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Short Article

Host Cell Phosphatidylcholine Is a Key Mediator of Malaria Parasite Survival during Liver Stage Infection

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SUMMARY

During invasion, *Plasmodium*, the causative agent of malaria, wraps itself in a parasitophorous vacuole membrane (PVM), which constitutes a critical interface between the parasite and its host cell. Within hepatocytes, each *Plasmodium* sporozoite generates thousands of new parasites, creating high demand for lipids to support this replication and enlarge the PVM. Here, a global analysis of the total lipid repertoire of *Plasmodium*-infected hepatocytes reveals an enrichment of neutral lipids and the major membrane phospholipid, phosphatidylcholine (PC). While infection is unaffected in mice deficient in key enzymes involved in neutral lipid synthesis and lipolysis, ablation of rate-limiting enzymes in hepatic PC biosynthetic pathways significantly decreases parasite numbers. Host PC is taken up by both *P. berghei* and *P. falciparum* and is necessary for correct localization of parasite proteins to the PVM, which is essential for parasite survival. Thus, *Plasmodium* relies on the abundance of these lipids within hepatocytes to support infection.

INTRODUCTION

Lipids play key roles in many biological processes; ranging from a structural role in membranes to signaling, in addition to being sources of metabolic energy (Bohdanowicz and Grinstein 2013; van Meer and Sprong 2004; van Meer et al., 2008).

Malaria infection is initiated when *Plasmodium* sporozoites enter the mammalian host through the bite of an infected female *Anopheles* mosquito. During a blood meal, ~10–100 sporozoites are deposited under the skin of the host and travel to the liver, where they infect hepatocytes. Each sporozoite resides in a hepatocyte for 2–14 days (2 days for *P. berghei* and ~7 days for *P. falciparum*), multiplying into >10,000 merozoites, which are then released in the bloodstream to infect red blood cells, initiating the symptoms of malaria (Prudêncio et al., 2006). The rapid replication of *Plasmodium* parasites in hepatocytes requires important lipid resources to support organelle and membrane neogenesis, the growth of the parasitophorous vacuole membrane (PVM), and possibly the maintenance of host cell and parasite homeostasis and survival (Prudêncio et al., 2006). Such demand is likely to be satisfied by import of hepatocyte lipids, as well as by de novo synthesis by the apicoplast fatty acid synthesis II (FAS II) system (Ralph et al., 2004) and the plethora of parasite-encoded phospholipid biosynthetic enzymes (Déchamps et al., 2010).

Transcriptomic studies revealed that the apicoplast-resident enzymes involved in the FAS II system are upregulated throughout liver stage infection (Tarun et al., 2008). While these and other enzymes of the pyruvate dehydrogenase complex are critical for the formation of infective merozoites, parasites lacking these enzymes initiate replication in the liver normally (Pei et al., 2010; Vaughan et al., 2009; Yu et al., 2008). Likewise, parasites deficient in octanoyl-ACP transferase or lipoic acid protein ligase (LipB), a limiting enzyme in the derivation of lipoic acid from a major FAS II product, octanoyl-ACP, show a similar phenotype. In addition, *P. yoelii* parasites deficient in glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate acyltransferase, key enzymes in the synthesis of the phospholipid precursor phosphatidic acid, develop normally, but again do not form merozoites (Lindner et al., 2014). These data imply that, despite the ability to synthesize fatty acids de novo, *Plasmodium* depends on host lipids during part or the entire pre-erythrocytic cycle.

Our previous work revealed that host genes involved in lipid metabolism are transcriptionally modulated during *Plasmodium* intrahepatic development (Albuquerque et al., 2009). Also, scavenger receptor binding protein 1, a membrane protein important for cellular cholesterol homeostasis, is key for in vitro infection (Rodrigues et al., 2008; Yalaoui et al., 2008). *Plasmodium* parasites scavenge cholesterol from the host irrespective of whether it has been internalized via the LDL receptor or synthesized de novo. Inhibition of either source of host cholesterol decreased the cholesterol content in merozoites but did not have any effect on liver stage development. On the other hand, scavenging of

lipoic acid from the host cell into parasite mitochondria was shown to be critical for *P. berghei* survival in hepatocytes (Allary et al., 2007; Deschermeier et al., 2012). Despite these advances, the contribution of host cell lipid metabolic pathway(s) to the establishment of a successful infection in hepatocytes is largely unexplored.

Aiming at understanding the dynamics of lipids during *Plasmodium* liver stage infection, we performed shotgun mass spectrometry analysis of the total cellular lipidome in *P. berghei*-infected versus noninfected cells at different points throughout infection. These analyses revealed major alterations in lipids involved in storage and membrane biogenesis, including phosphatidylcholine (PC), one of the major membrane phospholipids. Combining targeted silencing of host genes involved in de novo PC synthesis with visualization of host PC, we show that *Plasmodium* uptakes host-derived PC and that the activity of the two host de novo PC synthesis pathways is critical for the establishment of *Plasmodium* in the mammalian liver.

RESULTS

Lipid Composition of *P. berghei*-Infected Hepatocytes Is Altered during Infection

To assess whether changes in gene expression of major lipid biosynthetic pathways in the host and the parasite transcriptomes (Albuquerque et al., 2009; Tarun et al., 2008) correlated with changes in the total lipidome of hepatocytes upon infection with *Plasmodium* sporozoites, we performed quantitative Shotgun Lipidomics experiments on *P. berghei*-infected and noninfected Huh7 hepatoma cells (Figure 1A). Initial mass spectrometry analysis of noninfected Huh7 cells showed that the amount of lipids extracted was proportional to the number of cells used and that the minimal number of cells necessary to detect major lipid classes was 10^4 cells (Figure S1 available online). Due to the low infectivity of *Plasmodium* sporozoites, we isolated GFP-expressing *P. berghei*-infected and noninfected cells by fluorescence-activated cell sorting (FACS) (Prudêncio et al., 2008) at 25, 35, and 45 hr (h) after infection, which are representative time points of early parasite replication, liver schizogony, and the early cellularization process leading to the formation of individual merozoites. The number of cells used in this study ranged from 4.5 to 30 × 10^4 per sample. The relative abundance or total abundance of each lipid class was expressed as molar percentage of total lipidome or normalized as the total lipid per number of cells at any given time, respectively (Figure S1).

Major and significant alterations in the lipidome of *P. berghei*-infected cells were observed (see Table S1 for entire raw data). The neutral lipids triacylglycerol (TAG), diacylglycerol (DAG), and cholesterol esters (CEs) were increased at 25 hr after infection; however, at later time points their levels had subsided to those of control cells (Figures 1B and S1). Additionally, an enrichment of PC, the main structural membrane phospholipid, was observed in infected cells throughout infection, concomitant with a persistent and significant decrease in the levels of all anionic phospholipids: phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidic acid (PA), and phosphatidylinositol (PI) (Figure 1B). Altogether, the lipidomic data suggest that key aspects of hepatic lipid metabolism such as lipogenesis/lipolysis, in addition to phospholipid headgroup remodeling pathways, are actively engaged during *Plasmodium* infection of hepatocytes.

Plasmodium Liver Stage Infection Does Not Require Host De Novo Synthesized TAG and CE

Our lipidomic analyses revealed a significant enrichment in the neutral lipids TAG, CE, and DAG in *P. berghei*-infected cells at 25 hr after infection, although this was no longer the case at later time points (Figure 1B). Both TAG and CE are stored in organelles called lipid droplets (LDs) (Listenberger et al., 2003). During periods of increased lipogenesis, de-novo-synthesized or medium-derived fatty acids are channeled either into the glycerol-3-phosphate pathway to form DAG, which are then converted to TAG by DGAT1 or DGAT2 (Shi and Cheng 2009), or conjugated to free cholesterol by acyl-CoA: cholesterol acyltransferase (ACAT1/2) to form CE (Chang et al., 2001) (Figure S2). During periods of high-energy demand, fat stored in LDs is catabolized by neutral lipases to liberate free fatty acids (Figure S2). To assess the functional relevance of the observed changes, we first examined *P. berghei* infection in mice deficient in enzymes involved in CE and TAG synthesis. *P. berghei* load was similar in the livers of *ACAT1*$^{-/-}$ and *ACAT2*$^{-/-}$, when compared to littermate wild-type (WT) controls (Figure S2). Additionally, knockdown of *DGAT1* and *DGAT2* using siRNA in Huh7 cells did not influence the level of infection (Figure S2), in spite of a strong reduction in both TAG and CE in cells with reduced expression of DGAT2 (Figure S2). Finally, due to the decline in TAGs at 35 hr and 45 hr after infection, we also determined whether hydrolysis of TAGs in *P. berghei*-infected cells could provide building blocks for pathways active at later stages of liver stage infection. However, *P. berghei* liver infection was not affected in mice deficient in ATGL (Zimmermann et al., 2004) when compared to WT controls, in spite of the visible increase in LD size and a significant accumulation of TAG, as shown by oil red O staining and MS analysis, respectively (Figure S2). Taken together, these data suggest that *Plasmodium* liver stage infection is independent of host CE biosynthesis and TAG biosynthetic and lipolytic pathways.

Both Arms of Host Cell De Novo PC Synthesis Contribute to *Plasmodium* Liver Stage Infection Persistence

Since PC was highly enriched in infected cells (Figures 1B and S1), we next investigated whether this phospholipid could play a role during *Plasmodium* intrahepatic parasitism. The bulk of PC (as well as PE) is synthesized by the Kennedy pathway for which choline phosphate cytidyltransferase (PCYTα or CTα) is the rate-limiting enzyme (Kennedy and Weiss 1956) (Figure 2A). Strikingly, downregulation of host cell CTα using siRNA in Huh7 cells (Figure S3) greatly reduced *P. berghei* infection (Figure 2A). The parasite liver load 48 hr after intraveneous injection of *P. berghei* sporozoites was also significantly lower in CTα liver-specific deficient mice (CTα-LKO) (Jacobs et al., 2004), as compared to that of CTα-flox littermate mice (Figure 2B). We further characterized the effect of CTα depletion on infection by immunofluorescence microscopy analysis of thick liver sections. The number of *P. berghei* exo-erythrocytic forms (EEFs) was significantly reduced in CTα-LKO liver sections compared to CTα-flox controls (Figure 2C), with no differences in EEF

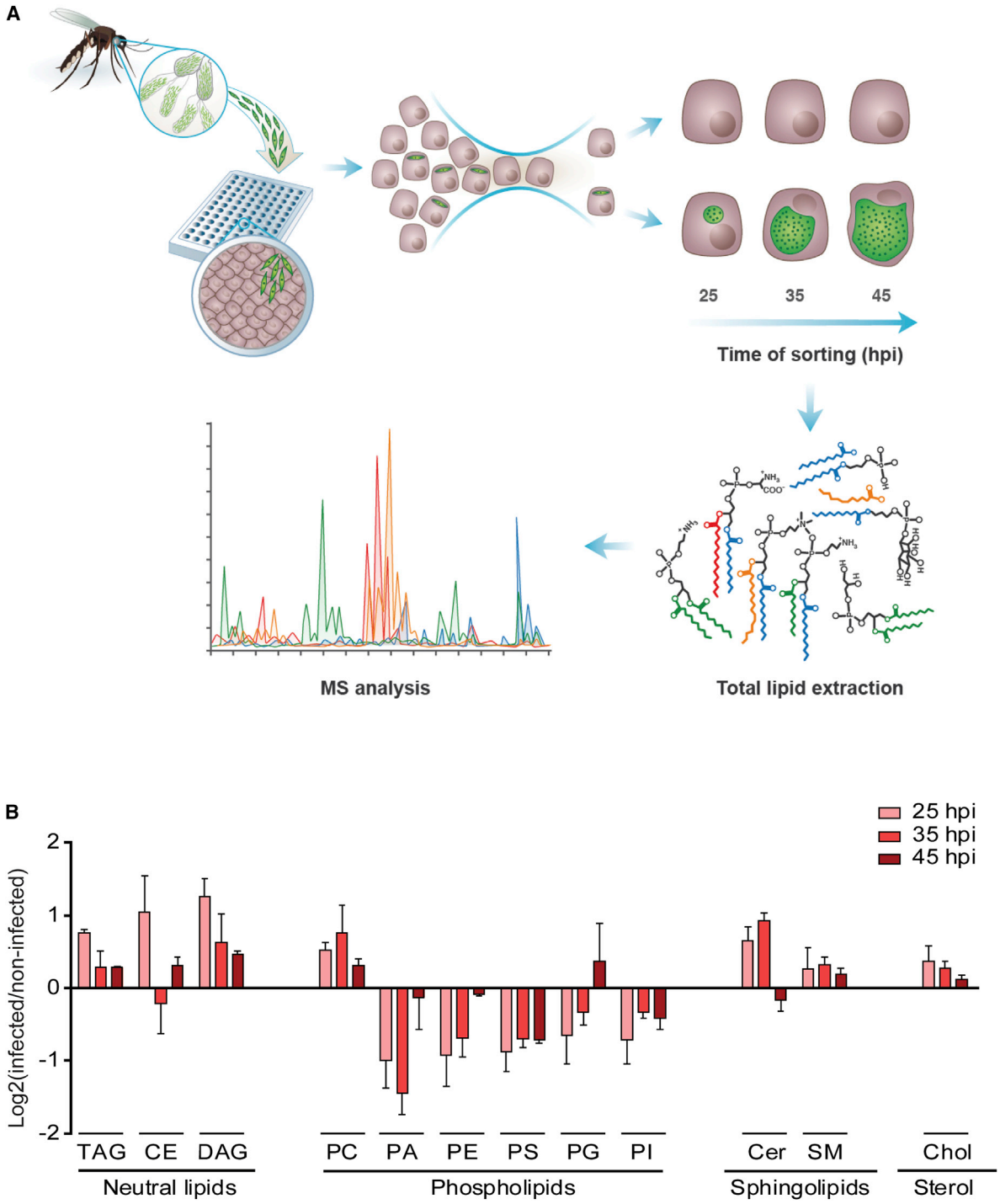

Figure 1. The Lipid Composition of *Plasmodium*-Infected Hepatoma Cells Is Altered during Infection

(A) Schematic representation of the approach for quantifying lipids in *P. berghei*-infected and noninfected cells: after FACS, cells were spiked with known concentrations of internal standards, and total cellular lipids were extracted and analyzed by shotgun ESI-MS.

(B) Relative abundance of major lipid classes in infected and noninfected cells at 25, 35, and 45 hr postinfection (hpi) are presented in $\log_2$ (infected/noninfected cells). Error bars represent SEM of each lipid from three to four biological replicates for each time point. Unpaired student t test was used to analyze statistical significance of differences in the abundance of each lipid in infected cells compared to noninfected cells at the same point: *$p < 0.05$

size (Figures 2D and 2E). Importantly, this effect on parasite numbers could not be ascribed to a defect in initial invasion of hepatocytes, as there was no difference in liver infection at 6 hpi between CTα-LKO and CTα-flox littermate mice (Figure 2F). The decrease in infection in CTα-LKO mice only became apparent at 24 hpi (Figure 2F).

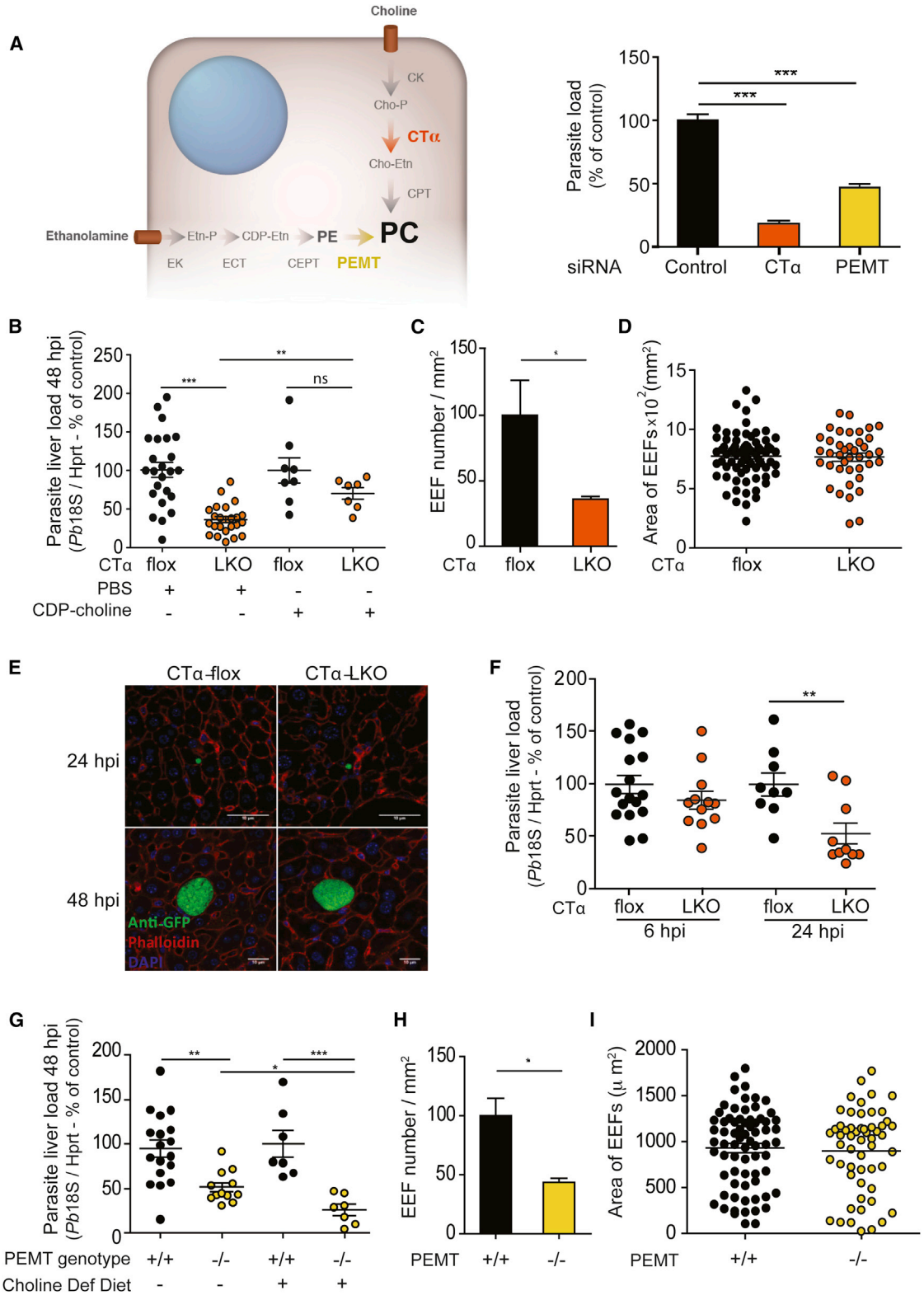

Figure 2. *P. berghei* Infection Is Significantly Impaired in CTα–Deficient Hepatocytes

(A) Huh7 cells were reverse transfected with control or siRNA specific to either CTα or PEMT, the two main enzymes involved in de novo host cell PC synthesis, and infected with luciferase-expressing *P. berghei* sporozoites. Parasite load (luminescence) was assessed after 48 hr and levels in CTα or PEMT knockdown cells were expressed as percentage of control siRNA-treated cells. Error bars represent SEM from three independent experiments. Mann Whitney test: ***p < 0.0001.

(legend continued on next page)

In addition to the Kennedy pathway, the sequential trimethylation of PE by phosphatidylethanolamine *N*-methyltransferase (PEMT), which is predominantly expressed in the liver, contributes about 20% of de novo PC synthesis (Ridgway and Vance 1987). Downmodulation of PEMT using siRNA in Huh7 cells (Figure S3) led to a decrease in *P. berghei* infection (Figure 2A). Similarly, the livers of mice deficient in PEMT ($PEMT^{-/-}$) showed a decreased parasite load compared to WT littermates, as measured by qRT-PCR of *P. berghei* 18S rRNA (Figure 2G). As before, this reduction in parasite load correlated with a significant decrease in *P. berghei* numbers in the livers of $PEMT^{-/-}$ mice (Figure 2H), without any effect on EEF size (Figure 2I). In an attempt to disrupt both routes of PC biosynthesis, we used $PEMT^{-/-}$ mice fed on a choline-deficient diet. While a choline-deficient diet alone (administered to $PEMT^{+/+}$ mice) did not affect infection, it had a significant impact on parasite liver load in $PEMT^{-/-}$ mice (Figure 2G). On the other hand, exogenous administration of CDP-choline to CTα-LKO mice (Niebergall et al., 2011) was sufficient to revert the impairment of *P. berghei* liver infection caused by depletion of CTα (Figure 2B). Altogether, and despite the ability of *Plasmodium* to synthesize PC from choline scavenged from the host cell (Déchamps et al., 2010), our data clearly show that de novo PC synthesis by the host cell is essential for *Plasmodium* hepatocyte infection.

Plasmodium Parasites Take Up Host PC during Intracellular Development

We next employed click chemistry detection to assess usage and distribution of PC in *Plasmodium* EEFs at different time points after infection (see Experimental Procedures; Figure S4). Prelabeled Huh7 cells (with propargylcholine, alkyne-tagged choline) infected with *P. berghei* sporozoites were analyzed by confocal microscopy. Choline-containing products were seen not only within the EEF (Figure S4) but also in the parasite plasma membrane (PPM) and PVM, as confirmed by colocalization with circumsporozoite (CS) (Figure S4) and UIS4 (upregulated in sporozoites), PPM, and PVM proteins, respectively. Intense staining of lipid-rich regions was observed at later time points (40–48 hr) after infection, and as the parasite underwent schizogony, each daughter nucleus was surrounded by choline-stained membranes (Figure S4).

While these observations show that the parasite uses choline or choline-containing products from the host cell, it is possible that the parasite might take up free choline (and not PC) previously hydrolyzed from choline-containing molecules. Indeed, propargylcholine is metabolized to PC, ether-lysophosphatidylcholine and lyso-phosphatidylcholine (lyso-PC) (Jao et al., 2009). Thus, we next used a nonhydrolyzable ether-lyso-PC to determine whether *Plasmodium* is capable of using host PC directly. The pattern of ether-lyso-PC distribution was similar to that of propargylcholine described above (Figure 3A). These results establish that the parasite takes up PC from the host cell throughout infection and without prior hydrolysis. Subcellular characterization of the lipid-rich domains within EEFs revealed that these structures likely correspond to parasite endoplasmic reticulum (ER) and not the apicoplast, as evidenced by the colocalization of ether-lyso-PC with the *P. berghei* ER-resident protein Bip (Figure 3B) but not with the apicoplast-resident protein ATG8 (Figure 3C). Additionally, ether-lyso-PC prelabeling of HepG2 cells, which support efficient merosome release upon PVM breakdown as it occurs in malaria infections in vivo, shows that host PC associates with PPM/PVM, intravacuolar membranous structures, and individual merozoites visualized by immune staining with anti-MSP1 antibodies (Figure S4). Click labeling on live detached merosomes also showed distinct propargylcholine staining of individual merozoites (Figure S4). Uptake of PC from the host cell into *P. berghei* EEFs was also observed in mouse primary hepatocytes (Figure S4). Similar results were obtained when an azido-tetramethylrhodamine was used in the click cyclo-addition reaction instead of azido-sulfo-bodipy, showing that the reporter fluorophore does not affect the pattern of PC distribution (Figure S4). Next, we assessed whether the use of PC from host cell also occurs during pre-erythrocytic development of the human pathogen *P. falciparum* in micropatterned human primary hepatocytes (March et al., 2013). Host-derived PC associated with *P. falciparum* EEFs as early as 1.5 days after infection, with a distinct perinuclear staining in all EEFs examined (Figure 3D).

Host Cell PC Contributes to PVM Integrity

We next sought to determine the mechanism by which host PC contributes to the establishment of parasite infection in

(B) CTα-floxed and CTα liver-specific knockout (LKO) mice were infected with 5×10^4 GFP-expressing *P. berghei* sporozoites, and the parasite load in the liver at 48 hr after infection was determined by qRT-PCR of *Pb*18S rRNA normalized to HPRT and expressed as percentage of controls. CTα-floxed n = 24, CTα liver-specific knockout (LKO) n = 24. Both CTα-floxed and CTα liver-specific knockout (LKO) mice were injected with CDP-choline (1mg/Kg mouse) intraperitoneally daily for 7 days prior to infection and daily after infection (CTα-floxed n = 8, LKO n = 7). Controls included mice that were injected with PBS (vehicle) intraperitoneally at the same times of CDP-choline administration. Mice were sacrificed at 48 hr after infection, and liver load was quantified as above. Error bars represent SEM. Mann Whitney test: $^{**}p < 0.001$ and $^{***}p < 0.0001$.

(C and D) Quantification of liver burden (number of EEFs per area of liver) and EEF size, respectively, at 48 hr after infection by fluorescence microscopy.

(E) Representative confocal images of EEFs in CTα-floxed versus CTα-LKO liver sections at 24 and 48 hpi. Parasites were stained with an anti-GFP antibody (green), F-actin was labeled with phalloidin 555 (red), and DNA was labelled with DAPI (blue). Scale bar, 10 μm.

(F) Parasite load in the livers of CTα-floxed versus CTα-LKO mice at 6 (n = 17 and n = 12, respectively) and 24 hr (n = 9, n = 10 respectively) after infection with 5×10^4 GFP-expressing *P. berghei* sporozoites, as measured by qRT-PCR of 18S rRNA normalized to HPRT and expressed as a percentage of controls. Mann Whitney test: $^{**}p < 0.001$, ns = not significant

(G) PEMT WT ($PEMT^{+/+}$) and PEMT-deficient mice ($PEMT^{-/-}$) on placebo (n = 18, n = 12 respectively) or choline-deficient diets (n = 7, n = 7 respectively) were infected with 5×10^4 GFP-expressing *P. berghei* sporozoites and parasite liver load quantified at 48 hr after infection by RT-PCR of 18S rRNA normalized to HPRT and expressed as percentage of WT in each condition. Error bars represent SEM. Mann Whitney test: $^{**}p < 0.001$ and $^{***}p < 0.0001$.

(H and I) Quantification of parasite burden and the area of EEFs in liver sections from $PEMT^{+/+}$ and $PEMT^{-/-}$ mice on placebo diet after immunostaining with anti-GFP antibodies and confocal imaging.

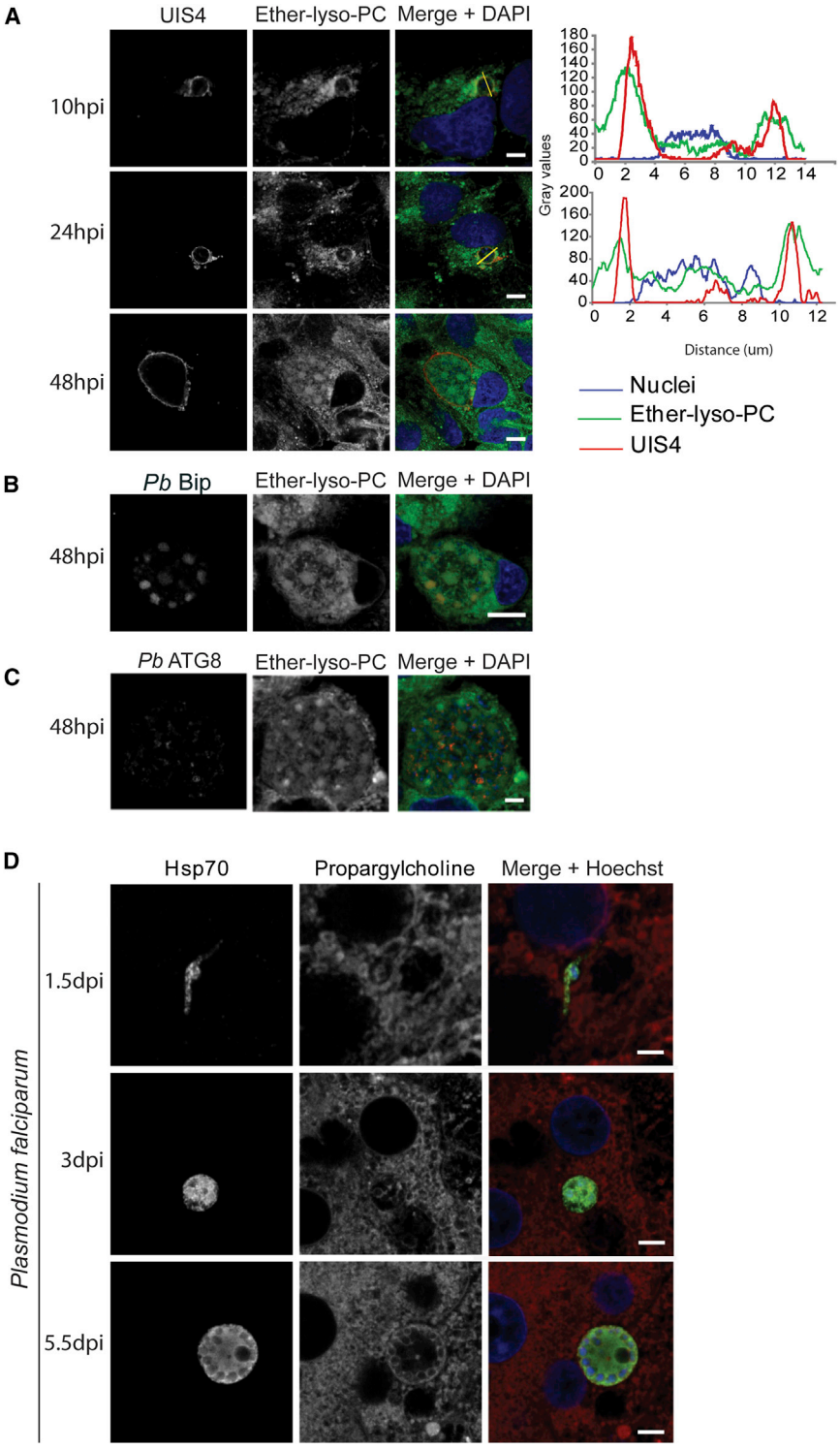

Figure 3. Ether-lyso PC/Choline-Containing Lipids from the Host Associate with *Plasmodium* Membranous Structures throughout Liver Stage Infection

(A–C) Huh7 hepatoma cells were prelabeled with ether-lyso-PC; infected with RFP-expressing *P. berghei* ANKA sporozoites; and fixed at 10, 24, and 48 hr after infection. The cells were immunostained with anti-UIS4 (red) (A), anti-*Pb*Bip (red) (B), anti-*Pb*ATG8 (C), and DAPI. Click labeling was performed with azido-bodipy (green) (see Figure S4A), and confocal images were acquired. Plot profiles of UIS4 (red), ether-lyso-PC (green), and DAPI (blue) intensity (gray values) distributions across EEFs are shown at 10 and 24 hpi.

(D) Primary human hepatocytes were prelabeled with propargylcholine; infected with *P. falciparum* sporozoites; and fixed at 1.5, 3, and 5.5 days postinfection (dpi). Parasites were immunostained with anti-*Pf*Hsp70 (green), and Click reaction was performed with Alexa-Fluor 594 conjugated azide (red). Confocal images were acquired with a laser scanning microscope. Scale bar, 10 μm.

hepatocytes. Several studies using distinct knockout parasites lines that exhibit defects in PVM formation and remodeling show a decrease in the number of parasites able to complete liver stage development (Aly et al., 2008; Ishino et al., 2005; Labaied et al., 2007; Mueller et al., 2005a, 2005b, Silvie et al., 2008; van Dijk et al., 2005; van Schaijk et al., 2008). Additionally, we have recently shown that treatment of liver stage parasites with a class of drugs called torins impairs trafficking of *Plasmodium* PVM-resident proteins resulting in elimination of those parasites (Hanson et al., 2013). Given our observations that host PC localizes to the PVM throughout infection and that parasite numbers decrease sharply when the PC-biosynthetic activity of the host cell is compromised, we hypothesized that reduction of PC at the PVM might impair the insertion and/or maintenance of *Plasmodium* PVM-resident proteins, leading to parasite elimination. Indeed, it is well established that the PC/PE ratio in membranes affects the membrane protein content (Li et al., 2006). Thus, we next analyzed the expression of UIS4, a *Plasmodium* protein known to localize to the PVM and to be essential during liver stage (Mueller et al., 2005b), in primary hepatocytes deficient for CTα. The amount of UIS4 was significantly reduced in the PVM of CTα-deficient hepatocytes, when compared to WT hepatocytes (Figures 4A and 4B), in spite of similar transcriptional expression of the *uis4* gene (Figure 4C). Thus, our data suggest that the insertion of host PC into the PVM is critical for the parasite to maintain the protein composition of this essential membrane and avoid host cell intrinsic elimination mechanisms.

DISCUSSION

How *Plasmodium* parasites modulate the host cell environment during the liver stage, in order to survive long enough to generate

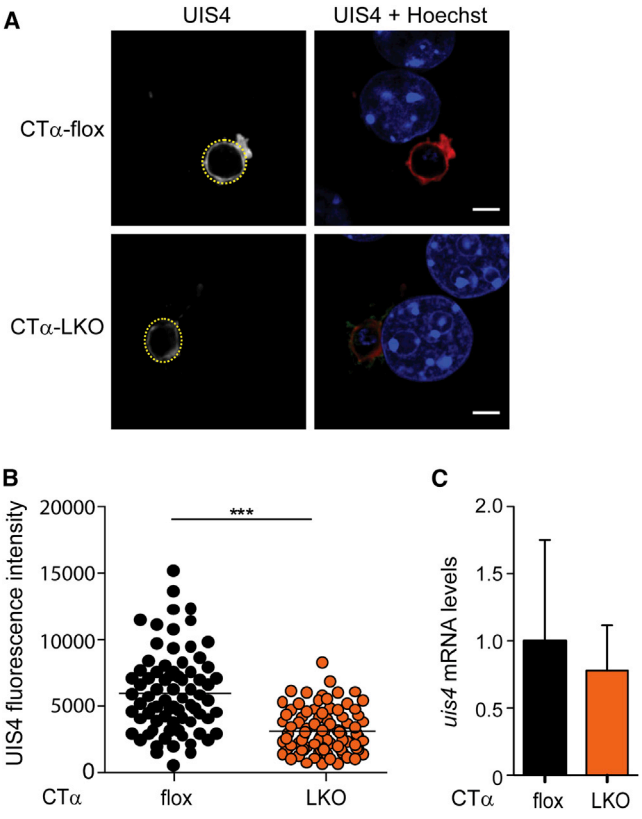

Figure 4. UIS4 Protein Levels Are Significantly Reduced in *Plasmodium* EEFs in Mouse Primary Hepatocytes with Impaired PC Biosynthesis

(A) Confocal images of *P. berghei* EEFs at 18 hpi in primary hepatocytes from CTα-flox and CTα-LKO mice; UIS4 (red) and nuclei (blue). Dotted circles around EEFs indicate the regions of interest for which UIS4 signal was measured ([B] below). Scale bar, 10 μm.

(B) UIS4 signal intensity on EEFs at 18 hpi in primary hepatocytes from CTα-flox and CTα-LKO mice. Mann Whitney test: ***$p < 0.0001$.

(C) Quantification of UIS4 transcriptional expression by qRT-PCR in the livers of CTα-flox and CTα-LKO mice infected with *P. berghei* parasites.

the large numbers of merozoites that are released into the blood, is still poorly understood. Inside each invaded hepatocyte, a single sporozoite generates thousands of new merozoites. This rapid proliferation rate implies that sufficient lipids are available to support both the enlargement of the PVM and membrane neogenesis for newly formed merozoites. We hypothesized that the inherent ability of hepatocytes to mobilize lipids may be critical during *Plasmodium* infection of the liver. Our quantitative determination of the lipid composition of *P. berghei*-infected hepatocytes at different time points after initial infection revealed that PC is the only phospholipid enriched in relative abundance in infected cells with a concomitant decline in the relative abundance of PE, PS, PA, and PI. In addition, confocal microscopy showed that *Plasmodium* parasites continuously accumulate host PC. Using target-specific siRNAs, in addition to CTα- and PEMT-deficient mice, we showed that the host de novo PC biosynthetic machinery is indispensable for *Plasmodium* intrahepatic infection, despite its ability to synthesize PC de novo (Déchamps et al., 2010).

Our data show that intracellular development of the parasite correlates with increased synthesis of the major structural phospholipid, PC, both through the Kennedy and the PEMT pathways, but PS decarboxylase activity does not seem to be required, as PE and PS levels declined at all time points examined. Considering that PC is the major structural phospholipid of eukaryotic membranes and that host PC is used by the parasite throughout infection, it was surprising that, despite a profound impairment in parasite survival, knockout of CTα did not affect parasite growth or merozoite formation. These observations suggest that compensatory PC biosynthetic pathways, such as the PEMT and Lands cycle (Lands 1958; Ridgway and Vance 1987), or other proteins either from the host or parasite side might be engaged during this process.

Also surprising is the fact that while the lipidomics data show a significant increase in lipids typically associated with LDs (namely TAG and CE), an organelle used by many pathogens as a source of structural lipids and energy (Chandak et al., 2010; Cocchiaro et al., 2008; Kumar et al., 2006; Miyanari et al., 2007), ablation of key enzymes involved in CE and TAG synthesis or lipolysis does not disturb any aspect of the infection. LD biogenesis represents a conserved cellular response to infection (Melo and Dvorak 2012) in macrophages but does not seem to play a direct role in *Plasmodium* infection of hepatocytes.

The exo-erythrocytic stage of *Plasmodium* infection occurs within host hepatocytes, a cell type with a phenomenal capacity to support lipid metabolism. Notably, lack of de novo synthesis of a single phospholipid, PC, in the host cell strongly affects parasite survival inside hepatocytes. We show that reduction of PC availability directly reflects on the protein composition of the PVM, as noted by a significant decrease on the levels of the PVM-resident protein UIS4, which implicates PC in PVM remodeling. Given that host PC was found to colocalize with the parasite ER, another plausible scenario is that PC depletion affects the trafficking of proteins to the parasite surface. We cannot exclude that other mechanisms might be in place to explain why host PC is so important for infection. Still, the PVM constitutes the critical interface between the parasite and a potentially hostile host cell environment, and the presence of *Plasmodium* proteins on the PVM was shown to be indispensable for the parasite to avoid elimination by the host cell (Hanson et al., 2013), providing a likely explanation as to why host PC plays such a critical role in parasite survival.

EXPERIMENTAL PROCEDURES

Mice

C57BL6 mice were purchased from Jackson laboratory, and all experiments were performed in strict compliance to the guidelines of our institution's animal ethics committee and the Federation of European Laboratory Animal Science Associations (FELASA).

Cells

HepG2, Huh7 cells, and primary hepatocytes were cultured in supplemented Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, and William's medium, respectively, as described in Liehl et al. (2014), and maintained in a 5% CO_2 humidified incubator at 37°C.

Parasites and Infections

GFP-, RFP-, or luciferase-expressing *P. berghei* sporozoites were dissected from salivary glands of infected female *Anopheles stephensi* mosquitoes into

DMEM medium prior to be added to cells or injected into mice for in vitro and in vivo infections. Infection in vitro was assessed as previously described using a multiplate reader (Infinite M200 from Tecan, Switzerland) or a BD LSR Fortessa cytometer (Franke-Fayard et al., 2004; Ploemen et al., 2009; Prudêncio et al., 2008).

Total Lipid Extraction and Quantitative Mass Spectrometry Analysis of Infected and Noninfected Cells

Noninfected and GFP-expressing *P. berghei*-infected cells were gated on the basis of their different fluorescence intensity as previously established (Albuquerque et al., 2009; Prudêncio et al., 2008). Cells were collected by FACS sorting, and total cellular lipid was extracted from sorted cells as previously described (Sampaio et al., 2011).

siRNA

Human Huh7 hepatoma cells were reverse transfected with 30 nM of target-specific or control siRNA sequences (Table S2) according to the manufacturer's instructions (Ambion, Life technologies). The efficiency of knockdown was assessed by quantitative RT-PCR (Table S3).

Imaging PC in *P. berghei*-Infected Hepatocytes

Host cells seeded on glass coverslips were metabolically prelabeled with 500 μM propargylcholine or 20 μM of a nonhydrolyzable PC, ether-lyso-PC (Kuerschner et al., 2012), for 8–12 hr. Cells were then infected with RFP-expressing *P. berghei* ANKA sporozoites, as previously described. After fixation, click cyclo-addition reaction with Sulfo-Azido-Bodipy was carried out as described elsewhere (Gaebler et al., 2013; Jao et al., 2009).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 software. Student t test was used for significance of differences observed for. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2014.11.006.

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