Plasmodium falciparum Atg18 localizes to the food vacuole via interaction with the multi-drug resistance protein 1 and phosphatidylinositol 3-phosphate

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Abbreviations. PfAtg18: Plasmodium falciparum Atg18; WIPI: WD-repeat protein interacting with phosphoinositides; PROPPIN: β-propellers that bind polyphosphoinositides; PI3P: Phosphatidylinositol 3-phosphate; PI3K: Phosphoinositide 3-kinase; Vps: Vacuolar Protein Sorting; LC3: Microtubule-associated protein 1A/1B-light chain 3; LIR: LC3-interacting region; AIM: Atg8-family interacting motif; MDR-1: multidrug resistance protein 1; PAS: phagophore assembly site; UTR: Untranslated region; GST: Glutathione S-transferase; DHFR: Dihydrofolate Reductase; CDD: C-terminal Destabilizing Domain; IC₅₀: half maximal inhibitory concentration; PDB: Protein Data Bank; PCV: packed cell volume; BSD: blasticidin
Abstract

Autophagy, a lysosome-dependent degradative process, does not appear to be a major degradative process in malaria parasites and has a limited repertoire of genes. To better understand the autophagy process, we investigated Plasmodium falciparum Atg18 (PfAtg18), a PROPPIN family protein, whose members like S. cerevisiae Atg18 (ScAtg18) and human WIPI2 bind PI3P and play an essential role in autophagosome formation. Wild type and mutant PfAtg18 were expressed in P. falciparum and assessed for localization, the effect of various inhibitors and antimalarials on PfAtg18 localization, and identification of PfAtg18-interacting proteins. PfAtg18 is expressed in asexual erythrocytic stages and localized to the food vacuole, which was also observed with other Plasmodium Atg18 proteins, indicating that food vacuole localization is likely a shared feature. Interaction of PfAtg18 with the food vacuole-associated PI3P is essential for localization, as PfAtg18 mutants of PI3P-binding motifs neither bound PI3P nor localized to the food vacuole. Interestingly, wild type ScAtg18 interacted with PI3P, but its expression in P. falciparum showed complete cytoplasmic localization, indicating additional requirement for food vacuole localization. The food vacuole multi-drug resistance protein 1 (MDR1) was consistently identified in the immunoprecipitates of PfAtg18 and P. berghei Atg18, and also interacted with PfAtg18. In contrast to PfAtg18, ScAtg18 did not interact with MDR1, which, in addition to PI3P, could play a critical role in localization of PfAtg18. Chloroquine and amodiaquine caused cytoplasmic localization of PfAtg18, suggesting that these target PfAtg18 transport pathway. Thus, PI3P and MDR1 are critical mediators of PfAtg18 localization.

Introduction

Malaria parasites undergo a multi-stage development in diverse intracellular and extracellular environments. Several of these stages are specialized for invasion, degradation of host cellular contents to obtain nutrients, generation of trafficking systems for import and export, and extensive reorganization of intracellular organelles to meet stage-specific needs. Since autophagy performs both degradative and biosynthetic functions, and several autophagy proteins have key roles in endosomal transport and organelle reorganization, investigation of autophagy during parasite development is warranted.

Autophagy involves degradation of dispensable cellular contents, including large protein complexes, protein aggregates, organelles and lipids in the lysosomes [1-3]. The cargo to be degraded is generally randomly selected, but certain cargo like mitochondria, peroxisomes and protein aggregates, is selectively recruited for autophagy [4]. About 35 Atg proteins participate in different stages of the autophagy process in S. cerevisiae and mammalian cells. The autophagy process can be broadly divided as: initiation by the Atg1 complex at a site known as the phagophore assembly site (PAS), assembly and vesicle
nucleation by the Vps34 complex at the PAS to form a double membrane cup-like structure called the phagophore, expansion of the phagophore by the Atg8 and Atg12 conjugation systems into a double membrane vesicle called the autophagosome, fusion of the autophagosome with lysosome, cargo degradation in the lysosome lumen and the efflux of degradation products into the cytoplasm for reuse [2, 4].

Atg8 is a popular marker for studying autophagy, and it is associated with membrane structures that are commonly referred as puncta [5]. Multiple independent studies focussing on the Atg8 protein indicate a limited autophagy repertoire in *Plasmodium* species [6-11]. It is not clear whether autophagy performs a degradative function in malaria parasites, as the majority of Atg8 puncta remain outside the food vacuole, a lysosome-like organelle wherein some of the essential and best studied biochemical processes, including haemoglobin degradation, occur [10]. Additionally, food vacuole is the site of chloroquine action, and mutations in the *P. falciparum* food vacuole membrane transporters chloroquine resistance transporter (PfCRT) and multi-drug resistance protein 1 (MDR1) have been shown to confer resistance to multiple antimalarials, including quinolines [12-19]. Autophagy may have a role in endosomal transport to the food vacuole, as some Atg8 puncta were observed near or within the food vacuole [8]. A role of autophagy in the biogenesis and/or the processes associated with apicoplast, a nonphotosynthetic plastid remnant that is essential for biosynthesis of isoprenoid precursors in *Plasmodium*, has been supported by colocalization of Atg8 with apicoplast, adverse effect on apicoplast proliferation upon overexpression of Atg8 during *P. berghei* liver stage development [20], and loss of apicoplast in Atg8 knock-down parasites [21, 22]. Although mechanistic details of the association of Atg8/autophagy pathway with apicoplast remain to be uncovered, autophagy may mediate transport of biomolecules and membranes to the apicoplast. Knockdown of Atg7 and chemical inhibition of Atg3, two essential enzymes of the Atg8 conjugation system, has been shown to impair parasite development, supporting an essential role of autophagy in parasite development [23, 24]. Autophagy pathway has also been associated with resistance to chloroquine and artemisinin [12, 25-27], but a link between autophagy and the associated drug resistance is not clear. Notably, a recent report showed that *Plasmodium* Atg12 and Atg5 form a noncovalent complex that mediated lipidation of Atg8 [28], revealing a non-canonical feature of *Plasmodium* autophagy, as the complex formation also requires Atg10 and Atg16 in the majority of other organisms. Thus, multiple lines of data conclude to an atypical, but essential, autophagy pathway in malaria parasites, which could offer attractive targets for new chemotherapeutic interventions.

All the studies focusing on Atg8 consistently indicated the presence of Atg8 puncta in malaria parasites [6-11], which look like autophagosomes in an immunoelectron micrograph [8], the hallmark of autophagy. The formation of autophagosome requires regulated and orderly action of multiple proteins, including the Vps34 complex, Atg18/WIPI4-Atg2 complex, Atg12-Atg5-Atg16 complex and its
conjugation system, and the Atg8 conjugation system [29-31]. Upon induction of autophagy, the Vps34 complex produces phosphatidylinositol 3-phosphates (PI3P) at the endoplasmic reticulum in mammalian cells and at a peri-vacuolar site in S. cerevisiae, leading to the generation of PI3P-rich structures, called the omegasomes or cradles [32, 33]. The omegasome gives rise to the phagophore that is elongated and expanded into the autophagosome. PI3P-binding proteins (Atg18, Atg21 and Hsv2 of yeast and WIPI1-4 of humans) are recruited at the omegasomes/cradles. These proteins belong to the PROPPIN family, and contain WD40 repeats and a conserved “FRRG” motif, which mediates binding to PI3P and phosphatidylinositol 3,5-bisphosphate [34-39]. The S. cerevisiae Atg18 (ScAtg18) is recruited to the PAS via interaction with PI3P [40-42]. WIPI1 and WIPI2 have also been shown to bind PI3P in PI3P-rich sites on the ER, phagophore and autophagosome [43]. WIPI2 has been shown to recruit the Atg12-Atg5-Atg16L complex at the phagophore, which facilitates conjugation of LC3 to phosphatidylethanolamine on the phagophore membrane that is crucial for its elongation and expansion into the autophagosome [43, 44].

All Plasmodium species encode for a highly conserved Atg18 homolog [10]. An earlier study showed that P. falciparum Atg18 (PfAtg18) is expressed in asexual erythrocytic stages and localizes to vesicular structures in the cytoplasm, and also colocalized with the apicoplast and the food vacuole [45]. This study also showed that PI3P is critical for localization of PfAtg18 to vesicular structures, and knockdown of PfAtg18 caused reduced conjugation of Atg8 to membranes and the loss of apicoplast, thereby indicated a crucial role of PfAtg18 in the regulation of apicoplast and autophagy. Using colocalization and inhibition approaches, a recent study reported that PfAtg18 has a role in the food vacuole dynamics and autophagy pathway [19]. Both PI3P and PI3K/Vps34 have been reported in P. falciparum erythrocytic stages [46, 47]. However, the homologs of several proteins that are essential for autophagosome formation, including the subunits of Vps34 complex (Atg6 and Atg14), Atg2, Atg10 and Atg16, are either absent or remain to be identified. Given that Atg8 puncta in Plasmodium species resemble autophagosomes [8], it is likely that Atg8 puncta are generated by a non-canonical mechanism in malaria parasites.

We hypothesized that investigation of PfAtg18 could provide insights into the formation of Atg8 puncta and the autophagy-associated drug resistance. Our data indicate that food vacuole localization is likely a conserved feature of Plasmodium Atg18, which is mediated via interaction with PI3P and MDR1, and altered upon treatment of parasites with chloroquine and amodiaquine.

**Experimental procedures**

**Materials**

All the biochemicals were from Sigma or Serva unless otherwise mentioned. The parasite culture reagents were from Lonza and Thermo Fisher Scientific. Restriction enzymes and DNA modifying enzymes were from New England Biolabs and Thermo Fisher Scientific. DNA isolation kits were from QIAGEN.
and MACHEREY-NAGEL. Dialysis membrane, ProLong Diamond Antifade Mountant, Hoechst, DAPI, LysoTracker® Red DND-99 and SuperSignal Chemiluminescent substrates were from Thermo Fisher Scientific. Antibodies were from Sigma and Thermo Fisher Scientific. WR99210 was a kind gift from David Jacobus (Jacobus Pharmaceutical, Princeton, USA). P. falciparum 3D7 and D10 strains, P. berghei ANKA strain and P. knowlesi (strain H) genomic DNA were obtained from the Malaria Research and Reagent Reference Resource centre (MR4). P. vivax genomic DNA was a kind gift from Dr. Kailash C Pandey of the National Institute of Malaria Research, New Delhi, India. Human blood was collected after obtaining written informed consent from all the subjects, processed according to the protocols (IEC-38/2015, IEC-38-R1/2015, IEC-38-R2/2015 and IEC-38-R3/2015) approved by the Institutional Ethics Committee of Centre for Cellular and Molecular Biology, and studies abide by the Declaration of Helsinki principles. Animals were housed in cabin type isolators at standard environmental conditions (22-25°C, 40-70% humidity, and 12:12 hour dark/light photoperiod). All animal experiments were carried out according to the protocols (13/2014, 5/2015, 38/2016, 4/2017 and 28/2018) approved by the Institutional Animal Ethics Committees (IAEC) of Centre for Cellular and Molecular Biology.

Sequence analysis and homology modelling

Sequences of selected characterized Atg18/WIPI proteins were obtained from the UniProt database (PaAtg18: Q5QA94; KmHsv2: J3QW34; DmAtg18a: Q9V5F0; ScAtg18: P43601; CeAtg18: O16466; DrWIP12: F1QLJ9; HsWIP12: Q9Y4P8). The sequences of PfAtg18 (PlasmoDB gene identifier: PF3D7_1012900) and other Plasmodium genes were obtained from PlasmoDB [48]. Sequence alignment was performed using T-Coffee (http://tcoffee.crg.cat/apps/tcoffee/do:mcoffee). The PfAtg18 sequence was analysed for conserved motifs and domains using the iLIR and WD40 repeat protein Structure Predictor (WDSP) programs [49, 50]. PDB was searched to identify the closest structural homologs of PfAtg18, which were used as templates (Kluveromyces marxianus Hsv2, PDB ID: 3VU4; Pichia angusta Atg18, PDB ID: 5LTD) for generating the modelled structure of PfAtg18 using the SWISS-MODEL server [51]. The model was viewed and edited using the PyMOL Molecular Graphics System (version 1.7.6.0 Schrödinger, LLC). Transmembrane helices in PfMDR1 (PlasmoDB gene identifier: PF3D7_0523000.1) were predicted using the TMHMM 2.0 server (http://www.cbs.dtu.dk/services/TMHMM-2.0/).

Parasite culture, isolation of nucleic acids and cDNA synthesis

P. falciparum 3D7 and D10 strains were grown in RPMI 1640-albumax medium with human erythrocytes at 2% haematocrit (RPMI 1640 supplemented with 2 g/l sodium bicarbonate, 2 g/l glucose, 25 µg/ml gentamicin, 300 mg/l glutamine, 100 µM hypoxanthine, 0.5% albumax II) [52]. Synchronization of
parasites was achieved by treatment with sorbitol when the majority of parasites were in the ring stage, and parasites were isolated from infected RBCs by lysis with saponin [10, 53]. Parasite pellets were used immediately or stored at -80°C till further use. Genomic DNA was isolated from late trophozoite/schizont stage parasites using the Puregene Blood Core kit according to the manufacturer’s protocol. For RNA isolation, the parasite pellet (~50 µl PCV) was resuspended in 300 µl Trizol solution, mixed with 200 µl chloroform, centrifuged to separate the upper layer, which was mixed with 300 µl isopropanol to precipitate total RNA. The RNA pellet was washed with 300 µl of 70% ethanol, resuspended in RNase-free water, and stored at -80°C until further use. cDNA was made from the total RNA using the SuperScript® III First-Strand Synthesis System as instructed by the manufacturer. Briefly, 5 µg of gDNA-free total RNA was mixed with random hexamer primers and dNTPs, heated at 65°C for 5 min, followed by immediate cooling in ice. 10 µl of this mixture was incubated with Reverse Transcriptase and RT buffer at 30°C for 10 min, followed by at 50°C for 1 hour. The reaction was terminated by incubating the mixture at 85°C for 5 min, and 1-2 µl of this sample was used in PCR.

For *P. berghei* parasites, 3-6 weeks old BALB/c mice were infected with a frozen stock of *P. berghei* ANKA and infection was monitored regularly by observing Giemsa stained blood smears of the tail snips of infected mice. The infected mice were euthanized at 10-15% parasitemia, blood was collected in Alsever’s solution (2.05% glucose, 0.8% sodium citrate, 0.055% citric acid and 0.42% sodium chloride) by cardiac puncture, parasites were purified by saponin lysis method, and the parasite pellets were processed for isolation of genomic DNA using the Puregene Blood Core kit according to the manufacturer’s protocol.

**Expression and purification of recombinant proteins**

The PfAtg18 C-terminal coding region (PfAtg18ct: 571-1143 bps) was amplified from *P. falciparum* cDNA using PfATG18-F2/Atg18expR primers (Table S2), and cloned into the pCR2.1 vector to obtain pCR2.1-PfAtg18ct. The insert was subcloned into pET32a at BamHI/HindIII sites to obtain pET32a-PfAtg18ct, which was transformed into BL21-CodonPlus(DE3)-RIL cells. pET32a-PfAtg18ct would express Thioredoxin/His-PfAtg18ct (Trx/His-PfAtg18ct) fusion protein, which was purified from IPTG induced cell under denaturing conditions. Briefly, the induced cell pellet was lysed in urea buffer (8M urea, 50 mM NaH₂PO₄, 500 mM NaCl, pH 8.0; 5 ml buffer/g pellet), sonicated using the SONICS Vibra Cell Ultrasonic Processor (9 secs pulses at 20% amplitude for 4 min), centrifuged, and the supernatant was separated. Imidazole (10 mM final), Triton-X 100 (0.5% final) and β-ME (5 mM final) were added to the supernatant, and incubated with Ni-NTA agarose resin (0.25 ml slurry/g weight of the initial pellet for 30 min at room temperature). The resin was washed with 50x column volume of wash buffer 1 (urea buffer with 30 mM imidazole, 0.5% Triton-X 100 and 5 mM β-ME) and wash buffer 2 (urea buffer with 50 mM imidazole). The bound proteins were eluted with elution buffer (250 mM imidazole in urea buffer) and
assessed for purity by SDS-PAGE. Elution fractions containing pure protein were pooled and refolded by dialyzing in a 10 kDa cut-off dialysis tubing against the refolding buffer (20 mM Tris pH 7.5, 1 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10% glycerol) at 4°C for 18 hrs with one change of buffer after 14 hours. The dialysed protein was concentrated in a 10 kDa cut off Amicon Ultra-15 (Millipore), quantitated using BCA, and used for immunizing rats.

A synthetic and codon optimised PfAtg18 gene was purchased from GenScript as a pUC57-PfAtg18syn plasmid, which contained PfAtg18 coding sequence flanked by the NotI/EcoRI sites. The PfAtg18syn insert was subcloned into the pGEX-6P-1 at EcoRI/NotI sites to generate pGEX/PfAtg18syn. PfAtg18 mutants of PI3P-binding motifs (PIPm, FRRG mutated to FAAG; ALCAm, WLCL mutated to ALCA) were generated by recombination PCR. PIPm was amplified from the pUC57-PfAtg18syn plasmid as two fragments using FAAG Syn F/M13R and M13F/FAAG Syn R primers, and the two fragments were recombined using M13F/M13R primers. Similarly, ALCAm was amplified from the pUC57-PfAtg18syn plasmid using the primer sets ALCA Syn F/M13R and M13F/ALCA Syn R, and the two fragments were recombined. The PIPm and ALCAm fragments were digested with EcoRI/NotI sites and cloned into similarly digested pGEX-6P-1 vector to obtain pGEX/PIPm and pGEX/ALCAm, respectively. The ScAtg18 coding region contains internal BamHI and BglII sites, which were sequentially eliminated without affecting the encoded-amino acids by recombination PCR. For elimination of BamHI site, ScAtg18 was amplified from the genomic DNA of wild type BY4741 strain as two fragments using SCATG18EXPF/SCATG18MBR and SCATG18MBF/SCATG18EXPR primer sets. The two fragments were recombined using the primers SCATG18EXPF/SCATG18EXPR and cloned into the pGT-GFPbsc vector at BamHI/XhoI sites to obtain pGT-GFPScAtg18-Bm. The pGT-GFPbsc plasmid has been described previously [54]. For elimination of BglII site, pGT-GFPScAtg18-Bm was used as a template using primer sets SCATG18EXPF/SCATG18MBGR and SCATG18MBGF/SCATG18EXPR. The two fragments were recombined using SCATG18EXPF/SCATG18EXPR primers and cloned into the pGT-GFPbsc plasmid at BamHI/XhoI sites to obtain pGT-ScAtg18-BmBgm. The ScAtg18BmBgm insert was subcloned into the pGEX-6P-1 vector at BamHI/XhoI sites to generate pGEX/ScAtg18. All inserts were sequenced at the Automatic DNA Sequencing Facility of CCMB to ensure that they were free of undesired mutations. pGEX/PfAtg18syn, pGEX/PIPm, pGEX/ALCAm and pGEX/ScAtg18 were transformed into BL21(DE3) E. coli cells, which would express the recombinant proteins as GST-fusions. pGEX-6P-1 was also transformed into BL21(DE3) E. coli cells to produce recombinant GST. IPTG induced cell pellets of all the five expression clones (pGEX/PfAtg18syn, pGEX/PIPm, pGEX/ALCAm, pGEX/ScAtg18 and pGEX6P1) were resuspended in lysis buffer (PBS with 1 mM DTT and 1 mg/ml lysozyme; at 5 ml/g weight of the pellet), incubated for 30 min at 4°C, and sonicated (5 secs pulses at 20% amplitude for 5-30 min depending on the sample volume). The lysate was centrifuged and the supernatant was incubated with
Protino® Glutathione Agarose 4B for 30 min at 4°C. The resin was washed three times with PBS and bound proteins were eluted (50 mM Tris, 20 mM GSH, pH 7.5). Elution fractions were assessed for purity by SDS-PAGE, fractions containing pure proteins were pooled and concentrated (Amicon Ultra centrifuge: 10 kDa cut off for GST, 50 kDa cut off for GST/PfAtg18syn, GST/PIpm, GST/ALCAm and GST/ScAtg18) with simultaneous buffer exchange to 20 mM Tris-Cl, 50 mM NaCl, pH 8.0 at 4°C. The proteins were quantitated using BCA and used for various assays.

PfMDR1 is a 1419 amino acid residue long multi-pass integral membrane transporter. It is predicted to contain 11 transmembrane helices, a large inside domain and a large cytoplasmic domain. The PfMDR1 coding regions corresponding to cytoplasmic (1054-1419 aa) and inside (341-788 aa) domains were amplified from *P. falciparum* genomic DNA using MDR1cyt-F/MDR1cyt-R and MDR1in-F/MDR1in-R primers, and cloned into the pGEX-6P-1 vector at BamHI-XhoI site to generate pGEX/MDR1cyt and pGEX/MDR1in plasmids, respectively. These plasmids were digested with KpnI-XhoI and ligated with a similarly digested 2× e-Myc-coding sequence to obtain pGEX/MDR1cyt-myc and pGEX/MDR1in-myc plasmids, which were transformed into BL21(DE3) *E. coli* cells for expression of recombinant cytoplasmic (GST/MDRCD) and inside (GST/MDRID) proteins, respectively. Recombinant GST/MDRCD and GST/MDRID domains were purified as described above for GST-fusion proteins.

**Generation of anti-PfAtg18 antibodies**

Two 4-6 weeks old male Wistar rats were immunized intraperitoneally with recombinant Trx/His-PfAtg18ct in complete (day 0) or incomplete (days 15, 30, 60, 90 and 120) Freund’s adjuvant. Sera were collected (on days 75, 105, 135 and 140), assessed for reactivity with the recombinant protein, and the day 140 serum was purified against the lysate of pET32a-transformed BL21-CodonPlus(DE3)-RIL cells to remove cross-reactive antibodies to *E. coli* proteins as has been described earlier [10]. The purified antibodies were stored in 50% glycerol with 0.01% sodium azide at -30°C.

**Western blotting**

For assessing reactivity and specificity of purified anti-Atg18 antibodies, the mock-transformed *E. coli* lysate, the total lysate of induced PfAtg18ct-expressing *E. coli*, purified Trx/His-PfAtg18ct, lysates of wild type and recombinant D10 parasites, and RBC lysate were resolved on 12% SDS-PAGE gel. The proteins were transferred onto the Immobilon-P membrane, which was incubated with blocking buffer (3% skim milk in TBST), followed by incubation with rat anti-Atg18 (at 1/5000 dilution in blocking buffer) or mouse anti-β actin (1/500 dilution in blocking buffer) antibodies. The membrane was washed with blocking buffer, incubated with secondary antibodies (goat anti-Rat IgG-HRP or goat anti-Mouse IgG-HRP at 1/20,000 dilution in blocking buffer), washed again with TBST, and the signal was developed using the
SuperSignal Chemiluminescent substrates. The β-actin band intensities were used as a loading control for samples in all western blots.

To determine expression of native PfAtg18 in different asexual stages of *P. falciparum* 3D7, parasite pellets of ring, early trophozoite, late trophozoite and schizont stages were lysed with SDS-PAGE sample buffer (1× sample buffer contains 20% v/v glycerol, 4% w/v SDS, 0.25% bromophenol blue, 0.1% v/v β-ME and 0.25 M Tris-Cl, pH 6.8), centrifuged at 23755g for 20 min, the supernatants were separated and equal amounts of supernatant samples were processed for western blotting using anti-Atg18 or mouse anti-β-actin antibodies, followed by appropriate secondary antibodies as described above.

**Construction of transfection plasmids**

The complete coding sequence of PfAtg18 was amplified from the p2.1-ATG18 plasmid using Atg18expF/PfAtg18-Rep primers, and cloned into the pGT-GFPbsc plasmid at BamHI/XhoI sites to generate pGT-GFP/PfAtg18 construct. The GFP/PfAtg18 insert was subcloned at BglIII/XhoI sites in pPICENv3 and pSTCII-GFP plasmids to obtain pCENv3-GFP/PfAtg18 and pSTCII-GFP/PfAtg18 plasmids, respectively. pPICENv3 and pSTCII-GFP plasmids have been described previously [54, 55]. The *P. vivax* Atg18 (PvAtg18, PlasmoDB gene identifier: PVX_094865) coding sequence was amplified from *P. vivax* genomic DNA using PvA18 F/PvA18 R primers, cloned into pGT-GFPbsc plasmid at BamHI/XhoI sites to generate pGT-GFP/PvAtg18 plasmid, from which the GFP/PvAtg18 insert was subcloned into pSTCII-GFP plasmid at BglIII/XhoI sites to obtain pSTCII-GFP/PvAtg18 transfection plasmid. The *P. knowlesi* Atg18 (PkAtg18, PlasmoDB gene identifier: PKNH_0812700) was amplified using PkA18-F/PkA18-R primers from the *P. knowlesi* genomic DNA, cloned into the pGT-GFPbsc vector at BamHI/XhoI sites to generate pGT-GFP/PkAtg18, which was further subcloned into pSTCII-GFP vector at BglIII/XhoI sites to obtain pSTCII-GFP/PkAtg18 transfection plasmid. The PvAtg18 and PkAtg18 genes contain a single intron, which would be spliced from the transcript by the parasite splicing machinery.

PIPm and ALCAm mutants of PfAtg18 were generated by recombination PCR method using primers containing the desired mutations (FRRG to FAAG in PIPm and WLCL to ALCA in ALCAm) [56]. PIPm was amplified as two fragments from the pSTCII-GFP/PfAtg18 plasmid using GFPseqF/PfA18-mPIP-R and PfA18-mPIP-F/PfAtg18-Rep primers. The two fragments were recombined using GFPseqF/PfAtg18-Rep primers. ALCAm was amplified as two fragments from the pSTCII-GFP/PfAtg18 plasmid using the primer sets GFPseqF/PfA18-mLIR-R and PfA18-mLIR-F/PfAtg18-Rep. The two fragments were recombined using GFPseqF/PfAtg18-Rep primers. PIPm and ALCAm fragments were digested with Ncol/XhoI and cloned into similarly digested pSTCII-GFP to obtain pSTCII-GFP/PIPm and pSTCII-GFP/ALCAm transfection plasmids. GFP/PIPm and GFP/ALCAm fragments were excised from the respective plasmids with BglIII/XhoI and ligated into the similarly digested pPICENv3 vector to obtain
The PfCRT (PlasmoDB gene identifier: PF3D7_0709000) coding region was amplified from *P. falciparum* cDNA using PfCRT-Fepi/PfCRT-Repi primers and cloned into the pGEM-T easy vector to generate pGEM-PfCRT plasmid. The mCherry coding region was amplified from the pmCherry-N1 (Clontech) plasmid using mCher-F/mCher-R primers and cloned into the pGT-GFPbsc plasmid at BglII/XhoI sites, replacing the GFP region, to obtain pGT-mCherry. The PfCRT insert was excised from the pGEM-PfCRT plasmid with BglII/KpnI and subcloned into the similarly digested pGT-mCherry plasmid to generate pGT-PfCRT/mCherry. The HB-DJ1KO plasmid was modified to express PfCRT/mCherry under 5′UTR and 3′UTR of *P. berghei* DNA damage inducible-1 protein (PbDdi1). The PbDdi1 5′ UTR and 3′ UTR were amplified from the *P. berghei* genomic DNA using PbDdi1-F1F/PbDdi1-F1R and PbDdi1-F2F/PbDdi1-F2R primers, respectively. The Ddi1 5′ UTR was digested with NotI/KpnI and the Ddi1 3′ UTR was digested with AvrII/KasI, and sequentially cloned into the similarly digested HB-DJ1KO plasmid to obtain HB-Ddi plasmid. The PfCRT/mCherry fragment was excised from the pGT-
PfCRT/mCherry plasmid with KpnI/XhoI and cloned into the similarly digested HB-Ddi plasmid to obtain HB-PfCRT/mCherry transfection plasmid.

For construction of a PfAtg18 knock-down plasmid, the PfAtg18 coding region (flank1) and 3’UTR (flank2) were amplified from *P. falciparum* genomic DNA. The internal KpnI site in flank1 was mutated by site-directed mutagenesis without changing the encoded amino acids, which involved amplifying it in two fragments (PFA18FL1-F/PFA18F1-RKUT and PFA18F1-FKUT/PFA18FL1-RKI primers), followed by recombination of the fragments using PFA18FL1-F/PFA18FL1-RKI primers. Flank 2 was amplified using PFA18FL2-F/PFA18FL2-R primers. The flank1 and flank2 were sequentially cloned into the HBA18/cDD_HAkd plasmid at NotI/KpnI and AvrII/KasI sites, respectively, to generate HBPF18/cDD_HA plasmid. A synthetic GlmSAc fragment (GenScript) was cloned into the HBPF18/cDD_HA plasmid at XhoI/AgeI site to generate HBPF18/cDD_HA/GlmSAc plasmid.

The coding regions and flanks in all the transfection plasmids were sequenced to ensure that they were free of undesired mutations, and the presence of different regions was confirmed by digestion with region-specific restriction enzymes. The transfection constructs were purified using the NucleoBond® Xtra Midi plasmid DNA purification kit.

**Transfection of *P. falciparum***

Early ring stage-infected RBCs from freshly thawed cultures of *P. falciparum* 3D7 and D10 strains were transfected with 50-100 µg of desired transfection plasmid DNAs (pCENv3-GFP/PfAtg18, pCENv3-GFP/PIpm, pCENv3-GFP/ALCaM, pCENv3-GFP/ScAtg18, HB-PfCRT/mCherry) as has been described earlier [58, 59]. The selection of transfected parasites was started with appropriate drugs (blasticidin at 1 µg/ml for pCENv3-based plasmids and WR99210 at 1 nM for HB-based plasmids) from day 2-12, followed by no drug till day 18, and then reaplication of the respective drug till the emergence of resistant parasites. Recombinant parasites were usually maintained in the presence of blasticidin, which was withdrawn during experiments. For co-transfection, the pCENv3-GFP/PfAtg18-transfected *P. falciparum* D10 parasites were cultured in the absence of blasticidin for 2 cycles. The ring stage-infected RBCs were transfected with HB-PfCRT/mCherry plasmid, and subjected to selection (0.5 µg/ml blasticidin and 1 nM WR99210) from day 2-12, and thereafter maintained in the presence of 1 nM WR99210 till the emergence of resistant parasites.

For obtaining PfAtg18 knock-down parasites, the *P. falciparum* D10 ring-stage parasites were transfected with HBPF18/cDD_HA/GlmSAc plasmid and selected with 1 nM WR99210 till the emergence of resistant parasites, and thereafter maintained in the presence of 10 µM trimethoprim.
Transfection of *P. berghei*

*P. berghei* ANKA was maintained in BALB/c mice as described in the parasite culture section. The infected mice were euthanized at 6-8% parasitemia and the blood was collected in Alsever’s solution by cardiac puncture. The trophozoite stage parasites were purified on a 65% Histodenz gradient at 360g with 0 deceleration using a swinging-bucket rotor, and cultured in RPMI 1640-FBS medium (supplemented with 2 g/litre sodium bicarbonate, 2 g/litre glucose, 25 µg/ml gentamicin, 300 mg/litre glutamine and 20% FBS) for 12-15 hrs at 35°C with shaking at 50 rpm. The culture was centrifuged at 1398g for 5 min and the parasite pellet, which mostly contained mature schizonts, was resuspended in 100 µl Nucleofector solution (Lonza) containing ~5 µg of desired circular (pSTCII-GFP/PfAtg18, pSTCII-GFP/PvAtg18, pSTCII-GFP/PkAtg18, pSTCII-GFP/Pm, pSTCII-GFP/ALCAm) or linear (HBA18KO, HBA18/GFPki and HBA18/cDDHakd) transfection plasmids. The content was electroporated using Amaza Nucleofector device as has been described previously [60]. 120 µl of RPMI 1640-FBS medium was added to the cuvette, and the entire sample was injected intravenously into a naive mouse. The mouse was given pyrimethamine in drinking water (70 µg/ml) for 7 days for selection of transfected parasites. For transfections with HBA18/cDDHakd, mice were given water with trimethoprim (400 µg/ml) for 2-3 hrs before the infection, and then given water containing trimethoprim+pyrimethamine unless otherwise mentioned. Pure clonal lines of HBA18/cDDHakd transfected parasites were obtained by the dilution cloning method, which involved intravenous infection of 10 mice, each with 0.5 parasite. The parasites were harvested for various analyses as mentioned elsewhere.

Localization of wild type and mutant Atg18 proteins in parasites

For localization of PfAtg18 and its mutants, GFP/PfAtg18-, GFP/Pm- and GFP/ALCAm-expressing *P. falciparum* and *P. berghei* parasites were processed for live cell imaging. 100 µl of parasite cultures (~5% parasitemia) were washed with PBS, stained with Hoechst (10 µg/µl in PBS; Invitrogen), layered on poly L-Lysine coated slides, unbound cells were washed off with PBS, coverslips were mounted over the slides, and the cells were observed under the 100× objective of a ZEISS Apotome microscope. Images were taken (Zeiss AxioCam HRm) and analysed with the Axiovision software. For Z-section images, GFP/PfAtg18-expressing *P. falciparum* parasites were washed with PBS, immobilized on a poly L-Lysine coated slide, and fixed (3% paraformaldehyde and 0.01% glutaraldehyde). The cells were permeabilized (0.5% Triton X-100 in PBS), blocked (3% BSA in PBS), and incubated with DAPI (10 µg/ml in PBS; Invitrogen). The slides were mounted with ProLong Gold anti-fade (Thermo Fisher Scientific), images were captured using the Leica TCS SP8 confocal laser scanning microscope and edited using the Leica Application Suite software.
The localization of GFP/PfAtg18, GFP/PIPm, GFP/ALCAm, GFP/PvAtg18 and GFP/PkAtg18 was assessed in *P. berghei* trophozoites and schizonts. For localization in trophozoites, 10-20 µl blood was collected in Alsever’s solution from the tail snips of infected-mice at 4-5% parasitemia, washed with PBS and processed for live cell fluorescence microscopy as described for GFP/PfAtg18-expressing *P. falciparum* parasites. For localization in schizonts, 50-100 µl blood was collected by retro-orbital bleeding and cultured in 10 ml RPMI 1640-FBS medium to obtain schizont stage as described in the transfection of *P. berghei* section. The culture was processed for live cell fluorescence microscopy as described for GFP/PfAtg18-expressing *P. falciparum* parasites.

PfAtg18 knock-down parasites would express PfAtg18/cDD<sub>HA</sub> under the endogenous PfAtg18 promoter. The uncloned population of PfAtg18 knock-down parasites was harvested at trophozoite stage, washed with PBS, immobilized on a poly-L-lysine slide, fixed (PBS with 3% para-formaldehyde and 0.01% glutaraldehyde), permeabilized (PBS with 0.01% Triton-X), and blocked (3% BSA in PBS). The slides were incubated with rabbit anti-HA antibodies (at 1/200 dilution in blocking buffer), followed by Alexa Fluor 488-conjugated donkey anti-Rabbit IgG (at 1/2000 dilution in blocking buffer with 10 µg/ml Hoechst). The slides were washed, air dried, mounted with Prolong<sup>TM</sup> Diamond antifade and observed under the 100× objective of AxioimagerZ.1 with Apotome.

Asynchronous cultures of GFP/PfAtg18-, GFP/PIPm-, GFP/ALCAm-, GFP/PvAtg18-, and GFP/PkAtg18-expressing *P. falciparum* and *P. berghei* were harvested at 10-15% parasitemia by saponin lysis. The pellets were processed for western blot using mouse anti-GFP (at 1/500 dilution in blocking buffer) or mouse anti-β-actin antibodies, followed by detection with appropriate secondary antibodies as described in the Western blotting section.

GFP/ScAtg18-expressing *P. falciparum* parasites were processed for live cell microscopy and western blotting as described for GFP/PfAtg18-expressing *P. falciparum* parasites.

**Colocalization of PfAtg18**

For colocalization with lysotracker, GFP/PfAtg18-expressing *P. falciparum* trophozoites were incubated with 200 nM lysotracker (Invitrogen) for 1 hour, washed with PBS, stained with Hoechst, and processed for live cell fluorescence microscopy. For colocalization with PfCRT, *P. falciparum* trophozoites transfected with pCENv3-GFP/PfAtg18 and HB-PfCRT/mCherry plasmids were processed for live cell fluorescence microscopy as described for GFP/PfAtg18-expressing *P. falciparum* parasites.

**PI3P binding assay**

20 µl slurry of PI(3)P Beads (Echelon Biosciences) was washed and equilibrated with 500 µl of cold binding buffer (50 mM Tris, pH 7.5, 150-200 mM NaCl, 0.25% NP-40). The beads were incubated
with 2-3 µg of recombinant GST/PfAtg18syn, GST/PIpM, GST/ALCAm, GST/ScAtg18 and GST proteins for 2 hrs at room temperature with gentle shaking. The beads were washed four times with the binding buffer and bound proteins were eluted by boiling the beads in 120 µl of 1× SDS-PAGE sample buffer for 10 min. Aliquots of the eluates, input, flow-through and washes were processed for western blotting using mouse anti-GST (at 1/1000 dilution; Invitrogen), followed by goat anti-Mouse IgG-HRP antibodies as described in the western blotting section.

Protein-protein overlay assay

For assessing interaction between PfAtg18 and PfMDR1, different amounts of purified recombinant GST, GST/PfAtg18syn and GST/ScAtg18 were immobilized on nitrocellulose membrane, the membrane was blocked (3% BSA in PBST), and then overlaid with purified recombinant GST/MDRCD or GST/MDR1ID (at 2.5 µg/ml in blocking buffer) for 12-13 hrs at 4°C. The membrane was washed with blocking buffer, incubated with mouse anti-myc antibodies, followed by HRP-conjugated goat anti-Mouse IgG as described in the western blotting section. Binding of GST/MDRCD with GST/PfAtg18syn, GST/PIpM and GST/ALCAm was compared by ELISA. Recombinant proteins (GST/PfAtg18syn, GST/PIpM, GST/ALCAm and GST) were coated to the wells of an ELISA plate (19.5 nM protein/well in 0.2 M bicarbonate buffer, pH 9.2) at 4°C for 8-10 hrs. The wells were washed with PBST (PBS with 0.1% Tween 20), and then filled with blocking buffer (3% BSA in PBST) for 2 hrs at room temperature. The blocking buffer was discarded, serial 2-fold dilutions of GST/MDRCD (in 200 µl of PBST) were added to the wells, the plate was incubated at 4°C for 12-13 hrs. The wells were washed 5 times with PBST, each time for 5 min. Mouse anti-myc antibodies (1/1000 in blocking buffer, 200 µl/well) were added to each well, and the plate was incubated for 1 hr at room temperature. The wells were washed 4 times with PBST, each time for 5 min, followed by once with blocking buffer. HRP-conjugated goat anti-mouse IgG (1/4000 dilution in blocking buffer, 200 µl/well) was added to the wells, incubated for 45 min, and washed 5 times with PBST, each time for 5 min. The reaction was developed using TMB/H2O2 for 15 min, stopped with 1N HCl, and absorbance was measured at 450 nm using the BioTek Power wave XS2 plate reader. The assay was done in triplicates for each protein concentration. The reactivity obtained with GST was subtracted from those of GST/PfAtg18syn, GST/PIpM or GST/ALCAm for the respective GST/MDRCD concentration to adjust for background reactivity. The mean of background-adjusted reactivity was plotted against GST/MDRCD concentrations using the GraphPad Prism.

CD spectroscopy

CD spectra for recombinant GST/PfAtg18syn and GST/ALCAm proteins (0.5 mg/ml in PBS) were recorded on a Jasco J-815 spectropolarimeter at room temperature. 0.02 cm and 1.0 cm path length quartz
cuvettes were used for far-UV and near-UV CD measurements, respectively. The instrument was set on wavelength spectrum scan mode and the spectra were recorded from 190 nm to 250 nm and 250 nm to 350 nm for far-UV and near-UV CD measurements, respectively. Five spectra were averaged to increase the signal-to-noise ratio, and readings were plotted using the Origin 2020b software.

**Inhibition experiments**

A variety of inhibitors and antimalarials were assessed for their effects on PfAtg18 localization. The concentrations of compounds were normalized to their IC50 concentrations, which were either determined in this study or taken from previous reports (Table S3). Highly synchronized GFP/PfAtg18-expressing *P. falciparum* parasites at late ring (16 hour post-synchronization) or early trophozoite stage were cultured in the presence of inhibitors (22 µM E64, 300 µM pepstatin A, 30.8 nM epoxomicin, 0.48 µM pristimerin, 95 µM LY294002, 52.4 µM orlistat; all at 4x IC50 concentration except E64 at 3x IC50 concentration) or antimalarials (236.4 nM artemisinin, 120 nM chloroquine, 81.6 nM amodiaquine, 124.8 nM mefloquine, 21.6 nM halofantrine, 532 nM quinine; all at 4x IC50 concentration) for 8 hours. For controls, parasites were grown with DMSO (0.06% v/v) under identical conditions. The parasites were washed with PBS and processed for localization of PfAtg18 using live cell imaging as described for GFP/PfAtg18-expressing *P. falciparum* parasites. The effect of treatment on PfAtg18 localization was scored by observing at least 200 cells, and experiments were repeated at least three times for each inhibitor. For scoring the effect of chloroquine and amodiaquine at different concentrations, treatment was carried out for 4 hrs. To assess if LY294002 exerts a dose-dependent effect on PfAtg18 localization, GFP/PfAtg18-expressing *P. falciparum* parasites at late ring stage (16 hour post-synchronization) were cultured in the presence of different concentrations of LY294002 (1x IC50 to 5x IC50, where 1x IC50 = 23.75 µM) or DMSO (0.45% v/v) for 8 hrs. The parasites were processed for live cell imaging as described earlier for GFP/PfAtg18-expressing *P. falciparum* parasites; at least 100 cells were observed for PfAtg18 localization, and the experiment was repeated three times. To assess the effect of inhibitors or antimalarials on PfAtg18 protein level, 20-25 ml cultures (at 10-12% parasitemia) of the control and treated parasites were harvested at the end of treatment, purified by saponin lysis and processed for western blotting with mouse anti-GFP and anti-β-actin antibodies, followed by appropriate secondary antibodies as described in the Western blotting section. For colocalization of PfAtg18 with PfAtg8, the E64-treated parasites were processed for immunofluorescence assay as described in the colocalization section. To assess the effect of endosomal transport inhibitors on PfAtg18 localization, highly synchronized GFP/PfAtg18-expressing *P. falciparum* parasites at late ring stage (16 hour post-synchronization) were cultured in the presence of bafilomycin (100 nM) or brefeldin A (20 µM) or NH4Cl (13.6 mM) for 8 hours, and parasites were processed for live cell imaging as described for localization of GFP/PfAtg18-expressing *P. falciparum* parasites. To assess food
vacuole integrity during inhibition experiments, PfCRT/mCherry-expressing *P. falciparum* parasites were treated at 16 hour post synchronization with chloroquine (120 nM), E64 (22 μM) or DMSO (0.08% v/v) for 8 hours. The parasites were processed for localization of PfCRT/mCherry using live cell imaging as described for GFP/PfAtg18-expressing *P. falciparum* parasites, and the effect of treatment was scored by observing at least 150-200 cells.

**Analysis of *P. berghei* Atg18 knock-in and knock-down parasites**

Mixed population of *P. berghei* Atg18/GFP knock-in (PbAtg18-KI) and a clonal line of *P. berghei* Atg18/cDDHA knock-down (PbAtg18-KD) were analysed for confirmation of integration of the transfection cassettes into the genome, localization and expression of Atg18/GFP or Atg18/cDDHA proteins. 6-8 weeks old BALB/c mice were infected with frozen stocks of wild type *P. berghei* ANKA, PbAtg18-KI or PbAtg18-KD parasites and infection was monitored regularly by observing Giemsa stained blood smears of the infected mice. Pyrimethamine (70 μg/ml in drinking water) was given to the mice infected with PbAtg18-KI parasites once parasites were observed in the blood smear, and continued throughout the infection. The mice infected with PbAtg18-KD parasites received trimethoprim (400 μg/ml drinking water) 2-3 hours before the infection, and were maintained under trimethoprim+pyrimethamine during the entire course of infection. The mice were euthanized at 10-15% parasitemia, parasites were purified, and the parasite pellets were processed for isolation of genomic DNA and western blotting as described in the parasite culture section. To confirm integration of transfection cassettes, genomic DNAs of wild type *P. berghei* ANKA, PbAtg18-KI and PbAtg18-KD parasites were used in PCR reactions containing primers specific for the wild type PbAtg18 gene (A18Con-5UF/A18Con-3UR), 5’-integration locus (PbAtg18-KD: A18Con-5UF/PvAc Con-R; Atg18-KI: A18Con-5UF/GFP seqR), 3’-integration locus (Hrp2-SeqF/A18Con-3UR), and the control MSP1 gene (PbMsp1-Fl1-F/PbMsp1-Fl1Rgpi). The PCR reactions were run on 0.8% agarose gel, and visualized with ethidium bromide.

PbAtg18-KI parasites were processed for localization of PbAtg18/GFP using live cell fluorescence microscopy as described for GFP/PfAtg18-expressing *P. berghei* parasites. The expression of PbAtg18/GFP in PbAtg18-KI parasites was determined using rabbit anti-GFP antibodies (1/1000 dilution in blocking buffer) as described in the Western blotting section. PbAtg18-KD parasites were assessed for expression of PbAtg18/cDDHA by western blotting using rabbit anti-HA (at 1/2000 dilution in blocking buffer), followed by HRP-conjugated goat anti-rabbit antibodies (at 1/10000 dilution in blocking buffer) as described in the Western blotting section. Mouse anti-β-actin antibodies were used to detect β-actin as a loading control. For localization of PbAtg18/cDDHA, 10-20 μl blood was collected from the tail-snip of PbAtg18-KD-infected mouse in Alsever’s solution, the cells were washed with PBS, immobilized on a poly-L-lysine slide, fixed (PBS with 3% para-formaldehyde and 0.01% glutaraldehyde), permeabilized
(PBS with 0.01% Triton-X), and blocked (3% BSA in PBS). The slides were incubated with rabbit anti-HA antibodies (at 1/200 dilution in blocking buffer), followed by Alexa Fluor 488-conjugated donkey anti-Rabbit IgG (at 1/2000 dilution in blocking buffer with 10 µg/ml DAPI). The slides were washed, air dried, mounted with Prolong™ Diamond antifade and observed under the 100× objective of AxioimagerZ.1 with Apotome.

For comparison of growth rates, 6 months old BALB/c mice were divided into three groups and infected intraperitoneally with wild type or PbAtg18-KD parasites (10⁶ parasites/mouse). One group was infected with wild type *P. berghei* ANKA, 2nd group was infected with PbAtg18-KD and not given trimethoprim (-TMP group) during the course of infection, the 3rd group was infected with PbAtg18-KD and maintained in trimethoprim (+TMP group) starting with 2-3 hours before the infection. Blood smears were regularly evaluated for parasitemia by counting at least 2000 cells, and parasitemia was plotted against days post-infection using GraphPad Prism. Growth rates of wild type *P. berghei* ANKA and PbAtg18-KD parasites were also compared in C57BL/6J mice exactly as described for BALB/c mice. The mice with >50% parasitemia were euthanized in all growth rate experiments.

To determine the effect of PbAtg18 knock-down on expression, 6 BALB/c mice were infected intraperitoneally with PbAtg18-KD parasites and given trimethoprim from 2-3 hours before the infection till the parasitemia reached to 10-15% parasitemia. Three mice were euthanized while they were still under trimethoprim and the blood was collected for isolation of parasites (+TMP parasites). Trimethoprim was withdrawn from the remaining 3 mice, and they were euthanized 24 hours later, blood was collected for isolation of parasites (-TMP parasites). The parasite pellets were processed for western blotting using rabbit anti-HA or mouse anti-β-actin antibodies, followed by appropriate secondary antibodies as described in the western blotting section.

**Immunoprecipitation of PbAtg18 and PfAtg18**

Parasites were isolated from asynchronous cultures of GFP-expressing and GFP/PfAtg18-expressing *P. falciparum* at 10-15% parasitemia as described in the parasite culture section. Wild type *P. berghei* ANKA and PbAtg18-KI parasites were maintained in 6-10 weeks old naïve BALB/c mice and parasites were isolated as described in the parasite culture section. The parasite pellet was resuspended in 5x pellet volume of the lysis buffer (10 mM Tris, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, pH 7.5, protease inhibitor cocktail), subjected to 5 cycles of freeze-thaw and 5 passages through a 26.5 G needle. The sample was incubated in ice for 30 min, centrifuged at 25000g for 30 min at 4°C, and the supernatant was transferred into a fresh tube. The pellet was re-extracted with 3x pellet volume of the lysis buffer as described above, and the supernatant was pooled with the first supernatant. The supernatant was incubated with GFP-Trap® MA (ChromoTek) antibodies; 15 µl slurry/2 mg protein for 2 hours at 4°C with gentle
shaking. The beads were washed with wash buffer 1 (10 mM Tris, 150 mM NaCl, 0.5 mM EDTA, pH 7.5, protease inhibitor cocktail), followed by wash buffer 2 (10 mM Tris, 300 mM NaCl, 0.5 mM EDTA, pH 7.5, protease inhibitor cocktail). The bound proteins were eluted by boiling the beads in 100 µl 2x SDS PAGE sample buffer for 15 min, and the eluate was processed for western blotting and mass spectrometry. Aliquots of the eluates were assessed for the presence of GFP/PfAtg18 or PbAtg18/GFP along with appropriate control samples (input, pellet post-extraction, flow through, washes, and beads after elution) by western blotting using rabbit anti-GFP antibodies as described above in the western blotting section.

Mass spectrometry of immunoprecipitates

The immunoprecipitate eluates were run on a 12% SDS-PAGE gel till the pre-stained protein ladder completely entered into the resolving gel. The gel was stained with coomassie blue, destained, and the gel region containing the protein band was excised. The gel piece was sequentially washed with 800 µl of 50 mM ammonium bicarbonate-acetonitrile solution (7:3 v/v), 300-800 µl of 50 mM ammonium bicarbonate, and 800 µl of acetonitrile. The gel piece was vacuum dried, resuspended in 200 µl of 10 mM DTT for 45 min at 37°C, and incubated in 200 µl of 50 mM ammonium bicarbonate-55 mM iodoacetamide solution for 30 min at room temperature. The gel piece was washed twice with 700 µl of 50 mM ammonium bicarbonate, followed by 700 µl of acetonitrile for 10 min. The gel piece was vacuum dried and treated with sequencing grade trypsin (15 ng/µl in 25 mM ammonium bicarbonate, 1 mM CaCl₂; Promega or Roche) at 37°C for 10-16 hours. The peptides were extracted with 5% formic acid-30% acetonitrile solution, the extract was vacuum dried, dissolved in 20 µl of 0.1% TFA, desalted using C18 Ziptips (Merck), and eluted with 40 µl of 50% acetonitrile-5% formic acid solution. The eluate was vacuum dried and resuspended in 11 µl of 2% formic acid. 10 µl of this sample was run on the Q Exactive HF (Thermo Fischer Scientific) to perform HCD mode fragmentation and LC-MS/MS analysis.

Raw data files were imported into the proteome discoverer v1.4 (Thermo Fischer Scientific), analysed and searched against the Uniprot databases of *P. berghei* ANKA (ID: UP000074855), *P. falciparum* (ID: UP00001450), *M. musculus* (ID: UP00000589) and *H. sapiens* (ID: UP000005640) using the HTSequest algorithm. The analysis parameters used were enzyme specificity for trypsin, maximum two missed cleavages, carbamidomethylation of cysteine, oxidation of methionine, deamidation of asparagine/glutamine as variable modifications. The precursor tolerance was set to 5 ppm and fragmentation tolerance was set to 0.05 Da. A 1% peptide FDR threshold was applied. The peptide spectral matches (PSM) and peptide identification groups of proteins were validated using the Percolator algorithm in proteome discoverer, and filtered to 1% FDR. The PSM and peptide groups passing through the FDR were exported to an excel file for analysis. The protein hits from the PbAtg18-KI sample were compared with those from the wild type *P. berghei* ANKA, and the protein hits identified in the GFP/PfAtg18 sample.
were compared with those in the GFP sample. Common proteins were excluded, and unique proteins common to 3 biological replicates with a minimum of 1 unique peptide were considered.

Results

*Plasmodium* Atg18 proteins localize to the food vacuole

PfAtg18 has been shown to be expressed in erythrocytic stages and localized to vesicular structures throughout the parasite, some of which also colocalized with the food vacuole and apicoplast [19, 26, 45]. To determine the expression of PfAtg18 in asexual erythrocytic stages, we produced the C-terminal region of PfAtg18 as a Trx/His-PfAtg18ct fusion protein and generated anti-PfAtg18 antibodies (Figure S1A). The antibodies detected a single band of the predicted size of Trx/His-PfAtg18ct (39.4 kDa) in the western blot of induced bacterial lysate and also reacted with purified Trx/His-PfAtg18ct, but did not react with the mock-bacterial lysate, indicating specificity for recombinant Trx/His-PfAtg18ct (Figure S1B). The anti-PfAtg18 antibodies recognized a single band of the size of native PfAtg18 (43.5 kDa) in the western blot of the lysates of different asexual erythrocytic stage parasites, indicating PfAtg18 expression in these stages (Figure S1C). However, the anti-PfAtg18 antibodies did not react in IFA, possibly due to failure to recognize the native PfAtg18, as the antibodies were generated against the in vitro-refolded C-terminal region of PfAtg18 that might have different conformation from that of native PfAtg18. Hence, for PfAtg18 localization studies in *P. falciparum*, it was expressed as a C-terminal fusion of GFP (GFP/PfAtg18) using an episomally maintained plasmid. The western blot of GFP/PfAtg18-expressing *P. falciparum* parasites with anti-Atg18 antibodies detected a band of the size of GFP/PfAtg18 (74.3 kDa) in addition to the native PfaTg18 (43.5 kDa), which was also detected in the lysate of wild type parasites (Figure 1A). Anti-GFP antibodies detected a single band of the size of GFP/PfAtg18 in the lysate of GFP/PfAtg18-expressing *P. falciparum* parasites, and it did not react with the lysate of wild type parasites (Figure 1B), confirming the expression of GFP/PfAtg18 in recombinant parasites. Both the antibodies did not react with uninfected RBC lysate, whereas anti-β-actin antibodies detected β-actin in all the lysates. Live cell microscopy of different stages of GFP/PfAtg18-expressing *P. falciparum* parasites showed fluorescence as a single dot in rings, whereas trophozoites and schizonts contained fluorescence around and within the food vacuole, indicating that PfAtg18 is localized to the food vacuole (Figure 1C). PfAtg18 colocalized with the lysosome marker dye lysotracker and the *P. falciparum* food vacuole membrane protein PfCRT (Figure S2), which further substantiated PfAtg18 localization to the food vacuole. A deeper observation of PfAtg18 localization in trophozoites, which have more pronounced food vacuole than other erythrocytic stages, revealed that it was localized both around the food vacuole (45.4% ± 9.8 parasites) and within the food vacuole (54.6% ± 9.8 parasites) (Figure S3).
PfAtg18 shares about 91% and 97% sequence identities with the Atg18 proteins of mouse and monkey/human malaria parasites, respectively [10]. However, food vacuole has two major morphologies in *Plasmodium* species: *P. falciparum* exhibits a single large food vacuole in all asexual erythrocytic stages, whereas other *Plasmodium* species, including *P. vivax*, *P. knowlesi* and *P. berghei*, exhibit multiple vesicles most of which fuse into a single large vesicle in the schizont stage [61]. Hence, we investigated the localization of selected *Plasmodium* Atg18 proteins in *P. falciparum* and *P. berghei*, which represent the two food vacuole morphologies.

For localization in *P. berghei*, GFP/PfAtg18 was expressed using an episomally maintained plasmid, and its expression was confirmed by western blot using anti-GFP antibodies (Figure S4A). These parasites showed GFP/PfAtg18 signal at multiple loci, which also contained the in-built food vacuole marker haemozoin, indicating that PfAtg18 localizes to food vacuoles, even in a heterologous parasite (Figure 2). Most of the food vacuoles present in *P. berghei* trophozoite stage merge into a single large food vacuole as the trophozoite matures into the schizont. Consistently, the majority of GFP/PfAtg18 foci merged into a large single food vacuole in schizonts. To rule out any effect of heterologous expression, we generated a *P. berghei* knock-in line (PbAtg18-KI) that expressed PbAtg18/GFP under its native promoter. PCR and western blot analysis of PbAtg18-KI parasites confirmed the replacement of wild type PbAtg18 gene with PbAtg18/GFP coding sequence and expression of PbAtg18/GFP, respectively (Figure S5). PbAtg18/GFP was predominantly associated with multiple haemozoin-containing food vacuoles in trophozoites, which coalesced into a single large food vacuole in schizonts (Figure 2), thereby ruling out the effect of heterologous expression. Consistently, the localization of PfAtg18 expressed under its endogenous promoter in *P. falciparum* was restricted to the food vacuole (Figure S6). We extended localization studies to Atg18 homologs of *P. vivax*, the most prevalent human malaria parasite, and *P. knowlesi*, a zoonotic malaria parasite. The GFP-fusions of *P. vivax* Atg18 (GFP/PvAtg18) and *P. knowlesi* Atg18 (GFP/PkAtg18) were expressed in *P. berghei* using episomally maintained plasmids. Both GFP/PvAtg18 and GFP/PkAtg18 were expressed (Figure S4B) and predominantly localized to food vacuoles (Figure 3), indicating that *Plasmodium* Atg18 proteins likely share the food vacuole localization. Earlier studies have shown that PfAtg18 is localized to vesicular structures in the cytoplasm and punctate structures around or close to the food vacuole [19, 45]. However, Atg18 localization was restricted to the food vacuole in our studies whether it was expressed in a homologous or a heterologous system (Figure 1C and Figure 2). Furthermore, both episomally and endogenously expressed Atg18 showed similar localization (Figure 2). One reason for this discrepancy could be the differences in the techniques used for localization: live-cell microscopy in our case and IFA of fixed cells in the published studies. The punctate Atg18 localization close or around the food vacuole could be due to the loss of cellular morphology during fixation, whereas better maintenance of cellular morphology in live-cell microscopy would have revealed complete food vacuole localization in our study.
To investigate if PfAtg18 follows endosomal transport to the food vacuole, we evaluated its localization in GFP/PfAtg18-expressing *P. falciparum* parasites upon treatment with the inhibitors of vesicle trafficking (brefeldin A and bafilomycin) and fusion (bafilomycin and NH$_4$Cl). None of the inhibitors significantly altered PfAtg18 localization (Figure S7), suggesting that it does not take endosomal trafficking route, which is in line with the absence of a signal/transmembrane sequence in PfAtg18. About 30% of the NH$_4$Cl-treated parasites showed PfAtg18 around an enlarged food vacuole (Figure S7). NH$_4$Cl has been shown to inhibit lysosomal degradation activity by alkalinizing the compartment [62], hence, the food vacuole enlargement could be due to accumulation of undegraded haemoglobin, as has been observed in parasites treated with E64 and pepstatin A [63-67]. Only a small fraction of NH$_4$Cl-treated parasites, particularly late ring and early trophozoite stages, contained multiple PfAtg18 foci near the parasite periphery or in the cytoplasm (Figure S7), which may be due to failure of vesicles to fuse to form the food vacuole, as has also been reported earlier [68]. The PfAtg18 vesicles in NH$_4$Cl-treated parasites may be part of the haemoglobin trafficking pathway, as a recent report proposed that PfAtg18 transport to the food vacuole uses haemoglobin trafficking pathway [19].

**FRRG and WLCL motifs are essential for PI3P-dependent food vacuole localization of PfAtg18**

PfAtg18 showed maximum sequence identity (34.7%) with the *Pichia angusta* Atg18 (PaAtg18) at amino acid level. Sequence analysis and homology modelling of PfAtg18 on the PaAtg18 structure (PDB id: 5LTD) predicted seven WD40 repeats in PfAtg18, which form a seven bladed β-propeller structure with two PI3P-binding sites, indicating that PfAtg18 is a PROPPIN family protein [34, 39], as are ScAtg18 and WIPI2 (Figure S8). The PI3P-binding sites are formed by the FRRG motif and amino acids from the 23$^{rd}$ β strand in blade 6 (Figure S8). PfAtg18 and other *Plasmodium* Atg18 proteins also share a positionally conserved “WLCL” motif that resembles the LC3-interacting region/Atg8-interacting motif (LIR/AIM) “WxxL” in a variety of proteins, which interact with Atg8. The LIR/AIM motif is mostly preceded by negatively charged amino acids [49], whereas the “WLCL” motif is not. Nonetheless, the “WLCL” motif is conserved at “WL” positions in the majority of Atg18 proteins (Figure S8) and forms the major part of 23$^{rd}$ β strand in blade 6 that contains the 2$^{nd}$ PI3P-binding site in *S. cerevisiae* Hsv2 [34, 39], suggesting an important role for this motif in Atg18 function.

ScAtg18 localizes to the PAS/phagophore, vacuole and endosomes. The PAS/phagophore localization requires association of ScAtg18 with Atg2 to form the Atg18-Atg2 complex, which binds PI3P through Atg18 [34, 39, 42, 69]. Similarly, WIPI2 localizes to PI3P-rich sites on the ER, phagophore and autophagosome through interaction with PI3P [34, 39, 43, 44]. An earlier study reported localization of PfAtg18 with vesicular structures in the cytoplasm and close to the food vacuole and colocalization with the apicoplast, which was mediated by interaction of PfAtg18 with PI3P [45]. Recently, PfAtg18 was
shown to localize to punctate structures close or around the food vacuole; based on co-localization of PfAtg18 with anti-PI3P antibodies and inhibition studies using the PI3K inhibitor wortmannin, this localization was suggested to be mediated by PI3P [19]. However, Atg18 localization was restricted to the food vacuole in our studies whether it was expressed in a homologous or a heterologous system (Figure 1C and Figure 2). Furthermore, both episomally and endogenously expressed Atg18 showed similar localization (Figure 2). Since GFP-2xFYVE, a routinely used marker for PI3P localization studies [70], indicated abundant PI3P in the food vacuole membrane of P. falciparum [46], we investigated if the food vacuole localization of PfAtg18 is mediated through its interaction with PI3P on the food vacuole membrane. We first treated GFP/PfAtg18-expressing P. falciparum parasites with the PI3K inhibitor LY294002 and scored the parasites for PfAtg18 localization. The treatment resulted in a dose-dependent diffuse localization of PfAtg18 throughout the parasite in a significantly larger number of parasites (18.4% ± 2.8 at 1×IC50 concentration to 59.8% ± 0.9 at 5×IC50 concentration) than in control parasites (1.6% ± 1.1) (Figure 4A), suggesting that PI3P is critical for PfAtg18 localization to the food vacuole. We next mutatet the FRRG motif to FAAG, and the mutant (PIPm) was expressed as a GFP-fusion protein (GFP/PIPm) in P. falciparum and P. berghei (Figure S4A and S4C). The GFP/PIPm was present outside the food vacuole in the cytoplasm of all P. falciparum (Figure 4A) and P. berghei parasites (Figure S9), indicating that FRRG motif is essential for localization of PfAtg18 to the food vacuole. We also mutated the “WLCL” motif to “ALCA” and expressed the mutant (ALCAm) as a GFP-fusion protein (GFP/ALCAm) in both P. falciparum and P. berghei (Figure S4A and S4C). The GFP/ALCAm was localised outside the food vacuole in the cytoplasm of P. falciparum (Figure 4A) and P. berghei (Figure S9), indicating that “WLCL” motif is essential for localization of PfAtg18 to the food vacuole. The localization of both GFP/PIPm and GFP/ALCAm was similar to that of a cytoplasmic GFP construct (GFPcyt) (Figure 4A), confirming that FRRG and WLCL motifs are essential for the food vacuole localization of PfAtg18.

To further investigate whether the loss of food vacuole localization of PfAtg18 mutants was due to their inability to interact with PI3P, recombinant GST/PfAtg18, GST/PIPm, GST/ALCAm, GST/ScAtg18 and GST were produced in BL21(DE3) cells (Figure S10), and assessed for binding with PI3P. GST/PfAtg18 bound with PI3P just like recombinant GST/ScAtg18 (Figure 4B), whereas GST/PIPm, GST/ALCAm and GST did not bind, confirming that FRRG and WLCL motifs are critical for PfAtg18 interaction with PI3P, and this interaction is essential for food vacuole localization. Since “WLCL” motif does not directly contribute to the 2nd PI3P-binding site, rather, it forms the major part of 23rd β strand in blade 6 that contains the 2nd PI3P-binding site in S. cerevisiae Hsv2, we compared CD spectra of GST/PfAtg18 and GST/ALCAm proteins. The far-UV CD spectrum of GST/ALCAm was different from that of GST/PfAtg18, whereas the near-UV CD spectra were similar, suggesting a local structural perturbation in GST/ALCAm resulting in the loss of binding to PI3P (Figure S11).
Interaction with PI3P is not the sole requirement for food vacuole localization of PfAtg18

The “FRRG” motif in ScAtg18 and WIPI2 is essential for interaction with PI3P and targetting to the PI3P-rich sites, and the loss of autophagic function of “FTTG” mutant of ScAtg18 can be fully restored by fusing it with the 2xFYVE domain, which specifically binds PI3P [69]. Hence, we investigated if the “FRRG” motif of ScAtg18 is sufficient to recruit it to the food vacuole of *P. falciparum*. GFP/ScAtg18 was expressed in *P. falciparum* (Figure S4D), and the parasites were assessed for localization of GFP/ScAtg18. To our surprise, GFP/ScAtg18 did not localize to the food vacuole, rather, it remained in the cytoplasm just like GFPcyt (Figure 5), indicating that interaction with PI3P alone is not sufficient and additional factors could be involved in recruitment of PfAtg18 to the food vacuole.

**MDR1 mediates PfAtg18 localization to the food vacuole**

To identify *Plasmodium* Atg18-associated proteins, we immunoprecipitated PbAtg18/GFP and GFP/PfAtg18 (Figure S12), and the samples were subjected to mass spectrophotometry. 10 unique proteins were identified in the GFP/PfAtg18 immunoprecipitate (Table 1) and 37 unique proteins were identified in the PbAtg18/GFP immunoprecipitate (Table S1). MDR1 and DnaJ proteins were common in both the samples, suggesting association of *Plasmodium* Atg18 with MDR1 and DnaJ. The GFP/PfAtg18 immunoprecipitate also contained HSP40, a *Plasmodium* exported protein, PHISTb, three ribosomal proteins, and a proteasome regulatory subunit. MDR1 was selected for further investigation as: 1) excluding the bait protein Atg18, it had the 3rd highest score in GFP/PfAtg18 immunoprecipitate (Table 1) and the highest score in the GFP/PbAtg18 immunoprecipitate (Table S1), 2) it is localized on the food vacuole membrane and associated with resistance to multiple antimalarials [13-16, 71]. MDR1 is a multi-pass transmembrane protein belonging to the ABC family of transporters; it is predicted to have 11 transmembrane helices, an inside domain and a cytoplasmic domain (Figure 6A). Hence, to investigate whether PfAtg18 directly interacts with MDR1, we produced recombinant GST-tagged inside (GST/MDRID) and cytoplasmic (GST/MDRCD) domains (Figure 6B and C), and tested both the domains for interaction with GST/PfAtg18, GST/ScAtg18 and GST using dot blot protein overlay assay. GST/MDRCD, but not GST/MDRID, interacted with GST/PfAtg18 (Figure 6D). Both the recombinant MDR domains did not interact with GST and GST/ScAtg18, indicating that PfAtg18-MDR1 interaction is direct and specific. We also compared interaction of wild type PfAtg18 and its mutants with GST/MDRCD by ELISA. The PIPm and ALCAm mutants showed significantly weaker interaction with GST/MDRCD than wild type Atg18 (Figure S13), which further substantiates PfAtg8-MDR1 interaction. Hence, MDR1, in addition to PI3P, plays a critical role in localization of PfAtg18 to the food vacuole.
**Food vacuole-localization of PfAtg18 is sensitive to chloroquine and amodiaquine**

The food vacuole of malaria parasites is the site of some of the essential and best studied biochemical processes, including haemoglobin degradation and haemozoin formation. Mutations in PfCRT and MDR1, which are also present in the food vacuole membrane, confer resistance to multiple antimalarials, including the quinolines [12-19]. Altered distribution of Atg8 puncta and Thr38Ile mutation of PfAtg18 have been associated with resistance to chloroquine and artemisinin, respectively [12, 25-27]. However, a direct link between autophagy and drug resistance is yet to be identified. Hence, we treated GFP/PfAtg18-expressing *P. falciparum* parasites with quinolines (chloroquine, amodiaquine, mefloquine, halofantrine and quinine) and evaluated for localization of PfAtg18. Chloroquine and amodiaquine caused diffuse localization of PfAtg18 throughout the parasite in a significant number of parasites, which became more pronounced with increasing drug concentrations, whereas mefloquine, halofantrine and quinine did not affect the localization (Figures 7 and 8), indicating that the effect of chloroquine and amodiaquine on PfAtg18 localization is specific. The immunoblot of quinoline-treated parasites did not show any noticeable change in PfAtg18 levels compared to that in control parasites (Figure S14A). The treatment of GFP/PfAtg18-expressing *P. falciparum* parasites with artemisinin neither affected PfAtg18 localization (Figure 7) nor the levels (Figure S14A). A recent study reported that PfAtg18 participates in food vacuole dynamics. Hence, altered food vacuole localization of PfAtg18 could affect multiple food vacuole-associated processes like haemoglobin degradation, which could subsequently affect the action of these drugs, as this degradation produces heme for quinoline action and iron for artemisinin activation.

**Food vacuole metabolism inhibitors affect PfAtg18 localization**

Haemoglobin degradation by asexual erythrocytic stage parasites provides amino acids for parasite protein synthesis and has also been proposed to keep the infected erythrocyte osmotically stable [72, 73]. It is majorly carried out by the cysteine protease falcipains and aspartic protease plasmepsins. Generic cysteine protease inhibitor E64 and aspartic protease inhibitor pepstatin A block haemoglobin degradation, resulting in the enlargement of food vacuole due to accumulation of undegraded haemoglobin [65-67], particularly in case of E64 [63-65, 74]. Although not yet reported in *Plasmodium*, lysosome is the primary site of lipid catabolism that involves transport of lipid droplets to lysosomes by lipophagy and subsequent degradation of lipid droplets by lysosomal lipases. Lipid droplets have been shown to be associated with the *Plasmodium* food vacuole [75], but specific food vacuole lipases are yet to be characterized according to our knowledge.

Hence, we treated GFP/PfAtg18-expressing *P. falciparum* parasites with several inhibitors of the food vacuole-associated metabolic processes and evaluated for PfAtg18 localization. E64 and pepstatin A caused a profound ring-like localization of GFP/PfAtg18 around the food vacuole, suggesting that it is
associated with the food vacuole membrane (Figure 9). The ring-like localization pattern was seen in a much larger number of parasites in case of E64 and pepstatin A than control parasites. The dot-like pattern might be of the PfAtg18 in the food vacuole lumen or due to processing of PfAtg18 on the luminal side by the food vacuole-resident proteases. A significant increase in the number of parasites with ring-like pattern upon treatment with E64 and pepstatin A supports this point. We also observed intense signal at a single site in the peri-food vacuole region, which was particularly evident in parasites treated with E64 and pepstatin A. Orlistat, an inhibitor of pancreatic lipases and fatty acid synthase [76, 77], has been shown to block the development of malaria parasites by inhibition of triacylglycerol hydrolysis [78]. A significant number of orlistat-treated parasites showed diffuse PfAtg18 localization all over the parasite, which could be due to decreased levels of fatty acids for membrane synthesis and phosphatidylinositol precursors (Figure 9). Localization of GFP/PfAtg18 in parasites treated with pristimerin (inhibitor of monoacylglycerol lipase and proteasome) and epoxomicin (proteasome inhibitor) was nearly identical to that in control parasites (Figure 9). The immunoblot of inhibitor treated-parasites and control parasites had similar PfAtg18 levels (Figure S14B), which ruled out any effect of treatment on PfAtg18 expression level. To address whether food vacuole integrity was intact during inhibitor treatment experiments, we treated the parasites expressing PfCRTmCherry, a food vacuole marker, with E64 and chloroquine, which inhibit haemoglobin degradation and haemozoin formation in the food vacuole, respectively. In both the cases, the number of parasites showing food vacuole-associated PfCRTmCherry was comparable to the DMSO control (Figure S15), indicating that food vacuole integrity was not compromised in our experiments. As observed in the case of PfAtg18, the E64-treated parasites also showed an enlarged ring of PfCRTmCherry due to enlargement of the food vacuole.

**Atg18 is crucial for parasite development**

To determine the role of Atg18 during parasite development, we attempted to knock-out and knock-down PbAtg18. Multiple attempts to generate knock-out parasites were not successful, whereas knock-down parasites (PbAtg18-KD) were readily obtained by replacing the wildtype PbAtg18 coding sequence with PbAtg18/cDDHA coding sequence (Figure 10A and B). cDD is a mutant of *E. coli* DHFR, and cDD-fusion proteins undergo degradation in the absence of trimethoprim (TMP) but not in the presence of TMP, thereby producing a knock-down phenotype at the protein level. The PbAtg18/cDDHA fusion protein was localized to haemozoin-containing vesicles in PbAtg18-KD parasites (Figure 10C), which also showed slightly lower Atg18 level in the absence of TMP than the parasites grown in the presence of TMP (Figure 10D), indicating a partial knock-down effect. The effect of knock-down on development of PbAtg18-KD parasites was assessed in BALB/c and C57/BL/6J mice. Without TMP, PbAtg18-KD parasites showed drastically reduced growth and were eventually cleared, whereas wild type and PbAtg18-KD (with TMP)
parasites grew similarly and the infected mice had to be euthanized or succumbed to high parasitemia (Figure 10E and F), indicating indispensability of PbAtg18 for parasite development.

Discussion

Plasmodium Atg8 is present as punctate structures throughout the parasite, which also co-localize with the food vacuole and apicoplast [19, 26, 45]. Altered distribution of Atg8 puncta and mutation in PfAtg18 have been observed in chloroquine and artemisinin resistant P. falciparum strains, respectively. We investigated the Plasmodium Atg18 to understand the mechanism of its localization and association with drug resistance. Our study reveals that Plasmodium Atg18 proteins localize to the food vacuole, which is mediated by interaction with PI3P and MDR1 on the food vacuole membrane. Chloroquine and amodiaquine altered PfAtg18 localization in a significant number of cells.

PfAtg18 and its orthologs in P. vivax, P. knowlesi and P. berghei localized to the food vacuole, indicating that food vacuole localization is likely a common feature of Plasmodium Atg18 proteins. Interaction of PfAtg18 with the food vacuole-associated PI3P was critical for food vacuole localization, as PfAtg18 mutants of PI3P-binding sites (PIPm and ALCAm) had complete cytosolic localization. Based on the PfAtg18 homology model, “WLCL” motif forms part of the 23rd β strand in blade 6, which contains the 2nd PI3P-binding site and the residues contributing to the PI3P-binding site are highly conserved in PROPPINs [34, 39], suggesting that “WLCL” motif is a critical structural requirement for the formation of 2nd PI3P-binding site in PfAtg18. The failure of ALCAm to bind PI3P and localize to the food vacuole could be due to disruption of the 2nd PI3P-binding site. Hence, the “WLCL” motif of PfAtg18 and the corresponding sequence in other PROPPINs represent a new sequence requirement for Atg18 functions. The PI3P-mediated localization of PfAtg18 in our study is consistent with previous reports of FRRG-mediated interaction of PROPPINs with PI3P, and the essentiality of this interaction for recruitment to the membrane structures [19, 34-39, 45, 69].

The Atg18 localization was restricted to food vacuole in our study regardless of the host used for expression, whereas previous studies have reported Atg18 localization as punctate structures all over the parasite, including colocalization with the apicoplast and some signal close or around the food vacuole [19, 26, 45]. This discrepancy in PfAtg18 localization between our and the previous studies may be due to differences in the procedures used for localization: live-cell imaging in our study and IFA of fixed parasites in the previous reports. The punctate Atg18 localization close or around the food vacuole could be due to the loss of cellular morphology during fixation and the subsequent immunofluorescence assay procedures, whereas better maintenance of cellular morphology and short duration of live-cell microscopy would have preserved the complete food vacuole localization in our study. In fact, PfAtg18 localization in our study is nearly identical to that in the live cell microscopy images of the previous study [19]. Nonetheless, multiple
lines of data support food vacuole localization of PfAtg18 in our study. 1) *P. falciparum* has been shown to have abundant PI3P in erythrocytic stages and localization studies using the PI3P probe GFP-2xFYVE indicated that PI3P is abundantly associated with the food vacuole membrane [46], which is consistent with PI3P-dependent localization of PfAtg18 to the food vacuole. 2) The localization was rendered all over the parasite upon treatment of parasites with the PI3K inhibitor LY294002, which might be due to decreased production of PI3P, hence, less PI3P on the food vacuole membrane. 3) PI3P and ALCAM neither bound PI3P nor localized to the food vacuole. 4) In *P. berghei* that contains multiple food vacuoles, the episomally expressed GFP/PfAtg18 and endogenously expressed PbAtg18/GFP showed identical localization with the food vacuoles, which ruled out any artefact of episomal expression and GFP fusion. 5) GFP fusions of ScAtg18 and WIPI have been extensively used in autophagy-related studies without any report of adverse effect on localization and functions of these proteins [34, 42, 69]. A previous study of the purified *P. falciparum* food vacuole proteome also contained PfAtg18 together with other food vacuole proteases and transporters, which further corroborates our result [79].

ScAtg18 has been shown to localize to endosomes, vacuole and autophagic membranes, and peri-vacuole membrane compartments in a PI3P-dependent manner [38, 69, 80, 81]. However, unlike *Plasmodium* Atg18 proteins investigated in this study, ScAtg18 showed complete cytoplasmic localization in *P. falciparum*, suggesting that interaction with PI3P is not the sole mediator for recruitment to the food vacuole. To identify the possible additional mediator of PfAtg18 localization, we carried out mass spectrometry of GFP/PfAtg18 and PbAtg18/GFP immunoprecipitates. MDR1, a food vacuole membrane protein [71], was reproducibly identified in both the immunoprecipitates and the recombinant cytoplasmic domain of PfMDR1 interacted with the recombinant PfAtg18. Recombinant ScAtg18 did not interact with the recombinant cytoplasmic domain of PfMDR1, though both PfAtg18 and ScAtg18 interacted with PI3P, which could be a reason why ScAtg18 failed to localize to the food vacuole. ScAtg18 interacts with Atg2, and the Atg18-Atg2 complex is recruited to PAS through interaction of Atg18 with PI3P [42]. The apparent absence of Atg2 homolog in *P. falciparum* and differences in binding affinities of PfAtg18 and ScAtg18 for PI3P on the food vacuole membrane may also contribute to the failure of ScAtg18 to localize to the food vacuole. Hence, interaction with both PI3P and MDR1 is critical for PfAtg18 localization to the food vacuole.

The altered PfAtg18 localization upon chloroquine and amodiaquine treatment is of particular significance because quinolines, particularly chloroquine, has been proposed to block heme polymerization to hemozoin in the food vacuole and alter Atg8 distribution in chloroquine sensitive strains as compared to that in resistant strains of *P. falciparum* [27, 82, 83]. In mammalian cells, quinolines have been shown to inhibit autophagy by alkalinizing the lysosomes, thereby inhibiting the activities of lysosomal hydrolases that are required for degradation of the autophagy cargo [84, 85]. However, the concentration required for
alkalinization is 1-2 orders of magnitude higher than the concentrations used in our study [83]. Also, treatment of parasites with other quinolines did not have any effect. Furthermore, E64 and chloroquine did not affect the food vacuole localization of PfCRTmCherry, a food vacuole membrane transport protein, indicating that food vacuole integrity was not compromised in our experiments. Taken together, the diffuse localization of PfAtg18 in parasites treated with chloroquine and amodiaquine is unlikely due to alkalinization or disruption of the food vacuole and these two drugs likely target Atg18 transport to the food vacuole, which may contribute to the overall antimalarial effect of these compounds. PfAtg18 has also been shown to participate in the food vacuole dynamics and it is transported via the haemoglobin trafficking pathway [19]. Quinolines have also been shown to have different effects on haemoglobin endocytosis and vesicle trafficking; in particular, inhibition of the transport of haemoglobin-containing vesicles to the food vacuole by chloroquine and amodiaquine [86]. It is possible that inhibition of the transport of haemoglobin-containing vesicles by amodiaquine and chloroquine resulted in the altered food vacuole localization of PfAtg18. However, the canonical vesicle trafficking inhibitors brefeldin A and bafilomycin did not affect PfAtg18 localization. This suggests for a different mode of inhibition of PfAtg18 localization by amodiaquine and chloroquine that might affect food vacuole-associated processes like haemoglobin degradation, which could subsequently affect the action of these drugs, as this degradation produces heme for quinoline action and iron for artemisinin activation. Further investigation is necessary to find out how chloroquine and amodiaquine affect the PfAtg18 localization.

The Plasmodium Atg18 gene is essential for parasite development [87, 88], and knock-down of PfAtg18 has been shown to cause the loss of apicoplast [45]. We generated PbAtg18-KD parasites, which did not show any noticeable change in the localization of PbAtg18, most likely because there was only a partial reduction in the PbAtg18 protein level. A more efficient knock-down approach will be required to define the role of Plasmodium Atg18 in autophagy and the functional importance of its association with the food vacuole.

Atg18 proteins of S. cerevisiae and P. pastoris are also required for selective degradation of peroxisomes by autophagy, which is known as pexophagy. The proteins are concentrated at one or more spots that resemble protuberances of the vacuole membrane [89]. Elegant microscopic images indicated colocalization of the protuberance with the peroxisome cluster [89, 90]. PfAtg18 also showed an intense spot on or near the food vacuole membrane, which tempts us to speculate if PfAtg18 functions in the engulfment of cellular contents directly by the food vacuole membrane. Although peroxisomes appear to be absent in Plasmodium [91], direct engulfment of cellular contents by the Plasmodium food vacuole membrane may occur. One such process could be the fusion of haemoglobin-containing vesicles with the food vacuole [92, 93]. Similar PfAtg18-enriched dot-like structure in the food vacuole periphery was also observed in a recent study, which the authors explained as a result of the food vacuole fission [19].
ScAtg18 has also been shown to be present as an intense spot in a peri-vacuole membrane compartment during starvation wherein it colocalizes with a number of autophagy proteins, and this compartment has been proposed to be a site for vesicle formation or membrane source [94]. The intense PfAtg18 spot in the peri-food vacuole region may be a platform where autophagy and some other pathways overlap. Further studies are required to gain insights into the nature and function of this peri-food vacuole compartment.

Thus, our data demonstrate that *Plasmodium* Atg18 proteins localize to the food vacuole, which is mediated by interaction with PI3P and MDR1. Additional study is needed to investigate the physiological significance of MDR1-PfAtg18 interaction.
Data availability: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD020847 and 10.6019/PXD020847.

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**Figure legends**

**Figure 1. PfAtg18 localizes to the food vacuole.** The GFP/PfAtg18-expressing *P. falciparum* D10 parasites were evaluated for expression (A and B) and localization (C) of GFP/PfAtg18. Western blots of the lysates of wild type parasites (WT), GFP/PfAtg18-expressing parasites (A18) and uninfected-RBCs (RBC) were probed using anti-PfAtg18 antibodies (ab-Atg18) in A and anti-GFP antibodies (ab-GFP) in B. β-actin was used as a loading control (ab-Ac). The protein bands around 43.5 kDa and 74.3 kDa correspond to native PfAtg18 and GFP/PfAtg18, respectively. The protein size markers are in kDa (M). C. Live GFP/PfAtg18-expressing parasites of the indicated stages were assessed for localization of GFP/PfAtg18 by fluorescence microscopy. The panels show GFP/PfAtg18 signal (Atg18), nucleic acid staining (Hoechst), bright field with RBC and parasite boundaries (DIC) and the overlap of all three images (Merged). The black substance in the DIC image is the food vacuole-resident pigment haemozoin, which serves as an in-built food vacuole marker.

**Figure 2. PfAtg18 and PbAtg18 show similar food vacuole localization in *P. berghei*.** The GFP/PfAtg18-expressing *P. berghei* (GFP/PfAtg18<sup>epl</sup>) and PbAtg18/GFP knock-in *P. berghei* (PbAtg18/GFP<sup>ki</sup>) parasites at trophozoite and schizont stages were evaluated for Atg18 localization by live cell fluorescence microscopy. The panels are for GFP/PfAtg18 or PbAtg18/GFP signal (Atg18), nucleic acid staining (Hoechst), bright field showing the boundaries of parasite and RBC (DIC), and the overlap of all three images (Merged). Note that GFP signal is associated with multiple haemozoin-containing foci in the trophozoite stage, which fuse into one big food vacuole in the schizont stage.

**Figure 3. PvAtg18 and PkAtg18 localize to the food vacuoles.** The GFP/PvAtg18- and GFP/PkAtg18-expressing *P. berghei* parasites at trophozoite and schizont stages were observed for localization of respective proteins by live cell fluorescence microscopy. The panels are as described in Figure 3. The GFP signal is primarily associated with multiple haemozoin-containing foci, which coalesce into one big food vacuole in the schizont stage.

**Figure 4. PfAtg18 localizes to the food vacuole through interaction with PI3P.** A. GFP/PfAtg18-expressing *P. falciparum* trophozoites were treated with DMSO (A18wt) or the PI3K inhibitor (LY294002) and assessed for PfAtg18 localization by live cell fluorescence microscopy. *P. falciparum* trophozoites expressing PfAtg18 mutants (PIPm and ALCAm) were assessed for the effect of mutations on PfAtg18 localization by live cell fluorescence microscopy. Parasites expressing GFP only served as a cytosolic localization control (GFPcyt). The panels from the left represent GFP signal (GFP), nucleic acid staining (Hoechst), boundaries of parasite and RBC (DIC), and the merge of all three images (Merged). Note that
the LY294002 treatment caused diffuse GFP/PfAtg18 localization all over the parasite and mutations rendered the mutant proteins outside the food vacuole in cytoplasm. B. Recombinant GST/PfAtg18 (PfA18), GST/PIPm (PIPm), GST/ALCAm (ALCAm), GST/ScAtg18 (ScA18) and GST were incubated for binding with PI3P-agarose beads, and the eluates (Elute) along with the input, flowthrough (FT), and washes were assessed for the presence of respective proteins by western blotting. The protein size markers are in kDa (M). The presence of signal in the eluate indicates interaction with PI3P.

**Figure 5 ScAtg18 does not localize to the *P. falciparum* food vacuole.** The *P. falciparum* 3D7 trophozoites expressing GFP/PfAtg18 (PfAtg18), GFP/ScAtg18 (ScAtg18) and a cytoplasmic GFP control (GFPcyt) were evaluated for localization of respective proteins by live cell fluorescence microscopy. The panels are for the GFP signal (GFP), nucleic acid staining (Hoechst), bright field showing the boundaries of parasite and RBC (DIC), and the overlap of all the three images (Merged). Contrary to the food vacuole localization of GFP/PfAtg18, GFP/ScAtg18 shows diffuse cytoplasmic localization outside the food vacuole, which is similar to that of GFPcyto.

**Figure 6. PfMDR1 interacts with PfAtg18.** A. Schematic of the predicted domain architecture of PfMDR1 shows transmembrane helices (filled bars), the inside domain (ID) and the cytoplasmic domain (CD). GST-fusions of the cytoplasmic (GST/MDRCD) and inside (GST/MDRID) domains were expressed in *E. coli*, and enriched for fusion proteins by purification. B. The coomassie stained PAGE gel contains lysates of uninduced (Un) and IPTG-induced (In) GST/MDRCD-expressing cells, soluble (Sol) and insoluble (Ins) fractions of the induced GST/MDRCD-expressing cells, purified GST/MDRCD (CD) and GST/MDRID (ID) proteins. C. The western blot shows GST/MDRCD (CD) and GST/MDRID (ID) proteins (indicated with an arrow head) in the purified samples. The protein size markers are in kDa (M). D. The blots containing spots of the indicated amounts of recombinant GST/PfAtg18 (PfA18), GST/ScAtg18 (ScA18) and GST were overlaid with recombinant GST/MDRCD (CD) or GST/MDRID (ID) proteins and probed with anti-GST antibodies. The blots show interaction of the PfMDR1 cytoplasmic domain with PfA18 in a dose-dependent manner.

**Figure 7. Effect of antimalarials on PfAtg18 localization.** Early trophozoite stage parasites were treated with DMSO (Con) or antimalarials (artemisinin, Art; halofantrine, HF; chloroquine, CQ; amodiaquine, AQ; quinine, QN; mefloquine, MQ) for 8 hours and assessed for PfAtg18 localization by live cell fluorescence microscopy. The panels from the left are for GFP/PfAtg18 signal (Atg18), nucleic acid staining (Hoechst), bright field showing the boundaries of parasite and RBC (DIC), and the overlap of all three images.
Compared with the food vacuole localization of GFP/PfAtg18 in control cells, treatment with chloroquine and amodiaquine caused diffuse localization all over the parasite.

**Figure 8. Effect of chloroquine and amodiaquine on PfAtg18 localization.** GFP/PfAtg18-expressing parasites at early trophozoite stage were treated with DMSO (control) or the indicated IC50 concentrations of antimalarials (chloroquine, CQ; artemisinin, Art; amodiaquine, AQ) for 4 hours. The graph shows the number of parasites with diffuse localization as times of the control parasites at different IC50 concentrations. The data is mean with SD of three independent experiments.

**Figure 9. PfAtg18 localization upon treatment with the inhibitors of food vacuole-associated processes.** Early trophozoite stage GFP/PfAtg18-expressing parasites were treated with DMSO (Con) or inhibitors (E64; pepstatin A, PepA; epoxomicin, Epox; pristimerin, Prist; orlistat, Orl) for 8 hours and assessed for PfAtg18 localization by live cell fluorescence microscopy. The panels for the indicated treatments represent GFP/PfAtg18 signal (Atg18), nucleic acid staining (Hoechst), bright field showing the boundaries of parasite and RBC (DIC), and the overlap of all three images (Merged). The %Representation column on the right indicates the %parasites showing that type of localization. The data is mean with SD (in the bracket) from three independent experiments. Compared to PfAtg18 localization in control parasites, treatment with E64 and pepstatin A produced a ring-like localization pattern and orlistat caused diffuse localization.

**Figure 10. Atg18 is critical for erythrocytic stage parasite development.** The endogenous PbAtg18 coding region was replaced with PbAtg18/cDDHA coding sequence, and a clonal PbAtg18-KD parasite line was evaluated for the presence of knock-down locus by PCR, localization of PbAtg18-cDDHA protein, effect of knock-down on the expression of PbAtg18/cDDHA protein level, and for comparison of growth rates with the wild type parasites. A. The schematic represents integration of the linear transfection construct (T cassette) into the endogenous PbAtg18 locus (WT locus), resulting into the generation of integration locus (Int locus). The coding regions for PbAtg18 (Atg18), cDD/HA, and hDHFR are represented by rectangular boxes. The flanking untranslated regions (5'U and 3'U), the location and orientation of primers (horizontal arrows), the restriction endonuclease sites, the regulatory regions in the linear transfection construct (PvAC3'U, PyaTb5'U and PfHrp2-3'U) are labelled. The wild type and integration loci can be distinguished by PCR using locus specific primers (5' integration, 5'-Int (P1/P2); 3' integration, 3'-Int (P3/P4); wild type locus, WTsp (P1/P4); MSP1 gene as a control, Con). B. The ethidium bromide stained agarose gel shows PCR products for the indicated regions from wild type (WT) and Atg18kd parasite gDNAs. C. The IFA images of PbAtg18-KD trophozoites show localization of
Atg18/cDDHA (Atg18), nucleic acid staining (DAPI), the bright field image showing boundaries of parasite and RBC (DIC), and the overlap of all three images (Merged). D. Western blot of WT and the PbAtg18-KD parasite lysates (grown in the presence (+T) or absence (-T) of trimethoprim) using antibodies to HA (ab-HA) and β-actin (ab-Ac). Naïve BALB/c (E) and C57BL/6J (F) mice were infected with equal number of wild type P. berghei ANKA (WT) or PbAtg18-KD (A18kd) parasites. The mice infected with PbAtg18-KD were kept under (A8kd, +T) or without (A18kd, -T) trimethoprim. The number of mice in each group is shown with “n”. The parasite growth is shown as %parasitemia over days post-infection.
Table 1. Proteins identified in the immunoprecipitate of GFP/PfAtg18-expressing *P. falciparum* parasites. The proteins listed are common in at least 3 of the 4 independent experiments, and contain at least 1 unique peptide. Shown are coverage (Cov), unique peptides (U Pep) and score (Sco) for each protein.

| Accession No | Proteins | Experiment-1 | | Experiment-2 | | Experiment-3 |
|--------------|----------|--------------|--------------|--------------|--------------|
|              |          | Cov | U Pep | Sco | Cov | U Pep | Sco | Cov | U Pep | Sco |             |
| C6KTC7 (PF3D7_0629200) | DnaJ protein, putative | 2.4 | 1 | 2.0 | 2.4 | 1 | 4.5 | 19.5 | 7 | 36.8 |
| Q7K6A5 (PF3D7_0523000) | Multidrug resistance protein 1 | 6.3 | 6 | 34.6 | 3.5 | 3 | 4.5 | 1.1 | 1 | 5.8 |
| Q8IL88 (PF14_0359) | HSP40, subfamily A, putative | 7.3 | 2 | 2.3 | 13.7 | 7 | 24.6 | 2.8 | 1 | 2.8 |
| Q8IDS6 (PF3D7_1341200) | 60S ribosomal protein L18a | 18.5 | 3 | 7.2 | 5.4 | 1 | 2.5 | 15.2 | 3 | 19.7 |
| O77395 (PF3D7_0316800) | 40S ribosomal protein S15A, putative | 6.2 | 1 | 4.4 | 6.2 | 1 | 2.2 | 33.1 | 5 | 16.3 |
| Q8IKH8 (PF14_0627) | 40S ribosomal protein S3 | 5.9 | 1 | 2.9 | 7.7 | 1 | 2.3 | 31.2 | 7 | 23.8 |
| Q8IEQ1 (PF3D7_1306400) | 26S protease regulatory subunit 10B, putative | 3.1 | 1 | 1.9 | 5.3 | 1 | 6.2 | 27.7 | 7 | 44.2 |
| Q8I490 (PF3D7_0501000) | Plasmodium exported protein | 8.1 | 2 | 12.2 | 4.2 | 1 | 3.1 | 13.9 | 3 | 14.9 |
| Q8I207 (PF3D7_0401800) | Plasmodium exported protein (PHISTb) | 11.3 | 8 | 28.2 | 2.7 | 1 | 2.9 | 20.7 | 13 | 59.9 |
| Q8IJR6 (PF10_0126) | Autophagy-related protein 18, putative | 49.0 | 18 | 184.1 | 49.2 | 19 | 235.8 | 6.6 | 2 | 7.1 |
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 9
Figure 10