PfPI3K, a Phosphatidylinsoitol-3 kinase in *Plasmodium falciparum*, is exported to the host erythrocyte and is involved in hemoglobin trafficking.

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Polyphosphorylated-Phosphoinositides (PIPs) are potent second messengers, which trigger a wide-variety of signaling and trafficking events in most eukaryotic cells. The role and metabolism of PIPs in malaria parasite-*Plasmodium* has remained elusive. Even though PIPs may not be very important for mature erythrocytes, their levels have been reported to increase significantly upon infection with malaria parasite. Our present studies suggest the reason for this increase may be PfPI3K, a Phosphatidyl-inositol-3-kinase (PI3K) in *Plasmodium falciparum*, which is exported to the host erythrocyte by the parasite in an active form. PfPI3K is a versatile enzyme as it possesses the ability to generate various PIPs. In the parasite, PfPI3K was localized in vesicular compartments near the membrane and in its food vacuole. PI-3 kinase inhibitor wortmanin turned out to be effective against PfPI3K and was used to study PfPI3K function. We found that PfPI3K may be involved in endocytosis from the host and trafficking of hemoglobin. Inhibition of PfPI3K resulted in entrapment of hemoglobin in vesicles in the parasite cytoplasm, which prevented its transport to the food vacuole, the site of hemoglobin catabolism. As a result, hemoglobin digestion, which is a source of amino acids necessary for parasite growth, was attenuated and caused the inhibition of parasite growth.

Plasmodium falciparum, a protozoan parasite, is the major causative agent of human malaria. The lack of a successful vaccine and wide-spread drug resistance contribute heavily towards the failure in eradication of this disease. A thorough understanding of molecular mechanisms that regulate the life cycle of *Plasmodium falciparum* may provide information useful for designing novel strategies to control malaria. Malarial parasites can propagate either asexually or undergo sexual differentiation. It is the asexual development in the erythrocytic phase of the life cycle, which causes pathology of severe malaria. Subsequent to erythrocyte invasion by merozoites, parasites first adopt a ring-like morphology and develop into trophozoites, which give rise to schizonts that contain several merozoites. During the trophozoite stage, the parasite endocytoses large quantities of the erythrocyte cytosol and digests its main constituent, hemoglobin[1]. The endocytosis of erythrocyte cargo occurs via cytosome invaginations and transport vesicles, which mediate the delivery of the material to the food vacuole, where hemoglobin is degraded by a battery of proteases and the residual heme is converted to hemozoin[2].

Phosphoinositides are important for regulating signaling and trafficking events in most eukaryotic cells. These phospholipids are generated by phosphorylation of 3', 4' or 5' hydroxyls of phosphatidylinositol head group by specific PI-kinases[3]. Phosphatidyl-inositol-3-kinase (PI-3K) are most well-characterized and catalyze the phosphorylation of Phosphatidylinositol (PI) Phosphatidyl-inositol-4 phosphate (PI4P), Phosphatidyl-inositol-4,5 bisphosphate [PI(4,5)P2] at 3' position of the inositol ring to generate Phosphatidyl-inositol-3 phosphate (PI3P), Phosphatidylinositol-3,4 bisphosphate [PI(3,4)P2] and Phosphatidyl-inositol-3,4,5 triphosphate [PI(3,4,5)P3] [3]. On the basis of

domain architecture and substrate specificity, PI-3 kinases have been classified into three major groups. Class I PI-3 kinases, which are heterodimeric complexes, can phosphorylate PI, PI(4)P or PI(4,5)P2 *in vitro*[3]. PI(3,4,5)P3, a product of these kinases binds PH domains present on a variety of proteins[4]. Class III PI-3 kinases are known to phosphorylate PI specifically resulting in formation of PI3P[3]. PI3P is present on vacuolar and endosomal membranes in yeast and on membranes of early endosomes and internal vesicles of multivesicular bodies in mammalian cells[5]. PI3P interacts with FYVE, or PX domains of proteins and recruits them to these intracellular locations and controls membrane and vesicular trafficking[6].

Given the important role 3'-phosphorylated PIPs play in signaling and trafficking events in most eukaryotes, it is worth investigating the function of PIPs in *Plasmodium*. This requires identification and characterization of enzymes/proteins involved in metabolism of PIPs and downstream targets of their products. Recently, a PI4P5 kinase was reported in *Plasmodium*, which is regulated by ARF[7] and catalyzes the formation of PI(4,5)P2. We have identified a FYVE domain protein (FCP), which is a likely downstream target of PfPI3K as it interacts with PI3P. Studies performed on this protein resulted in identification of a Cvt pathway in the parasite[8]. In the present study, we report the identification of a PI3-kinase homologue from *Plasmodium falciparum*, PfPI3K. This enzyme is versatile as it catalyzes the formation of PI3P, PI(3,4)P2, PI(3,4,5)P3 from their respective precursors. PfPI3K is present in the food vacuole and in vesicular compartments at the parasite membrane (PM)/parasitophorous vacuole membrane (PVM). Surprisingly, PfPI3K is exported to the host erythrocyte by the parasite and is located at its periphery. PI-3 kinase inhibitor wortmanin inhibited PfPI3K activity. Using

this inhibitors, we demonstrate that PfPI3K may play a key role in hemoglobin endocytosis and its trafficking in the parasite and its activity may be important for parasite development.

Experimental Procedures

P. falciparum cultures

P. falciparum strain 3D7 was cultured at 37^{o}C in RPMI 1640 medium using either AB^{+} human serum or 5% Albumax II (GIBCO) as previously described [9]. Cultures were gassed with 7%CO_2, 5% O_2 and 88% N_2. Synchronization of parasites in culture was achieved by sorbitol treatment.

Parasite growth inhibition assays

Synchronized parasites were cultured either in amino acid free RPMI1640 supplemented with isoleucine (Ile media) for 96 hours. Parasite cultures were diluted with Ile-medium to ~1% parasitaemia in 6 well culture plates. For experiments performed in complete medium, the media was replaced with complete RPMI1640 after 12h (trophozoite stage parasites). At this point, pepstatin A (30μM) or desired concentration of wortmannin were added to the cultures, while parasites were treated with DMSO/MeOH in control experiments. Thin blood smears were stained with giemsa and parasites were visualized microscopically to determine the parasitemia.

Cloning, expression and generation of antisera against PfPI3K

Total RNA was isolated from asynchronous *P. falciparum* 3D7 parasites and reverse transcription was performed using random hexamers provided in the Thermoscript RT-PCR kit (Invitrogen). Based on the sequence information, PCR primers were designed to amplify cDNA corresponding to the helical and the catalytic domain of PfPI3K.

The helical domain of PfPI3K was cloned by using the following primer set PfPI3KHD1 (Forward) ATGTTATCACCAACTATAAACGAGATTAAAAAC and PfPI3KHD1 (Reverse) GATAATTTTATTTTTTCTAATATTAGAAGTCAT. PCR products were cloned directly in pQE-UA bacterial expression vector. For protein expression, plasmid DNA was transformed in BL21-RIL *E. coli* strain (Stratagene). Cultures were grown in LB media containing 100μg/ml ampicillin and 25μg/ml chloramphenicol. Protein expression was induced by addition of 1mM IPTG either at 37^{o}C for 4 h or at 18^{o}C for 14 h. Bacterial cells were harvested by centrifugation at 4000g for 30 min and suspended in Buffer A [50 mM Tris pH 7.4, 6M Urea, 300mM NaCl] followed by sonication for 8 cycles of 1 min each. Cell lysates were clarified by centrifugation at 20,000g for 30 min at 4^{o}C. Subsequently, supernatant was incubated with Ni-NTA agarose resin (Qiagen) with end-to-end shaking for 12 h at 4^{o}C. Following washes with Buffer A, recombinant 6xHis tagged proteins were eluted in 25 mM Tris pH 8.0 containing 300mM NaCl, 300mM Imidazole and 1mM DTT, 6M Urea. Eluted proteins were renatured by dialysis against buffer containing reduced amounts (4M-0M) of urea. Refolded recombinant proteins were analyzed by SDS-PAGE.

To raise anti-sera against PfPI3K helical domain (PfPI3KHD), 100μg of recombinant proteins was emulsified with complete Fruend's adjuvant and used to raise antisera in rabbits and mice by using standard protocols.

Immunoblotting and Immunofluorescence

Parasites were released from infected erythrocytes by 0.05% (w/v) saponin treatment as described earlier[10]. Unless indicated, cell-free protein extracts from specific parasite stages were prepared by suspending parasite pellets in 2% SDS. After separation of lysate proteins on SDS-PAGE gels, proteins were transferred to a nitrocellulose membrane. Immunoblotting was performed using anti-PfPI3K antisera and HRP labeled anti-rabbit IgG. WestDura enhanced chemiluminescence (ECL) substrate (Pierce) was used to develop blots following manufacturer's instructions. Hemoglobin quantitation in parasites by Western blotting with anti-hemoglobin antiserum was carried out as described previously[11,12].

Immunofluoroscence assays (IFA) were performed on thin blood smears as described previously [8]. Briefly, samples were fixed with methanol for 10 min and permeablized with 0.05% Triton X-100 for 5 min. After blocking with either 3% BSA or 2% gelatin (prepared in PBS), smears were incubated with primary antibodies/antisera for 16h at 4^{o}C. Control experiments were performed using pre-immune bleeds or by using uninfected eythrocyte lysates. After washing with PBS, incubations with anti-mouse or anti-rabbit IgG conjugated to either FITC or Texas red were performed. Hoechst 33342 was used for nuclear localization. Cells were visualized using a Zeiss LSM510 confocal microscope equipped with cooled CCD camera or a Zeiss Axio Imager fluorescence

microscope. Immunofluorescence localization of hemoglobin in saponin-treated, aldehyde-fixed and Triton X-100 permeabilized parasites was carried out as described previously [12,13].

Horseradish peroxidase (HRP) endocytosis assay

Enriched schizont-stage parasites were added to HRP-loaded erythrocytes and following invasion, HRP content in the subsequent trophozoite-stage parasites was determined colorimetrically as described previously [12,13].

Immunoprecipitation and assay of lipid kinase activity

Parasites in mid-trophozoite stage were collected by saponin lysis (see above) and washed several times to remove RBC contamination. Subsequently, parasite lysates were prepared in Buffer A [10mM Tris, 100mM Nacl, 5mM EDTA, 1% Triton X-100,
100 µM sodium orthovanadate, 20 µM sodium fluoride, 20 µM beta glycerophosphate and 1x protease inhibitor cocktail (Roche), 10% glycerol] and were clarified by centrifugation at 20,000g at 4^{o}C. Typically, 50µg of parasite lysates were incubated with anti-PfPI3K antisera with end-to-end shaking at 4^{o}C for 12h. Protein A/G agarose beads were added to this mix and further incubation was done for additional 6 hours. The protein A/G agarose beads were washed several times with Buffer A and resuspended in kinase assay buffer.

10µl of immunoprecipitate (IP) was used for performing kinase assays in a 20µl reaction volume in a buffer containing 20mM HEPES, 5mM $MgCl_2$, 0.45mM EGTA, 70µM ATP (γ^{32}P- 6µCi /reaction) and 200µM substrate PI or PIP in the presence or absence of

wortmanin (Biomol). Reaction mix was incubated at 30°C for 30 min and reaction was terminated by addition of 1M HCl. Lipid extraction was first done using 200µl $CHCl_3$: MeOH (1:1) and then with 80 µl of 1M HCl : MeOH (1:1). Phospholipids were separated by Thin Layer Chromatography (TLC), which was performed on pre-activated Silica Gel 60 plates (Merck) in a $CHCl_3$: Methanol : 4N NH_4OH (9:7:2) solvent system. Radiolabeled phosphoinositde products were detected by using a Fuji FL-5000 phosphorimager.

Results

PfPI3K, a PI3-kinase orthologue in *P.falciparum*

BLAST search performed with sequences of mammalian and yeast PI3-kinases against *P. falciparum* genome database resulted in only one significant match on chromosome 5, which was named PfPI3K. PfPI3K corresponds to PlasmoDb annotated gene PFE0765w, which may not have any introns and is predicted to encode a 2133 amino acid protein. PfPI3K shares high homology with the catalytic and signature helical domain of PI3Ks (Supplementary Material, Table I).

PI3-kinases are classified based on their domain architecture, mechanism of regulation and their substrate affinities[3]. Comparison of vacuolar protein sorting protein 34 (vps34), a class III PI3-kinase and PI3Kγ, a class I PI3 Kinase to PfPI3K suggests that reasonable sequence homology exists in the helical domain region and the catalytic domain of these enzymes (Supplementary material, Table I). Helical domain anchors the other important

regions of PI3Ks in an orientation conducive for the catalytic domain to function properly[14]. PfPI3K also has a putative C2 domain, which is known to interact with calcium and lipids (Fig. 1A). PfPI3K lacks any other known protein domains like the Ras Binding Domain (RBD) or Pleckstrin Homology (PH) domain that are found in class I or class II enzymes[3]. PfPI3K has NKNDDD repeats near its N-terminal end, which is common in several *Plasmodium* proteins[15]. The comparison of amino acid sequence and domain organization suggests that PfPI3K may be closest to class III PI3Ks like vps34.

The crystal structure of PI3Kγ exhibits a catalytic domain that has a fold similar to that of protein kinases, predominantly β-sheet containing N-terminal lobe and a helical C-terminal lobe [14]. Structure-based sequence comparisons with PI3Kγ revealed that all 10 β-sheets and 12 α–helices found in PI3Kγ catalytic domain may be present in PfPI3K (Supplementary Fig. I). Interestingly, the catalytic domain of PfPI3K is interrupted by three additional insertions (Supplementary Figure IB).

The helical domain of PI3Kγ has 10 α-helices which form five A/B type pairs. While seven of these helices were reasonably well conserved (Supplementary Figure IA), the sequence of the last three helices of PI3Kγ does not pair up with the corresponding region in PfPI3K. While helical and catalytic domains of PI3Kγ are almost contiguous, they are separated by a ~110 amino acid linker in PfPI3K. Despite the similarity between regions of PfPI3K with other PI3Ks, there are interesting differences, which may suggest a different mode of regulation of this enzyme.

PfPI3K expression during intraerythrocytic parasitic stages

Attempts to express the full-length PfPI3K either in *E. coli* or mammalian cells were not successful. However, the helical domain was expressed with a 6xHis tag (Supplementary Figure II) and was used to raise anti-sera against PfPI3K. A ~ 210 kD band, which was close to the expected size of PfPI3K, was observed in trophozoite lysates and not in control uninfected erythrocyte lysates (Fig. 1B). PfPI3K was present at very low-levels in other intra-erythrocytic stages (data not shown). The transcriptome data (www.PlasmoDb.org) suggests that PfPI3K may also be expressed in sporozoites.

Demonstration of PfPI3K activity in P. falciparum

It was important to have an active PfPI3K to elucidate its substrate selectivity. We were not able to express an active form of recombinant PfPI3K. However, PfPI3K was immunoprecipitated successfully from the parasite lysate. PfPI3K immunoprecipitate (PfPI3K-IP) was used to phosphorylate PI, PI4P or PI(4,5)P2 in a kinase assay mix. Upon incubation with PI, a product corresponding to the size of PIP was observed. Since this product was not observed when synthetic PI3P was used in assays, it is safe to attribute this to PI3P (Fig. 1C, left panel). When PI4P was used as a potential substrate, a radiolabeled product with expected slower mobility corresponding to PI(3,4)P2 was observed. In addition, we found that PI(4,5)P2 may also be converted to PI(3,4,5)P3 by PfPI3K (Fig 1C, right panel). Mock immunoprecipitates made from RBC lysates did not show any kinase activity (data not shown here). These data indicate that PfPI3K can generate PI3P, PI(3,4)P2, PI(3,4,5)P3. These results were surprising since the sequence

and domain architecture comparisons suggest that PfPI3K is closest to class III PI-3 kinases, which mainly form PI3P from PI [3,5].

Attempts to obtain *P. berghei* lacking PI3-kinase gene failed as this kinase is essential for the parasite [16]. Although gene disruption has not been done in *P. falciparum*, PfPI3K can be expected to be indispensable for *P. falciparum* as well. We used a pharmacological approach to evaluate PfPI3K function. Wortmanin, a widely used classical inhibitor for PI3Ks, was tested against PfPI3K. Incubation with wortmanin caused a significant decrease in PfPI3K activity (IC50 ~ 0.5μM) (Fig 1D). In further studies, we have used wortmanin to elucidate the role of PfPI3K in the parasite life cycle.

Localization and trafficking of PfPI3K

Since determination of PfPI3K cellular localization would determine the site of its action in the parasite where it may generate 3'PIP products, immunofluorescence assays were performed. PfPI3K was detected inside the food vacuole (Fig. 2A), which fits well with the presence of a FYVE domain containing protein (FCP) in this compartment. FCP interacts with PI3P [8] and therefore is a likely effector of PfPI3K signaling. In addition, PfPI3K staining was detected in vesicular structures concentrated near the PVM/PM and the food vacuole membrane (Fig. 2 Aa,b). Strikingly, PfPI3K was also found in the host erythrocyte (Fig 2A,B). It was present near the erythrocyte periphery as confirmed by co-staining with an antibody against the conserved C-terminal of VAR gene products (VARC) (Fig. 2B), which are part of knob- structures [17]. We could not detect an obvious PEXEL or HTS motifs in PfPI3K sequence, which target parasite proteins to the host.

To determine if PfPI3K transport to the host is dependent on secretory pathways, parasites were treated with brefeldin A (BFA). BFA, which inhibits post-golgi events like vesicle budding in secretion, blocks the classical secretory pathway as well as the alternate secretory pathways that send the parasite proteins to the host erythrocyte [18,19]. BFA caused a dramatic loss in PfPI3K staining on the PVM/PM and also in the host erythrocyte (Fig. 2B). In BFA-treated parasites, PfPI3K was retained inside the parasite. These data indicate the BFA-sensitive secretory pathway is responsible for exporting PfPI3K to parasite surface and to the host erythrocyte.

PfPI3K, a source of PIPs in infected erythrocytes.

It was important to assess if PfPI3K retains its ability to produce 3'-PIPs in the host. The host erythrocyte cytoplasmic proteins were separated from the parasite by streptolysin A, which may create pores in the membrane of infected Red Blood Cell (iRBC) and cleaves it without modifying the PVM [20]. In addition, PV and iRBC proteins were separated from the parasite proteins by treatment with 0.15% saponin [20]. Consistent with the IFA data, western blotting on these fractions revealed the presence of PfPI3K both in iRBC as well as parasite fractions (Fig. 2C). The presence of PfPI3K in the supernatant of streptolysin-treated iRBC suggested that it is most likely present on the cytosolic side of iRBC membrane, which is consistent with immunofluorescence results. PfPI3K was immunoprecipitated from these fractions and assayed for kinase activity using PI4P as the substrate. PfPI3K was active in the erythrocyte as well as the PV fractions as evidenced by the formation of PI(3,4)P2 (Fig. 2C). These observations suggest that PfPI3K secreted to the host may generate 3'-PIPs, which may be important

for controlling signaling/trafficking events in the host erythrocyte. Interestingly, it has been reported that the levels of PIP and PIP2 increase dramatically in erythrocytes after infection with *P. falciparum* and parasite machinery was speculated to contribute to this process [21]. Our finding that the parasite exports active PfPI3K to the RBC provides an explanation for this observation.

PfPI3K inhibition leads to accumulation of hemoglobin in the parasite

Malaria parasites endocytose hemoglobin from the host erythrocyte, which is an important for their development. Morphologically, several cellular mechanisms have been implicated in hemoglobin trafficking to the parasite[22]. However, molecular mechanisms that regulate hemoglobin trafficking are still unclear[12]. Since PI3K and its products are important players in endocytosis and trafficking events in most eukaryotes, it was worth studying the role of PfPI3K in hemoglobin transport to the parasite. To investigate this, parasites were incubated with wortmanin and the amount of hemoglobin present inside the parasite was determined by western blotting. Wortmanin treated parasites exhibited significantly increased hemoglobin levels (Fig. 3A) compared to the solvent control. In contrast, mefloquine, a known hemoglobin endocytosis inhibitor significantly reduced hemoglobin levels (data not shown) as previously reported [11]. Based on these observations, it is reasonable to suggest that wortmanin either causes a stimulation of hemoglobin endocytosis or attenuates of hemoglobin digestion which results in increased levels of hemoglobin in the parasite.

PfPI3K may be involved in uptake of material from the host

Firstly, efforts were made understand if PfPI3K is involved in trafficking of material from the host. To this end, uptake of horseradish peroxidase (HRP), a standard non-digestible fluid-phase endocytic tracer, was evaluated. Erythrocytes infected with mature parasites were enriched and incubated with HRP-loaded erythrocytes in culture to allow invasion of the latter. HRP uptake from the infected pre-loaded erythrocytes by parasite fluid-phase endocytosis was quantitated by calorimetrically measuring HRP enzymatic activity in isolated trophozoites. Wortmanin caused a significant decrease in HRP levels in the parasites indicating an inhibition of endocytosis (Fig. 3B).

PfPI3K inhibitor block food vacuole trafficking of hemoglobin.

PfPI3K inhibition blocks endocytosis of HRP from the host (Fig. 3B). While this observation is indicative of a role of PfPI3K in the parasite, it does not explain the increase in the levels of hemoglobin levels upon wortmanin treatment. To explore this frther, changes in hemoglobin trafficking in the parasite was followed. After treatment with wortmanin, parasites were released from RBCs by saponin treatment, immobilized on coverslips and fixed with paraformaldehyde/glutaraldehyde. Subsequently, immunofluorescence assays were performed with commercial anti-hemoglobin antiserum to determine the subcellular localization of hemoglobin in the parasite. Expectedly, hemoglobin staining was observed inside the food vacuole and occasional punctate structures in the cytoplasm, which likely represent previously described hemoglobin-laden endocytic vesicles[11,12] (Fig. 4A). Treatment with wortmanin resulted in apparent decrease in food vacuole hemoglobin. More strikingly, the inhibitor treated parasites

exhibited an increase in the number of hemoglobin positive vesicles (Fig. 4A). This was confirmed by counting the number of extra-digestive vacuolar fluorescent foci in randomly selected parasites. Parasites treated with wortmanin contained ~5 fold more vesicles (Fig. 4B). In contrast to these results, mefloquine treatment resulted in a marked decrease in the vesicle number (data not shown).

These data suggest that the inhibition of PfPI3K may cause defects in endocytic vesicle trafficking of hemoglobin to the food vacuole, the site of hemoglobin digestion. Hemoglobin may consequently be trapped in endocytic vesicles upon PfPI3K inhibition, which prevents efficient transport to the food vacuole resulting in impaired catabolism.

To confirm the hemoglobin IFA results, wortmannin treated parasites were further visualized by electron microscopy. As suggested by the IFA images (Fig. 4A), treated parasites contained increased numbers of hemoglobin transport vesicles, often strikingly so in individual parasites (Fig. 4C). Average vesicle number per parasite profile increased from 1.58 ± 0.12 to 3.83 ± 0.23. There appeared to be a concurrent increase in vesicle diameter, from an average of 322 ± 33 nm in controls to 543 ± 26 in treated parasites. Vesicle size increase was also suggested by the IFA images (compare Fig. 4A "C" and "W").

Inhibition of PfPI3K-mediated transport blocks parasite growth.

The endocytosis of hemoglobin to the food vacuole and its catabolism is important for parasite growth as it is a major source of most amino acids for the parasite. When hemoglobin proteolysis is blocked, the parasite can get the supply of amino acids from extracellular milieu [23]. Isoleucine is the only amino acid that is not present in human

hemoglobin for which the parasite relies almost completely on acquisition from extracellular medium [23,24]. Since hemoglobin endocytosis is a prerequisite for its degradation in the parasite food vacuole and PfPI3K regulates hemoglobin uptake, we checked if wortmanin alters parasite growth. These experiments were done simulateously in two sets; in one set, the parasite was cultured in complete RPMI 1640 medium, in the other, a medium lacking all amino acids except Ile (Ile-medium), was used. In the latter situation, parasite was almost completely dependent on hemoglobin as a source for other amino acids [23].

The parasites were plated at low parasitemia in the presence or absence of wortmannin and pepstatin A, a aspartic protease inhibitor which is known to stall parasite growth [23]. It was evident that the growth of parasites, when cultured in complete medium for 6 days in the presence of wortmanin, was inhibited significantly (Figure 5A), ~2.5 μM wortmannin caused ~50% reduction in parasite growth. When parasites were treated with wortmannin in Ile-medium, a strikingly higher sensitivity towards wortmannin was observed. The parasite growth was almost completely blocked by ~2.5μM wortmannin (Figure 5B). Treatment with protease inhibitor pepstatin A resulted in similar trends and indicated that this inhibitor is also more effective in Ile-medium as reported previously [23]. These observations suggest that since the amino acid supply to the parasite in Ile-medium is almost completely dependent on hemoglobin catabolism, the inhibition of PfPI3K mediated endocytosis by wortmannin causes even more severe defects in parasite growth. Based on these data, we conclude that PfPI3K may control trafficking pathway(s) that are involved in hemoglobin transport to the parasite food vacuole (Figure 6), a crucial step for its catabolism and therefore is necessary for parasite growth. This is consistent

with gene disruption studies carried on PI3K in *P. berghei*, which suggsted that it is an essential gene [16].

Discussion

The presence of enzymes like PI synthase [21], PfPI4P5K [7], PfPI3K and other PIP kinases (Pushkar Sharma, unpublished results) in *Plasmodium* suggests that PIPs may have a major role to play in parasite signaling and trafficking as is the case in most eukaryotes. 3'-phosphorylated PIPs are generated by the action of PI-3 kinases on PI, PI4P and/or PI(4,5)P2, are major players in this process [3,25]. One of the major targets of PI-3 kinases in eukaryotes is protein kinase B (PKB), which is activated and translocated to the cell surface following binding to PI(3,4)P2 or PI(3,4,5)P3 via a PH domain present at its N-termius. PfPKB, a PKB like kinase in *P. falciparum* lacks a PH domain and is not regulated directly by PIPs [26,27]. This was indicative of one of the many differences that may distinguish signaling events in this parasite from other eukaryotes. PfPI3K seems to be the only PI-3 kinase in *Plasmodium falciparum* and shares characteristics with class III PI3Ks like vps34. In contrast to vps34, which generates mainly PI3P from PIP[3,5], PfPI3K phosphorylates both PI and PI4P to yield PI3P, PI(3,4)P2. Attempts to disrupt PfPI3K homologue gene in *P. berghei* were futile as it is essential for parasite growth[16]. Based on this, it is reasonable to assume that PfPI3K may have a similar role in *P. falciparum*. The classical PI3K inhibitor wortmannin was effective against PfPI3K and served as an effective tool to elucidate PfPI3K function in the parasite. There are no concrete reports that suggest that PI3- kinases may have an active role in mature

erythrocytes, which was also supported by the presence of low levels of PIPs in uninfected erythrocytes[21]. Therefore, it is reasonable to attribute the effects of wortamanin to PfPI3K inhibition.

PIPs are known to regulate membrane and vesicular trafficking by interacting with FYVE, PX, PH and other domains and target proteins that contain them to various cellular compartments. Very often, this targeting is to cellular organelles like endosomes, MVB, cytoplasmic vesicles, which are involved in protein and membrane trafficking [4,28,29]. We recently identified a FYVE domain containing protein (FCP) that binds PI3P [8], which makes it a target of PfPI3K. FYVE domain proteins in higher eukaryotes localize mainly on endocytic organelles and compartments like early endosomes [29]. In contrast, FCP was present mainly inside the food vacuole of the parasite. Its targeting to the food vacuole was independent of the PI3P-interacting FYVE domain and via Cvt-like pathway [8]. The co-existence of FCP and PfPI3K inside the food vacuole suggests that production of PI3P may be inside the food vacuole, where, it interacts with FCP. Our results indicate that PfPI3K is exported to erythrocytes in vesicle-like organelles and to the PVM/PM. PfPI3K activity in the host erythrocyte raised the possibility that it may regulate host signaling and trafficking events. Interestingly, it has been reported that the levels of 3'-phosphorylated PIPs increase significantly in erythrocytes upon infection, which was attributed to the parasite but not the host machinery [21]. These observations may be explained on the basis of our results which demonstrate that PfPI3K is exported to the host by the parasite in an active form.

In *Dictyostelium*, PI3K is required for macromolecule uptake by macropinocytosis[30], while Wortmannin and LY294002 have been reported to inhibit fluid-phase endocytosis within mammalian cells[31]. PI-3 kinases also act as regulators of phagocytosis and are essential players in phagosome formation and maturation[32]. Based on the reported participation of PI3K endocytic events in other eukaryotes, we investigated whether PI-3 kinase inhibition would downregulate endocytosis of host cytoplasm in malaria parasites**.** Consistent with this hypothesis, parasite HRP accumulation through fluid-phase endocytosis from infected host erythrocytes was decreased by wortmannin. However, earlier Western blotting results had indicated an increase in hemoglobin content in parasites exposed to the inhibitors, suggesting additional effects on the parasite endocytic pathway and/or digestion of endocytosed material in the food vacuole. Immunofluorescence localization of hemoglobin in wortmannin treated parasites showed a significant increase in endocytic hemoglobin transport vesicles, suggestive of a block in endocytic trafficking. The inhibition of hemoglobin transport and delivery to the food vacuole likely impairs the digestion of internalized hemoglobin in the latter compartment and produces the increase in hemoglobin levels in the treated parasites. This conclusion is consistent with the reported function of PI-3 kinases in post-endosomal sorting and degradation in mammalian cells [33], genetic evidence for the role of PI-3 kinases in sorting to yeast vacuoles [34], and PI-3 kinase involvement in phagosome maturation [32]. In addition, the association of PfPI3K and a downstream effectors like FCP with the parasite food vacuole may have direct bearing on the inability of inbound hemoglobin transport vesicles to fuse with this compartment in the presence of PI3-kinase inhibitors.

Interestingly, previous studies have reported a similar role for actin in hemoglobin trafficking in malaria parasites, evidenced by an increase in volume and number of hemoglobin-filled vesicles in parasites treated with actin disruptors [12, 22, 35]. However, PfPI3K appears to affect both hemoglobin uptake as well as subsequent trafficking, while actin inhibitor effects are focused primarily on the latter. The principal mode of hemoglobin endocytosis in trophozoites is via cytostomes, invaginations of the parasite plasma membrane characterized by an electron-dense collar at the neck [22]. In a manner reminiscent of classic clathrin-dependent endocytosis in mammalian cells, the cytostomes presumably pinch off to form endocytic vesicles that traffic hemoglobin to the digestive vacuole in an actin and PfPI3K-dependent manner. A detailed ultrastructural study of endocytosis identified three additional modes of hemoglobin uptake that supplement the cytostome pathway: a "big gulp" in the ring stage that forms a nascent digestive vacuole, large hemoglobin-filled "phagotrophs" that are likely formed by a cytostome-independent mechanism and elongated "cytostomal tubes" that are morphologically distinct from the more spherical standard hemoglobin transport vesicles [22]. We have not attempted to distinguish between these modes in addressing the effects of PfPI3K, but overt evidence of cytostomal tubes and phagotrophs in addition to the hemoglobin transport vesicles in our IFA and EM images were not found. In contradiction to the above ultrastructural study, an alternative model of the hemoglobin endocytic pathway was proposed, in which the cytostomes do not pinch off to form independent vesicles, but elongate and fuse directly with the digestive vacuole[35]. By extension, an increase in "vesicle" number in parasites compromised in actin or PfPI3K activity would represent an increase in the convolutions of individual cytostomes [35].

In addition to actin, hemoglobin trafficking may also be dependent on Rab5a, a known mediator of mammalian early endosome function although, the detailed underlying molecular mechanisms remain unknown [22]. It is known that Rab GTPases participate in PI3K-mediated vesicular trafficking in several cell types and several members of the Rab family are encoded in *Plasmodium* genome [35,36]. Therefore, it will be worth testing if PfPI3K controls hemoglobin endocytosis via Rabs.

Hemoglobin catabolism, which is important for the parasite growth, is carried out by aspartic proteases plasmepsins and cysteine proteases falcipains [37]. Interestingly, gene disruption of individual enymes or even in combination only marginally slowed the growth of the parasite [23]. It was demonstrated that the parasite could grow in a medium containing only a single amino acid, Ile, which is missing from human hemoglobin [23]. Ile, which is necessary for protein synthesis and parasite survival, is acquired from extracellular milieu by ATP dependent and independent processes [24]. Several protease knock-out lines and protease inhibitor treatment exhibited marked impairment of parasite growth in this medium, which highlighted the importance of hemoglobin degradation in parasite survival in this situation. Our observation that wortmannin inhibits parasite growth in complete medium indicates the importance of PfPI3K in hemoglobin endocytosis to the parasite food vacuole. Although, it is possible that PfPI3K may play additional roles in parasite development (summarized in Fig. 6). Since the effects of wortmannin were significantly more dramatic in Ile-medium, it is reasonable to suggest that hemoglobin uptake is a prominent PfPI3K- dependent process.

Acknowledgements

The funding for this work was supported in part by the FP7 programme (MALSIG project) from EU and Swaranajayanti fellowship to P.S. A.V. and R.R. were supported by the Junior Research Fellowship by the Council for Scientific and Industrial Research, India. Authors would like to thank Dr. Amit Sharma for VARC antisera.

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Figure Legends:

Figure 1: A. Schematic diagram showing domain architecture of PfPI3K. PfPI3K has a helical domain upstream of the catalytic domain and these domains are separated by a ~110 amino acid linker. In addition, a C2 domain is present near the N-terminal end.

B. Equal amount of cell lysates prepared from uninfected erythrocytes (E) and trophozoite stage parasites (Pf) were electrophoresed on a 7% SDS-PAGE gel and western blot analysis was performed using anti-PfPI3K antisera.

C. PfPI3K was immunoprecipitated from trophozoite lysates using anti-PfPI3K antisera and equal amount of PfPI3K-IP was used in a lipid kinase assay wherein PI, PI3P, PI4P, PI(4,5)P2, were used as substrate. Phospholipids were separated on a TLC along with phosphoinositide standards and radiolabeled lipid products were detected by using a phosphorimager.

D. The activity of PfPI3K-IP from trophozoite lysates was assayed using PI4P as substrate (as described in C) in the presence (+) of 0.6 μM wortmannin or DMSO (-).

For calculating the IC50 of wortmanin, various concentrations of wortmanin were used in a kinase assay and the densitometry of the radiolabelled PI(3,4)P2 product was performed. The IC50 for PfPI3K inhibition was ~0.5μM.

Figure 2. A. Intracellular-localization of PfPI3K and its trafficking to the host.

A. IEF was performed for localizing PfPI3K, FCP (a) and VARC (b). FCP (red) is located inside the food vacuole of mature trophozoites. PfPI3K (green) exhibits "vesicular" staining on PVM/PM (black arrows) and the host erythrocyte (white arrows)

and in the food vacuole, which can be identified by the presence of black hemozoin. Panel b, PfPI3K co-localizes with VARC at the erythrocyte surface.

B. Parasites were treated either with 5mg/ml (BFA) or DMSO and immunofluorescnce was performed using anti-PfPI3K antisera. BFA treatment blocked the transport of PfPI3K to the erythrocyte.

C. Trophozoite stage parasites were either treated with streptolysin (SLO) or saponin (SAP). The soluble (SUP) and pellet (PEL) fractions were used for western blot (bottom panel) and immunoprecipitation of PfPI3K. PfPI3K-IP was used to assay the activity with PI4P as the substrate as described above.

Fig. 3. **Hemoglobin accumulation in malaria parasites as result of PfPI3 K inhibition**

A. Parasite were incubated either with DMSO (Control), 10μM wortmannin for 14h. After releasing the parasite from the erythrocytes, parasite lysates were prepared and Western blotting performed using anti-hemoglobin antiserum.

B. Erythrocytes were preloaded with HRP, infected with parasites, and treated with wortmannin for 14 hours. After releasing parasites from infected erythrocytes by saponin treatment, HRP levels associated with parasites was determined with a colorimetric enzyme assay. Absorbance values were normalized to the solvent controls set at 100. Error bars indicate standard error of the mean.

Fig. 4. **The effect of PfPI3K inhibition on hemoglobin trafficking.** A. Parasites were treated with DMSO or wortmannin. Saponin treatment was used to release excess non-parasitic hemoglobin, followed by attachment of parasites onto poly-lysine-coated glass cover slips. Following fixation in paraformaldehyde/glutaraldehyde and Triton X-100 permeabilization, immunofluorescence assays were performed using anti-hemoglobin antibody and DAPI was used to stain the parasite nucleus. A single erythrocyte-free parasite can be seen in each phase contrast image (PC) with its food vacuole (indicated by the prominent haemozoin crystal) denoted with a large arrow. Corresponding hemoglobin immunofluorescence images (Hb) contain punctate vesicle-like structures indicated by the narrow arrow. B. Average transport vesicle counts per parasite were compared between wortmannin and DMSO control. Error bars represent SEM. C. Transmission electron micrographs of DMSO and 10μM Wortmannin treated trophozoite stage malaria parasite. Each micrograph shows a single parasite located inside a host erythrocyte. The cytoplasm of the host cell is darkly stained due to the preponderance of electron-dense hemoglobin. Labelled structures inside the parasites are the parasite nucleus (N), food vacuole (Fv), and hemoglobin transport vesicles (V).

Figure 5. **PfPI3K inhibition stalls parasite growth**. Ring stage parasite cultures in complete medium (A) or Ile-medium (B) were incubated with DMSO/MeOH (control), indicated concentrations of wortmannin or pepstatin A (peps). Parasite growth was monitored by counting the parasite infected erythrocytes after 6 days. The % growth of parasite in drug-treated cultures in comparison to control (100%) is indicated. Error bars represent S.E.M.

Figure 6. A model for role and regulation of PfPI3K in malaria parasite.

After synthesis in the parasite, PfPI3K is trafficked to vesicular compartments at PM/PVM and the food vacuole, and exported to the host RBC. PfPI3K may regulate the function of FCP, a PI3P binding protein, which is present in the food vacuole [8]. PfPI3K inhibition blocks endocytosis of hemoglobin to the food vacuole and blocks parasite growth. PfPI3K exported to the host RBC is active and may trigger signaling and trafficking pathways that may contribute to additional functions.

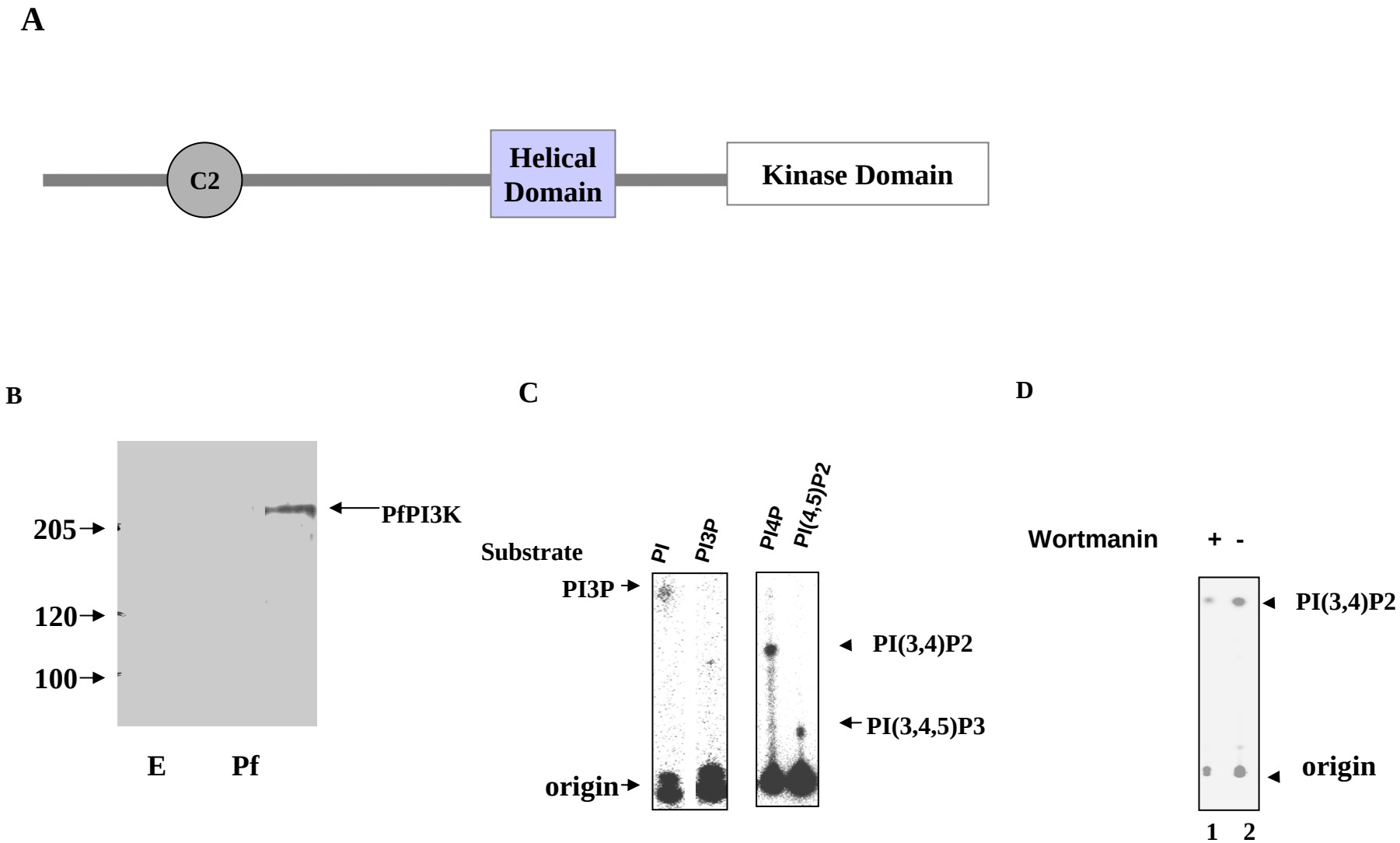

A
C2
Helical Domain
Kinase Domain
B
205
120
100
PfPI3K
E
Pf
C
Substrate
PI
PI3P
PI4P
PI(4,5)P2
PI3P
PI(3,4)P2
PI(3,4,5)P3
origin
D
Wortmanin
+ -
PI(3,4)P2
origin
1 2

Fig. 1

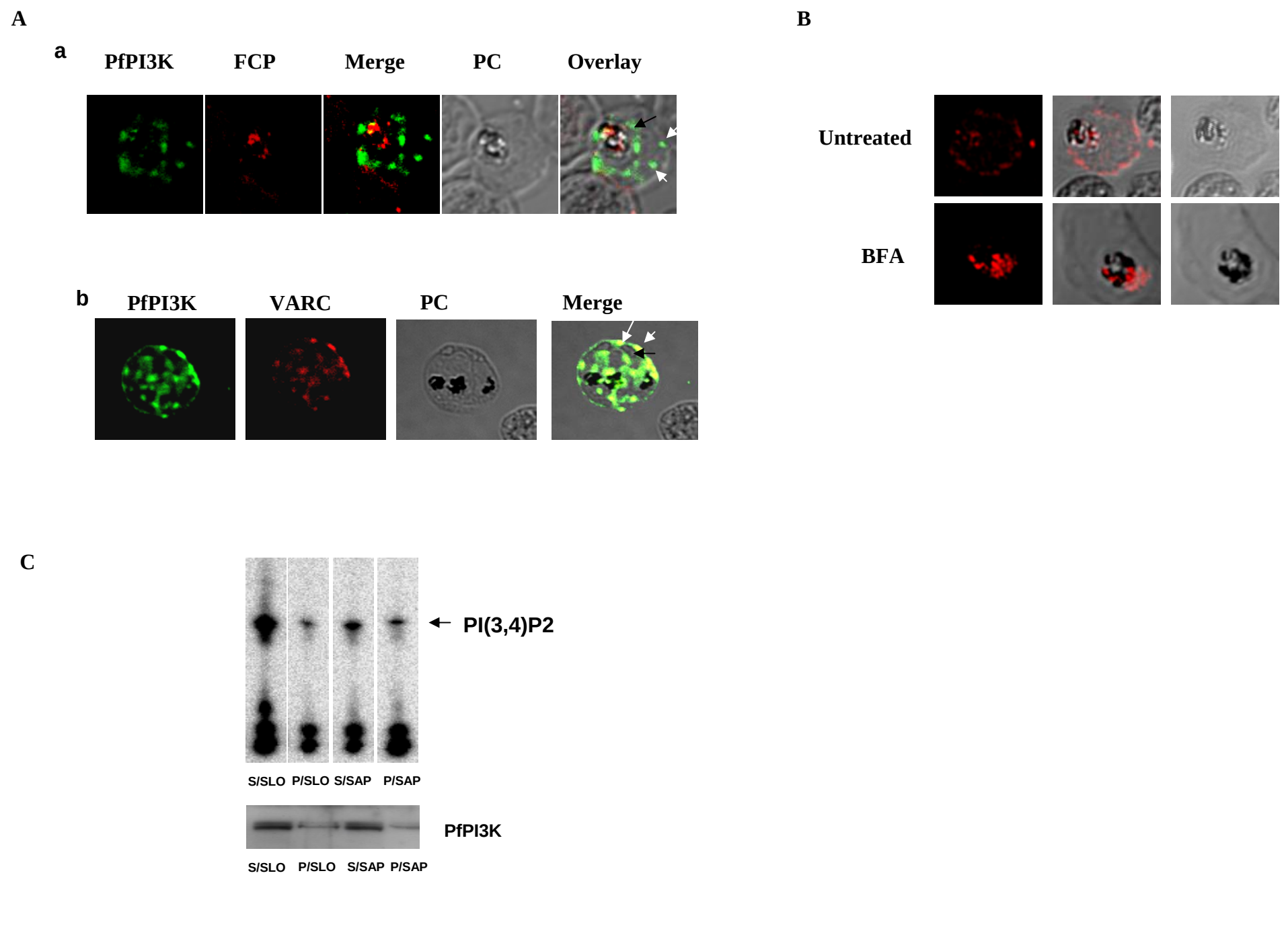
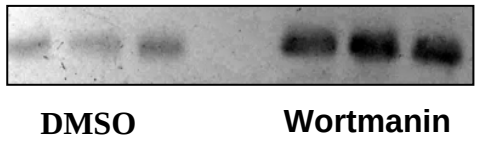

A
a
PfPI3K
FCP
Merge
PC
Overlay
b
PfPI3K
VARC
PC
Merge
B
Untreated
BFA
C
PI(3,4)P2
S/SLO P/SLO S/SAP P/SAP
PfPI3K
S/SLO P/SLO S/SAP P/SAP

Fig. 2

A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Wortmanin

B

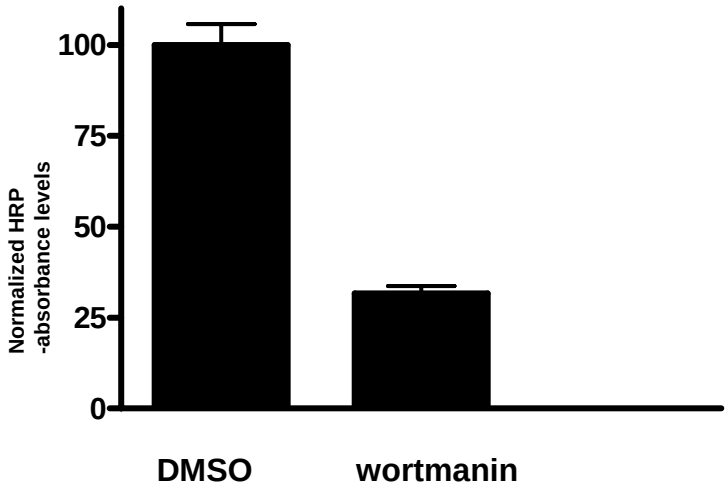
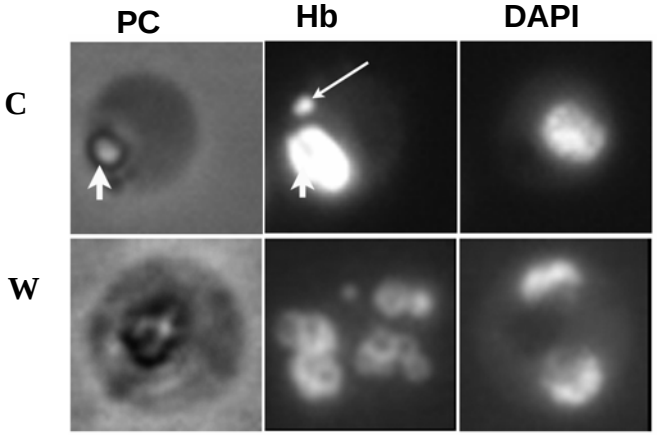
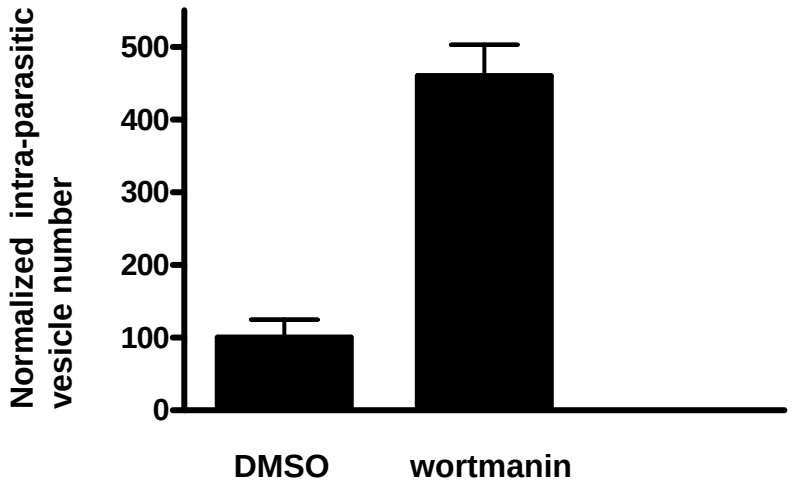

Normalized HRP
-absorbance levels
100
75
50
25
0
DMSO
wortmanin

Fig. 3

A


PC
Hb
DAPI
C
W

B


Normalized intra-parasitic vesicle number
500
400
300
200
100
0
DMSO
wortmanin

C

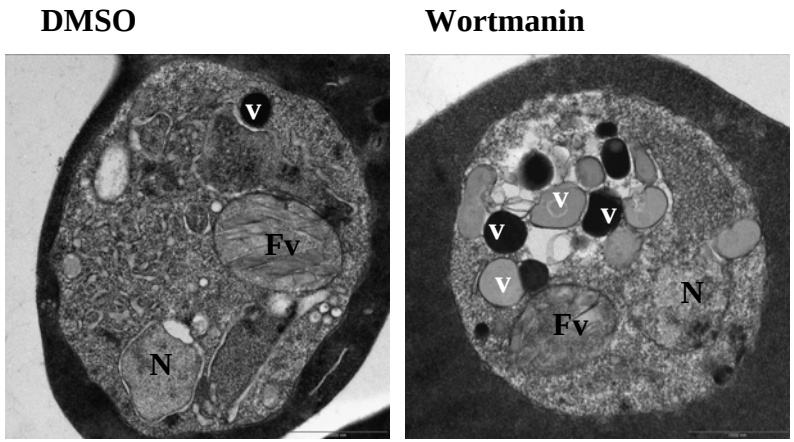
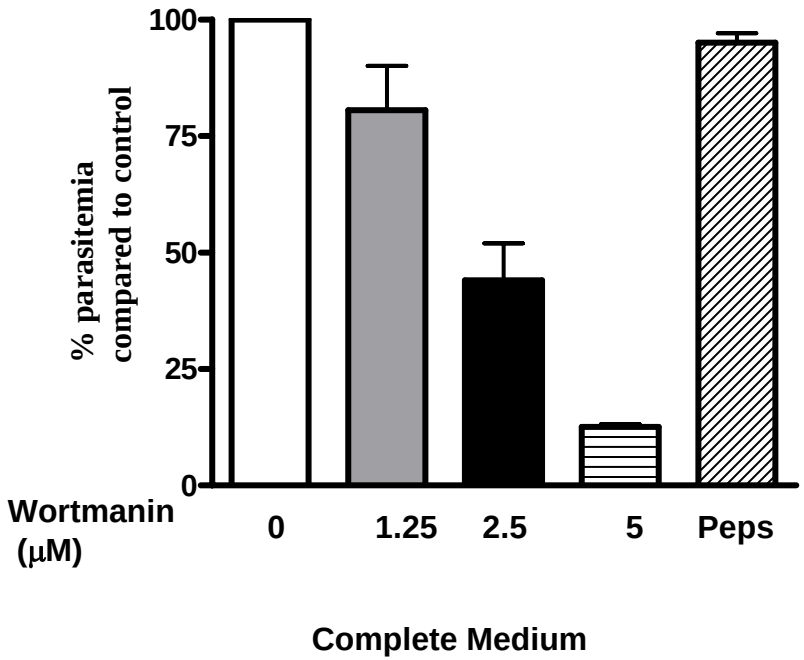

DMSO
Wortmanin
V
Fv
N
V
V
V
V
Fv
N

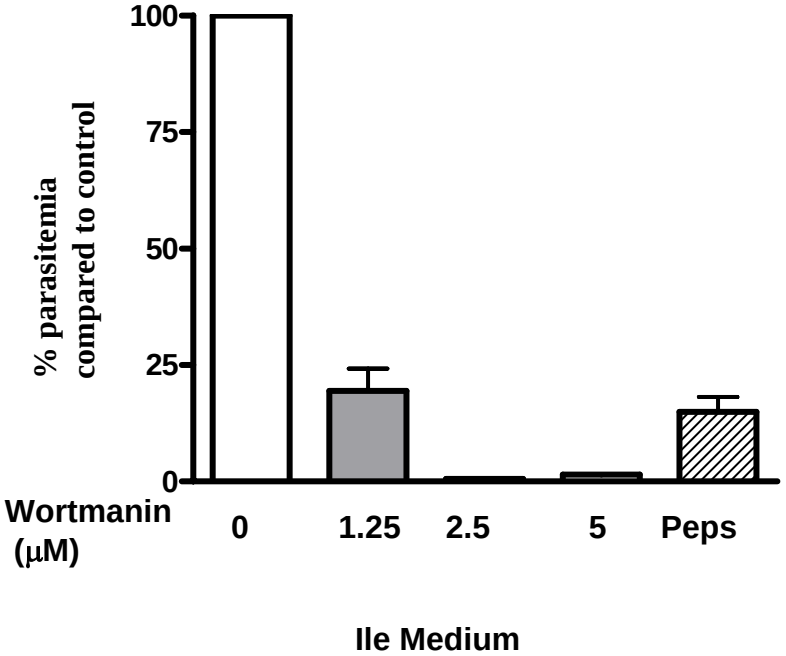
Fig.4

A



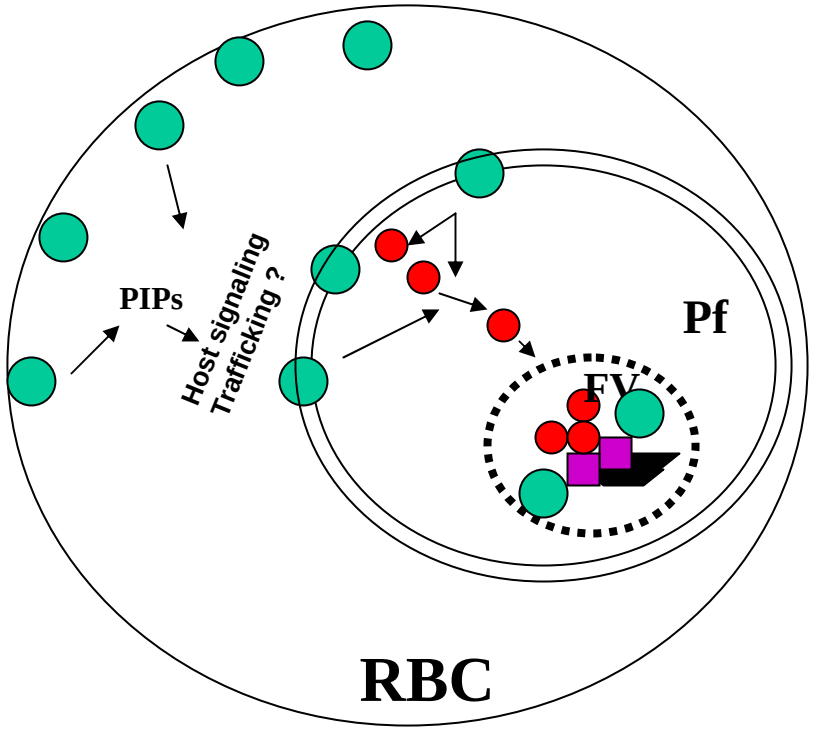
Complete Medium

B



Ile Medium

Fig. 5



Hb-vesicles

PfPI3K

FCP

Fig. 6