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A role for adaptor protein complex 1 in protein targeting to rhoptry organelles in *Plasmodium falciparum*

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ABSTRACT

The human malaria parasite *Plasmodium falciparum* possesses sophisticated systems of protein secretion to modulate host cell invasion and remodeling. In the present study, we provide insights into the function of the AP-1 complex in *P. falciparum*. We utilized GFP fusion constructs for live cell imaging, as well as fixed parasites in immunofluorescence analysis, to study adaptor protein mu1 (Pfμ1) mediated protein trafficking in *P. falciparum*. In trophozoites Pfμ1 showed similar dynamic localization to that of several Golgi/ER markers, indicating Golgi/ER localization. Treatment of transgenic parasites with Brefeldin A altered the localization of Golgi-associated Pfμ1, supporting the localization studies. Co-localization studies showed considerable overlap of Pfμ1 with the resident rhoptry proteins, rhoptry associated protein 1 (RAP1) and Cytoadherence linked asexual gene 3.1 (Clag3.1) in schizont stage. Immunoprecipitation experiments with Pfμ1 and PfRAP1 revealed an interaction, which may be mediated through an intermediate transmembrane cargo receptor. A specific role for Pfμ1 in trafficking was suggested by treatment with AlF_4, which resulted in a shift to a predominantly ER-associated compartment and consequent decrease in co-localization with the Golgi marker GRASP. Together, these results suggest a role for the AP-1 complex in rhoptry protein trafficking in *P. falciparum*.

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1. Introduction

Despite increased preventative measures and renewed interest in the eradication of malaria, global mortality has been estimated at 1.2 million in 2010 [1]. Though five species cause infections in humans, *Plasmodium falciparum* can induce lethal cerebral malaria and is associated with the highest mortality rates. *Plasmodium* belongs to a phylum of obligate intracellular parasites known as the Apicomplexa, that also includes such prolific organisms as *Toxoplasma gondii* and *Cryptosporidium spp*. The phylum is so named because of the apical complex, a sophisticated cytoskeletal structure located at the apical end of the cell, and the associated organelles; micronemes and rhoptries. Discharge of these organelles, as well as the non apically localized dense granules, assists in all stages of host cell invasion and remodeling, and they are essential for parasite viability [2]. Micronemes and rhoptries likely represent highly divergent endolysosomal organelles [3,4], but they are distinct in terms of morphology and protein content. Assuming this relationship to be true, trafficking to apical organelles should bear similarities to that of endosomes and lysosomes in model systems.

Recent studies in *T. gondii* have deciphered some of the machinery involved in trafficking to apical organelles (reviewed in [5]). Early studies noted the existence of an intermediate compartment in the trafficking of micronemal proteins [6], which was subsequently shown to be an endosome-like compartment for the removal of microneme propeptides [7]. Rhoptry and microneme biogenesis in *T. gondii* occur from the fusion of post-Golgi vesicles, whose scission is likely regulated by a dynamin related protein (DrpB). Ablation of DrpB in *T. gondii* results in the absence of distinct micronemes and rhoptries [8]. Rab GTPases, specifically Rab5a and 5c, are involved in targeting at least a subset of micronemal and rhoptry proteins [9]. Additionally, for soluble rhoptry and microneme contents, transmembrane receptors such as TgSORTLR are required for appropriate targeting [10]. The current paradigm seems to be a re-purposing of trafficking pathways traditionally involved in endocytic processes to facilitate trafficking to the secretory organelles (reviewed in [11]). This still needs to be verified in *P. falciparum*, as it appears that micronemes and rhoptries may form

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directly from the Golgi apparatus [12–14], though an endosome-like compartment was just described [15], and the mechanisms governing similar processes in other Apicomplexa remain unclear as well. One set of potentially important machinery that has not been investigated thoroughly to date in *P. falciparum* is the adaptor protein (AP) complexes.

Five such adaptor protein complexes exist, and are involved in cargo recognition in the formation of post-Golgi trafficking vesicles [16]. AP-1 and AP-2 interact with the scaffolding protein clathrin, while AP-4 and AP-5 do not; the interaction of AP-3 with clathrin remains uncertain [17]. In mammalian cells, AP-1 is involved in trafficking between the TGN and endosomes, AP-2 is involved in clathrin-mediated endoyctosis at the plasma membrane, and AP-3 is involved in trafficking to lysosomes and lysosome-related organelles (reviewed in [18,19]). The more recently discovered AP-4 and AP-5 do not have well-defined roles, but appear to play a role in endosomal trafficking of specific cargoes [17,20]. APs are heterotetrameric complexes composed of two large (γ, α, δ, ε, ζ, and β1–5), one medium (μ1–5), and one small (σ1–5) subunit. Large subunits are involved in binding to the target membrane and mediating interactions with clathrin and other cargo adaptors [18]. The small subunits are thought to be involved in complex stability [21], and also form binding interfaces for dileucine-based cargo motifs [22]. Medium subunits are involved in the recognition of transmembrane cargo adaptors by binding to specific motifs in their cytoplasmic tail, notably the canonical YXXphi motif [23].

A previous bioinformatic study identified components of the AP-1–4 complexes in the genomes of *Plasmodium spp.* and *T. gondii* [24]. However, while *T. gondii* was reported to possess the recently described AP-5 complex, *P. falciparum* did not [16]. Though AP-3 is involved in trafficking to lysosomes and LROs [25], it is not present in *Theileria*, *Babesia*, *or Cryptosporidium*, and hence is a less attractive candidate for trafficking to micronemes and rhoptries. AP-1 was previously implicated in trafficking to the rhoptries in *T. gondii*. The authors demonstrated localization of Tgμ1 to the Golgi, endosome like compartment, and to maturing and mature rhoptries utilizing both immunofluorescence and electron microscopy [26]. Additionally, the authors showed that a D176A mutation, predicted to alter binding to the YXXphi motif, alters rhoptry morphology and arrests ROP2 trafficking in intermediate endosomal compartments [26]. However, their additional suggestion that Tgμ1 interacts directly with the C-terminal, cytoplasmic, portion of the rhoptry bulb protein ROP2 was subsequently discredited by the lack of a transmembrane domain in its structure, and the subsequent finding of association with membranes being mediated through N-terminal amphipathic helices [27,28]. More recent studies suggest that AP-1 in *T. gondii* mediates trafficking of at least a subset of apical organelle proteins through interactions with the sortilin-like receptor TgSORTLR [10,15]. Consequently, the full extent and nature of AP-1 involvement in trafficking to invasion organelles in Apicomplexa are unclear. This is hampered by the fact that this question has only been examined in the model system of *Toxoplasma*. To address this gap on the promising basis of the previous work in *Toxoplasma* and the role of AP-1 in functionally homologous organelles in other eukaryotes, we chose to investigate the role of AP-1 in protein trafficking to the apical organelles in *P. falciparum*.

In the present work, we have cloned the μ subunit of AP-1 and generated a Pfμ1–GFP transgenic parasite line to study Pfμ1 mediated vesicular transport processes. Our results show that Pfμ1 is associated with the Golgi in early trophozoite stages, and co-localizes with resident rhoptry proteins RAP1 and Clag3.1 in later asexual stages, but not with the microneme marker EBA175, suggesting a specific role in rhoptry trafficking. These results are further supported by co-immunoprecipitation studies showing interaction between Pfμ1 and RAP1, and a substantial re-distribution of Pfμ1 upon AlF_4 treatment, which suggests a specific role for the complex in trafficking. Our results suggest that the AP-1 complex is involved in trafficking to the rhoptry organelles in *P. falciparum*.

2. Results

2.1. Cloning and expression analysis of the mu subunit of the P. falciparum AP-1 complex

To get insight into the role of the AP-1 complex in erythrocytic stages of the malaria parasite, we cloned a C-terminal fragment of Pfμ1 (277–437aa) with Plasmodb gene ID PF3D7_1311400, and expressed it in *E. coli*. The recombinant protein was purified by affinity chromatography, as shown in Fig. 1A. We raised antibodies in mice and rats against the purified recombinant $Pf\mu1_c$ protein. The specificity of the anti-$Pf\mu1_c$ antibody was assessed by western blot analysis of *P. falciparum* strain 3D7 parasite lysate. As shown in Fig. 1B, anti-$Pf\mu1_c$ antibody was able to detect the full length Pfμ1 protein band of ~50 kDa in 3D7 lysate, the size of the band corresponding to the size of native Pfμ1. Immunolocalization studies using anti-$Pf\mu1_c$ antibodies at asexual blood stages of the parasite showed well-defined punctate structures in schizont stage of the parasite, a pattern characteristic of staining for apical secretory organelles (Fig. 1C). These results demonstrate the specificity and reactivity of the anti-$Pf\mu1_c$ antibodies raised in the present study.

2.2. Generation of a chimeric GFP line and sub-cellular localization of Pfμ1 throughout the intraerythrocytic lifecycle of P. falciparum

To study the localization of the *P. falciparum* AP-1 complex and elucidate its role in protein trafficking within the parasite, a transgenic parasite line expressing the AP subunit Pfμ1 as a chimeric protein, C-terminally tagged with GFP, was generated. Fig. 2A shows a schematic of the fusion construct used for transfection. Expression of the fusion protein was confirmed by western blot analysis and fluorescence microscopy. A western blot of transgenic parasite lysate was stained using either anti-GFP or α-$Pf\mu1_c$ antibodies. The anti-GFP antibody detected a ~76 kDa band, corresponding to the expected size of the GFP fusion construct in transgenic, but not 3D7, lysate (Fig. 2Bi). The α-$Pf\mu1_c$ antibody recognized two bands in the transgenic line; one corresponding to the GFP fusion protein and the other to the native Pfμ1 protein (Fig. 2Bii, lane 2). Staining of 3D7 lysate using α-Pfμ1c antibody detected only the native Pfμ1 protein (Fig. 2Bii, lane 1). Fig. 2B iii shows loading control lanes probed by an ER resident protein PfBiP (~70 kDa)

We investigated the subcellular localization of Pfμ1–GFP by fluorescence microscopy of live cells at various time points throughout the intracellular lifecycle. In early asexual blood stages, 10–18 h post-invasion, Pfμ1–GFP was observed as a single spot in a small compartment in close proximity to the nucleus (Fig. 2Ci). After further development, in young trophozoite stages (20–24 h post invasion), two to four fluorescent puncta were observed adjacent to the nucleus (Fig. 2Cii–iv). As nuclear division commences (~32 h post invasion), Pfμ1–GFP was localized in multiple compartments, ensuring that each merozoite inherits one such spot (Fig. 2Cv). At the mature schizont stage, a well-defined punctate staining was seen, typical of apical organelle distribution of proteins in *Plasmodium* (Fig. 2Cvi).

2.3. Pfμ1 resides near the Golgi compartment and is involved in post-Golgi trafficking in early trophozoites

Localization of Pfμ1–GFP to a single loci adjacent to the nucleus in early (10–18 h post invasion) stages of parasite development suggested its association with a single compartment. In order to define this compartment, we performed immunofluorescence assays to stain for Pfμ1–GFP, as well as the ER marker binding immunoglobulin protein (BiP) and a Golgi marker Golgi re-assembly stacking protein (GRASP). As shown in Fig. 3A & B, Pfμ1 partially co-localized with PfGRASP and PfBip, showing very similar correlation coefficients with each marker (~0.56). As detailed in previous EM studies (e.g. [14]), the ER (which is contiguous with the nuclear envelope) and Golgi in developing merozoites are very closely juxtaposed, and dense vesicular traffic occurs between these two

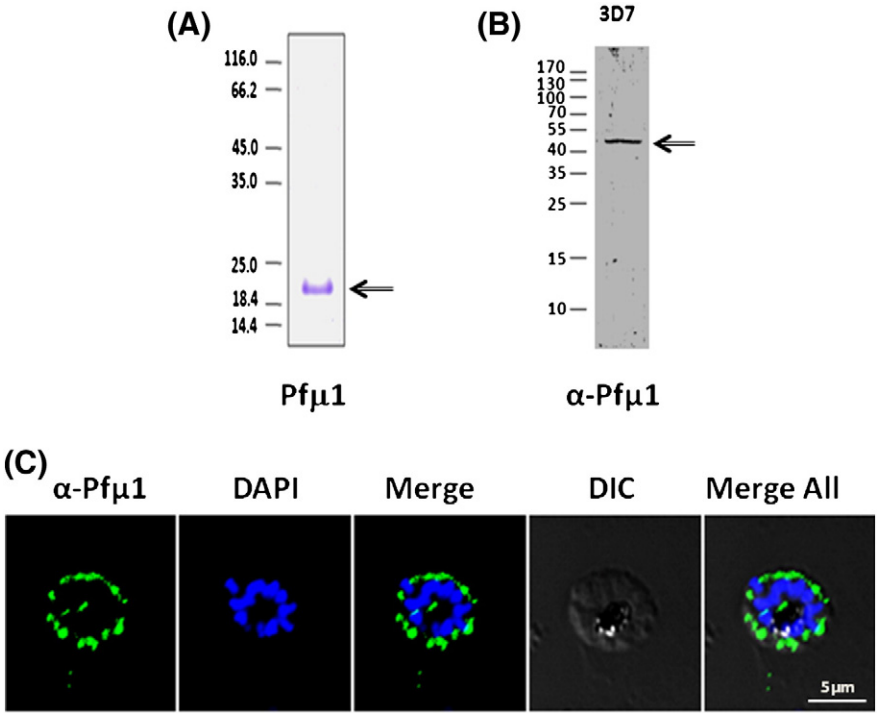

Fig. 1. Expression and localization of Pfμ1 protein in *P. falciparum*. (A) Purified recombinant Pfμ1$_c$ protein, showing a band of expected size at ~20 kDa. (B) Immunoblot analysis of whole cell 3D7 strain parasite lysate using antibodies raised against Pfμ1, demonstrating detection of a single ~50 kDa band corresponding to the native Pfμ1 protein. (C) Immunofluorescence analysis using the anti-Pfμ1 antibody, demonstrating discreet punctate structures in schizont stage parasites. Parasite nuclei were stained with DAPI; scale bars denote 5 μm.

compartments, as well as between nascent rhoptries. Moreover, we analyzed the localization of Pfμ1–GFP with respect to PfRab7, a marker for the recently described endosome in *P. falciparum* using anti-PfRab7 antibody. We failed to observe any overlap between the two proteins. This suggests that Pfμ1–GFP and Rab7 do not co-localize, and hence that Rab7 is not involved in trafficking at this stage (Figs. 3C and S1). These results thus suggest the presence of Pfμ1 in the Golgi–ER network during the early stages of intra-erythrocytic development.

Brefeldin A is a fungal metabolite that affects the GDP–GTP exchange factors of the ARF family of small GTPases. AP-1, as well as AP-3 and AP-4 have been shown to be sensitive to treatment with brefeldin A in model systems, and so we sought to identify whether *Plasmodium* AP-1 was also affected [17,29,30]. Therefore, we tested the effect of BFA addition on the distribution of Pfμ1–GFP in transgenic parasites. After 16 h treatment with 5 μg/mL BFA, Pfμ1 exhibited a diffused staining (Fig. 4A) in comparison to the control parasites wherein staining was confined to well defined punctate structures in close association with the parasite nuclei (Fig. 4B). To further characterize the effect of BFA treatment on the localization of Pfμ1, we performed co-localization studies with antibodies to ERD2 (a cis-Golgi marker) and BiP, as well as the resident rhoptry protein RAP1 and the cytosolic protein Sel2. Pfμ1 did not appear to show a similar pattern as Bip or Sel2, beyond the overlap expected in a cell of this size. However, Pfμ1 showed a substantially similar staining pattern as ERD2 which is a Golgi marker that has been previously shown to get re-distributed upon BFA addition [31] (Fig. 4Ci–iii). Pfμ1 also did co-localize with RAP1 in BFA treated parasites (Fig. 4Civ). These observations are consistent with Pfμ1 being Golgi associated at this life stage, consistent with its similar dynamics of redistribution upon brefeldin treatment

2.4. Pfμ1 co-localizes with resident rhoptry proteins in schizonts

Localization of punctate staining in the apical end of the parasite at the late schizont stage suggested a role of Pfμ1 in trafficking to the apical organelles. To further characterize the potential targets of Pfμ1 mediated trafficking events, we performed IFA with antibodies to rhoptry (RAP1 and Clag3.1), microneme (EBA175), and surface markers (MSP1). IFA with anti-MSP1 antibody showed no overlap in staining between MSP1 and Pfμ1 (Fig. 5A). Similar results were seen with antibodies to EBA175 (Fig. 5B). Importantly, anti-RAP1 and anti-Clag3.1 showed co-localization with the Pfμ1–GFP chimeric protein (Fig. 5C and D), suggesting a potential role for Pfμ1 in rhoptry trafficking. These results were confirmed using anti-RAP1 and anti-Pfμ1 antibodies (Figs. S2 and S3). Co-localization between Pfμ1 and RAP1 was first observed ~24 h post invasion in budding vesicles near the Golgi. As nuclear division commenced (32 h), Golgi multiplication occurred as well, and this resulted in apical distribution of Pfμ1 along with RAP1 in the rhoptries. Two confocal imagery based movies of merozoites (Movie S1) and schizonts (Movie S2) showing co-localization of Pfμ1 and RAP1 have been uploaded with this manuscript. The co-localization between rhoptry proteins and Pfμ1 was further quantified by Pearson's correlation coefficient analysis. Substantial correlation was observed between Pfμ1, and both RAP1 (Figs. 5C and S4) and Clag3.1 (Fig. 5D). Additionally, we performed co-immunoprecipitation (IP) studies pulling down with antibodies against RAP1 and Pfμ1 (Fig. 6A). Immunoprecipitation using anti-RAP1, followed by incubation with anti-Pfμ1 showed two bands at ~50 and ~70 kDa, corresponding to the size of the native and GFP-tagged Pfμ1 proteins in the transgenic parasites (Fig. 6A). Similarly, immunoprecipitation using anti-Pfμ1 allowed detection of the ~90 kDa native RAP1 protein, suggesting that Pfμ1 and PfRap1 interact (Fig. 6A). In addition, we carried out immuno-precipitation using anti-GFP antibody and transgenic parasites followed by LC/MS/MS analysis; a number of adaptin complex proteins, clathrin and rhoptry proteins were identified in the sample (Table S1). Our data thus demonstrates the spatiotemporal relationship of Pfμ1 with immature and mature rhoptries, suggesting a role for the AP-1 complex in association with the rhoptries.

2.5. Pfμ1 localization to rhoptries is dependent on vesicular trafficking

Though our localization studies demonstrate Pfμ1 in close association with the rhoptry organelles in the late trophozoite/schizont stages of the

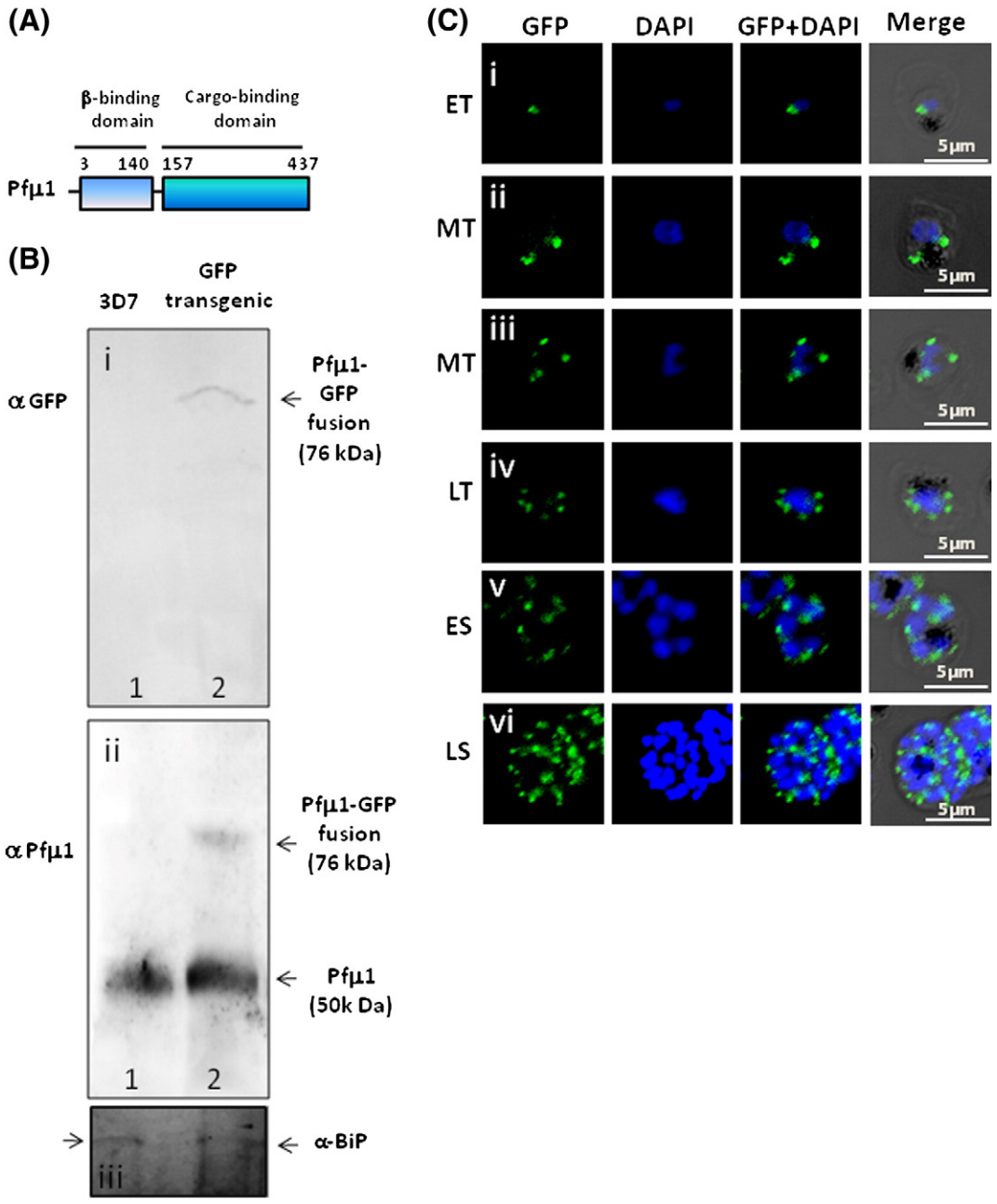

Fig. 2. Localization of Pfμ1 in different intracellular stages of *P. falciparum*. (A) Schematic diagram of the wild type Pfμ1 (PF3D7_1311400) showing location of β-binding domain and cargo-binding domain. The complete gene was cloned in frame with GFP in the pARL1a + vector under the control of chloroquine resistant transporter gene promoter (crt 5′ UTR) and dhfr terminator (3′ UTR). (B i) Immunoblot analysis of whole cell lysates of trophozoite-stage 3D7 and transgenic parasites expressing Pfμ1–GFP by α-GFP antibody shows a band at ~76 kDa. (B ii) Immunoblot analysis of whole cell lysates of trophozoite-stage 3D7 and transgenic parasites expressing Pfμ1–GFP by α-Pfμ1 antibody. A band at ~50 kDa, representing the native protein is recognized by the Pfμ1 antibody in lanes 1 and 2, while another band at ~76 kDa (lane 2), representing the Pfμ1–GFP fusion, was recognized in the transgenic line only. (B iii) Loading control lane as probed by anti-Pf BiP. (C) Live cell imaging of transgenic parasites expressing the Pfμ1–GFP fusion protein from early trophozoite to late schizont stages. Parasite nuclei were stained with DAPI; scale bars denote 5 μM. ET, Early Trophozoite; MT, Mid Trophozoite; LT, Late Trophozoite; ES, Early Schizont; LS, Late Schizont.

parasite, we could not rule out the potential that Pfμ1 is simply a resident protein at these stages. To address this question, we utilized AlF_4 treatment, which functions as a general blocker of vesicular trafficking, i.e., it inhibits intra-Golgi transport as well as anterograde cargo trafficking between ER and Golgi [32]. As shown in Fig. 6B, control trophozoites showed typical ER–Golgi distribution for Pfμ1 as evident from its partial co-localization with PfBip and PfGRASP markers. After AlF_4 treatment, Pfμ1 labeling was restricted to a small compartment, which appeared by correlation analysis to be more closely associated with the parasite ER. This drastic redistribution, demonstrated in the inset micrographs and supported by quantification, demonstrates the reliance of Pfμ1 localization on proper vesicular trafficking in early development stages (Fig. S5A and B). Together with the observation that Pfμ1–GFP redistributes upon BFA addition, these results argue against a role for Pfμ1 as a resident protein at any stage, and suggest that its localization is dependent on vesicular traffic. This further suggests that the eventual rhoptry localization of Pfμ1 is a direct result of post-Golgi Pfμ1-mediated trafficking of rhoptry proteins. Confocal imagery-based movies of trophozoites showing co-localization of Pfμ1 (Green) and PfBip (Red) before and after AlF_4 treatment (supplementary movies S3 and S4) along with movies S5 and S6 showing co-localization of Pfμ1 (Green) and PfGRASP (Red) before and after AlF_4 treatment have been uploaded with this manuscript.

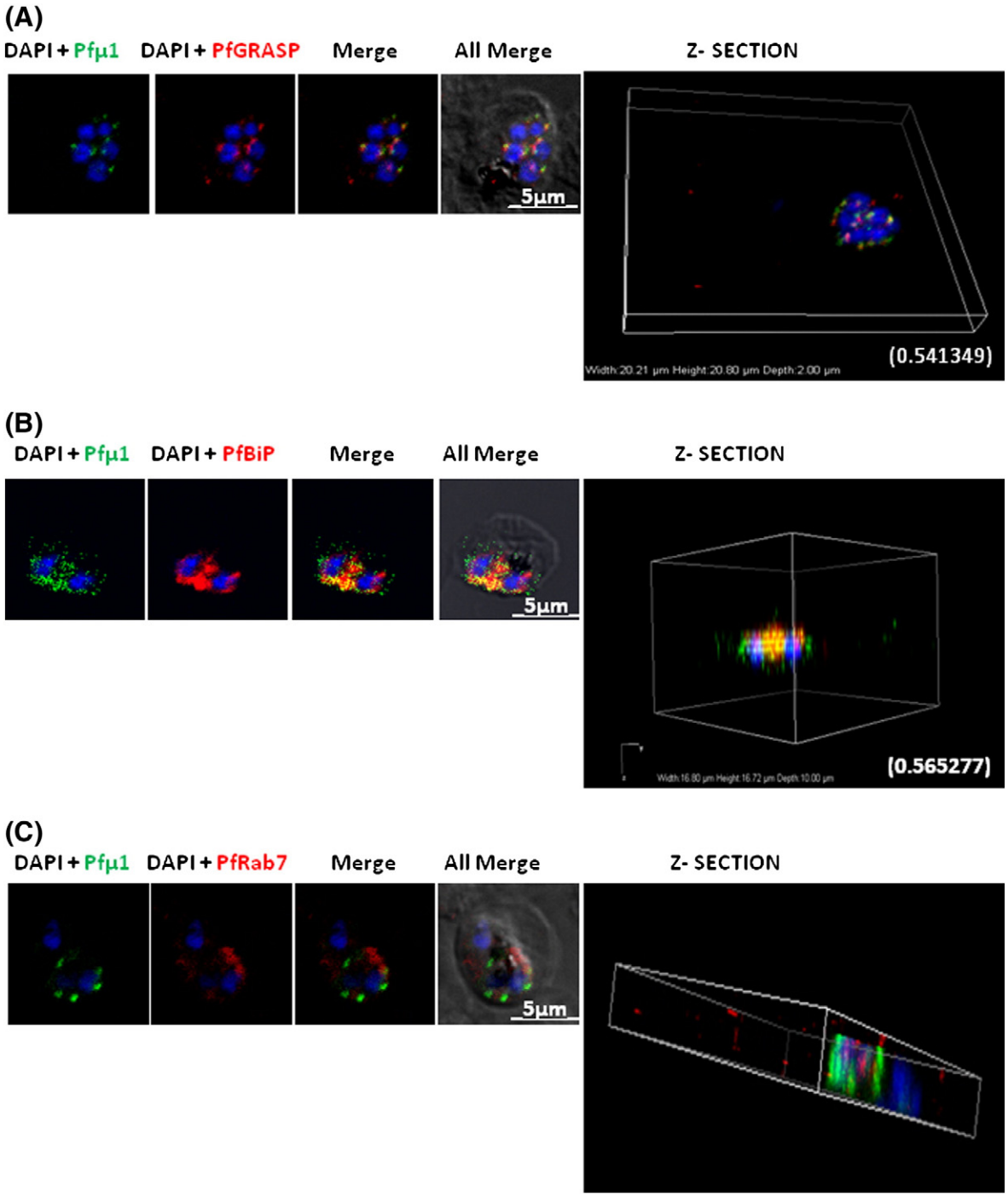

Fig. 3. Pfμ1 is closely associated with the Golgi and ER in early trophozoite stages. Transgenic parasites expressing Pfμ1–GFP protein at trophozoite stages were immunostained with anti-PfGRASP (A), anti-PfBiP (B) and anti-PfRab7 (C) antibodies. The parasite nuclei were stained with DAPI and slides were visualized by confocal laser scanning microscopy. Values in brackets show Pearson correlation coefficients; scale bars denote 5 μm.

3. Discussion

Though numerous studies to date have implicated the role of various trafficking components in trafficking to the apical organelles, including dynamin-like proteins [8], Rabs [9], and trans-membrane cargo receptors [10], much remains to be deciphered about these critical processes. One of the main questions is how post-Golgi cargo sorting and coat recruitment are involved in the specificity of trafficking to the rhoptries, and distinct sub-populations of micronemes. A previous study suggested the role of AP-1 in trafficking to the rhoptries, but determining the full extent of AP-1 involvement in the process was hampered by the later discovery that TgROP2 does not possess a transmembrane domain, and hence cannot interact directly with the AP-1 mu subunit as the author's claimed [26]. However, this early study did provide convincing immunofluorescence and immuno-EM data for the localization of Tgμ1 to post-Golgi vesicles, immature, and mature rhoptries as well as direct functional data from disruption of AP-1 function through point mutations and gene disruption. Additionally, immunoprecipitation experiments with TgSORTLR revealed interactions with AP1 subunits and clathrin, suggesting that this interaction may be important for the forward translocation of soluble rhoptry cargo [10], at least in *Toxoplasma*. More recent immunolocalization data not only confirms

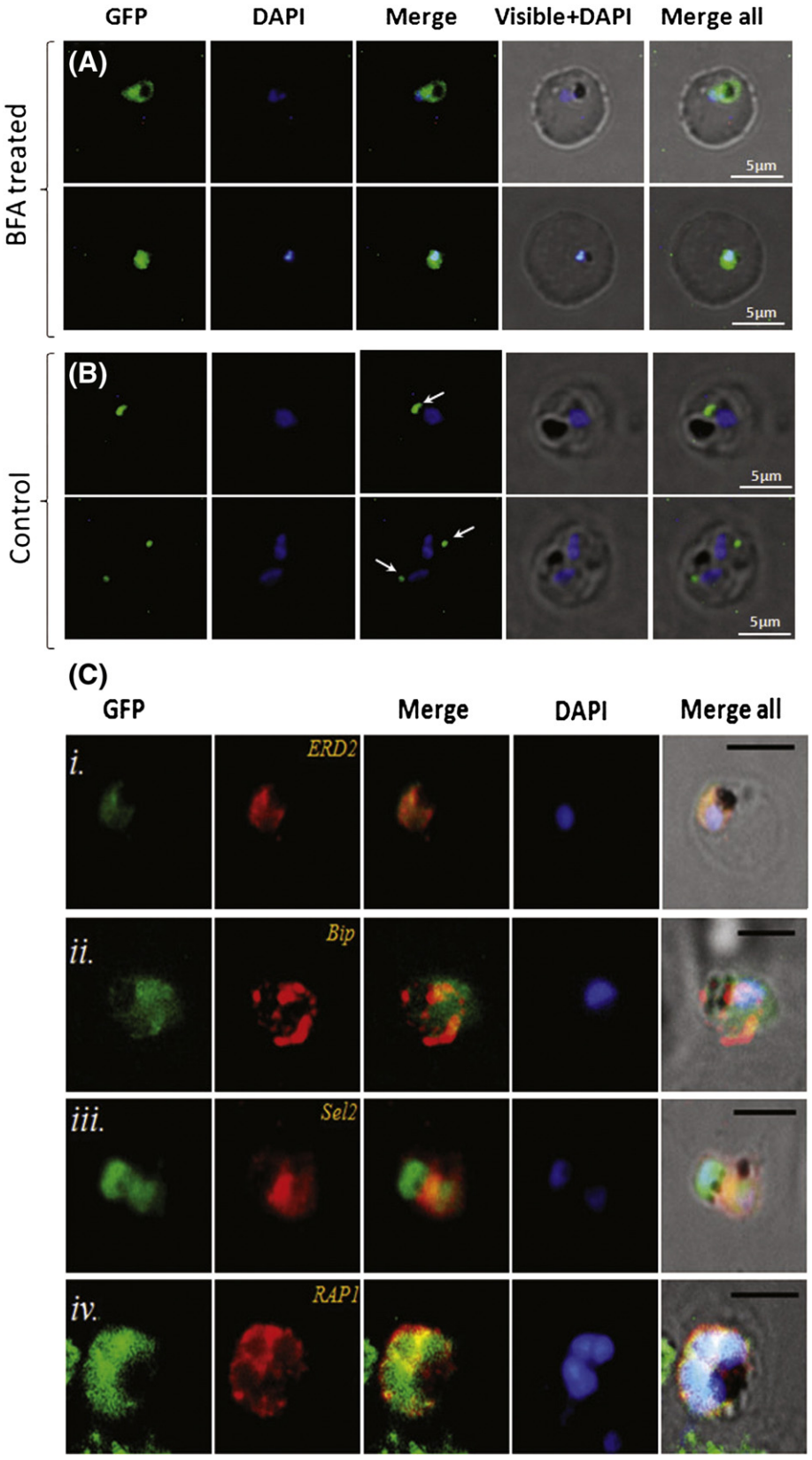

Fig. 4. Brefeldin A treatment disrupts Golgi localization of Pfµ1. Transgenic parasites expressing Pfµ1–GFP were treated with Brefeldin-A (at 5 µg/ml), or DMSO alone (control). Live cell imaging of BFA-treated (A) or DMSO-treated (B) transgenic parasites at trophozoite stage after the treatment. (C) Transgenic parasites expressing Pfµ1–GFP were treated with Brefeldin A (BFA) and immunostained with antibodies specific to cis-Golgi apparatus marker ERD2 (i), endoplasmic reticulum marker, Bip (ii), cytoplasm localized, Sel2 (iii) and RAP1 (iv). The Pfµ1–GFP fusion protein colocalized with Sel2 as well as ERD2 in the parasite cytoplasm upon BFA treatment [C (i & iii)]. Parasite nuclei were stained with DAPI; scale bars denote 5 µm.

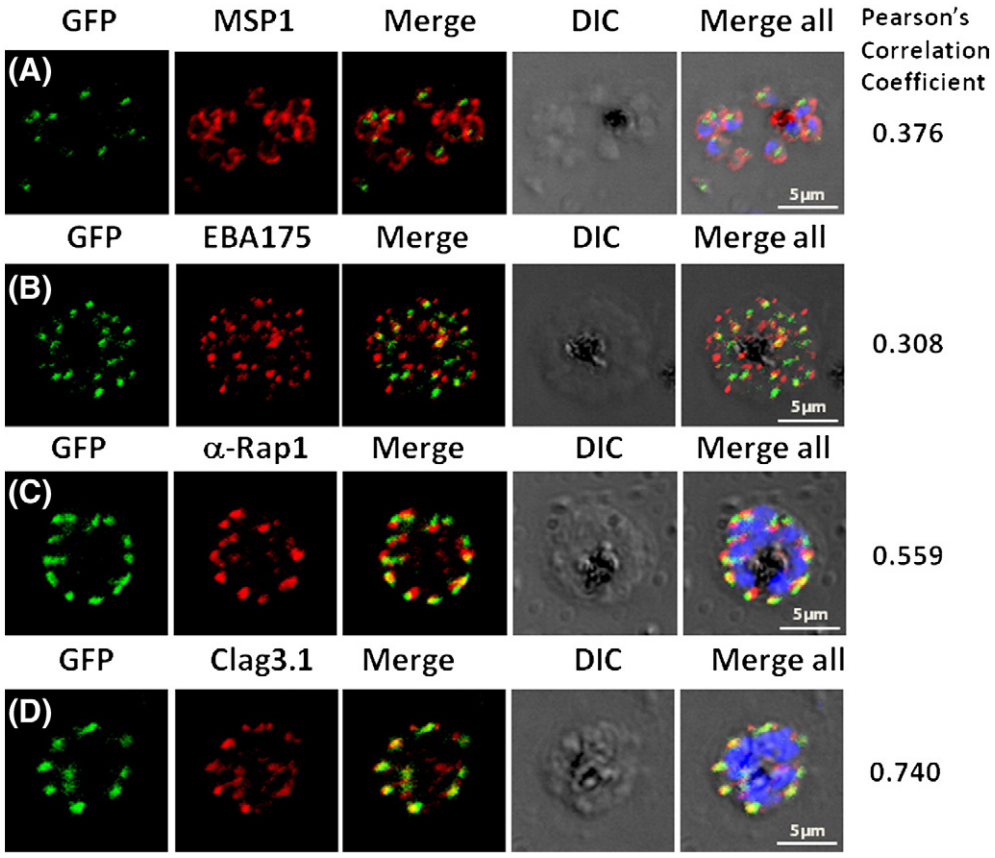

Fig. 5. Pfμ1 co-localizes with rhoptry marker proteins in schizont stage parasites. Transgenic parasites expressing Pfμ1–GFP were immunostained with antibodies specific to the Merozoite surface localized MSP1 (A), Microneme localized EBA175 (B), and Rhoptry localized RAP1 (C) and Clag3.1 (D). The parasite nuclei were stained with DAPI and slides were visualized by confocal microscopy. Representative images are shown for each antibody, together with DIC images; scale bars denote 5 μM. To quantify co-localisation, Pearson correlation coefficients of the individual stains were calculated and are shown in the right panel of each image.

the AP-1:SORTLR interaction in *T. gondii*, but also suggests the involvement of the retromer complex in this process, as TgSORTLR exhibits similar co-localization with the apicomplexan homolog of Vps26 [5]. A recent study in *P. falciparum* may tie these observations together with our own. The authors describe an endosome-like compartment ([15] and Fig. 7), adjacent to, but distinct from the ER and Golgi, which stains positive for Rab7 and components of retromer, and suggest that vesicular traffic may occur through this organelle.

Surprisingly, we did not detect co-localization of Pfμ1–GFP with Rab7 in our analyses. Instead, we observed Rab7 localization in other parts of the infected cell, possibly coincident with the digestive vacuole, which is a lysosome-like organelle. It is possible that Rab7 is involved in diverse trafficking processes throughout the intracellular cycle, such that we were unable to observe cells in which Pfμ1–GFP and Rab7 are acting at similar steps. Further work will be required to clarify the role of Rab7 and the retromer complex in trafficking to apical organelles in *Plasmodium*. Our data are, however, consistent with the notion that AP-1 is likely involved in rhoptry biogenesis and trafficking of proteins to mature rhoptries in *Plasmodium* (Fig. 7). Pfμ1–GFP localizes to structures consistent with the Golgi apparatus in early trophozoite stages, before rhoptries begin to form, and this interaction is sensitive to treatment with BFA. Rhoptry biogenesis begins roughly halfway through the intracellular stage, and occurs via the fusion of specific Golgi-derived vesicles, consistent with our localization data (Fig. 7A). As the intracellular cycle progresses through late trophozoite and schizont stages, Pfμ1–GFP localizes to distinct punctae at the apical end of developing merozoites, consistent with rhoptry localization (Fig. 7B). Furthermore, Pfμ1–GFP co-localizes with two resident rhoptry proteins, RAP1 and Clag3.1, but not with known markers of micronemes (EBA-175) and the parasite surface (MSP1). The incomplete nature of this co-localization may be explained by the presence of endosome-like compartments in close proximity to rhoptries in schizont stage parasites, through which trafficking of rhoptry proteins may occur [15]. We demonstrate a specific interaction between one of these proteins, RAP1, and Pfμ1, consistent with the notion that Pfμ1 is likely involved in trafficking RAP1 to the rhoptries. Additionally, treatment with AlF_4, which affects Arf GTP exchange and acts to disturb normal trafficking processes, disrupts established localization of Pfμ1, resulting in a punctate distribution. Previous studies have demonstrated fragmentation of the Golgi upon AlF_4 treatment, which is consistent with our observations [33]. Together, these results demonstrate sub-cellular localization for Pfμ1 consistent with a protein involved in rhoptry biogenesis and trafficking throughout the intracellular lifecycle of *P. falciparum*, and suggest that this localization is dependent on trafficking events.

We provide co-IP data demonstrating a weak but measurable interaction between RAP1 and Pfμ1. PfRAP1 does not contain putative transmembrane domains, which suggests that the interaction is not directly mediated by any of the putative YXXϕ or LL motifs present in PfRAP1, but occurs through at least one other protein in a complex. One potential identity for this protein is the *P. falciparum* homolog of TgSORTLR, as it has been shown to interact with clathrin and adaptor protein subunits [10], but it is possible that other transmembrane cargo receptors could be involved in trafficking to micronemes and rhoptries in apicomplexan parasites, and that escorter proteins such as RAMA may also be involved [34]. A recent analysis of clathrin function in *T. gondii* demonstrated that functional ablation of clathrin caused defects in trafficking of the microneme protein MIC3 and the rhoptry protein ROP5, as well as affecting the morphology of the Golgi [35]. Their results are consistent with clathrin being involved in post-Golgi trafficking, but they could find no evidence of a role for clathrin in endocytosis, again

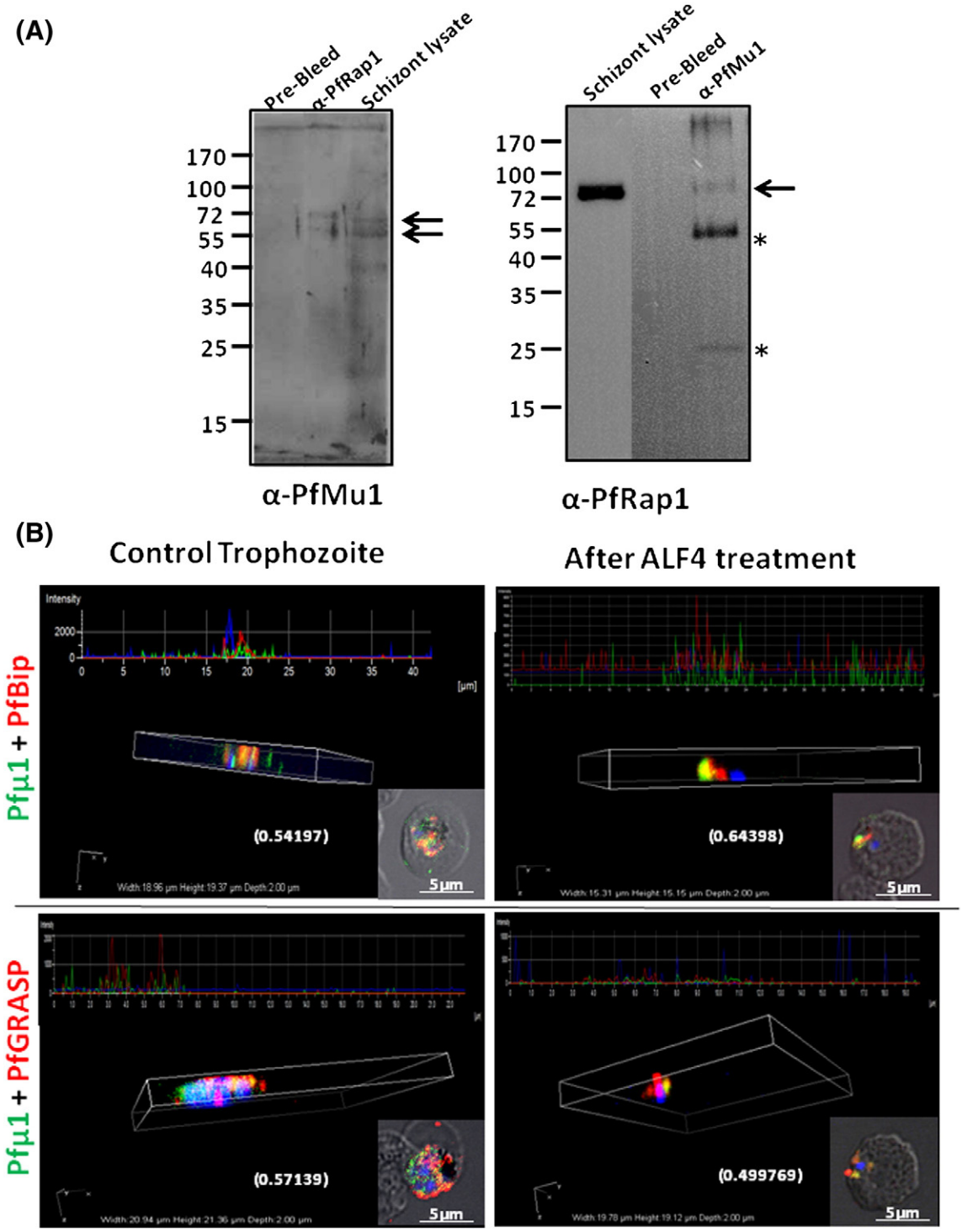

Fig. 6. Pfμ1 interacts with RAP1 in a trafficking-specific manner. (A) Immuno-precipitation of *P. falciparum* 3D7 schizont protein extracts was performed with anti-μ1 and anti-RAP-1 antibodies separately. The interaction of both the proteins was confirmed with western blot. Immuno pull-down of schizont total protein lysate with anti-RAP1 antibody recognizes two bands of Mu1 corresponding to a molecular weight of 70 and 55 kDa, while immunoprecipitation with anti-PfMu1 antibody recognized Rap1 at a band of 90 kDa. The asterisk indicates the heavy chain of IgG eluted from the antibody affinity beads. (B) *P. falciparum* 3D7 infected RBCs were treated with 100 μm $AlCl_3$ and 30 mM NaF in RPMI for 1 h at 37 °C. The washed parasites were then stained with anti-GFP antibody (green) and anti-PfBip antibody (red). The parasite nuclei were stained with DAPI and slides were visualized by confocal laser scanning microscopy. Values in brackets show Pearson correlation coefficients; scale bars denote 5 μm.

consistent with the hypothesis of alteration of endocytic machinery in Apicomplexa.

Our data thus provide evidence for the role of AP-1 in trafficking to the rhoptry organelles of *P. falciparum*, a function which is likely conserved with *T. gondii*, and potentially all other Apicomplexa. A previous study demonstrated that AP-1 is universally conserved in Apicomplexa, suggestive of a conserved and essential function [24]. Assuming that rhoptries and micronemes are modified endolysosomal organelles as hypothesized, one obvious additional candidate for mediating trafficking would be the AP-3 complex. However, *Babesia*, *Theileria*, and *Cryptosporidium* lack all subunits of this complex, suggesting that it does not provide a conserved function in all Apicomplexa. Additionally, the presence of other lysosome-like organelles, such as the *T. gondii* lytic vacuole [36], and the *Plasmodium* digestive vacuole [37], provides a tantalizing prospect for the involvement

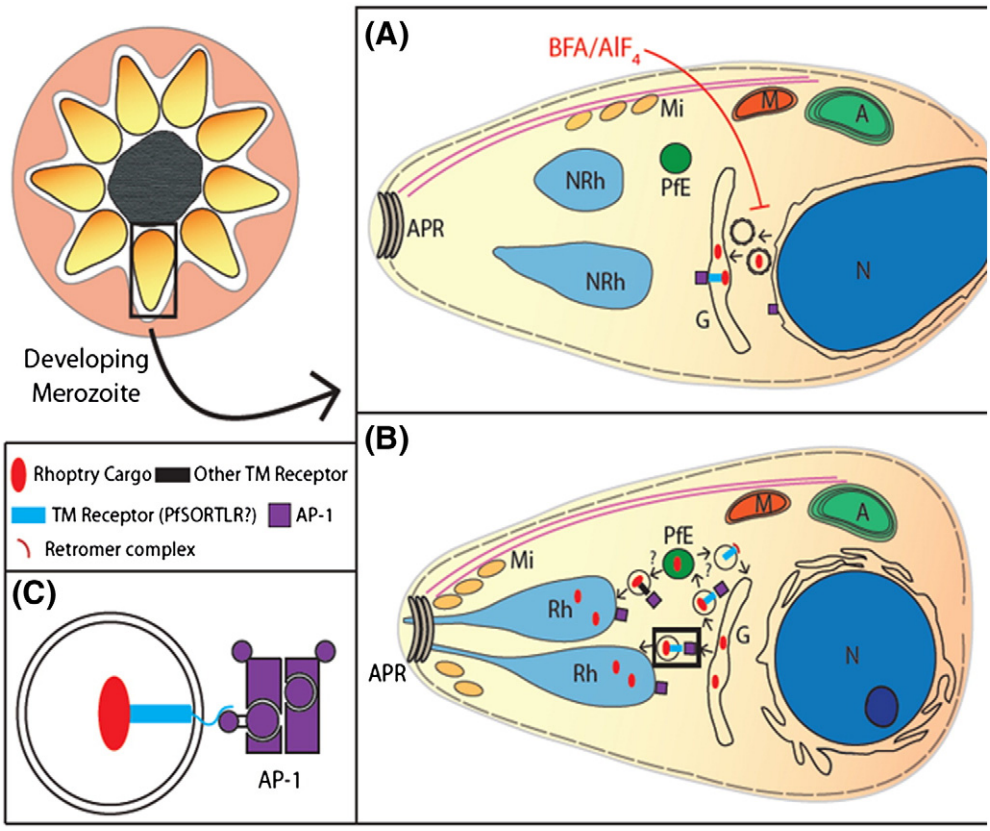

Fig. 7. Proposed model of Pfμ1-mediated rhoptry trafficking in *P. falciparum*. (A) Early in merozoite development, coated vesicles transport rhoptry proteins between the ER and Golgi, a process that can be inhibited by addition of BFA/AlF_4. Adaptor proteins are synthesized at the ER face and proceed to recognize cargo at the Golgi through transmembrane cargo adaptors. (B) As development progresses, vesicular traffic, which may be clathrin-mediated (not shown), progresses from the Golgi to rhoptries. The cargo may change receptors and recycling can occur via the retromer complex. (C) Enlargement of the vesicle boxed off in panel (B), showing the binding of AP-1 to soluble rhoptry cargoes through a transmembrane receptor, as described in the text. A = apicoplast, APR = apical polar rings, G = Golgi apparatus, M = mitochondrion, Mi = microneme, N = nucleus, NRH = nascent rhoptry, PfE = *Plasmodium falciparum* endosome, and Rh = rhoptry.

of AP-3. In fact, a recent screen for novel chemotherapeutic agents against Apicomplexa revealed that an inhibitor resulted in the complete absence of the lytic vacuole in *T. gondii*, which could be partially mitigated by mutations in AP-3β, suggesting that AP-3 may play a role in trafficking to this organelle [38]. These data have implications for post-Golgi trafficking, and specifically adaptor protein function, in organisms possessing multiple lysosome-related organelles. Apicomplexa may prove an interesting model system to study these processes moving forward.

Our data suggest that AP-1 is not involved in trafficking to micronemes, or at least that EBA175 trafficking is AP-1-independent. It is now widely known that micronemes do not represent a homogeneous population, and that different microneme proteins require different trafficking pathways. AP-1 may be involved in trafficking of a subset of microneme proteins, though this remains to be tested. Alternatively, or perhaps in conjunction, the AP-4 and/or AP-2 complexes represent potential candidates for this role, as they are also universally conserved in Apicomplexa.

In conclusion, we have provided data suggestive of a function for the AP-1 complex in trafficking to the rhoptries in *P. falciparum*. Pfμ1–GFP localizes to the Golgi in early trophozoite stages, an association which is sensitive to BFA, and assumes an apical localization by schizont stages. Pfμ1–GFP displays correlated co-localization with two resident rhoptry proteins, but not with known markers for the micronemes or the merozoite surface, and this interaction is dependent on trafficking processes. Additionally, Pfμ1 displays a consistent interaction with RAP1 via co-immunoprecipitation. This provides corroborating evidence to the work in other experimentally tractable apicomplexan model organisms. Though the exact mechanisms involved in these trafficking events remain to be deciphered, mounting evidence should now put some of the controversy to rest and support the hypothesis that adaptor proteins, especially AP-1, do play important roles in trafficking to the apical organelles of apicomplexan parasites.

4. Materials and methods

4.1. Parasite culture and transfection

P. falciparum strain 3D7 parasites were maintained in culture using O positive human RBCs (4% hematocrit) in RPMI 1640 media (Invitrogen) supplemented with 10% Albumax (GibcoBRL) following standard protocols [39]. To generate the transfection vector constructs, the full length Pfμ1 gene (437aa) was amplified from genomic DNA using forward primer 5′ Cg ggA TCC AgA TTA gAC AAA ATg gCA TgT ATA Ag 3′ and reverse primer 5′ C CCT Agg ggA CAT TCT gAC CTg ATA gTC 3′. The amplified fragments were digested with BamH1 and AvrII and ligated into the pHH2 vector [40] using the BglII and AvrII sites to place the gene in frame with the 3′ appended mut2 eGFP sequence. The amplified Pfμ1 gene was sub-cloned into the XhoI site of the transfection vector pARL1a [41] and analysed for correct orientation. Parasite cultures were synchronized by two consecutive sorbitol treatments at 4-hour intervals following previously described protocols [42]. Tightly synchronized ring stage parasites were collected by centrifugation and washed with incomplete cytomix [43]. 200 μl of these parasites was then resuspended in a solution containing 370 μl of incomplete cytomix and 30 μl of Plasmid DNA (100 μg) and transfected by electroporation (310 V, 950 μF). After electroporation, parasites were immediately transferred to 10 ml of pre-warmed complete culture medium supplemented with 200 μl of uninfected RBCs. The transfected parasites were selected

on 2.5 nMol WR99210, an antifolate drug that selects for the presence of the human dhfr gene present in the plasmid [44].

4.2. Cloning and expression of recombinant Pfμ1 protein and generation of polyclonal anti-sera

The C-terminal fragment of the Pfμ1 gene was PCR amplified from *P. falciparum* genomic DNA using forward primer 5′ CCC ATg ggA TCC ACg TAT CgT CTA AgT ACT CAT g 3′) and reverse primer 5′ gC gTC gAC ggA CAT TCT gAC CTg ATA gTC 3′. The amplified PCR products were ligated into the pET28a vector (Novagen) using the NcoI and SalI sites. The expression construct was transformed into expression cells BL21 (DE3) which were grown in Luria broth containing kanamycin (25 μg/ml) at 37 °C in a shaking rotor and induced with isopropyl-β-thiogalactopyranoside (IPTG) at 1 mM final concentration. The cultures were further grown at 37 °C for 4 h and the *E. coli* cells were harvested by centrifugation. The cell pellet was suspended in lysis buffer (20 mM Tris pH 8.0, 500 mM NaCl, 1 mM phenyl methyl sulphonyl flouride, and 1% Tween 20), and the bacterial cells were lysed by sonication (Torebeo Ultrasonic Processor 36800, Cole Parmer). The lysate was centrifuged at 15,000 ×*g* for 30 min at 4 °C, and the supernatant was discarded. The pellet was dissolved in 8 M urea (50 mM Tris pH 8.0, 300 mM NaCl) and incubated with Ninitrilotriacetic acid (Ni^{2+}-NTA) agarose resin (Qiagen), pre-equilibrated with 8 M urea buffer pH 8.0, at room temperature for 1 h. The suspension was applied to a column and washed with 10 bed volumes of the wash buffer (8 M urea pH 8.0, 50 mM Tris, 300 mM NaCl). The bound protein was eluted with 10 bed volumes of elution buffer containing between 50 and 500 mM imidazole gradient (8 M urea pH 8.0 in 50 mM Tris, 300 mM NaCl). The eluted fractions were analyzed on SDS-PAGE and the fractions containing the recombinant protein with a clear single band were pooled and dialysed to remove imidazole and urea. The protein concentration was determined using the bicinchoninic acid assay (BCA method) using a standard curve of bovine serum albumin. Rat antiserum was raised against the C-terminal end of Pfμ1 protein and the titre was measured using ELISA.

4.3. Immunoflourescence and microscopy

Parasite fixation for indirect immunoflourescence assays and GFP localization was performed as described previously [45]. Briefly, thin smears of *P. falciparum* iRBCs were made on glass slides, and subsequently washed and fixed with fixation solution containing 4% paraformaldehyde and 0.0075% glutaraldehyde in PBS for 30 min. After washing with PBS, slides were subjected to permeabilization with 0.1% Triton X-100 and treated with 0.1 mg/ml $NaBH_4$ to remove free aldehyde groups; each for 10 min. Parasites on slides and in solution were blocked using 3% BSA in PBS for 1 h. After blocking, slides were incubated with appropriate primary antibodies (Rat anti-μ1 1:100, other antibodies 1:250 for 1 h at 37 °C). After proper washing with PBS, slides were incubated with appropriate secondary antibodies conjugated to fluorescent dye (FITC 1: 250 or Cy3 1:500) for 1 h at 37 °C. Incubation with DAPI (2 μg/ml for 30 min at 37 °C) was used to stain the nucleus. After 3 consecutive 1 × PBS washes, slides were mounted with cover slips in the presence of anti-fade mounting media (Bio-Rad). The stained 3D7 and transgenic parasites were imaged on a Nikon TE 2000-U fluorescence microscope. The images were analyzed by NIS elements software (Nikon).

4.4. Western blotting

For the western blot analyses, 3D7 and Pfμ1–GFP transgenic parasites were isolated from tightly synchronized cultures at schizont stage by lysis of iRBCs with 0.15% saponin. Parasite pellets were washed with PBS, suspended in 4% SDS sample buffer containing β-mercaptoethanol, boiled, and centrifuged, and the supernatant obtained was separated on a 12% SDS–PAGE gel. The fractionated proteins were transferred from gel onto a high-protein-binding-capacity hydrophobic polyvinylidene difluoride (PVDF) membrane (Amersham) and blocked in blocking buffer (1% PBS, 0.1% Tween-20, 3% BSA) for 2 h. The blot was washed and incubated for 1 h with primary antibody (Rat anti-Pfμ1 1:250; rabbit anti-GFP 1:500) diluted in dilution buffer (1X PBS, 0.1% Tween-20, and 1% BSA). Later, the blot was washed and incubated for 1 h with appropriate secondary antibody (anti-mice, rat or rabbit antibodies 1:3000) conjugated to HRP, diluted in dilution buffer. Bands were visualized by using the ECL detection kit (Amersham).

4.5. Brefeldin A treatment

Parasite cultures were treated using Brefeldin A (BFA) following the procedure described in a previous study [46]. Briefly, *P. falciparum* cultures were synchronized by two consecutive sorbitol treatments at 4 hour intervals and cultured further for 40 h and allowed to re-invade. Brefeldin A was added to the culture at a concentration of 5 μg/ml from a stock solution of 10 mg/ml BFA in DMSO; a control culture was maintained using the equivalent amount of DMSO to ensure the solvent had no effect on growth or cell morphology. After 16 h, BFA was removed from culture by washing twice and the parasites were cultured for another 24 h in BFA free media to ensure viability of cells after BFA treatment.

4.6. Co-immuno-precipitation

Infected erythrocytes containing late stage *P. falciparum* (40–42 h post-infection) were harvested by centrifuging at 2000 rpm for 5 min and treated with 1.5 vol 0.15% saponin (in PBS) for 10 min at 4 °C. Cells were again centrifuged at 7000 rpm for 15 min to separate intact parasites from the lysed erythrocytes. The parasite pellet was washed several times with PBS. Parasite lysate was prepared by treating the pellet with IP Lysis Buffer (provided with the Pierce Crosslink Immunoprecipitation Kit-Product #26147) containing protease inhibitors, for 15–20 min at 4 °C with intermittent mixing. Released cellular contents were separated from the debris by centrifugation at 13,000 rpm for 20 min. Total protein content of the lysate was determined by the BCA Protein estimation assay kit (Pierce). The co-immunoprecipitation experiments were carried out according to the manufacturer's instructions. Briefly, 1 mg of total protein was incubated overnight at 4 °C with 10 μg of anti-Pfμ1 (Rat) and/or anti-PfRap1 (Mouse) antibodies cross-linked to 10 μl of Protein A/G sepharose beads. An equal amount of protein was allowed to interact with beads conjugated and cross-linked to pre-immune mouse or rat sera to serve as a control. After binding, beads were washed with the Wash Buffer and the bound proteins were eluted from the beads using the Elution Buffer. The elutes were used to perform the immune-blotting using anti-PfRap-1 and anti-Pfμ1 antibodies.

4.7. AlF_4 treatment

Parasite cultures were treated using AlF_4, as described previously [47]. Briefly, *P. falciparum* 3D7 iRBCs were treated with 100 μm $AlCl_3$ and 30 mM NaF in RPMI for 1 h at 37 °C. The RBCs were then washed thrice by iRPMI and smears were prepared for confocal microscopy, as described under "Immunofluorescence and microscopy".

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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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