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Structural characterization and inhibition of the *Plasmodium* Atg8-Atg3 interaction

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Abstract

The autophagy-related proteins are thought to serve multiple functions in *Plasmodium* and are considered essential to parasite survival and development. We have studied two key interacting proteins, Atg8 and Atg3, of the autophagy pathway in *P. falciparum*. These proteins are vital for the formation and elongation of the autophagosome and essential to the process of macroautophagy. Autophagy may be required for conversion of the sporozoite into erythrocytic-infective merozoites and may be crucial for other functions during asexual blood stages. Here we describe the identification of an Atg8 family interacting motif (AIM) in *Plasmodium* Atg3, which binds *Plasmodium* Atg8. We determined the co-crystal structure of PfAtg8 with a short Atg3¹⁰³⁻¹¹⁰ peptide, corresponding to this motif, to 2.2 Å resolution. Our *in vitro* interaction studies are in agreement with our x-ray crystal structure. Furthermore they suggest an important role for a unique Apicomplexan loop absent from human Atg8 homologues. Prevention of the protein-protein interaction of full length PfAtg8 with PfAtg3 was achieved at low micromolar concentrations with a small molecule, 1,2,3-trihydroxybenzene. Together our structural and interaction studies represent a starting point for future antimalarial drug discovery and design for this novel protein-protein interaction.

Keywords

malaria; autophagy; x-ray crystallography; surface plasmon resonance; fragment library; protein-protein interaction; fragment-based drug discovery; *Plasmodium*

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A.U.P.H. and J.B. designed research. A.U.P.H., R.R.W., D.R.C., B.J., I.C., and J.B. performed research. J.B. and A.U.P.H. wrote paper. H.H., D.R.C., R.R.D., and D.R.M.G. contributed new reagents/analytical tools. A.U.P.H., D.R.C., and J.B. analyzed data. All authors read and commented on the manuscript.

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1. Introduction

Malaria kills over a million people each year, primarily affecting children (Murray et al., 2012). The continual threat of drug resistance necessitates identification of novel antimalarial drug targets. Appreciation for the role of autophagy in differentiation, development, and survival under stress in pathogenic parasites is increasing (Alvarez et al., 2008; Besteiro, 2012; Besteiro et al., 2006; Besteiro et al., 2011; Brennand et al., 2011; Picazarri et al., 2008; Sinai and Roepe, 2012) and evidence suggests that autophagy for organelle turnover is functional and necessary in the malaria parasite, *Plasmodium* (Brennand et al., 2011; Duszenko et al., 2011).

Autophagy is an intracellular degradation system highly conserved within eukaryotes. In macroautophagy, hereafter referred to as autophagy, cellular contents are enclosed in a double membrane structure called the autophagosome and digested through fusion of the autophagosome with the lysosome or vacuole (Yang and Klionsky, 2010). Over thirty Atg proteins involved in autophagy have been identified in yeast and mammals including two ubiquitin-like conjugation systems (the Atg12~Atg5 system and the Atg8~phosphatidylethanolamine (PE) system), which are important for autophagosome formation and expansion (Ichimura et al., 2000; Mizushima et al., 1998). While a limited repertoire of these proteins has been identified in *Plasmodium*, the Atg8- PE conjugation system is fully intact (Rigden et al., 2009). Most Atg8 orthologues require proteolytic processing of one or several amino acids to expose a C-terminal glycine; in contrast Atg8 is synthesized in an active form in *Plasmodium* (Brennand et al., 2011). The exposed C-terminus of Atg8 is attached to its E1 activating enzyme, Atg7, through a thioester bond that requires activation by ATP hydrolysis. Atg8 is then transferred to its E2 conjugating enzyme, Atg3, to form another thioester bond before being transferred to PE in the phagophore membrane. In addition to thioester formation with Atg3, Atg8 interacts noncovalently with Atg3 prior to substrate attachment (Yamaguchi et al., 2010). *In vitro*, Atg8 lipidation is accelerated through the E3-like action of the Atg12-Atg5 conjugate (Hanada et al., 2007). It is not known if a functional E3 exists in *Plasmodium*; clear putative orthologues for the Atg12 system have only been identified for Atg7, the shared E1 activating enzyme, and Atg12, which does not contain the C-terminal glycine needed for conjugation (Duszenko et al., 2011).

Atg8 is expressed during all life stages in *Plasmodium* and is essential for parasite survival as demonstrated in gene knockout experiments (Duszenko et al., 2011; Le Roch et al., 2003), making it an attractive candidate for antimalarial drug design. While autophagy has been implicated in microneme disposal in the liver stage (Duszenko et al., 2011), its function in the blood stage remains unknown. Recently PfAtg8 was shown to localize to the apicoplast, a plastid-like organelle unique to Apicomplexa (Kitamura et al., 2012). Here we report biochemical and structural evidence that *P. falciparum* (Pf) Atg8 binds PfAtg3 in a conserved mechanism as reported in other species, including humans. Despite this conservation we have discerned regions in the parasite protein that can be exploited by small molecules to specifically target the *Plasmodium* Atg8-Atg3 interaction and have identified several promising molecules for drug optimization.

2. Methods and Materials

All reagents, unless specified, were purchased from Sigma-Aldrich. Primers used for cloning and mutagenesis are found in Table S1.

2.1 Cloning, Expression, and Purification

PfAtg8 was amplified from 3D7 cDNA using primers A8MF and A8R, containing NcoI and BamHI restriction sites, and ligated into a modified pRSF vector with an N-terminal Maltose binding protein (MBP) tag and Tobacco etch virus (TEV) protease cleavage site. An N-terminal His₆-tagged construct was made using primers A8HF and A8R, containing NcoI and BamHI sites and hexahistidine tag, and ligated into a pRSF-1b vector (Novagen). Plasmids were transformed into *E. coli* Rosetta2 cells containing a rare codon plasmid. A single colony was used to inoculate TB media containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol and grown at 37°C. At an O.D. 600 of 3.3, protein expression was induced with 0.3 mM IPTG and grown for 16 h at 20°C under vigorous shaking.

Cells were harvested by centrifugation at 4,000 x g, 30 min, 4°C and then resuspended in 50 mM HEPES pH 8.0, 500 mM NaCl, 1 mM MgCl₂, 10% glycerol, a tablet of Roche Complete EDTA-free protease inhibitor and 500 U benzonase and lysed using an EmulsiFlex C5 cell disruptor at 15,000 PSI. After centrifugation at 35,000 x g for 30 min, MBP-PfAtg8 lysate was incubated with amylose resin (NEB) and washed with lysis buffer before eluting with 50 mM HEPES pH 8.0, 20 mM maltose, 10% glycerol. Protein was dialyzed with homemade MBP-TEV protease at 1:100 in 50 mM MES pH 6.5, 10% glycerol, 0.5 mM EDTA, 1 mM DTT. Cleaved protein was separated from TEV and MBP through cation exchange over a RESOURCETM S column (GE Healthcare) using an ÄKTA purifier system. Pure fractions were dialyzed in 50 mM MES pH 6.5, 50 mM NaCl, 10% glycerol overnight and concentrated using a filter device with 3 kDa MWCO (Amicon). All purification steps were conducted at 4°C.

After centrifugation of His₆-PfAtg8 lysate, protein incubated with Cobalt-charged TALON resin (Clontech) was washed with lysis buffer and eluted in 50 mM HEPES pH 8.0, 300 mM imidazole, 10% glycerol. Elutions were further purified with RESOURCETM S column as for MBP-PfAtg8.

Cloning, Expression, and Purification of PbAtg8—For all protein purifications sample, purity was assessed with SDS-PAGE, visualizing with either Gelcode blue (Thermo Scientific) or coomassie stain.

Full length *PbAtg8* was cloned from cDNA with primers Pb1F and Pb1R (SIT3) and ligated into the PQE-30 vector (Qiagen, Hilden, Germany). His-tagged PbAtg8 was expressed in *E. coli* SG13009, grown in LB-media to an OD₆₀₀=0.6, and induced with 1 mM IPTG for 3 h at 37°C. After centrifugation at 3000 x g, bacteria were resuspended in buffer A, containing 20 mM phosphate (pH of 7.2), 100 mM NaCl, 10 mM imidazole and lysed by sonication with 60 pulses at 50% duty cycle and an output of 50 followed by 1- min on ice. Lysate was cleared by centrifugation at 1,100 x g for 10 min and the pellet was resuspended in buffer A containing 6 M urea. Lysate was loaded onto an HP Nickel Column (HiTrap chelating HP from GE Healthcare, USA), washed with 6 column volumes of buffer A containing 20 mM imidazole and His₆-PbAtg8 was eluted with 250 mM imidazole. Fractions were separated on a 15% acrylamide gel, coomassie blue stained, and the band corresponding to His-PbAtg8 was used for immunization in rat to raise antibodies (Covance, Princeton, NJ).

Cloning, Expression, and Purification of PfAtg3—PfAtg3 was cloned from *P. falciparum* 3D7 cDNA using primers containing either a His₆ tag and NcoI and BamHI restriction sites (A3HF and A3R (SIT3)) and ligated into a pET45b vector or just NcoI and BamHI sites (A3MF and A3R (SIT3)) ligated into a modified pET45b vector to have an N-terminal MBP tag followed by a TEV protease cleavage site. Constructs were verified with DNA sequencing and transformed into *E. coli* Rosetta2 cells. A single colony was used to inoculate TB and grown at 37°C. Protein expression was induced at an O.D. 600 of 3.5 with

1 mM IPTG and expressed at 20°C for 16 h. Cells were harvested and the bacterial pellet resuspended in 50 mM Tris pH 7.0, 500 mM NaCl, with a Complete EDTA-free protease inhibitor cocktail tablet (Roche) and benzonase (Sigma). Cells were lysed with an EmulsiFlex C5 cell disruptor at 15,000 PSI and cleared with 35000 x *g* for 35 min.

All purification steps were conducted at 4°C. MBP-PfAtg3 was applied to amylose resin and washed with lysis buffer. Bound protein was eluted in 50 mM Tris pH 7.0, 20 mM maltose. His₆-PfAtg3 was purified with TALON™ resin (Clontech). Following a washing step with lysis buffer, PfAtg3 was eluted in 50 mM Tris pH 7.0, 300 mM imidazole. Both constructs were further purified using anion exchange chromatography over a RESOURCE™ Q column (GE Healthcare).

Expression and Purification of hLC3—MAP1LC3B (hLC3) was amplified from an expression vector (generous gift of Dr. Marie Hardwick, Johns Hopkins University) and ligated into a modified pET45b vector to contain an MBP N-terminal tag and TEV protease cleavage site using primers L3MF and L3MR (SIT3) containing NcoI and BamHI restriction sites. MBP-LC3 was expressed and purified following the same protocol as MBP-PfAtg8.

Site-directed mutagenesis—Amino acid changes were introduced into constructs using the protocol and reagents in the QuickChange® Lightning site-directed mutagenesis kit (Agilent) (Supplementary Table 1 for primers). A previously published, modified protocol was followed (Deng et al., 2007) to delete residues 68-76 from PfAtg8.

2.2 Crystallization of PfAtg8^{CM}-PfAtg3¹⁰³⁻¹¹⁰

MBP-PfAtg8^{CM} was expressed and purified as specified. Pure fractions from the RESOURCE™ S column were dialyzed over night into 50 mM MES pH 6.5, 7% glycerol, 1 mM MgCl₂, 2 mM DTT and concentrated and further purified on a HiLoad S75 column (GE Healthcare) in 50 mM MES pH 6.5, 7% glycerol, 1 mM MgCl₂, 2 mM DTT. Equimolar PfAtg3¹⁰³⁻¹¹⁰ peptide was added to PfAtg8^{CM} at 2 mg/ml and concentrated in a 3 kDa MWCO filter device (Amicon) to 8 mg/mL. More peptide was added to a final concentration of 2.5 mM. Original crystals were identified from a screen set up with 200 nL of protein and 200 nL reservoir using a Mosquito crystallization robot (TTP Labtech) containing 100 mM MES pH 6, 0.2 M Ca(OAc)₂, 20% w/v PEG 8000 equilibrated at 20°C in a 96-well sitting drop INTELLI-PLATE® (Art Robbins Instruments). Conditions were further optimized to obtain larger crystals using a 24-4 sitting drop INTELLI-PLATE® (Art Robbins) with reservoir containing 100 mM MES pH 6.5, 100 mM Ca(OAc)₂, 20% w/v PEG 8000 and sitting drops containing 2 µL protein at 8 mg/mL and 2 µL reservoir, equilibrated at 20°C. Crystals were mounted in a 0.3 mm nylon cryoloop (Hampton Research) and flash frozen directly in liquid nitrogen before shipping to the Stanford Synchrotron Radiation Lightsource. Data sets were collected at SSRL beamline 12-2 at λ 1.033 Å using a microfocus beam of 6 × 9 µm and the Berkeley Advanced Light Source beamline 5-01 at λ 0.977 Å with a 100×100 µm beam.

2.3 Data Collection and Structure Determination

The crystals belonged to the space group P2₁ with cell dimensions of a=33.30 Å, b=111.08 Å, c=57.09 Å and β=92.58°. Data reduction and scaling was carried out with XDS/XSCALE (Kabsch, 1993). The refined twin fraction was 9.6% as determined by Refmac (Murshudov et al., 1997). The asymmetric unit contained three monomers resulting in a solvent content of 48%. We initially failed to obtain a molecular replacement solution with various models using Phaser (McCoy et al., 2007), MOLREP (Vagin and Teplyakov, 2010), and BALBES (Long et al., 2008). Only the combination of 20 models including homology models generated by Robetta (Kim et al., 2004) yielded a molecular replacement solution using

phenix.ensemble (Wang and Snoeyink, 2008) together with phenix.automr (McCoy et al., 2007). Manual rebuilding and refinement was carried out in Coot (Emsley and Cowtan, 2004). TLS refinement was performed with TLS groups determined by the TLSMD server (Painter and Merritt, 2006) using Phenix (Adams et al., 2011; Terwilliger et al., 2008). PfAtg8 was refined to a crystallographic R_{work} of 20.5% and an R_{free} of 27.9% (Brünger, 1992). The final structure was analyzed with validation tools in Coot (Emsley and Cowtan, 2004) as well as MOLPROBITY (Lovell et al., 2003), indicating zero Ramachandran outliers (Ramakrishnan and Ramachandran, 1965).

2.4 Plasmodium infection and Immunological assays

Blood stage parasites were obtained from anesthetized infected mice with a parasitemia above 3% by cardiac puncture. Blood was collected into syringes coated with a stock solution of 25,000 U/ml of heparin to prevent clotting and washed with PBS prior to saponin lysis.

Full length *PbAtg8* was used to generate rat antibodies against the protein. The specificity of anti-PbAtg8 antibodies was verified by western blot at a dilution of 1:1000 against blood stage parasite lysates. To prepare lysates, blood stage parasites were isolated by saponin lysis and resuspended in buffer consisting of 50 mM Tris HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF and 1x Complete Protease Inhibitor Cocktail (Roche, Germany). Laemmli buffer was added to a final concentration of 1x and samples were boiled for 5 min and centrifuged briefly prior to separation by SDS-PAGE on a 4-15% Tris-HCl gradient gel (Bio-Rad, Hercules, CA). Proteins were transferred to nitrocellulose and blocked with 2% dehydrated milk and 0.1% Tween-20 in PBS. Anti-Rat-HRP, diluted at 1:10,000 in blocking buffer, was used to detect bound antibody (GE Healthcare, Uppsala, Sweden). HRP was visualized using ECL Plus (GE Healthcare). Antibodies against PbAtg8 revealed the presence of a unique band at the expected size of 13 kDa when lysates were separated on 4-15% acrylamide gels.

For immunofluorescence assays (IFA), *P. berghei*-infected red blood cells were fixed in a solution consisting of 4% paraformaldehyde (Polysciences, Inc., Warrington, PA) and 0.02% glutaraldehyde in PBS for 15 min, washed with PBS, permeabilized with 0.3% Triton X-100 for 5 min and washed 3 times with PBS. Samples were then blocked with 3% BSA dissolved in PBS for 45 min and probed with anti-PbAtg8 antibodies diluted 1/100 in blocking buffer for 1-2 h. Samples were then washed 3 times and probed with secondary antibodies (Anti-Rat Alexa Fluor 568 antibody from Life Technologies) diluted 1/1000 in blocking buffer for 45 min. Coverslips were mounted onto glass microscope slides using ProLong Gold anti-fade mounting solution with or without DAPI (Invitrogen). Images were viewed with a Nikon Plan Apo 100x objective using a Nikon 90i microscope and pictures were taken using a Hamamatsu ORCA-ER camera and Velocity software. Images (a z-stack per field) were processed using iterative restoration (confidence limit 98% and iteration limit 25) and further processing was done using Adobe Photoshop software (Adobe Systems Inc.).

2.5 In Vitro Assays

Pulldown Assays—All studies were performed at 4°C. For the interaction study between PfAtg8 mutants and PfAtg3, purified MBP-PfAtg3 was immobilized on amylose resin. Columns were incubated with purified wild type and mutant His₆-PfAtg8 for 20 min and washed with 50 mM HEPES pH 7.5, 50 mM NaCl, 10% glycerol. Wash buffer containing 20 mM maltose was used to elute bound protein, which was visualized with SDS-PAGE and analyzed using ImageJ (Abramoff et al., 2004).

For interaction studies between PfAtg3 mutants and PfAtg8, His₆-PfAtg8^{CM} was co-expressed with WT and mutant MBP-PfAtg3 in *E. coli*, grown in TB, induced with 1 mM IPTG and expressed at 20°C for 16 h. Cells were harvested with centrifugation at 3000 x g and lysed in 50 mM HEPES pH 7.4, 50 mM NaCl, 1 mM MgCl₂ as stated elsewhere. Cleared lysate was incubated with amylose resin, washed, eluted, and analyzed as above.

To identify endogenous binding partners of PfAtg8, a 50 mL culture of asynchronous *P. falciparum* 3D7 blood stage parasites, primarily trophozoites, was separated from RBCs through saponin treatment and centrifugation. The parasite pellet was resuspended in 1x PBS with Complete EDTA-free protease inhibitors (Roche) and sonicated on ice for 5-30 sec pulses, output power 15%. Lysate was centrifuged for 20 min at 3000 x g. Supernatant was split between Ni-NTA (Qiagen) columns with and without immobilized His₆-PfAtg8. The columns were washed with 1.06 mM KH₂PO₄, 5.6 mM Na₂HPO₄, 154 mM NaCl, pH 7.4. Bound proteins were eluted in wash buffer containing 50 mM HEPES pH 7.4, 300 mM Imidazole, 10% glycerol and visualized using SYPRO[®] Ruby stain (Invitrogen) with SDS-PAGE using a typhoon 9410 scanner (GE Healthcare). The experiment was repeated for PfAtg8^{CM} with the same protocol except that parasites were synchronized to the trophozoite stage, parasitemia of 7.5%, and equimolar purified His₆-PfMTIPΔ60 (Bosch et al., 2007; Bosch et al., 2006) was used as a control for nonspecific binding instead of Ni-NTA resin only.

Thermal Shift Assay—Mutant and wild type proteins were tested at 1mg/mL in a reaction containing 30 μL of protein and 1 μL of a 1:30 dilution of SYPRO[®] orange dye. Fluorescent measurements were done in triplicates in a CFX96 thermal cycler (BioRad) from 20°C to 80°C over a period of 60 min and the melting temperature was determined from the maximum of the first derivative of the melting curve. Because MBP has its own melting curve only His₆-tagged variants were tested and were expressed and purified concurrently to those in same experiment.

Peptides: 20 μg of PfAtg8 in 50 mM MES pH 6.5, 0.2 M NaCl, 1 mM MgCl₂, 10% glycerol, 2 mM DTT was added to each well of an opaque 96well plate (Eppendorf) containing varying concentrations of peptide, normalized with peptide buffer (50 mM MES pH 6.5) to match buffer conditions at each concentration, for a total reaction volume of 25 μL (final protein concentration of 55 μM). 1 μL of a 1:30 dilution of SYPRO[®] orange dye (Sigma) was added to each well. Fluorescent measurements were done in triplicates in a CFX96 thermal cycler (BioRad) from 20°C to 80°C over a period of 60 min and the melting temperature was determined as before.

Fragments: 5 μL of His₆-PfAtg8^{CM} at 2 mg/mL with a 1:350 dilution of SYPRO[®] 6 orange dye in 50 mM HEPES 7.4, 50 mM NaCl was added to 5 μL of solution containing 0-2 mM fragment in the same buffer, normalized so all conditions contained 0.5% DMSO. Fluorescence was measured as above in triplicates at each fragment concentration.

Dynamic Light Scattering—A DynaPro[™] DLS instrument (Protein Solutions[™]) with a 30 μL sample cell cuvette (PROTERION) was used to measure the hydrodynamic radius of His₆-PfAtg8, His₆-PfAtg3, and the complex at 300 μM in 50 mM HEPES pH 7.4, 50 mM NaCl, 10% glycerol, 1 mM MgCl₂, 2 mM DTT. The molecular weight was calculated from the radius using the Dynamics V6 acquisition and analysis software (Protein Solutions[™]).

Analytical Gel Filtration—His₆-PfAtg8 and His₆-PfAtg3 were injected over a Superdex 75 10/300 GL column (GE Healthcare) at 0.3 mL/min in 50 mM MES pH 6.5, 50 mM NaCl, 10% glycerol, 1 mM MgCl₂ 10 mM DTT alone and together at 4°C. Relative molecular

weights were determined by comparison with a gel filtration standard (Biorad) run under identical conditions. Identity of peaks was confirmed with SDS-PAGE.

2.6 Mass Spectrometry Analysis

Gel bands corresponding to regions of interest were excised, washed, reduced (10 mM DTT in 20 mM NH_4HCO_3 for 1 hr at 56 °C) and alkylated (55 mM iodoacetamide in 20 mM NH_4HCO_3 in the dark at room temperature for 45 min) and digested (using 25 μL of 10 ng/ μL proteomics grade trypsin in 20 mM NH_4HCO_3 (Promega)). Subsequent overnight digests were extracted, concentrated and injected onto an Agilent 6520 Q-TOF with a Chip Cube interface using standard gradients. Acquisition parameters were previously described (Parish et al., 2011). Acquired spectra were processed and searched against NCBI subset databases (Benson et al., 2009; Sayers et al., 2009) using the Mascot Daemon search tool (Matrix Science). Results were curated and visualized using Scaffold 2.0 (Proteome Software). Hits with at least 2 matched peptides above the significance threshold present in the Atg8 sample and none in the control were considered significant.

2.7 Surface Plasmon Resonance

SPR runs conducted on a Biacore 3000 instrument (GE Healthcare). Unless otherwise stated, runs were conducted in running buffer (RB) containing 354 μM KH_2PO_4 , 1.87 mM Na_2HPO_4 , 51.5 mM NaCl, pH 7.4, 0.01% v/v P20, 0.2 mg/ml BSA at 25°C (Quality Biologicals). Bound analyte was dissociated from the chip using 1 injection of 1 M MgCl_2 followed by 2 blank injections of RB between each concentration. Binding and equilibrium constants were determined with Scrubber (BioLogic™). A double referencing method was applied to correct for nonspecific binding to the chip with interspersed blank injections correcting for baseline drifts.

The surface chip was pre-conditioned with multiple injections of 20 mM glycine pH 2.2 prior to pH scouting for identification of optimal binding conditions of Atg3 to the sensor chip. MBP-PfAtg3, dissolved in 10 mM Na Acetate pH 4.0, was immobilized by amine coupling to the surface of a CM3 sensor chip (GE Healthcare).

Determination of dissociation constants for PfAtg8 WT and CM: His₆-PfAtg8^{CM} and His₆-PfAtg8^{WT} were injected over MBP-PfAtg3-immobilized CM3 chip at 20 $\mu\text{L}/\text{min}$ with 6 2-fold dilutions at the lowest concentration, increasing to 250 nM. Injection volumes were 40 μL and 60 μL and dissociation times were 120 sec and 150 sec, for CM and WT, respectively.

Measuring inhibition of PfAtg8-PfAtg3 interaction by PfAtg3¹⁰³⁻¹¹⁰ and PfAldolase peptides: A dilution series was set up with His₆-PfAtg8^{CM} 6 at a constant concentration of 200 nM and 9 3-fold dilutions of peptide starting at 400 μM in RB. His₆-PfAtg8^{CM} without peptide was run at the beginning and end of each concentration series to check for chip decay or decreases in protein activity. Each concentration was run in triplicate at 20 $\mu\text{L}/\text{min}$, with 60 μL injections and a 150 sec dissociation time.

Measuring inhibition of PfAtg8-PfAtg3 by fragments: His₆-PfAtg8^{CM} at a constant final concentration of 250 nM was injected in the presence of a 2-fold dilution series of fragments starting at 1 mM, with eight concentrations in RB supplemented with 3% DMSO (no BSA). His₆-PfAtg8^{CM} without fragments was run at the beginning, middle, and end of each concentration series to control for loss of protein activity over time. Each concentration was run in triplicate at 50 $\mu\text{L}/\text{min}$ with 30 μL injections and a 40 sec dissociation time starting with the lowest fragment concentration.

Determination of binding of PfAtg8^{CM} and hLC3: Binding was measured using purified His₆-PfAtg8^{CM} and hLC3 with 7 2-fold dilutions in 1.06 mM KH₂PO₄, 5.6 mM Na₂HPO₄, 154 mM NaCl, pH 7.4, 0.01% v/v P20, 0.2 mg/mL BSA (RB2) starting at 625 nM injected at 40 µL/min at 37°C.

Identification of fragments that bind Atg8 near the cargo receptor site: His₆-PfAtg8 WT and K19E/K47E were immobilized onto different flowcells of an NTA-Chip (GE Healthcare) preconditioned with nickel. A library of 352 fragments (library 1 from Zenobia Therapeutics, Inc.) was screened for binding at a concentration of 0.1 mM diluted in running buffer containing 50 mM HEPES pH 8, 50 mM NaCl, 3% DMSO, 0.005% v/v P20. 50 µL of each compound was injected at 20 µL/min and dissociation time of 150 sec. Data was processed in Scrubber (BioLogic™ Software) using double referencing method and correcting for the change in refractive index from DMSO with a DMSO calibration curve.

3. Results

Recombinant *Plasmodium* Atg8 expressed in *E. coli* showed very low solubility in all species tested (PbAtg8, PvAtg8, and PfAtg8). While a mutation from Lys26 to Pro was found to prevent aggregation while retaining full autophagic activity for ScAtg8 (Kumeta et al., 2010) proline is already present at this position in *Plasmodium*. Through site-directed mutagenesis and thermal shift assays (TSAs), a triple cysteine mutant (C37I, C119S, C122S) was identified that had significantly increased solubility and is referred to here as the cysteine mutant (CM) (S1). Because Cys37 was predicted with homology modeling to be buried within the Ub-like core, we mutated this residue to the hydrophobic amino acid, Ile. Exposed free cysteines are often a source of aggregation and in previous otherwise refractory proteins, mutagenesis has been successful in yielding crystals (Derewenda, 2010). Similar procedures to decrease aggregation, such as cysteine carboxymethylation, were not found to alter the overall protein structure when compared to native protein (Eiler et al., 2001).

An Atg8 family interacting motif (AIM) has been identified in Atg8's conjugating enzyme, Atg3 as well as in Atg8-binding proteins involved in the cytoplasm-to-vacuole targeting (Cvt) pathway in yeast and selective autophagy in mammals. The motif is ΘXXΓ, where Θ is aromatic and Γ is hydrophobic, with nearby acidic residues preferred (Noda et al., 2010; Noda et al., 2008; Rozenknop et al., 2011). We identified a potential AIM in PfAtg3 (Figure 1A) and mutated Trp105 in this region to alanine. PfAtg3 was recombinantly expressed with an MBP tag and binding to PfAtg8 was tested through MBP pulldown assays. Atg8 binding, as quantified with ImageJ (Abramoff et al., 2004), was decreased by 35% in MBP-Atg3^{W105A} as compared to wild type (Figure 1B). TSAs confirmed the decreased binding in the W105A mutant is not due to destabilization of the tertiary structure (S2). We next tested whether residues 103-110 corresponding to the AIM was sufficient for binding PfAtg8 through a synthetic peptide. The PfAtg3¹⁰³⁻¹¹⁰ peptide led to a concentration-dependent positive shift in the T_m of PfAtg8 in TSAs, indicating binding and stabilization of PfAtg8, while a control peptide of PfAldolase³⁶⁴⁻³⁶⁹ did not lead to a significant shift in T_m (Figure 2C). To exclude nonspecific stabilization by the peptide, we evaluated whether the PfAtg3¹⁰³⁻¹¹⁰ peptide could compete with full length PfAtg3 for PfAtg8 binding in SPR studies. PfAtg8 was injected over a chip containing immobilized MBP-PfAtg3 with increasing concentrations of PfAtg3¹⁰³⁻¹¹⁰ or PfAldolase³⁶⁴⁻³⁶⁹ peptides and the SPR response was measured. The PfAtg3¹⁰³⁻¹¹⁰ peptide inhibited interaction with full length PfAtg3 in a concentration-dependent manner, whereas the control peptide did not have an effect (Figure 2D), supporting the identification of a conserved AIM in PfAtg3, WLLP, responsible for PfAtg8 binding.

3.1 Overall structure of the PfAtg8^{CM}-PfAtg3¹⁰³⁻¹¹⁰ complex

Protein crystals of PfAtg8^{CM} were obtained in the presence of the PfAtg3¹⁰³⁻¹¹⁰ peptide (Figure 2). The structure of the complex was solved by molecular replacement with an ensemble of 20 models using Phaser (McCoy et al., 2007) as described in the materials and methods section. The structure was refined to an $R_{\text{work}}/R_{\text{free}}$ combination of 20.5/27.9% with a twin fraction of 9.4% and zero Ramachandran outliers (Table 1). The asymmetric unit contained three copies of Atg8 and three copies of the bound peptide corresponding to a 1:1 ratio of protein to peptide (Figure 2, S3). The buried surface area between any two given chains of PfAtg8 is approximately 1,330 Å², not indicative of a stable multimer in solution. The three copies of PfAtg8 superimpose with an r.m.s.d. of 0.02 Å for 120 C_α-atoms. The PfAtg3¹⁰³⁻¹¹⁰ peptide also superimposes well except for the N- and C-terminal residues, which adopt different orientations in the crystal lattice. (S4). PfAtg8 contains an N-terminal domain composed of two alpha helices attached and stabilized via hydrogen bonding to a C-terminal ubiquitin (Ub)-like domain as has been visualized in other Atg8 homologues (Knight et al., 2002; Noda et al., 2008; Paz et al., 2000; Sugawara et al., 2004). Our structure additionally contains two short beta strands, connected by a beta turn between $\alpha 3$ and $\beta 5$ which is absent from all other homologue structures (Figure 2A, S5). *Plasmodium* Atg8 contains an insertion of nine residues only conserved within *Apicomplexa* that comprise $\beta 3$ and the turn in the loop. $\beta 3$ forms numerous hydrogen bonds with the antiparallel $\beta 4$ strand. The secondary structure observed in this region may be due to crystal contacts as the loop forms numerous hydrogen bonds with the PfAtg3¹⁰³⁻¹¹⁰ peptide of another chain and may explain why we were unable to obtain crystals without the peptide (Figure 2C).

3.2 The cargo-receptor site

Two hydrophobic pockets in PfAtg8 are responsible for binding the PfAtg3¹⁰³⁻¹¹⁰ peptide similarly to the Atg8-Atg19^{peptide} and microtubule-associated protein 1 light chain 3 (LC3)-p62^{peptide} structures (Ichimura et al., 2008; Noda et al., 2008) (Figure 3A, B, S6). These pockets are termed the W- and L-site and together make the cargo receptor site (Ho et al., 2009; Noda et al., 2010). The side chain of PfAtg3 W105 binds deeply inside the W-site of PfAtg8, which consists of residues L4, E16, T17, I20, P29, V30, V31, K47, F48, L49, and Y112. P108 of the Atg3 peptide fits in the L-site, consisting of residues R27, F48, L49, V50, P51, M54, E58, F59, I62 and M99 (Figure 3B). The peptide additionally makes hydrogen bonds with the side chains of K45, E58, and H66 surrounding the W- and L-site. The overall conservation of the binding site makes it a challenging task to design specific inhibitors targeting *Plasmodium*, however differences exist in amino acid composition (Figure 3B), electrostatic surface potential and cargo receptor pocket size. For example, the W-site of *Plasmodium* is ~100 Å³ larger than the corresponding site in the human orthologues, microtubule-associated protein 1 light chain 3 (LC3), and Golgi-associated ATPase enhancer of 16 kDa (GATE-16) (Figure 3C). Figure 3D compares the electrostatic surface on the Atg3-binding face of PfAtg8 with GATE-16 and LC3. All three proteins have a prominent positively charged band on the cargo receptor site face. Relative to PfAtg8, the orientation of this band is tilted 40° and 55° in GATE-16 and LC3, respectively. Both PfAtg8 and GATE-16 show striking differences compared to LC3, where the positive charge is concentrated around the central area of the protein and $\alpha 1$ and $\alpha 2$ form an acidic patch above the W-site that is basic in the other proteins. The L-site of GATE-16 is predominately positively charged whereas PfAtg8 is more neutral in this region. An acidic patch formed between $\alpha 3$ and the loop between $\beta 1$ and $\beta 2$ in both GATE-16 and LC3 is basic and neutral in our structure and forms a deeper groove due to the $\beta 3$ strand inserted in the PfAtg8 structure.

3.3 PfAtg8 is expressed in the parasite blood stage and recombinant PfAtg8 interacts with endogenous proteins in the blood stage

To study expression and localization of Atg8 in the parasite, we used the rodent model *P. berghei* (Pb) as PbAtg8 has 98% sequence similarity to PfAtg8. Immunofluorescence assays (IFA) in *P. berghei*-infected red blood cells showed high expression of PbAtg8 associated with well-defined structures that were proximal to the nucleus in all stages, from ring forms (2-h post-infection (p.i.)) to schizonts (48-h p.i.) (Figure 4A). We next tested whether recombinant PfAtg8 could interact with the endogenous autophagy machinery proteins from *P. falciparum* blood stages. PfAtg8^{WT} was immobilized via a hexahistidine tag and incubated with blood stage parasite lysate. A control column containing only Nickel-NTA resin was also incubated with lysate. Bound proteins were eluted with imidazole and separated using SDS-PAGE. Bands enriched in the Atg8 bait sample were cut out and analyzed with tandem mass spectrometry (MS/MS) (Figure 4B). A significant number of unique peptides matching PfAtg3, representing 11% sequence coverage, were present in the PfAtg8-immobilized sample but not in the control (S7). PfAtg3 is predicted to be 35 kDa, however the peptides were identified in a band migrating at a molecular weight (MW) of 20 kDa and therefore likely represents a degradation product. The location of the peptides indicates the protein was C-terminally degraded. In addition peptides for small GTP binding protein sar1 (PlasmoDB accession code PF3D7_0416800) were identified in this band, corresponding to 16% sequence coverage (data not shown). PfAtg3 peptides, representing 10% coverage, were also identified when PfAtg8^{CM} was used as bait in an independent experiment and were detected in a band migrating at 35 kDa (S7). These results strongly suggest that PfAtg8 WT and PfAtg8^{CM} interact with PfAtg3 and that our recombinantly expressed proteins are properly folded. A direct interaction between PfAtg8 and PfAtg3 was confirmed through co-migration with size exclusion chromatography and dynamic light scattering and indicate a 1:1 complex, in agreement with our crystal structure (Figure 5A,B). We next measured the affinity of the interaction using SPR. PfAtg8^{CM} was injected at six twofold serial dilutions over a CM3 chip containing MBP-PfAtg3 and response versus time was measured (Figure 5C). A K_D of 326 nM was determined for PfAtg8^{CM}, indicating a strong interaction with Atg3. Similar results were obtained from the WT construct (K_D =290 nM), validating the use of the PfAtg8^{CM} construct in our studies (Figure 5D). Our co-crystal structure revealed that PfAtg8 binds PfAtg3¹⁰³⁻¹¹⁰ with a pocket well conserved across species; we therefore tested whether PfAtg3 could bind the human homologue LC3. The SPR response from hLC3 and PfAtg8^{CM} injected over the MBP-PfAtg3 CM3 chip was compared. Surprisingly hLC3 did not show concentration-dependent binding indicating the human and *Plasmodium* systems are not interchangeable (Figure 5E).

3.4 Identification of Atg3-interacting residues

To confirm the importance of the pockets implicated in our crystal structure, as well as to identify other regions important for the Atg3 interaction, we mutated residues in (K47E, L49A, and R27E) and near (K47E/K19E, E44A/K45S/K46A) the cargo receptor site. A variant with deletion of residues 68 to 76, which formed a β -hairpin in our structure and is only present within *Apicomplexa* was also tested (Figure 6A, B). PfAtg8 variants were tested for binding using an MBP pulldown assay with immobilized MBP-PfAtg3 as before (Figure 6C). R27E, K47E, and L49A W-site mutations all reduced binding to MBP-PfAtg3. The triple mutant Atg8^{E44A/K45S/K46A}, containing mutations near the W-site showed moderate decreases in binding. These results are in agreement with our structural studies where mutation of K45S in the E44A/K45S/K46A mutant eliminates a salt bridge with D104 of Atg3. In one of the peptide orientations, N103 hydrogen bonds with K47 of PfAtg8, which would be disrupted in our K47E mutant. Additionally, shortening L49 to alanine creates a less hydrophobic environment for Atg3^{W105}. TSAs suggest PfAtg8^{R27E} is less stable than WT (S8), our structure confirms that the residue is involved in the Atg3

interaction (Figure 3B). The destabilization in the mutant can be partially explained by repulsive electrostatic forces between E27 and the backbone carbonyl of L49 in the Ub-like core of the mutant. While the structure verifies that K19 is surface accessible, the Atg8^{K19E/K47E} mutant exhibited similar binding as Atg8^{K47E}, suggesting this region of $\alpha 2$ is not extensively involved in binding Atg3. Strikingly, PfAtg8^{68-76 Δ} binding was reduced by 80%, as quantified with ImageJ (Abramoff et al., 2004), suggesting a large interaction surface between PfAtg8 and PfAtg3 (Figure 6C). These results confirm that in addition to the importance of the cargo receptor site, the unique Apicomplexan loop of *Plasmodium* Atg8 is necessary for interaction with PfAtg3 (Figure 6D). This may in part explain why hLC3 did not interact with PfAtg3 in our SPR studies (Figure 5E) despite a 74% similarity between PfAtg8 and hLC3 for 19 residues in the cargo receptor site (Figure 3B, 6A).

3.5 Identification of inhibitors against the PfAtg8-PfAtg3 interaction

We sought to identify molecules that could be optimized to inhibit the Atg8-Atg3 interaction in *Plasmodium*. A library of 352 small molecular fragments with an average molecular weight of 150 Da from Zenobia Therapeutics was chosen because of the high solubility, non-toxic nature, and functional diversity of the compounds as well as the compatibility of the library with fragment-based co-crystallization experiments. The library was screened for binding to PfAtg8 with SPR. To enrich for molecules binding in or near the W-site of PfAtg8, we immobilized His₆-PfAtg8^{WT} and His₆-PfAtg8^{K19E/K47E} onto a Nickel-NTA chip. Molecules were sequentially injected over the immobilized proteins and the response between wild type and the W-site mutant compared (Table 2, S9). Hits eliciting 1.5 x's the response (RU) over noise and 1.5 x's greater normalized binding for wild type than mutant were further validated for PfAtg8 binding with TSAs (Figure 7A). Likely due to the high internal fluorescence of F410 and F470, we were not able to measure the effect of these compounds on the T_m of PfAtg8. Three fragments, F268, F408, and F537, showed significant binding in the TSA and were tested, along with F410, for their ability to inhibit the PfAtg8-PfAtg3 interaction in SPR competition studies. PfAtg8^{CM} was injected at a constant concentration over the MBP-PfAtg3 chip with an increasing concentration of fragments F268, F408, F410, and F537. While F268 showed no inhibition and F537 showed only slight inhibition even at 1 mM (S10), F410, 1,2,3-trihydroxybenzene (common name pyrogallol), and F408, 1,2,4-trihydroxybenzene, decreased the SPR response in a concentration-dependent manner, indicating inhibition of the PfAtg8-PfAtg3 interaction with an IC₅₀ of 150 μ M and 1 mM, respectively (Figure 7B). However injection of PfAtg8 with the highest concentration of F410 led to a slight increase in the response and injection of F410 alone over the chip led to a concentration-dependent response, indicating F410 binds either PfAtg3 or the MBP tag in addition to PfAtg8 (S11). Therefore it is likely the actual IC₅₀ may be lower. The site of interaction on MBP-PfAtg3 has not yet been determined, however the decrease in PfAtg8 binding in the SPR inhibition studies indicates that at least one important interaction site for F410 is preventing formation of the *Plasmodium* Atg8-Atg3 complex.

4. Discussion

Our biochemical and structural studies indicate that PfAtg8 binds an AIM in PfAtg3. To date, the crystal structure of Atg3 has only been elucidated in yeast. It interacts with the W- and L-site of Atg8 through an AIM located in the handle region of Atg3 (Noda et al., 2010). The handle region is severely truncated in *Plasmodium* Atg3, similarly as in the human homologue (S12). Additionally, the PfAtg3 AIM differs from known motifs in yeast and humans by the presence of proline, rather than leucine or isoleucine at the fourth position of the motif (Θ XXI). Prolines are often found at the edges of beta-strands suggesting the possibility this region forms a beta strand in solution. Recent NMR and x-ray studies on

yeast Atg7 and Atg8 suggest that substrates can bind the Atg8 cargo receptor site without forming an intermolecular beta sheet (Noda et al., 2011). Crystallization of full length *Plasmodium* Atg3 will help elucidate the secondary structure of the AIM while solution NMR experiments may reveal the different states of this region in the presence or absence of a substrate.

PfAtg8 shows the greatest sequence and structural similarity, with minimal r.m.s.d., to GATE-16, implicated in intra-Golgi trafficking and macroautophagy in mammals. Like GATE-16, it does not contain the extra beta strand located between $\beta 3$ and $\alpha 4$ in LC3 and TbAtg8. Considering the 74% sequence similarity between the cargo receptor site of the human homologue LC3 and *Plasmodium* Atg8, it is surprising that LC3 cannot interact with PfAtg3. However, differences in the extent and orientation of charges, as well as the larger size of the pocket in PfAtg8 may explain the lack of binding in the heterologous system. These charge and size differences can be exploited to selectively target the parasite autophagy system. Additionally, our structure contains a semi-ordered insertion of two beta strands between $\alpha 3$ and $\beta 5$ with respect to structures in other species. Our mutagenesis studies suggest an important role for the Apicomplexan-specific loop in mediating interactions between PfAtg8 and PfAtg3. Together our structural and biochemical studies implicate a unique pocket formed between the loop and the Ub-like core, which could potentially be targeted with a small molecule inhibitor. Further studies are required to determine how this region interacts with PfAtg3 and if it is amenable to drug design. While the cargo receptor site is well conserved across Atg8 homologues, there is more divergence on the other side of the protein, as well as regions just outside the receptor site including $\alpha 1$, which may be able to serve as anchoring points during drug design (S13).

Autophagy is increasingly being recognized as an important target in many diseases, including cancer, neurodegeneration, and other protozoan infections (Brennan et al., 2011; Levine and Kroemer, 2008; Levine et al., 2011). The Atg8-Atg3 interaction has emerged as an important modulator of Atg8 lipidation and autophagy. The viral FLICE-like inhibitor protein (FLIP) (Lee et al., 2009) binds Atg3 and competes for LC3 interaction to prevent LC3 lipidation and autophagy-mediated host cell death. We envision a similar strategy in developing a compound that will disrupt the Atg8-Atg3 interaction in the malaria parasite. Previous studies have looked at the role of autophagy in the liver stage of infection and implicate a role for autophagy in microneme disposal (Duszenko et al., 2011). Our IFA studies and parasite lysate pulldown assays together confirm protein expression of Atg8 and Atg3 in the asexual blood stage. Our results in *P. berghei* are in agreement with recent findings in *P. falciparum* showing punctate expression in the blood stage (Kitamura et al., 2012). Kitamura et al. describe co-localization of PfAtg8 with the apicoplast markers histone-like protein, heat unstable (HU) and apicoplast localizing protein, (ACP) suggesting a hitherto unpredicted function distinct from autophagy. Although the function of Atg8 at the apicoplast membrane is unclear, their work shows it is lipidated and thus still requires interaction with its conjugating enzyme, Atg3. Therefore, in addition to its therapeutic application, a drug targeting the Atg8 conjugation step would help elucidate Atg8's role in the different stages of the malaria life cycle.

We have identified a fragment that inhibits the *Plasmodium* Atg8-Atg3 interaction with an IC_{50} of 150 μM , which can serve as a guide for subsequent antimalarial drug design studies. Fragment screening is increasingly being appreciated in the drug discovery realm for its ability to more efficiently sample chemical space, saving time and money (Ciulli and Abell, 2007). Fragment screening has been successfully employed in the identification of inhibitors against beta-secretase for Alzheimer's disease (Edwards et al., 2007; Geschwindner et al., 2007; Murray et al., 2007), Urokinase (Frederickson et al., 2008), Phosphodiesterase 4 (Card et al., 2005), Thrombin (Howard et al., 2006), and the Aurora-kinase inhibitor (Warner et

al., 2006). Although drug design has long focused on targeting single enzyme active sites, PPIs are increasingly considered the next generation of drug targets (Mullard, 2012). Recent successes include the completion of Phase II clinical trials of navitoclax (Rudin et al., 2012) as well as the promising targeting of the BRD4/histone interaction in squamous carcinomas (Filippakopoulos et al., 2010) and the MDM2-p53 interaction to slow tumor growth (Rew et al., 2012). Though the cancer field has led the way in PPI drug development, the strategy is progressively being sought in other diseases, including HIV and parasitic infections (Mullard, 2012; Taylor et al., 2011). Our studies of the *P. falciparum* Atg8-Atg3 system provide the basis for a structure-based drug design campaign with already identified fragments near the cargo receptor site. The observed inhibition from these fragments falls well within the expected range for small molecule fragments (Ciulli and Abell, 2007; Schulz and Hubbard, 2009). Future studies will elucidate the molecular binding site of the hits, providing insight for optimization through tethering into drug-like molecules with increased inhibition and specificity.

5. PlasmoDB and protein data base accession codes

The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 4EOY).

PlasmoDB gene accession numbers

PfAtg8 (PF3D7_1019900), PfAtg3 (PF3D7_0905700.2), PfAtg12 (PF3D7_1470000), PfAtg7 (PF3D7_1126100), PfAtg4 (PF3D7_1417300)

Ethics statement

5–8 week old female Swiss-Webster were purchased from Taconic (Hudson, NY). All animal procedures were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University (Protocol Number MO11H146) following the National Institutes of Health guidelines for animal housing and care.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Pf	<i>P. falciparum</i>
Pb	<i>P. berghei</i>
Pv	<i>P. vivax</i>
SPR	surface plasmon resonance
TSA	thermal shift assay

EMSA	electromobility shift assay
WT	wild type
CM	cysteine mutant
AIM	Atg8 family interacting motif
T_m	melting temperature
Atg	autophagy
RU	response units
SD	standard deviation
Ub	ubiquitin
RBC	red blood cell
IFA	immunofluorescence assays
p.i.	post-infection
r.m.s.d.	root-mean-square deviation

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Figure 1. Identification of an AIM in PfAtg3

A. Alignment of known AIMs in other species with potential AIMs in PfAtg3. Aromatic residues at position 1 are denoted in green, hydrophobic residues at position 4 in blue, and acidic residues in red. B. *In vitro* MBP pulldown assay. His₆-PfAtg8^{CM} was co-expressed with MBP-PfAtg3 variants and purified with an amylose affinity column. Left: Bound proteins were subjected to SDS-PAGE and visualized with coomassie stain. Asterisk denotes MBP-PfAtg3 and plus sign denotes His₆-PfAtg8. Gel represents one of three independent experiments. Right: Binding was quantified with ImageJ as the ratio of PfAtg8 to PfAtg3, normalized to WT binding levels. Error bars represent means and standard deviation (SD) from three independent experiments. C. TSA with peptides. His₆-PfAtg8^{CM} was incubated with increasing concentrations of PfAtg3 and PfAldolase peptides and fluorescence of SYPRO® Orange dye was measured from 20–80°C over 60 min. D. SPR competitive binding assay. His₆-PfAtg8^{CM} was injected over an MBP-PfAtg3 conjugated chip with increasing concentrations of PfAtg3 and PfAldolase peptide and the binding response was measured.

Figure 2. The overall structure of PfAtg8^{CM}-PfAtg3¹⁰³⁻¹¹⁰

A. The overall structure of PfAtg8^{CM} is shown as a cartoon representation with the PfAtg3-peptide as a stick model. Beta sheets are colored yellow and alpha helices colored red. B. Stereo figure of PfAtg3¹⁰³⁻¹¹⁰ peptide bound to cargo receptor site of PfAtg8, shown with surface representation. 2Fo-Fc map electron density for peptide is shown at 1 σ (blue) and Fo-Fc difference map is shown at 5 σ (red) and 3 σ (green) level with peptide as a stick model in cyan. C. Contacts between Atg3¹⁰³⁻¹¹⁰ peptide (cyan) and PfAtg8 in the crystal structure. The peptide, shown as sticks in cyan, makes numerous hydrogen bonds, number denotes distance in angstroms, with the loop region (residues 76-82) of the nearest symmetry mate (red) as well as several hydrogen bonds and hydrophobic interactions with the cargo receptor site of PfAtg8 (green), shown in inset.

Figure 3. Comparison of the cargo receptor site in *Plasmodium* and human Atg8 homologues

A. The overall structure of PfAtg8 displayed as a cartoon with the cargo receptor site of PfAtg8 shown as a surface representation. The PfAtg3-peptide is represented as a stick model in cyan. Residues identical in the nearest human homologue, GATE-16, are shown in yellow, similar residues are colored orange, and different residues are colored red. B. Close-up of cargo receptor site. Residues composing the W-site (top) and L-site (bottom) of PfAtg8 are shown as sticks with the same color coding for conservation as in A. C. Comparison of the cargo receptor site pocket of Atg8 homologues. The W- and L-site of PfAtg8 (blue, 511 Å³), human GATE-16 (green, 308 Å³) and human LC3 (brown, 403 Å³) are viewed from side-, top- and bottom with the PfAtg3¹⁰³⁻¹¹⁰ peptide represented in sticks for orientation. Pocket calculation and visualization were performed with Fred Receptor and Vida from the OpenEye suite (www.eyesopen.com). PDB codes of structures used for calculation are shown in parentheses. D. Electrostatic surface potential of Atg8 homologues. Dashed line indicates orientation of positively charged band in Atg8 homologues. The band is tilted 56° in PfAtg8, 15° in GATE-16, and 2° in LC3 from the x-axis.

Figure 4. *Plasmodium* Atg8 expression, localization, and interaction with endogenous parasite proteins

A. IFA of *Plasmodium berghei* blood forms from ring to late schizonts isolated from parasitized mice, stained with anti-PbAtg8 antibodies and DAPI, reveals high expression of PbAtg8 throughout the blood stage. B. Parasite lysate pulldown assay. His₆-PfAtg8 was attached to Ni-NTA resin and incubated with RBC stage *P. falciparum* lysate. Bound proteins were subjected to SDS-PAGE and visualized with SYPRO® Ruby staining. Asterisk denotes band in which PfAtg3 peptides were identified through MS/MS.

Figure 5. *In vitro* PfAtg8 and PfAtg3 Interaction Studies

A. Size exclusion chromatography of His₆-PfAtg8 (green), His₆-PfAtg3 (blue), and the complex (brown) compared with a molecular weight standard in red, run under identical conditions. Inset shows presence of PfAtg8 and PfAtg3 in complex peak (brown), visualized with SDS-PAGE. B. Dynamic light scattering analysis of His₆-PfAtg8, His₆-PfAtg3, and the complex with the radius and predicted MW for a globular protein. C. SPR-based Biacore studies. MBP-PfAtg3 was immobilized on a CM3 chip and PfAtg8^{CM} was injected at 6 twofold serial dilutions in triplicate. Sensorgram shows response units over time of run. Orange lines indicate fit used by Scrubber (BiologicTM Software) data processing software to derive an association rate of $5.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, dissociation rate of 0.17 s^{-1} , and equilibrium dissociation constant of 326 nM. D. SPR binding studies of PfAtg8^{WT} to PfAtg3. His₆-PfAtg8^{WT} was injected at 6 twofold dilutions in triplicate. An association rate of $54.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, dissociation rate of 0.13 s^{-1} , and equilibrium dissociation constant of 290 nM was derived for the interaction with MBP-PfAtg3. E. SPR studies to determine PfAtg8 and hLC3 binding to PfAtg3. Binding to the MBP-PfAtg3 CM3 chip was determined by SPR with triplicate injections of PfAtg8^{CM} (in blue) and hLC3 (in green) at seven concentrations. Inset: SPR response versus concentration of PfAtg8^{CM} and hLC3.

Figure 6. Identification of Atg3-interacting residues in PfAtg8

A. Multiple structural sequence alignment of *Apicomplexa*, human, and yeast Atg8 homologues. Abbreviations: *Plasmodium* (P), *Toxoplasma* (T), *Cryptosporidium* (C), *Eimeria* (E), and *Babesia* (B) Atg8 homologues, from *Apicomplexa*, were aligned with *Homo sapiens* LC3 and *Saccharomyces cerevisiae* Atg8 using 3D-Coffee (www.tcoffee.org) with the PfAtg8, hLC3, and ScAtg8 structures (PDB codes 1EOY, 2ZJD, and 2ZPN, respectively), visualized with Esript (<http://esript.ibcp.fr/ESPrpt/>). Structural elements shown above sequence are based on the PfAtg8 crystal structure. Loop region unique to *Apicomplexa* is highlighted in yellow and cargo receptor site residues are denoted with asterisks. B. Mutations made to PfAtg8 are color-coded on the crystal structure. C. *In vitro* MBP pulldown assay. Immobilized MBP-PfAtg3 was incubated with His₆-PfAtg8 mutants. Left: Bound proteins were subjected to SDS-PAGE and stained with coomassie stain. Asterisk denotes MBP-PfAtg3 and plus sign denotes His₆-PfAtg8. Gel is representative of three experiments. Right: Binding was quantified with ImageJ as the ratio of bound PfAtg8 to PfAtg3, normalized to WT levels. Error bars represent the mean and SD of three experiments. D. Surface representation of PfAtg8 highlighting groove formed between loop deletion (68-76 Δ) in yellow and the E44A/K45S/K46A mutant in orange which are involved in the Atg3 interaction. PfAtg3¹⁰⁵⁻¹¹⁰ peptide shown as sticks in cyan.

Figure 7. Identification of fragments that inhibit the PfAtg8-PfAtg3 interaction

A. TSA of SPR hits with His₆-PfAtg8^{CM}. The melting temperature of His -PfAtg8^{CM} 6 was measured with increasing concentration of fragments. B. SPR-based inhibition assay. His -PfAtg8^{CM} 6 was injected over the MBP-PfAtg3-conjugated chip with increasing concentrations of fragment. Solid line indicates mean response with no fragment for reference. Horizontal dashed line indicates half the normal response and vertical line denotes the IC₅₀.

Table 1

Data collection, phasing and refinement statistics

	Native	
Data collection	SSRL12-2	ALS5-01
Space group	P2 ₁	P2 ₁
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	33.30, 111.08, 57.09	33.26, 110.33, 56.57
α , β , γ (°)	90, 92.58, 90	90, 92.49, 90
Wavelength	1.0332	0.9774
Resolution (Å)	31 - 2.2 (2.24-2.18)	20 - 2.25 (2.3-2.25)
<i>R</i> _{merge}	21.5 (91.8)	10.1 (44.7)
<i>I</i> / σ <i>I</i>	7.3 (1.6)	9.05 (2.2)
Completeness (%)	87.6 (45.0)	88.2 (46.2)
Redundancy	2.1 (2.2)	1.13 (1.54)
Wilson B (Å ²)	29.08	23.94
Beam size (μm)	6 × 9	100 × 100
Refinement		
Resolution (Å)	31 - 2.2	20 - 2.2
No. reflections	21538	15705
<i>R</i> _{work} / <i>R</i> _{free}	20.5/27.9	23.8/28.7
<i>Refined twin fraction</i> (%)	9.6	40.5
No. atoms		
Protein	3289	3289
Ligand/ion	23	23
Water	331	331
<i>B</i> -factors (Å ²)		
Protein	31.6	19.2
Ligand/ion	51.4	57.6
Water	46.2	54.77
R.m.s deviations		
Bond lengths (Å)	0.008	0.012
Bond angles (°)	1.197	1.306
<i>Ramachandran main chain dihedral analysis</i> **		
favored	96.9% (370/382)	97.1% (371/382)
allowed	3.1% (12/382)	2.9% (11/382)
outliers	0	0
<i>Molprobability side chain rotamer analysis</i> **		
bad rotamers	1.3% (5/372)	0.81 (3/372)

* Highest resolution shell is shown in parentheses.

** According to Molprobability (Lovell et al., 2003).

Table 2

Binding of selected fragment hits from first pass fragment screen. Fragments were screened against PfAtg8 WT and K19E/K47E (Mut) with SPR. Columns show response from fragments divided by overall noise for WT and Mut. Last column indicates binding of fragments to WT response over noise divided by Mut response over noise. The threshold for a hit was at least 1.5 x's response over noise for WT and at least 1.5 x's normalized response for WT than Mut.

Fragment	IUPAC Name	WT _{RU/NOISE}	Mut _{RU/NOISE}	(WT _{RU/NOISE})/(Mut _{RU/NOISE})
408	1,2,4-benzenetriol	5.33	1.49	3.56
410	1,2,3-benzenetriol	22.79	3.68	6.19
537	4-nitrocatechol	2.39	1.25	1.90
792	1,3-indandione	2.38	1.11	2.15
268	6,7-dihydroxycoumarin	1.79	0.48	3.73
470	4-chloro-7-nitrobenzofurazan	6.44	4.22	1.52