A *Plasmodium falciparum* lysophospholipase regulates fatty acid acquisition for membrane biogenesis to enable schizogonic asexual division

Pradeep Kumar Sheokand^a, Yoshiaki Yamaryo-Botté^b, Vandana Thakur^a, Mudassir M. Banday^a, Mohd Asad^a, Cyrille Y. Botté^{b#} and Asif Mohammed^{a#}

^a International Centre for Genetic Engineering and Biotechnology, New Delhi 110 067, India.

^b ApicoLipid Team, Institute for Advanced Biosciences, CNRS UMR5309, Université Grenoble Alpes, INSERM U1209, Grenoble, France

Running title: Lysophospholipase regulates schizogony in *Plasmodium*

Corresponding Authors: *E-mail* address: cyrille.botte@gmail.com; amohd@icgeb.res.in

Abstract

Phospholipid metabolism is crucial for membrane biogenesis and homeostasis during the intracellular life cycle of *Plasmodium falciparum*. To generate large amounts of phospholipids required during blood stages, the parasite massively scavenge, recycle and reassemble host lipids. *P. falciparum* possesses an unusual large number of lysophospholipases. However, their functional roles and importance remain to be elucidated. Here, we functionally characterized one of *P. falciparum* lysophospholipase (*Pf*LPL3) (Gene ID PF3D7_1476800), to reveal its critical role in parasite propagation during asexual blood stages. We generated a transgenic parasite line using GFP-*glmS* C-terminal tagging approach, for localization as well as inducible knockdown of *Pf*LPL3. *Pf*LPL3 displayed a dynamic localization throughout asexual stages, mainly localizing in the host parasite interface: parasitophorous vacuole space and expanding into the tubulovesicular network within the host cell. Inducible knock-down of *Pf*LPL3 hindered normal intraerythrocytic cycle, specifically causing disruption in parasite development from trophozoites to schizont, as well as reduction in number of merozoites progenies. Thus, down-regulation of *Pf*LPL3 significantly inhibited parasite growth suggesting its critical role for proper parasite propagation during blood stages. Detailed lipidomic analyses showed that *Pf*LPL3 generates fatty-acids for the synthesis of neutral lipids DAG and TAG, whilst controlling the timely synthesis of phospholipids that are crucial for membrane biogenesis required for merozoite development during asexual cycle. Setting up an *in vitro* activity based screening of ‘Malaria Box’ allowed identification of specific inhibitors of *Pf*LPL3 having potent parasitological efficacies. These compounds are pertinent both as anti-malarial drug candidates and chemical tools specifically targeting membrane biogenesis during asexual blood stages.

Introduction

Malaria is a vector-transmitted parasitic disease, which remains a major medical problem in tropical and sub-tropical areas of the world. Malaria leads to ~500,000 to 1 million deaths globally every year (1) WHO, (2). In absence of efficient vaccine and due to rapid spread of drug resistant parasites strains, there is urgent need to identify of new drug-targets and development of new drugs against the disease. Understanding of key metabolic pathways in the parasite, which are essential for parasite survival, is a crucial pre-requisite to identify unique and potent targets in the parasites. The pathological symptoms of malaria are caused by repeated blood stage asexual cycles of the parasite in host erythrocytes. Different parasite development stages during the asexual life cycle involve extensive coordinated lipid synthesis, modification of host cell membrane, and biogenesis membrane needed for parasite division and propagation (3). This high demand of lipids/phospholipids required for extensive membrane synthesis is fulfilled by: scavenging from the host milieu, recycling and modifying host lipids, *de novo* synthesis and re-shuffling of those through parasite lipid synthesis machinery (4, 5). The high demand in lipids by the parasite is perfectly illustrated in *P. falciparum* asexual blood stages where lipid content increases by 200-300% through the acquisition and synthesis of a large number of lipid species for growth and proliferation (3, 6). Phospholipids are the major structural components of parasite membranes and are used to make new daughter cells. PC is the most abundant phospholipid in *Plasmodium* membranes during both liver stages and blood stages, attesting its importance for parasite division and propagation (4, 7). The parasite possesses the typical eukaryotic *de novo* phospholipid synthesis machineries to generate most (lyso)phospholipids classes and their precursors from fatty acids (FA), and polar heads, i.e. the so-called Kennedy pathway and the CDP-DAG pathway. *P. falciparum* blood stages notably relies on the scavenging of lysophosphatidylcholine (LPC), especially its polar head phosphocholine, to fuel this *de novo* synthesis of PC and maintain

high asexual division rates (8, 9). Absence of LPC in the extracellular media drives parasite sexual differentiation into gametocytes, likely by lack of PC used as building material for active asexual division. Since LPC plays an important role in parasite development and differentiation, enzymes involved in its hydrolysis are considered as attractive targets for antimalarial development. The catabolism of lipid metabolites from the host must involve phospholipases to manipulate the scavenged lipids and generate the proper precursors (i.e. FA and polar heads). Indeed, parasite contains a high number of phospholipases, harbouring α/β hydrolase domain, including a family of lysophospholipase (LPL). The LPLs play key role recycling of lipids by hydrolysis of acyl chains from lysophospholipids, which are the intermediated in metabolism of membrane phospholipids.

In the present study, we have functionally characterized one of the *P. falciparum* lysophospholipase, *PfLPL3* (Gene ID PF3D7_1476800) that is essential for blood stage schizogonic division through recycling of host lipids. We used a GFP-*glmS* ribozyme approach (10), which allowed the endogenous localization of the protein as well as inducible-transient down-regulation during the parasite blood stages. Confocal analyses showed that *PfLPL3* has a dynamic localization at interfaces between the parasite and the host cell, at the parasitophorous vacuole, as well as extending into the tubulovesicular network space within the host erythrocytes, more specifically during late-trophozoite and schizont stages. Transient knock-down of *PfLPL3* caused dramatic hindrance in schizont development as well as significant decrease in number of merozoites progenies generated in each schizont. To determine the function of *PfLPL3* for merozoite formation and parasite survival, we performed state-of-the-art lipidomic analyses, which showed that disruption of *PfLPL3* induced drastic changes in parasite lipid composition and homeostasis, impacting fatty acids (FA), phospholipids and neutral lipid contents. Specifically, lipidomic analyses revealed that *PfLPL3* generates free FA (FFA) that are normally fuelling the DAG-TAG synthesis pathway for lipid

storage, and in turn regulate the active synthesis of phospholipids, which are essentially required for membrane biogenesis of merozoites during schizogony. We developed an *in vitro* activity assay using recombinant *Pf*LPL3 that confirmed LPL activity for the enzyme. Using this activity assay, we screened the existing MMV “Malaria Box” compounds chemo library. We were able to identify two specific inhibitors of *Pf*LPL3 enzymatic activities with potent parasitological efficacies (<1.0 μ M range). Importantly, treatment of blood stage parasites phenocopied the disruption of *Pf*LPL3 in the parasite, confirming their selectivity for *Pf*LPL3. Based on the importance of *Pf*LPL3 for blood stage parasite development, these compounds could be further developed as new antimalarials and/or chemical tools specifically targeting lipid homeostasis machinery in malaria parasite.

Results

Endogenous tagging of *pflPL3* gene and localization of fusion protein in transgenic parasites: We tagged the endogenous *Pf*LPL3 for physiological localization studies as well as to study its function essentially by conditional knock-down strategy. We used GFP-*glmS* ribozyme system (10) for C-terminal tagging of the native *pflPL3* gene, so that the fusion protein gets expressed under the control of native promoter (Fig. 1A). Integration of the tag at the C-terminus of the gene was confirmed in selected clonal parasite population using PCR-based analyses (Fig. 1B). Expression of fusion protein of calculated size of ~70 kDa was detected by western blot analysis using anti-GFP antibody (Fig. 1C). This band was not detected in wild type 3D7 parasites. These transgenic parasites, *Pf*LPL3-GFP-*glmS*, were studied to determine the physiological localization of the *Pf*LPL3-GFP fusion protein by fluorescence and confocal microscopy approaches. The chimeric protein was expressed throughout all stages of the asexual blood life cycle of the parasite (Fig. 1D). In early development stages, ring and mid-trophozoite stages, GFP fluorescence was mainly observed

in the cytosol. As parasites matured into late-trophozoite and schizonts stages, the GFP labelling was observed at the parasite periphery in the parasitophorous vacuole (PV) region. Intriguingly, during the late trophozoite stages, *PfLPL3* was also present in the parasite extension of host-parasite interface structure: within the tubulovesicular networks (TVN) (Fig. 1D; Supplementary Fig. S2A). A 3D reconstruction of trophozoite stage parasite images clearly confirmed presence of GFP staining around the parasite periphery and in the TVN (Fig. 2A). To find out if the *PfLPL3* is present outside the parasite plasma membrane, infected RBCs from transgenic parasite culture were fractionated by saponin lysis. *PfLPL3* was detected in the supernatant as well as in the pellet fraction by western blot analysis, which confirmed the presence of *PfLPL3* in the PV as well as in parasite cytosol (Fig. 2B), correlating with the fluorescence microscopy results.

To further investigate these localization patterns, membrane labelling and immunostaining studies were carried out. The GFP tagged parasites were co-stained with the lipid membrane probe BODIPY-TR ceramide, which stains all membranous structures. The BODIPY-Ceramide probe labeled parasite periphery, which include plasma membrane and PV membrane (PVM) (Fig. 2C). In addition, the tubulovesicular extension of the PVM were observed in the erythrocyte cytosol for a section of the late trophozoite/schizont stage parasite population (Fig. 2B, C, D, 1D). The *PfLPL3*-GFP labelling showed overlap with BODIPY, in the PV region as well as in the TVN (Figure 2C and S2B). Further, co-labeling of transgenic parasites with one of the PV-resident protein, SERA5 (11), confirmed the localization of *PfLPL3*-GFP protein in the PV (Fig. 2D and S2C).

Selective degradation of *PfLPL3* inhibits parasite growth: To understand the functional significance of *PfLPL3* and its possible involvement in lipid metabolism, we utilized the *PfLPL3*_GFP_*glmS* parasite line for inducible knock-down of *PfLPL3* expression using *glmS*

ribozyme. In presence of glucosamine (GlcN), the *glmS*-ribozyme cleaves itself, which in turn leads to degradation of the associated *PfLPL3* mRNA. Selective knock-down of *PfLPL3* protein was assessed in transgenic parasite treated with different GlcN concentrations (1.25mM, 2.5mM, 5mM and 10mM). The parasites showed GlcN concentration dependent reduction in *PfLPL3* levels (60-90%) in treated sets (Fig. S3A). To avoid any potential GlcN-mediated toxicity observed at high GlcN concentrations, all further experiments were carried out using 1.25mM of GlcN. To study the effect of the inducible knock-down of *PfLPL3* (*PfLPL3*-iKD) on parasite growth, total parasitemia was determined at different time points (24h, 36h, 48h) after GlcN treatment. The *PfLPL3*-iKD set showed ~70% inhibition of parasite growth compared to untreated parasites (Fig. 3A). There was no deleterious effect of 1.25mM GlcN on the growth of the wild type parasite line.

***PfLPL3* is important for parasite intracellular development and crucial for normal schizogonic division:** To study the effect of selective degradation of *PfLPL3* on parasite development and morphology, the *PfLPL3*-iKD set and control set were monitored after different time intervals (Fig. 3B, C and D). In control set, during each intra-erythrocytic cycle, the parasites usually develop from ring to trophozoites to mature schizonts, and subsequent merozoites released from these schizonts can invade new erythrocytes. Together, the intra-erythrocytic cycle can effectively increase the total parasitemia for about 5-6 fold/48h cycle in our culture conditions (Fig. 3A). In the *PfLPL3*-iKD set, parasite growth could only increase up to two folds (Fig. 3). Morphological observations on parasite intracellular development showed no effect on development of ring stages in to trophozoite stages in *PfLPL3*-iKD as compared to control set (Fig. 3B). However, aberrant development of late trophozoites and schizonts were observed in *PfLPL3*-iKD, having smaller, aberrant, or sick-looking parasites compared to control (Fig. 3B); a large number of trophozoites were not able develop into

schizonts, which caused ~50% reduction in number of schizonts as compared to control (Fig. 3C, D). Indeed, at 48 hpi (hours post invasion), a number of stressed parasites were observed in *PfLPL3*-iKD set as compared to control set (Fig. 3B, C), which were not viable to develop into schizonts (Fig. 3D). Further, the *PfLPL3*-iKD parasites were more specifically affected at the replicative fitness of these schizonts. During schizogony *P. falciparum* typically undergoes multiple rounds of nuclear divisions resulting in 16-32 merozoites. In the control set, majority of the schizonts were found to contain 16 or more merozoites (Fig. 4A). However, in the *PfLPL3*-iKD parasite set, mean number of merozoites per schizonts was significantly reduced (Fig. 4A). This decrease in replicative fitness of schizonts resulted in ~25% decrease in total number of merozoites with respect to segmented schizonts (Fig. 4B). It is to be pointed out this effect is in addition to hampering of parasite development in the *PfLPL3*-iKD set, and a number of parasites were developmentally stuck even before the segmental stages; therefore, only few of the parasite could develop into segmented schizonts, which also harbored reduced number of merozoites as compared to control. However, immunostaining of these schizonts with anti-MSP1 antibody showed that there was no defect in the merozoites membrane in the *PfLPL3*-iKD set, which suggested that the merozoite generated had fully developed plasma membrane (Fig 4C). Overall, we show that *PfLPL3*-iKD not only inhibited schizont development (>50%), but it also reduced replicative fitness of parasites (~25%), which resulted in >70% parasite growth inhibition.

Downregulation of *PfLPL3* disrupts lipid homeostasis through decrease of neutral lipid content, concomitant increase of free fatty acids and phospholipids relative abundance:

To assess the role of *PfLPL3* for parasite membrane biogenesis, we conducted exhaustive mass spectrometry-based lipidomic analyses on total lipid extracted from *PfLPL3*-iKD set of parasites. Based on the predicted lysophospholipase function of *PfLPL3*, i.e. releasing FA from

existing (lyso)phospholipids, total FA content of parasites was initially assessed. Logically, the disruption of *Pf*LPL3 induced a significant reduction of total FA abundance (in nmol) (Fig. 5A) as quantified by lipidomic approach. Detailed analysis of the FA composition of total FA revealed significant reductions of the abundance of C16:0, C18:0 and C18:1 (Fig. 5B), all of which are known to be massively scavenged from the host (4)(12). We then sought to determine which lipid species were potentially affected the loss of *Pf*LPL3 and the reduction in FA content. We therefore analyzed the content of major FA-containing lipids, i.e. (a) neutral lipids making the bulk of lipid bodies/storage lipids: diacylglycerol (DAG) and triacylglycerol (TAG), (b) phospholipids (PL), major membrane lipids, and (c) free FA that are used as building blocks for both neutral lipids and phospholipids. The abundance of TAGs, DAGs, and PL was significantly lower in absence of *Pf*LPL3, whilst the abundance of FFA was significantly increased, together confirming the important role of *Pf*LPL3 for parasite membrane biogenesis (Fig. 5C). Importantly, the relative abundance profiles (in mol %) revealed that there was a significant decrease of TAGs and DAGs whereas FFA and phospholipids were significantly increasing in the absence of *Pf*LPL3 (Fig. 5 D1, 2, 3). This indicates that the downregulation of *Pf*LPL3 induces an accumulation of FFA, a decrease in the synthesis of TAGs and DAGs in favor of the synthesis of PL instead. Further TAG, DAG and PL composition analyses confirmed that it was the bulk of each lipid class that was impacted by the loss of *Pf*LPL3 rather than specific molecular species (Fig. S4), again emphasizing the central role of *Pf*LPL3 for parasite membrane homeostasis. Further analysis of the neutral lipid content shows that both the real and relative abundance of the TAG/DAG ratio is significantly reduced in the absence of *Pf*LPL3 (Fig. 5E), showing that a decrease in TAG following *Pf*LPL3 induces a concomitant decrease in parasite DAG content.

Developing a robust lysophospholipase enzymatic assay and identification of potent

inhibitors against *Pf*LPL3: To determine the enzymatic activity of *Pf*LPL3, we expressed a recombinant protein corresponding to its lysophospholipase/hydrolase domain (41aa-338aa). The corresponding recombinant protein was purified, and which migrated on SDS-PAGE at the predicted size of ~70kDa (Fig. S5A). The purified recombinant *Pf*LPL3 was used to standardize a fluorescent-based activity assay to quantify its putative lysophospholipase activity, using LPC as a standard substrate (Fig. S5B). The recombinant *Pf*LPL3 showed concentration dependent lysophospholipase activity in the assay (Fig. S5C), confirming the predicted activity of *Pf*LPL3, and more specifically on LPC. The K_m and V_{max} values for the *Pf*LPL3 were found to be 49.25 μ M and 13113 nM/min/mg respectively (Fig. S5D). This result confirmed the lysophospholipase activity of the enzyme. The recombinant MBP (maltose binding protein) purified in same way as was used as a negative control and did not show any enzymatic activity. The Z-value for the activity assay was found to be ~0.9, showing the robustness of this activity assay, thus also suggesting it could be used for high throughput screening of compounds (Fig. S5E, F). Therefore, to identify putative inhibitors of *Pf*LPL3, we decided to screen the existing MMV antimalarial compounds, “Malaria Box”. Interestingly, two compounds MMV009015 and MMV665796, named as PCB6 and PCB7 respectively, were initially identified with a >70% inhibition of *Pf*LPL3 enzyme activity at 5 μ M concentration. We then determined the corresponding IC_{50} values of these compounds to be 1.26 μ M and 0.92 μ M respectively. Their K_i were found to be 0.634 μ M and 0.464 μ M respectively (Fig. 6A)

***Pf*LPL3 Inhibitors block *P. falciparum* schizont development:** The two “Malaria Box” compounds PCB6 and PCB7 are known to show parasitocidal efficacies with EC_{50} at low μ M range (Fig. 6A). We confirmed the EC_{50} of PCB6 and PCB7 compounds to be 1.26 μ M and

0.9 μ M respectively (Fig. 6A). Both inhibitors were assessed for their effect throughout parasite developmental cell cycle during asexual blood stages. It was found that treatment with compound PCB6 and PCB7 led to normal parasites development from ring to trophozoites/late-trophozoite, similar to the solvent control (Fig. 6B). However, more than 50% of these trophozoites were not able to fully develop into mature schizonts with their morphology appeared deeply affected. The morphological changes of parasites treated by PCB6 and PCB7 both timely coincided and phenocopied the morphological effect observed in *PfLPL3* knock-down (Fig. 6B). These results strongly indicate the specificity of these compounds for *PfLPL3*. Both compounds therefore displayed promising anti-malarial activity and their mechanism of action likely targeting *PfLPL3* and its central function for parasite membrane biogenesis and progeny formation during schizont stages.

Discussion

A number of studies highlight that metabolic pathways linked with lipid synthesis, catabolism and trafficking are essential for survival of malaria parasite (13)(14)(15, 16). During the intra-erythrocytic cycle of *P. falciparum* there is large increase in the phospholipid, neutral lipid and lipid-associated FA contents in the infected red blood cells (4, 6, 17). Indeed, the parasites require large amount of lipids to synthesise new membrane-bound organelles as well as for the assembly of new merozoites after cell division (18–20). To fulfill this massive requirement of lipids, the parasites *de novo* synthesize as well as scavenge lipids/phospholipids from host milieu during the erythrocytic cycle. Phospholipids play a central role in the progression of the life cycle of every parasite and its pathogenesis. Lysophospholipids, especially LPC, are highly present in the host cell external environment and are actively scavenged by intra-erythrocytic parasites. Importantly, LPC serve as major substrates for phospholipid synthesis via the Kennedy pathway through the generation of choline for further PC synthesis and concomitant

de-acylation (i.e. loss of their FA chain) putatively catalyzed by action of unknown lysophospholipase (9) (8). The nature of the involved phospholipases and their exact function during parasite development are currently unknown. Interestingly, an unusual high number of LPLs have been identified in *P. falciparum* genome, however their putative roles, functions and localizations remain to be elucidated.

Here, to further understand lipid homeostasis and membrane biosynthesis in the parasite, we explored the role of a putative LPL in *P. falciparum*, *PfLPL3*, during asexual life cycle of the parasite. *PfLPL3* is a member of lysophospholipase family in the parasite having α/β hydrolases domain and the GX SXG motif, which is the characteristic feature of lysophospholipases. We carried out detailed localization and functional studies to understand role of *PfLPL3* in parasite. A number of genetic tools have been developed for transient down regulation of target proteins in *Plasmodium* by tagging native genes (21–23). Here we have tagged the native gene at the C-terminus with GFP and a ribozyme system (10), which was used for both localization and transient knock-down of the target. *PfLPL3* was localized at the parasitophorous vacuole (PV) as well as in the tubulovesicular networks (TVN) extensions within host erythrocyte. The PV regions are at the interphase of host-parasite interaction during intracellular growth and are site of active transport of material from host to parasite through PVM. The PVM act as a molecular sieve for the parasite whereas TVN are extensions of PVM, which increases the surface area of the PVM to facilitate the more nutrient uptake and protein transport. Based on its putative function, the presence of *PfLPL3* in PV is quite intuitive and reveals that the PV/TVN regions are also sites for phospholipid uptake and metabolism. Glycerophosphodiesterase are enzymes that can act downstream of LPL and removes the choline moiety from glycerophospholipids. Recent study (24) shows tripartite distribution of glycerophosphodiesterase in the cytosol, PV and food vacuole. Presence of glycerophosphodiesterase as well as lysophospholipase, *PfLPL3*, in the PV therefore of the

parasite confirms this area as a major site for the catabolism of exogenously imported phospholipids into FA and respective head-group.

Transient down regulation of *Pf*LPL3 caused severe disruption in the development of trophozoites into schizonts stages. Trophozoite stage of the parasite is metabolically most active stage in which parasite generates and utilizes the available raw materials for organelle biogenesis, daughter merozoite cell formation, cytokinesis and karyokinesis; all these processes require extensive amount of lipids and membrane material. Those parasites in the *Pf*LPL3-iKD set which were able to develop from trophozoite into schizonts, were heavily compromised on number of merozoites developed, although these merozoites had properly developed membrane. In *P. berghei*, a phospholipase (PbPL) is also shown to be present in the PV and its depletion caused defect in the egress from the host hepatocytes (25). The *Pf*LPL3-iKD inhibited schizont development as well as reduced replicative fitness of parasites, which resulted in significant parasite growth inhibition.

Intracellular growth and segregation of *Plasmodium* requires lipid synthesis, which is essentially needed for new membrane development. Phospholipids are *de novo* synthesized/assembled by the parasite; however, their precursors (FA and polar heads) are scavenged from the host milieu. It has been shown that depletion in the serum LPC leads to decrease in the number of merozoites in rodent malaria model (8). Host scavenged LPC is essential for Phosphatidylcholine (PC) synthesis in the parasite; PC is generated by Kennedy pathway from cytidine diphosphate (CDP)-choline (26, 27), whereas in absence of choline, PC is generated via triple methylation of PE using ethanolamine and serine as external precursors. Our lipidomic analysis revealed that *Pf*LPL3 is pivotal for the generation of FA and subsequent lipid synthesis for parasite development. Specifically, we found that *Pf*LPL3 functions as a regulator to channel the high rate in FA influx from the host towards neutral lipid DAG and TAG synthesis. Its absence leads to an uncontrolled increase in phospholipid content, most

likely blocking the normal development of trophozoite into schizonts and future merozoites. The neutral lipids play important role in eukaryotic cell cycle; DAG is known to activates protein kinase C by acting as second messenger (28), it also serves as a precursor for synthesis of TAG and major phospholipids. TAG is stored into cytosolic lipid droplets that detoxify and sequester FAs; whereas, TAG degradation by specific lipase can release these FAs to be utilized for membrane assembly (29). In asexual stage parasites, neutral lipids are stored in a lipid-body associated with the food-vacuole (30, 31). During parasite growth, hydrolysis of TAG can rapidly produce FAs to utilize in membrane synthesis (32). A general lipase inhibitor, Orlistat, was suggested to block utilization of stored TAGs, which inhibited parasite growth and caused developmental arrest in late stages (6). In similar way, reduction in TAG levels in *PfLPL3*-iKD conditions, caused arrest of parasite in late stages, in addition, some of the parasite showed reduced number of merozoites, which points at hindrance in membrane biogenesis. Our data reveals that during blood stages, *P. falciparum* controls lipid synthesis and consequent formation of daughter cells by: (1) favoring massive generation and storage of FA building blocks through the synthesis of DAG and TAG; and (2) timely allowing the generation of structural phospholipids for merozoite formation through the utilization of FA from DAG and TAG, rather than constantly accumulating FFA and/or synthesize phospholipid from them, both of which being lipotoxic for the parasite.

One of the important motives to understanding the biology of the parasite is to identify functionally important metabolic pathways as drug targets, and subsequently design their specific inhibitors to develop as new antimalarials. Our study involving detailed localization, gene knock-down and lipidomic analyses clearly show that *PfLPL3* plays critical role in growth and segregation of the parasite during asexual cycle. We also showed that *PfLPL3* harbor enzymatic activity for lysophospholipase. Furthermore, a robust *in vitro* activity assay was established to screen the compound libraries. Medicine for Malaria Venture (MMV)

established an open collaborative initiative in search of novel drugs for neglected tropical diseases. Based upon extensive screening strategy, they identified a set of drug-like compounds having anti-malarial activity, and labelled this set as ‘Malaria Box’(33). These compounds are new leads to develop further. However, there is need to identify potential targets for these compounds in the parasite, which may help medicinal chemistry approaches to design diversity library and identify potent antimalarial. Screening of the “Malaria Box” by using the *PfLPL3* activity assay identified two hit compounds, labelled as PCB6 and PCB7; both the compounds have parasitocidal efficacy and block the schizont development in *P. falciparum*, which phenocopies the gene knock-down phenotype, and thus consistently confirms the role of *PfLPL3*. Further, both the compounds showed very close efficacies to inhibit enzyme activity and parasite growth, IC_{50} and EC_{50} respectively, in the low micromolar range. These results suggest that both the compounds specifically targeting the *PfLPL3* in the parasite. Hence, these can be further developed through medicinal chemistry campaign to develop potent antimalarials, in addition of being specific molecular tools to study lipid synthesis and membrane biogenesis during schizogony and merozoite formation.

Materials and Methods:

Parasite culture, plasmid construct and parasite transfection: *Plasmodium falciparum* strain 3D7 was cultured in RPMI media (Invitrogen) supplemented with 0.5% (w/v) AlbumaxTM (Invitrogen) 4% haematocrit. Culture was kept static in a gas mixture of 5% carbon dioxide, 5% oxygen and 90% nitrogen at 37°C. To generate *PfLPL3*_GFP_*glmS* construct, a C-terminal fragment (702 base pair) of *pfLPL3* gene was amplified using specific primers 1222A (gcACTAGTAGTATTTTAGAATCTGAAACG) and 1223A (gcGGTACCTTTCTTTTCTTTTCTTTTGC) and cloned in to GFP_*glmS* vector. The C-terminal fragment was cloned in frame to the N-terminus of GFP in the *SpeI* and *KpnI*

restriction enzymes sites. Parasite cultures were synchronized by repeated sorbitol treatment and 100 µg of purified plasmid DNA (Plasmid Midi Kit, Qiagen, Valencia, CA) was transfected in *P. falciparum* by electroporation (310 V, 950 µF) (Crabb et. al. 2004). Transfected parasites were selected over 2.5 µg/ml blasticidin (Calbiochem) and subsequently subjected to on and off cycling of blasticidin drug to promote integration of the plasmid in the main genome. Integration was confirmed by PCR using 1270A and 1234A and by western blotting also by using anti-GFP antibody.

Conditional knock-down and in-vitro growth assays analysis: To assess the effect of knock-down of *PfLPL3* on parasite, *PfLPL3*_GFP_ *glmS* transgenic parasites were tightly synchronized with 5% sorbitol and different concentration (0mM, 1.25mM, 2.5mM, and 10mM) of glucosamine (Sigma-Aldrich) was added in 24 well plates containing 4% hematocrit and 1% parasitemia. For microscopic analysis and morphology of the parasites, Giemsa stained smears were prepared at every 8 hr interval, from both glucosamine treated and control sets. Parasite growth was assessed at 48 and 96 hours after the addition of glucosamine by flow cytometry using BD FACS Calibur (Beckton Dickinson); briefly, cells were incubated with EtBr for 30 minutes at 37 C in dark and after two washing with PBS, 100000 events were acquired using BD FACS Calibur system (Beckton Dickinson). To assess the effect of glucosamine on the down regulation of *PfLPL3* at protein level, *PfLPL3*_GFP_ *glmS* ring stage parasites were grown in presence of various concentration of glucosamine (1.25mM, 2.5mM, 5mM, 10mM), harvested at schizonts stage and subjected to the western blotting.

Parasite fractionation and Western blot analysis: Parasites were harvested at trophozoite stage using 0.15% saponin for RBC lysis, the supernatant was collected, and parasites pellet

was lysed by freeze-thaw cycle. Laemmli buffer was added to both the fractions and proteins were separated in 12% SDS-PAGE; the proteins were transferred to PVDF membrane (Millipore) and incubated with blocking buffer (4 % skim milk in $1 \times$ PBS) followed by incubation with the primary antibody: (monoclonal anti-GFP mice 1:5000 (Roche), rabbit anti-GFP 1:10000, rabbit anti-BiP 1:15000 or monoclonal anti-spectrin 1:1000). Blots were washed 5 times with 1 PBS, probed with HRP conjugated secondary antibody (1:100000) and visualized using ECL detection kit (Thermo-scientific).

Immuno-fluorescence assay (IFA) and fluorescent microscopy: The parasites were fixed in 4% paraformaldehyde. After permeabilization with 0.1 % TritonX-100, 10% bovine serum was used for blocking; cells were incubated with rabbit polyclonal anti-GFP (1:500) or mice polyclonal anti-SERA5 (1:500) for 2 hours and subsequently with Alexa Flour-488 and- 594 labelled secondary anti-body. Labeled parasites were washed three times with $1 \times$ PBS. Parasite nuclei were stained with DAPI at a final concentration of 5 μ g/ml. The membrane structures in parasitized erythrocytes were labelled with BODIPY TR-ceramide (Invitrogen) with a final concentration of 1 μ M. Images were captured by Nikon A1 confocal laser scanning microscope and analysed by Nikon-nis element software (version 4.1). The 3D images were constructed by using series of Z-stack images and IMARIS 7.0 (Bitplane Scientific) software.

Lipid extraction from *P. falciparum* and liquid chromatography-mass spectrometry analysis: Total lipids were extracted from treated (*Pf*LPL3-iKD) and control parasites. First parasites (4×10^8 cell equivalents) were harvested by using 0.15% saponin treatment. The total lipid samples were spiked with 20 nmol C21:0 phosphatidylcholine, and extracted by chloroform: methanol, 1:2(v/v) and chloroform: methanol, 2:1 (v/v). The pooled organic phase was subjected to biphasic separation by adding 0.1% KCl and was then dried under N_2

gas flux prior to being dissolved in 1-butanol. For the total fatty acid analysis, an aliquot of the lipid extract was derivatized on-line using MethPrep II (Alltech) and the resulting FA methyl esters were analyzed by GC-MS as previously described. For the quantification of each lipid, total lipid was separated by 2D HPTLC using chloroform/methanol/28% NH₄OH, 60:35:8 (v/v/v) as the 1st dimension solvent system and chloroform/acetone/methanol/acetic acid/water, 50:20:10:13:5 (v/v/v/v/v) as the 2nd dimension solvent system. Each lipid spot was extracted for quantification of fatty acids by gas chromatography-mass spectrometry (Agilent 5977A-7890B) after methanolysis. Fatty acid methyl esters were identified by their mass spectrum and retention time and quantified by Mass Hunter Quantification Software (Agilent) and the calibration curve generated with fatty acid methyl esters standards mix (Sigma CRM47885). Then each lipid content was normalized according to the parasite cell number and a C21:0 internal standard (Avanti Polar lipids). All analyses were performed in triplicate or more ($n=3$). The p values of ≤ 0.05 from statistical analyses (Student's t -test) obtained from GraphPad software analysis were considered statistically significant.

Cloning, Expression and purification of recombinant protein: Hydrolase domain of *PfLPL3* was amplified by using specific primers 1270A (gcGGATCCACCATGGAAGTATCATAATATTAATACATGGGTTAGC) and 1271A (gcCCTAGGTCCTTTTTCGATTGTTA TAACATGAC) and cloned in to pETM41 vector between *NcoI* and *XhoI* sites to give pETM41-His6-MBP-*PfLPL3* construct. The recombinant protein *PfLPL3* was expressed as soluble protein in the cytosol of the *E. coli* BL21(DE3) codon⁺ cells. For expression, bacterial cells having pETM41-His6-MBP-*PfLPL3* was grown in of LB medium supplemented with kanamycin at 37°C to an absorbance (A_{600}) of ~0.8-1.0. Expression of the fusion proteins was induced using 1mM isopropyl β -D-thiogalactoside (IPTG) overnight at 16°C. Next day cells were harvested and resuspended into resuspension

buffer (25mM Tris, pH7.4, 500mM NaCl, 10mM Imidazole) and lysed by sonication (Vibra cell sonicator). The supernatant was subjected a combination of Ni-NTA affinity chromatography followed by amylose affinity chromatography. Eluted fractions were subjected to SDS PAGE and western blot analysis to assess the purity of the purified recombinant protein.

Lysophospholipase (LPL) activity assay and enzyme kinetics: For assessing the enzymatic activity of the recombinant *Pf*LPL3, a lysophospholipase (LPL) activity assay was established using lysophosphatidylcholine as substrate. The activity assay reaction mixture (200 μ l total volume) contained 16:0 lysophosphatidylcholine (LPC) as substrate, 20 μ g (285pmol) of recombinant protein, 0.1 unit glycerophosphodiesterase (Sigma), 0.2 U/ml choline oxidase (Sigma 26978), 2U/ml horseradish peroxidase (Sigma P8125), 100 μ M Amplex Red (Invitrogen) in a reaction buffer (50mM Tris pH 8.0, 5mM CaCl₂). The *Pf*LPL3 cleaves the acyl chain from LPC resulting the generation of glycerophosphocholine on which glycerophosphodiesterase acts and cleaves the choline moiety. This choline was oxidized by choline oxidase to produce betaine and H₂O₂. Finally, H₂O₂ in presence of horseradish peroxidase, reacts with Amplex Red reagent in 1:1 stoichiometry to generate the highly fluorescent product resorufin, which has absorption and fluorescence emission maxima of approximately 571 nM and 585 nM respectively. The LPL activity was monitored by measuring fluorescence intensities (530 ex/590 em) for 6 hrs using spectramax M2 microplate reader. The assay was performed by varying the amount of protein and substrate to get optimum LPL activity. The kinetic constant *K*_m and *V*_{max} were determined by fitting Michaelis-Menten curve using the Graph Pad Prism V5.0 software package.

Standardization of a robust lysophospholipase (LPL) activity assay and screening of compound library: Robustness of the activity assays was assessed by conducting HML test for a period of two days in accordance for statistical test to calculate the Z-value. The HML assay employed three different reaction wells: **H**igh activity well i.e. lysophospholipase activity well with 20µg enzyme; **M**edium activity well i.e. in the presence reduced amount of enzyme, (10µg protein); and the **L**ow activity well that is the substrate control well without the enzyme; this set of three wells (HML) was repeated throughout the 96-well plate and the assay was repeated for two consecutive days. The Z-value was calculated based on the variations in activity between the wells/plates (34).

To identify the *Pf*LPL3 targeting antimalarial compounds, “Malaria Box” compound library obtained from Medicines for Malaria Venture (MMV, Switzerland), was screened using the standardized *Pf*LPL3 activity assay; briefly, the recombinant enzyme (20µg) was incubated with different concentrations of each of the compound or DMSO alone; the reactions were initiated by addition of the activity assay reaction mixture to a final volume of 200µl and the substrate hydrolysis was monitored as described above. To assess the effect of selected *Pf*LPL3 inhibitors on parasite growth and morphology, synchronized parasite cultures at 1% ring stage parasites were treated with varied concentrations of each of the selected compounds (5.0 to 0.2µM), parasite growth was estimated in next cycle by flow-cytometry. DMSO was used as a control.

Statistical Analysis

The data sets were analysed using GraphPad Prism ver 5.0 to calculate K_m , V_{max} , IC_{50} and EC_{50} values, and the data were compared using unpaired Student’s *t*-test.

Acknowledgements

We are grateful to Philip J. Shaw for providing vector pGFP_*glmS* and useful suggestions. We thank Rotary blood bank, New Delhi for providing the RBCs. PKS is supported by research fellowship from University Grant Commission, Govt. of India. VT is supported by BioCARE Women Scientist fellowship from Department of Biotechnology, Govt. of India. The research work in AM's laboratory is supported by Centre Of Excellence grant (BT/COE/34/SP15138/2015) Flagship Project grant (BT/IC-06/003/91) from Department of Biotechnology, Govt. of India. CYB and YYB are supported by Agence Nationale de la Recherche, France (Grant ANR-12-PDOC-0028-Project Apicolipid), Finovi programs, LABEX ParaFrap, France (grant number ANR-11-LABX-0024), the Université Grenoble Alpes (IDEX ISP) Apicolipid) and Région Auvergne Rhone-Alpes for the lipidomics analyses platform (Grant IRICE Project GEMELI). The research work was supported by Indo-French Collaborative Research Program Grant (Project 6003-1) to AM and CYB by the CEFIPRA. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

References:

1. Hay SI, Okiro EA, Gething PW, Patil AP, Tatem AJ, Guerra CA, Snow RW. 2010. Estimating the Global Clinical Burden of Plasmodium falciparum Malaria in 2007. PLoS Med 7:e1000290.
2. World Health Organization. World malaria report 2019.
3. Déchamps S, Wengelnik K, Berry-Sterkers L, Cerdan R, Vial HJ, Gannoun-Zaki L. 2010. The Kennedy phospholipid biosynthesis pathways are refractory to genetic disruption in Plasmodium berghei and therefore appear essential in blood stages. Mol Biochem Parasitol 173:69–80.

4. Botté CY, Yamaro-Botté Y, Rupasinghe TWT, Mullin KA, MacRae JI, Spurck TP, Kalanon M, Shears MJ, Coppel RL, Crellin PK, Maréchal E, McConville MJ, McFadden GI. 2013. Atypical lipid composition in the purified relict plastid (apicoplast) of malaria parasites. *Proc Natl Acad Sci U S A* 110:7506–7511.
5. Amiar S, Katris NJ, Berry L, Dass S, Duley S, Arnold CS, Shears MJ, Brunet C, Touquet B, McFadden GI, Yamaro-Botté Y, Botté CY. 2020. Division and Adaptation to Host Environment of Apicomplexan Parasites Depend on Apicoplast Lipid Metabolic Plasticity and Host Organelle Remodeling. *Cell Rep* 30:3778-3792.e9.
6. Gulati S, Ekland EH, Ruggles K V., Chan RB, Jayabalasingham B, Zhou B, Mantel PY, Lee MCS, Spottiswoode N, Coburn-Flynn O, Hjelmqvist D, Worgall TS, Marti M, Di Paolo G, Fidock DA. 2015. Profiling the Essential Nature of Lipid Metabolism in Asexual Blood and Gametocyte Stages of *Plasmodium falciparum*. *Cell Host Microbe* 18:371–381.
7. Itoe MA, Sampaio JL, Cabal GG, Real E, Zuzarte-Luis V, March S, Bhatia SN, Frischknecht F, Thiele C, Shevchenko A, Mota MM. 2014. Host cell phosphatidylcholine is a key mediator of malaria parasite survival during liver stage infection. *Cell Host Microbe* 16:778–786.
8. Brancucci NMB, Gerdt JP, Wang CQ, De Niz M, Philip N, Adapa SR, Zhang M, Hitz E, Niederwieser I, Boltryk SD, Laffitte MC, Clark MA, Grüning C, Ravel D, Blancke Soares A, Demas A, Bopp S, Rubio-Ruiz B, Conejo-Garcia A, Wirth DF, Gendaszewska-Darmach E, Duraisingh MT, Adams JH, Voss TS, Waters AP, Jiang RHY, Clardy J, Marti M. 2017. Lysophosphatidylcholine Regulates Sexual Stage Differentiation in the Human Malaria Parasite *Plasmodium falciparum*. *Cell* 171:1532-

1544.e15.

9. Wein S, Ghezal S, Buré C, Maynadier M, Périgaud C, Vial HJ, Lefebvre-Tournier I, Wengelnik K, Cerdan R. 2018. Contribution of the precursors and interplay of the pathways in the phospholipid metabolism of the malaria parasite. *J Lipid Res* 59:1461–1471.
10. Prommana P, Uthaipibull C, Wongsombat C, Kamchonwongpaisan S, Yuthavong Y, Knuepfer E, Holder AA, Shaw PJ. 2013. Inducible Knockdown of Plasmodium Gene Expression Using the glmS Ribozyme. *PLoS One* 8.
11. Stallmach R, Kavishwar M, Withers-Martinez C, Hackett F, Collins CR, Howell SA, Yeoh S, Knuepfer E, Atid AJ, Holder AA, Blackman MJ. 2015. Plasmodium falciparum SERA5 plays a non-enzymatic role in the malarial asexual blood-stage lifecycle. *Mol Microbiol* 96:368–387.
12. Mitamura T, Hanada K, Ko-Mitamura EP, Nishijima M, Horii T. 2000. Serum factors governing intraerythrocytic development and cell cycle progression of Plasmodium falciparum. *Parasitol Int* 49:219–229.
13. Hanada K, Palacpac NMQ, Magistrado PA, Kurokawa K, Rai G, Sakata D, Hara T, Horii T, Nishijima M, Mitamura T. 2002. Plasmodium falciparum phospholipase C hydrolyzing sphingomyelin and lysocholinephospholipids is a possible target for malaria chemotherapy. *J Exp Med* 195:23–34.
14. Mitamura T, Palacpac NMQ. 2003. Lipid metabolism in Plasmodium falciparum-infected erythrocytes: Possible new targets for malaria chemotherapy. *Microbes Infect.* Elsevier Masson SAS.
15. Mamoun C Ben, Prigge ST, Vial H. 2010. Targeting the lipid metabolic pathways for

the treatment of malaria. Drug Dev Res. Drug Dev Res.

16. Choubey V, Maity P, Guha M, Kumar S, Srivastava K, Puri SK, Bandyopadhyay U. 2007. Inhibition of Plasmodium falciparum choline kinase by hexadecyltrimethylammonium bromide: A possible antimalarial mechanism. Antimicrob Agents Chemother 51:696–706.
17. Nawabi P, Lykidis A, Ji D, Halder K. 2003. Neutral-lipid analysis reveals elevation of acylglycerols and lack of cholesterol esters in Plasmodium falciparum-infected erythrocytes. Eukaryot Cell 2:1128–1131.
18. Pessi G, Kociubinski G, Ben Mamoun C. 2004. A pathway for phosphatidylcholine biosynthesis in Plasmodium falciparum involving phosphoethanolamine methylation. Proc Natl Acad Sci U S A 101:6206–6211.
19. Witola WH, El Bissati K, Pessi G, Xie C, Roepe PD, Mamoun C Ben. 2008. Disruption of the Plasmodium falciparum PfPMT gene results in a complete loss of phosphatidylcholine biosynthesis via the serine-decarboxylase- phosphoethanolamine-methyltransferase pathway and severe growth and survival defects. J Biol Chem 283:27636–27643.
20. Ramakrishnan S, Serricchio M, Striepen B, Bütikofer P. 2013. Lipid synthesis in protozoan parasites: A comparison between kinetoplastids and apicomplexans. Prog Lipid Res. Prog Lipid Res.
21. De Koning-Ward TF, Gilson PR, Crabb BS. 2015. Advances in molecular genetic systems in malaria. Nat Rev Microbiol. Nature Publishing Group.
22. Armstrong CM, Goldberg DE. 2007. An FKBP destabilization domain modulates protein levels in Plasmodium falciparum. Nat Methods 4:1007–1009.

23. Meissner M, Krejany E, Gilson PR, De Koning-Ward TF, Soldati D, Crabb BS. 2005. Tetracycline analogue-regulated transgene expression in *Plasmodium falciparum* blood stages using *Toxoplasma gondii* transactivators. *Proc Natl Acad Sci U S A* 102:2980–2985.
24. Denloye T, Dalal S, Klemba M. 2012. Characterization of a glycerophosphodiesterase with an unusual tripartite distribution and an important role in the asexual blood stages of *Plasmodium falciparum*. *Mol Biochem Parasitol* 186:29–37.
25. Burda PC, Roelli MA, Schaffner M, Khan SM, Janse CJ, Heussler VT. 2015. A *Plasmodium* Phospholipase Is Involved in Disruption of the Liver Stage Parasitophorous Vacuole Membrane. *PLoS Pathog* 11:1–25.
26. Tischer M, Pradel G, Ohlsen K, Holzgrabe U. 2012. Quaternary ammonium salts and their antimicrobial potential: Targets or nonspecific interactions? *ChemMedChem*. *ChemMedChem*.
27. Flammersfeld A, Lang C, Flieger A, Pradel G. 2018. Phospholipases during membrane dynamics in malaria parasites. *Int J Med Microbiol*. Elsevier GmbH.
28. Goñi FM, Alonso A. 1999. Structure and functional properties of diacylglycerols in membranes. *Prog Lipid Res*. *Prog Lipid Res*.
29. Athenstaedt K, Daum G. 2006. The life cycle of neutral lipids: Synthesis, storage and degradation. *Cell Mol Life Sci*. *Cell Mol Life Sci*.
30. Jackson KE, Klonis N, Ferguson DJP, Adisa A, Dogovski C, Tilley L. 2004. Food vacuole-associated lipid bodies and heterogeneous lipid environments in the malaria parasite, *Plasmodium falciparum*. *Mol Microbiol* 54:109–122.

31. Asad M, Yamaryo-Botté Y, Hossain ME, Thakur V, Jain S, Datta G, Botté CY, Mohammed A. 2019. A lysophospholipase plays role in generation of neutral-lipids required for hemozoin formation in malaria parasite. bioRxiv. bioRxiv.
32. Palacpac NMQ, Hiramane Y, Mi-Ichi F, Torii M, Kita K, Hiramatsu R, Horii T, Mitamura T. 2004. Developmental-stage-specific triacylglycerol biosynthesis, degradation and trafficking as lipid bodies in *Plasmodium falciparum*-infected erythrocytes. *J Cell Sci* 117:1469–1480.
33. Spangenberg T, Burrows JN, Kowalczyk P, McDonald S, Wells TNC, Willis P. 2013. The Open Access Malaria Box: A Drug Discovery Catalyst for Neglected Diseases. *PLoS One* 8.
34. Zhang JH, Chung TDY, Oldenburg KR. 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* 4:67–73.

Figure 1

Figure 1: Generation of *PflLPL3*-GFP-*glmS* transgenic parasite line: (A) A schematic representation of single crossover homologous recombination showing the integration of the *LPL3*-GFP-*glmS* plasmid at the C-terminus of the endogenous *pflpl3* gene. (B) PCR based analyses of the selected clonal parasite culture and wild type 3D7 parasite lines, locations of primers used are marked in the schematic. Lane 1 and 4 (primers 1078A and 1234A) showing amplification only in the integrants; lane 2 and 4 (primers 1222A and 1234A) show amplification only in the integrants; lane 3 and 6 (primers number 1222A and 1223A) show amplification in both integrants and the wild-type line. (C) Western blot of

transgenic parasites probed with anti-GFP antibody. The fusion protein band was detected in the transgenic parasites, *Pf*LPL3-GFP-*glms* only (lane 1) and not in the 3D7 parent parasite (lane 2). Blot ran in parallel and probed with anti-BiP was used as loading control. (D) Fluorescent microscopic images of live transgenic parasites at ring, trophozoite, and schizont stages. The parasite nuclei were stained with DAPI and parasites were visualized by confocal laser scanning microscope. In young parasites, the fusion protein was observed in the cytosol of the parasite, but as the parasite matures, the fluorescence was observed towards the periphery of the parasite.

Figure 2

Figure 2: Sub-cellular localization of *PfLPL3* in parasitophorous vacuole (PV) and tubulovesicular network: (A) The 3D image constructed by using series of Z-stack images of the transgenic parasites with IMARIS 7.0, shows the GFP-fusion protein present in the PV and tubulovesicular network (TVN) region. (B) Western blot of infected erythrocyte fractions after saponin lysis showing that the *PfLPL3*-GFP fusion protein is present in soluble fraction representing content of parasitophorous vacuole (PV) and host cytosol; blot ran in parallel and probed with anti-BiP and anti-SERA5 antibody were used as a negative and positive controls respectively. (C) Fluorescent microscopic images of transgenic parasites immunostained with anti-

SERA5 antibody showing colocalisation of *Pf*LPL3 with SERA5 in PV. (D) Fluorescent microscopic images of transgenic parasites stained with membrane probe BODIPY-TR ceramide, showing GFP fusion protein at the parasite periphery in late parasite stages. Parasite nuclei were stained with DAPI and images were acquired by confocal laser scanning microscope.

Figure 3

Figure 3: Inducible knock-down of *Pf*LPL3 hampers parasite growth and survival: (A) Graph showing percentage parasitemia in *Pf*LPL3-GFP-*glmS* transgenic parasite cultures grown with 2.5 mM glucosamine (iKD and control), as estimated by new ring stage parasites after 2 and 4 days of growth. Western blot analysis showing reduction in the fusion protein in presence of glucosamine is shown in inset. (B) Giemsa stained images of parasites showing effect on parasite morphology at different time points (0-48h) for control and *Pf*LPL3-iKD sets. Parasite stage progression curve for (C) control and (D) *Pf*LPL3-iKD sets.

Figure 4

Figure 4: *PfLPL3* knock-down effect parasite schizogony: (A) Frequency distribution of number of merozoites per schizont in the control and *PfLPL3*-iKD parasite cultures. (B) Graph showing reduction in the total number of merozoites in *PfLPL3*-iKD parasite line in comparison to the control ($n=50$ segmented schizonts). ** $p < 0.01$ (C) Fluorescent images of control and *PfLPL3*-iKD parasites immunostained with anti-MSP1 antibody suggesting that there is no defect in the merozoites membrane in the *PfLPL3*-iKD set.

Figure 5

Figure 5: Lipidomic analyses reveal the role of *PfLPL3* to generate FAs and regulate DAG-TAG synthesis vs phospholipid synthesis: Synchronous transgenic parasites at ring stages were grown till late trophozoite stages (32h post infection) and lipid composition were assessed by mass spectrometry-based lipidomic analyses from *PfLPL3*-iKD and control sets. Data is shown from six independent biological replicates. (A). Total fatty acid abundance (in

nmol lipid/10⁷parasites) reveal a significant decrease of total lipids in *PfLPL3-iKD*. (B) Profile of fatty acid species in the 3D7 control and *PfLPL3-iKD* strains. There was a significant decrease in C16:0, C18:0 and C18:1 in the *PfLPL-iKD*. (C) Quantification of the abundance (nmol) in TAG, DAG, FFA and PL between *PfLPL-iKD* and control parasites revealed (D) Relative abundance of TAG, DAG, FFA, and PL showed significant decreases in TAG and DAG whereas there was a significant increase in FFA and PL. Right panels are enlarged sections of the left panel (E) Relative abundance of TAG to DAG ratio in *PfLPL3-iKD* parasites as compared to control set. For all the assays P values are marked: * for $p \leq 0.05$, and ** for $p < 0.005$

Figure 6

Figure 6: Identification of *Pf*LPL3 specific inhibitors from MMV Malaria Box: A robust *in vitro* activity assay was used to screen the ‘Malaria Box’ and potent inhibitors of *Pf*LPL3 are identified having parasitocidal efficacies. (A) Concentration dependent inhibition of enzymatic activity as well as inhibition of asexual stage parasite growth by PCB6 (MMV009015) and, and PCB7 (MMV665796) compounds respectively. The IC_{50} values (enzyme inhibition) and EC_{50} values (parasite growth inhibition) for both compounds are indicated. (B) Effect on the morphology of *P. falciparum* parasites at different time points after treatment with *Pf*LPL3 inhibitors PCB6 and PCB7 at a concentration of EC_{50} values.

Figure 7

Figure 7: Model of *PfLPL3* function in *Plasmodium falciparum* asexual blood stage cycle:

Schematic diagram showing metabolic pathways of lipid metabolism associated with proposed function of *PfLPL3*. In the left panel, *PfLPL3* level is maintained in absence of glucosamine. At the late trophozoite stage, *PfLPL3* localizes in the PV of the parasite which cleaves the acyl chain from the lysophospholipids (acquired from the host or generated from phospholipid catabolism) results in generation of G-3-P and FAs. Which ultimately required for DAG and TAG synthesis. In presence of *PfLPL3* neutral lipid and membrane biogenesis are normal

which results normal schizogony. On the right panel, consequences of *Pf*LPL3 knockdown are shown. The decreased level of *Pf*LPL3 level in presence of glucosamine leads to desregulation of fatty acid content, which disrupts phospholipid synthesis and development of new membranes. Therefore *Pf*LPL3 knock-down disrupts schizogony and cause reduction in number of merozoites in schizonts. PLs (Phospholipids), PLA (Phospholipase A), FA (Fatty acid), LPLs (Lysophospholipids), LPL3 (Lysophospholipase 3), G-3-PC (Glycero-3-Phosphocholine), G-3-P (Glycero-3-Phosphate), PA (Phosphatidic acid), DAG (Diacylglycerol), TAG (Triacylglycerol), GPAT (Glycerol-3-phosphate acyltransferases), PAP (Phosphatidate phosphatase), DGAT (Diglyceride acyltransferase), FV (Food Vacuole), N (Nucleus).

A Plasmodium falciparum lysophospholipase regulates fatty acid acquisition for membrane biogenesis to enable schizogonic asexual division

Pradeep Kumar Sheokand^a, Yoshiaki Yamaro-Botté^b, Vandana Thakur^a,
Mudassir M. Banday^a, Mohd Asad^a, Cyrille Y. Botté^{b#} and Asif Mohammed^{a#}

Supplementary Figures:

Figure S1: Domain organisation and multiple sequence alignment of *Plasmodium* LPL3 homologues: (A) Schematic diagram of *Pf*LPL3 showing 262 amino acids long hydrolase domain, location of active site residues is marked. (B) Multiple amino acid sequence alignment of *Pf*LPL3 with its homologs in other organisms (*T. gondii*, *P. knowlesi*, *P. berghei*, *P. vivax*) showing the characteristic motif and active site residues are conserved among these proteins. Red boxes indicate the conserved motifs and asterisk indicates the catalytic residues.

Figure S2

Figure S2: Co-localization of *Pf*LPL3 with PV and PVM markers: (A) Fluorescent microscopy image of the transgenic parasites shows that the GFP-fusion protein present in the PV and tubulovesicular network (TVN) region. (B) Fluorescent microscopic images of transgenic parasites stained with membrane probe BODIPY-TR ceramide, showing GFP fusion protein at the parasite periphery in late parasite stages. (C) Fluorescent microscopic images of transgenic parasites immunostained with anti-SERA5 antibody showing colocalisation of *Pf*LPL3 with SERA5 in PV region. Parasite nuclei were stained with DAPI and images were acquired by confocal laser scanning microscope.

Figure S3

Figure S3: (A) Schizont development and merozoite segregation is hindered in *PfLPL3*-iKD parasites. Giemsa stained smears from the *PfLPL3* knock-down parasites set showing that the glucosamine treated parasites have stressed parasites as well as reduced number of merozoites per schizont as compared to control set which has mature schizonts with higher number of merozoites per schizonts. (B) Effect of *PfLPL3* knock down on merozoite membrane. Fluorescent images of the wild type and the *PfLPL3*-iKD parasites immuno-stained with anti-MSP1 antibody. Although the number of merozoites were reduced in *PfLPL3*-iKD, these merozoites have intact plasma membrane as in case of control. Parasite nuclei were stained with DAPI and images were acquired by confocal laser scanning microscope.

Figure S4

35 **Figure S4: Alteration in lipid composition by inducible knock-down of *PfLPL3* in the**
36 **parasites:** (A) Graphs showing compstion (Mol%) of different clssses of fatty acids, there
37 was no alteration in abundance in different fatty acids classes between *PfLPL3*-iKD and
38 control. (B) Graphs showing compstion (Mol%) of different clssses of phosopholipids, there
39 was no alteration in abundance of different classes except minor changes in PE.

40

41

Figure S5

Figure S5: Standardization of lysophospholipase specific activity of the recombinant *PfLPL3* for screening of MMV ‘Malaria Box’ compound library: (A) Coomassie stained SDS-PAGE showing purified recombinant *PfLPL3*. (B) Representative diagram of reaction and components involved in the LPL activity assay. LPC was used as a substrate. The *PfLPL3* cleaves the acyl chain from LPC resulting in the generation of glycerophosphocholine on which glycerophosphodiesterase acts and cleaves the choline moiety. This choline was oxidized by choline oxidase to produce betaine and H_2O_2 . Finally, H_2O_2 in the presence of horseradish peroxidase, reacts with Amplex Red reagent in 1:1 stoichiometry to generate the highly fluorescent product resorufin, and fluorescence of resorufin was measured. (C) Graphical

representation of LPL activity of *Pf*LPL3 in presence of varying amount of protein. (D) Line graph of Michelis-Menten fit for *Pf*LPL3 activity in presence of the 20 μ g (85pmoles) of recombinant protein. The K_m and V_{max} values of *Pf*LPL3 were found to be 49.25 μ M and 13113nM/min/mg respectively. (E) & (F) Robustness of the activity assay was assessed by estimating Z-value carried out in HML format in 96-well plate for two consecutive days, the Z-value was found to be $\sim$ 0.9, suggesting robustness of the assay. The screening was carried out at 5 μ M concentration, two compounds were identified to inhibit >70% enzyme activity which are labelled as PCB6 and PCB7.