Eps15 homology domain containing protein of *Plasmodium falciparum* (PfEHD) associates with endocytosis and vesicular trafficking towards neutral lipid storage site

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**ABSTRACT**

The human malaria parasite, *Plasmodium falciparum*, takes up numerous host cytosolic components and exogenous nutrients through endocytosis during the intra-erythrocytic stages. Eps15 homology domain-containing proteins (EHDs) are conserved NTPases, which are implicated in membrane remodeling and regulation of specific endocytic transport steps in eukaryotic cells. In the present study, we have characterized the dynamin-like C-terminal Eps15 homology domain containing protein of *P. falciparum* (PfEHD). Using a GFP-targeting approach, we studied localization and trafficking of PfEHD in the parasite. The PfEHD-GFP fusion protein was found to be a membrane bound protein that associates with vesicular network in the parasite. Time-lapse microscopy studies showed that these vesicles originate at parasite plasma membrane, migrate through the parasite cytosol and culminate into a large multi-vesicular like structure near the food-vacuole. Co-staining of food vacuole membrane showed that the multi-vesicular structure is juxtaposed but outside the food vacuole. Labeling of parasites with neutral lipid specific dye, Nile Red, showed that this large structure is neutral lipid storage site in the parasites. Proteomic analysis identified endocytosis modulators as PfEHD associated proteins in the parasites. Treatment of parasites with endocytosis inhibitors obstructed the development of PfEHD-labeled vesicles and blocked their targeting to the lipid storage site. Overall, our data suggests that the PfEHD is involved in endocytosis and plays a role in the generation of endocytic vesicles at the parasite plasma membrane, that are subsequently targeted to the neutral lipid generation/storage site localized near the food vacuole.

**1. Introduction**

Malaria remains a major parasitic disease in the tropical and sub-tropical countries causing 1–2 million deaths globally every year [1,2]. All the pathological symptoms of malaria are due to asexual stage life cycle of the parasite in the host erythrocytes. The intra-erythrocytic parasite grows within the parasitophorous vacuole, divides to form new merozoites, which are released by rupture of the host cell and subsequently invade new erythrocytes. During this growth cycle, the parasite takes up and degrades large amount of hemoglobin – an essential requirement for its growth – from the host cell. The uptake of host cytosol and hemoglobin occurs via cytosomal vesicles that traverse to the food vacuole [3]. In addition, for its survival the parasite also requires the uptake of material from the host milieu through endocytosis, which include uptake of several nutrients and building blocks [4]. Trafficking of variety of these substances, from outside as well as within the parasite, is essential to support its growth and multiplication. A detailed understanding of the process of endocytosis may therefore be helpful to design novel anti-malarial strategies.

The eukaryotic Eps15-homology domain containing proteins (EHDs) play an important role in the regulation of specific endocytic transport steps including endocytic membrane trafficking, endosomal recycling and intracellular sorting through protein–protein interactions [5–7]. Among these, the endocytic recycling is proposed to be one of the major roles of EHD protein that involves recycling of membrane receptors and lipids [8–10]. The four EHD paralogues in mammalian cells are known to regulate different but partly overlapping endocytic transport events [6,7]. EHD1 is associated with endocytic recycling compartments primarily through its interaction with Rab effector proteins [11]. In addition, it is also known to be involved in internalization of low density lipoprotein receptor and cell adhesion molecules [12]. Once internalized, the small vesicles containing the internalized cargo fuse with...
early endosomes (also known as sorting endosomes), and the receptors are either degraded by lysosomal pathway or recycled back to the plasma membrane, where they may participate in additional rounds of internalization [11]. EHD3, on the other hand, has been implicated in regulating transport of not only early-endosome to recycling-endosome but also in regulating the endosome-to-Golgi transport [13, 14]. In addition, EHDs have also been shown to be involved in endosomes-to-Golgi retrograde transport from as well as in regulation of receptor transport from early endosomes to the lysosomal degradation pathway [15]. However, the key parasite proteins regulating this process are not well characterized. Here, we characterize the \textit{Plasmodium falciparum} homolog of dynamin-like C-terminal Eps15 homology domain containing protein (PfEHD); our studies suggest that the PfEHD plays a role in endocytosis-like events as well as trafficking of vesicles towards neutral lipid storage site in the parasite.

2. Results

2.1. Identification and sequence analysis of \textit{P. falciparum} EHD homolog

In our attempts to further understand the process of uptake and trafficking of host material into the parasite, we characterized a putative dynamin-like C-terminal Eps15 homology domain containing protein (PfEHD) of \textit{P. falciparum} (PfEHD; Gene ID: PF3D7_0304200). The PfEHD is a 533 amino acid long protein that possesses a dynamin-N domain of 159 amino acids (Pfam E-value $2.5 \times 10^{-22}$) towards N-terminus and an Eps15 homology domain (Pfam E-value $2.9 \times 10^{-10}$) of 90 amino acids at the C-terminus (Fig. 1A); the Eps15 homology domain (EHD) is a known substrate for tyrosine kinase [16,17]. A BLAST search analysis showed that the EHD domain of PfEHD has high sequence homology with EHD domains of \textit{Acromyrmex echinatior} (95%), \textit{Toxoplasma gondii}

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Fig. 1. Expression and localization of the PfEHD fusion protein with GFP (PfEHD-GFP) in transgenic parasites. (A) Schematic diagram showing domain structure of PfEHD. The predicted N terminal-dynamin like domain, EF hand-domain and the calcium-binding region are shown along with their respective amino acid positions. Region cloned for expression of recombinant protein for lipid–protein binding assay is also marked. (B) Immunoblot analysis of wild-type and transgenic parasites expressing PfEHD-GFP using anti GFP antibodies. An ~85 kDa band representing the GFP fusion protein is recognized in the transgenic parasites (PfEHD-GFP), but not in the wild type (WT). Blot ran in parallel and probed with anti-PfBiP antibodies detected native PfBiP protein (~70 kDa) in both parasite lines. (C) Confocal microscopy images of live transgenic parasites showing localization of PfEHD-GFP fusion protein through ring, trophozoite and schizont stages of the parasite. (D) Immunoblot analysis of pellet and supernatant fractions of infected erythrocytes after saponin lysis. The anti-GFP antibodies detected PfEHD-GFP in pellet fraction.
(98%), Harpegnathos saltator (95%), Bombyx mori (98%), Drosophila melanogaster (98%) and Eimeria tenella (96%). Furthermore, this region was also found to be highly conserved among different species of Plasmodium (Fig. S1). The PfEHD domain contains helix-loop-helix motifs (EF hands) as well as the conserved lysine critical for phosphatidylinositol binding during the endocytosis process (Figs. S1 and S2). In addition, amino acid sequence alignment of the EF hand region of PfEHD with that of EHD homologs from other organisms showed that a number of conserved residues are present in PfEHD proteins (Fig. S2). This analysis suggests that PfEHD may be involved in endocytic processes in the parasite as in case of EHD proteins in other organisms.

2.2. Localization of PfEHD in the transgenic parasites by GFP targeting

To localize the PfEHD protein and to understand its potential role in the parasite, we generated transgenic parasites expressing PfEHD-GFP fusion protein. Western blot analysis of total lysates of the transgenic parasites using anti-GFP antibodies showed a band of ~85 kDa corresponding to the fusion protein (Fig. 1B). The transgenic parasites were used for fluorescence and confocal microscopy studies to localize the PfEHD-GFP fusion protein in different developmental stages of the asexual cycle. In young trophozoite stages the fluorescence was observed as distinct foci close to the parasite boundaries; each parasite usually one-to-three such distinct foci were seen (Fig. 1C). In mature trophozoites several fluorescent foci/vesicle-like structures were observed in the parasite that are distributed in the cytosol (Figs. 1C and S3A). In late trophozoite stage parasites, a brightly fluorescent GFP labeled structure was also observed in the parasite cytosol juxtaposed to the food-vacuole, which was larger than other GFP labeled vesicles (Fig. 1C, panel 3; Fig. S3B). To assess any possibility of protein trafficking to the parasitophorous-vacuole, the saponin fractions of the infected erythrocyte cytosol were also observed (Fig. 2A). Additionally, the association of PfEHD with the parasite plasma membrane was determined using confocal microscopy. Some of the parasites also showed GFP foci dispersed in the parasite cytosol away from membrane labeling (Fig. 2A, panel 2). The association of PfEHD with the parasite membrane in specific foci may suggest their involvement in endocytosis-like vesicle-mediated uptake of material from parasitophorous vacuole.

Further, we also analyzed any possible association of the PfEHD labeled vesicles with Rab proteins that are known to be involved in vesicular trafficking. We used specific antibodies to PIRab5A, PIRab5B and PIRab7 for immuno-staining of transgenic parasites expressing PfEHD-GFP. Immuno-staining of all the three anti-Rab antibodies showed vesicular punctate structure in the parasites (Fig. 3); however there was no co-localization with Rab5B or with Rab7. The Rab5A staining was observed in close association and juxtaposed to PfEHD (Fig. 3), which suggest that the Rab5A associates with PfEHD labeled endosomal vesicles. We have also calculated Pearson's correlation coefficient (PCC) as a statistic for quantifying colocalization of PfEHD and Rab5A in several images (n = 20), and mean was found to be 0.31, suggesting partial colocalization of two proteins; whereas, for PfEHD and Rab7, the coefficient was 0.01 suggesting that there is no overlap.

To ascertain the association of PfEHD with vesicular structure and its possible involvement in endocytic pathways, we assessed membrane association of PfEHD in the parasites by sequential solubilization studies. In parasite lysate, using Tris-buffer, the PfEHD was completely insoluble and detected in pellet fraction; further suspension of this pellet in carbonate buffer was not able to solubilize PfEHD and it was detected in pellet fraction (Fig. 4A). Treatment of this pellet with Triton X-100 containing buffer only solubilized the PfEHD and it was detected in supernatant fraction (Fig. 4A), suggesting that the PfEHD is a membrane bound protein in the parasite. Since PfEHD protein does not contain any trans-membrane domain, we assessed its membrane association by Triton X-114 phase separation technique. Using this protocol, the peripheral associated membrane proteins are obtained in aqueous phase and integral membrane proteins are obtained in detergent phase when the solubilized protein samples are exposed to temperature above the ‘cloud point’ for the detergent [18]. The PfEHD protein was detected in the aqueous phase after Triton X-114 phase separation supporting the point that it is membrane associated protein and not an integral membrane protein (Fig. 4B).

To further confirm membrane association of PfEHD, we assessed its ability to directly interact with lipids. The C-terminal lipid-binding region of PfEHD was expressed as recombinant protein (Fig. 4C upper panel) and its interaction with PI/PtdIns was evaluated on a nitrocellulose membrane. The PfEHD-C protein showed major interaction with PI (3, 4, 5) P3 as well as with PI (3, 4) P2. In addition a weak interaction was observed with PI (4) P (Fig. 4C lower panel). PfEHD did not show any detectable binding with PI in the same assay.

2.4. PfEHD-vesicles are targeted to multi-vesicular like structures near food-vacuole

To further comprehend the possible association of PfEHD in endocytosis-like events in the parasites, we studied the localization and movement of PfEHD-GFP tagged vesicles in the transgenic parasites by time-lapse confocal microscopy. The time-lapse imaging of parasites expressing PfEHD-GFP, at trophozoite stages, showed different phases of vesicles development and movement in the parasites (Fig. 5A–C, Movie M2, M3 and M4). In the first set of images, an early trophozoite stage parasite is shown with the GFP fluorescence at parasite boundaries; the GFP fluorescence levels in these foci continuously increased in a time dependent manner, clearly showing the development and maturation of a vesicle like structure close to the parasite boundary (Fig. 5A; Movie M2). In the second set of images, GFP labeled vesicular structures are present near the parasite membrane as well as in the cytosol of the parasite, away from food-vacuole; in due course, one of the cytosolic vesicles was observed to migrate towards the food-vacuole and subsequently localized in close proximity to the food-vacuole.
In the third set of images, a number of GFP labeled vesicles are seen originating from the parasite membrane. These vesicles subsequently migrated as a trail in the parasite cytosol and ultimately culminated as a large structure near the food-vacuole (Fig. 5C; Movie M4). Overall these results show sequential events of generation of PfEHD tagged vesicles at the parasite plasma membrane, their migration in the parasite cytosol and culmination into a multi-vesicular like body near food-vacuole.

2.5. PfEHD associates with components of endocytic trafficking machinery

In order to ascertain the role of PfEHD in endocytosis-like process, we tried to identify its interacting protein partners by utilizing a co-immunoprecipitation approach using anti-GFP antibodies and transgenic parasites expressing PfEHD-GFP protein; the co-immunoprecipitated interacting partners were identified using mass spectrometry-based proteomic identification. The identified proteins were considered positive when these were detected in at least two of the three experimental replicates, and remained absent in the control set. As summarized in Table 1, proteins identified as putative PfEHD interacting partners include: Vacuolar ATPase (V-ATPase), coronin, coronin-binding protein, Bet3 transporter protein and a conserved hypothetical protein (PF3D7_0315300). The Vacuolar ATPase (V-ATPase) and coronin are known to play role in endocytosis-like events in eukaryotic cells [19,20]. The conserved hypothetical protein is predicted by the MPMP (Malaria Parasite Metabolic Pathways) database and by gene ontology (GO) analysis to be part of tubulin assembly for microtubule cytoskeleton organization; tubulin assembly is known to play role in cell-motility, intracellular trafficking and signaling in eukaryotic cells. The Bet3 transporter protein plays role in Golgi mediated protein trafficking [21].

In order to confirm the direct interactions of PfEHD with these identified proteins we utilized transgenic mammalian cell expression system (Figs. 6, S5). The PfEHD was cloned in pFLAG-CMV-6a vector (pCMV-EHD) and its interacting partners, Bet3 transporter protein, Vacuolar ATP synthase subunit b, coronin and, coronin binding protein, were expressed in fusion with GFP. Expression of respective recombinant protein in each case was confirmed by western blotting with anti-GFP or anti-PfEHD antibodies (Fig. 6) in single or double transfected cells. Eluates from Co-IP with cell lysates using anti-GFP antibodies were analyzed by western blotting with anti-GFP and anti-EHD antibodies. The PfEHD was found to co-precipitate with Vacuolar ATP synthase subunit b, coronin and, coronin binding protein. These results indicate that these three proteins have direct interaction with EHD; however the Bet3 transporter protein did not show any direct
interaction with EHD and it may have indirect interaction involving other parasite proteins.

2.6. PfEHD labeled endocytic vesicles culminate into lipid storage sites juxtaposed to food-vacuole

As stated earlier, in most of the mature trophozoite stage parasites one or two bright distinct GFP foci were seen near the food vacuole having distinct hemozoin crystals. In addition, time-lapse microscopy showed that PfEHD-labeled vesicles are targeted to a multi-vesicular like structure near the food-vacuole. To ascertain localization of these structures and to understand their association with the food-vacuole, we immuno-stained the parasite with anti-PfCRT antibodies. The CRT protein is a food-vacuole membrane protein, and therefore as expected the anti-PfCRT antibodies stained food-vacuole membrane in the parasite, whereas the large GFP labeled structures were observed outside the food-vacuole in close proximity of the food-vacuole membrane (Fig. 7A). The 3-D image that was reconstructed based upon Z-stack images, showed that these GFP-labeled structures are in close association but distinct and outside the food-vacuole (Fig. 7B). In a similar experiment we labeled the parasite with LysoTracker dye which labels the acidic food-vacuole within the parasite. The LysoTracker stained the food-vacuole region in the parasite around hemozoin, whereas the large GFP labeled structures were observed in close proximity of the LysoTracker stained food-vacuole (Fig. S6A). The 3-D image showed that the GFP-labeled structures are in close association with the food-vacuole but outside its boundaries (Fig. S6B).

The location of these large multi-vesicular like structures near food-vacuole showed resemblance with the neutral lipid bodies known to be intimately associated with the food-vacuole in *P. falciparum* [22]. To find
out if PfEHD labeling is associated with these lipid bodies, we co-stained the transgenic parasites with Nile Red. Nile Red is a hydrophobic probe that tends to concentrate in neutral lipid droplets in the cell including stores of Tri Acyl glycerols (TAGs) and cholesterol esters [23]. Labeling of parasites with Nile Red showed intensely stained neutral lipid bodies associated with food vacuole; the intense GFP foci clearly overlapped with the Nile Red staining (Figs. 7C and S6C). These results along with the life microscopy results suggest that PfEHD-labeled vesicles originating at the parasite boundary ultimately culminate in to a multi-vesicular like lipid storage structure near food-vacuole in the parasite. The 3-D image reconstructed based upon Z-stack images showed that the large GFP labeling is around the Nile Red stained lipids (Fig. 7D) suggesting its association on the surface of the lipid storage body in the parasite.

2.7. Endocytosis inhibitors block development and targeting of PfEHD labeled vesicles

To further confirm the association of PfEHD-tagged vesicles with endocytosis-like events, we treated parasites with compounds that are known to block different steps of endocytosis process, and followed sub-cellular distribution of these vesicles. The parasites were treated at concentration close to IC50, but for a short period (~8 h) so that the parasites are not killed by this treatment (Fig. S7). As mentioned above, in control set the vesicles originated at the parasite plasma membrane were able to migrate towards food-vacuole and culminated into the multi-vesicular like lipid storage body (Figs. 8A, S8A). In these cultures, about 60% of the parasites showed GFP in close association with the food-vacuole at the lipid-storage site and about 40% parasites showed GFP labeled in transit vesicles in the cytosol. In first set parasites were treated with jasplakinolide, an actin inhibitor, which stabilizes the actin filament and thus enhances polymerization [24]. In the jasplakinolide treated parasites, the development PfEHD-GFP tagged vesicles at the parasite boundary were observed. However, the migration of these vesicles from plasma membrane was inhibited, and in >80% of parasites these vesicles were found to be accumulated close to the plasma membrane. In these parasites no GFP labeling was seen close to the food-vacuole (Figs. 8B, S8B). In addition, Nile Red staining was also reduced/absent in these parasites suggesting that storage of neutral lipids is also affected in these parasites. In the second set, the parasites were treated with wortmannin, a PfPI3K inhibitor, which is shown to block uptake of material form host by inhibiting the trafficking of early endosome in the parasite cytosol [25]. In wortmannin treated parasites, the PfEHD-GFP tagged vesicles were observed to accumulate in the parasite cytosol, these vesicles were not able to migrate towards food-vacuole. About 90% of the parasites showed accumulation of GFP vesicles in the cytosol. In the treated parasites, the GFP-labeling as well as the Nile Red staining was not observed near the food-vacuole (Figs. 8C, S8C). In third set the parasites were treated with bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, which is known to alter intracellular trafficking by preventing early endosome acidification along the endocytic pathway [26]. In bafilomycin treated parasites, the GFP vesicles were seen close to parasite boundary or in parasite cytosol; however, their targeting to the multi-vesicular like structure near food-vacuole was hindered. In bafilomycin treated cultures, about 60% of parasites showed GFP vesicles at the parasite boundary. In these parasites, development of the GFP-tagged multi-vesicular structure was not observed and poor staining of Nile Red was seen near food-vacuole (Figs. 8D, S8D). In summary, treatment with endocytosis inhibitors blocked development of PfEHD vesicles in the parasite and their targeting to multi-vesicular like lipid-storage site near food-vacuole.

3. Discussion

During the asexual stage cycle the malaria parasite remains inside the host erythrocyte and extensively performs endocytosis to take up host cytosol and other material from the milieu. The molecular mechanisms and key parasite proteins that mediate endocytosis and trafficking of endosomes are not well known. In the present study, we have characterized the P. falciparum homolog of a dynamin like Eps15 homology domain-containing proteins (EHD). EHD proteins are
known to be associated with regulation of endocytic pathways, and it has been shown that most of the EHD-domain containing proteins, despite possessing other functional domains, carry out defined regulatory roles in endocytic membrane transport through their direct or indirect interaction with membrane lipids [27]. Structurally the EH domain are known to contain two sets of EF-hands that bind with calcium as well as proteins containing tripeptide asparagine–proline–phenylalanine (NPF) [14,28]. It has been also shown that the EH domains bind with Phosphatidylinositol through a conserved lysine residue (Lys-483) [14]. In mammalian cells, EHD1 has been shown to be involved in regulation of cholesterol homeostasis, and it affects generation of cholesterol as well as triglyceride lipid bodies in the cell [12]. The PfEHD protein contains conserved N-terminal dynamin like domain, C-terminal Eps15 homology domain and the lysine residues required for phosphatidylinositol binding, which may point towards its role in endocytosis-like events. Confocal microscopic studies using transgenic parasites expressing PfEHD-GFP fusion protein showed association of PfEHD with small vesicular structures that originate at the parasite boundary. In addition, PfEHD binds with PIPs confirmed that it is a membrane-associated protein. The association of PfEHD with the parasite membrane in specific foci suggests their involvement in endocytosis-like vesicle-mediated uptake of material, as in case of the four EHD paralogues in mammalian cells [8,14,29]. The availability of crystal structure of EHD protein has provided key evidence in support of its role as membrane binders and benders, in turn leading to a model that explains its role in endocytic transport [30]. According to this model, the EHD protein forms a dimer that gets inserted into the membrane by utilizing a highly curved interface. This results in bending of the membrane towards the dimer. Subsequently, hydrolysis of ATP by oligomeric EHD induces conformational changes leading to membrane destabilization, ultimately resulting in membrane scission and fission of endocytic vesicles [30]. The PfEHD harbors the conserved

Table 1

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Fig. 5. Consecutive images from time-lapse microscopy of PfEHD-GFP expressing transgenic parasites showing localization and migration GFP labeled vesicles. (A) Sequential images of a parasite over a time interval of 17 min showing generation and development of a GFP labeled vesicle at the parasite plasma membrane. (B) Sequential images of a parasite over a time interval of 36 min showing GFP labeled vesicle in parasite cytosol (marked with arrowhead) and its migration towards the food vacuole. (C) Sequential images of a parasite over a time interval of 41 min showing several GFP labeled vesicle originating at the parasite boundary, their migration through cytosol as a trail and accumulation of GFP in a vesicular structure close to food-vacuole.
shown in mammalian cells that EHD protein interact with Rab protein and be involved in the cargo trafficking. It also points towards a possible role in vesicle trafficking, suggesting that there is no recycling of the EHD protein, and it may remain associated with the vesicles till they fuse to form a multi-vesicular like structure, which might have been observed due to in-cell transport of PfEHD protein.

In our studies we found that PfRab5A show partial overlap with PfEHD association of PfEHD suggest that the it may be involved in pinching of vesicles in eukaryotic cells. The microscopic studies and membrane association of PfEHD suggest that the it may be involved in pinching of endocytic vesicles at the parasitophorous vacuole; PfEHD remains attached with these vesicles migrating towards the food-vacuole and to fuse with the multi-vesicular structure present there. In eukaryotes, EHD proteins serve as endocytic adaptors and have been implicated both in clathrin mediated and non-clathrin mediated endocytosis by virtue of their interaction with a diverse array of proteins. The EHD proteins are shown to associate with the nascent vesicles at the time of vesicle fission. Upon internalization, endocytic vesicles fuse rapidly with early endosomes. The EE regulate degradation of cargo molecules or recycling to the plasma membrane via endocytic recycling compartment (ERC). However, the EHD proteins are also found to be associated with EE while in complex with other proteins. We utilized co-immunoprecipitation approach using anti-GFP antibodies and transgenic parasites combined with proteomic analysis to identify PfEHD interacting partners; similar strategy has been used earlier to ascertain protein–protein interaction in the parasite. Our studies identified a few important components of endocytic vesicular trafficking, namely, V-ATPase, coronin and coronin-binding protein. V-ATPase are evolutionarily conserved proteins that play crucial roles in various cellular processes including endocytosis, vesicular trafficking, protein recycling etc. Vesicular acidification mediated by V-ATPase is required for transition from early endosome to late endosome. Indeed, the homolog of V-type ATPase is found to be distributed in parasite cytosol and also localized in RBC membrane. Coronin is a conserved component of the actin cytoskeleton found in all eukaryotes localizing to sites of dynamic actin assembly. In Dictyostelium discoideum, coronin mutants display defects in cell migration, cytokinesis, phagocytosis, and fluid phase endocytosis. Further, we also identified a putative coronin binding protein, which is suggested by MPMP database to play a role in actin dynamics. Interaction of PfEHD with these crucial endocytosis modulators indicates its possible involvement in the endocytosis process. In addition, we also identified a conserved hypothetical protein which is also predicted to be involved in microtubule organization according to MPMP database. Tubulin polymerization and microtubule organization is essential for intracellular trafficking and signaling in eukaryotic cells. Our studies also yielded a component of Transport Protein particle (TRAPP), Bet3, as a PfEHD interacting partner. TRAPP is involved in vesicular trafficking to and through the Golgi complex. It is possible that this interaction might have been observed due to in-cell transport of PfEHD protein. We confirmed the interaction of PfEHD with these identified protein partners by transient expression in mammalian cell. Our results showed direct interaction of the PfEHD with the putative partners, Vacular ATP synthase subunit b, coronin and, coronin binding protein, which were identified by proteomic approach.

The parasite is known to take up content of host erythrocyte cytosol, especially the host hemoglobin. The parasite acquires host hemoglobin through double membrane bound vesicles, cytosomal vesicles, which subsequently fuse with the food vacuole. In our studies we found that PfRab5A show partial overlap with PfEHD labeled vesicles, again pointing out association of PfEHD in endosomal pathway.

![Fig. 6. Transient expression and interaction of PfEHD with its interacting partners. Huh-10 cells were transfected with pEGFP-X and pCMV-EHD vector constructs alone or in combination; pEGFP-X represents vector construct for each of the interacting partners, 1: Bet3 transporter protein; 2: Vacuolar ATP synthase subunit b; 3: Putative coronin protein and; 4: Coronin binding protein, respectively. Western blot analysis of cell lysate show expression of each of the protein in single and double transfected cells (inputs). Western blot analysis after immuno-precipitated of these lysate with ant-GFP antibodies (IP-α-GFP), showed interaction of PfEHD proteins with respective partner proteins.](image-url)
outer membrane of these vesicles fuses with the food-vacuole membrane, while the inner membrane possibly gets degraded by phospholipases. However, in our study the PfEHD-GFP vesicles were not found to fuse with the food-vacuole, and neither was the PfEHD-GFP staining observed in the food-vacuole; rather, the PfEHD-GFP vesicles fused with the multi-vesicular like structure in close proximity to the food-vacuole. The location of these large vesicular structures showed resemblance with the neutral lipid bodies known to be intimately associated with the food vacuole [22]. These lipid storage bodies are suggested to play important role in hemozoin formation [22]. Our results showed clear association of Nile Red stained neutral lipid bodies with the PfEHD-GFP near food-vacuole, suggesting that the PfEHD coated vesicles terminate into the lipid storage site in the parasites. These lipid bodies are the sites of storage of di- and tri-acylglycerols (DAGs and TAGs); the neutral lipids are possibly obtained by the parasite by scavenging or these are generated by digestion of phospholipids during membrane recycling [44,45]. It is proposed that the membrane phospholipids, which remained after fusion of double membrane vesicles with the food-vacuole, get broken down into neutral lipids, TAGs and DAGs, which are subsequently stored in the lipid body [22]. This process may involve a number of enzymes such as phospholipases and acyltransferase, which need to be trafficked to this multi-vesicular structure.

The intracellular protein sorting and their targeting to different organelles in the Plasmodium is a complex process; a number of signal...
sequences and different trafficking pathways are defined in the parasites [46]. A number of food vacuole targeting proteases, including plasmepsins and falcipains, have been shown to utilize conventional signal sequences that target these proteins to parasitophorous vacuole. Subsequently these proteins are targeted to the food-vacuole through the endomembrane vesicular system [47,48]. Our data suggests that the PfEHD may be involved in an endocytic-like process to traffic cargo from parasite membrane to lipid conversion/storage site. Thus it may be involved in the trafficking of enzymes, which are needed to convert complex phospholipids into neutral lipids, to the multi-vesicular like structure by utilizing the vesicular system. Indeed, EHD proteins are known to be associated with regulation of endocytic pathways associated with lipid metabolism; in mammalian cells EHD1 is shown to be involved in regulation of cholesterol homeostasis, and it affects generation of cholesterol as well as triglyceride lipid bodies [12].

The process of endocytosis and endosomal recycling involve pinching of vesicle from plasma membrane along with the cargo, trafficking of endocytic vesicle, maturation of early endosome to late-endosome and ultimately fusion with the lysosomal organelle. A number of compounds are shown to inhibit different molecular events during this process, and are therefore used to study endocytosis processes. To ascertain the association of PfEHD with endocytosis, we utilized these inhibitors on Plasmodium and studied the subcellular localization of PfEHD in the parasites. The localized polymerization of F-actin is essential to recruit the endocytic machinery at the plasma membrane; any disruption of actin assembly can also disrupt endocytosis [49–51]. Jasplakinolide is an actin inhibitor, which stabilizes the actin filament and thus enhances polymerization [24]. Treatment of P. falciparum with jasplakinolide is shown to block endocytic events in the parasite with accumulation of endocytic vesicles near plasma membrane [52]. We found that the jasplakinolide treatment caused accumulation of PfEHD labeled vesicles at parasite plasma membrane and prevented their migration and subsequent targeting to the lipid storage site. Similarly, PPE65 inhibitor wortmannin is shown to block uptake of material form host by inhibiting the trafficking of early endosome in the parasite cytosol [25]: we also found that wortmannin...
treatment blocked trafficking of PFEHD labeled vesicles, which stayed in the parasite cytosol as early-endosomes. Wortmannin effect also suggest some kind of PI/PIP association of PFEHD, our results with lipid binding assays also showed its interaction with PIPs. Bafilomycin A1, a specific inhibitor of vacuolar-type H(+) -ATPase, alters intracellular trafficking by preventing early endosome acidification along the endocytic pathway [26]. In certain cell types, bafilomycin treatment arrests the transport of all markers in early endosomes and thus affects the transport of endocytosed material from early to late endocytic compartments [53]. Bafilomycin treatment in *P. falciparum* is shown to disrupt vesicular trafficking towards the cellular organelle, apicoplast, which causes accumulation of vesicles in the cytosol possibly by disruption of vesicle fusion [54]. We found that the bafilomycin treatment inhibited PFEHD vesicle fusion into multi-vesicular structure causing accumulation of vesicles in the parasite cytosol. Our proteomic studies also identified V-type H(+) -ATPase to be associated with PFEHD. Thus our studies suggest that the vacuolar-type H(+) -ATPase may be involved in maturation of PFEHD endosome and their targeting. Overall, the endocytosis inhibitors effect analyses combined with time-lapse microscopic studies confirmed the association of PFEHD in endocytic process in the *P. falciparum*.

In summary, our results show that the PFEHD protein may be involved in generation/fission of small protein trafficking vesicles that are targeted towards lipid storage body near food vacuole, and thus it may play a role in the development of neutral lipid storage body. Detailed understanding of trafficking of key enzymes involved in generation of neutral lipids may help us in designing novel anti-malarial strategies.

### 4. Material and methods

#### 4.1. Parasite culture, plasmid construct and parasite transfection

*P. falciparum* strain 3D7 was cultured with 4% hematocrit in RPMI media (Invitrogen) supplemented with 0.5% Albumax using a protocol described previously [55]. Parasite cultures were synchronized by repeated sorbitol treatment following Lambros and Vanderberg [56].

To generate a transfection vector construct, a full length gene encoding amino acid residues 1 to 533 of PFEHD was cloned in the vector pSSPF2 vector [57] at the BglII and AvrII restriction sites to create a fusion of PFEHD with green fluorescence protein (GFP). This vector contains the full length gene in fusion with GFP gene under the control of hsp86 promoter. Synchronized *P. falciparum* 3D7 ring stage parasites were transfected with 100 μg of purified plasmid DNA (Plasmid Maxi Kit, Qiagen, Valencia, CA) by electroporation (310 V, 950 μs) [58] and the transfected parasites were selected over 2.5 nM of WR99210 drug.

#### 4.2. Isolation of parasites, differential extraction of parasite proteins and immuno-blotting

Expression of PFEHD-GFP fusion protein in transgenic *P. falciparum* blood stage parasites was confirmed by western blotting. Briefly, parasites were isolated from mixed stage culture by lysis of infected erythrocyte with 0.15% saponin, the supernatant and washed pellets were suspended in Laemmli buffer, boiled, centrifuged, and the supernatant obtained was separated on 12% SDS-PAGE. The fractionated proteins were transferred from gel onto the PVDF membrane (Amersham, Piscataway, NJ, USA) and blocked in blocking buffer (1 × PBS, 0.1% Tween-20, 5% milk powder) for 2 h. The blot was washed and incubated for 1 h with primary antibody (mouse anti-GFP (1:1000); rabbit anti-BIP (1:2000); rabbit anti-SERAS (1:1000)) diluted in dilution buffer (1 × PBS, 0.1% Tween-20, and 1% milk powder). Subsequently, the blot was washed and incubated for 1 h with appropriate secondary antibody (anti-rabbit or anti-mouse, 1:2000) conjugated to HRP. Bands were visualized by using ECL detection kit (Amersham).

To characterize the membrane association of PFEHD protein, differential protein extraction was carried out following [59,60]. Infected erythrocyte were suspended in 7.5 mM Tris–HCl pH 7.4 and lysed by freeze–thaw cycles, the pellet (containing membrane bound and associated proteins, Tris-insoluble fraction) was washed twice in 7.5 mM Tris–HCl (pH 7.4) and divided into four aliquots. The first aliquot was extracted with 6 M urea in 7.5 mM Tris–HCl (pH 7.4) for 1 h at room temperature, the second with 100 mM sodium carbonate (pH 11.5) for 1 h on ice, the third with 1% Triton X-100 in PBS for 1 h at room temperature, and the fourth with 2% SDS and 1% Triton X-100 in PBS for 1 h at room temperature. Samples were subsequently centrifuged at 100,000 × g for 60 min to separate soluble and insoluble proteins. The supernatant fractions (containing solubilized proteins) were centrifuged again to remove any insoluble remnants, and the pellet fractions (containing insoluble proteins) were washed twice in respective extraction buffer. All the samples were separated on SDS-PAGE and analyzed by immuno-blotting. For Triton X-114 phase separation assay, the transgenic parasites were isolated from infected erythrocytes with 0.15% saponin and phase separation in the presence of Triton X-114 was carried out as described earlier [18]. Briefly, the pellet was lysed in the presence of ice-cold 0.5% Triton X-114 (Sigma Aldrich), then centrifuged to remove insoluble materials, and the supernatant obtained was loaded onto a cushion of 6% sucrose in 0.06% Triton X-114. Phase separation was conducted by incubation at 37 °C for 5 min followed by centrifugation at 500 × g for 5 min. The aqueous phase was washed with Triton X-114 three times to remove any hydrophobic material, and the detergent phase was washed three times with phosphate-buffered saline to deplete any hydrophilic material. The resulting samples were separated on SDS-PAGE and analyzed by immuno-blotting.

#### 4.3. Fluorescent labeling of parasites and fluorescence microscopy

PFEHD-GFP transgenic parasites were synchronized by two consecutive sorbitol treatments 4 h apart. Parasites at different developmental stages were collected from the culture for fluorescence microscopy and stained with DAPI at a final concentration of 2 μg/ml for 30 min at 37 °C prior to imaging.

To visualize the mitochondria, the PFEHD-GFP transgenic parasites were stained with MitoTracker Red CMXRos (Invitrogen) at a final concentration of 20 nM in 1 × PBS for 15 min at 37 °C. Cells were washed twice with 1 × PBS and subsequently fixed in 4% paraformaldehyde. To label the parasite food-vacuole, the parasites were stained with 100 nM LysoTracker Red-DND99 (Life Technologies) and DAPI (2 μg/ml) for 40 min at room temperature and then the cells were washed twice with 1 × PBS. To visualize the lipid membranes, parasitized erythrocytes were labeled with BODIPY-TR-ceramide (Molecular Probe) as described earlier [48,61]. Briefly, parasitized erythrocytes were resuspended in complete media (5% parasitemia, 4% hematocrit) and incubated with 1 μM BODIPY-TR-ceramide at 37 °C for 60 min, washed in complete media three times and examined by fluorescence microscopy. Parasitized erythrocytes were labeled with the lipid probe, Nile Red, as described earlier [22]. Briefly, infected erythrocytes (5–10% parasitemia, 4% hematocrit) were incubated in complete culture media containing Nile Red (Molecular Probes) at final concentration of 1 μg/ml. The infected erythrocytes were examined within 30 min of staining.

The GFP expressing parasites and the parasite stained with different labeling dyes were viewed using a Nikon A1 confocal laser scanning microscope. Observations were limited to 30 min to ensure parasite viability throughout the analyses. The 3D images were constructed by using series of Z-stack images using IMARIS 7.0 (Bitplane Scientific) software.

For endocytic inhibitors studies, the tightly synchronized transgenic parasite culture at late ring stage/early trophozoite stage (20–22 hpi) were treated with wortmannin (2.5 μM), jasplakinolide (7.5 μM), bafilomycin A1 (25 nM) or solvent (DMSO) alone for 8 h. The parasites were observed by confocal laser scanning microscopy as described above.
4.4. Time-lapse microscopy

Time-lapse live cell imaging was performed using the Nikon A1 confocal laser scanning microscope integrated with live setup imaging. Images were acquired using a Nikon 100 × 1.4 numeric aperture objective lens and the Nikon NIS element software. Tightly synchronized PfEHD-GFP parasites stained with DAPI (concentration of 2 μg/ml for 30 min at 37 °C) were placed in cover glass bottom dishes in the imaging chamber; conditions inside the chamber were maintained at 5% CO2 at 37 °C and parasites were observed at regular intervals. Image processing was performed using NIS elements AR 4.00.12 and Imeris 7.6.8.

4.5. Indirect immunofluorescence assay

Indirect immunofluorescence assays were performed on P. falciparum transgenic parasite lines as described earlier [62,63]. Briefly, the parasite samples were fixed with 4% paraformaldehyde; fixed parasites were incubated with a primary antibody [rabbit anti-PfClpP (1:200), rabbit anti-PICRT (1:500), mice anti-Rab5A (1:200), mice anti-Rab5B (1:200) or mice anti-Rab7 (1:200) antibody] diluted in 3% BSA, 1 × PBS, and subsequently with Alexa-594 linked goat anti-rabbit anti-Rab5B (1:200) or mice anti-Rab7 (1:200) antibody diluted in 3% BSA, 1 × PBS, and subsequently with Alexa-594 linked goat anti-rabbit or anti-mice antibody (1:250, Sigma) as secondary antibody, with intermittent washing. The antibody nuclei were stained with DAPI (2 μg/ml). The immunostained parasites were viewed using a Nikon A1 confocal laser scanning microscope.

4.6. Co-immunoprecipitation and protein identification by mass spectrometry

Tightly synchronized transgenic parasites, PfEHD-GFP, at trophozoite stages were harvested by saponin lysis and immuno-precipitation was carried out against anti-GFP antibody using Crosslink-IP kit (Pierce) following manufacturer’s instructions. A pre-immune rabbit serum was used as control. Briefly, the antibody was covalently linked with protein A/G beads using DSS cross-linker. The parasites were lysed in lysis buffer, lystate was pre-cleared with agarose beads and was subsequently incubated O/N with these beads at 4 °C; the protein complexes bound with the beads were eluted following three washes. The samples were directly processed for LC-MS/MS analysis by trypsin digestion as described earlier [39]. The peptides generated from control and test samples were loaded onto Acclaim PepMap RSLC (50 μm × 15 cm) column using NanoLC-1000 (ThermoFisher). The peptide mixture was separated with a linear gradient of solvent B (95% acetonitrile + 0.1% formic acid) from 5–90% in 80 min. The eluting peptides were directly injected into the Orbitrap Velos Pro MS (ThermoFisher). Full-scan MS spectra of peptides (m/z 350–1800) were acquired in Orbitrap mass analyzer with a resolution of 60,000. The twenty most abundant ions in each cycle were fragmented by Collision Induced Disassociation (CID) in ion trap using collision energy of 35 and activation time of 20 ms. The data acquired was then analyzed using Proteome Discoverer software (PD 1.4) (ThermoFisher). Proteins were identified by searching against the P. falciparum proteome databases using SEQUEST algorithm and peptide matches were validated using Percolator.

4.7. Huh-10 cell culture, plasmid constructs and transfection

Huh-10 cells were grown in DMEM media supplemented with 10% fetal bovine serum and transfected using jet Prime reagent (Polyplus transfection) according to the manufacturer’s instructions. Cells were cultured at 37 °C for 36 h before processing.

The generation transfection constructs the full-length pfEHD was cloned in plasmid vector pFLAG-CMV-6a (Sigma Aldrich) using NolI and Smal sites and pEFP-N1 (to code for YFP fusion protein) using Xhol and SacII sites. Full length genes for putative interacting partners: Bet3 transporter protein; Vascular ATP synthase subunit-PF3D7_0406100; putative coronin protein (PF3D7_1352200); coronin binding protein (PF3D7_0623100) were cloned in plasmid vector pEFP-N1 vector using Xhol and SacII sites to code for GFP fusion proteins.

4.8. Co-immunoprecipitation and microscopy in Huh-10 cell line

Transfected cells were harvested after 36 h and immunoprecipitation was carried out with anti-GFP rabbit antibody using Crosslink-IP kit (Pierce) following manufacturer’s instructions. A pre-immune rabbit serum was used as control. Briefly, the antibody was covalently linked with protein A/G beads using DSS cross-linker. The pre-cleared cell lysate was incubated O/N with anti-GFP antibody conjugated beads at 4 °C and the protein complex was eluted following three washes. The eluates were analyzed by western blot using anti-PfEHD and anti-GFP mice antibodies.

For fluorescence microscopy, the Huh cells were grown on cover glasses in 24 well plate, transfected with pEFP-N1-PfEHD and each of the pEFP-N1 plasmid constructs as described earlier, and grown at 37 °C. After 36 h cells were fixed with 4% (vol/vol) paraformaldehyde in PBS for 30 min at 4 °C. After washing with PBS, the cover-slips were mounted with Prolong antifade reagent (Life Technologies). Fluorescence images were acquired using Nikon A1 confocal microscope (Nikon) by using a 60× numerical aperture objective with appropriate filters.

4.9. Expression of PfEHD and protein–lipid binding assay

Fragment of pfEHD gene corresponding to lipid binding region (263–523 aa) was cloned into the Ssol and SacII restriction sites of pET-28a plasmid (Novagen), recombinant protein His-tagged at the N-terminus were expressed using Escherichia coli BL21(DE3) codonplus (Strategene) cells. Briefly, expression of the recombinant protein, His-PfEHD-C, was induced with 1 mM isopropyl-thio-β-D-galactoside (IPTG) at OD600 nm for 4 h, at 37 °C. His-PfEHD-C purification was carried out at 4 °C by affinity chromatography. All buffers were supplemented with an EDTA-free proteases inhibitor cocktail (Roche). The bacterial pellet was resuspended with of ice-cold sonication buffer (50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 10 mM imidazole). After sonication, the lysate was centrifuged (12,000 ×g, 30 min), and the soluble fraction, diluted twice into the ice-cold sonication buffer, was purified by affinity through a Ni-NTA column. His-PfEHD-C was eluted with washing buffer containing 250 mM imidazole and concentrated by centrifugation with Centricon-10 (Millipore Corp.).

A dot blot based lipid binding assay was used for the detection of direct protein–lipid interaction. Various phosphoinositides lipids [PI, PI(3)P, PI(4)P, PI(3,4)P2, PI(4,5)P2 and PI(3,4,5)P3] were diluted in chloroform/methanol and 100 μM of each was spotted on a nitrocellulose membrane. The membrane was air dried, incubated with 3% fat free-ultrapure BSA in TBST (50 mM Tris pH 7.4, 150 mM NaCl and 0, 1% tween 20) for 2 h at room temperature, washed with TBST and subsequently incubated with 0.5 μg/ml purified recombinant PfEHD-C histidine tagged protein overnight at 4 °C. After washing with TBST, the membranes were probed using mouse anti-Histidine conjugated to HRP and visualized by enhanced chemiluminescence.

4.10. Generation of antisera

To generate polyclonal antisera against PfEHD, female BALB/c mice were immunized (on day 0) with the purified recombinant protein (25 μg) formulated in complete Freund’s adjuvant (Sigma, USA). The mice were administered two booster doses (day 14 and 28) of the proteins formulated in Freund’s incomplete adjuvant. The mice serum was collected 10 days after the second boost. Two New Zealand white rabbits (3 months old) were immunized in the same way with 250 μg of recombinant protein (on day 0) and administered two booster doses (on day 28 and 49). The rabbit sera were collected on day 56.
To generate specific anti-PfRAB7 antibodies two peptides (sequences: FALNQSEQKMYKSR and FLIQASKPDKENFF), anti-PfRab5A antibodies three peptides (sequences: MEKSSKVTGLVE, NEKNNINNINDN and IVKNIINNNNTSNNK), anti-PfRab5B peptide (SPAAQQKKNAAQDTKV, GDGSVGGSSALYC and ASRYSRFEVLYGN), respectively with KLH conjugated were synthesized, mixed and used to immunize in mice following the above.

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Conflict of interest statement

The authors declare no conflict of interest.

Transparency Document

The Transparency document associated with this article can be found, in the online version.

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Following the above.

GDSGVGKSSIALYLC and ASRIYSRFKEVLYYN), respectively with KLH three peptides (sequences: MEKKSSYKTVLLGES, NEKNNNINSINDDNN and TNIYNNICSKALCMLN). The authors declare no conflict of interest.

References


