The Study of Autophagy in *Plasmodium falciparum*

Dissertation

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By

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Abstract

The *Plasmodium falciparum* genome encodes a limited number of putative autophagy genes, specifically the four genes involved in Atg8 lipidation, an essential step in formation of autophagosomes. In other eukaryotic systems, Atg8 lipidation requires the E1-type ligase Atg7, an E2-type ligase Atg3, and a cysteine protease Atg4. We have confirmed that these four putative *P. falciparum* ATG (PfATG) genes are transcribed during the parasite’s erythrocytic stages. We expect that these putative autophagy genes are the essential players of a functional Atg8 lipidation pathway in *P. falciparum*. To assess PfAtg8 localization, we have expressed mCherry tagged PfAtg8 in the parasite and observed an unusual tubular localization. This has recently been confirmed as localization to the apicoplast, a unique organelle in apicomplexan parasites. This leads to our hypothesis of PfAtg8 involvement in the maintenance (e.g., turnover, expansion, segregation) of this essential organelle, an interesting and novel role for an autophagy pathway. Recent efforts by our lab have focused on dissecting the biochemistry of this pathway. We have genetically engineered parasites to allow for regulatable expression of the activating enzyme PfAtg7. Upon PfAtg7 attenuation, parasites exhibit slow growth in culture, indicating the essentiality of this enzyme for normal growth. Furthermore, we have recently acquired a stable
parasite line harboring a transposable element upstream of PfATG7 that also results in slow parasite growth due to a reduction in PfATG7 expression as confirmed by quantitative PCR. We have also modified the PfATG7 locus to introduce a C-terminal hemagglutinin (HA) tag. Detection of PfAtg7-HA by western blot has revealed the presence of two species, one at the predicted 150kDa and one at 65kDa. Experiments are in process to determine which form(s) are active and to confirm E1-type ligase activity.

To assess the role of PfAtg7 in apicoplast maintenance, we are evaluating inhibitors of the apicoplast's isoprenoid biosynthesis pathway. Parasites with attenuated levels of PfATG7 are more sensitive to fosmidomycin, a drug that targets an enzyme in the non-mevalonate pathway. This suggests that PfAtg7 is involved in maintaining properly functioning apicoplasts. Measuring the cell cycle time, number of merozoites, and doubling time indicates a delayed death-like phenotype in parasites with attenuated PfAtg7, which suggests an apicoplast defect. Experiments are ongoing to determine the exact mechanism responsible for slow growth of these parasites. As to translational implications of this research, it has been shown possible to develop specific inhibitors for E1-type ligases. Such inhibitors, like the mammalian NEDD activating enzyme, are currently in clinical trials as anticancer therapeutics. We propose a similar strategy in the development of specific and selective PfAtg7 inhibitors. If successful, these inhibitors would represent a novel class of antimalarials.
Dedication
This document is dedicated to Fred Drew and his heroic battle against diamond-blackfan anemia, with hope that when he is a famous astronaut this will just be the first of many challenges that he has conquered in life.
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First and foremost, I would like to acknowledge my advisor Mark Drew, without whom this work would not have come to fruition. Many factors contributed to my decision to join the Drew lab 6 years ago. The deciding factor for me was based on the advisor-mentee relationship. And although it was new and raw, I could sense a growing rapport between Mark and me. The passion that he has for his research is grossly evident anytime you “talk science” with him. One thing I have probably told every new member of the Drew lab is if you are getting discouraged about your research, the best thing to do is schedule a meeting with Mark. I don’t know how many times I walked into his office feeling nothing but aggravation and left with a sense of hope and excitement about upcoming experiments and the prospects of my work. And even if I silently rolled my eyes every time he said, “this is an easy experiment” or “we should have a publication out really soon,” I always appreciated and looked up to him for his optimism and passion for science.

In life, as in science, he has this optimistic spirit. He maintains a healthy balance between lab life and his personal life and has allowed me to do the same throughout graduate school, a fact that allowed me work efficiently and to avoid “burn-out.” Mark has faced so many personal and professional trials and
remained this optimistic enthusiastic researcher, which is often hard to find these days. For these reasons and so many more, Mark has not only been an advisor to me, but someone I will look up to for the rest of my life and someone I will try to emulate when I face adversity. Thanks Mark, for all the memories and for helping me grow into an independent research scientist and overall a better person.

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For the last year and a half of my graduate school career Mark was in and out of Columbus for his son’s medical needs. It was really during this time that, after a lot of struggle, I learned to stand on my own two feet in lab. But no one walks alone and there were times that I needed extra guidance and council. In these times, I was not always the best at asking for help. Two faculty members
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“It takes a village to” …produce a dissertation. Thank you to every member of “my village.”
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**Fields of Study**

*Major Field:* Biomedical Sciences

*Area of Emphasis:* Microbial Pathogenesis
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List of Abbreviations

ACT – artemisinin combination therapies
AMA-1 – apical membrane antigen-1
AMP – ampicillin
ART – artemisinin resistance transporter
Atg – autophagy-related
ATP – adenosine triphosphate
BCA – bicinchoinic acid
CRT – chloroquine resistance transporter
CSP – circumsporozoite protein
Cvt – cytoplasm to vacuole targeting
DHFR – dihydrofolate reductase
EBL – erythrocyte-binding-like proteins
EDTA – Ethylenediaminetetraacetic acid
ETOH – ethanol
FDA – Food and Drug Administration
GFP – green fluorescent protein
HA – hemagglutinin
IPP – isopentenyl diphosphate
kDa – kilo dalton
LB – Lysogeny broth
Lm – *Leishmania major*
MEP – non-mevalonate pathway
MIC – microneme protein
MJ – moving junction
MLN – Millennium Pharmaceuticals, Inc.
NAE – NEDD8 activating enzyme
NEDD8 – neural precursor cell expressed, developmentally down-regulated 8
Nu – neutral
ORF – open reading frame
PAGE – polyacrilamide gel electrophoresis
PAS – phagophore assembly site
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PE – phosphatidylethanolamine
Pf – *Plasmodium falciparum*
PL – phospholipid
PMV – plasmepsin V
PVDF – polyvinylidene fluoride
qPCR – quantitative PCR
RBC – red blood cell
RBL – reticulocyte-binding-like proteins
Chapter 1: Introduction

1.1 Malaria

Malaria is a disease caused by protozoan parasites of the genus *Plasmodium*. These parasites have two hosts: the mosquito vector and a mammal, with disease transmitted by the bite of a female anopheline mosquito. These parasites have a complex life cycle, involving four niches: the mosquito midgut and salivary gland, human hepatocytes in the liver, and human erythrocytes in the bloodstream (Fig. 1.1). Malaria is prevalent in tropical and subtropical regions due to the warm temperature, rainfall and stagnant waters available as breeding grounds for mosquitoes. The spread of this disease is dependent on high human population density, high mosquito density and high transmission rates between humans and mosquitoes. Although malaria is preventable and curable, the disease causes hundreds of thousands of deaths each year [1]. This is partially due to the low economic status of the regions where malaria is endemic.

1.1.1 A Global Health Concern

Malaria is a major global health concern affecting nearly 40% of the world’s population. According to the World Malaria report of 2012, there are 104
Figure 1.1. Life Cycle of *Plasmodium* parasites. A. The exo-erythrocytic cycle occurs in the liver. (1) Sporozoites transfer from mosquito saliva to human lymph and blood system when an infected mosquito takes a blood meal. (2) After infecting liver cells, sporozoites undergo multiple rounds of mitosis until development into schizonts (3) and rupture from the liver cells (4) releasing merozoites into the bloodstream. B. The erythrocytic cycle (asexual) is responsible for symptoms of disease. (5) Merozoites invade erythrocytes developing into rings, trophozoites, and finally mature schizonts (d). (6) Erythrocytes rupture and release merozoites into the bloodstream to re-invade new erythrocytes. (7) Some parasites mature into the sexual stages, male (microgametocyte) and female (macrogametocyte) gametocytes, and are taken up by uninfected mosquitoes during a blood meal (8). C. Sexual reproduction occurs during the sporogonic cycle. (9) In the midgut of the mosquito the microgametocytes penetrate the macrogametocytes and form a zygote. (10) The zygote elongates into an ookinete and invades the midgut wall to develop into an oocyst (11). (12) When the oocyst ruptures it releases sporozoites that travel to the salivary gland and released into a human when the mosquito takes its next blood meal. Figure accredited to CDC - DPDx/ Alexander J. da Silva, PhD, and Melanie Moser, permission for use granted through the Public Health Image Library.

malaria endemic countries where 150-275 million cases of malaria are reported with 655,000 deaths, most of which are children under the age of 5 in sub-Saharan Africa [1]. This deadly disease costs billions of dollars worldwide in antimalarial efforts, including development of new antimalarial drugs, development of vaccines, and distribution of bed nets to endemic areas. In 2012 alone, the US contributed $1.84 billion towards international malaria control [1].
Malaria incidence and deaths have decreased in the last decade mainly due to the distribution of bed nets as a preventative measure. Despite this, the numbers are still astronomically high.

Malaria is still considered a curable disease, with many drugs on the market to combat it. The most effective antimalarial currently on the market is artemisinin and artemisinin combination therapies (ACTs), which are strictly controlled by the World Health Organization (WHO) [2]. Resistance to antimalarials is continually spreading and resistance to artemisinin has now been reported in 4 Southeast Asian countries: Cambodia, Myanmar, Thailand, and Vietnam [1]. With hope on the horizon for a vaccine, drug development remains a critical tool to combat malaria and decrease morbidity and mortality due to this disease.

1.1.2 Symptoms and Diagnosis

Five species of *Plasmodium* cause human disease, these include *P. falciparum, P. vivax, P. malariae, P. ovale* and, shown recently to infect humans, *P. knowlesi* [3]. Symptoms of malaria begin 8-30 days following infection through a mosquito bite, depending on the species of *Plasmodium* and whether or not the person was taking prophylaxis medication [4]. Common symptoms include: headache, fever, fatigue, muscular back or joint pain, nausea and vomiting, chills, sweating, dry cough, jaundice, hemolytic anemia, spleen enlargement, hemoglobin in the urine, retinal damage and convulsions [5,6]. These symptoms resemble other conditions such as the flu, septicemia, gastroenteritis and other
viral diseases that are common in the tropics [7]. One key distinction is the cyclic nature of malaria fevers, which is due to the erythrocytic stage of the parasites’ life cycle. An infected person will go through symptoms of fever and sweating that will subside and then repeat every 2-3 days, the timing of which is dependent on the infective species of Plasmodium. The most severe form of malaria is caused by P. falciparum and is called falciparum or cerebral malaria. Along with the other symptoms listed above, a person infected with P. falciparum will also exhibit neurological symptoms that include: conjugate gaze palsy, involuntary eye movement, seizures, opisthotonus, abnormal posturing, or coma [6]. Cerebral malaria is responsible for the majority of deaths (90%) due to malaria [1].

The most common method of diagnosing malaria is microscopic examination of giemsa stained blood films from the patient as each of the four major species (P. ovale, P. malariae, P. vivax and P. falciparum) have distinctive characteristics [2]. Thick films allow the technician to determine parasitemia even if it is extremely low. However, in a thick film the parasites are distorted and hard to identify. A thin blood film presents the parasites more clearly for identification. Thus, it is important to examine both a thin and thick blood film for the best diagnostic outcome. Diagnosis by microscopy is difficult in many of the underdeveloped regions where malaria is common because it is dependent on equipment availability as well as the skill of the technician, two things often lacking in many rural areas [2].
*P. malariae* and *P. knowlesi* look so similar under the microscope that it is hard to distinguish between the two species. However, *P. knowlesi* causes more severe disease, thus it is imperative that a quick diagnosis be made and treatment be administered. Other methods of diagnosis can be helpful for such cases. Molecular tests, such as polymerase chain reaction (PCR), can distinguish species but not necessarily overall parasitemia due to infected erythrocytes that are sequestered in capillaries [2]. Moreover, such tests depend on a more specialized laboratory with the appropriate equipment. There are kits that have been developed, called malaria rapid diagnostic tests or antigen capture assay [2]. These kits only require a drop of blood from the patient and only take 15-20 minutes to complete but vary in sensitivity. These work well in the field but are only qualitative. Again, parasitemia is unable to be determined with these antigen kits.

1.1.3 History of Malaria Research

The term malaria originates from the Latin “malus aria” or the Italian “malaria” meaning “bad air.” References to the disease are included in writings from many regions, with reference to cyclic fevers as far back as 2700 BC in China and continually from Rome, Greece, Assyria, India and other European countries up to the 19th century [8]. Malaria was associated with marshlands and swamps and was commonly referred to as “ague” or “marsh fever.” In 2010 an autopsy on King Tutankhamun, the pharaoh of Egypt in the 18th dynasty from 1332-1323 BC, showed that, among other hereditary diseases and ailments, King Tut
suffered from severe malaria that may have contributed to his death along with an infected broken leg [9]. A study on the mitochondrial genome of *P. falciparum* in 2003 determined that the parasite originated 50,000-100,000 years ago, but not until the introduction of agriculture 10,000 years ago did the parasite increase in human populations [10]. This was supported by the genetic examination of glucose-6-phosphate dehydrogenase gene in humans [11]. Some variants of this enzyme allow for resistance to malaria infection, verifying malaria as a selective force in human genetics occurring within the last 10,000 years.

The founder of tropical medicine was Sir Patrick Manson who elucidated the transmission of *Filaria bancrofti*, the causative agent of the human disease filariasis, by the mosquito during the years of 1877-1879 [12]. In 1880, Alphonse Laveran made a blood smear from a patient that died of malaria to discover that the cause of malaria was a protozoan parasite [13]. These two discoveries, as well as the collaborative effort with Sir Manson, enabled Sir Ronald Ross to fully elucidate the life cycle of avian malaria in the mosquito [14]. Ross isolated parasites from the salivary glands of mosquitoes after they fed on infected sparrows. He studied malaria from 1882-1899 and was awarded the Nobel Prize in Medicine for his work on malaria in 1902 [8]. During the same time period Italian parasitologists Giovanni Battista Grassi, along with colleagues Amico Bignami and Giuseppe Bastianelli, also described the developmental stages of malaria. Their discovery was more relevant to human health as they described the life cycle of *P. falciparum*, *P. vivax* and *P. malariae* as well as pinpointing the
species of mosquito: the female anopheles mosquito, though their work came out a year later [8].

For over 50 years after this discovery of mosquito transmission, researchers were still stunted over where the parasite resided for the 8-10 days following a bite before the parasite showed up in the blood. It wasn’t until 1947, while working in London, that Henry Shortt and Cyril Garnham discovered the parasite dividing in liver cells prior to invading red blood cells [15]. Working with Garnham’s team was an American clinician Wojciech Krotoski, who described *P. vivax*’s ability to lay dormant in the liver for months after initial infection [16].

### 1.2 Features of Apicomplexan Parasites

Apicomplexa is derived from the Latin words *apex* or top and *complexus* or in folds. Parasites of this group include *Plasmodium, Toxoplasma, Isospora, Cyclospora, and Cryptosporidium*, each causing specific human diseases. This group has two defining features: an apical complex structure and an apicoplast organelle. The apical complex structure is involved in invasion into the host cell. This complex includes secretory organelles that are essential for this penetration of the host cell. These organelles include rhoptries, micronemes, polar rings (with or without conoid) and dense granules. The apicoplast is a unique organelle that has prokaryotic origin. Not all protists of this group have an apicoplast, for example *Cryptosporidium*. The use of Apicomplexa as a biological classification is discouraged in use due to its biological invalidity, however the term is still widely used to describe these parasites.
1.2.1 The Apical Complex and Invasion

Orientation of the parasite to its apical end approximating the host cell membrane then occurs followed by tight junction formation through high-affinity interactions between parasite ligands and host cell receptors [17]. In *Plasmodium*, two families of proteins, the erythrocyte-binding-like (EBLs) protein and the reticulocyte-binding-like (RBLs or *P. falciparum* Rhs) proteins, have been well recognized as major parasite ligands responsible for binding and invasion and have been shown to play a cooperative role in invasion, as functional loss of the former leads to the increased transcription of the latter [18]. Both are localized to apical organelles and are released onto the parasite surface during invasion. EBLs and RBLs both appear to have specific receptors on the surface of erythrocytes, such as EBL-175 binding predominantly to glycophorin A [19]. In *P. falciparum* five EBLs and six RBLs have been described.

In both *Plasmodium* and *Toxoplasma* species, the contents of the micronemes are secreted first during the initial contact with the host cell. This has been studied extensively in *Toxoplasma gondii* but is similar in *Plasmodium*: the microneme secrete the full length cellular form of microneme protein 2 (MIC2), which binds specifically to the host cell [20]. MIC2, along with MIC1, MIC4, MIC6 and MIC8, is involved the interaction between the parasite and the host cell [21,22]. As the parasite invades the target cell these microneme proteins are proteolytically cleaved from the surface of the parasite. The rhoptries then secrete rhoptry neck proteins (RON). RON2 is inserted into the host cell
membrane of the host cell being invaded, while RON4, 5, 8, 9, and 10 form a complex at membrane surface of the membrane [23-27]. *T. gondii* tachyzoites then actively invade similar to *Plasmodium* merozoites, with the formation of the moving junction and a motor complex.

**Figure 1.2. Overview of molecular mechanisms utilized by *Plasmodium* for invasion of the erythrocyte.** *Plasmodium* merozoites bind to the surface of the erythrocyte through the interaction of surface antigens (A). The merozoite is re-oriented so that the apical end of the merozoite is adjacent to the surface of the host cell in order to form the tight junction (B). The tight junction includes interactions of merozoite surface protein 1 (MSP-1) and erythrocyte Band 3, erythrocyte binding-like (EBL) and reticulocyte binding-like (RBL) proteins to unknown erythrocyte surface proteins (expanded panel B). Another known interaction specific to *P. falciparum* is of PfRh4 to complement receptor 1 (CR1). As the moving junction forms the parasite pulls itself into the erythrocyte. Subtilisin-like serine proteases (SUBs) and rhomboid proteases (ROMs) cleave the surface antigens from the parasite that were needed for initial binding (C). Apical membrane antigen 1 (AMA1) is secreted by the micronemes and the rhoptry neck protein 2 (RON2) is secreted from the rhoptry. RON2 is inserted into the erythrocyte membrane (expanded panel C). AMA1 and RON2 bind the cytoskeletal elements of the parasite and the host cell, respectively. AMA1 binds to adolase, which is bound to parasite actin. RON2 interacts with the host cytoskeleton. Parasite myosin bound to actin, binds to microtubules (MT) and performs its power stroke, pulling the parasite into the erythrocyte. As the parasite invaginates, a parasitophorous vacuole is formed from the erythrocyte membrane (D) with mechanisms that the parasite engages to exclude host membrane proteins (not shown). Figure drawn by Dawn Walker, illustrated by Patricia Ferrer Beals. [17]
Following tight junction formation between the parasite and the host cell surface, parasites forcibly enter through invagination of the host cell membrane, eventually forming and residing within parasitophorous vacuoles. The process involves a tight junction “motor” termed a moving junction (MJ) (Fig. 1.2). This junction can be thought of a “ring of contact” or “polar ring” between the invading merozoite and the erythrocyte and is used by the parasite to “pull” itself into the cell. Once the parasite is completely inside the host cell, the MJ disappears as the parasitophorous vacuole resolves. No host proteins have been identified in the MJ although studies suggest host cytoskeleton components are involved [28,29]. The most widely studied parasite protein involved in MJ formation is the apical membrane antigen-1 (AMA1) which is highly conserved across Plasmodium species [30-33] and T. gondii [26,34]. AMA1 is secreted by micronemes and interacts with RON2, RON4 and RON5 [23,35] as well as aldolase, an F-actin binding protein. In T. gondii, MIC6 also interacts directly with aldolase [21]. The RON proteins are part of a parasite derived protein complex termed the RON complex, which is secreted into the host cell upon MJ formation. The interaction between AMA1 and the RON complex is essential for parasite invasion [35]. In fact, antibodies to AMA1 can block invasion [36] and a 20-residue AMA1 binding peptide also specifically blocks invasion [37,38], providing evidence that AMA-1 could be a potential vaccine target. As the MJ progresses around the parasite during invasion many proteins are shed from the surface of the parasite [39-41], including AMA-1 [42] and PTRAMP [43], a process required for invasion [40,44,45]. Recent work in Plasmodium has identified a subtilisin-
like serine protease, PfSUB2, as the “sheddase” responsible for this indispensible event [46]. In addition to the role of SUB2 as a sheddase, a number of intramembrane proteases called rhomboids (ROM) are also implicated [41,47-49]. In *T. gondii*, ROM4 is implicated in cleaving surface proteins to produce a gradient of adhesins, these including MIC2, AMA1, and MIC3 [50].

1.2.2 The Apicoplast

The apicoplast is derived from a plastid bearing red or green algae that has undergone a double endosymbiotic event, resulting in a unique four membraned organelle [51-53]. More evidence supports the origin of the apicoplast to be red algae [54-56]. Although it is prokaryotic in origin, it has lost all photosynthetic function over time. During the course of evolution the apicoplast has also transferred most of its genome to the nucleus and developed a protein-targeting pathway to localize over 500 gene products to the apicoplast [57]. The identification of the apicoplast-targeted products elucidated the metabolic functions of the organelle: biosyntheses of fatty acids, isoprenoids, iron-sulphur clusters and heme [58]. The small 35kb apicoplast genome encodes about 50 housekeeping genes, those mostly being involved in transcription and translation [55,59]. The organelle is essential for parasite survival, making it an interesting target for development of novel antimalarials as well as treatment with antibiotics that are already approved by the Food and Drug Administration (FDA).
The antimalarial feature of antibiotics has been known since the 1940s [60]. Only antibiotics that inhibit prokaryotic translation or transcription, but not cell wall synthesis, have an effect on parasites; this was observed in 1983 [61]. It was assumed that these antibiotics were targeting the mitochondrion until the early 1990s when the genomes of Apicomplexan parasites were sequenced [62]. The 6kb genome that encoded mitochondrial proteins was sequenced and the 35kb genome, previously thought to be the mitochondrial genome, was discovered to have features of chloroplast genomes. The antibiotics with antimalarial activity were slow acting and in 1997 Fichera and Roos discovered the “delayed death” phenotype of these drugs [63]. The parasites were not killed by antibiotic treatment, but rather their progeny would die, even if antibiotics were removed prior to re-invasion of this progeny. The merozoites could re-invade and complete most of the second cycle of development but are unable to form functional merozoites during schizogony. Initial hypotheses described a segregation defect, in which some of the progeny simply did not receive an apicoplast. However, this was disproved by ultrastructural studies [64]. Instead, each merozoite of the first generation has a non-functional apicoplast that is unable to elongate and branch during its next cycle. The antibiotics block apicoplast housekeeping functions, such as nucleic acid and protein translation, more commonly the latter.

Apicoplast functions have been proposed, based on the identification of the proteins targeted to the apicoplast from the nucleus, but the exact essential mechanism of the apicoplast during the disease-causing erythrocytic cycle has
been a source of debate for years. Fatty acid synthesis was thought to be the essential mechanism until it was proven that it was only essential for the liver stages of the parasite's life cycle [65,66]. It wasn't until Yeh and DeRisi demonstrated that parasites could lose their apicoplast when cultured with isopentenyl diphosphate (IPP), one of the products of the apicoplast's isoprenal precursor biosynthesis pathway, that the essential function of the organelle during the erythrocytic stages was revealed [67]. However, other functions of the apicoplast are still being studied and appear to have importance in the parasites liver and blood stages.

1.3 *Plasmodium* background

Five species of *Apicomplexan* parasites of the genus *Plasmodium* infect humans to cause malaria and, like many parasites, they develop through discrete stages, each particularly adapted to one of its two hosts (the human and the female anopheline mosquito). These include *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and, shown recently to infect humans, *P. knowlesi* [3]. The most prevalent in causing human disease is *P. falciparum*, which is responsible for about 75% of malaria cases, with the second most common is *P. vivax*, which is responsible for about 20% of malaria cases [7]. *P. vivax* is able to remain dormant in the liver causing recurrent infections well after initial treatment. *P. ovale* and *P. malariae* are responsible for a more mild case of malaria. *P. knowlesi* has recently made the jump from infecting primarily macaques to infecting humans in areas of Southeast Asia [3].
1.3.1 *Plasmodium* Life Cycle

A striking feature of these parasites is their ability to invade a wide variety of cell types during this complex life cycle (Fig. 1.2). This cycle can be broken down into eight steps: 1) In the midgut of an infected female mosquito, the definitive host, diploid zygotes migrate through the chitinous, cell-free peritrophic matrix and penetrate the midgut epithelium, taking up residence between the epithelium and the midgut basal lamina; 2) The zygotes then develop into oocysts that mature and release motile sporozoites that travel through the mosquito’s hemocoel, attaching to and traversing through salivary gland cells to access the interior of the gland; 3) Upon the female mosquito taking a blood meal from the mammalian host, sporozoites are deposited in dermal tissue and use gliding motility to reach dermal vessels, crossing the endothelium into the bloodstream; 4) Sporozoites then rapidly (within minutes) migrate to and cross the sinusoids of the liver by traversal through resident macrophages, called Kupffer cells. Once in the liver parenchyma, sporozoites trans-migrate through a number of hepatocytes by a membrane wounding process, after which they eventually invade a hepatocyte through invagination of the hepatocyte membrane; 5) Within the hepatocyte, sporozoites develop into exoerythrocytic forms that differentiate and replicate to produce haploid merozoites, which are then released into the peripheral bloodstream; 6) Merozoites invade erythrocytes, initiating the erythrocytic cycle of infection. During this cycle, parasites grow and
replicate through several developmental stages: ring, trophozoite and schizonts. The erythrocyte ruptures, releasing more merozoites into the bloodstream, which re-invade uninfected erythrocytes in a 48-72 hour cycle, dependent on the species; 7) During this cycle of development and re-invasion of erythrocytes, some parasites transform into sexual gametes; 8) Gametocytes are taken up by a mosquito during a blood meal, and fertilize to become zygotes, thus completing the life cycle.

1.3.2 Plasmodium falciparum

The most deadly form of malaria is caused by *P. falciparum*, which has the highest rate of complications and mortality, responsible for 90% of the deaths due to malaria [68]. After infecting erythrocytes, *P. falciparum* exports adhesins to the surface of the erythrocyte that change the morphology of the cell through an exomembrane system [69]. These adhesins clump up and the erythrocyte becomes more rigid and "knob"-like in appearance [70]. Proteins that are responsible for this change are termed knob proteins and knob-associated cytoadherence proteins. Cytoadherence refers to the property of knobbed erythrocytes to adhere to the microvasculature, preventing clearance of infected erythrocytes through the spleen [70]. This feature of *P. falciparum* infected erythrocytes is also responsible for the clinical symptoms of disease and dysfunction of multiple organs due to the disruption in microcirculation. Dysfunction of the brain due to cytoadherence is termed cerebral malaria, which is responsible for the high mortality rate of *P. falciparum*. 
The standard method of diagnosis of \textit{P. falciparum} is a blood smear (Fig. 1.3). When a blood sample is taken from an infected patient, only early rings and gametocytes are seen in the peripheral blood due to sequestration of the later stage infected erythrocytes in tissues and blood vessels. This is one of the hallmarks of a falciparum infection during diagnosis.

\textit{P. falciparum} is the only species able to be cultured in an \textit{ex vivo} environment. Developed by Trager and Jenson in 1976, successful continuous culturing of \textit{P. falciparum} in human red blood cells and nutrient medium with serum or serum substitutes in a nitrogen rich or CO$_2$ rich gas mixture, the latter of which is achieved by culture in a CO$_2$ incubator or a candlejar \cite{71}. This culturing method has allowed \textit{P. falciparum} research to escalate and has lead to the discovery and development of new antimalarial drugs and has aided in the vaccine effort as well.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Blood smear of \textit{P. falciparum} parasites in culture shows all stages of the erythrocytic cycle. Parasites within erythrocytes from asynchronous laboratory cultures contain early rings (A), late rings (B), early trophozoites (C), late trophozoites (D), and schizonts (E), whereas, a blood film from an infected patient would only contain rings and gametocytes (not shown) as trophozoites and schizonts would adhere to microvasculature.}
\end{figure}
1.4 Vaccine Efforts

Recent vaccine strategies focusing on antigens crucial to invasion of hepatocytes or erythrocytes aim at impairing the ability of the sporozoite/merozoite to invade these cells rendering the parasite avirulent. Targets of hepatocytes invasion include circumsporozoite protein (CSP) and targets of erythrocyte invasion include EBL and RBL proteins. Due to functional redundancy and antigen switching, multi-antigen vaccine strategies are more likely to act synergistically, reducing the chance of resistance and showing a more effective immune response.

Targeting hepatocyte invasion could be a great tool for prophylaxis drugs, vaccines or treatments to prevent recurrent malaria, as in the case of a *P. vivax* infection. The vaccine RTS,S, which is based on CSP from *P. vivax*, was recently reported in phase 3 clinical trials in Africa to be effective, with every 100 vaccinations averting 65 cases of malaria; however, vaccine efficacy decreased over time and with increased exposure to malaria [72]. Serum from rabbits treated with this *P. vivax* CSP based vaccine has shown cross-species recognition of *P. falciparum* and *P. berghei* sporozoites, which is promising for the use of RTS,S against malaria caused by the various species of *Plasmodium* or even mixed infections [73].

Targeting erythrocytic invasion proteins could also serve as a useful vaccine strategy. Studies of polymorphisms of invasion-related ligands PfEBL and PfRh in field isolates from Columbia, Peru and Brazil highlight the
importance of understanding the variety of invasion pathways being utilized by parasites in different geographical regions [74]. This needs to be taken into account by developing multi-targeted vaccines, which might overcome “vaccine resistance” and be effective against parasites from different regions, different species, or mixed infections. As an additional example of this, antibodies against PfRh5 used in combination with seven other merozoite antigens, most notably PfRh4, have been shown to act synergistically in inhibiting growth of *P. falciparum* in culture [75].

A recent successful vaccine effort offers protection against malaria by the intravenous administration of 4-6 doses of non-replicating *P. falciparum* sporozoites (PfSPZ) [76]. All test patients that were administered 5 doses of the vaccine by IV were completely protected against contracting malaria. This attenuated PfSPZ vaccine elicits a specific antibody and T cell response that is dose dependent. Due to cryopreservation and multiple IV administration, this is not a suitable vaccine for malaria endemic regions, which are mostly developing countries. However, it may be suitable for travelers to these regions of the world.

### 1.5 Antimalarial Drugs

There are many drugs that treat malaria, the most recently developed and most effective being artemisinin combination therapies (ACTs). However, to date resistant parasites have emerged for all current antimalarials [77], highlighting the great need for continued drug development. Table 1.1 is a summary of antimalarial drugs currently being used to combat malaria.
The first effective antimalarial compound quinine was discovered in Peru from the bark of the cinchona tree [78]. The indigenous peoples of Peru made tinctures with this bark to control fevers and shivering. They eventually ground up the bark and added it to sweet water to offset its bitter taste, creating tonic

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug Class</th>
<th>Mechanism of Action</th>
<th>Administration</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amodiaquine</td>
<td>4-aminoquinolone</td>
<td>similar to chloroquine</td>
<td>oral</td>
<td></td>
</tr>
<tr>
<td>Artemisinin</td>
<td>sesquiterpene lactone</td>
<td>unclear; reacts with heme causing free-radical damage to membranes</td>
<td>oral</td>
<td>multiple multi-drug therapies</td>
</tr>
<tr>
<td>Atovaquone</td>
<td>hydroxynaphtho -quinone</td>
<td>selectively inhibits cytochrome electron transport</td>
<td>oral</td>
<td>proguanil</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>macrolide antibiotic</td>
<td>inhibits apicoplast protein synthesis-binds to 50S ribosomal unit</td>
<td>oral</td>
<td>CQ, quinine or artemisinin derivatives, but only as last resort</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>4-aminoquinolone</td>
<td>unclear; accumulates in food vacuole and inhibits hemoglobin crystallization</td>
<td>oral</td>
<td>quinine or chloroquine</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>lincosamide antibiotic</td>
<td>inhibits apicoplast protein synthesis-binds to 50S ribosomal unit</td>
<td>oral</td>
<td>quinine</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>tetracycline antibiotic</td>
<td>inhibits apicoplast protein synthesis-binds to 30S ribosomal unit</td>
<td>oral, intravenous, intramuscular</td>
<td>quinine</td>
</tr>
<tr>
<td>Fosmidomycin</td>
<td>derivative of phosphonic acid</td>
<td>inhibits enzymes of isoprenoid synthesis pathway of the apicoplast</td>
<td>limited oral bioavailability</td>
<td>clindamycin</td>
</tr>
<tr>
<td>Halofantrine</td>
<td>phenanthrene methanol</td>
<td>unclear; binds to hematin and pasmpesin</td>
<td>oral</td>
<td></td>
</tr>
<tr>
<td>Mefloquine</td>
<td>4-methanolquinoline</td>
<td>forms toxic heme complexes that damage parasitic food vacuoles</td>
<td>oral</td>
<td></td>
</tr>
<tr>
<td>Primaquine</td>
<td>8-aminoquinolone</td>
<td>unknown; thought to block oxidative metabolism</td>
<td>oral</td>
<td></td>
</tr>
<tr>
<td>Proguanil</td>
<td>biguanine</td>
<td>inhibits dihydrofolate reductase thus inhibiting the biosynthesis of purines and pyrimidines</td>
<td>oral</td>
<td>Atovaquone</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td></td>
<td>inhibits dihydrofolate reductase thus inhibiting the biosynthesis of purines and pyrimidines</td>
<td>oral</td>
<td>sulfonamide and folic acid</td>
</tr>
<tr>
<td>Quinine</td>
<td>alkaloid</td>
<td>accumulates in food vacuole and inhibits hemoglobin crystallization</td>
<td>oral, intravenous, intramuscular</td>
<td></td>
</tr>
<tr>
<td>Sulfadoxine</td>
<td>sulfonamide</td>
<td>specific inhibitor of dihydroterato synthetase in the tetrahydrofolate synthesis pathway</td>
<td>oral</td>
<td>Pyrimethamine</td>
</tr>
</tbody>
</table>

* compiled from [2,80,81]
water in the process. This treatment was introduced by Jesuits to Europe, especially to Rome where surrounding swamps made malaria quite rampant [79]. Quinine was the main treatment until the 1940s when other antimalarials began to be developed. The mechanism of action is mostly unknown, although it is thought to act in a similar way as chloroquine, by disruption of hemozoin formation as it localizes to the food vacuole [82].

Chloroquine was discovered in 1934 but wasn’t administered for clinical use until 1947 due to initial fear of its toxicity [83]. The US government increased antimalarial drug development during World War II as American soldiers were stationed in malaria endemic regions. During this research chloroquine was found to be an effective antimalarial drug as well as a prophylaxis treatment. However, due to high usage of chloroquine, resistant strains of Plasmodium spread from East and West Africa to Southeast Asia and South America in the late 1960s to early 1970s [84,85]. The proposed mechanism of action of chloroquine involves it’s accumulation in the food vacuole of the parasite to disrupt hemozoin formation preventing biocrystalization and leading to a build of toxic heme [86]. Resistance to chloroquine’s mechanism of action involves efflux of chloroquine from the food vacuole, the best studied resistance mechanisms are through mutations in the transmembrane protein P. falciparum chloroquine resistance transporter (PfCRT) gene [86].

Several antibiotic classes of drugs work as antimalarials including the lincosamide, macrolide, and tetracycline antibiotics [87]. The common mechanism of action of these drugs involves targeting apicoplast protein
synthesis by binding to either the 30S or 50S subunit of ribosomal unit [64]. Most antimalarials are recommended to be taken as combination therapy for better effectiveness and to combat resistance (see Table 1.1 for examples). Malarone is a combination of proguanil and atovaquone, neither of which can be issued as a monotherapy [88]. Proguanil is metabolized by the body to cycloguanil, which inhibits dihydrofolate reductase. Atovaquone works by selectively inhibiting the electron transport chain. An oral dose for adults usually contains about 100 mg of proguanil hydrochloride and 250 mg of atovaquone.

The current “gold standard” antimalarial is artemisinin (ART) and its derivatives, usually administered as ACTs, again to combat the development of resistance. This natural product was first extracted from the Artemisia annua, or sweet wormwood plant, by Chinese herbalists dating as far back as 200BC. ART has a endoperoxide bridge moiety that is unusual in natural products and is likely responsible for the mechanism of action of the drug [89]. Many semi synthetic derivatives of ART have been developed for ACTs. Like proguanil, artemisinin is a prodrug; the body metabolizes it into its active form: dihydroartemisinin. The exact mechanism of action of artemisinin and its derivatives is unknown, however several studies have elucidated potential targets or mechanisms. Hemoglobin degradation, likely a product of this process, is essential for ART activity. This is supported by the reduced ART activity in the presence of an iron chelator or hemoglobinase [90-92]. Other validated parasite targets include translationally controlled tumor protein (TCTP), sarco/endoplasmic reticulum membrane calcium ATP-ase (SERCA), and NADH dehydrogenase [93-95]. Whatever the
exact target, it is generally agreed upon that generation of reactive oxygen species disrupts the membrane potential of parasite mitochondria [96,97]. Understanding the exact mechanism of action might aid in fighting resistance development, which has been seen in four countries in South East Asia so far, as well as refinement of drug potency/toxicity. ACT and other multi-drug therapies represent the best strategy to combat resistance in the field and the WHO carefully regulates these regimens.

1.6 Autophagy Overview

With drug resistance on the rise it is necessary to study and understand the basic biological pathways in *P. falciparum* that contribute to disease in an effort to identify novel drug targets. A recent interest of many drug discovery efforts, especially in fields like cancer and neurodegenerative diseases, focus on cellular homeostasis [98,99]. There are two main catabolic pathways responsible for ridding the cell of misfolded proteins, protein aggregates, damaged organelles, or even intracellular pathogens. Without a disposal system these components can become toxic and eventually lead to cell death. These are the ubiquitin-proteosome system and lysosomal catabolism. The former uses the conjugation of ubiquitin to target proteins through the enzymatic activity of E1-, E2-, and E3- type ligases to “mark” a protein for degradation via the proteosome. Lysosomal catabolism involves a process called autophagy, where the cell degrades cellular material by packaging it into double membraned vesicles called autophagosomes that delivers their cargo to the lysosome for degradation by
lysosomal hydrolysis. The degraded cargo components can then be used by the cell, a recycling process essential for survival in times of nutrient limitation or starvation.

Disruption or misregulation of autophagy in humans is known to contribute to many diseases, including cancer, neurodegenerative diseases, heart disease, and autoimmune conditions, as well as protection against intracellular pathogens [100-104]. In fact, autophagy has been proven conserved and essential in every eukaryotic system studied. Three types of autophagy have been well described in the literature: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy is the pathway that is generally referred to as just “autophagy.” Interestingly, autophagy utilizes ubiquitin-like (UBL) pathways for the formation of autophagosomes. Two of the UBL pathways that have been well studied in yeast and that are essential in vivo for autophagosome formation are the autophagy-related protein 8 (Atg8) lipidation pathway and the Atg5-Atg12 conjugation pathway [105].

1.6.1 Atg8 Lipidation and Atg5-Atg12 Conjugation Pathways

Autophagy has been characterized in yeast, mammalian, and plant systems; however, there have been limited published studies investigating autophagy in Plasmodium [106-108]. Studying the Atg8 lipidation pathway in P. falciparum may lead to new avenues of disrupting its pathogenesis. A growing number of autophagy-related (ATG) genes are being discovered in other systems that play crucial roles in the process of autophagy. To date there are over 68
yeast genes that have an important function in the autophagy, approximately 30 of those are ATG genes that appear to be exclusively involved in autophagy [109]. The Atg8 phospholipidation pathway is one of the best-described components of autophagy and is a well-conserved mechanism across eukaryotes.

This pathway, consisting of four ATG genes, plays an integral role in de novo formation of the autophagosomes, essential structures for autophagy activation. The conjugation of the small ubiquitin-like modifier Atg8 to a phospholipid, typically phosphatidylethanolamine (PE), localizes it to phagophores, which then form autophagosomes, which then package cellular content for delivery to the lysosomal machinery for catabolism (Fig 1.4A). In yeast, under nutrient rich conditions, autophagy occurs at low basal levels. Under starvation conditions, however, autophagy becomes highly activated as the E1- and E2- type ligases Atg7 and Atg3 conjugate Atg8 to PE, converting it to its active form. Atg4 is then necessary to deconjugate Atg8 from PE, a step necessary for autophagosome closure (Fig. 1.4B) [110]. Atg8 is the typical autophagy marker in the field since it remains attached to the autophagosome until fusion to the lysosome [111].

The Atg12-Atg5 conjugation pathway also plays a role in autophagosome formation. Atg12 is the first ubiquitin-like Atg protein discovered [112]. The only known target for Atg12 to bind is Atg5. The Atg12-Atg5 conjugate is formed through the action of E1- type ligase Atg7 and E2- type ligase Atg10. There is no known E3- type ligase involved in Atg12-Atg5 conjugation. This conjugate is
formed constitutively and is irreversible as there is no identified protease to cleave the isopeptide bond joining the two proteins. Atg12-Atg5 also forms a complex with a coiled coil protein Atg16 that specifically interacts with Atg5. This complex localizes to the phagophore-assembly site (PAS) although it is removed prior to the autophagosome formation. Thus, it does not make a good marker for autophagy [111].

Figure 1.4. Atg8 lipidation in yeast. (A) Role of Atg8 lipidation in autophagosome formation. Conjugation of Atg8 to phosphatidylethanolamine (PE) localizes Atg8 to phagophores, initializing autophagosome formation. Atg8 must be deconjugated by Atg4 for autophagosome closure and subsequent fusion to the lysosome. (B) Biochemistry of Atg8 lipidation. In yeast, activation of Atg8 occurs through conjugation to PE. Atg4 cleaves off the C-terminal arginine residue. This leaves the glycine residue exposed and the E1- and E2-type ligases Atg7 and Atg3 are able to conjugate Atg8 to a phospholipid (PL), typically PE. Atg8-PL can be deconjugated to its inactive form by the isopeptidase activity of Atg4.

Studies have shown that Atg12-Atg5 can serve as an E3-type ligase for the conjugation of Atg8 to PE accelerating the transfer of Atg8 from Atg3 to PE, although Atg12-Atg5 lacks any E3-type ligase domains [113]. Mutants affecting
Atg12-Atg5 conjugation show fewer autophagosomes and localization of Atg8 to phagophores is impaired. Although Atg12-Atg5 conjugation is essential for autophagosome formation, it has been shown to be dispensable *in vitro* [114]. In Atg8 mutants the Atg12-Atg5-Atg16 complex still localizes to the PAS and it seems the only role of the complex is to aid in Atg8 lipidation and localization to the phagophore [115].

1.6.2 Autophagy in Protists

Autophagy has not been well studied in protozoa until recently. Current efforts have focused on autophagy in different species of the phyla Apicomplexa and Kinetoplastida and putative ATG genes have been found in some of these organisms’ genomes (Table 1.2). This table is a general overview of ATG genes present in Apicomplexan parasites *P. falciparum* and *T. gondii* and Kinetoplastids *Trypanosoma brucei* and *T. cruzi* and *Leishmania major*.

<table>
<thead>
<tr>
<th>Organism</th>
<th>ATG Gene</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Plasmodium falciparum</em></td>
<td>3, 4, 7, 8, 12(?), 18</td>
<td>Walker <em>et al.</em>, 2013 \ Duszenko <em>et al.</em>, 2011 \ Brennand <em>et al.</em>, 2011</td>
</tr>
<tr>
<td><em>Toxoplasma gondii</em></td>
<td>1, 3, 4, 5, 6, 7, 8, 9, 18, 21</td>
<td>Besteiro <em>et al.</em>, 2011</td>
</tr>
<tr>
<td><em>Trypanosoma brucei</em></td>
<td>3, 4, 6, 7, 8, 9, 15, 18, 24</td>
<td>Herman <em>et al.</em>, 2006 \ Brennand <em>et al.</em>, 2011</td>
</tr>
<tr>
<td><em>Trypanosoma cruzi</em></td>
<td>3, 4, 7, 8, 9, 15, 18, 24</td>
<td>Herman <em>et al.</em>, 2006 \ Brennand <em>et al.</em>, 2011</td>
</tr>
<tr>
<td><em>Leishmania major</em></td>
<td>3, 4, 6, 7, 8, 9, 15, 18, 24</td>
<td>Herman <em>et al.</em>, 2006 \ Willams <em>et al.</em>, 2009</td>
</tr>
</tbody>
</table>

*Table includes genes with strong similarity to known yeast autophagy-like genes (ATG), excluding multiple genes in a family (i.e. Leishmania has ~25 genes in the ATG8 family) and other genes involved in autophagy processes (i.e. TOR kinase, Bcl, vsp34, etc.) Highlighted genes represent those in common to all protozoa listed.*
Several genomic differences in the ATG genes of these anciently diverging eukaryotic organisms, such as reduced complexity and nutrient insensitivity, indicate that studying these processes in such systems may elucidate an early, initial autophagy pathway, prior to specialization by higher eukaryotic organisms such as yeast. Interestingly, a common subset to each protozoan species includes: ATG3, ATG4, ATG7, ATG8, and ATG18 (Table 1.2, highlighted). Four of the five of these is involved autophagosome formation through conjugation of Atg8 to a phospholipid (PL).

Each of these parasites has a complex life cycle and two hosts: an insect vector and a vertebrate, with the exception of *Toxoplasma*, which doesn’t have an insect vector. These complex life cycles involve adapting to many microenvironments (including different temperatures, nutrient availability, cell types, host immune factors, etc) and thus require differentiation to forms adapted to each microenvironment. In *Trypanosoma* and *Leishmania* species, autophagy is observed not only as a survival mechanism in times of starvation, but seems to be required for cell differentiation [116,117]. Ultrastructural studies in *L. mexicana* reveal autophagosome abundance during differentiation, especially conversion from metacyclic forms to amastigotes [118]. ATG gene deletions in *L. major* also reveal parasites that are unable to differentiate and have reduced ability to survive starvation and reduced virulence [117,119,120]. In *T. brucei*, this autophagy pathway is involved in stress response, differentiation, and glycosome removal during differentiation and plays a role in stress response and differentiation in *T. cruzi* [116,121,122].
The role of this pathway in Apicomplexan parasites is only beginning to be elucidated. Initial work in *Toxoplasma* indicated a role of Atg8 lipidation (specifically TgAtg3 activity) in mitochondrial integrity and intracellular development of tachyzoites [123]. Starvation of *T. gondii* parasites leads to mitochondrial fragmentation that is abrogated by treatment with 3-methyladenine, a known autophagy inhibitor, suggesting a role for autophagy as a cell death mechanism [124]. However, a recent finding in *T. gondii* and *P. falciparum*, is Atg8’s localization to the apicoplast, suggesting a role in apicoplast function and/or maintenance [106,125]. A TgAtg4 conditional expression mutant demonstrates a severe growth phenotype of the parasite as well as alterations in the mitochondria and ablation of the apicoplast upon TgAtg4 attenuation [125].

Early work in *Plasmodium* suggests the Atg8 lipidation pathway is involved in the clearance of micronemes in *P. berghei* due to observed localization of PbAtg8 to micronemes while parasites are differentiating; however, despite being repeatedly reported in reviews, no experimental data has yet been published [126-129]. The field now seems divided as to whether this pathway has a role in classical autophagy, forming autophagosomes during starvation, or during differentiation. One study demonstrates that PbAtg8 does not localize to any autophagosome structures in liver stages but only to the apicoplast, suggesting only a role for autophagy in apicoplast biology [130]. Another recent study argues a role of PfAtg8 in both apicoplast biology and autophagosome formation, although this study is focused on the blood stages of infection and is a different species of *Plasmodium* [131]. Whatever the role of the pathway, our data on
PfAtg7 attenuation in parasites suggests the pathway is essential during the blood stages (presented in Chapter 3), making the pathway potentially interesting for drug development [132].

The role of Atg8 lipidation in protists is still unclear but overall it has been suggested to play a role in classical autophagy, intracellular development, differentiation between stages of the parasite within its life cycle, a cell death mechanism, playing a role in mitochondrial or apicoplast biology, or disposal of organelles between stages. Most of these roles are novel and unique to parasite biology and other roles have been either partially or completely observed in other eukaryotic systems. Overall, most of the data in the field strongly suggests that this pathway is essential for parasite survival and/or virulence. Some species of these parasites even exhibit redundancy in these genes, such as the multiple (~25) genes in the Atg8 family in L. major or TcAtg4.1, TcAtg4.2, TcAtg8.1 and TcAtg8.2 of T. cruzi [116,133].

1.7 Activating Enzymes as Drug Targets

The ubiquitin-proteasome system (UPS) and ubiquitin-like (UBL) conjugation pathways have been well studied and have many roles in cellular homeostasis and proteolytic capacities. Dysfunction of these pathways has been known to cause various human diseases due to the disruption of their key cellular and biological functions. These diseases, such as cancer, immuno-inflammatory disease, cardiovascular disease, viral or infectious disease, and neurological disorders, elicit the search for small molecule inhibitors to selectively block these
pathways [134]. These pathways all have a three step mechanism that include a ubiquitin or ubiquitin like protein that is covalently linked to a target protein by the action of three enzymes: (1) an activating enzyme or E1-type ligase, (2) a conjugation enzyme or E2-type ligase, and (3) a ligase or E3-type ligase [135]. The final outcome of the pathway can be proteolysis via the proteosome, endocytosis, autophagy, or non-degradation functions, such as DNA repair, initiation of inflammatory response, function of transcription factors, or ribosomal function, depending on the specific pathway in play.

The E1-type ligase or activating enzymes of these pathways serve as the initial interaction with ubiquitin or ubiquitin-like proteins, first forming an adenylate intermediate that is attacked by the activating enzyme's catalytic cysteine to form a thiol ester in an adenosine triphosphate (ATP) dependent mechanism [135]. Activating enzymes have low affinity for their ubiquitin or UBL protein prior to binding MgATP. A fully loaded enzyme contains two activated ubiquitin or UBL molecules: the thiol ester and the adenylate (Fig. 1.5A). This reaction is very efficient and the maximum turnover of 1-2 s⁻¹, whereas the catalytic rate of the overall reaction is 10- to 100- fold slower [136]. Thus, the concentration of E1-type enzymes is less than the concentration of the other enzymes of the pathway [137].

Targeting ubiquitin or UBL conjugation pathways has already been proven as a useful therapeutic approach, as the proteosome inhibitor bortezomib was FDA approved in 2003 as a cancer drug [138]. Recent efforts to target the activating enzymes of such pathways have led to the development of MLN4924,
Figure 1.5. Mechanism of activating enzyme to form ternary complex with ubiquitin.  (A) The activating enzyme’s mechanism broken into three steps.  (1) ATP binds to the binding pocket of the activating enzyme (AE) and forms an adenylate with ubiquitin (UB), releasing a pyrophosphate (PPi).  (2) The catalytic cysteine of the activating enzyme attacks the adenylate to form a thioester linkage with UB, releasing AMP.  (3) The activating enzyme can repeat the reaction.  A fully loaded activating enzyme had the thioester and adenylate bound UB.

(B) Inhibition of NEDD8 activating enzyme (NAE) by specific compound MLN4924.  MLN4924 binds in the ATP binding pocket and attacks the thioester bound NEDD8 to form an inactive adduct with NEDD8, inactivating downstream steps of the pathway.  Figure adapted from Chen et al. 2011.

an UBL protein (NEDD8) activating enzyme (NAE) inhibitor [139].  This particular inhibitor works by a novel mechanism that forms an NEDD8-MLN4924 adduct that halts the downstream conjugation of NEDD8 to its target protein (Fig. 1.5B).  After the thiol ester of NEDD8 and NAE has formed, MLN4942 competes with ATP for the ATP binding pocket and attacks the thiol ester to form a NEDD8-MLN4924 adduct [140].  This adduct is not able to transfer NEDD8 to the E2-type ligase, halting the pathway and disrupting its overall function.  This inhibitor was designed by Millennium Pharmaceuticals, Inc. (MLN) and a library of such
compounds exist that can be exploited in other systems for drug development (personal communication with James Brownell and Larry Dick). If a selective inhibitor to PfAtg7 or other *P. falciparum* activating enzymes can be discovered, they could represent a novel class of antimalarials.

**1.8 Summary**

Due to resistance to the many effective antimalarials, new drugs and new drug targets need to be discovered in *P. falciparum* to combat disease and death caused by cerebral malaria. The Drew lab has a handful of projects that either tackle the biochemistry of basic pathways in the parasites in an attempt to uncover new drug targets or screen multiple small compound libraries in high throughput assays to discover effective antimalarials. This dissertation is a summary of the work done on the former, a study of a classic autophagy pathway: Atg8 lipidation.

The hypothesis of this work is that the putative members of the Atg8 lipidation pathway found in the genome of *P. falciparum* are the active members of an Atg8 lipidation pathway that functions in apicoplast maintenance, such as in the expansion, segregation or turnover of this essential organelle. Furthermore, we hypothesize that PfAtg7 is the activating enzyme of the pathway and is a potential drug target for novel antimalarials.

This study includes a thorough bioinformatic analysis of autophagy genes in the *Plasmodium falciparum* genome. Genetic and molecular methods were utilized to investigate the biochemistry of PfAtg7 and the function of the PfAtg8
lipidation pathway. The Atg8 lipidation pathway is conserved in *P. falciparum*'s genome and appears to be expressed according to published microarray data [141,142]. This pathway has many interesting features, mainly that the three enzymes of the pathway are potential drug targets. Recent work shows that *P. falciparum* Atg8 (PfAtg8) localizes to the apicoplast, a unique and essential organelle of Apicomplexan parasites. This is indicative of this pathway having a novel and essential function in these parasites, which has generated much interest in this pathway across the parasitology field. Using a parasite line with 70% attenuation of PfAtg7 expression levels, we examine the effect of PfAtg7 attenuation on growth and use it as a tool to assess the role Atg8 lipidation may play in apicoplast function or maintenance. These results are presented in this dissertation.
Chapter 2: Materials and Methods

All reagents were purchased from Sigma-Aldrich unless otherwise stated. Human O\(^-\) erythrocytes, from anonymous donors, were purchased from Interstate Blood Bank (Nashville TN).

2.1 Bioinformatic analysis

Annotated yeast autophagy-related (Atg) protein sequences were obtained from the Saccharomyces Genome Database (www.yeastgenome.org). Putative P. falciparum proteins were identified by BlastP through PlasmoDB (www.plasmodb.org) using default parameters. Alignments were performed using ClustalW (www.ebi.ac.uk/tools/msa/clustalw2) with default alignment parameters. Percent identity and similarity were calculated by hand using the ClustalW alignment.

2.2 Plasmid Construction

PfAtg7-HA: A targeting fragment comprising a portion of the 3’ end of the PfATG7 gene was amplified using the primers:

CTCGAGGGTGATAATGTGTTATGTGA and
CCTAGGGTCCAATATTATAACATCATT,
with Platinum Taq (Invitrogen) and cloned using the TOPO-TA for Sequencing kit (Invitrogen). DNA sequencing was performed (Nucleic Acid Sequencing facility at the Ohio State University) to confirm proper sequence, followed by release of the insert through digestion with XhoI and AvrII and ligated into pPM2GT-HA [143]. For the PfAtg7-RFA construct, the PfATG7 targeting fragment (above) was cloned into a modified version of pGDB in the same manner [144]. The PfAtg7-HA construct consists of a small hemogluttanin tag, whereas the PfAtg7-RFA construct tags PfAtg7 with a regulatable affinity tag that consists of HA, GFP, and a dihydrofolate reductase (DHFR) degradation domain, which is stabilized with trimethoprim (TMP), allowing for regulatable protein attenuation.

2.3 Parasite Culture, Transfection, and Selection
Parasites were maintained and synchronized by standard methods [71,145]. Culture media included: RPMI (plus L-glutamine, without NaHCO₃), 0.5% albumax, 0.37 mM hypoxanthine, 27 mM NaHCO₃, 11 mM glucose, 10 µg/ml gentamicin. Parasites were cultured in O⁻ human erythrocytes at 2% hematocrit under 5% CO₂, 5% O₂, and 90% N₂, 37° C. For transfections, 400 µl of 50% hematocrit RBCs infected with ring stage parasites was transfected with 100 µg of purified plasmid DNA by electroporation, as previously described [146]. Atg7-HA was transfected into 3D7 (wildtype) parasites and PfAtg7-RFA was transfected into the PM1 cell line, which expresses human DHFR conferring resistance to TMP, its parental line being 3D7 parasites [144,147]. Transfected
parasites were selected with selection agents WR99210 or blasticidin (against selectable marker human DHFR in Atg7-HA and blasticidin-S deaminase in Atg7-RFA, respectively) with Atg7-RFA parasites maintained continuously in 5 µM TMP. Following two rounds of on-off drug cycling to enrich for integrants, clonal lines were obtained by limiting dilution into 96-well plates.

2.4 Cloning, Recombinant Expression and Nickel Column Purification

The PfAtg8 gene was amplified from genomic DNA using the primers 5’-GGATCCCCATCGCTTAAGACGAAG-3’ and 5’-GTCGACTTATCCTAGACAACCTCACC-3’ with Platinum Taq (Invitrogen, USA) and cloned using the TOPO-TA for Sequencing kit (Invitrogen, USA). DNA sequencing was performed (Nucleic Acid Sequencing facility at the Ohio State University) to confirm proper sequence, followed by release of the insert through digestion with BamHI and SalI and ligation into PQE30 vector using T4 ligase (Invitrogen, USA) with a vector to insert ratio of 1:3. 2µl of ligation product was transformed into 200µl of JM109 competent cells (Stratagene, USA). Colonies (12) were miniprepped and digested with BamHI and HindIII. DNA sequencing was performed (Nucleic Acid Sequencing facility at the Ohio State University) to verify successful transformation and glycerol stocks were stored at -80 degrees.

Induction of PfAtg8 recombinant expression was performed as described by the QIAexpressionist handbook and all reagents are from Qiagen unless otherwise noted. E. coli (JM109) was grown overnight at 37°C in 25ml of LB+AMP media. This volume was increased to 1L with LB+AMP media and
monitored over 3 hours until OD<sub>600</sub> reached 0.5-0.7. The culture was then induced by addition of IPTG to a final concentration of 1mM. Prior to induction, a sample was taken as a non-induced control. Cells were lysed by sonication and centrifugation to collect soluble and insoluble protein. Protein solubility was determined by SDS-PAGE and comassie stain. Recombinant PfAtg8 (rPfAtg8) was found in inclusion bodies under these conditions. Soluble rPfAtg8 (srPfAtg8) was obtained by induction at room temperature (RT) overnight. Protein was purified via Ni-NTA affinity chromatography utilizing the 6xHis tag added to PfAtg8 through the PQE30 vector. Protein concentration was determined by BCA assay (Thermo Scientific, USA).

2.5 PfAtg8 Antisera Production and Antibody Affinity Purification

1.2 mg/ml of rPfAtg8 was injected into rats to produce antisera (Cocalico Biologicals, Inc.). To affinity purify PfAtg8 antisera, approximately 10 µg of rPfAtg8 was resolved on a criterion SDS-PAGE gel and transferred to a methanol activated polyvinylidene fluoride (PVDF) membrane (Millipore) using the criterion wet transfer system. The membrane was stained with Ponceau S to identify the rPfAtg8 band, which was cut out of the membrane. This was blocked with 5% milk in TBST for 20 min at RT and then incubated with 500 µl of PfAtg8 antisera for 1 hour at RT. The membrane was rinsed with PBS and stripped of affinity purified antisera through 2 30 sec washes with 5mM glycine, 150mM NaCl, pH 2.4 followed by neutralization with 1M NaPO4, pH 8.0 in a separate tube. Sodium azide (0.01%) was added to avoid bacteria contamination. Affinity purified
PfAtg8 antisera was stored at 4°C.

**2.6 Parasite Purification, Western Blotting, and Antibody Dilutions**

All incubations were performed at RT unless otherwise noted. Parasites were harvested for western blot analysis using saponin release [132]. To avoid proteolysis, all harvests were performed rapidly on ice and parasites washed with ice-cold PBS with Complete protease inhibitor (Roche USA).

Criterion Western Blot – Protein samples were prepared by addition of loading buffer (250mM Tris-HCl, pH 6.8, 6% SDS, 20% β-mercaptoethanol, 0.04% bromophenol blue, 40% glycerol), boiled for 5 min and centrifuged at 14,000 RPM for 5 min, with the resulting supernatant used for analysis. The amount of lysate representing 1X10^7 parasites was resolved on 4-20% Criterion SDS-PAGE gels (Bio-Rad) and transferred to methanol-activated PVDF membrane (Millipore) using transfer buffer (20 mM Tris-HCl, 192 mM glycine, 20% methanol) via the TransBlot Semi-Dry Transfer system (Bio-Rad). The membrane was blocked with 5% milk in 1X TBST (20mM Tris-HCl, 150 mM NaCl, 0.1% Tween20) for 30 min. The primary antibody was diluted in 5% milk in TBST and incubated with the membrane overnight at 4°C. Secondary antibodies were also diluted in 5% milk in TBST and incubated with the membrane for 1 hour at RT. Membrane was washed between and after antibody incubations three times for 10 min in TBST at RT. Immunoblots were developed using either the SuperSignal West Dura chemiluminescent substrate (Thermo Scientific) or SuperSignal West Femto chemiluminescent subrstate (Thermo Scientific), as
Neutral-PAGE Western blot, non-reducing conditions – Protein samples were prepared with Nu-PAGE loading buffer, heated for 10 min at 70°C, and cleared by centrifugation at 14,000 RPM for 5 min. Lysates representing $1 \times 10^7$ parasites were resolved on 4-12% Bis Tris Nu-PAGE gels (Invitrogen) using either MES SDS running buffer or MOPS SDS running buffer (allows proteins to run slower if better resolution needed) and transferred to methanol-activated PVDF membrane (Millipore) using Nu-PAGE transfer buffer via the Nu-PAGE XCell II™ Blot Module wet transfer system (Invitrogen). Blocking conditions, antibody dilutions and detection was performed as described for the Criterion system, stated above.

Neutral-PAGE Western blot, reducing conditions – Same as above with following modifications: the reducing agent 5% β-mercaptoethanol added to Nu-PAGE loading buffer prior to gel electrophoresis and 500ul of Nu-PAGE antioxidant added to both the running buffer and the Nu-PAGE transfer buffer.

An independent transgenic *P. falciparum* line expressing an unrelated HA tagged protein (UCH) was used as a control for HA western blotting. A 1:1000 dilution of recombinant expressed PfAtg8 was used as a control for Atg8 western blotting. Antibodies and dilutions were: affinity purified anti-HA, rabbit, 1:5000 (Rockland); anti-rabbit IgG horseradish peroxidase-linked whole antibody, 1:10000 (GE Healthcare); anti-rPfAtg8, 1:5000 (Drew Lab); anti-PfAtg8, 1:2000 (Mizushima Lab, Tokyo Medical and Dental University, Tokyo, Japan); anti-Plasmodium plasmepsin V, 1:1000 (Goldberg Lab, Washington University, St.
Louis MO), α-mouse IgG horseradish peroxidase-linked whole antibody, 1:5000 (GE Healthcare).

2.7 Densitometry

Percent attenuation of PfAtg7 was assessed by densitometry and normalized to *P. falciparum* plasmepsin V (PMV) to account for loading. Pixel intensities of scanned images were determined using ImageJ software (NIH).

2.8 Membrane Fractionation

High percentage trophozoites stage parasites were harvested for membrane fractionation, followed by western blot analysis. Parasites were released from erythrocytes by saponin release (0.025% in PBS) on ice and rinsed with PBS with complete protease inhibitor cocktail (Roche). Parasites were resuspened in lysis buffer (50mM Tris pH 7.5, 250mM Sucrose, 1mM EDTA, protease inhibitor cocktail) and sonicated 6X for 10 sec at maximum amplitude and placed on ice between sonication for 1 min. An aliquot of total lysate was saved for western blot analysis. Parasite lysate was centrifuged for 10 min at 1,000G and supernantant decated into a separate tube. The remaining nuclear fraction pellet was resuspended in lysis buffer with 1% TritonX and saved for western blot analysis. The supernatant was centrifuged further at 100,000xG for 1 h at 4°C and saved as the soluble fraction for western blot analysis. The remaining insoluble fraction pellet was resuspended in lysis buffer with 1% TritonX. SDS-loading buffer was added to a fraction of each sample and boiled
for 5 minutes prior to reducing Nu-PAGE and western blot detection.

2.9 Amplification of mRNA and cDNA Synthesis

RNA was isolated using Trizol (Invitrogen) and cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen). Reaction conditions are as follows: 10 µg total RNA along with oligo-dT and dNTPs were heated to 65°C for 5 min followed by addition of RT buffer and incubated at 42°C, 2 h. DNase-free RNase was added and incubated for 30 min, 37°C. The reaction was inactivated by heating at 65°C for 10 min.

Standard PCR conditions were used with annealing performed at 50°C. Primers were (forward and reverse, respectively):
PfATG4: 5'-AGATCTTCCGACTAGTCAGAGGAA-3' and 5'-ATCGATAACTCCATGGCGCTGGTT-3'.
PfATG3: 5'-CCATGGTCAGAGGAAATTTTTGAGGA-3' and 5'-CCTAGGTCATAATCCTGCATTGCTAT-3'.
PfATG7: 5'-CCATGGTTTTTGCTCCCCATATAGAC-3' and 5'-CCTAGGTCATTCCAATATTATAACAT-3'.
PfATG8 5'-GGATCCCCATCGCTTAAGAGACGAA-3' and 5'-GTCGACTTTATCCTAGACAAACTCTCAC-3'.

Genomic DNA was purified from parasites using QIAamp DNA blood mini kit (Qiagen) and used as a control. Predicted sizes of amplified products:
PfATG3: 282 bp for cDNA, 643 bp for gDNA; PfATG4: 740 bp for cDNA, 1030 bp
for gDNA; PfATG7: 333 bp for cDNA, 512 bp for gDNA; PfATG8: 375 bp for both cDNA and gDNA.

2.10 Growth Analysis

A comparative analysis between parental (PM1 and 3D7) and transgenic parasite lines (PfAtg7-RFA and PB-57) to determine growth rate were initiated at 1% parasitemia, 1 or 2% hematocrit in appropriate culture media. Experiments were carried out with or without TMP to test for a growth phenotype and with or without amino acids, glucose or isoleucine to test for induction of autophagy or change in growth. Parasitemia was monitored daily by flow cytometry. To verify results, growth experiments were repeated several times, each in triplicate.

2.11 Flow Cytometry

Flow cytometry was used to assess the percentage of infected erythrocytes, or parasitemia, by staining live cells with 1.5 µg/ml acridine orange in PBS, 5 min, RT. Acridine orange stains nucleic acid, allowing differentiation between human red blood cells, which don’t have any nucleic acid, and red blood cells infected with parasites. The increase in fluorescence of infected RBCs was detected using a FACSCanto II flow cytometer (BD Biosystems) and parasitemia enumerated by gating of the high fluorescence cell population. 30,000 cells were analyzed for each sampling.
2.12 Cell Cycle Analysis

Highly synchronized parasites cultures were used to initiate growth assays in triplicate 12 mL volumes at 0.5% parasitemia. 5 µl was sampled every 2 hours for 4 days for analysis by acridine orange staining using flow cytometry. PfAtg7-RFA cultures were grown with either 5 µM trimethoprim (TMP) or no TMP. PB-57 and 3D7 parasites were grown in normal media conditions. Percentage of schizont stage parasites was plotted over time using the software program Prism (GraphPad Software).

2.13 Merozoite Counting

Thin peripheral blood smears of fully segmented schizont stage parasites were analyzed for the number of daughter merozoites per parasite line. Slides were giemsa stained and blind counts of approximately 300 schizonts were performed.

2.14 mCherry-PfAtg8 Localization – The PfATG8 open reading frame (ORF) was cloned 3’ of the mCherry ORF lacking the stop codon by PCR amplification, ligated into pM2GT, and transfected into 3D7 parasites as previously described (section 2.3). The mCherry-PfATG8 episomally expressed plasmid was selected for by WR99210. Live imaging was performed on a Zeiss Axio Scope fitted with a 100x, 1.3 NA oil immersion objective.
2.15 Southern Blot

Genomic DNA for southern blots was isolated by QIAamp DNA Blood mini kit (Qiagen). For Southern blots, 1.5 µg of DNA was digested and analyzed by agarose gel electrophoresis as previously described [147]. The single cross-over integrations were screened by AvrII/BclI digestion and probed using PfAtg7 ORF 3’ end (1kb). Probe was synthesized by PCR using the AlkPhos direct Labeling system (GE Amersham) with the following primers and developed using CDPStar developing kit (Roche Diagnostics):

For: 5’-CTCGAGGGGTGATAATGTGTTATGTGA-3’
Rev: 5’-CCTAGGTTCCAATATTATAACATCATT -3’

2.16 Semi-quantitative PCR

Amplification of mRNA, gDNA, and cDNA synthesis performed as previously described in section 2.9. Standard PCR conditions were used on a dilution series of the cDNA with annealing performed at 47° C. Primers were (forward and reverse, respectively):
PfATG7 (PF3D7_1126100): 5’-CCTCCTTTAGGTATTACGGTAG -3’ and 5’-CCTAGGTCATTCCAATATTATAACAT-3’.
Ribosomal protein (PF3D7_1126200): 5’-GTCACTCAAAGTAATAGATAATAATG -3’ and 5’-CTCCTTTCTTTTGGCAACAC -3’.
Predicted sizes of amplified products: PfATG7: 566 bp for cDNA, 745 bp for gDNA; ribosomal protein: 464 bp for cDNA and 700 bp for gDNA.
For quantification of Semi-quantitative PCR, Pixel intensities of scanned images were determined using ImageJ software (NIH) as previously described in 2.7.

2.17 Quantitative PCR (qPCR)

To investigate expression data of PfATG7 from the parental line 3D7 and the clonal PB57 line, qPCR was preformed as follows, RNA was isolated and synthesized to first-strand cDNA (see section 2.9). The qPCR was preformed in triplicate 20 µl reactions containing 33.3 ng of cDNA, 10 µl of 2× iQ SYBR Supermix (BIO-RAD, CA), and 10 µM of gene-specific primer. The reactions were preformed on a CFX96 real-time PCR instrument (BIO-RAD, CA) under the following conditions: 95°C for 5 minutes; 35 cycles of 95°C for 30 seconds, 56°C or 57.8°C for 30 seconds, and 72°C for 15 seconds. Fluorescence readings were taken after each extension step. The melt curve was set from 50°C to 90°C and a plate read was taken in 1°C increments, holding for 3 seconds each time. CFX manager software was used to obtain expression level of each sample. Primers used are listed below (forward and reverse, respectively):

PfATG7 (PF3D7_1126100): 5’-TGTGTATCATGGGGAATACAACA-3’ and 5’-TTTAAATGGCCAGGCATAGG-3’

Ribosomal protein (PF3D7_1126200): 5’-CCCTGATTGGTTTTAACACAGG-3’ and 5’-CTCCTTTCTTTTGGCAACAC-3’

PfActin (PF3D7_1246200): 5’-AAAGAAGCAGCAGGAATCCA-3’ and 5’-TGATGGTGCAAGGGGTTGTA-3’
2.18 Fosmidomycin Dose Response Curves

Plasmodium falciparum 3D7 parasites were grown under standard conditions as previously described (section 2.3). PB-57 parasites were grown in 5 µM farnesol, a downstream product of the apicoplast’s isoprenoid biosynthesis pathway, for 8 days prior to fosmidomycin dose response. Fosmidomycin targets DXP reductoisomerase, a key enzyme of the non-mevalonate pathway of isoprenoid biosynthesis. All assays were performed in triplicate in a total volume of 100 µl with ETOH-only incubations as a negative control. Parasitemia was determined following 72 h of incubation by flow cytometry.

The Fosmidomycin dose-response curves were performed in a 96-well plate format using asynchronous parasite culture at 1% parasitemia. Fifty percent inhibitory concentrations (IC₅₀s) were determined by scoring parasite growth in three separate wells over a 2-fold dilution series starting at 50 µM fosmidomycin. Percent inhibition was calculated by comparison to parasites grown in the EtOH controls from each plate. Chloroquine IC₅₀ was determined as a positive control. Averages for the triplicates were calculated, and dose-response curves were fitted and IC₅₀s determined using the software program Prism (GraphPad Software).

2.19 Transmission Electron Microscopy

Asynchronous parasites were fixed in red blood cells with 2% paraformaldehyde/2.5% glutaraldehyde in 100 mM phosphate buffer. Samples
were processed and sectioned by the The Ohio State Campus Microscopy and Imaging Facility and images were taken on the FEI Tecnai G2 Spirit TEM.

2.20 Statistical Analysis

All culture work for growth curves, cycle time, dose response curves, doubling time and counts for merozoite number were performed in triplicate. Standard deviation, student’s T-test, curve fit to graphs and all other statistical analysis were calculated using GraphPad Prism software.
Chapter 3: PfAtg7 Attenuation and Growth Defect

3.1 Abstract

Analysis of the *Plasmodium falciparum* genome by BlastP and sequence alignments reveals a limited number of putative autophagy genes, specifically the four genes involved in autophagy-related protein 8 (Atg8) lipidation, an essential step in formation of autophagosomes. In yeast, Atg8 lipidation requires the E1-type ligase Atg7, an E2-type ligase Atg3, and a cysteine protease Atg4. These four putative *P. falciparum* ATG (PfATG) genes are transcribed during the parasite’s erythrocytic stages. PfAtg7 has relatively low identity and similarity to yeast Atg7 (14.7% and 32.2%, respectively), due primarily to long insertions typical of *P. falciparum*. Excluding the insertions the identity and similarity are higher (38.0% and 70.8%, respectively). Considering this and the fact that key residues are conserved, including the catalytic cysteine and ATP binding domain, we hypothesize that PfAtg7 is the activating enzyme of PfAtg8. To assess the role of PfAtg7 we have generated two transgenic parasite lines. In one, the PfATG7 locus was modified to introduce a C-terminal hemagglutinin (HA) tag. Western blotting reveals two distinct protein species, one migrating near the predicted 150 kDa and one at approximately 65 kDa. The second transgenic line
introduces an inducible degradation domain into the PfATG7 locus, allowing us to rapidly attenuate PfAtg7 protein levels. Corresponding species are also observed in this parasite line at approximately 200 kDa and 100 kDa. Upon PfATG7 attenuation parasites exhibit a slow growth phenotype indicating the essentiality of this putative enzyme for normal growth.

3.2 Introduction

Autophagy (from Greek *auto*, self + *phagein*, eating) has recently become accepted as an important lysosome-mediated catabolic process in eukaryotes. It serves a diversity of cellular roles such as nutrient acquisition during starvation, protein trafficking (cytosol to vacuole targeting or Cvt pathway), anti-apoptosis, and remarkably, the clearance of relatively large macromolecular structures such as protein aggregates, mitochondria, peroxisomes, nuclei, and intracellular pathogens [104,148-157]. Rapid induction of autophagy during starvation provides essential amino acids and energy to synthesize proteins necessary for survival [158]. Misregulation of autophagy has been shown to be involved in human diseases, such as cancer and neurodegenerative diseases [98-100,159].

Many of the proteins involved in autophagy have been identified and characterized in *Saccharomyces cerevisiae*, including approximately 30 autophagy-related (ATG) genes [109]. Despite ATG genes being highly conserved across eukaryotes, there is seemingly only a subset conserved among parasitic protozoa including *Leishmania, Toxoplasma, Trypanosoma and Plasmodium* [127-129]. In common to these protozoa are members of the Atg8
lipidation pathway, an essential pathway for formation of autophagosomes, the
double membrane vesicles that envelop cargo for delivery to the lysosome. As
described in yeast, the small ubiquitin-like modifier Atg8 plays an integral role in
this process [160]. Under certain conditions, such as nutrient starvation,
autophagy becomes highly upregulated as the E1- and E2- type ligases Atg7 and
Atg3 activate cytosolic Atg8 by conjugating it to phosphatidylethanolamine (PE)
[161], with a result in an increase in autophagosome formation [162].

*Plasmodium falciparum* is the causative agent of the most deadly form of
human malaria and, like many parasites, has multiple developmental stages that
are adapted to its two hosts (the human and the anopheline mosquito).
Autophagy proteins have been studied in liver stages of the rodent malaria
parasite *P. berghei*, where autophagosome-like structures are present and appear
to eliminate organelles such as micronemes and mitochondria [163]. To date,
there are no published data regarding the essentiality of autophagy proteins in
*Plasmodium*, especially during the disease-causing erythrocytic stages of *P.
falciparum*.

Our genomic analysis has revealed *P. falciparum* to have a limited
repertoire of putative ATG genes present in the genome, the most identifiable
being the members of the Atg8 lipidation system. The Atg8 lipidation pathway
also appears to be present in other protozoan parasites [127-129].

Atg7 is a ubiquitin-related modifier, namely an E1-type activating enzyme.
The mechanism of Atg8 lipidation mimics that of protein ubiquitination, which has
been well characterized in systems such as yeast and mammals [135]. Briefly,
during ubiquitination (or autophagy), a thioester intermediate is formed between the E1 (Atg7) and ubiquitin (Atg8). Ubiquitin (Atg8) is then transferred to the catalytic cysteine residue of the ubiquitin-conjugating enzyme or E2 (Atg3). The final step includes transfer of ubiquitin (Atg8) to its target protein (PE) forming a covalent bond through an isopeptide linkage. This can occur directly by the E2 or through a third ubiquitin-protein ligase or E3 (Atg5-Atg12).

In this study we show that the putative Atg8 lipidation pathway members PfATG3 (PF3D7_0905700.2), PfATG4 (PF3D7_1417300), PfATG7 (PF3D7_1126100) and PfATG8 (PF3D7_1019900) are transcribed in erythrocytic stage parasites. We focus on the putative PfAtg7 because as the activating enzyme of PfAtg8 lipidation, PfAtg7 could have an interesting biological role in the parasite, as well as the potential to be a good drug target, having notable differences from its mammalian counterpart. We confirm PfAtg7 expression by modifying the gene locus to add a C-terminally encoded epitope tag (HA), which reveals the presence of two PfAtg7 species. This suggests a post-translational processing of PfAtg7. We are able to attenuate levels of endogenous PfAtg7 through integration of a C-terminal regulatable fluorescent affinity (RFA) tag that allows for rapid destabilization of the fusion protein, PfAtg7-RFA. Attenuation of PfAtg7-RFA results in a marked reduction in parasite growth, demonstrating the requirement of PfAtg7 during *P. falciparum*’s erythrocytic cycle for normal growth.
3.3 Results

3.3.1 Atg8 Lipidation Genes are Expressed During *P. falciparum* Erythrocytic Stages.

Over 30 ATG genes and other molecular components of autophagy have been discovered, largely in *Saccharomyces cerevisiae* [109]. To identify putative autophagy machinery in *P. falciparum*, Atg protein sequences from *S. cerevisiae* were used in homology searches through the PlasmoDB database. The results of this search were generally consistent with other bioinformatic studies [127-129]; however, a number of differences were obtained in our analysis which yielded a limited repertoire of putative autophagy genes (Fig. A1 and Table A1 in Appendix A). This limited number of ATG genes included the four genes involved in Atg8 lipidation: ATG8, ATG4, ATG7, ATG3. This ubiquitin-like conjugation pathway is involved in autophagosome formation [112,161]. The comparison between yeast and *P. falciparum* Atg8 yields similarity of the four proteins: Atg8, Atg4, Atg3, Atg7. Both PfAtg4 and PfAtg7, when compared to yeast, are predicted to encode long insertions, which are commonly found in *P. falciparum* proteins [164-167].

We were unable to detect a similar Atg5 in *P. falciparum* despite the presence of a low similarity, putative Atg12 (Fig. A5 in Appendix A). However, the C-terminal glycine needed for conjugation of Atg12 to Atg5 is absent in the putative PfAtg12, indicating it as non-functional. In yeast, Atg5-Atg12 conjugation is essential for autophagosome formation but has been shown dispensable *in vitro* [114]. The presence of a PfATG12 but not other members of this arm of the
autophagy pathway suggests they were lost evolutionarily or other gene products have assumed their function. The related apicomplexan parasite *Toxoplasma gondii* also appears to lack the homologs to the Atg5 and Atg12 [123,124].

Published microarray data indicates all four putative autophagy proteins to be expressed during erythrocytic stages of *P. falciparum* infection, with PfAtg8 and PfAtg4 peaking 40 hours post invasion (schizont stage), PfAtg7 peaking 25 hours post invasion (early trophozoite stage) and PfAtg3 remaining constant throughout all the erythrocytic stages [141,168]. To confirm that these genes are transcribed, we purified total RNA from asynchronous 3D7 parasites and synthesized cDNA. Amplification of the cDNA across a predicted intron confirms transcription of PfATG3, PfATG4 and PfATG7 (Fig. 3.1). PfATG8 has no introns so primers amplify the entire gene.

![Figure 3.1. PfAtgs 3, 4, 7, 8 are expressed in P. falciparum erythrocytic stage parasites.](image)

Genomic DNA (gDNA) and RNA was extracted from asynchronous erythrocytic stage parasites, the latter used for cDNA production. Primers flanking introns were chosen, except for PfATG8, which has no predicted introns. No RT control shows lack of gDNA contamination of RNA preparation.
3.3.2 Bioinformatic Analysis Indicates Conservation of PfAtg7

PfAtg7 appears to be significantly larger than E1-type ligases in other systems (Fig. 3.2A). The enzyme in *Plasmodium* is predicted to be 156.6 kDa whereas in yeast ScAtg7 is 71.4 kDa. ScAtg7 has an N-terminal domain comprising the first 288 amino acids, a 6 amino acid linker region, and a 336 amino acid C-terminal domain containing the catalytic core and dimerization domain [169]. Taking *P. falciparum’s* apparent insertions out of consideration,

**Figure 3.2. PfAtg7 has an unusual primary structure.** (A) PfAtg7 contains long insertions within the C-terminal and N-terminal domains. A schematic of PfATG7 and ScATG7 domains illustrates the presence of insertions in PfAtg7 as compared to the well-described C-terminal and N-terminal domains of ScAtg7. (B) Alignment of ScAtg7 and PfAtg7 C-terminal regions reveals conservation of key motifs. Alignment between yeast (Sc) and *P. falciparum* (Pf) ATG7 C-terminal domain illustrate similarity, with conservation of the ATP binding domain (black bar), the catalytic cysteine (box), amino acids required for hydrogen bonding (black arrowheads), and salt bridges (open arrowheads) between ATG7 and ATG8 (for complete alignment see Appendix A).
PfAtg7 has 30.8% identity and 68.1% similarity to ScAtg7. The C-terminal domain itself, again taking the two long insertions out of consideration, has 38.0% identity and 70.8% similarity (Fig. 3.2B). The C-terminal 123 amino acids, required for dimerization, is well conserved, although there is a unique 29 amino acid insertion present in *P. falciparum* that is absent in yeast [169]. Also conserved is the catalytic cysteine, in yeast at position 507 (1177 in *P. falciparum*) within the essential C-terminus. The ATP binding domain is conserved with a motif of GxGxxGCx at position 890-897. In yeast the residues Y486, R443, and S466 are indicated to be necessary for the formation of hydrogen bonds between Atg7 and Atg8 [169]. The corresponding residues Y1156, R1008, and S1140 are conserved in PfAtg7. In yeast, residues significant for salt bridge formation between Atg7 and Atg8 include: D490 and R550. Only the aspartic acid at 1160 is conserved in *P. falciparum*.

### 3.3.3 PfAtg7 is Detected as Two Protein Species

To begin our studies of PfAtg7 in the parasite, a transgenic line was generated in which we modified the gene locus, via homologous recombination, to encode for a C-terminal hemagglutinin (HA) epitope tag on the protein. For this, a targeting plasmid was constructed which included approximately 1 kb of the 3’ end of the gene followed in-frame by the coding sequence to add the HA tag. Clonal integrant lines C1 and F2 were isolated by limiting dilution and proper integration into the PfATG7 locus was confirmed by Southern blotting (Fig. 3.3).
Western blotting of total parasite lysate against the small 9 amino acid HA epitope of the resultant fusion protein, under control of the native promotor, confirms expression of the protein during the erythrocytic stages of the parasite’s life cycle (Fig. 3.4A). Unexpectedly, we consistently detect two species, one near the predicted size of the full-length protein at ~150 kDa and one at ~65 kDa. Despite all efforts to minimize proteolysis (see Material and Methods), these two species are consistently and repeatedly detected. We have yet to confirm which species has enzymatic activity. It is notable that these species might be artifactual as a result of the HA epitope tag or that this could represent a processing event of PfAtg7. An in vitro activity assay will assess both of these possibilities, which is further discussed in the Discussion (section 3.4).
3.3.4 Attenuation of PfAtg7 Results in Slow Parasite Growth

In a strategy similar to that described in 3.3.3, we tagged PfAtg7 with a regulatable fluorescent affinity tag (RFA), an approach that has been successful for regulated protein attenuation in *P. falciparum* [144,170]. This construct adds the RFA tag to the C-terminus, which results in the Atg7 protein fused to an attenuable destabilization domain (in addition to GFP and HA), which is stabilized by the presence of the folate analog trimethoprim (TMP) in the growth medium. In the absence of TMP the fusion protein becomes misfolded and rapidly degraded via the proteasome (Fig. 3.5A). The PfATG7-RFA construct was successfully integrated into the genome of the parental PM1 cell line which expresses human dihydrofolate reductase (DHFR) conferring resistance to TMP.
Clonal cell lines A1 and B4 were isolated for further analysis and proper integration was confirmed by southern blot (Fig. 3.3).

Figure 3.5. Use of regulatable fluorescent affinity (RFA) tag in *P. falciparum* to attenuate PfAtg7. (A) RFA tagging scheme. A fragment representing ~1 kb of the 3’ end of the gene of interest (GOI) is amplified by PCR and cloned into an integration plasmid designed to undergo homologous recombination with the parasite’s endogenous GOI, resulting in the addition of extra coding sequence at the 3’ end of the gene. This construct adds a C-terminal RFA tag which results in the GOI fused to an attenuatable destabilization domain, which is regulated by trimethoprim (TMP) concentration, in addition to a GFP tag and an HA epitope tag. (B) Western blot of PfATG7-RFA transgenic parasites confirm protein attenuation. Following 48 hours post TMP washout during the growth assay (Fig. 5D), PfAtg7 protein levels were attenuated. The ER-resident Plasmepsin V (PMV) was used as a control to normalize for loading. Densitometry normalized against PMV indicates that the ~200kDa band in clone A1 was attenuated by 67.6% (±4.0%) and the ~100kDa band was attenuated 17.0% (±2.4%). In clone B4 the ~200kDa band was attenuated by 36.1% (±8.5%) and the ~100kDa band was attenuated 8.0% (±1.9%).

Western blotting against the HA epitope present within the RFA tag, reveals two species at ~200 kDa and ~100 kDa. Given the addition of the larger 47kDa tag this corresponds to the two forms observed in the Atg7-HA cell lines (Figs. 3.4B, 3.5A,B), which decreases the speculation that these are merely artifacts. Both protein species were rapidly and sustainably lost upon removal of TMP, with attenuation observed as quickly as 4 hours post TMP removal (data
not shown) and this loss was shown to be sustained for 48h (Fig. 3.5B). It is likely the loss of PfAtg7 was sustained throughout the growth experiment.

Loss of PfAtg7 results in a slow growth phenotype, as seen by monitoring parasitemia in culture over time (Fig. 3.6). Clonal cell lines A1 and B4 were washed free of TMP and parasitemia was measured by flow cytometry for short periods of time without splitting the parasites (Fig. 3.6A,B, and C) or for a longer growth analysis requiring dilutions every three days (Fig. 3.6D and E). For these experiments, the overall dilution was taken into account, resulting in “cumulative growth” (Fig. 3.6D and E).

The Adams lab studied gene disruption using random piggyBac transposon mutagenesis and identified growth phenotypes in over 200 independent clones [171]. One of these clones, PB-57, the transposable element was inserted 705 bp upstream of the PfATG7 gene and was reported to have a 3.7 fold decrease in growth of *P. falciparum* as a result [172]. We have acquired this clone from the Adams lab and confirmed the slow growth phenotype (Fig. 4.5) as well as PfAtg7 attenuation by semi-quantitative PCR and qPCR (Fig. 3.7). The qPCR results confirm a 30% decrease in PfAtg7 expression in PB-57 as compared to 3D7 wildtype parasites. This clonal PB-57 parasite line phenocopies the growth phenotype seen with our PfAtg7-RFA clones.
Figure 3.6. PfAtg7 is essential for normal parasite growth. Short-term growth experiment reveals slow growth phenotype upon TMP removal. PfAtg7-RFA clone A1 (A) B4 (B) and parental PM1 (C) were washed free of TMP and parasitemia was monitored by flow cytometry every 24h for 3 days. Parasitemia in the absence of TMP (white bars/circles) for clones A1 and B4 was significantly reduced compared to growth in the presence of TMP (black bars/circles; **p<0.001, student’s T test). Parental line (PM1) shows significant growth difference at 48h (*p<0.05, student’s T test) but no significant difference in the presence and absence of TMP at the 24h or 72h timepoints. (D) PfATG7 attenuation results in a sustained slow growth phenotype. Two independent PfATG7-RFA transgenic clones, A1 and B4 (not shown), were monitored every 24 h by staining with acridine orange and enumeration of parasitemia by flow cytometry for up to 8 days. Dilutions were performed on parasite cultures every three days to maintain the parasites at optimal parasitemia and avoid parasite death. Overall dilutions were factored in, resulting in “cumulative growth.” (E) PM1 parental parasites exhibit no significant change in growth over the 8 day period.
Figure 3.7. Semi-quantitative and quantitative PCR confirms lower expression levels of PfAtg7 in PB-57 parasites. Standard PCR conditions were used to establish expression levels of PfAtg7 (A) and the ribosomal protein upstream of PfAtg7 (B) across a dilution series of cDNA (nanogram amounts) for 3D7 and PB-57 parasites, with quantification also shown (C, D). The ribosomal protein is used as a control as the transposable element was inserted between it and PfAtg7. Predicted sizes of amplified products: PfATG7: 566 bp for cDNA, 745 bp for gDNA; ribosomal protein: 464 bp for cDNA and 700 bp for gDNA. Real time qPCR confirms lower expression levels of PfAtg7 in PB-57 parasites (E). Standard qPCR conditions were used to establish expression levels of PfAtg7 using actin as a control. Results show normalized expression of PfAtg7 in 3D7 and PB-57 parasites, with a decrease of 70% expression in PB-57 parasites.

Continued
3.4 Discussion

The study of autophagy in eukaryotes has revealed a functional complexity well beyond the initial discoveries of its roles in lysosomal protein trafficking and as a defense against nutrient starvation. It is now appreciated as a tightly regulated and selective catabolic machine capable of delivering a remarkable variety of cargo to the lysosome. In a homeostatic role, long-lived and damaged proteins, as well as whole organelles are degraded via incorporation into autophagosomes. Selective delivery of lysosomal resident proteins and other cargo can also occur through what is known as the cytoplasm-to-vacuole (Cvt) targeting pathway [150]. Cellular defense, to both internal and external insult, also appears to be an important role for autophagy. Potentially harmful protein aggregates are enveloped and catabolized, essential nutrient is scavenged from the cytoplasm during periods of metabolic stress, and
intracellular pathogens are killed via autophagic mechanisms [104,153,158].

Defects in, inhibition of, or overstimulation of autophagy can be either deleterious or protective in a variety of the pathologies caused by cancers, neurodegenerative disorders, heart disease, autoimmune conditions, and intracellular pathogens [100,102-104,173].

The function of autophagy in protozoan parasites is only beginning to be understood. A response to nutrient deprivation and a possible role in endocytosis has been observed for ATG8 in *L. major*, regulation of cellular differentiation in *T. brucei*, and a role in mitochondrial maintenance and tachyzoite development have been described in *T. gondii* [122-124,133]. In *T. gondii*, TgAtg8 localizes to autophagosomes upon starvation and down regulation of TgAtg3 impairs normal development of tachyzoites [123]. Mitochondrial defects are also apparent in *Toxoplasma* upon PfAtg3 attenuation, suggesting a mitophagy-related function [124]. Recent localization of lipidated Atg8 to the apicoplast of *P. falciparum* [106] suggests to us a role in the turnover, maturation, or segregation of this essential organelle.

In contrast to autophagy in more complex eukaryotes, protozoan parasites appear to encode a comparatively small repertoire of autophagy genes. Through bioinformatic analysis, the only autophagy genes common across protozoa appear to be those encoding Atg8 and its lipidation pathway. *Leishmania major* contains four different ATG8 genes, with apparent roles in starvation induced autophagy as well as endocytosis [133]. *L. major* also encodes members of the Atg5-Atg12 conjugation system. Although dispensable *in vitro*, it has been
reported that Atg5-Atg12 can act as an E3-type ligase that enhances Atg8 conjugation to PE [114,115]. Apicomplexan parasites such as *T. gondii* and *P. falciparum*, do not appear to have homologs of the Atg5-Atg12 pathway (a low similarity gene in *P. falciparum*, gene ID PF3D7_147000 could be an ATG12) in their genomes but do have putative Atg8 lipidation proteins [127-129].

Our expression studies detected mRNA for ATGs 3, 4, 7, and 8 during the erythrocytic stages of *P. falciparum* (Fig. 3.1), which agrees with published microarray analysis [141,168]. Each transcript was easily detected and splicing appears to be as predicted by PlasmoDB (with exception of ATG8 which contains no predicted introns). PfAtg7, though still putative in function, has significant domains and key amino acid residues conserved to suggest its functional enzymatic role as an E1-type ligase in PfAtg8 lipidation. Ignoring the long insertions, PfAtg7 has 38.0% identity and 70.8% similarity to ScAtg7, with conservation of the catalytic cysteine and the ATP binding domain.

To begin our functional studies on Atg7 in *P. falciparum*, we employed a molecular genetic strategy. Western blotting of our transgenic Atg7-HA cell lines revealed the presence of two protein species (Fig. 3.4A). Despite careful attention to minimize the chances of non-specific proteolysis (see Materials and Methods), the smaller species persisted in all experiments. We also observed the same pattern in our ATG7-RFA cell lines (Fig. 3.4B) consistent with the addition of the larger 47 kDa RFA tag. We speculate that the smaller 65 kDa species could represent a specific processing event of Atg7, possibly retaining catalytic activity, although we have no direct evidence of this. Pulse-chase experiments
and *in vitro* activity assays to examine processing and catalytic activity of these two species will be performed to assess these questions.

Attenuation experiments in our cell lines displayed rapid reduction of PfAtg7 and a significant reduction in parasite growth rate, demonstrating the necessity of PfAtg7 for normal parasite growth. This could be detected in clonal lines as early as 24 h and persisted for as long as 8 days (Fig. 3.7). This finding is also supported by recent work from the Adams lab who report a ~3.7-fold reduction in growth of a clonal line of 3D7 *P. falciparum* (the same strain used as a background in our studies) into which a single random integration of a transposable element occurred 705 bp upstream of the start codon for the ATG7 gene [174]. We confirmed by semi-quantitative RT-PCR and qPCR that insertion of the transposon results in reduced ATG7 transcription (Fig. 3.8). It is notable that the parasites did not die upon PfAtg7 attenuation and we speculate this to be due to insufficient attenuation of PfAtg7, as evidence suggests that PfAtg7 is not entirely knocked out in either the PfAtg7-RFA-TMP or PB-57 parasites. The PB-57 cell line should be valuable in future functional studies as it is more stable than the PfAtg7-RFA clones.

Recently a specific inhibitor of the human E1-type ligase NAE (NEDD8 activating enzyme) has been developed that takes advantage of E1's enzymatic mechanism [175,176]. This inhibitor, MLN4924 (Millenium Pharmaceuticals), has led toward development of an anti-cancer drug targeting the NEDD8 pathway that is currently in clinical trials. MLN4924 is an adenosine sulfamate analog that binds to the ATP binding domain, forming an adduct with NEDD8, a small
ubiquitin-like protein (as is Atg8). The adduct MLN4924-NEDD8 mimics the NEDD8-adenylate intermediate and binds at the adenylation site of NAE forming a tight binary complex that inhibits NAE from binding ATP or NEDD8, inhibiting conjugation of NEDD8 to its target protein (Fig. 1.5B). Thus, it is not unreasonable to suggest that PfAtg7, also an E1-type ligase, may in similar fashion be a druggable target for antimalarial drug discovery.

The role of autophagy in *Plasmodium* has yet to be elucidated. Recent studies in *Apicomplexa* point to both the apicoplast and mitochondrion as sites of localization of Atg8, leading to our hypothesis of Atg8 involvement the maintenance (e.g., turnover, expansion, segregation) of these essential organelles [106,125]. The findings of our study support these hypotheses by validation of the activating enzyme PfAtg7 and, therefore the PfAtg8 lipidation pathway, as essential for normal growth of the parasite. Our current research is addressing this apicoplast hypothesis. In other systems, Atg7 is also involved in the Atg5-Atg12 conjugation pathway; however, our bioinformatic analysis has confirmed the absence of a PfAtg5 in the genome. However, this does not discount that PfAtg7 may be involved in other roles and it is our goal to elucidate the role of PfAtg7 in erythrocytic stages of *P. falciparum*s life cycle.

Whether such an apparent low complexity pathway in these anciently diverging eukaryotes represents the evolutionary origins of autophagy or a divergence from other eukaryotes is still not known, but it is likely that a detailed understanding of “simple autophagy” in *Plasmodium* has potential to enrich our
understanding of its role in this medically important parasite as well as benefit our understanding of more complex autophagic systems as in humans.

3.5 Acknowledgments

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Chapter 4: Functional Analysis of Atg8 lipidation in *P. falciparum*

4.1 Abstract

In classic autophagy, the Atg8 phospholipidation pathway ligates the small ubiquitin-like protein Atg8 to phosphatidylethanolamine, a step necessary for the formation of autophagosomes. Analysis of *P. falciparum*’s genome reveals four genes similar to members of the yeast Atg8 pathway: PfATG8, E1-/E2-type ligases PfATG7 and PfATG3, and dual-role protease/isopeptidase PfATG4. A notable difference between PfATG8 and ATG8s of other systems is a predicted lack of C-terminal amino acid(s) that are proteolytically removed by the isopeptidase activity of Atg4 revealing the glycine to which PE is ligated. This suggests the activating role of PfATG4 is unnecessary, and predicts unregulated pfATG8 lipidation. To explore the activity and function of the PfAtg8 pathway in erythrocytic stage parasites we have episomally-expressed PfAtg8 fused to the fluorescent mCherry protein. Rather than autophagosomal puncta as seen in yeast, live-cell imaging reveals an unexpected localization. Recent work has confirmed this as localization to the apicoplast, a unique and essential organelle that is responsible for fatty acid and isoprenoid precursor biosynthesis. We have generated antisera to pfATG8 and have successfully shown PfAtg8 as two
species, with the lower species migrating in the insoluble fraction, suggesting PfAtg8 phospholipidation occurs in the erythrocytic stages. To assess the role of PfAtg8 pathway in apicoplast maintenance, we assessed wildtype and ∆Atg7 clones for their sensitivity to fosmidomycin, an inhibitor of the non-mevalonate (MEP) pathway within the apicoplast. Parasites with attenuated levels of PfAtg7 (PB-57 and PfAtg7-RFA –TMP) are more sensitive to fosmidomycin, showing a significantly lower IC₅₀. This indicates a slight association of PfAtg7 to apicoplast function. Further assessment of the slow growth phenotype of PB-57 parasites suggests growth impairment similar to a delayed death phenotype, which is characteristic of an apicoplast impairment previously shown by antibiotic activity against Apicomplexan parasites. If PfAtg8 phospholipidation is essential to apicoplast maintenance, then inhibition of PfAtg7 could similarly be an effective way of killing parasites without harming the host, making it a promising drug target.

4.2 Introduction

The Atg8 phospholipidation pathway is best described in the yeast Saccharomyces cerevisiae [105]. In yeast, Atg8 proteins are ubiquitously expressed; however, under certain stress conditions, such as starvation, expression is upregulated. Prior to conjugation to PE, the Atg8 carboxy-terminal (C-terminal) is cleaved by the cysteine protease Atg4 to expose the C-terminal glycine. Atg4 is specific to Atg8 and serves a dual role by deconjugating Atg8 from PE on the surface of the autophagosome. Atg8 is conjugated to PE through
sequential activities of Atg7 and Atg3, by the action of catalytic cysteine Cys507 of Atg7 and Cys133 of Atg3. Atg8-PE is highly associated with phospholipids on the surface of membranes and maintains a tight association with mature autophagosomes, making it a good marker for autophagosome formation [111].

The localization of Atg8 has been examined in protozoa in attempt to define the function of this pathway in parasite biology; those discussed further are the parasites of the genus Leishmania, Trypanosoma, Toxoplasma and Plasmodium.

In Leishmania major there are 4 families of ATG8-like genes: ATG8, ATG8A, ATG8B, and ATG8C, all of which complement yeast Atg8 deletions strains, demonstrating conservation of function [133]. There are approximately 25 Atg8-like proteins in total in the genome; this redundancy of genes is typical in L. major as gene duplication events are prevalent [118]. LmAtg8A primarily localizes to autophagosomes through the action LmAtg4.2, whereas LmAtg8, LmAtg8B, and LmAtg8C are processed by LmAtg4.1 [133]. LmAtg8 localizes to autophagosomes but not to the extent of LmAtg8B. LmAtg8B and LmAtg8C localize to the flagellar pocket, perhaps playing a role in endocytosis or exocytosis [133]. Despite previous bioinformatic analysis reporting the absence of an ATG12 and ATG5 in L. major’s genome, Williams et al. indicate an active Atg12-Atg5 conjugation pathway that was probably undetectable due to high divergence from the yeast genes [128,133].

In Trypanosoma there is a full set of Atg8 lipidation proteins, but Atg10, Atg12, and Atg5 are not found in the genome. Bioinformatics reveals two Trypanosoma Atg8 proteins, Atg8.1 and Atg8.2. In T. cruzi, only TcAtg8.1
complemented yeast Atg8 deletion strains and localize to autophagosomes upon starvation [116]. Other members of the TcAtg8 lipidation pathway, TcAtg4.1 and TcAtg4.2 have been shown complement yeast deletion strains [116]. In *T. brucei* both TbAtg8.1 and TbAtg8.2 localize to autophagosomes upon starvation or induction of autophagy with wortmannin and chloroquine [121]. The TcAtg8 lipidation pathway is active and is crucial for survival in times of starvation, as well as playing a role in differentiation between the life stages of the parasites.

Work in *Toxoplasma gondii* initially showed TgAtg8 localization to the mitochondria of the parasite [123]. More so, starving *T. gondii* causes fragmentation of the mitochondria, suggesting the occurrence of mitophagy and a role of autophagy in regulated cell death [124]. However, studies in *Plasmodium* reveals localization of PfAtg8 to the apicoplast of the parasite during blood and liver stages, shown by either fluorescent microscopy, immunoEM, or both [106,130]. Subsequently, recent work in *T. gondii* confirmed TgAtg8 localization to the apicoplast [125], not the mitochondrion, an understandable oversight, as the two organelles are in close proximity to each other in the cell. This localization of Atg8 in Apicomplexan parasites suggests a novel role for the pathway.

The apicoplast is an essential organelle of Apicomplexan parasites, a group that consists of *Plasmodium, Toxoplasma, Babesia, Cryptosporidium, Cyclospora,* and *Isospora* species. The apicoplast is derived from a plastid bearing red algae undergoing a secondary endosymbiotic event [53]. Several features of this organelle make it both biologically fascinating and an attractive
therapeutic target. It is prokaryotic in origin, containing pathways that have no counterpart in the human host, making it a potential source of highly selective drug targets. During the course of evolution the apicoplast has lost its photosynthetic function and transferred most of its genome to the nucleus, requiring a protein-targeting pathway to localize the majority of over 500 gene products into the organelle [177]. The resident 35kb apicoplast genome encodes approximately 50 housekeeping genes. The exact essential mechanism of the apicoplast was unknown until Yeh and DeRisi demonstrated that when supplemented with isopentenyl diphosphate (IPP), one of the products of the apicoplast’s isoprenal precursois biosynthesis pathway, over multiple cycles, parasites became dependent on the exogenous supply of IPP and had lost their apicoplast. This loss of the apicoplast resulted in death upon the removal of IPP, evidence that isoprenoid biosynthesis is the essential function of the organelle during the parasites’ erythrocytic stage [67].

The unexpected localization of PfAtg8 to the apicoplast in *P. falciparum* parasites suggests a novel role of the PfAtg8 phospholipidation pathway in this parasite. The biochemistry of the lipidation pathway seems to be consistent with that seen in yeast and other eukaryotic systems due to the presence of a fast and slow migrating PfAtg8, the enzymatic activity of PfAtg7, and the ability of PbAtg3 and PbAtg8 to form a thioester linkage [108]. However, the function seems to differ in many respects. Other than localizing to the apicoplast, it also appears that PfAtg8 primarily migrates faster on SDS-PAGE, indicating it is in the phospholipidated form whether or not autophagy is induced.
Drugs that target the apicoplast do not kill the treated parasites, but rather these parasites’ progeny, a feature which is called a “delayed death” phenotype [64]. These drugs are labeled “slow acting” due to this delayed parasite death and work well in combination with fast-acting antimalarials. These apicoplast targeting drugs are non-antifolate antibiotics, which include the drugs classes: lincosamides, tetracycline, and macrolides. Table 1.1 summarizes the mechanisms of action and dosage of the drugs found in each of these drug classes (see clindamycin, doxycycline and azithromycin). Fluoroquinolones exhibit moderate antimalarial activity but are not discussed further.

Fosmidomycin, a derivative of phosphonic acid, also exhibits antimalarial properties by inhibiting DOXP reductoisomerases and 2C-methyl-D-erythritol-4-(cystidine-5-diphospho) transferase of the isoprenoid biosynthesis pathway [178]. Unlike the other apicoplast targeting drugs, fosmidomycin acts more rapidly, not seeming to cause a delayed death phenotype, but must be co-administered with clindamycin for the best results [179].

In this study we present evidence that PfAtg8 has high similarity to yeast Atg8 and confirm PfAtg8 is present two migrating forms in the erythrocytic stages of P. falciparum’s life cycle. We also show that the faster insoluble species is detectable in parasite lysate without induction of autophagy, suggesting that the pathway is activated at a basal level. Due to the unusual localization of PfAtg8 to the parasite’s apicoplast, the function of the pathway’s effect on apicoplast metabolism was assessed by fosmidomycin sensitivity assays. Fosmidomycin is an inhibitor of DXP reductoisomerase, an important enzyme in the non-
mevalonate pathway of isoprenoid biosynthesis, the only necessary metabolic function of the apicoplast during erythrocytic stages. These results confirm a possible role in apicoplast function or maintenance as attenuated levels of PfAtg7 result in an increased sensitivity to this inhibitor. Furthermore, despite the slow growth phenotype of PB-57, these parasites develop the same number of daughter merozoites as the parental line and an unaltered cycle time. These observations are typical of a delayed death phenotype due to apicoplast impairment. These results indicate a possible link between autophagy and the non-mevalonate pathway of isoprenoid biosynthesis in *P. falciparum* parasites.

4.3 Results

4.3.1 Bioinformatic Analysis Indicates Conservation of PfAtg8

The Atg8 gene family encodes ubiquitin-like proteins that share a conserved structure, including two amino-terminal α helices and carboxy-terminal ubiquitin-like core [180]. This core consists of four stranded central sheets, two central strands that are parallel to each other and two that are antiparallel to the central strands [181]. Genomic analysis has revealed a single PfATG8 gene with 40% amino acid identity and 59% similarity to yeast ATG8. Interestingly, PfAtg8 lacks C-terminal masking residues, a feature not only found in *Plasmodium* but in other Apicomplexan species (Fig. 4.1). In all other characterized systems ATG8 genes encode amino acid(s) that mask a glycine residue, which must be exposed at the C-terminus in order for ATG7 and ATG3 to ligate ATG8 to PE. It is through
the protease activity of Atg4 that these masking amino acid(s) are removed prior to ligation to PE. However, in Apicomplexa the C-terminal glycine of newly translated PfAtg8 is accessible for PfAtg8-PE ligation by the E1- and E2- type ligases PfAtg7 and PfAtg3.

Figure 4.1. PfAtg8 alignment. Apicomplexa (shown in figure: Plasmodium falciparum and Toxoplasma gondii) have a lack of C-terminal masking amino acids, which can be seen in yeast and mammals (human LC3 above). Arrowheads mark key residues proven to play an important role in autophagic function, some of which are conserved in P. falciparum (black arrowheads) and others that are not conserved (white arrowheads). Other key residues (at the P2, P3 and P4 position) are also not conserved across eukaryotes, these having potential significance for Atg4 binding, suggesting that PfAtg4 has a different catalytic pocket than mammalian or yeast Atg4. This could be important for selective inhibitors as potential drugs, as in such proteases specificity is often strongly associated with the residues N-terminal of the cleavage site.

Residues that have been determined essential for normal autophagy function in yeast Atg8 are not fully conserved in P. falciparum. For example, two hydrophobic resides, Phe77 and Phe79, are required for Atg4 binding but in P. falciparum only Phe79 is conserved [182]. PfAtg8 contains a tyrosine at this position, a neutral amino acid. Additionally, other residues necessary for the catalytic activity of Atg4 are not conserved in PfAtg8, including residues at the P2, P3 and P4 positions upstream of the cleavage site. This suggests that PfAtg4 has a different catalytic pocket than mammalian Atg4 or yeast Atg4. This
could be important in the design of selective inhibitors as potential drugs, as in such proteases specificity is often strongly associated with the residues N-terminal of the cleavage site. Residues that are required for autophagic function in yeast that are conserved in *P. falciparum* include Arg28, Lys49, Leu51, and Ph79 (Fig. 4.1 black arrowheads) [182,183]. Residues that are important autophagic function in yeast but are not conserved in PfAtg8 are Y51F, L55M, and P77Y (Fig. 4.1 white arrowheads) [182,183].

### 4.3.2 PfAtg8 Localizes to the Parasite’s Apicoplast

In order to study ATG8 conjugation in *P. falciparum* we constructed an N-terminal mCherry fluorescently tagged PfATG8 under the regulation of the PfHsp86 promoter (Fig 4.2A). This construct was transformed into *P. falciparum* to create an episomally expressed mCherry-PfAtg8 parasite line. Upon activation of autophagy in other systems, fluorescently tagged Atg8 have shown punctate localization, representing Atg8-PE labeled autophagosomes [184-186]. In *P. falciparum*, however, we observe localization of mCherry-PfATG8 to tube-like structures extending between the food vacuole and the parasite membrane (Fig 4.2B). The unusual localization of PfATG8 suggests an interesting and possibly novel role for autophagy in *P. falciparum*. Recent work in the Mizushima lab provides proof that PfAtg8 co-localizes with apical membrane antigen 1 (AMA-1), an apicoplast marker, using fluorescent microscopy. This was also confirmed at the ultrastructural level through immuno-EM, where PfAtg8 and AMA-1 gold-labeled particles were found to co-localize on the surface of the apicoplast.
4.3.3 PfAtg8 is Primarily Lipidated in Erythrocytic Stage Parasites

In order to determine the lipidation status of PfAtg8, anti-sera against recombinant PfAtg8 (rPfAtg8) was generated. Experiments using the affinity-purified rPfAtg8 antisera indicate PfAtg8 was detectable via western blot analysis in crude parasite lysate (Fig. 4.3B). The PfAtg8 antisera was used to immunoprecipitate PfAtg8 from parasite lysate at different stages of the erythrocytic life cycle of *P. falciparum* and western blot analysis using affinity purified PfAtg8 antibody reveal that we are able to pull down what appears to contain both lipidated and non-lipidated PfAtg8 (Fig. 4.3C).
Membrane fractionation of parasite lysate was performed and the dominant PfAtg8 band is shown to migrate with the insoluble fraction, further evidence that this is could represent the phospholipidated species (Fig 4.4). This confirms other reports in the literature that in *P. falciparum*, PfAtg8 is primarily lipidated, consistent with its presence in the PfAtg8-PE form [106].
Figure 4.4. Membrane fractionation reveals faster migrating band is membrane-associated. 3D7 parasites were purified by saponin-mediated release and lysed by sonication in lysis buffer. Nuclear, Soluble and Insoluble fractions were isolated and re-suspended in lysis buffer + 1% TritonX. Samples were separated by SDS Nu-PAGE and blotted with rPfATG8 antisera.

4.3.4 PfAtg7 Attenuation Results in an Intermediate Delayed Death Growth Phenotype

To assess the slow growth phenotype of parasites with attenuated levels of PfAtg7 we examined the PB-57 parasite cell line (see 3.3.4, Fig. 3.7) including growth rate, examining merozoite count, cycle time, and doubling time.

According to Adams, the PB-57 parasites have a 3.7 fold decrease in parasitemia [174]. To confirm this observed phenotype, PB-57 parasites were grown over a 72 hour time period and parasitemia was recorded over time (Fig. 4.5).
Peripheral blood smears were made from synchronized cultures containing fully mature schizonts of 3D7 and PB-57 parasites and daughter merozoites counted from approximately 300 schizonts. Counts were performed by two individuals in a blinded fashion. No significant difference in merozoite number was found between 3D7 and PB-57 parasites. 3D7 schizonts contained 14.165 ± 0.085 and PB-57 schizonts contained 14.96 ± 1.274.

The cycle time (invasion→development→egress→invasion) for PB-57 and 3D7 parasites was determined by monitoring growth of highly synchronized cultures every 2 hours for 4 days by flow cytometry. The percentage of ring stage parasites was plotted over time to reveal the cycle time of the parasites. Results indicate an approximate 44-hour cycle time for both 3D7 and PB-57, showing that attenuation of PfAtg7 has no significant affect on cycle time of the parasites erythrocytic stages (Fig. 4.6).

The doubling time of parasites was also calculated for 3D7 and PB-57 parasites using the equation:

\[ T(d) = \frac{(T2-T1) \times \log(2)}{\log(N2-N1)} \]

where \(T(d)\) is doubling time, \(T\) is timepoint, and \(N\) is number of cells

This difference in doubling time between 3D7 and PB-57 may be able to account for slow growth. The doubling time is significantly different for the two cells lines. The doubling time of 3D7 parasites is 17.9 ± 0.156 hours and for PB-57 parasite is 20.25 ± 0.609 hours v, with a p-value=0.006.
Cell cycle time for PB-57 and 3D7 parasites is unaltered. Synchronous cultures of 3D7 and PB-57 parasites were split to low parasitemia and flow cytometer readings were taken of both cultures every 2h for >80h to assess cycle time of each parasite line. PB-57 parasites, despite 70% decrease in PfAtg7 expression and a severe growth phenotype, showed a similar cell cycle time to wildtype parasites, 3D7.

4.3.5 PfAtg7 Attenuation Results in an Increased Sensitivity to Fosmidomycin

Fosmidomycin is an inhibitor of DXP reductoisomerase, an important enzyme in the non-mevalonate pathway of isoprenoid biosynthesis, the only necessary metabolic function of the apicoplast during erythrocytic stages. To address the role of PfAtg8 lipidation in apicoplast function, we determined the sensitivity of 3D7 and PB-57 parasites to fosmidomycin. For PB-57 parasites, where PfAtg7 is transcriptionally repressed, the IC$_{50}$ of fosmidomycin is decreased (2.8 µM for 3D7 vs. 1.4 µM for PB-57 parasites) (Fig. 4.7A). If PB-57 was preincubated with farnesol, a downstream product of isoprenoid biosynthesis, prior to the fosmidomycin sensitivity assay the IC$_{50}$ exceeds that of
Figure 4.7. PfAtg7 attenuation leads to an increase in fosmidomycin sensitivity. Fosmidomycin targets the isoprenoid biosynthesis pathway of the apicoplast. (A) Fosmidomycin dose response curve on 3D7 and PB-57 parasites +/- farnesol. A dose response assay reveals the same change in sensitivity to this inhibitor. 3D7 wildtype parasites have an IC_{50} of 2.8µM and an IC_{50} of 1.4µM in PB-57 parasites (PfAtg7 attenuated). When farnesol, a downstream product of the isoprenoid biosynthesis pathway, is added to PB-57 parasites for 8 days and then removed prior to the fosmidomycin assay the IC_{50} is 3.4µM. (B) Fosmidomycin dose response curve on PfAtg7-RFA parasites +/-TMP. A dose response assay reveals a change in sensitivity to this inhibitor upon PfAtg7 attenuation, with an IC_{50} of 3.0µM when PfAtg7 is at normal levels and an IC_{50} of 1.8µM upon PfAtg7 attenuation.

3D7 (3.4 µM). Although farnesol does not rescue the growth phenotype of PB-57 parasites, it appears to rescue the change in sensitivity to fosmidomycin in these parasites. The experiment was also repeated with PfAtg7-RFA parasites +/- TMP. In the presence of TMP (normal PfAtg7 levels) the IC_{50} is 3.0 µM but upon removal of TMP (reduced PfAtg7 levels) the IC_{50} is 1.8 µM (Fig. 4.7B). This is further evidence that when PfAtg7 is reduced the parasite is more sensitive to fosmidomycin, suggesting a role for PfAtg8 lipidation in apicoplast function.

4.3.6 A Model for PfAtg8 Lipidation Function

The discovery that PfAtg8 localizes to the apicoplast strongly suggests that the function of PfAtg8 lipidation in *P. falciparum* erythrocytic and exo-
erythrocytic stages has a functional role in apicoplast biology, the details of which have not been elucidated. The slow growth phenotype of PfAtg7-RFA-TMP and PB-57 parasites, both having an attenuation of PfAtg7 levels, confirms that this pathway has a crucial function needed for normal parasite growth and survival. Densitometry analysis of PfAtg7-RFA +/-TMP western blots confirms a decrease in PfAtg7 protein levels by up to 67.6±4.0% (Fig. 3.5B) and PB-57 qPCR confirms a 70% decrease in PfAtg7 expression (Fig. 3.7E). Our model is that this pathway played a role in either 1) piecemeal autophagy of apicoplast (apicophagy), perhaps in times of organelle damage, 2) expansion of the apicoplast during development, or 3) segregation of the apicoplast during schizogony (Fig. 4.8). The cell cycle data and merozoite counts supports the latter, that PfAtg8 function is involved in apicoplast segregation and the attenuation of PfAtg7 is leading to a segregation defect.

To further test our model of apicoplast maintenance as a role for Atg8 lipidation we are utilizing the PB-57 cell line. The first experiment to prove a role in apicoplast biology is to attempt a rescue of the growth phenotype using isopentenyl diphosphate (IPP), the apicoplast product proven essential to the parasite [67]. This rescue was attempted using downstream products of the MEP pathway, farnesol and geranylgeranyl, but these compounds were found to be toxic at high concentrations and failed to rescue growth at lower concentrations (data not shown). If IPP rescues PB-57 growth, we can conclude that Atg8 lipidation has a function in maintaining healthy apicoplasts in P. falciparum parasites.
Since the cell cycle time of PB-57 and 3D7 parasites are roughly equal and both appear to produce an equal number of merozoites, the nature of the slow growth phenotype due to PfAtg7 attenuation is unknown. An invasion assay is currently in progress to differentiate whether the slow growth phenotype of PB-57 parasites is due to invasion defect or a delayed death type phenotype where parasites do invade but then die during intracellular development. For this assay, blood smears of highly synchronized parasites during invasion at 0, 4, 8, 12, and 24 hours are counted for newly infected RBCs. If there is an invasion impairment, we would expect to see a decrease in parasitemia at the first timepoint and every ring in a erythtocyte would develop normally at subsequent timepoints. If slow growth is not due to an invasion impairment and is more like a delayed death phenotype, then we will see normal invasion but subsequent parasite death during intracellular development.

In addition to the invasion assay, fluorescent microscopy using mitotracker and the apicoplast marker apical membrane antigen-1 (AMA-1) will indicate whether there is an organellar segmentation defect during schizogony. Mitotracker may suggest Atg8 lipidation to be involved in apicoplast segregation given the proximity of the two organelles [187]. If a mitochondria segregation defect is observed, it may indicate that the apicoplast is also not segregating. This would explain PB-57 slow growth, as the merozoites without an apicoplast would be able to invade but not develop during the erythrocytic cycle. An initial look at the ultrastructure of both PfAtg7-RFA +/- TMP and PB-57 parasites was explored via
transmission electron microscopy; however, TEM failed to extrapolate a statistically significant phenotype (Appendix B).

Figure 4.8. Model for PfAtg8 lipidation biochemistry and function. (A) Due to the lack of C-terminal masking amino acids, PfAtg4 is not needed for lipidation of PfAtg8. Through the action of PfAtg7 and PfAtg3 PfAtg8 is phospholipidated to membrane components of the apicoplast. PfAtg4 is able to free PfAtg8 from the membrane. (B) Our proposed model is that PfAtg8 may be involved in one of three functions at the apicoplast’s surface: (1) Piecemeal autophagy of the apicoplast, (2) expansion of the apicoplast membrane during expansion, or (3) segregation of the apicoplast during daughter merozoite development.

4.4 Discussion

The biochemistry of the Atg8 lipidation pathway is typical of a UBL pathway. The small ubiquitin-like protein Atg8, however, is conjugated to a phospholipid, instead of a protein target, through the activity of an E1- and E2-
type ligases, Atg7 and Atg3. Atg12-Atg5 can act as an atypical E3-type ligase that aids in transferring Atg8 from Atg3 to PE. This system, first described in yeast, has now been validated in many eukaryotic systems, including mammals, plants and protists. Protists have unique features of this pathway, including absence of C-terminal glycine masking amino acids, localization to organelles, and seemingly unique functions. In *P. falciparum*, all current data, presented here (Fig. 4.4) and in the literature, supports that PfAtg8 is constitutively in an active state, conjugated to a phospholipid [131]. Furthermore, PfAtg8 localizes to the parasite’s apicoplast, an essential organelle absent from all other eukaryotic systems other than Apicomplexan parasites [106].

As we have reported, PfAtg7 attenuation causes a severe slow growth phenotype indicating that the pathway is likely essential in the parasite, but the exact function of the pathway is still unclear [132]. Recent evidence has shown that the Atg8 lipidation pathway may play a classical autophagy role involved in a survival response to starvation [131]. Although no PbAtg8-marked autophagosomes were detected in liver stages, it is possible that the classic role of autophagosome formation may be functional in the blood stages [130,131]. However, it seems unlikely as malaria parasites do not encounter starved state in vivo, as the parasites reside in a niche where they continually have a source of nutrients, mainly hemoglobin from the red blood cells and glucose levels, even in malnourished patients, do not vary greatly in the blood serum. Despite this, PfAtg8 is still shown in vitro to localize to autophagosome structures when starved of amino acids [131].
The aim of this work has been to examine the role of PfAtg8 lipidation. If apicoplast maintenance or function is the primary role of the PfAtg8 lipidation pathway this could indicate autophagy as a potential drug target. By attenuating PfAtg7 there may be decreased PfAtg8 at the organelle’s surface, which may impair the overall function of the apicoplast, either directly or indirectly. Impairing apicoplast function in parasites is known to cause a delayed death phenotype. Originally the hypothesis behind the delayed death phenomenon was a segregation defect, where not all merozoites of the next progeny received an apicoplast. It turned out the true mechanism included normal segregation but of non-functional apicoplasts, where the housekeeping transcription and translation machinery was impaired [64]. However, in these parasites, where PfAtg7 is attenuated, we know that the slow growth is not due to an altered cell cycle time or change in merozoite number, allowing for speculation that either an invasion defect, apicoplast segmentation defect, or apicoplast functional defect may be responsible for slow parasite growth. The latter being described as a classical delayed death as seen in parasites treated with antibiotics [188]. Since PB-57 parasites have a 70% decrease of PfAtg8 expression levels (Fig. 3.7E), it is possible that PfAtg8 is still conjugated to PE at a low rate, resulting in an intermediate delayed death-like phenotype. The three assays described above in section 4.3.6 will address all three of these options and should give us a clear answer to the what is happening in these slow growing parasites.

The fosmidomycin assay seems to support the third possible mechanism behind PB-57 slow growth, that PfAtg8 lipidation plays a role in keeping
apicoplast functionally active. The change in IC$_{50}$ indicates that parasites with attenuated PfAtg7 are more sensitive to fosmidomycin, suggesting that their apicoplasts may be less functional. Thus, exposing parasites to this MEP pathway-targeting drug, a decreased concentration of drug is needed to elicit death. Based on observation of PB-57 blood smears (data not shown), it seems that parasites are invading normally but dying relatively quickly. This would support a segmentation defect, as parasites with non-functional apicoplast tend to survive until they develop into schizonts. The invasion assay and fluorescent microscopy with mitotracker and AMA-1 will explain the mechanism, both of which are in progress.

In protists this pathway has been shown to affect differentiation, organelle biology, through association with the mitochondria and apicoplast, a cell death mechanism, as well as the classic role: survival in response to stress, such as starvation [128,129]. Similar mechanisms have been seen in other systems, such as mitochondria turnover, especially in times of damage to the organelle to avoid apoptosis [189]. The connection of autophagy to apoptosis in eukaryotes is well described and the role of autophagy as a survival mechanism is clear. The Atg8 lipidation pathway appears to function in a way that is needed for parasite survival in the different niches where parasites reside. Clearance of organelles during differentiation or during development doesn’t seem novel, but the localization of PfAtg8 to the apicoplast is novel since this is a unique organelle, which does not contribute to cellular death mechanisms like the mitochondria.
Understanding the exact function of the PfAtg8 lipidation pathway, especially in relation to the apicoplast, is crucial for many reasons. First, the pathway includes three enzymes that could be potential drug targets, as it appears the pathway is essential. Knowing the exact function of the pathway will also aid in possible combination therapies for such drugs for the avoidance of development of resistance mechanisms by the parasite. Furthermore, examining this pathway in these anciently diverging parasites might lend insight to the evolution of the pathway across eukaryotes, which could aid in autophagy research for cancer and other human disease.

4.5 Acknowledgments

Mark Drew is accredited for the cloning and transfection of mCherry-Atg8 parasites and the 2006 Biology of Parasitism class for their live imaging. I gratefully acknowledge Maribeth Spangler who contributed greatly to experimental design, execution, and critical discussion on this chapter, as well as for all the editing that she performed on this entire dissertation.
Chapter 5: Discussion

Malaria is a major global health concern that affects nearly half the world’s population. Although curable, the prevention, control, and treatment of such a devastating disease is dynamic and complex, tightly interwoven with socioeconomic factors. Malaria endemic areas are most often developing countries, which is problematic for the distribution of bed-nets and treatments due to the vast rural areas that encounter malaria infected mosquitoes. Families are often too poor to pay for treatments, especially since children often contract the disease 2-3 times a year until they develop immunity to the parasite, a weak immunity that only lasts upon remaining in the endemic area. Public health measures are suffering in these areas, only adding to the problem. To date there hasn’t been development of a successful vaccine, but there are many effective antimalarials able to cure the disease. Unfortunately, every antimalarial on the market has encountered some level of parasite resistance. This underscores the importance of drug discovery research. New drugs also need to be inexpensive and easily accessible to be able to reach the rural areas where they are needed most.
Of the five species of *Plasmodium* parasites that cause human disease, including *P. ovale, P. malariae, P. vivax, P. knowlesi,* and *P. falciparum,* the last is the most devastating in terms of disease severity and death. Fortunately, in regards to research, *P. falciparum* is the only species that we are able to culture *ex vivo* in a laboratory setting [71]. Studying the basic biology of *P. falciparum* may reveal novel targets or pathways for drug development or how to utilize current drugs and drug combination therapies to combat cerebral malaria. One such biological pathway is autophagy. Until recently, autophagy was unexplored in protists, especially in *P. falciparum.* Previous to 2012, the only work on *P. falciparum* autophagy reported in the literature was found in protist autophagy reviews [126-128]. *Plasmodium* autophagy was discussed in these reviews without the publication of experimental data and mostly based on non-peer reviewed observations and speculation. Since 2012, five research studies on *Plasmodium* autophagy have now been published, mainly focused on the players of the Atg8 lipidation pathway and its function [106,108,130-132]. This is truly the frontier of autophagy research in malaria parasites.

The term autophagy is the general term used primarily for what is now known as macroautophagy. This process was originally named after it’s first discovered function; its Greek name means “self eating.” What was thought of as a simple mechanism to survive cellular starvation is now understood as a much more complex process with multiple functions. In fact, it is now known that there are three main arms of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. The functions of these pathways in eukaryotes
include: 1) recycling of cytosolic components as a nutrient source during starvation, 2) a mechanism to degrade/recycle protein aggregates and misfolded proteins, 3) a mechanism to degrade/recycle organelles (for example pexophagy, the degradation of peroxisomes, or piecemeal autophagy of the nucleus), 4) avoidance of apoptosis through mitophagy, the degradation of the mitochondria, which assures that apoptotic factors won’t lead to cell death, and 5) clearance of intracellular pathogens. This list of functions continues to expand as autophagy is explored in other eukaryotes and disease states.

The Atg8 lipidation pathway is explored in this work for the reason that it is seemingly the only complete set of autophagy proteins in *P. falciparum*. This lipidation pathway is the most basic pathway needed for autophagosome formation. The conjugation of Atg8 to phosphatidylethanolamine (PE) localizes the protein to the phagophore assembly site (PAS). This conjugation event occurs through the activity of three enzymes, previously described in yeast, the cysteine protease Atg4, the activating enzyme Atg7 (E1), and the conjugating enzyme Atg3 (E2). Atg4 cleaves the C-terminus of newly transcribed Atg8 to expose a glycine that can be conjugated to a phospholipid by a peptide bond. After this cleavage event, Atg7 forms a thioester linkage to Atg8, transfers Atg8 to Atg3 for conjugation to PE. This localization of Atg8 to the PAS begins the nucleation process and expansion of the autophagosome. After autophagosome closure, Atg4 cleaves Atg8 off of the outer membrane, allowing for its fusion to the lysosome and recycling Atg8 back to the cytosol. It is unknown whether the biochemistry of these enzymes differ much in *P. falciparum*, there are however,
notable differences in primary structure. These include the lack of glycine masking amino acids on the C-terminal of PfAtg8, the hypothesized difference in enzyme catalytic pockets based on primary structure of the enzymes and Atg8, and the difference in length and composition of each player. Exploiting these differences in the development of specific inhibitors that could represent a novel class of antimalarial compounds.

To assess this pathway in the parasites’ erythrocytic cycle, several strategies were executed. This work focuses on the activating enzyme of the pathway, PfAtg7. Tagging PfAtg7 with hemogluttanin (HA) allowed us to visualize the protein on western blot and discover two species, one at the predicted 150kDa and one at 65kDa (Fig. 3.4A). Atg7 in other eukaryotic systems is typically around 70kDa. Although it is typical of Plasmodium genes to have long stretches of non-conserved sequence within known genes, a processing event could play a role in this smaller species presence. This processing event could represent a novel mechanism of autophagy regulation or it could also represent a proteolytic event. A conjugation assay of the E1-type PfAtg7 enzyme is currently being explored to investigate which species of PfAtg7 is active. Details of this assay consist of isolating active PfAtg7-HA by immunoprecipitation, followed by incubation with ATP and recombinant PfAtg8. The binding event of PFA tg7-PfAtg8 can be visualized as a band shift by comparing reducing and non-reducing Nu-PAGE gels.

To attenuated protein levels of PfAtg7 a regulatable fluorescent affinity tag (RFA) was added to the C-terminal end. Densitometry analysis of western blot
detection of PfAtg7-RFA +/−TMP confirmed that even a 30% decrease in PfAtg7 levels reduced parasites growth significantly. Another method of gene attenuation, the piggybac transposon system, was successfully developed in *P. falciparum* [172]. A mutant, PB-57, generated by this approach, inserted a transposable element 705 base pairs upstream to PfAtg7, causing a 3.7 fold decrease in growth, similar to the growth defect observed in PfAtg7-RFA parasites [174]. Semi-quantitative PCR confirmed that PfAtg7 was attenuated but the gene upstream, a ribosomal protein, was not affected (Fig. 3.7A-D). Furthermore, qPCR confirm that PfAtg7 expression levels are 70% lower than in 3D7 wildtype parasites, normalized to PfActin (Fig. 3.7E). These results confirm that PfAtg7 is essential for normal growth and suggest that a full knock-out of PfAtg7 may kill parasites.

Two novel discoveries about PfAtg8 lipidation attributed to the hypothesis and model: the localization of PfAtg8 to the apicoplast and that PfAtg8 is constitutively activated without the induction of autophagy or any other activation stimulus [106,131]. Although PfAtg8 may also play a role in autophagosome formation, the main function of PfAtg8 lipidation seems to involve the apicoplast [131]. The model proposed is that PfAtg8 functions in apicoplast maintenance in three possible ways: 1) turnover of apicoplast by piecemeal autophagy, 2) expansion of apicoplasts during branching/development, and 3) segmentation of apicoplasts into daughter merozoites during schizogony. To explore this model and to identify the exact function of PfAtg8, the cause of slow growth in parasites with decreased PfAtg7 is being examined. The PB-57 parasites have the same
cycle time and the same number of merozoites per segmented schizont as 3D7 wildtype parasites. One difference seen in PB-57 parasites is the doubling time, which is about 2 hours longer in PB-57 parasite than wildtype. These results support a delayed death-like phenotype, which is the typical mechanism of parasitic death due to an apicoplast defect [64]. Furthermore, fosmidomycin dose response assays reveal a decreased sensitivity to this apicoplast-targeting drug when PfAtg7 is attenuated.

These pieces of data support a role of PfAtg8 lipidation in apicoplast biology. To further elucidate this role, future experiments involving fluorescent microscopy and immunofluorescent assays will allow for the visualization of mitochondria and apicoplast segmentation during schizogony in mutant and parental parasite lines. An apicoplast rescue experiment utilizing IPP, the sole essential product of the apicoplast, will also provide additional evidence of PfAtg8 lipidation in apicoplast biology [67]. Finally, an invasion assay will differentiate between an invasion defect or a delayed death-like phenotype.

Overall, the data presented here contributes to an emerging field of autophagy in protists. This field has been expanding for the past decade, initially investigated in Leishmania and appearing in the literature in 1999, followed by Trypanosoma and Toxoplasma data in 2005 and 2006, respectively, and finally by Plasmodium in 2012 [106,190-192]. This exploration of autophagy in protists has added the following functions of autophagy to the growing list: 1) clearance of organelles during parasite differentiation, such as glycosomes in Trypanosoma [122], 2) a controlled death mechanism involving mitochondrial fragmentation, as
in *Toxoplasma* [124], 3) parasite development during differentiation, as seen with tachyzoite development in *Toxoplasma* [123], and 4) an undefined role in apicoplast biology, as observed in both *Toxoplasma* and *Plasmodium* [106,125]. The study of autophagy in parasites is interesting for drug development as previously discussed, but it also has implication in the evolution of such a basic and well-conserved pathway. By exploring autophagy in these anciently diverging eukaryotes, we may be able to bring to light unknown mechanisms that we can relate to human disease due to autophagy defects or an overall understanding of a pathway that was initially thought to be so simple but turned out to be so complex.
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## Appendix A: Bioinformatic Analysis

### A

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**Figure A1.** Alignments show similarity between yeast and P. falciparum Atg proteins. Atg8 (A), Atg4 (B), Atg7 (C) and Atg3 (D) alignments between P. falciparum and S. cerevisiae performed through ClustalW. PfAtg protein sequences identified by a PlasmoDB blastp using ScAtg protein sequences as described in section 2.1 of the Dissertation.

### B

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**Contd**
Fig. A1 continued

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| PfATG3/1-313 | 47 DFLVKFKWEWQ EADKDA VV YLPENKOF LTMNVFCKQR KDKL 91 |
| ScATG3/1-310 | 38 DVLCMFPTK WNEE SBDISRDFLPK NQFLII KVRCCRAEQ 83 |
| PfATG3/1-313 | 92 NN IVDNLKLIVDNLWP SVEFDTNP DIXEYLPSNEYTI NDKNIYN 137 |
| ScATG3/1-310 | 84 --------------- V VECPD VIMNGEADGDDEDDDVL EYIGSETEHVQ 118 |
| PfATG3/1-313 | 138 YEE EEDDNC TEAI D INNFYMNLI KEHDP A S I N STSCY SKNMLH 183 |
| ScATG3/1-310 | 119 --------------- TAGGT I S I DDI DELIDQMEIRED E NDD TEEFNA KGGLA 160 |
| PfATG3/1-313 | 184 DNLMKI RTYDVSTTYDKYQT PRIWLFGYNKGDPLKLSEIFEDIL 229 |
| ScATG3/1-310 | 161 KDMAQ E FYDLHYA S TSSRVPKMYLVGFNSAGS PLLRMFDI S 206 |
| PfATG3/1-313 | 230 SDYSDKTVYDPHPCT G - - VMTASIHPCKHA - - - - - - - 258 |
| ScATG3/1-310 | 207 A DYRTKTAT I EKLPEY KNSLVSL S I HPC HHNVMK I LLDKVVVR VQ 252 |
| PfATG3/1-313 | 259 - - - - - EAI LNVVNNWI S EKEP - - KHDLYLLFLLK FIEGVI 292 |
| ScATG3/1-310 | 253 RRRKELEQE EQLEDGVD WDLEDD I DDSRLRQVYLIVFL FITE SVT 298 |
| PfATG3/1-313 | 293 PTIELYDFT TDIEIPRDSAGL 313 |
| ScATG3/1-310 | 299 PIQHDYGME CW - - - - - 310 |
**Figure A2.** Alignment shows similarity between yeast and protist Atg3 proteins. Atg3 alignments between *S. cerevisiae* Atg3 and the putative Atg3s of *Toxoplasma gondii* (Tg), *Plasmodium falciparum* (Pf), *Trypanosoma brucei* (Tb), *Trypanosoma cruzi* (Tc), and *Leishmania infantum* (Li) performed through ClustalW. Atg3 protein sequences identified by a PlasmoDB blastp using ScAtg3 protein sequences as described in section 2.1 of the Dissertation.
Figure A3. Alignment shows similarity between yeast and protist Atg4 proteins. Atg4 alignments between S. cerevisiae (yeast) Atg3 and the putative Atg4s of L. infantum (Li), T. brucei (Tb), and P. falciparum (Pf) performed through ClustalW. Atg4 protein sequences identified by a PlasmoDB blastp using ScAtg4 protein sequences as described in section 2.1 of the Dissertation.
Figure A4. Alignment shows similarity between yeast, human, and protist Atg7 proteins. (A) Atg7 alignment between S. cerevisiae (Sc), Human (Hs) Atg7 and the putative Atg7s of Plasmodium falciparum (Pf), Plasmodium berghei (Pb), and Plasmodium vivax (Pv) performed through ClustalW. Plasmodium Atg7 protein sequences identified by a PlasmoDB blastp using ScAtg7 protein sequences as described in section 2.1 of the Dissertation. (B) The C-terminal of Atg7 (A, zoomed in) shows higher conservation in the C-terminal than the protein as a whole. (C) Human (Hs) Atg7 and putative PfAtg7 aligned.
**Fig. A4 continued**

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Continued
Fig. A4 continued

C

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Figure A5. Alignment shows similarity between yeast and protist Atg12 proteins. Atg12 alignments between S. cerevisiae (yeast) Atg12 and the putative Atg12s of Plasmodium falciparum (Pf), Trypanosoma brucei (Tb), and Trypanosoma cruzi (Tc) performed through ClustalW. Atg12 protein sequences identified by a PlasmoDB blastp using ScAtg12 protein sequences as described in section 2.1 of the Dissertation.
Figure A6. Alignment shows similarity between yeast and protist Atg5 proteins. Atg5 alignments between S. cerevisiae (yeast) Atg5 and the most similar putative Atg5s of Trypanosoma brucei (Tb), Trypanosoma cruzi (Tc), Plasmodium falciparum (Pf), Toxoplasma gondii (Tg), and Leishmania infantum (Li) performed through ClustalW. Atg5 protein sequences identified by a PlasmoDB blastp using ScAtg5 protein sequences as described in section 2.1 of the Dissertation.
Table A1. Results of bioinformatic analysis of ATG genes in *P. falciparum*. See M&M in main manuscript for search parameters.

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### Upstream signalling

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Appendix B: Transmission Electron Microscopy

Figure B1. Transmission electron microscopy (TEM) images from PM1 parasites in the presence of trimethoprim (TMP) serve as a control for ultrastructural analysis. Six representative images (A-F) of the parental parasite line PM1 reveal no obvious defect in parasite morphology.

Continued
Figure B2. TEM images from PfAtg7-RFA parasites in the presence of TMP reveal no obvious ultrastructural phenotype. Six representative images (A-F) of PfAtg7-RFA parasites +TMP reveal no statistically significant difference compared to PfAtg7 -TMP parasites.

Continued
Fig. B2 continued

D

E

F

500 nm
Figure B3. TEM images from PfAtg7-RFA parasites post TMP removal reveal no obvious ultrastructural phenotype. Six representative images (A-F) of PfAtg7-RFA parasites -TMP reveal no statistically significant phenotype. A small percentage of parasites exhibit an electron dense food vacuole (E) but this was not phenocopied in the PB-57 parasites (Fig. B5).

Continued
Figure B4. TEM images from 3D7 parasites serve as a control for ultrastructural analysis. Six representative images (A-F) of the wildtype parasite line 3D7 reveal no obvious defect in parasite morphology.

Continued
Fig. B4 continued

E

F

500 nm
Figure B5. TEM images from PB-57 parasites reveal no obvious ultrastructural phenotype. Six representative images (A-F) of PB-57 parasites reveal no statistically significant difference compared to 3D7 parasites.

Continued
Fig. B5 continued

E

F

500 nm