the first time the importance of a metabolic pathway throughout the parasite life cycle. We demonstrated that apicoplast-targeted FAS II is only necessary for Plasmodium late liver stage development. Thus, in all other life cycle stages either the parasite synthesizes fatty acids by a yet unidentified de novo pathway or the parasite is able to scavenge all the fatty acids it requires from the host. Moreover, FAS II is only needed late in liver stage schizogony. We have previously shown that the liver stage PV membrane-resident protein UIS3 interacts with the hepatocyte lipid carrier liver-fatty acid binding protein (Mikolajczak et al., 2007). It is possible that this interaction allows for the transfer of lipids from the hepatocyte cytosol to UIS3 and subsequently to the developing liver stage and this concept has recently been buoyed by the fact that P. falciparum UIS3 cocrystallizes with the lipid phosphatidylethanolamine (Sharma et al., 2008). Thus, the parasite liver stage might have developed a means of directly transferring lipids from its host. Nevertheless, host lipids alone are clearly not sufficient for completion of liver stage development. In the malaria model under study, no difference was seen in the first half of liver stage development (up to 24 h pi) between WT and fabb/f− parasites and it was only after 24 h that the fabb/f− liver stages showed growth retardation, accompanied by lack of cytomere formation and subsequent merozoite differentiation. Thus, during the complete P. yoelii life cycle, FAS II is required only for the final stages of parasite transition from its first site of infection in the liver to the blood. We do not currently know why FAS II is only necessary for late liver stage development. It is possible that the sheer amount of membrane biogenesis required for the formation of tens of thousands of merozoites (Baer et al., 2007) cannot be met by host lipid scavenging and thus also relies on parasite-derived fatty acid synthesis to give a final boost to the formation of the merozoite membrane phospholipid bilayers. Alternatively, FAS II could be necessary to provide a particular fatty acid that is necessary for late liver stage development. In the sleeping sickness parasite, Trypanosoma brucei, the bloodstream form evades the host’s immune response by expressing continually switching variant surface glycoprotein molecules (Donelson, 2003). The variant surface glycoprotein is attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor whose fatty acids are exclusively myristate (Ferguson and Cross, 1984). The continuous supply of myristate cannot be met by the bloodstream and T. brucei has a unique de novo fatty acid synthesis pathway to supply its myristate needs (Lee et al., 2006). Perhaps Plasmodium FAS II is fulfilling a similar need by supplying particular fatty acids necessary for late liver stage schizogony that the parasite cannot obtain from its host. It is of interest to note that MSP1 is a GPI-anchored protein (Gerold et al., 1996). However, we currently do not know if GPI biosynthesis is abrogated in FAS II-deficient liver stages. Notwithstanding, we observed lack of MSP1 in liver stages of FAS II knockout parasites, suggesting that MSP1 could play an essential role in the formation of exo-erythrocytic merozoites.

Our data show that some FAS II enzymes are initially expressed in the salivary gland sporozoite based on both epitope tagging of FabI and our qRT-PCR data. This also revealed a high transcript abundance for FabI and FabB/F in salivary gland sporozoites although this was not the case for FabG and FabZ. We currently do not understand the functional significance of these increases; nevertheless, the pathway is clearly not necessary in this life cycle stage as both fabb/f− and fabz− sporozoites infected the mosquito salivary glands and were able to initiate liver stage infection. Previously, it was shown that depletion of
Plasmodium type II fatty acid synthesis

UIS3 and UIS4 (Mueller et al., 2005a,b), proteins that are initially expressed in sporozoites and localize to the PV membrane during liver stage development, as well as the sporozoite proteins P52 and P36 (van Dijk et al., 2005; Ishino et al., 2005; Labaied et al., 2007), and the sporozoite asparagine-rich protein 1 (Aly et al., 2008), cause arrest early in liver stage development. However, depletion of these proteins, as shown here for FabB/F and FabZ, does also not affect salivary gland sporozoite maturation. Furthermore, the liver stage phenotype for fabb/f− parasites is unique, when compared with the above as the parasites do not arrest early in liver stage development but grow substantially and undergo schizogony. At 52 h pi, the fabb/f− liver stages still seemed viable, based on the presence of an intact PV membrane (visualized by Hep17 expression). Nevertheless, liver stage development is not completed and the parasites might be cleared by host defense mechanisms although this requires further investigation.

Our studies have also shown that FAS II is not necessary for either the mosquito stage or blood stage of the P. yoelii life cycle. It is currently not known how the parasite utilizes host lipids whilst developing on the mosquito midgut but it is well documented that host serum fatty acids are utilized by parasite blood stages for growth (Krishnegowda and Gowda, 2003; Mi-Ichi et al., 2006). Our results appear to contradict previous work demonstrating that triclosan, an inhibitor of FabI (McMurry et al., 1998), is able to kill cultured P. falciparum blood stages and in vivo rodent blood stage infections of P. berghei (Surolia and Surolia, 2001). Others have also concluded that FAS II inhibitors are directly interacting with their apicoplast targets in Plasmodium blood stages (Surolia et al., 2004; Jones et al., 2005; Tasdemir et al., 2006; Goodman et al., 2007), thereby inhibiting parasite growth. However, it is possible that the FAS II inhibitors used are having off target effects on parasite growth. This has previously been shown in T. brucei, where it was concluded that triclosan killing may be due to a non-specific perturbation of subcellular membrane structure leading to dysfunction in sensitive membrane-resident biochemical pathways (Paul et al., 2004). Furthermore, studies of the effect of triclosan on several microorganisms have concluded that the interaction of triclosan with the bacterial cell is complex and its lethality cannot be explained solely by the inhibition of metabolic pathways such as FAS II (Escalada et al., 2005). Thus, triclosan could be killing P. falciparum blood stages by inhibiting a vital process other than FAS II. It has recently been shown based on transcriptional data obtained from malaria patient isolates, that there appears to be three distinct P. falciparum blood stage physiological states (Dailly et al., 2007). These three states closely resemble (i) active growth; (ii) starvation; and (iii) environmental stress. In the starvation state, the authors noted an upregulation of FAS II genes when compared with active growth. These data suggest that under active glycolytic growth conditions, which were similar to the P. falciparum 3D7 growth conditions in vitro for which transcriptome profiles have been previously published (Bozdech et al., 2003; Le Roch et al., 2003), FAS II is not significantly involved in blood stage replication.

This study has concentrated on the effect of FAS II depletion on liver stage development in the rodent malaria parasite P. yoelii. However, we have also shown that the deletion of FabI from the human malaria parasite, P. falciparum has no apparent effect on blood stage replication when compared with WT parasites. This result, which converges on the results we obtained for the deletions of P. yoelii FabB/F and FabZ, demonstrates that FAS II is not required in blood stages. Future work might determine whether deletion of FabI affects P. falciparum sporozoite infectivity in humans; however, this requires a clinical investigation. Collectively, the data suggest that the metabolic pathways present in the Plasmodium apicoplast are not always necessary to support parasite progression through the specific parts of the life cycle. Thus, this unique organelle is likely to perform its critical metabolic functions at only certain time points during the Plasmodium life cycle and the functions it performs will be directly related to the needs the parasite cannot fulfil by nutrient uptake from the host. The Plasmodium apicoplast, as well as being the centre for FAS II, is also thought to harbour the only pyruvate dehydrogenase complex the parasite possesses (Foth et al., 2005). It is possible that pyruvate dehydrogenase is solely required by the apicoplast for the formation of acetyl CoA which is subsequently utilized by FAS II. Further studies are needed to address this issue.

Although our findings concerning liver stage development were generated with the rodent malaria parasite P. yoelii, the high conservation of FAS II among Plasmodium species (Carlton et al., 2002) suggests that FAS II is also essential for P. falciparum liver stage development. This might have consequences for the direction of anti-malaria FAS II inhibitor drug development. Rather than concentrating on the P. falciparum blood stage, research into FAS II inhibitors should concentrate on their efficacy against the initial, clinically silent liver stage of infection. This might significantly contribute to the goal of eradicating malaria.

**Experimental procedures**

**Experimental animals**

Six- to eight-week-old female Swiss Webster (SW) mice and female BALB/c mice were purchased from Harlan (Indianapolis, IN). Animal handling was conducted according to institutional animal care and use committee-approved protocols.
Parasite isolation

*Plasmodium yoelii* (17XNL) liver stage-infected hepatocytes were isolated at four time points post infection from infected mice: 12 h (LS-12), 24 h (LS-24), 40 h (LS-40) and 50 h (LS-50). Sporozoites were isolated from mosquito salivary glands at day 15 after infectious blood meal. Contaminating mosquito tissue was removed from sporozoite preparation by passing the extract over a DEAE cellulose column. For the preparation of parasites in the mixed blood stages, blood was harvested from infected SW mice when parasitaemia was at 5–10%. Lymphocytes were removed by passing the infected blood through a Sephadex column. Puriﬁed blood stage schizonts were prepared from NycoDenz puriﬁcation of *P. yoelii* infected blood cultured for 12 h.

Quantitative real-time PCR

Total RNA from each sample was extracted using Trizol (Invitrogen) and DNase treated using Turbo-DNA free (Ambion). Total RNA was then subjected to two rounds of linear ampliﬁcation using the Amino Allyl Message Amp II aRNA Ampliﬁcation Kit (Ambion) according to manufacturer’s directions. First-strand cDNA was synthesized from 500 ng of ampliﬁed RNA (aRNA) using the Superscript III Platinum RT kit (Invitrogen). The resulting cDNA was diluted 1:5 with nuclease-free water. Primers (Table S2) were designed using Primer Express v3.0 (Applied Biosystems). Designs were based on the mRNA sequence of the genes available at PlasmIDB. Amplicons were set to be between 100 and 200 bp. Real-time PCR analysis was performed on ABI prism 7300 Sequence Detection Systems using the SYBR Green PCR Master Mix (Applied Biosystems). The PCR reaction consisted of 12.5 μl of SYBR Green PCR Master Mix, 20 pmole of forward and reverse primers and 5 μl of diluted cDNA in a total volume of 25 μl. PCR cycling conditions were performed using the default conditions of the ABI Prism 7300 SDS Software.

Quantification of gene expression was done using the Relative Standard Curve Method (Applied Biosystems bulletin). The standard is prepared from a mixture of aRNA (salivary gland sporozoites, blood stages and liver stages) in a 1:1:1 ratio. First-strand cDNA is prepared from the standard and dilutions of 1:1, 1:5, 1:10, 1:20 and 1:50 of the resulting cDNA was used as templates for real-time PCR for each primer pair. The relative quantity of gene in the cDNAs from the seven aRNA samples (salivary gland sporozoites, LS-12, LS-24, LS-40, LS-50, mixed blood stages and blood stage schizonts) is interpolated from the correspond-}

In vitro analysis of *PyFabZ-myc, PyFabG-myc and PyFabZ-myc liver stages

*In vitro* assays were conducted using the human hepatoma cell line HepG2 expressing the tetraspanin CD81 (HepG2:CD81) cultured in Dulbecco’s modiﬁed Eagle’s medium with 10% fetal calf serum at 37°C and 5% CO2. Infections were done by adding 5 × 10^6 sporozoites to individual chambers of an 8 well chamber slide (Laboratory-Tek Permanox eight-well chamber slide; Nalge Nunc International, Rochester, NY) which had been seeded with 10^5 subconfluent HepG2:CD81 cells the previous day. The slide was then centrifuged at 500 g for 2.5 min to aid sporozoite infection. Sporozoites which had failed to invade cells were removed after 2 h and the media were replaced. For the liver stage develop-}

Generation of *P. yoelii* transgenic parasites expressing FabI-myc, FabZ-myc and FabG-myc

To epitope tag FabI, a quadruple (4×) myc tag sequence followed by a stop codon was introduced into the b3D.DT^H^D vector (Catalog # MRA-80 in the MR4 Malaria Research and Refer-}

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using *P. yoelii* 17XLN genomic DNA as a template. The two fragments were cloned into the b3D.DT+H-D targeting vector between the *T. gondii* DHFR/TS gene, which allows selection of recombination events with pyrimethamine. The plasmid was transfected into *P. yoelii* 17XLN blood stage schizonts using standard procedures (Labaied et al., 2007). Two independent clones of fabb/f− and fabz− parasites were obtained by limited dilution of parentals from independent transfection experiments. A graphic representation of the construction of the plasmid and its subsequent transfection into *P. yoelii* blood stages is given in Fig. 3 and the primers used is Table S2.

**Phenotypic analysis of blood stage *P. yoelii* fabb/f− and fabz− parasites**

To assay growth of non-letal *P. yoelii* 17XLN WT, fabb/f− and fabz− blood stages, blood was removed from infected SW mice when parasitaemia was between 0.5% and 1.5%. The blood was diluted in RPMI-1640 media (HyClone, Logan, UT) so that 100 μl contained either 10^5 or 10^6 parasites. SW mice (four in each group) were then injected iv with 10^5 or 10^6 parasites (WT or fabb/f−). Percentage parasitaemia was followed as often as daily until clearance, by assay of Giemsa-stained blood smear.

**Phenotypic analysis of *P. yoelii* fabb/f− and fabz− parasites in the mosquito**

*Anopheles stephensi* mosquitoes were infected with *P. yoelii* WT, fabb/f+ or fabz− parasites by blood feeding for 6 min on the first and second day using infected SW mice and subsequently maintained under a cycle of 12.5 h light/11.5 h dark and 70% humidity at 24.5°C. Gametocyte exflagellation capacity was evaluated microscopically before mosquito blood meal. Infected mosquitoes were dissected (at least 20 mosquitoes for each dissection) at days 10 and 14 (after the first infectious blood meal) to determine the presence of midgut oocyst sporozoites and the numbers of salivary gland sporozoites respectively.

**In vivo analysis of *P. yoelii* fabb/f− liver stage development**

To analyse in vivo sporozoite infection and liver stage development, BALB/c mice were injected iv with 10^6 WT or fabb/f− sporozoites. For each parasite population, the livers were harvested from euthanized mice at several time points post infection (12, 24, 44 and 52 h). Livers were perfused with PBS, washed extensively with PBS and then fixed in 4% paraformaldehyde. Liver lobes were cut into 50 μm sections using a Vibratome apparatus (Ted Pella Inc., Redding, CA). For IFA, sections were permeabilized in Tris buffered saline (TBS) containing 3% H2O2 and 0.25% Triton X-100 for 30 min at room temperature. Sections were then blocked in TBS containing 5% dried milk (TBS-M) at least 1 h and incubated with primary antibody in TBS-M at 4°C overnight. Primary antibodies used were mouse monoclonal anti-circumsporozoite protein, mouse monoclonal anti-Hep17 (Charoenavit et al., 1995) and rabbit polyclonal anti-MSP1. For washing in TBS, secondary antibody was added in TBS-M for 2 h at room temperature in a similar manner as above. After further washing, the section was incubated in 0.06% KMnO4 for 10 min to quench background fluorescence. The section was then washed with TBS and cells were stained with DAPI to visualize the DNA and mounted with FluoroGuard anti-fade reagent (Bio-Rad, Hercules, CA). Preparations were analysed as above for fluorescence with the addition of acquisition of a differential interference contrast image.

**Comparison of WT and fabb/f− liver stage growth**

To compare the sizes of parasite liver stages at 44 h pi, liver sections labelled with Hep17 (see above) were sequentially scanned using Nikon fluorescence microscopy. The greatest diameter for each liver stage detected was determined by adjusting the z plane of the liver section and the area of the parasite was subsequently determined. For the WT, 251 liver stages were assayed and for the fabb/f−, 147. All parasite segments were less than 3000 μm^2 and the total numbers of parasites were divided into quartiles by area.

**Generation of *P. falciparum* fabi− parasites**

Targeting sequences 5′ and 3′ to *P. falciparum* Fab1 (PFF0730c) were cloned into plasmid pC71 to facilitate positive–negative selection (Maier et al., 2006) (Fig. S4). Restriction sites in the multiple cloning site were SacI/SpeI for the 5′ flank and AvrII/EcoRI for the 3′ flank. Sequencing was performed to confirm inserts and primers used are detailed in Table S2. Plasmid DNA was extracted by maxi prep kit (Qiagen). The NF54 line *P. falciparum* parasites were synchronized at ring stage with sorbitol 2 days prior to transfection. On the following day trophozoites were selected for WT cytoadherence properties by incubation in RPMI plus Gelofusine (Braun). Transfection of *P. falciparum* ring stages with 100 μg of DNA was performed by electroporation at 0.31 kV and 950 μF with a Bio-Rad Gene Pulser (Bio-Rad, La Jolla, CA). Cultures were placed on the positive selection drug WR99210 (Jacobus Pharmaceuticals, Princeton, NJ) 6 h post transfection and maintained as described (Crabb et al., 2004). This was followed by negative selection against the cytosine deaminase/uracil phosphoribosyl transferase gene product with 5-fluorocytosine in order to obtain a parental line with double cross-over homologous recombination, which results in specific FabI gene deletion.

Two individual clones with a FabI deletion were isolated (E6 and G8) and genotypic analysis was confirmed by Southern Blot (Fig. S4). Genomic DNA from WT NF54 and knockout lines was digested for 2–16 h with the following enzymes: 5′ test: BglII/BamHI and 3′ test: BglII/BamHI. Digested DNA was run on a 1% TAE agarose gel at 15 V for 18 h and transferred to Hybond-N membrane (Amersham) overnight at room temperature, UV cross-linked and pre-hybridized with herring sperm DNA for 2.5 h. A digoxigenin-labelled probe was prepared by PCR per supplier protocol (Roche) using the cloning primers. Hybridization was carried out for 18 h at 55°C. The blot was exposed to film for 10–60 min and developed per standard protocol.

**Assessment of *P. falciparum* growth**

Parasite cultures of both the WT NF54 and the fabi− clone E6 containing mainly ring stages were synchronized twice within 4 h
using sorbitol (Lambros and Vanderberg, 1979). Parasite density was determined and the culture was diluted to 0.8% parasitaemia, 5% haematocrit. Cultures were maintained under an atmosphere of reduced oxygen at 37°C and medium was refreshed every 24 h. Growth was monitored by Giemsa-stained thin blood smears every 24 h and for each determination of percentage parasitaemia, the number of infected erythrocytes per at least 2000 erythrocytes was recorded.

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References

Aly, A.S., Mikolajczak, S.A., Rivera, H.S., Camargo, N., Jacobs-Lorena, V., Labaied, M., et al. (2008) Targeted deletion of SAP1 abolishes the expression of infectivity factors necessary for successful malaria parasite liver infection. Mol Microbiol 69: 152–163.

Baer, K., Klotz, C., Kappe, S.H., Schnieder, T., and Frevert, U. (2007) Release of hepatic Plasmodium yoelii merozoites into the pulmonary microvasculature. PLoS Pathog 3: e171.

Bahl, A., Brunk, B., Crabtree, J., Fraunholz, M.J., Gajria, B., Grant, G.R., et al. (2003) PlasmoDB: the Plasmodium genome resource. A database integrating experimental and computational data. Nucleic Acids Res 31: 212–215.

Bozdech, Z., Linhas, M., Pulliam, B.L., Wong, E.D., Zhu, J., and DeRisi, J.L. (2003) The transcriptome of the intraerythrocytic developmental cycle of Plasmodium falciparum. PloS Biol 1: E5.

Carlton, J.M., Angiuli, S.V., Suh, B.B., Kooij, T.W., Pertea, M., Silva, J.C., et al. (2002) Genome sequence and comparative analysis of the model rodent malaria parasite Plasmodium yoelii yoelii. Nature 419: 512–519.

Charoenvit, Y., Mellouk, S., Sedegah, M., Toyoshima, T., Leef, M.F., De la Vega, P., et al. (1995) Plasmodium yoelii: 17-kDa hepatic and erythrocytic stage protein is the target of an inhibitory monoclonal antibody. Exp Parasitol 80: 419–429.

Cowman, A.F., and Crabb, B.S. (2006) Invasion of red blood cells by malaria parasites. Cell 124: 755–766.

Crabb, B.S., Rug, M., Gilberger, T.W., Thompson, J.K., Triglia, T., Maier, A.G., and Cowman, A.F. (2004) Transfection of the human malaria parasite Plasmodium falciparum. Methods Mol Biol 270: 263–276.

Daily, J.P., Scanfeld, D., Pochet, N., Le Roch, K., Plouffe, D., Kamal, M., et al. (2007) Distinct physiological states of Plasmodium falciparum in malarial-infected patients. Nature 450: 1091–1095.

van Dijk, M.R., Douradinha, B., Franke-Fayard, B., Heussler, V., van Dooren, M.W., van Schaikj, B., et al. (2005) Genetically attenuated, P36p-deficient malarial sporozoites induce protective immunity and apoptosis of infected liver cells. Proc Natl Acad Sci USA 102: 12194–12199.

Donelson, J.E. (2003) Antigenic variation and the African trypanosome genome. Acta Trop 85: 391–404.

van Dooren, G.G., Marti, M., Tonkin, C.J., Stimmier, L.M., Cowman, A.F., and McFadden, G.I. (2005) Development of the endoplasmic reticulum, mitochondrion and apicoplast during the asexual life cycle of Plasmodium falciparum. Mol Microbiol 57: 405–419.

Escalada, M.G., Russell, A.D., Maillard, J.Y., and Ochs, D. (2005) Triclosan–bacteria interactions: single or multiple target sites? Lett Appl Microbiol 41: 476–481.

Ferguson, D.J., Campbell, S.A., Henriquez, F.L., Phan, L., Mui, E., Richards, T.A., et al. (2007) Enzymes of type II fatty acid synthesis and apicoplast differentiation and division in Eimeria tenella. Int J Parasitol 37: 33–51.

Ferguson, M.A., and Cross, G.A. (1984) Myristylation of the membrane form of a Trypanosoma brucei variant surface glycoprotein. J Biol Chem 259: 3011–3015.

Foth, B.J., Ralph, S.A., Tonkin, C.J., Struck, N.S., Furnholz, M., Roos, D.S., et al. (2003) Dissecting apicoplast targeting in the malaria parasite Plasmodium falciparum. Science 299: 705–708.

Foth, B.J., Stimmier, L.M., Handman, E., Crabb, B.S., Hodder, A.N., and McFadden, G.I. (2005) The malaria parasite Plasmodium falciparum has only one pyruvate dehydrogenase complex, which is located in the apicoplast. Mol Microbiol 59: 39–53.

Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., et al. (2002) Genome sequence of the human malaria parasite Plasmodium falciparum. Nature 419: 498–511.

Gerold, P., Schofield, L., Blackman, M.J., Holder, A.A., and Schwarz, R.T. (1996) Structural analysis of the glycosylphosphatidylinositol membrane anchor of the merozoite surface proteins-1 and -2 of Plasmodium falciparum. Mol Biochem Parasitol 75: 131–143.

Goodman, C.D., Su, V., and McFadden, G.I. (2007) The effects of anti-bacterials on the malaria parasite Plasmodium falciparum. Mol Biochem Parasitol 152: 181–191.

Gornicki, P. (2003) Apicoplast fatty acid biosynthesis as a target for medical intervention in apicomplexan parasites. Int J Parasitol 33: 885–896.

Ishino, T., Chinzei, Y., and Yuda, M. (2005) Two proteins with 6-cys motifs are required for malarial parasites to commit to infection of the hepatocyte. Mol Microbiol 58: 1264–1275.

Jones, S.M., Urch, J.E., Kaiser, M., Brun, R., Harwood, J.L., Berry, C., and Gilbert, I.H. (2005) Analogues of thiolytocin as potential antimalarial agents. J Med Chem 48: 5932–5941.

Kohler, S., Delwiche, C.F., Denny, P.W., Tilney, L.G., Webster, P., Wilson, R.J., et al. (1997) A plastid of probable green algal origin in apicomplexan parasites. Science 275: 1485–1489.

Krishnegowda, G., and Gowda, D.C. (2003) Intraerythrocytic Plasmodium falciparum incorporates extraneous fatty
acids to its lipids without any structural modification. Mol Biochem Parasitol 132: 55–58.
Labaied, M., Harupa, A.,Dumpit, R.F., Coppens, I., Mikolajczak, S.A., and Kappe, S.H. (2007) Plasmodium yoelii sporozoites with simultaneous deletion of P52 and P36 are completely attenuated and confer sterile immunity against infection. Infect Immun 75: 3758–3768.
Lambros, C., and Vanderberg, J.P. (1979) Synchronization of Plasmodium falciparum erythrocytic stages in culture. J Parasitol 65: 418–420.
Le Roch, K.G., Zhou, Y., Blair, P.L., Grainger, M., Moch, J.K., Haynes, J.D., et al. (2003) Discovery of gene function by expression profiling of the malaria parasite life cycle. Science 301: 1503–1508.
Lee, S.H., Stephens, J.L., Paul, K.S., and Englund, P.T. (2006) Fatty acid synthesis by elongases in trypanosomes. Cell 126: 691–699.
McCurry, L.M., Oethinger, M., and Levy, S.B. (1998) Tri-closan targets lipid synthesis. Nature 394: 531–532.
Maier, A.G., Braks, J.A., Waters, A.P., and Cowman, A.F. (2006) Negative selection using yeast cytosine deaminase/uracil phosphoribosyl transferase in Plasmodium falciparum for targeted gene deletion by double crossover recombination. Mol Biochem Parasitol 150: 118–121.
Mazumdar, J., and Striepen, B. (2007) Make it or take it: fatty acid metabolism of apicomplexan parasites. Eukaryot Cell 6: 1727–1735.
Mazumdar, J., Wilson, E.H., Masek, K., Hunter, C.A., and Striepen, B. (2006) Apicoplast fatty acid synthesis is essential for organelle biogenesis and parasite survival in Toxoplasma gondii. Proc Natl Acad Sci USA 103: 13192–13197.
Menard, R., and Janse, C. (1997) Gene targeting in malaria parasites. Methods 13: 148–157.
Mi-Ichi, F., Kita, K., and Mitamura, T. (2006) Intraerythrocytic Plasmodium falciparum utilize a broad range of serum-derived fatty acids with limited modification for their growth. Parasitology 133: 399–410.
Mikolajczak, S.A., Jacobs-Lorena, V., MacKellar, D.C., Camargo, N., and Kappe, S.H. (2007) L-FABP is a critical host factor for successful malaria liver stage development. Int J Parasitol 37: 483–489.
Mueller, A.K., Camargo, N., Kaiser, K., Andorfer, C., Frevert, U., Matuschewski, K., and Kappe, S.H. (2005a) Plasmodium liver stage developmental arrest by depletion of a protein at the parasite–host interface. Proc Natl Acad Sci USA 102: 3022–3027.
Mueller, A.K., Labaied, M., Kappe, S.H., and Matuschewski, K. (2005b) Genetically modified Plasmodium parasites as a protective experimental malaria vaccine. Nature 433: 164–167.
Paul, K.S., Bacchi, C.J., and Englund, P.T. (2004) Multiple triclosan targets in Trypanosoma brucei. Eukaryot Cell 3: 855–861.
Prudencio, M., Rodriguez, A., and Mota, M.M. (2006) The silent path to thousands of merozoites: the Plasmodium liver stage. Nat Rev Microbiol 4: 849–856.
Ralph, S.A., van Dooren, G.G., Waller, R.F., Crawford, M.J., Fraunholz, M.J., Foth, B.J., et al. (2004) Tropical infectious diseases: metabolic maps and functions of the Plasmodium falciparum apicoplast. Nat Rev Microbiol 2: 203–216.
Sato, S., and Wilson, R.J. (2005) The plastid of Plasmodium spp. a target for inhibitors. Curr Top Microbiol Immunol 295: 251–273.
Sharma, S., Sharma, S.K., Modak, R., Kamodiya, K., Surolia, N., and Surolia, A. (2007) Mass spectrometry-based systems approach for identification of inhibitors of Plasmodium falciparum fatty acid synthase. Antimicrob Agents Chemother 51: 2552–2558.
Sharma, A., Yogavel, M., Akhouri, R.R., Gill, J., and Sharma, A. (2008) Crystal structure of soluble domain of malaria sporozoite protein UIS3 in complex with lipid. J Biol Chem 283: 24077–24088.
Shortt, H.E., Fairley, N.H., Covell, G., Shute, P.G., and Garnham, P.C. (1951) The pre-erythrocytic stage of Plasmodium falciparum. Trans R Soc Tropl Med Hyg 44: 405–419.
Silvie, O., Rubinstein, E., Franetic, J.F., Prenant, M., Belnoue, E., Renia, L., et al. (2003) Hepatocyte CD81 is required for Plasmodium falciparum and Plasmodium yoelii sporozoite infectivity. Nat Med 9: 93–96.
Snow, R.W., Guerra, C.A., Noor, A.M., Myint, H.Y., and Hay, S.I. (2005) The global distribution of clinical episodes of Plasmodium falciparum malaria. Nature 434: 214–217.
Suhrbier, A., Holder, A.A., Wiser, M.F., Nicholas, J., and Sinden, R.E. (1989) Expression of the precursor of the major merozoite surface antigens during the hepatic stage of malaria. Am J Trop Med Hyg 40: 351–355.
Surolia, A., Ramya, T.N., Ramya, V., and Surolia, N. (2004) ‘FAS’T inhibition of malaria. Biochem J 383: 401–412.
Surolia, N., and Surolia, A. (2001) Triclosan offers protection against blood stages of malaria by inhibiting enoyl-ACP reductase of Plasmodium falciparum. Nat Med 7: 167–173.
Tarun, A.S., Dumpit, R.F., Camargo, N., Labaied, M., Liu, P., Takagi, A., et al. (2007) Protracted sterile protection with Plasmodium yoelii pre-erythrocytic genetically attenuated parasite malaria vaccines is independent of significant liver-stage persistence and is mediated by CD8+ T cells. J Infect Dis 196: 608–616.
Tarun, A.S., Peng, X., Dumpit, R.F., Ogata, Y., Silva-Rivera, H., Camargo, N., et al. (2008) A combined transcriptome and proteome survey of malaria parasite liver stages. Proc Natl Acad Sci USA 105: 305–310.
Tademedir, D., Lack, G., Brun, R., Ruedi, P., Scazzozza, L., and Perozzo, R. (2006) Inhibition of Plasmodium falciparum fatty acid biosynthesis: evaluation of FabG, FabZ, and FabI as drug targets for flavonoids. J Med Chem 49: 3345–3353.
Tsuij, M., Mattei, D., Nussenzweig, R.S., Eichinger, D., and Zavala, F. (1994) Demonstration of heat-shock protein 70 in the sporozoite stage of malaria parasites. Parasitol Res 80: 16–21.
Vial, H.J., and Ancelin, M.L. (1992) Malarial lipids. An overview. Subcell Biochem 18: 259–306.
Wallier, R.F., Keeling, P.J., Donald, R.G., Striepen, B., Handman, E., Lang-Unnasch, N., et al. (1998) Nuclear-encoded proteins target to the plastid in Toxoplasma gondii and Plasmodium falciparum. Proc Natl Acad Sci USA 95: 12352–12357.
Wiesner, J., and Seeber, F. (2005) The plastid-derived organelle of protozoan human parasites as a target of established and emerging drugs. Expert Opin Ther Targets 9: 23–44.
Yeoh, S., O’Donnell, R.A., Koussis, K., Dluzewski, A.R., Ansell, K.H., Osborne, S.A., et al. (2007) Subcellular discharge of a serine protease mediates release of invasive malaria parasites from host erythrocytes. *Cell* **131**: 1072–1083.

**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Integration of a second copy of FabI fused to a quadruple myc tag into the *P. yoelii* genome (PyFabI-myc). **(A)** The FabI gene, including 1 kb upstream of the start methionine and up to but not including the stop codon was amplified and ligated upstream of the quadruple myc tag (4× myc) in the 4× myc tag integration vector. Following linearization of the vector with BsaI, the construct was transfected into *P. yoelii* blood stage schizonts which were subsequently injected into mice. The vector contains the *T. gondii* DHFR/TS mutated gene as a pyrimethamine selectable marker (TgDHFR) and integrants were selected for by pyrimethamine treatment. **(B)** Ethidium bromide-stained agarose gel showing the integration of the FabI-myc vector into the *P. yoelii* genome. Only PyFabI-myc is positive for this test (‘int test’), whereas, as expected, both PyFabI-myc and wild-type parasite genomic DNA is positive for the FabI-specific open reading frame test (‘ORF test’). Note that the selected parasites have two copies of FabI, the wild-type copy and the 4× myc tagged copy, both with their endogenous promoter. PyFabI-myc should express a second copy of FabI with a myc epitope and the expression of the tagged protein should mimic that of the endogenous copy. The same integration strategy was used to generate PyFabB-myc and PyFabZ-myc.

**Fig. S2.** Lack of expression of quadruple-myc epitope-tagged FabI in the (A) developing midgut oocyst sporozoites and (B) free midgut sporozoites of the transgenic *P. yoelii* parasite PyFabI-myc. PyFabI-myc was generated to express a second copy of FabI under the control of its endogenous promoter with a C-terminal quadruple-myc tag. Expression of FabI in PyFabI-myc was monitored by immunofluorescence assay (IFA) using a rabbit anti-myc antibody. The oocyst and midgut sporozoites were detected with a mouse anti-circumsporozoite protein (CSP) antibody. Fluorescent staining was achieved with Alexa Fluor conjugated secondary antibodies (Alexa Fluor 488, green and Alexa Fluor 594, red) specific to rabbit and mouse IgG. Nuclear staining was achieved with 4′,6-diamidino-2-phenylindole (DAPI). Differential interference contrast (DIC) and fluorescent images were captured and processed using deconvolution microscopy and a merge of the captured images is presented on the far right pane (merge). Scale bar is 5 μm. FabI-myc expression was not detectable during sporozoite development in the mosquito.

**Fig. S3.** Successful deletion of *P. yoelii* FabB/F and FabZ by double cross-over homologous recombination to generate *P. yoelii* fabb/F and fabz− knockout parasites. **(A)** DNA fragments of approximately 800 base pairs spanning the 5′ and 3′ UTR of the *FabB/F* gene (PY04452) and *FabZ* gene (PY01586) were ligated into the b3D.DT^H.^D vector. The vector contains the *T. gondii* DHFR/TS mutated gene as a pyrimethamine selectable marker (TgDHFR). The vector was linearized with KpnI and SacII and transfected into *P. yoelii* blood stage schizonts which were then injected into SW mice. Double cross-over homologous recombination was selected for with pyrimethamine and confirmed by a positive PCR result using the primer sets ‘test 1’ and ‘test 2’ and a negative PCR result for the deleted gene (‘wt test’). **(B)** Ethidium bromide-stained agarose gel showing PCR products from the amplification of parasite genomic DNA from two clonal populations (clone 1 and clone 2) of the *FabB/F* gene deletion and wild-type (wt). **(C)** Similar to (B) but for the *FabZ* gene. The PCR results demonstrate the successful deletion of the genes because the ‘test 1’ and ‘test 2’ primer sets are positive only for the knockout population. Similarly, the wild-type test (‘wt test’) is positive only for the wild-type population. The result shows that the *FabB/F* and *FabZ* genes have been successfully deleted from blood stage *P. yoelii* parasites.

**Fig. S4.** Deletion of FabI in *P. falciparum*. **(A)** Targeting sequences 5′ and 3′ to *P. falciparum* FabI (PFF0730c) were cloned into plasmid pCC1 to facilitate positive-negative selection (Maier et al., 2006). Restriction sites in the multiple cloning site were SacII/SpeI for the 5′ flank and AvrII/EcoRI for the 3′ flank. Genomic DNA from WT NF54 and knockout lines (clones E6 and G8) were assayed by Southern Blot and using the 5′ and 3′ flanks as probes and the expected restriction fragments resulting from enzymatic digestion of the WT loci and KO loci are shown. **(B)** The fragments recognized by the 5′ probe of KpnI/BglII restricted DNA are 6.1 kb for the WT locus and 1.3 kb for the KO locus. **(C)** The fragments recognized by the 3′ probe of BglII/BamHII restricted DNA are 4.6 kb for the WT locus and 4.1 kb for the KO locus. **Table S1.** *P. yoelii* fabb/F and fabz− parasites develop normally in the mosquito.

Table S1. Oligonucleotide primers used in the study.

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