Role of a patatin-like phospholipase in *Plasmodium falciparum* gametogenesis and malaria transmission

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Transmission of *Plasmodium falciparum* involves a complex process that starts with the ingestion of gametocytes by female *Anopheles* mosquitoes during a blood meal. Activation of gametocytes in the mosquito midgut triggers "rounding up" followed by egress of both male and female gametes. Egress requires secretion of a perforin-like protein, PfPLP2, from intracellular vesicles to the periphery, which leads to destabilization of peripheral membranes. Male gametes also develop flagella, which assist in binding female gametes for fertilization. This process of gametogenesis, which is key to malaria transmission, involves extensive membrane remodeling as well as vesicular discharge. Phospholipase A2 enzymes (PLA2) are known to mediate membrane remodeling and vesicle secretion in diverse organisms. Here, we show that a *P. falciparum* patatin-like phospholipase (PfPATPL1) with PLA2 activity plays a key role in gametogenesis. Conditional deletion of the gene encoding PfPATPL1 does not affect *P. falciparum* blood stage growth or gametocyte development but reduces efficiency of rounding up, egress, and exflagellation of gametocytes following activation. Interestingly, deletion of the *PfPATPL1* gene inhibits secretion of PfPLP2, reducing the efficiency of gamete egress. Deletion of *PfPATPL1* also reduces the efficiency of oocyst formation in mosquitoes. These studies demonstrate that PfPATPL1 plays a role in gametogenesis, thereby identifying PLA2 phospholipases such as PfPATPL1 as potential targets for the development of drugs to block malaria transmission.

malaria | *Plasmodium* gametogenesis | malaria transmission | patatin-like phospholipases

The malaria parasite, *Plasmodium falciparum*, has a complex life cycle that includes distinct stages in the human host and mosquito vector. Transmission of malaria parasites is dependent on differentiation of some blood-stage parasites into gametocytes in a process called gametocytogenesis (1). Gametocytes require uptake by a female *Anopheles* mosquito in a blood meal to continue their life cycle. Within minutes of being ingested, gametocytes are activated in the mosquito midgut and differentiate into gametes in a process termed gametogenesis. In this process, banana-shaped, mature, stage V gametocytes rapidly transform into rounded-up forms and egress from host rest blood cells (RBCs) to be released as male and female gametes. Male gametes develop flagella in a process referred to as exflagellation, which assists in binding to female gametes for fertilization.

"Activation" of male and female gametocytes is triggered by signals in the mosquito midgut and can be simulated in vitro by dropping the temperature to 25 °C, raising the pH, and exposing gametocytes to the mosquito midgut-derived factor xanthurenic acid (XA) (2). Although the receptors for XA are not known, it has been shown that XA activates membrane guanyl cyclases in gametocytes, leading to the synthesis of cyclic guanosine monophosphate (cGMP), which, in turn, triggers the cGMP-dependent protein kinase G (PKG) (3). Inhibition of PKG prevents gametocytes from transitioning from stage V into the rounded-up forms, the first step in gametogenesis (3). Intracellular calcium (Ca^{2+}) also plays a role in regulating gametogenesis (3). Treatment of gametocytes with the cell permeant Ca^{2+} chelator 1,2-Bis(2-aminophenoxy) ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester (BAPTA-AM) does not affect gametocyte rounding up, but blocks gamete egress (3). A rise in cytosolic Ca^{2+} triggers secretion of a perforin-like protein, PfPLP2, which is localized in cytosolic vesicles that translocate to the periphery and discharge their contents, leading to host membrane permeabilization to enable gamete egress (4).

Gametogenesis involves extensive membrane remodeling and regulated vesicular discharge following activation of gametocytes in the mosquito midgut. The molecular players and signaling mechanisms that mediate these processes are not clearly understood. The rise in intracellular Ca^{2+} before gamete egress has

Significance

Malaria transmission involves a complex process that is initiated when *Plasmodium* gametocytes ingested during a blood meal are activated in the mosquito midgut and transform into gametes. The process of gametogenesis involves rounding up of activated gametocytes, exflagellation of male gametes, and gamete egress. Here, we have used a genetic approach to identify a patatin-like phospholipase, PfPATPL1, that mediates gametocyte rounding up, exflagellation, and secretion of a perforin-like protein, PfPLP2, which ruptures peripheral membranes to enable egress. We demonstrate that genetic depletion of PfPATPL1 reduces the transmission efficiency of malaria parasites in mosquitoes. Our study identifies PfPATPL1 as a player in the process of gametogenesis and suggests that phospholipases could be targeted to inhibit transmission and help eliminate malaria.

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been linked to the phosphatidyl inositol–phospholipase C (PI-PLC) pathway in *Plasmodium berghei* (5). PLC catalyzes the conversion of PI biphosphate to inositol triphosphate (IP_3) and diacyl glycerol, which leads to release of Ca^{2+} from the endoplasmic reticulum, presumably via an IP_3 receptor (5). Another phospholipase family comprises phospholipase A2 (PLA2) enzymes, which catalyze the release of fatty acids and lysophospholipids by hydrolyzing the sn2 position of the glycerol backbone of phospholipids (6) to mediate pathways for membrane remodeling and vesicle secretion in diverse organisms (7–9). A recent bioinformatics study in *P. falciparum* identified 22 proteins with lipolytic activity (10). Out of these, 12 were predicted to be lysophospholipases, 4 were predicted to be patatin-like phospholipases, and 1 each had a PC:sterol-O-acyltransferase domain, PI-PLC domain, sphingomyelin phosphodiesterase domain, and PLA/lipase domain (10). In *P. berghei*, egress of merozoites from liver-stage schizonts requires a dual activity phospholipase (PbPL), with PLA2 activity as well as lecithin:cholesterol acyltransferase activity (11). PbPL-deficient parasites were less efficient at egressing from liver-stage schizonts, but PbPL appeared dispensable for gametogenesis (11). Bioinformatics analysis revealed 4 putative PLA2-like proteins in *P. falciparum* that belong to the patatin-like phospholipase family. Patatins are storage proteins in potatoes that possess acyl-hydrolase activity (12). Patatins are thought to signal during stress or defense against pathogens (13). Although first identified in potatoes, patatin-like domain-containing proteins are ubiquitously distributed in nature (InterPro domain IPR002641, https://www.ebi.ac.uk/interpro/entry/IPR002641) (14). For example, patatin-like domain-containing PLA2s are widely distributed in prokaryotic pathogens and symbionts, suggesting they play a role at the host–pathogen interface (15).

Here, we show that a *P. falciparum* patatin-like phospholipase (PlasmoDB ID code Pf3D7_0209100) with PLA2 activity plays a role in the process of gametogenesis. Conditional deletion of the gene encoding PfPATPL1 does not affect asexual growth of blood-stage parasites or the formation of gametocytes. However, loss of PfPATPL1 results in reduced efficiency of rounding up, gamete egress, and exflagellation. Moreover, we demonstrate that PfPATPL1 plays a role in translocation of PfPLP2 to the periphery of gametes to enable egress. Importantly, loss of PfPATPL1 in gametocytes reduces transmission efficiency in mosquitos, identifying PfPATPL1 as a player in the process of gametogenesis and a potential target for development of drugs to block malaria transmission.

Results

Selection of a *P. falciparum* Patatin-Like PLA2 for Functional Analysis. Initial scanning of the genome of *P. falciparum* at PlasmoDB (https://plasmodb.org/plasmo/) revealed 3 putative patatin-like phospholipases—PF3D7_0209100, PF3D7_1358000, and PF3D7_0924000. Of these, Pf3D7_1358000 and Pf3D7_0924000 are predicted to localize to the apicoplast (https://plasmodb.org/plasmo/plasmoap.jsp). Two additional genes encoding conserved PLA2 domains, namely, PF3D7_0218600 and PF3D7_0629300, were further identified by bioinformatic analysis using Hidden Markov Model (HMM) profiling based on a broad range of PLA2 sequences (see *Methods*). PF3D7_0629300 is a homolog of *P. berghei* PbPL, which has both PLA2 and lecithin:cholesterol acyltransferase activities and plays a role in merozoite egress from hepatocytes (11). However, PbPL was not essential for *P. berghei* gametogenesis (11), suggesting that PF3D7_0629300 is unlikely to play a role in this process. Both Pf3D7_0218600 and Pf3D7_0209100 are predicted cytosolic phospholipases. Domain analysis using Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de) (16) shows that they both contain patatin-like PLA2 domains (*SI Appendix*, Fig. S1). Of these 2 patatin-like PLA2s, only Pf3D7_0209100, which we refer to as PfPATPL1, contains the serine/aspartate catalytic dyad as well as the oxyanion loop motif that are conserved in other plant and human patatin-like PLA2s (*SI Appendix*, Fig. S1). We have therefore focused our attention on PfPATPL1 (Pf3D7_0209100), a conserved 679-amino acid protein that has orthologs in other *Plasmodium spp*. and is expressed in *P. falciparum* blood stages and gametocytes (https://plasmodb.org/plasmo/). It is not known whether PfPATPL1 or its *P. berghei* homolog is essential for blood stage parasite growth or sexual stage development.

Conditional Deletion of *PfPATPL1* Gene and Functional Analysis in Blood Stages. We used dimerizable Cre recombinase (DiCre)–*loxP* technology (17, 18) to achieve conditional deletion of exon 3 of the *PfPATPL1* gene, which encodes the patatin-like domain with the predicted PLA2 active site. CRISPR-Cas9–based genome editing was used to replace the wild-type *PfPATPL1* gene in the *P. falciparum* NF54 DiCre line (kindly shared prepublication by Marta Tiburcio and Moritz Treeck, Francis Crick Institute, London, United Kingdom) with a modified *PfPATPL1* gene containing recodonized exon 3 with a C-terminal triple hemagglutinin tag (3-HA) followed by *Hsp86 3′UTR* (Fig. 1*A*). In addition, *loxP* sequences were introduced in the intron preceding exon 3 and downstream of the *Hsp86 3′UTR* in the modified *PfPATPL1* construct (Fig. 1*A*). Replacement of the wild-type *PfPATPL1* gene with modified *PfPATPL1-3HA* in *P. falciparum* NF54 DiCre *PfPATPL1-3HA* clone C4 was confirmed by PCR (Fig. 1*B*) using primer pairs shown in Fig. 1*A*. PfPATPL1-3HA is expressed in the cytosol of *P. falciparum* NF54 DiCre *PfPATPL1-3HA* rings, trophozoites, and schizonts as detected by immunofluorescence assay (IFA) using an anti-HA rat monoclonal antibody (anti-HA mAb) (*SI Appendix*, Fig. S2). Synchronized blood-stage cultures of *P. falciparum* NF54 DiCre *PfPATPL1-3HA* were treated with rapamycin (100 nM) at ring stage for 12 h and analyzed for excision of exon 3 of *PfPATPL1* at schizont stage. Addition of rapamycin promotes assembly of functional Cre recombinase dimers that excise *PfPATPL1 exon 3*. PCR analysis of genomic DNA from parasites cultured with rapamycin showed excision resulting in a truncated *PfPATPL1* gene (Fig. 2*A*). Treatment of synchronized *P. falciparum* NF54 DiCre *PfPATPL1-3HA* with rapamycin (100 nM) at ring stage resulted in greater than 95% reduction in expression of full-length PfPATPL1-3HA at schizont stage as determined by Western blotting using anti-HA mouse monoclonal antibodies (anti-HA mAbs) (Fig. 2*B*). IFA using anti-HA mAbs also demonstrates depletion of PfPATPL1-3HA in schizonts from rapamycin-treated cultures (Fig. 2*C*). Growth of *P. falciparum* NF54 DiCre *PfPATPL1-3HA* blood-stage parasites with and without rapamycin treatment was studied to investigate whether PfPATPL1 was essential for growth. Synchronized *P. falciparum* NF54 DiCre *PfPATPL1-3HA* parasites were treated with rapamycin or dimethyl sulfoxide (DMSO) at ring stage for 12 h, and parasite growth was observed over 3 subsequent cycles. The growth of *P. falciparum* NF54 DiCre *PfPATPL1-3HA* treated with rapamycin (Rap+) was identical to controls treated with DMSO (Rap−), indicating that PfPATPL1 is not essential for blood-stage growth (Fig. 2*D*).

Conditional Deletion of *PfPATPL1* Gene and Development of Gametocytes. IFA using polyclonal anti-PfPATPL1 mouse sera shows that PfPATPL1 is localized in the cytosol of stage II to stage V gametocytes (*SI Appendix*, Fig. S3). PfPATPL1 does not colocalize with Pfg377 (*SI Appendix*, Fig. S4), which is located in osmiophilic bodies in stage V female gametocytes and is secreted to the gametocyte periphery following activation by exposure to low temperature (25 °C) and XA (19). PfPATPL1 also does not colocalize with perforin-like protein, PfPLP2 (*SI Appendix*, Fig. S4), which is localized in intracellular vesicles and is also secreted to the periphery following gametocyte activation (4). Unlike Pfg377 and PfPLP2, there is no change in the localization of PfPATPL1 following gametocyte activation (*SI Appendix*, Fig. S4).

It is not known whether PfPATPL1 is required for gametocytogenesis. Synchronized *P. falciparum* NF54 DiCre *PfPATPL1-3HA* blood-stage cultures at around 2% parasitemia were treated with

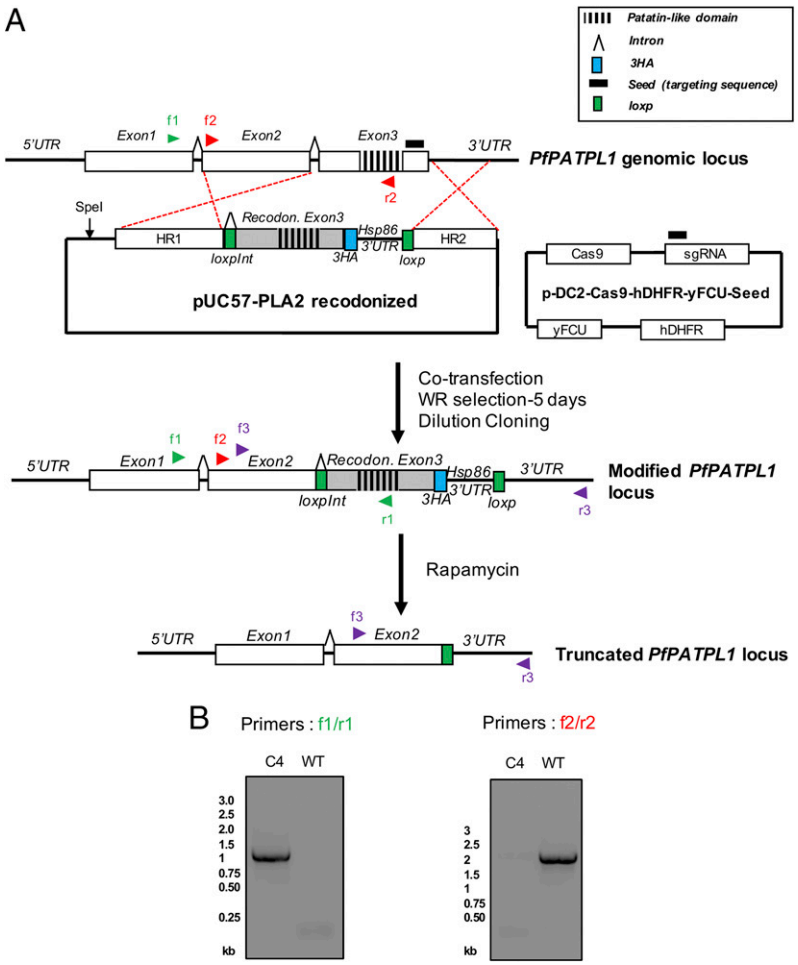

Fig. 1. Conditional deletion of *PfPATPL1* using DiCre–loxP. (*A*) Schematic illustration for the creation of *P. falciparum* NF54 DiCre *PfPATPL1-3HA* line. A "rescue plasmid," pUC57-PLA2 recodonized, contains a region of *PfPATPL1* gene that includes recodonized Exon 3 (Recodon. Exon 3) fused to a 3HA tag (blue) and *Hsp86 3′UTR* flanked by homologous regions 1 and 2 (HR1 and HR2). A *loxp* sequence (green) was introduced in the intron between exons 2 and 3. Another *loxp* sequence was introduced downstream of *Hsp86 3′UTR*. Plasmid "p-DC2-Cas9-hDHFR-yFCU-Seed" contains genes encoding human dihydrofolate reductase (hDHFR) and a bifunctional protein that combines yeast cytosine deaminase and uridyl phosphoribosyl transferase (yFCU), Cas9 endonuclease, and a single-guide RNA (sgRNA) that targets exon 3 of *PfPATPL1*. Plasmid p-DC2-Cas9-hDHFR-yFCU-Seed was used together with the rescue plasmid pUC57-PLA2 recodonized for transfection of *P. falciparum* NF54 DiCre. Transfectants were selected for resistance to WR99210 for 5 d posttransfection and cultured until stable transfectants were observed. The stable pool was subcloned by limiting dilution to derive transgenic clone *P. falciparum* NF54 DiCre *PfPATPL1-3HA* Clone 4 (C4). (*B*) PCR analysis to confirm creation of a modified *PfPATPL1* locus in *P. falciparum* NF54 DiCre *PfPATPL1-3HA* C4. Genomic DNA samples isolated from *P. falciparum* NF54 DiCre (WT) and C4 were analyzed by PCR using primer sets, f1/r1 and f2/r2. PCR with genomic DNA isolated from C4 yielded a fragment of expected size with primer set f1/r1, but no signal was detected with primer set f2/r2. In contrast, PCR with genomic DNA from WT yielded a DNA fragment of the expected size using primer set f2/r2, but no signal was detected with primer set f1/r1.

rapamycin (Rap+) or DMSO (Rap–) at ring stage for 12 h and allowed to grow to high parasitemia (10 to 15% parasitemia), when they spontaneously start forming male and female gametocytes. At this point, parasite cultures were treated with *N*-acetyl-D-glucosamine to remove asexual blood-stage parasites, leaving behind only sexual stages, which were allowed to progress to stage V over 10 to 12 d. PCR analysis of genomic DNA isolated from day 10 gametocytes confirmed that gametocytes that develop from blood-stage cultures that were transiently treated with rapamycin have a truncated copy of the *PfPATPL1* gene (Fig. 3*A*). Analysis of RNA transcripts in day 10 gametocytes derived from Rap– and Rap+ cultures demonstrates that, while short transcripts spanning exons 1 and 2 are detected in both Rap– and Rap+ gametocytes, full-length PfPATPL1 transcripts spanning exons 1 to 3 are detected only in Rap– gametocytes (*SI Appendix*, Fig. S5). Detection of PfPATPL1-3HA by Western blotting using anti-HA mouse mAbs confirmed that expression of full-length PfPATPL1-3HA was depleted by more than 95% in Rap+ gametocytes compared with Rap– controls (Fig. 3*B*). IFA using anti-HA mAbs also demonstrates complete depletion of PfPATPL1-3HA in Rap+ gametocytes (Fig. 3*C*).

PfPATPL1-3HA was immunoprecipitated from Rap– and Rap+ gametocyte lysates using beads coated with anti-HA mAbs and tested for PLA2 activity. Eluted fractions from the beads were incubated with L-α-dipalmitoyl phosphatidylcholine [2-palmitoyl-1-^{14}C] and released ^{14}C-labeled palmitic acid was measured using a scintillation counter. The release of ^{14}C-labeled palmitic acid from the sn2 position of ^{14}C phosphatidylcholine indicates the presence of PLA2 activity. Significant release of ^{14}C palmitic acid was detected when immunoprecipitation (IPT) elutes from Rap– gametocyte lysates were incubated with ^{14}C phosphatidylcholine (*SI Appendix*, Fig. S6). In contrast, no ^{14}C palmitic acid release was detected when IPT elutes from Rap+ gametocyte lysates were incubated with ^{14}C phosphatidylcholine (*SI Appendix*, Fig. S6). This is in line with the complete loss in expression of PfPATPL1 observed in Rap+ gametocytes (Fig. 3 *B* and *C*). Also, no ^{14}C palmitic acid release was detected when control beads that were not coated

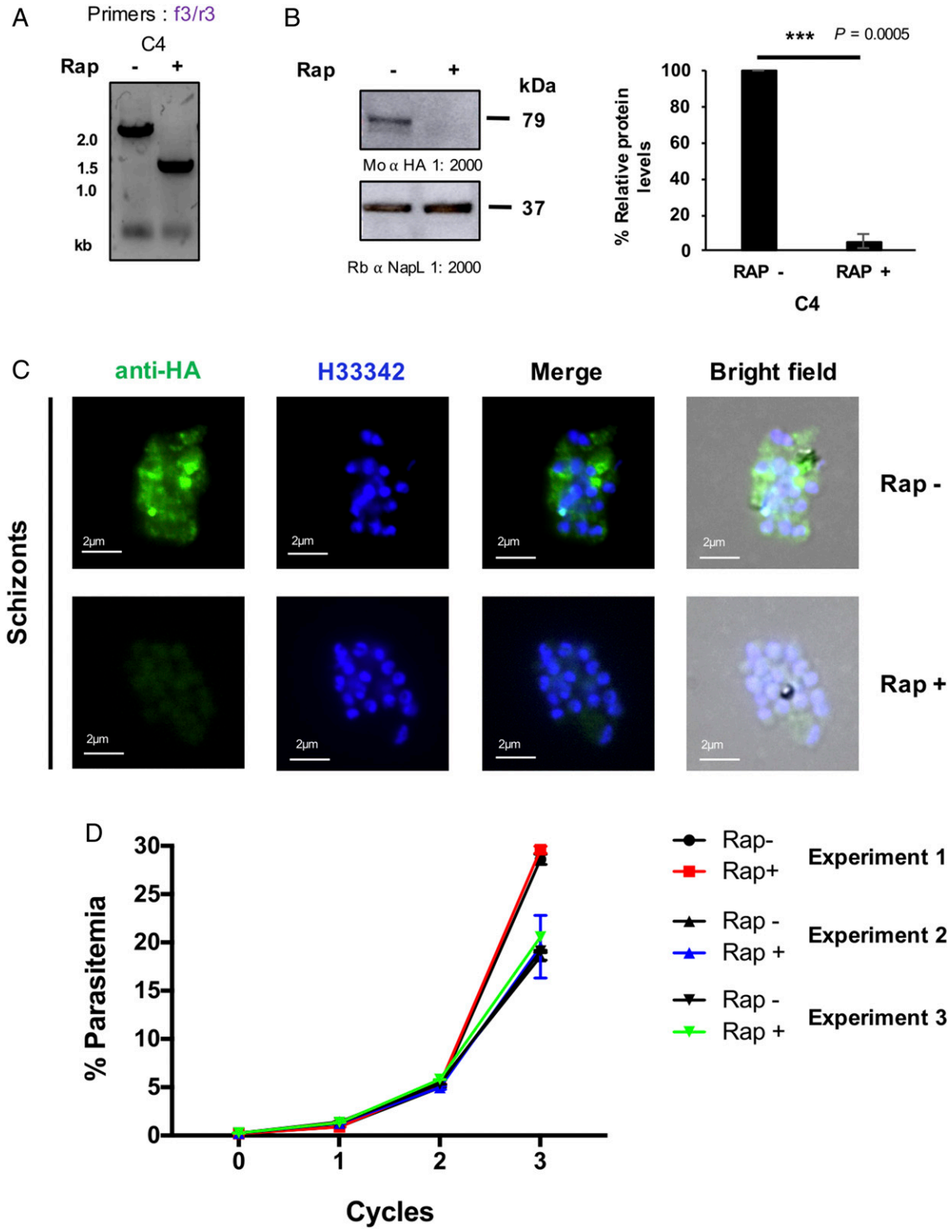

Fig. 2. Conditional deletion of *PfPATPL1-3HA exon 3* and effect on blood-stage growth. (*A*) Conditional deletion of *PfPATPL1-3HA exon 3* in blood stages. *P. falciparum* NF54 DiCre *PfPATPL1-3HA* C4 cultures were treated at ring stage with rapamycin (Rap+) or DMSO (Rap−) for 12 h. Genomic DNA extracted from Rap+ and Rap− schizonts was analyzed by PCR. PCR with primer set f3/r3 (*SI Appendix*, Table S1 and Fig. 1) demonstrates truncation of gene encoding *PfPATPL1-3HA* in Rap+ parasites. (*B*) Depletion of PfPATPL1-3HA in C4 schizonts following rapamycin treatment. Rap+ and Rap− schizont lysates were analyzed for presence of PfPATPL1-3HA using an anti-HA mouse mAb (Mo α HA). PfPATPL1-3HA is more than 95% depleted in Rap+ parasites. Rabbit antiserum raised against cytoplasmic protein NapL (Rb α NapL) was used as control. (*C*) Detection of PfPATPL1-3HA in Rap+ and Rap− schizonts by IFA. Rap+ and Rap− schizonts were probed with anti-HA rat mAbs to detect PfPATPL1-3HA by IFA. PfPATPL1-3HA is completely depleted in Rap+ parasites. Nuclear DNA was stained with DNA-binding fluorescent dye H33342. (*D*) Growth of Rap+ and Rap− blood-stage parasites. Growth of Rap+ and Rap− blood-stage cultures was followed for 3 cycles by staining with SYBR-Green and determining parasitemia (mean ± SD) by fluorescence-activated cell sorter. Results from 3 independent experiments are shown.

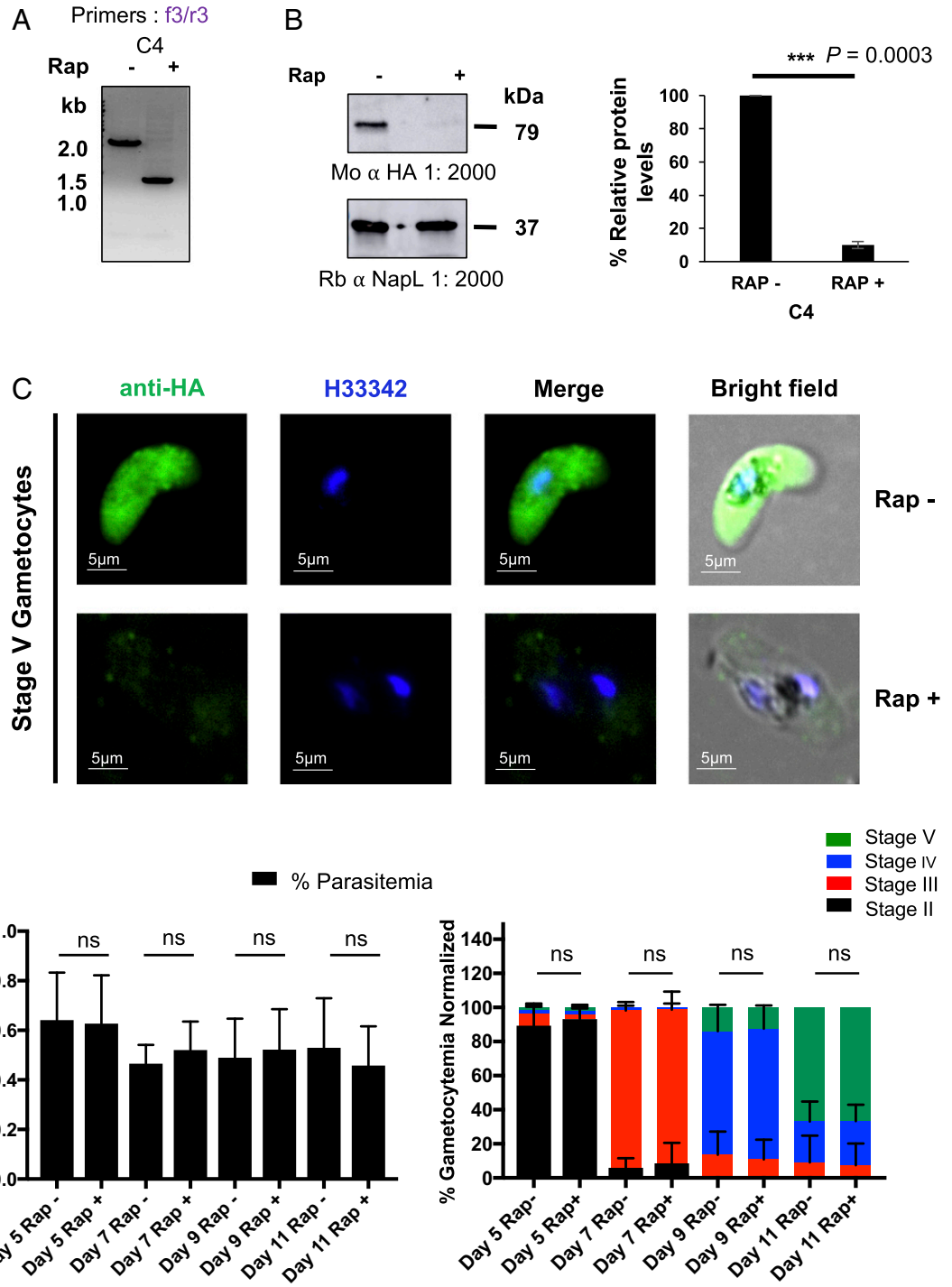

Fig. 3. Conditional deletion of *PfPATPL1 exon 3* and effect on gametocytogenesis. (*A*) Conditional deletion of *PfPATPL1 exon 3* in stage V gametocytes. Blood-stage cultures of *P. falciparum* NF54 DiCre *PfPATPL1-3HA* C4 clone were treated at ring stage with rapamycin (Rap+) or with DMSO (Rap–) for 12 h and cultured to high parasitemia (10 to 15%) to induce gametocyte formation. Blood-stage parasites were cleared by treatment with *N*-acetyl-D-glucosamine. Genomic DNA isolated from stage V Rap+ and Rap– gametocytes collected 10 to 12 d post–*N*-acetyl-D-glucosamine treatment was analyzed by PCR for deletion of *PfPATPL1-3HA exon 3*. PCR using primer set f3/r3 (Fig. 1*A*) demonstrates truncation of gene encoding *PfPATPL1-3HA* in Rap+ gametocytes. (*B*) Depletion of PfPATPL1-3HA in Rap+ gametocytes. Lysates of stage V Rap+ and Rap– gametocytes were analyzed by Western blotting for presence of PfPATPL1-3HA using Mo α HA mAb. Rabbit serum against cytoplasmic protein PfNapL (Rb α NapL) was used as a control. PfPATPL1-3HA is more than 95% depleted in Rap+ gametocytes compared with Rap– control gametocytes. (*C*) Detection of PfPATPL1-3HA in Rap+ and Rap– gametocytes by IFA. Stage V Rap+ and Rap– gametocytes were probed with anti-HA rat mAbs by IFA to detect PfPATPL1-3HA. PfPATPL1-3HA is detected in Rap– gametocytes but is completely depleted in Rap+ gametocytes. Nuclear DNA was stained with DNA-binding fluorescent dye H33342. (*D*) Effect of conditional deletion of *PfPATPL1-3HA exon 3* on gametocyte production. Rap+ and Rap– gametocyte cultures collected on alternate days starting on day 5 post–*N*-acetyl-D-glucosamine treatment were scored for gametocyte density and distribution of stage II to stage V gametocytes by microscopy. Around 4,000 RBCs were scored in each condition to determine gametocyte density and gametocyte stage distribution. There are no differences in total number of gametocytes or distribution of gametocytes across different stages (stages II to V) during gametocyte development (days 5 to 11) between Rap+ and Rap– cultures ($P > 0.05$, ns, using Student's *t* test). Results show mean ± SD from 3 independent experiments ($n = 3$). ns, not significant.

with antibodies were used for IPT from Rap+ or Rap− gametocyte lysates, demonstrating the specificity of the assay (*SI Appendix*, Fig. S6). These results demonstrate that PfPATPL1 has PLA2 activity. Moreover, the PLA2 activity of PfPATPL1 is completely depleted in Rap+ gametocytes compared with Rap− gametocytes.

To determine whether PfPATPL1 is required for gametocytogenesis, both total gametocytemia and progression of gametocytes from stages II to V were scored by light microscopy of Giemsa-stained smears of Rap+ and Rap− gametocytes made on alternate days from day 5 to day 11 post–*N*-acetyl-D-glucosamine treatment (Fig. 3*D*). Depletion of PfPATPL1 did not affect either gametocytemia or progression of gametocytes from stages II to V (Fig. 3*D*). These results demonstrate that PfPATPL1 does not play a role in gametocytogenesis.

Conditional Deletion of *PfPATPL1* Gene and Gametogenesis. *P. falciparum* NF54 DiCre *PfPATPL1-3HA* gametocytes derived from blood-stage cultures transiently treated with rapamycin (Rap+) or DMSO (Rap−) were activated by exposure to low temperature (25 °C) and XA, and the frequency of rounding up of gametocytes was scored by light microscopy of Giemsa-stained smears (Fig. 4*A*). There was a ∼45% drop in frequency of rounding up of Rap+ gametocytes compared with Rap− gametocytes (Fig. 4*A*). Activated gametocytes were also analyzed for egress by staining nuclei with H33342 and scoring for presence or loss of RBC membrane using wheat germ agglutinin–Texas Red (WGA-Texas Red) (Fig. 4*B*). Around 76% of Rap− control gametocytes egressed, as determined by loss of the RBC membrane. Frequency of egress was ∼50% lower in Rap+ gametocytes compared with Rap− control gametocytes (Fig. 4*B*). The exflagellation efficiency was also scored for Rap+ and Rap− male gametocytes (Fig. 4*C*). Exflagellation efficiency was lower by ∼54% in Rap+ gametocytes compared with Rap− gametocytes (Fig. 4*C*). PfPATPL1 thus appears to play an important role in mediating the rounding up, egress, and exflagellation of activated gametocytes.

Finally, we used transmission electron microscopy (TEM) to detect ultrastructural changes in peripheral membrane layers following activation of Rap+ and Rap− gametocytes. Before activation, stage V gametocytes contain 4 peripheral membranous layers including the erythrocyte membrane (EM), parasitophorous vacuolar membrane (PVM), parasite plasma membrane (PPM), and inner membrane complex (IMC) (Fig. 5*A*). The morphology of Rap+ and Rap− stage V gametocytes looks similar before activation, indicating that loss of PfPATPL1 does not affect gametocyte development. Around 20 min following activation, gametes lose the EM and PVM and are left with just 2 peripheral membrane layers, the PPM and IMC (Fig. 5*A*). The frequency of gametocytes that retain the EM and do not egress following activation is significantly higher in Rap+ gametocytes compared with Rap− gametocytes as determined by TEM (Fig. 5*B*).

PfPATPL1 Plays a Role in Dynamic Relocalization of PfPLP2-Containing Vesicles to the Periphery of Gametocytes during Egress. Egress of mature gametes from host erythrocytes is mediated by PfPLP2 (4). Following activation, intracellular vesicles containing PfPLP2 translocate to the gamete periphery, where PfPLP2 is secreted and perforates the PVM and EM to enable gamete egress (4). We investigated whether loss of wild-type *PfPATPL1* gene impairs the dynamic translocation of PfPLP2 vesicles to the periphery of gametes. The localization of PfPLP2 was examined by IFA using anti-PfPLP2 mouse sera 15 min postactivation of Rap+ and Rap− gametocytes (4). PfPLP2 was localized at the periphery of ∼78% of rounded-up Rap− gametocytes following activation. The frequency of translocation of PfPLP2 to the periphery was significantly lower in Rap+ activated gametocytes compared with Rap− activated controls (Fig. 4*D*). PfPLP2 remains localized in internal vesicular structures in the majority of Rap+ activated gametocytes 15 min postactivation (Fig. 4*D*), indicating that PfPATPL1 plays a role in translocation of PfPLP2 to the periphery during gamete egress. In additional experiments, secretion of PfPLP2 to the gametocyte periphery was scored by examining colocalization of PfPLP2 in activated gametocytes with antibodies to RBC membrane protein band 3 (*SI Appendix*, Fig. S7). The frequency of localization of PfPLP2 with band 3 at the RBC membrane was lower in Rap+ gametocytes compared with Rap− gametocytes (*SI Appendix*, Fig. S7), confirming that PfPATPL1 plays a role in optimal secretion of PfPLP2 to the gametocyte periphery.

Deletion of *PfPATPL1* Inhibits Transmission of *P. falciparum* in Anopheline Mosquitoes. The transmission efficiency of Rap+ and Rap− gametocytes in *Anopheles stephensi* mosquitoes was tested. Female *A. stephensi* mosquitoes were fed with human blood containing similar numbers of Rap+ and Rap− gametocytes, and the development of oocysts on the outer face of mosquito midguts was scored on the tenth day after feeding. Oocyst densities in mosquitoes fed on blood with Rap− gametocytes and Rap+ gametocytes were compared (Fig. 6). Median of oocyst densities (±95% confidence interval) in mosquitoes fed with Rap− and Rap+ gametocytes are plotted in Fig. 6. Comparison of oocyst densities by Mann–Whitney test demonstrates a significant drop in oocyst numbers in mosquitoes fed with Rap+ gametocytes compared with Rap− gametocytes (Fig. 6). Reduction in oocyst densities in mosquitoes fed with Rap+ gametocytes compared with Rap− gametocytes indicates that depletion of PfPATPL1 reduces the number of oocysts that develop on the mosquito midgut (Table 1). Thus, PfPATPL1 plays an important role in efficient transmission of malaria parasites.

Discussion

In this study, we have examined the role of a patatin-like phospholipase, PfPATPL1 (Pf3D7_0209100), in gametogenesis. We used a conditional genetic knockout strategy based on DiCre–loxP technology to investigate the potential role of PfPATPL1 in gametogenesis. Treatment of *P. falciparum* NF54 DiCre *PfPATPL1-3HA* ring-stage cultures with rapamycin for 12 h results in excision of *PfPATPL1 exon 3*, and depletion of full-length PfPATPL1-3HA protein levels by >95% in both blood stages and gametocytes (Figs. 1–3). Despite >95% depletion of PfPATPL1 protein levels, blood-stage growth is identical to control parasites with normal PfPATPL1 expression. Thus, PfPATPL1 does not play an essential role in growth of *P. falciparum* blood-stage parasites. Loss of PfPATPL1 also does not appear to affect the ability of the parasites to form gametocytes (Fig. 3). The number of gametocytes formed and progression kinetics of gametocyte development in absence of PfPATPL1 is similar to control parasites with PfPATPL1 (Fig. 3). The morphology of Rap+ gametocytes is also identical to Rap− control gametocytes as observed by electron microscopy (Fig. 5). PfPATPL1 thus does not appear play a role in gametocytogenesis.

In contrast, depletion of full-length PfPATPL1 has an effect on development of gametes following gametocyte activation. Loss of PfPATPL1 results in reduced efficiency of rounding up and gamete egress following gametocyte activation (Fig. 4). Depletion of PfPATPL1 also results in reduced efficiency of exflagellation in male gametes (Fig. 4). A key step in the egress of gametes involves the secretion of the perforin-like protein PfPLP2 from internal vesicles to the periphery, where it destabilizes the vacuolar and RBC membranes (4). Depletion of PfPATPL1 inhibits translocation of PfPLP2 to the membrane periphery (Fig. 4 and *SI Appendix*, Fig. S7). These observations identify a mechanistic role for PfPATPL1 in mediating PfPLP2 secretion, a key step in gamete egress.

Based on bioinformatic analysis, PfPATPL1 was predicted to be a patatin-like phospholipase with PLA2 activity. We have used anti-HA mAbs coated on agarose beads to immunoprecipitate PfPATPL1-3HA from *P. falciparum* DiCre *PfPATPL1-3HA* Rap+ and Rap− gametocyte lysates. The IPT elutes were incubated with L-α-dipalmitoyl [2-palmitoyl-1-^{14}C], and the release of ^{14}C palmitic acid from sn2 position of the phospholipid was measured as an

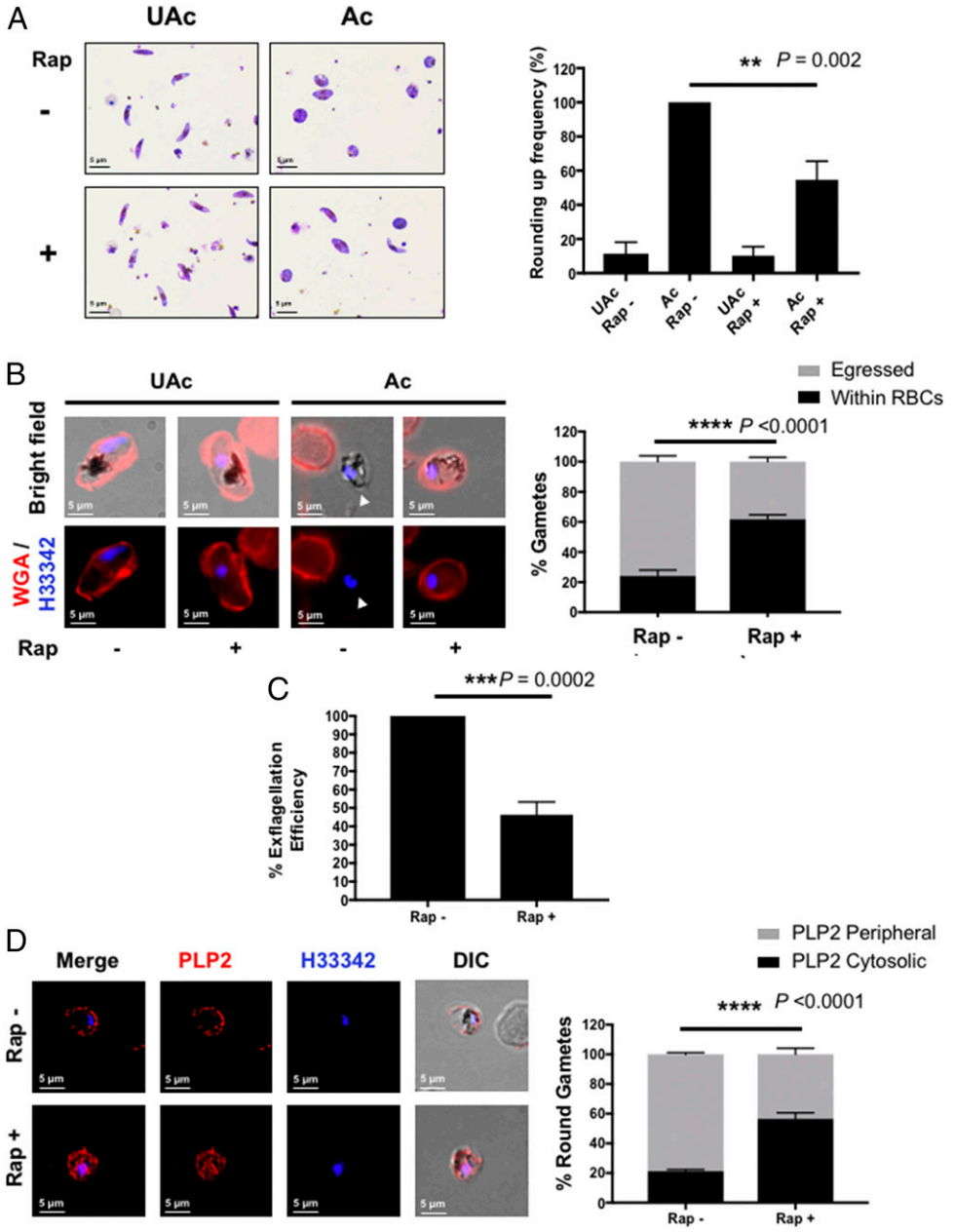

Fig. 4. Conditional deletion of *PfPATPL1 exon 3* inhibits different steps of gametogenesis. (*A*) Conditional deletion of *PfPATPL1 exon 3* inhibits gametocyte rounding up following activation. Rap+ and Rap− stage V gametocytes were activated by incubation at 25 °C in activation medium containing XA to induce gametogenesis. Giemsa-stained smears of Rap+ and Rap− gametocytes made 5 min postactivation were analyzed under light microscopy. Around 200 to 250 gametocytes were scored to determine the frequency of rounding up in each condition. The frequency of rounded-up gametocytes in Rap+ gametocyte cultures following activation (Ac Rap+) is lower than the frequency of rounded-up gametocytes in Rap− gametocyte cultures (Ac Rap−). Unactivated Rap− (UAc Rap−) and Rap+ (UAc Rap+) gametocytes were used as negative controls. Results shown (mean ± SD) are derived from 3 independent experiments (n = 3). Statistical significance was examined using Student's t test (P = 0.002). (*B*) Conditional deletion of *PfPATPL1 exon 3* reduces efficiency of gamete egress. Gamete egress was scored using WGA-Texas Red to detect RBC membrane (red) 20 min after activation of stage V Rap+ and Rap− gametocytes. Gametes that remain trapped within RBCs postactivation stain positive for both WGA Texas-Red (red) and fluorescent DNA-binding dye H33342 (blue). Gametes that egress normally postactivation stain positive only for H33342 (blue). Images from one of 3 independent (n = 3) experiments are shown. Around 150 to 170 gametes were scored for egress under each condition. The percentage of gametes with RBC membrane (within RBC, black) or without RBC membrane (egressed, gray) in Rap− and Rap+ gametocyte cultures is shown. The frequency of egress is lower in Rap+ gametocytes compared with Rap− gametocytes. The plot depicts results (mean ± SD) from 3 independent experiments (n = 3). ****P < 0.0001 as examined using 2-way ANOVA. (*C*) Conditional deletion of *PfPATPL1 exon 3* inhibits exflagellation of male gametes. The number of exflagellation centers was scored 15 min postactivation in 30 optical fields at 40× magnification by light microscopy. Exflagellation efficiency of Rap+ and Rap− gametocytes (mean ± SD) from 3 independent experiments (n = 3) is shown. Statistical significance of differences was evaluated by Student's t test (P = 0.0002). Rap+ gametocytes have lower exflagellation efficiency than Rap− gametocytes. (*D*) Conditional deletion of *PfPATPL1* impairs relocalization of PfPLP2 to gamete periphery upon activation. Gametes from Rap+ and Rap− cultures were fixed 15 min postactivation and probed with anti-PfPLP2 antibodies and H33342 by IFA. Representative images shown here are derived from one of 3 independent (n = 3) experiments. The percentage of rounded-up gametes with PfPLP2 at the periphery (gray) or in the cytosol (black) in Rap+ and Rap− cultures was scored. Cases in which PfPLP2 was located at the periphery and in the cytosol were scored as peripheral localization. Results (mean ± SD) were obtained from 3 independent experiments (n = 3). Statistical significance of differences was estimated by 2-way ANOVA. ****P < 0.0001. The efficiency of translocation of PfPLP2 to the periphery is lower in Rap+ gametocytes compared with Rap− gametocytes.

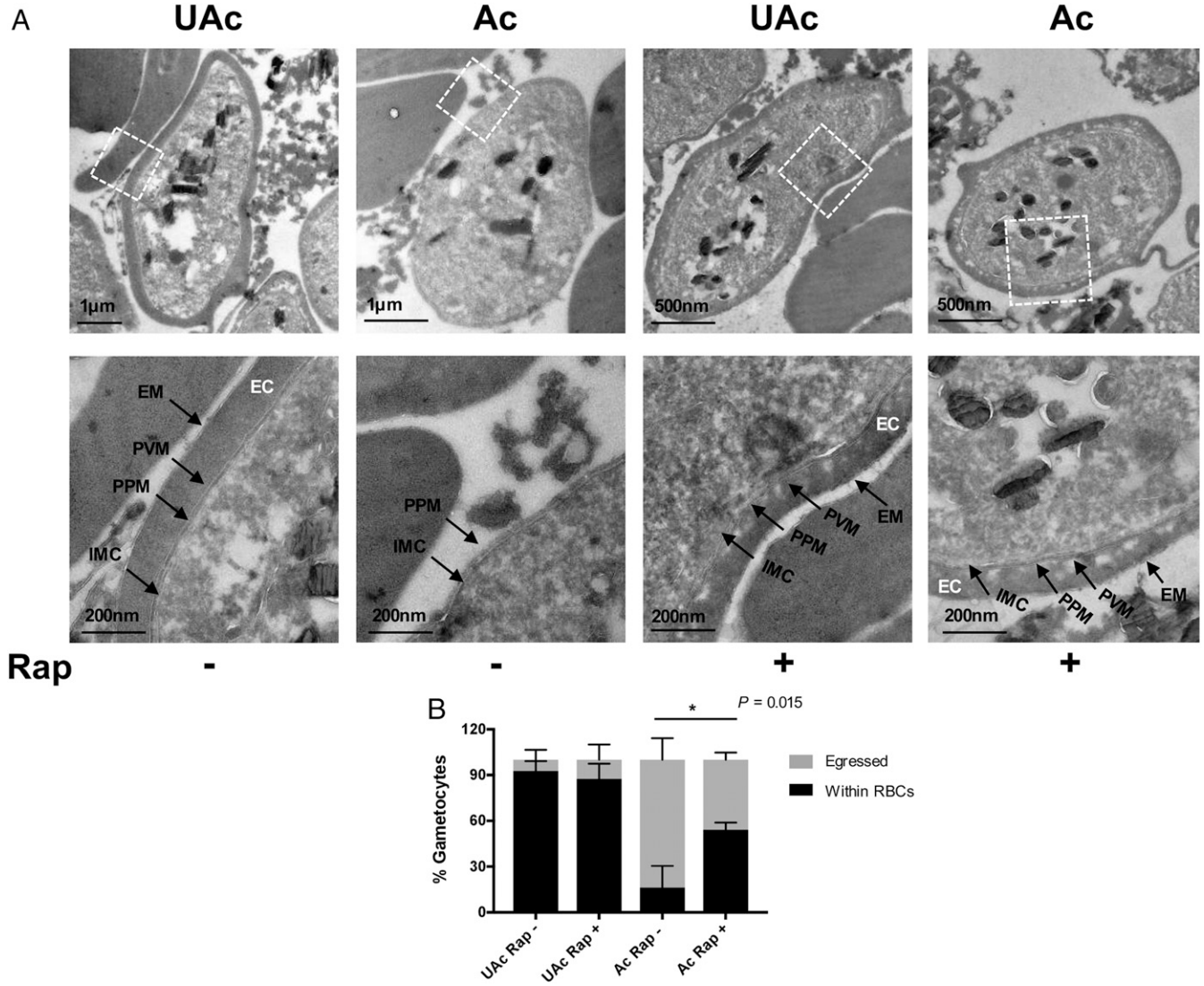

Fig. 5. Conditional deletion of *PfPATPL1 exon 3* inhibits rupture of PVM and EM to prevent egress. (*A*) Ultrastructural changes in gametocytes following conditional deletion of *PfPATPL1 exon 3*. Rap+ and Rap− gametocyte cultures were activated by incubation at 25 °C in activation medium containing XA for 20 min. TEM was used to follow ultrastructural changes in unactivated (UAc) and activated (Ac) Rap+ and Rap− gametocytes. Unactivated Rap− (UAc Rap−) and Rap+ (UAc Rap+) gametocytes contain 4 peripheral membranous layers including the outermost host EM followed by a PVM, PPM, and the IMC. Activated Rap− gametes (Ac Rap−) contain only PPM and IMC. A significant proportion of activated Rap+ gametes (Ac Rap+) remain surrounded by all of the 4 peripheral membranous layers, i.e., EM, PVM, PPM, and IMC after activation. (*B*) The number of gametes with host EM (within host RBCs [black]) or without EM (egressed [gray]) in activated Rap+ (Ac Rap+) and activated Rap− (Ac Rap−) cultures was scored. All Rap− and Rap+ gametocytes retain the EM before activation (UAc Rap− and UAc Rap+). Results (mean ± SD) from 3 independent experiments are reported ($n = 3$). Statistical significance of differences was estimated by 2-way ANOVA. *$P < 0.05$. The percentage of Rap+ gametocytes that lose their EM membrane and egress following activation is lower compared with Rap− gametocytes.

indicator of PLA2 activity. IPT elutes from Rap− gametocytes released ^{14}C palmitic acid, confirming that PfPATPL1 has PLA2 activity (*SI Appendix*, Fig. S6). In contrast, IPT elutes from Rap+ gametocytes lack PLA2 activity due to deletion of *PfPATPL1* (*SI Appendix*, Fig. S6). Despite deletion of *PfPATPL1* in Rap+ gametocytes, the resulting inhibition of rounding up, egress, and exflagellation was partial (Fig. 4). It is unlikely that residual PfPATPL1 is responsible for the partial phenotype observed. A more likely reason for the partial phenotype is the potential of redundancy in phospholipases, in that PfPATPL1 may be functionally replaced by other PLA2 homologs. We examined the expression of 3 other putative *P. falciparum* patatin-like PLA2s, Pf3D7_1358000, Pf3D7_0924000, and Pf3D7_0218600, as well as expression of Pf3D7_0629300, which is a homolog of *P. berghei* phospholipase predicted to have PLA2 activity as well as acyltransferase activity. RNA isolated from *P. falciparum* 3D7 stage V gametocytes was reverse-transcribed, and the resulting complementary DNA was used as a template for PCR, with specific primers targeting these phospholipases (*SI Appendix*, *Methods*). While the 3 patatin-like phospholipases, Pf3D7_1358000, Pf3D7_0924000, and Pf3D7_0218600, are expressed in gametocytes, Pf3D7_0629300 was not expressed in sexual stages (*SI Appendix*, Fig. S8). Of these, Pf3D7_1358000 and Pf3D7_0924000 are localized to the apicoplast, and Pf3D7_0218600 is cytosolic. Pf3D7_0218600 may provide redundancy in case PfPATPL1 is depleted. As a result, deletion of PfPATPL1 is not lethal for development of gametes following gametocyte activation. However, the significant reduction observed in different steps of gametogenesis, such as rounding up and PfPLP2 secretion, leads to a substantial reduction in efficiency of gamete egress, exflagellation,

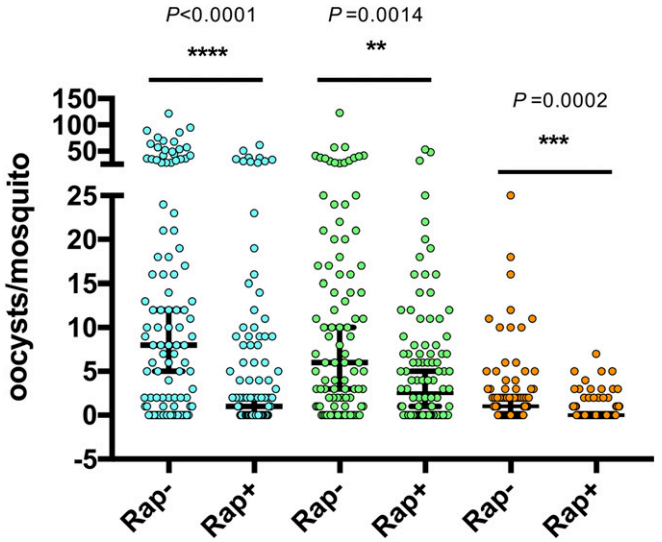

Fig. 6. Conditional knockout of *PfPATPL1* reduces oocyst numbers during transmission in *A. stephensi*. Rap+ and Rap− stage V gametocytes were added to blood used to feed *A. stephensi* mosquitoes. The number of oocysts per mosquito midgut was scored on day 10 postfeeding. The plot depicts the number of oocysts per mosquito fed with blood containing Rap+ and Rap− gametocytes from 3 independent experiments ($n = 3$). Median oocyst numbers per experimental feed (±95% confidence interval) determined on day 10 postfeeding are shown on the plot. Statistical significance of differences in oocyst densities was estimated by Mann–Whitney test. *P* values ****$P < 0.0001$ for experiment 1, **$P = 0.0014$ for experiment 2, and ***$P = 0.0002$ for experiment 3 were calculated.

and mosquito transmission, indicating that PfPATPL1 plays an important role in the process of gametogenesis.

Gametogenesis includes multiple steps, such as rounding up of gametocytes, secretion of PfPLP2 from internal vesicles to the gametocyte periphery leading to the rupture of EM, and gamete egress. These steps involve membrane remodeling, cell shape changes, vesicle secretion, and membrane fusion. The molecular mechanisms that mediate these processes are not known. Phospholipases can modify phospholipids to enable changes in membrane curvature, membrane fusion, rupture, and turnover. Through their diverse actions on phospholipids, phospholipases can mediate dynamic processes such as membrane budding, vesicle secretion, and fusion, which are key to many physiological processes in eukaryotic cells. It is thus not surprising that PfPATPL1, which we have shown displays PLA2 activity, is implicated in processes such as gametocyte rounding up and vesicle secretion during gametogenesis. Rounding up and egress require membrane remodeling to enable events such as change in cell shape and vesicle secretion. PLA2s can modify lipid composition of membranes, leading to changes in membrane curvature, which may promote vesicle fusion (20) and may be important for cell shape changes such as rounding up of gametocytes following activation. PLA2s have also been implicated in regulating vesicle docking by mediating the synthesis and release of fatty acids such as arachidonic acid (21, 22). Arachidonic acid can enable vesicular docking by promoting SNARE (soluble NSF attachment protein [SNAP] receptor) complex formation between membranes (23–25). These mechanisms may be responsible for the role of PfPATPL1 in secretion of PfPLP2-containing vesicles following gametocyte activation.

When female *A. stephensi* mosquitoes were fed with *P. falciparum* NF54 DiCre *PfPATPL1-3HA* Rap+ and Rap− gametocytes, a significant reduction in oocyst prevalence and densities was observed in mosquitoes fed with Rap+ gametocytes compared with Rap− controls (Fig. 6 and Table 1). Our study has identified a patatin-like phospholipase, PfPATPL1, with demonstrated PLA2 activity that plays a key role in gametogenesis and transmission. These observations open the possibility of developing drugs that target such phospholipases to block malaria transmission.

Methods

Bioinformatic Analysis of PLA2s in *P. falciparum*. To identify putative PLA2s in *P. falciparum*, we started with a set of 25 annotated PLA2 sequences from diverse organisms ranging from bacteria to plants and humans. The National Center for Biotechnology Information CD-Search tool (26) was used to identify conserved sequence motifs in these PLA2 sequences. All identified conserved sequences were used to build 11 HMM profiles using HMMer (27). Each of these profiles was used to search for putative PLA2s in the *P. falciparum* 3D7 proteome.

In Vitro Culture of *P. falciparum* Blood-Stage Parasites and Gametocytes. *P. falciparum* NF54 and NF54 DiCre (kindly shared prepublication by Marta Tiburcio and Moritz Treeck, Francis Crick Institute, London, United Kingdom) blood-stage cultures were maintained at 4% hematocrit in human O^+ erythrocytes in Rosewell Park Memorial Institute (RPMI) 1640/Hepes medium (Gibco) supplemented with 0.5% Albumax II (Gibco), hypoxanthine (CC Pro), and gentamicin (Sigma) under mixed gas (5% O_2, 5% CO_2, and 90% N_2). To initiate *P. falciparum* gametocyte cultures, synchronized asexual blood-stage cultures were grown to a parasitemia of 10 to 15%, treated with 50 mM *N*-acetyl-D-glucosamine (Sigma-Aldrich) containing medium for 4 d to remove asexual stages, and maintained in complete RPMI/Hepes (Gibco) to allow gametocyte development.

Cloning of DNA Constructs and Generation of *P. falciparum* NF54 DiCre *PfPATPL1-3HA* Parasite Line. The targeting plasmid, pDC2-Cas9-hDHFR-yFCU-Seed, containing the seed sequence for exon 3 of PfPATPL1, and the rescue plasmid, pUC57-PLA2, containing the following 5 components, 1) the 5′ homology box (HR1) consisting of the Pf3D7_0209100 gene sequence 569 to 1194 (exon 2; 625 bp in length), 2) a synthetic intron including *loxp* sequence and the in-frame recodonized *exon 3* without the stop codon, 3) a sequence encoding a triple HA (3-HA) tag followed by a stop codon, 4) an Hsp86 5′ UTR sequence followed by another *loxp* sequence, and 5) the 3′ homology box (HR2) consisting of a 600-bp sequence directly downstream of the Pf3D7_0209100 gene (Fig. 1*A*), were constructed as described in *SI Appendix*. The linearized rescue plasmid and targeting plasmid were cotransfected into ring-stage NF54-DiCre parasites, and transfectants with integrated rescue plasmid were selected and cloned as described in *SI Appendix*. Genomic DNA from the clones was tested for integration and lack of the wild-type locus by PCR (Fig. 1). Clone C4 was selected for further analysis. For excision of *PfPATPL1 exon 3*, 100 nM rapamycin was added to ring-stage C4 culture for 12 h (Rap+). Parasites were harvested at schizont stage, and genomic DNA was tested by PCR for gene excision using primer set f3/r3 (Fig. 2*A*).

Table 1. Transmission of Rap+ and Rap− gametocytes in *A. stephensi*

	Experiment 1		Experiment 2		Experiment 3	
Transmission parameters	Rap−	Rap+	Rap−	Rap+	Rap−	Rap+
n (no. of mosquitoes scored)	104	108	101	100	96	95
Oocyst prevalence (%)	77.9	54.6	77.2	62	55	30.5
Median oocyst density (no. of oocysts per mosquito)	8	1	6	2.5	1	0
Mean oocyst density	17.9	6.1	12	5.8	2.4	0.7
% reduction in oocyst density based on median	—	87.5	—	58.3	—	100
% reduction in oocyst density based on mean	—	65.9	—	52	—	69.6

Testing Effect of PfPATPL1 Depletion on *P. falciparum* Blood-Stage Growth and Gametocytogenesis. Synchronized cultures of clone C4 were treated at ring stage with 100 nM rapamycin (Rap+) or DMSO (Rap−) for 12 h and analyzed for asexual growth over 3 cycles by flow cytometry after staining with SYBR-Green (28). Rap+ and Rap− parasites were induced to form gametocytes as described above. Giemsa-stained smears of Rap+ and Rap− cultures prepared on alternate days from day 5 to day 11 post–*N*-acetyl glucosamine treatment were scored for gametocyte density and distribution of gametocyte stages (stages II to V).

Purification of Recombinant PfPATPL1 Protein and Generation of Anti-PfPATL1 Mouse Sera. Recombinant PfPATPL1 with 6-His tag was expressed in *Escherichia coli* BL21 (Invitrogen), purified from inclusion bodies by Ni^{2+}-NTA (nitrilotriacetate) chromatography under denaturing conditions, separated on 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) and eluted from the gel (*SI Appendix*, Fig. S3). PfPATPL1 formulations with Freund's complete and incomplete adjuvant were used to raise sera in mice using standard protocols.

Western Blotting Analysis. Asexual blood stages (late trophozoites and schizonts) and stage V gametocytes were purified using VarioMACS magnetic separator (Miltenyi Biotech) and treated with 0.1% saponin. Saponin-treated parasite pellets were lysed in parasite lysis buffer (10 mM Tris pH 8, 0.4 M NaCl, 1 mM (ethylenedinitrilo)tetraacetic acid, 1% SDS) for 5 min, separated by SDS/PAGE and analyzed by Western blotting using anti-HA mouse mAb (Clone 12CA5; Roche) (1:1,000) or anti-PfNAPL rabbit serum (1:1,000) followed by anti-mouse IgG (1:2,000) and anti-rabbit IgG secondary antibodies (1:2,000) (Promega) conjugated to horse radish peroxidase. Signal from anti-PfNAPL rabbit serum was used for normalization.

IFAs. Rap+ and Rap− blood-stage or gametocyte cultures were smeared onto glass slides, fixed with 4% paraformaldehyde (Electron Microscopy Sciences) and 0.0075% gluteraldehyde (Sigma) for 20 min at room temperature, and used for IFA with anti-HA rat mAb (Clone 3F10; Roche) (1:500) followed by Alexa 488 conjugated goat anti-rat IgG antibodies (1:500) (Life Technologies) and Hoechst H33342 (1:1,000) (Thermofisher Scientific).

Measuring PLA2 Activity of PfPATPL1 in Gametocytes. Lysates of mature Rap+ and Rap− stage V gametocytes were prepared (29) and used for IPT using agarose beads coated with anti-HA mouse mAbs (Clone 12CA5; Abcam) as described in *SI Appendix, Methods*. IPT elutes from Rap+ and Rap− gametocyte lysates were analyzed for presence of PLA2 activity as described in *SI Appendix, Methods*.

Activation of *P. falciparum* Gametocytes for Gametogenesis and Scoring for Rounding Up, Egress, and Exflagellation. Stage V Rap+ and Rap− gametocytes were incubated with activation medium (100 nM XA, 20% AB^+ human serum in RPMI1640/Hepes [Gibco]) at 25 °C. Activated gametocytes were spread on glass slides 5 min after activation, fixed with methanol, and stained with Giemsa (RAL Diagnostics). Around 200 to 250 gametocytes were scored to determine the percentage of rounded-up gametocytes. Frequency of rounding up in Rap+ activated gametocytes relative to frequency of rounding up in Rap− cultures (mean ± SD) from 3 independent experiments is reported. The number of exflagellation centers was scored in Rap+ and Rap− gametocyte cultures by light microscopy at 40× magnification in 30 optical fields 15 min after activation. The exflagellation efficiency of Rap+ gametocytes relative to the exflagellation efficiency of Rap− gametocytes (mean ± SD) from 3 independent experiments is reported. To score egress efficiency, gametocytes were spread on glass slides 20 min after activation, fixed with 1% paraformaldehyde, and stained with WGA conjugated to Texas Red (WGA-Texas Red) (5 μg/mL) and DNA intercalating dye Hoechst H33342 (1:1,000). Around 150 to 170 rounded-up gametes were scored for egress. Egressed gametes were identified based on the absence of WGA-Texas Red staining (due to loss of host EM) and presence of nuclear staining (Hoescht H33342). Similarly, unegressed gametocytes were identified based on presence of both WGA-Texas Red staining and H33342. The frequency of egressed gametes in Rap+ gametocyte cultures relative to frequency of egressed gametes in Rap− gametocyte cultures (mean ± SD) from 3 independent experiments is shown.

PfPLP2 Secretion. Stage V Rap+ and Rap− gametocytes were examined 12 min postactivation for presence of PfPLP2 at the periphery of rounded-up gametocytes by IFA using anti-PfPLP2 rabbit sera as described in *SI Appendix, Methods*. Mouse mAb to band 3 was used as a marker for RBC membrane to colocalize PfPLP2 at the gametocyte periphery after activation, as described in *SI Appendix, Methods*. Frequency of peripheral localization of PfPLP2 in Rap+ and Rap− gametocytes after activation is reported.

Electron Microscopy. Stage V Rap− and Rap+ unactivated and activated gametocytes collected 20 min postactivation were fixed with 0.1% gluteraldehyde (Sigma G5882) and 4% paraformaldehyde (EMS 15714) and prepared for imaging by electron microscopy as described in *SI Appendix, Methods*. Images were recorded with TECHNAI SPIRIT 120 Kv (with bottom-mounted EAGLE 4Kx4K camera). Around 40 to 50 gametocytes were scored in each condition for presence of RBC membrane. Results (mean ± SD) from 3 independent experiments are shown.

Mosquito Infection Using Standard Membrane Feeding Assay. Rap− and Rap+ gametocytes were resuspended in human serum AB^+ and fresh Rh^+ erythrocytes (vol/vol) and fed, using a membrane feeder, to 2- to 4-d-old female *A. stephensi*. Mosquitoes were kept at 26 °C, 70% humidity, and 10% sucrose. The midguts of mosquitoes were dissected on day 10 postfeeding, stained with 0.25% fluorescein, and scored for the number of oocysts per mosquito midgut. Prevalence of oocysts and median as well as mean of oocyst densities from 3 independent experiments are reported.

Statistical Analysis. The statistical analysis for all of the plots was performed using GraphPad Prism 7 software. Gametocyte rounding up and male gamete exflagellation experiments were analyzed using unpaired Student's *t* test (assuming equal SD), and $P \leq 0.05$ was considered significant. The graphs were plotted with mean ± SD of the population. Gamete egress and PfPLP2 redistribution was analyzed by 2-way ANOVA test, and $P \leq 0.05$ was considered significant. The graphs were plotted with mean ± SD of the population. Standard membrane feeding assays performed with transgenic Rap+ and Rap− gametocytes were analyzed using a Mann–Whitney test. Median oocyst density in mosquitoes fed with Rap− and Rap+ gametocytes are plotted showing 95% confidence intervals. *P* values were calculated for comparison of number of oocysts produced in mosquitoes fed with Rap− and Rap+ gametocytes using a Mann–Whitney test.

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