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A novel RCC1-like protein is a crucial regulator of the intraerythrocytic cycle of the human malaria parasite, *Plasmodium falciparum* .

Marcus Davon Skaflen
James Madison University

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A novel RCC1-like protein is a crucial regulator of the intraerythrocytic cycle of the human malaria parasite, *Plasmodium falciparum*.

Marcus D. Skafren

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Dedication

This thesis is dedicated to my family. To my wife and my children for all of their support, and understanding of my long hours and late nights. Their belief and curiosity was a boon that kept me at it long into the night, and well into the morning. My brothers continually inspired me when I was growing up, driving me to learn more so that I might keep up with them. Finally I must thank my high school biology teacher Mr. Myron Blosser who instilled me with a deep interest and respect for biology.

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Table of Contents

Dedication	ii
Acknowledgments	iii
List of Figures	v
List of Tables	vi
Abstract	vii
I. Introduction	1
Importance.....	1
Disease History.....	2
Life Cycle.....	3
Pathology.....	6
<i>Plasmodium falciparum</i> Cell Cycle.....	9
<i>Plasmodium</i> Genomics.....	14
Mutant Identification.....	16
Project Goals.....	19
Specific Aims.....	20
II. Materials and Methods	21
Cell Culture.....	21
Ring Stage Culture Synchronization.....	21
Late Stage Parasite Purification	22
Genomic DNA Extraction.....	22
RNA Extraction.....	22
RT-PCR.....	23
Proliferation Assay.....	23
Flow Cytometry.....	24
Recombinant Protein Expression.....	24
Western Blot	26
Immunohistochemistry.....	26
Transfection of Degradation Domain and Selection.....	27
Southern Blot	28
III. Results	29
Bioinformatic Analysis.....	29
Protein Expression.....	32
Mutant Characterization.....	35
Conditional Knockout.....	38
Cell Cycle.....	40
IV. Discussion	46
V. List of References	54

List of Figures

Figure 1. Life cycle of <i>P. falciparum</i>	5
Figure 2. The intraerythrocytic cycle of <i>P. falciparum</i>	7
Figure 3. Cross species chromosomal alignment.....	17
Figure 4. PFRLP1 protein sequence motifs.....	30
Figure 5. Recombinant protein purification and antiserum specificity.....	33
Figure 6. PFRLP1 expression and location.....	34
Figure 7. Intraerythrocytic life cycle analysis.....	36
Figure 8. Intraerythrocytic proliferation assay.....	37
Figure 9. Microscopic analysis of merozoite formation.....	39
Figure 10. Degradation domain merozoite formation analysis.....	41
Figure 11 Nuclear division time course assay.....	42
Figure 12. Nuclear division time course analysis.....	44
Figure 13. Panel of antiserum and α tubulin staining of the three intraerythrocytic stages...45	
Figure 14. Comparison of mitosis between a typical eukaryotic cell and <i>Plasmodium</i>	52

List of Tables

Table 1. Comparative protein sequence analysis.....	31
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Abstract

Malaria is a deadly infection caused by a single celled protozoan of the *Plasmodium* genus. *Plasmodium spp.* are transmitted to humans by mosquitoes, and initially invade the liver, but the disease is caused by the blood stage of the infection. Approximately 500 million cases of malaria are documented annually and over 1 million of those result in death. *Plasmodium falciparum* is the most lethal of five species known to infect humans. To further compound this problem, drug-resistant parasite strains have been documented for every currently available antimalarial drug, making the need to identify new drug targets more urgent than ever. Modern genetics have found that more than 50% of the *Plasmodium* genome codes for proteins of unknown functions, with no significant sequence homology to any known eukaryotic genes. Recent advances in forward genetics and the use of transposable elements to manipulate the genome of *P. falciparum* have made tremendous contributions to discovering the functions of these unknown genes, which is critical to rapidly advance antimalarial drug development.

In this study we have identified a gene of unknown function, PF3D7_1143500, that is significant for intraerythrocytic development of *Plasmodium*. This gene exhibits weak similarities to the human regulator of chromatin condensation 1 protein (RCC1) and appears to belong to the class of RCC1-like proteins that perform diverse functions in eukaryotes. A thorough cellular and molecular analysis of an insertional knockout mutant of PF3D7_1143500 in *P. falciparum* has revealed a critical role for this gene in the production of merozoites during the intraerythrocytic cycle. The insertional mutant parasite strain displays a significant delay in initiating nuclear division, which results in a 40% reduction in the number of merozoites produced at the end of the intraerythrocytic cycle, thereby severely attenuating the parasite growth rate. PF3D7_1143500 localizes to the microtubule

organization centers within the nucleus during the early stages of parasite development, suggesting it functions in regulating mitosis. Since cell cycle regulatory mechanisms are largely unknown in *Plasmodium*, the identification of this novel RCC1-like protein promises to offer new insights into this critical biological pathway that has high potential as an antimalarial drug target.

Introduction:

Importance

Malaria is a parasitic infection that has been causing disease in humans for thousands of years (Neghina *et al.* 2010). Malaria is widespread, pervasive, costly, and rapidly becoming resistant to modern drugs. There are 300-500 million cases of malaria reported annually, with more than 1 million of these cases resulting in death. Most cases of malaria occur in tropical regions, home to more than half of the world's population. However, modern travel has allowed the disease to escape tropical regions. In 2009 there were more than 1400 cases in the United States (Mali *et al.* 2011). The majority of those infected in the United States acquired the disease following travel to endemic regions. Worldwide, malaria has a tremendous monetary cost. There is a substantial direct economic cost of running and stocking treatment facilities with effective drugs. The total direct monetary cost is estimated to be over 12 billion dollars annually according to the Center for Disease Control (CDC). The indirect cost on economic growth is believed to be several times that number. For example, many sick people are unable to work or are required to spend time with sick children in lieu of working.

There are currently several anti-malarial drugs on the market but drug resistant strains of *Plasmodium falciparum* have been on the rise. The previous first line drug, chloroquine, has been compromised by the development of resistant strains. Resistance is a result of the mutation of a protein pump that is now capable of transporting the drug out of the cytosol (Ecker *et al.* 2012). Multiple drug regimens have been established in order to combat the rise of antimalarial resistant strains. Drug regimens are specifically tailored to optimize effectiveness based on the prevalent resistant strains. The development of resistance has been determined to rise from communities where there is low transmission and high population density. The hosts in these areas provide selective conditions for the expansion of resistant

strains. Over time these conditions have led to the development of significant resistance to previously effective drug regimens (White and Pongtavornpinyo 2003). The newest antimalarial drugs have already encountered resistant strains, and it is the rise in resistant strains that makes the need for identifying new drug targets paramount in antimalarial research. The World Health Organization monitors the identification and spread of resistant strains. However, the coordination and standardization of tests and sample analysis has complicated the accurate identification and tracking of the latest drug resistant strains (Talisuna *et al.* 2012). The drive for new drug targets could be mitigated by the development of vaccines against *P. falciparum*, however to date there is no effective vaccine available. There are several candidates being developed and tested, but so far none have proven successful (Heppner 2013). A better understanding of *Plasmodium* biology is crucial for the development of new drug targets to counter the rapid development of resistant strains and the development of effective vaccines.

Disease History

The protozoan organism *Plasmodium* is a microscopic parasite transmitted by the bite of the female *Anopheles* mosquito. Early medical writing in the Middle East and China described the unique periodic fevers that are characteristic of malaria. In the fifth century BC Hippocrates described the different fever cycles indicative of the disease showing just how long mankind has been subject to this infection (Neghina *et al.* 2010). The word malaria comes from the Latin words “mal” meaning bad, and “aria” meaning air. The fevers that come with the parasitic infection were first associated with dank misty swamp areas, which is the ideal breeding ground for mosquitoes. Attempting to combat the presumed source of the disease they would fill in the swamps, subsequently halting the spread of the disease by destroying the mosquito’s breeding ground. The actual cause of malaria, *Plasmodium*, was

discovered in 1880 with the use of microscopes. The first parasite was visualized in 1880 by a French army doctor named Charles Louis Alphonse Laveran. He described a foreign body inside the red blood cells (RBCs) of a sick soldier suffering from malaria. By 1898 scientists had proven that the mosquito was the vector responsible for the spread of the disease (Hafalla *et al.* 2011).

Life Cycle

Five different species of *Plasmodium* have been found that can infect humans: *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. Each of the different species behave similarly, yet are different enough that they can be distinguished as separate species pathologically. When an infected female *Anopheles* mosquito takes a blood meal from a human the *Plasmodium* sporozoites are transmitted by the saliva. Once inside the human host, the sporozoites travel through the bloodstream to the liver, and enter the non-erythrocytic life cycle. In the liver they actively invade the resident parenchymal cells and form parasitophorous vacuoles inside of the parenchymal cells (Hafalla *et al.* 2011). Inside the liver each species of *Plasmodium* behaves differently, but with the common goal of proliferation. The sporozoites go through a process of binary fission to reproduce rapidly. Each species has a different parasite yield and time span during this process. The structures formed by these rapidly dividing parasites in the parenchymal cells are known as cryptozoites. *P. vivax* and *P. ovale* have the ability to create dormant forms known as hypnozoites. The cryptozoites fill the hijacked parenchymal cell with vesicles packed with parasites known as merozoites. The number of merozoites produced during this stage, and the time it takes to rupture the cell, is indicative of each *Plasmodium* species. The rupture of the infected liver cells results in the release of hundreds of merozoites from the cryptozoite into the bloodstream. The merozoites travel to the lungs where they rupture

and release the merozoites packed inside. The release of merozoites into the bloodstream initiates the intraerythrocytic cycle of *Plasmodium spp.* All symptoms and pathology are caused by this particular cycle of the infection (Baer *et al.* 2007, Prudencio *et al.* 2011). Once inside the bloodstream merozoites invade RBCs. *P. falciparum* has no preferred erythrocyte stage to invade while *P. vivax* prefers young RBCs known as reticulocytes (Mons, 1990). Once inside the RBC the parasite begins to quickly replicate utilizing hemoglobin as its food source. Entrance into the intraerythrocytic cycle is identified by the penetration of the RBC and the formation of the parasitic vacuole. After the initial invasion the parasite develops into a trophozoite. The early trophozoite is called the ring stage due to its morphology. As the parasite feeds it develops into the trophozoite stage, losing the ring morphology. Lastly the trophozoite commences replication and matures into a schizont. The schizont stage is characterized by the development of multiple merozoites and ending when the RBC ruptures. The newly developed merozoites enter the bloodstream to infect new RBCs and repeat the cycle. The parasite can also undergo a sexual proliferation cycle where the parasite replicates sexually. This occurs when gametes, known as microgametocytes and macrogametocytes, are formed instead of a schizont. When the RBC ruptures it frees these haploid gametes into the circulation. These gametocytes are ingested by the female *Anopheles* mosquito during a blood meal. Inside the stomach of the mosquito the microgametocytes and macrogametocytes merge and form a zygote. The sexual recombination of the two gametocytes in the gut of the mosquito leads to genetic recombination (Walliker *et al.* 1987). During the sexual reproduction stage the zygotes develop into oocysts. These oocysts form in the wall of the mosquito's gut where they mature and produce sporozoites. These sporozoites migrate to the salivary glands of the mosquito where they are eventually transmitted to the human during the next blood meal via the salivary secretions (Fig. 1).

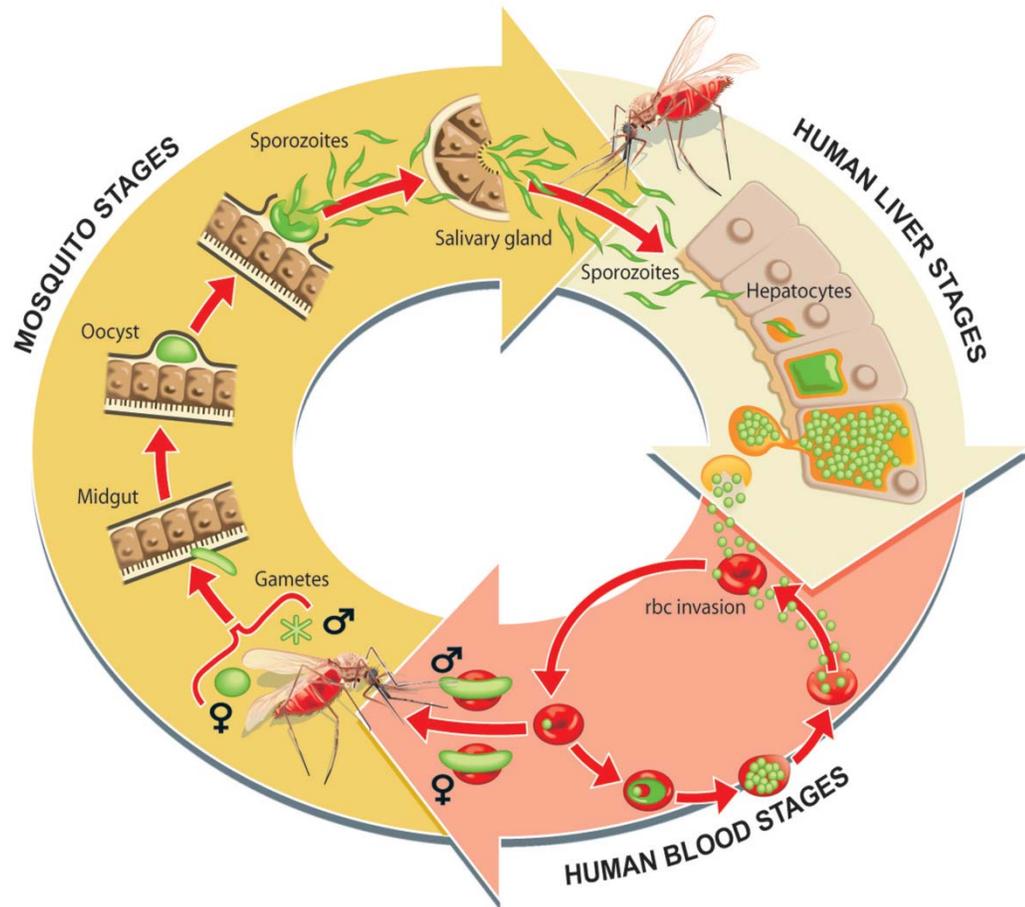
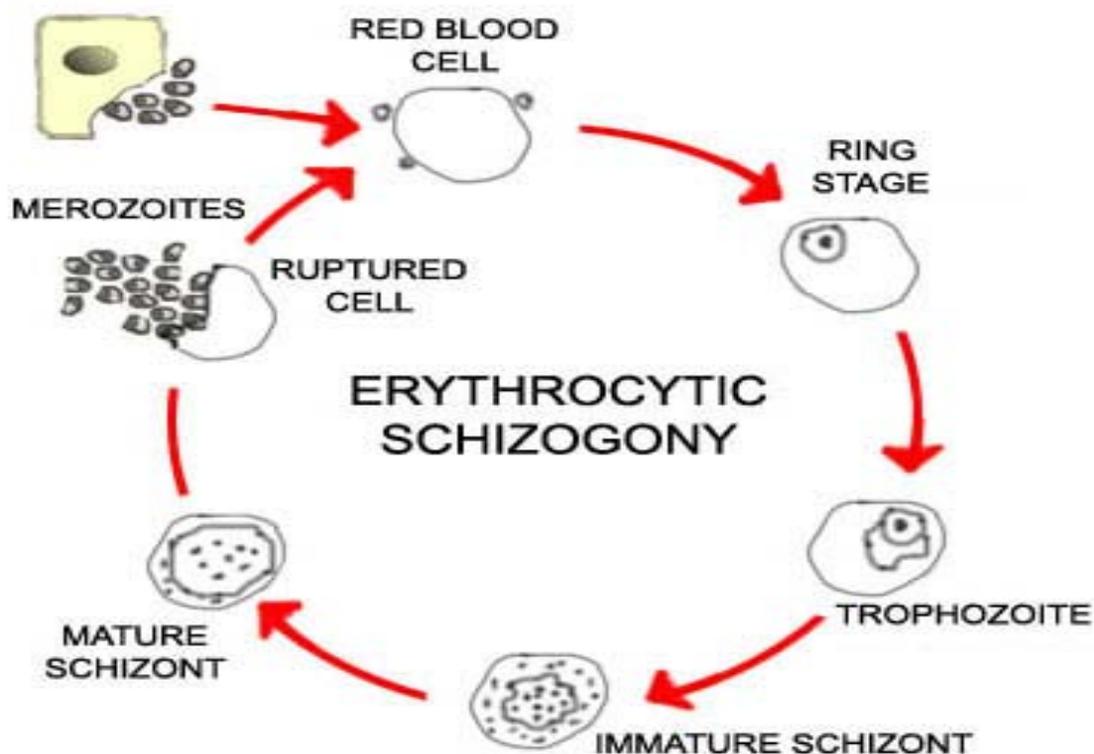


Figure 1. Life cycle of *Plasmodium*. *Plasmodium* has two different hosts and three different stages of development. Asexual reproduction occurs in two separate stages in the human host and sexual reproduction occurs in the gut of the mosquito host (Derbyshire *et al.* 2011).

Pathology

The clinical symptoms of malaria can take more than a week to manifest after the initial infection. The liver stages of infection do not typically cause pathology, it is only after the intraerythrocytic cycle begins that the infected individual shows signs of illness. Major symptoms include fever, chills, arthralgia, vomiting, jaundice, and anemia. The classic symptoms are chills followed with fever and sweating lasting four to six hours and cycles every two to three days depending upon the species of *Plasmodium* causing the infection (Akinosoglou *et al.* 2012). This study focuses on *Plasmodium falciparum*, which typically causes a cyclic fever that recurs every 36-48 hours. The rupture of the RBC leads to the classic clinical signs of malaria; anemia, fever, and chills. When the infected RBC ruptures it releases metabolic waste products and merozoites into the bloodstream. In the intraerythrocytic cycle *Plasmodium* goes through several cycles of invading RBCs, replicating inside of them, consuming the hemoglobin, bursting the cell, and invading new RBCs (Fig. 2). Ruptured RBCs release metabolic waste products from the consumption of hemoglobin by *P. falciparum*. The metabolic wastes released overwhelm the liver and lead to jaundice. The ruptured RBCs inundate the bloodstream with merozoites causing a fever as the immune system reacts to the sudden load of non-self parasite antigens. The newly released parasites quickly invade RBCs, essentially removing themselves from detection by the immune system, and the fever subsides. Each passage through the intraerythrocytic cycle consumes a greater number of RBCs and leads to anemia (Gerald *et al.* 2011). As the RBC is invaded it undergoes specific physical changes induced by the parasite and general physical changes in response to being invaded. The membrane of the RBC is extremely strong and flexible compared to most animal cells because of the high pressures and physical stresses it endures while traversing small capillaries. The strength and flexibility of the cell is due to the cytoskeleton containing protein complexes largely composed of an actin and $\alpha\beta$ spectrin



London school of Hygiene and Tropical Medicine

Figure 2. The intraerythrocytic cycle of *Plasmodium falciparum*. Merozoites infect a RBC and mature. The first stage, the ring stage, is identified by a small ring-like structure in the RBC. The second stage, the trophozoite, is characterized by an expanding cytoplasm and increased size of the parasite within the RBC. The third stage, the schizont, is characterized by multiple nuclei forming in the RBC as the parasite completes replication and prepares to rupture the RBC.

dimer-based network connected to the cell membrane by anchor proteins (Zuccala and Baum 2011). Parasitic invasion causes the rearrangement of the cytoskeleton during the formation of the parasitic vacuole and the subsequent alterations cause a loss in the flexibility of the RBC membrane.

The spleen is responsible for removing old and damaged RBCs from the bloodstream under normal conditions (Buffet *et al.* 2011). The vasculature of the spleen is split into two pathways; fast flow and slow flow. The fast flow region bypasses the majority of the spleen's functions and returns the blood to the rest of the body. The slow flow route is specially designed to slow the flow of red blood cells and bring them into close contact with endothelial cells and macrophages. This pathway exposes the RBC to the smallest capillary beds in the body. These capillary beds test the flexibility and integrity of the cell membrane. Macrophages comprise half of the cell population in these low flow/high stringency vessels, allowing close contact and long exposure time between the macrophages and the RBCs. The lengthened time and increased proximity allows the macrophages to engulf compromised RBCs through ligand-receptor mediated mechanisms. The expression of phosphatidylserine (PS) on the surface of RBCs is the prominent ligand-receptor mediated process for macrophage binding and phagocytosis. Structural abnormalities that compromise the flexibility of the membrane cause the RBC to rupture in the high stress conditions of the slow flow route (Buffet *et al.* 2011). Infected RBCs undergo oxidative stress from the invasion of *Plasmodium*. The oxidative stress leads to the initiation of cell death and results in the exposure of PS on the outer membrane of the RBC in a calcium-dependent manner (Foller *et al.* 2009), (Nguyen *et al.* 2011). The Tim-4 receptors on the surface of the macrophage bind to the PS and initiate phagocytosis. This ligand-receptor pathway is responsible for clearing damaged RBCs from the bloodstream. The increase in the number of RBCs being cleared during infection puts strain on the spleen. The red pulp of the spleen becomes overwhelmed

with infected RBCs and the white pulp becomes disorganized as an increase in macrophages compromises the structure. This strain causes the spleen to enlarge and creates a condition known as splenomegaly (Chua *et al.* 2013).

P. falciparum has developed a method to avoid clearance by the spleen. The parasite attaches cysteine rich proteins onto the outer surface of the RBC membrane. Inside the smaller vessels of the vasculature these cysteine rich domains interact with CD-36 expressed on the surface of the endothelial cells lining the blood vessel. This interaction allows the parasitized RBC to bind to the capillary wall and avoid clearance by the spleen (Fonager *et al.* 2012).

Cerebral malaria is defined as altered consciousness, spasms, coma, and eventually death as the blood flow to the brain becomes compromised. Interaction between the infected RBC (iRBC) and the endothelium of the brain are driven by ICAM1 and CD-36 expression on the surface of the endothelial cells and the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) expressed on the surface of the iRBC. There are also interactions between Pfemp-1 and complement receptor 1 (CR-1) on the surface of erythrocytes allowing the iRBC to bind to uninfected RBCs, forming rosettes in the bloodstream. The combination of iRBCs binding to uninfected RBCs, and the endothelial cells lining blood vessels, leads to aggregates that can block the flow of blood in the brain and cause cerebral malaria (Dondorp *et al.* 2004).

Plasmodium cell cycle

Plasmodium falciparum is a single-celled eukaryote. The parasite undergoes the normal stages of the eukaryotic cell cycle; G₀, G₁, S, G₂, and M. G₀ is the nomenclature used for the constitutive processes that the cell undergoes to maintain its functioning state. Once the cell has received the appropriate signals to divide, it reaches G₁. During this stage it

prepares to replicate its genome by synthesizing the appropriate cellular machinery required. Once the cell has enough of the appropriate enzymes it begins to synthesize a new genome, entering the S phase. When the genome has been duplicated it enters the M stage and the DNA is mechanically separated and the cytoplasm cleaves in two, completing the cell cycle.

The cell cycle is strictly controlled by a series of checkpoints that monitor each stage. The regulation of the cell cycle is conducted by a highly conserved family of proteins known as cyclin dependent kinases (Cdks). The Cdks regulate the cell cycle by phosphorylating specific target proteins. Targeting and activation of the Cdk is regulated by the cyclin protein bound to it. In this manner a single kinase can have different effects and targets at different stages of the cell cycle. This mechanism for regulating cell cycle progression is conserved across all eukaryotes. Over time there has been a divergence in the timing and targets of the cyclin proteins. One cyclin may be used in fungi to initiate G₁ growth, while mammalian cells might use that same cyclin to initiate the S phase. The core functional domains have remained conserved, but over time variation has led to large families of Cdks and cyclins with targets and effects that can vary by organism. (Cross *et al.* 2011).

Coinciding with the tightly regulated progression of the cell cycle are the stage specific requirements of the cell cycle. The progression of each stage instigates a coordinated array of molecular mechanisms to conduct the mechanical processes required by that stage. G₁ requires the production and duplication of the cellular organelles and all of the subsequent protein structures required to support and produce those organelles. The S phase requires the up-regulation of DNA synthesis and the expression of appropriate proteins for organizing and packaging the duplicated genome. The M phase requires another set of specific proteins and enzymes for mitosis and cleavage.

There are several different factors that can be used to determine the initiation of mitosis. One of these factors is the organization of the mitotic spindle and the migration of

the centrioles to the proper positions on the nuclear membrane. The centriole is then responsible for physically separating the genomes. Centrioles are structures consisting of nine microtubule triplets. This configuration of microtubules is present in many structures within the cell, including centrosomes and cilia. Centrosomes are the main microtubule-organizing center (MTOC). The MTOC is comprised of two centrioles, which are considered to be the main bodies involved in the separation of DNA during mitosis (Debec *et al.* 2010). At the core of the centrosomes are the centrioles. During mitosis the centriole replicates and forms a daughter centriole within the centrosome. The centrosomes then migrate along the nuclear envelope and form the poles of the bipolar spindle assembly, which separates the chromosomes during mitosis. Disruptions to the centrosome duplication cycle can cause chromosome instability and lead to genetic instability. These consequences require the cell to tightly regulate the replication and division of centrosomes and their subsequent migration across the nuclear envelope to establish the mitotic spindles (Mardin and Schiebel 2012).

Plasmodium species are eukaryotic apicomplexans that share many similarities with mammalian cells. While they have the same general cell cycle, there are several distinct differences that are poorly understood. The life cycle of *Plasmodium* includes two different host organisms and three distinct cycles. This study is focused on the intraerythrocytic cycle of *P. falciparum* that occurs in humans. During typical eukaryotic mitosis the DNA is condensed into chromosomes, centrosomes localize to the nuclear envelope, replicate, and then migrate to the poles. Microtubules from the MTOC connect to the chromosomes at the kinetochore during metaphase. Then the opposing centrioles separate the genome as the microtubules are shortened during anaphase. Nuclear membranes form around the now separated chromosomes during telophase and the daughter cell is soon cleaved from the mother cell. *P. falciparum* does not undergo chromosome condensation, but it does have kinetochores that attach the DNA to the MTOC. The nuclear membrane remains intact during

mitosis. This means that the nuclear envelope must be divided among several daughter cells after replication has occurred. A single merozoite produces multiple progeny during schizogony. This means that several genomes are being synthesized, and then replicated, inside of the nuclear membrane during schizogony. The stages of S/M can be asynchronous between the developing progeny. One genome can be undergoing mitotic separation while another, within the same schizont, may be in S phase. Cleavage of the nuclear membrane is indicated by furrows developing in the membrane around the separate daughter cells and affects all progeny simultaneously. The simultaneous initiation of cleavage suggests that there is a mechanism in place that governs the end of schizogony and brings all of the genomic progeny to completion in a timely fashion (Gerald *et al.* 2011).

The eukaryotic cell cycle is rigidly regulated by the interplay between Cdks and the cyclins they bind. To date there is still very little known about the regulation of the cell cycle in *P. falciparum*. There are only two proteins known to regulate nuclear division (Fu *et al.* 2007, Mahajan *et al.* 2008). One of these proteins is a centrin. Centrins are proteins known to play roles in regulating nuclear division and centrosome duplication. Four centrins were identified in *P. falciparum* using comparative genetics and analyzed for their role in nuclear division. Centrins contain calcium-binding sites and are responsible for driving the contractile mechanics of the microtubules during mitosis. Four proteins in *Plasmodium* share homology with mammalian centrin proteins. Subsequent investigation found that one of the *P. falciparum* centrin (PfcEN) molecules had a role in cell cycle regulation. PfcEN3 co-localized with the MTOC during nuclear division and the dysfunction of this protein resulted in the arrest of cell cycle progression (Mahajan *et al.* 2008).

The only other protein known to regulate the intraerythrocytic cell cycle is an aurora related kinase (Fu *et al.* 2007). There are several different families of kinases that are crucial during the cell cycle. Each of these kinase families play roles in driving the mechanics

involved in the duplication of centrosomes, their migration to the poles of the nucleus, the separation of the genome, the contraction of microtubules, the formation of new nuclei, and membrane cleavage. Aurora kinases (ARKs) are serine/threonine-phosphorylating enzymes that are crucial for the appropriate segregation of chromatid during mitosis. Aurora kinases were first discovered in *Drosophila*. The name aurora comes from the mono-polar spindle formation, reminiscent of the aurora borealis, which results when the gene is mutated and the centrosome does not divide properly. Mammalian cells have 3 different ARKs; A, B, and C. These three ARK proteins are significantly similar, with 70% amino acid sequence homology. The variable portions of the three proteins are the binding domains of the ARKs. ARK A is primarily found in pericentriolar material during the S and G1 phase where it attaches to the proximal ends of the spindle microtubules. ARK A is an important component in spindle assembly formation and ensuring that the centrosome functions properly. ARK B is known to bind to three different partners, and the binding partner determines the localization and function of the kinase. ARK B has been shown to be crucial for proper kinetochore-microtubule attachment, a spindle checkpoint kinase, and a regulator of chromatin modification. The two ARKs, A and B, are involved in mitosis and the third ARK is involved in meiosis. In mammals ARK C is mainly found in the testes, where it serves a regulatory role in (Fu *et al.* 2007). Genomic analysis of the *P. falciparum* identified three homologues to aurora kinases. One ARK in particular shows conservation across the different *Plasmodium* species and a conserved orthologue in other apicomplexans. *P. falciparum* aurora kinase (PfARK-1) was shown to co-localize with the centrosome body during the formation of bipolar mitotic spindle bodies. This makes it more similar to ARK A in mammals with its localization to the centrosome. The genetic disruption of this protein resulted in an inability of the parasite to grow in culture, identifying it as a crucial gene for the intraerythrocytic cycle (Reininger *et al.* 2011).

Plasmodium genomics

The rise of drug resistant strains coupled with little progress in managing malaria over the last century led to a joint project to sequence the genome of *P. falciparum* in hopes of revealing a better understanding of the parasite (Gardner *et al.* 2002). The collaboration of several labs over several years completed the genome sequence for the *P. falciparum* strain 3D7 in 2002. After sequencing and homology analysis the collaborators determined that 60% of the genome was made of genes of unknown function. The majority of the genome was composed of genes with no significantly homologous genes in other known genomes (Elyazar *et al.* 2011). Modern genetic techniques have been adapted to investigate the function of these unknown genes. There are now several databases and genomic tools that can be used by investigators. Scientists use these techniques and databases to determine similarities with other genes and make predictions about the structure or function of their gene of interest. This information is used to infer protein function and aid them in their investigations (Di Girolamo *et al.* 2005).

The new databases and online software platforms have been extremely useful in predicting the roles of unknown genes in *Plasmodium*. Another approach for understanding the function of *P. falciparum* genes is through genetic manipulation. Genetic experimentation to determine the role of a gene *en vivo* can be done by two different methods; forward genetics and reverse genetics.

The classic approach to determine the function of a gene of interest is reverse genetics. The technique compares the sequence of a gene of interest against other sequences in a comprehensive database. The resulting list of homologous genes allows the investigator to determine the most likely function of their gene of interest. The gene is then disabled to verify that the gene of interest does in fact perform a function similar to the known homologous genes. Homologous recombination is used to disable the gene of interest. The

insertion of a strand of foreign DNA into the sequence of interest functionally disables the gene. The function of the gene of interest can be characterized by the effects of its absence. The quantity of unknown genes in the *Plasmodium* genome makes reverse genetics an ineffective approach for understanding this complex and deadly parasite.

Forward genetics is a process where a collection of mutants is created by randomly disabling a single gene in multiple individuals. The mutants are then subjected to a phenotypic screen. Analysis of the screen identifies mutants with a phenotype of interest. Once a mutant with a significant phenotype is identified the genome is sequenced to determine which gene was disrupted.

The use of forward genetic tools to analyze the proteome of *P. falciparum* is a difficult process largely because of low transfection efficiency. Transfection efficiency is a measure of what number of the intended target organisms are actually being transfected. Standard transfection techniques used on *Plasmodium spp.* yields an efficiency of $\sim 1 \times 10^{-6}$. When attempting to insert genome-altering foreign DNA constructs into malaria, the material has to cross the membrane of the RBC, the membrane of the parasitic vacuole, the membrane of the parasite, and then enter the nucleus of the parasite (Balu and Adams 2007). The development of transposon based mutagenic techniques has allowed the field to overcome the difficulties of forward genetic transfection because of the increased number of locations where homologous recombination can occur. Transposable elements (TE), or 'jumping genes', are highly conserved elements found in a wide variety of genomes. The TEs have binding sequences of low complexity making integration by the transposase enzyme more likely because of an increase in the number of locations for insertion and a decrease in the length of homologous sequences. This low complexity strategy supplies the TE with a plethora of viable insertion sights in any genome (Balu *et al.* 2005, Fonager *et al.* 2011). The *piggyBac* transposable element has an insertion sequence of TTAA, and there are

approximately 300,000 possible sites for insertion into the genome of *P. falciparum*. The insertion of the *piggyBac* element has been found to be completely random, showing no preferences between genes or chromosomes in *P. falciparum* (Balu 2012). This modification increased the transfection efficiency of experiments from as little as 10^{-6} in typical reverse genetic transfection experiments to as high as 1-10 insertions per transfection with the *piggyBac* method, making the forward genetics screening process both viable and efficient. The increased transfection efficiency of using the *piggyBac* transposable element to mutate the genome of *P. falciparum* has allowed scientists to overcome the difficulty of manipulating its genome (Balu *et al.* 2009).

Mutant Identification

Utilizing the *piggyBac* method a forward genetic screen for attenuated growth identified several *P. falciparum* NF54 insertional mutants, one in particular was a mutation of the gene PF3D7_1143500 (Balu *et al.* 2010). PF3D7_1143500 is a gene of unknown function from the cloned strain of *P. falciparum* identified as 3D7 and it is located on chromosome 11. The gene itself is constitutively conserved across all *Plasmodium spp*, consisting of 2774 base pairs (bp), with a coding sequence of 2674 bp, and the gene has a 20% GC content in *P. falciparum*. Not only is the sequence highly conserved, but so is its location (Fig. 3). This gene has a partial sequence homology to the Regulator of Chromatin Condensation 1 (RCC1) domain from human cells.

RCC1 proteins are widely varied and highly conserved proteins organized into superfamilies. The superfamilies are organized by structural similarity and function. Each member of the RCC1 family contains homologous repeats of a 51-68 amino acid residue motif that adopts a seven-bladed β -propeller fold, which has been shown to have a wide range of functions. Members of the RCC1 superfamily perform a myriad of cellular

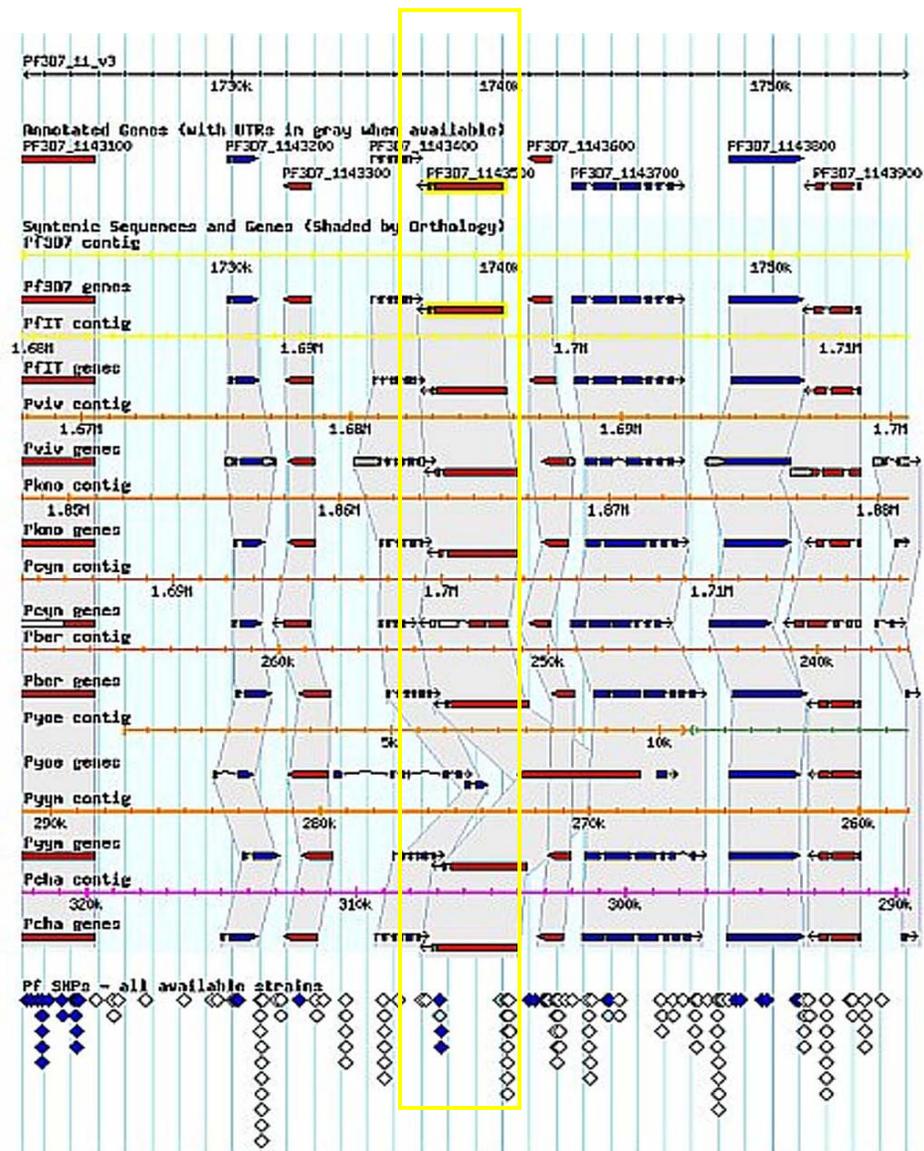


Figure 3. Cross species chromosomal alignment. Diagram of the location of PF3D7_1143500 on chromosome 11 with homologue alignment from other *Plasmodium spp.* Genome browser view from plasmdb.org of PF3D7_1143500 and the homologues across multiple species of *Plasmodium*, including members that do not infect humans. The highlighted box shows the synteny of the gene across multiple species. (http://plasmdb.org/plasmo/showRecord.do?name=GeneRecordClasses.GeneRecordClass&source_id=PF3D7_1143500&project_id=PlasmoDB)

functions: guanidine exchange, enzyme inhibition, DNA binding, interactions with proteins or lipids. Members of the superfamily have a variable number of repeats of the homologous sequence and have been further categorized into five groups (Hadjebi *et al.* 2008). This novel protein, PF3D7_1143500, which will be referred to as the *P. falciparum* RCC1-Like Protein 1 (PFRLP1), contains three repeats of the RCC1 domain. While the specific function of PFRLP1 is unknown the phenotypic screen suggests that it plays a role in proper mitosis and intraerythrocytic schizogony.

In humans, RCC1 is a critical protein in the regulation of transporting proteins across the nuclear membrane. This process is strictly regulated by a large variety of protein interactions in the eukaryotic Ran-RCC1 pathway. This pathway utilizes a GDP gradient to drive the transfer of proteins across the nuclear membrane. To regulate and facilitate protein import into the nucleus proteins targeted for the nucleus bind to importin proteins. The bound importin then interacts with Ran-RCC1 proteins and are then shuttled across the nuclear membrane. Several members of the importin family of proteins are known to bind to target ligands and the RanGDP protein, then via the Ran-RCC1 pathway, transport these proteins into the nucleus. Once inside the nucleus RCC1 acts on the RanGDP-importin complex. RCC1 phosphorylates Ran GDP to Ran GTP which causes the release of the imported protein from importin. The process of exporting is regulated in a similar manner. There are families of exportin proteins that bind to specific ligands inside the nucleus. Then the exportin complex interacts with Ran GTP to be exported from the nucleus. Along with the families of proteins that bind the targets for import and export there are a few crucial proteins that work within the Ran-RCC1 pathway to facilitate the cycle in eukaryotic cells. All known eukaryotes have homologues to these proteins, which infers the importance of this pathway to mediate the import and export of proteins to and from the nucleus. *Plasmodium* does not yet

have any known homologues to these proteins (Frankel and Knoll 2009), (Hadjebi *et al.* 2008).

There are several RCC1 like proteins that have homology with the different motifs of RCC1. There are proteins that utilize the guanidine nucleotide exchange factor motif of RCC1 but have no role in nuclear membrane transport. There are several ways in which cells can use a GTP gradient to perform a variety of functions. This motif is known to regulate the proper functioning of motor neurons by stimulating the enlargement of endosomes and regulating endosomal dynamics in cortical neurons (Otomo *et al.* 2003). Other RCC1 like proteins have been found to play roles in regulating the progression of mitosis. The NIMA-like kinase Nercc1 shares homology with RCC1s binding site for Ran-GTPase. Nercc1 has been shown to be capable of arresting the progression of mitosis under experimental conditions (Roig *et al.* 2002). Another RCC1 like protein, DelGEF, has been characterized and identified as an important gene in hearing. The expression of splice variants and over expression of DelGEF have been shown to cause deafness in humans (Uhlmann *et al.* 1999). RCC1 has a guanidine nucleotide exchange motif that is shared among many proteins and this well-conserved functional motif is utilized by a variety of different organisms to conduct a wide diversity of cellular functions.

Project Goals

The rise, and spread, of drug resistant strains has created a need for a better understanding of *P. falciparum*. The cryptic *Plasmodium* genome has made progress elusive, however the development of modern genetic tools has greatly aided this endeavor. The *piggyBac* transposable element was used to create a library of mutants and a phenotypic screen revealed a mutation with a significant impact on parasite proliferation *in vitro*. This

study proposes to identify the role that this crucial gene, PF3D7_1143500, plays in the intraerythrocytic proliferation of *P. falciparum*.

The specific aims of this project are as follows:

Aim1- Characterize the protein product for PF3D7_1143500. Perform comparative bioinformatic analysis to determine the presence of any domains of interest and develop methods for visualizing the protein product. The bioinformatic analysis was performed using online software and databases to identify sequence homologies with known domains or protein motifs. Partial homologies were identified and used to gain insight into the possible functional pathway in which the protein interacts. Analysis of the predicted amino acid sequence was done to determine a sequence of optimal antigenicity for the development of antibodies against PFRLP1. The antiserum developed from the identified sequence was used to characterize the expression timing and sub-cellular localization of the protein.

Aim2- Perform a comparative analysis of the intraerythrocytic cycle to characterize the phenotype of the mutant strain for insight into the function of PF3D7_1143500. Assays were conducted to identify the role that the PF3D7_1143500 gene product has in the regulation of intraerythrocytic proliferation. The differences in the intraerythrocytic cycle between mutant and wild type parasites were determined using microscopic visualization, flow cytometry, immunohistochemistry, and *in vitro* assays.

Materials and Methods:

Cell culture conditions

P. falciparum clones were maintained in human RBC culture as previously described (Trager and Jensen 2005). Cultures were incubated at 37°C in an environment of 5% O₂, 5% CO₂, and 90% N₂. Culture media was RPMI 1640 (Invitrogen) with a 5% hematocrit of human blood supplemented with 0.5% Albumax I (Invitrogen) or 10% human AB sera (VWR scientific), 0.25% sodium bicarbonate and 0.01 mg/mL gentamicin. Human blood was acquired from Virginia blood services and washed 3 times in RPMI 1640. A week's supply of fresh blood was washed three times in RPMI 1640 and resuspended to a 50% hematocrit and stored at 4°C.

Ring stage culture synchronization

To synchronize parasites in the early trophozoite ring stage, *P. falciparum* infected RBC (iRBC) cultures were centrifuged at 1200 x g for 5 minutes at room temperature (RT). The supernatant was decanted and the pellet containing iRBCs was resuspended in an equal volume of 5% sorbitol (Sigma) in RPMI 1640. The mixture was incubated at RT for 10 minutes with intermittent mixing, then centrifuged at 2000 x g for 5 minutes at RT. The supernatant was removed and the pellet containing iRBCs was resuspended in normal complete culture media to the initial volume and returned to the incubator at the previously mentioned conditions. The procedure was repeated 8 hours later to yield cultures of highly synchronized ring stage parasites and was confirmed by microscopy.

Late stage parasite purification

The purification of parasites in the late stage of the intraerythrocytic cycle were purified for *in vitro* assays. High parasitemia cultures (approximately 5-10% parasitemia) were passed through a MACS magnetic column (Miltenyi Biotech) attached to a high power magnet. The iron built up in the parasite from the metabolism of hemoglobin anchored late stage iRBCs to the magnetized column while RBCs infected with early stage parasites passed through the column. The column was washed with 30 mL of incomplete RPMI 1640 to wash out early stage iRBCs and uninfected RBCs. The column was removed from the magnet and the late stage parasites bound to the previously magnetized column were eluted in 20 mL of incomplete RPMI 1640 and centrifuged at 1200 x g for 5 minutes. The pellet containing purified iRBCs was washed in complete media and then resuspended in 5 mL of complete media at 5% hematocrit.

Genomic DNA extraction

Frozen parasite pellets were re-suspended in tris sodium EDTA (TSE) buffer (50mM Tris pH. 7.5, 0.1M NaCl, 0.1MM EDTA), washed three times in TSE buffer, and then lysed in 300 μ L of 2% sodium dodecyl sulphate (SDS) in TSE. Following the addition of 200 μ L of 7.5% ammonium acetate, DNA was precipitated using an equal volume of isopropanol and centrifuged at 12,000 x g and washed with 70% EtOH. The DNA pellet was air dried and dissolved in 20 μ L of tris EDTA (TE) buffer.

RNA extraction

Parasites extracted from 5ml cultures were re-suspended in 0.5ml of Trizol followed by the addition of 100 μ L of chloroform and then centrifuged at 12000 x g for 5 minutes. The upper phase was mixed with 300 μ L of isopropanol and centrifuged at 16000 x g for 45

minutes. The RNA pellet was then washed three times in 70% EtOH, air dried, and dissolved in H₂O.

RT-PCR

RT-PCR was performed to check for the presence of PFRLP1 gene expression. The RNA isolated from parasite cultures was first treated with TURBO DNA-free™ (am1907) kit. SuperScript® III One-Step RT-PCR System with Platinum® Taq High Fidelity (Invitrogen, 12574-030) was used for the reverse transcription reaction and PCR with specific primers for the PFRLP1 transcript. Actin was used as a reference gene for a positive control. DNase treated RNA was used as template for a negative control with Platinum Taq DNA Polymerase High Fidelity (Invitrogen, 11304-011) and actin primers. The size of the PFRLP1 transcript PCR product was confirmed by gel electrophoresis.

Proliferation assay

iRBC cultures were purified using the late stage purification to isolate late trophozoites and schizonts. The purified iRBCs were then introduced into fresh un-parasitized RBC culture. The culture was seeded with the purified iRBCs to an initial 1% parasitemia and cultured for eight days. Cultures were diluted as needed to maintain a healthy level of parasitemia (0.5% - 2%). Smears of the iRBC cultures were made daily to monitor the parasitemia level. The smears were air dried, stained with Geimsa for ten minutes, rinsed in H₂O, and then air dried. Slides were visualized using a microscope and 1000 cells were counted to determine the percentage of iRBCs.

Flow cytometry

Culture samples were taken every 4 hours over an 80 hour time span and processed for flow cytometry to determine the intraerythrocytic cycle time. For each sample 200 μ L of iRBC cultures were centrifuged at 450 x g for 3 minutes then the media was removed from the pellets. The pellets were then resuspended in 200 μ L of 0.05% glutaraldehyde in PBS and mixed by pipetting up and down 4 times. The cells were incubated for 10 minutes at RT and then centrifuged at 450 x g for 3 minutes. The supernatant was removed and pellets were resuspended in 200 μ L of permeabilization buffer (0.3% TritonX-100 (Sigma)) in PBS. The iRBCs were incubated for 10 minutes at RT and centrifuged at 450 x g for 3 minutes. The supernatant was removed and then 200 μ L of PBS was added to wash the cells. The iRBCs were centrifuged at 450 x g for 3 minutes, after which 200 μ L of supernatant was removed and then 200 μ L of 0.5mg/mL pancreatic RNase in PBS was added and mixed by pipetting. After incubating at 37°C for 1 hour the cells were centrifuged at 450 x g for 3 minutes. The supernatant was removed and 200 μ L of 0.1 mg/mL ethidium bromide in PBS was added and mixed by pipetting. The cells were incubated at 37°C for 1 hour in dark, then centrifuged at 450 x g for 3 minutes. The supernatant was then removed and 200 μ L of PBS was used to resuspend the cells and used for flow cytometry. The fluorescence of processed samples was quantitated on an Accuri flow cytometer. The fluorescent intensity of ethidium bromide was used to distinguish single nuclei early stage parasites from multi-nucleated schizonts.

Recombinant protein expression

EpiC online software (Bioware) was used to identify portions of the 878 amino acid sequence of PFRLP1 with high isoelectric points and high antigenic properties ideal for eliciting an immune response from a host animal. A trio of 177 amino acid sequences with high antigenic properties and high likelihoods of inducing the generation of antibodies were

identified. In order to increase the specificity of any antibodies developed the amino acid sequences were compared to the proteome of *P. falciparum*. Candidate sequences with multiple amino acid sequences prevalent in the proteome of the organism were excluded. The results were used to identify an optimal sequence with low homology to other *P. falciparum* genes and a high antigenicity score. A codon optimized plasmid from DNA2.0. was procured. The construct contained an Isopropyl β -D-1-thiogalactopyranoside (IPTG) induction vector, the optimal sequence previously mentioned, a hemagglutinin (HA) tag, and a 6 x histidine tag was purchased from DNA2.0 for expression in *E.coli*.

E. coli was transformed with the plasmid. Cultures were grown in Luria broth (LB) in a shaking incubator at 37 °C until optical density at 600 nm (OD_{600}) of 0.6 was reached. Then plasmid expression was induced with 0.5M IPTG and incubating at 30 °C for 8 hours. Cultures were then centrifuged at 6,000 x *g* for 10 minutes. The supernatant was decanted, and the bacterial pellet was flash frozen in liquid nitrogen.

The pellet of 1-liter of cultured bacteria was resuspended in 10 mL of lysis buffer (B-per Pierce 78266) with 1:100 dilution of protease inhibitor (Sigma p8340). The suspension was then shaken for 15 minutes at RT. The suspension was put on ice and pulse sonicated at 55% power 5 times for 10 seconds with one minute intervals between each burst. The lysed suspension was centrifuged for 20 minutes at 25,000 x *g* and the supernatant was collected. Nickel cartridges from Pierce scientific (Thermo Scientific 90098) were used to affinity purify the protein of interest. The interaction of the 6 x His tag on the C-terminus of the protein and the charge of the nickel column were used to isolate the protein. Samples were processed as per the manufacturer's protocol. The collected elute was then concentrated using 50 kDa spin columns (Vivaspin). The concentrated recombinant protein was collected for polyclonal anti-serum. Finally 300 μ g of the concentrated recombinant protein was used by Harlan laboratory for polyclonal anti-serum development in a rat.

Western Blot

To determine the timing and level of expression of PFRLP1 parasite lysate samples of synchronized culture of the three blood stages were analyzed by western. Samples were heated to 95 °C for 5 minutes in laemmli sample buffer (BioRad) and resolved on a 10% SDS stacking gel, then transferred to a nitrocellulose membrane at 300 mA. The membrane was immersed in blocking buffer (0.05% tween, 5% nonfat milk in PBS) for 1 hour at RT. Rat polyclonal α -PFRLP1 or anti α -tubulin (Pierce 32230) was diluted 1:250 in blocking and then incubated over night at 4°C. The membrane was then washed 3 x with 0.05% tween in PBS (PBST) for 5 minutes at RT. The horseradish peroxidase (HRP) conjugated IgG secondary (Pierce Scientific), diluted 1:2000 in PBST, was added and incubated at RT for 1 hour. The membrane was then washed 3x in PBST for 10 minutes, developed with chemiluminescence (ECL plus, Pierce Scientific), and imaged with a Bio Rad gel doc system.

Immunohistochemistry

An immunofluorescence assay (IFA) was conducted to visualize the formation of microtubule organizing centers (MTOCs). Cultures of purified late stage iRBC were transferred to fresh culture media to invade fresh RBCs. Smears of iRBC cultures were acquired every 2 hours from 20 hours post invasion to 38 hours post invasion for α -tubulin staining for the first experiment. The smears were air dried, and processed as previously described (Mahajan et al. 2008). Slides were incubated overnight at 4°C in the primary antibody (α -tubulin (Sigma) and/or rat antiserum) diluted 1:100 in blocking buffer. The secondary, Dylight 488 anti-mouse (KPL) diluted 1:100 in PBS and Hoechst 10 μ g/mL, was added to the slide and incubated in the dark at room temperature for 1 hour then rinsed. After

rinsing off the secondary the slide was mounted and analyzed as below. The co-localization experiment required another stain after the α -tubulin staining. Smears were made from samples taken from a non-synchronized culture, then stained with α -tubulin as previously described. After the α -tubulin secondary was rinsed off of the slide the smears were labeled with rat polyclonal α -PFRLP1 primary diluted 1:100 in blocking buffer. The secondary, goat anti-rat PE was diluted 1:100 in PBS with Hoechst 10 μ g/mL, added to the slide and incubated for 1 hour at RT in the dark. . The slides were then washed 3 x in PBS at RT for 5 minutes, mounted and cover-slipped, then stored in the dark until visualized a Nikon TE 2000-E microscope using fluorescence at 600X magnification

Transfection of Degradation Domain and Selection:

A conditional knockout study was conducted in order to confirm that the phenotype of the mutant was solely caused by the dysfunction of PFRLP1. PFRLP1 null parasites were ring stage purified as described above (Lambros and Vanderberg 1979). The synchronized culture was grown in culture through two intraerythrocytic cycles, and the schizonts from the second cycle were isolated via magnetic column (Miltenyi Biotec) as previously described (Balu et al. 2005). The schizonts were used to invade RBCs containing the degradation domain plasmid. The plasmid contains the functional PFRLP1 gene, degradation domain, and a human dihydrofolate reductase (DHFR) selection sequence. Erythrocytes were loaded with 150 μ g of plasmids containing the degradation domain construct, and electroporated as previously described (Balu *et al.* 2005, Deitsch *et al.* 2001). Four million schizonts were added to the culture of transfected RBCs. After four days of growth the cultures were treated with the antimalarial drug WR99210 (sigma W1770) which selectively inhibits parasite DHFR, not human DHFR, and halts nucleotide synthesis. The selection cassette allows

parasites that have been successfully transfected to proliferate in the presence of WR99210. However, *P. falciparum* can retain the plasmid without genomic integration. To select for the parasites with genomic integration the cultures were treated with and without WR99210, at two-week intervals, over several months to enrich the culture with successfully transfected parasites.

Southern blot:

The correct location of the insertion of the degradation domain in the genome was verified for the conditional knockout study. Genomic DNA was isolated as described above. Briefly, 5 µg of genomic DNA was digested overnight at 37°C with either *EcoRV* or *Bgl* II (New England Bio). The digested DNA was separated via electrophoresis on a 0.8 % Agarose gel at a constant 100V for 2 hours. Digestion of the DNA was confirmed by ethidium bromide staining. The gel was depurinated for 5 min in a solution of 0.25 M HCl at RT, then transferred to a solution of 1.5M NaCl and 0.2N NaOH for 20 minutes at RT. The gel was equilibrated in transfer solution (1M Ammonium Acetate, 0.02N NaOH) for 30 minutes at RT and then blotted as previously described (Adams *et al.* 1990). The blot was processed using a southern hybridization kit (Thermo scientific 17097) and biotinylated primers (Integrated DNA technologies). Primer sequences specific for human DHFR present in the selection cassette of the degradation domain construct, forward sequence 5' – ATGGTTGGTTCGCTAAACTG-3' and reverse sequence 5'- TTAATCATTCTTCTCATATACTTCAAAA-3', were hybridized with the blot overnight at 60°C and processed per kit protocol. The blot was visualized with Bio Rad gel doc system and Image Lab software (Bio Rad).

Results:

PF3D7_1143500 (PFRLP1) displays partial homology to human RCC1

The majority of the genome of *P. falciparum*, including PF3D7_1143500, does not have any homologous genes in the genomic databases. In order to gain insight into the possible function of PF3D7_1143500 (PFRLP1) a bioinformatics analysis was performed to identify partial homologies to known sequences, domains, or motifs. Protein sequence analysis of PFRLP1 using NCBI conserved domain search database revealed a partial sequence homology to the protein RCC1 (Fig. 4). The predicted amino acid sequence of PFRLP1 contains a nuclear localization signal and two identical sequences that are partially homologous to the DNA binding domain of the human RCC1 protein. RCC1 is a highly conserved protein with two well-defined motifs. It is known to regulate protein transfer across the nuclear membrane. The presence of only one motif in PFRLP1 implies that it is unlikely to be the as yet un-identified RCC1 homologue in *P. falciparum*. A comparative sequence analysis of the human RCC1 sequence and the entire proteome of *P. falciparum* identified PFRLP1 to be one of ten proteins with significant partial sequence homology to RCC1 (Table 1). The original RCC1 protein is vital in regulating protein transport into and out of the nucleus. RCC1 contains two dynamic motifs, the first is a guanidine transfer motif, and the second is a DNA binding motif. These motifs are utilized by hundreds of proteins to perform a variety of functions. The gene PF3D7_1143500 is one of ten genes to share homology with human RCC1, it is also one of the least homologous. The nuclear localization signal and the DNA binding motif of RCC1 infers a role in the nucleus, but the lack of the second RCC1 domain make it an unlikely homologue for RCC1.

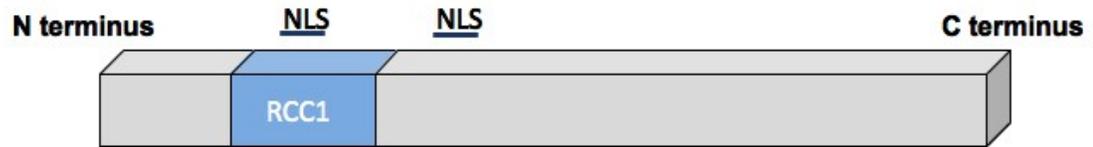


Figure 4. PFRLP1 protein sequence motifs. The representation of the predicted 878 amino acid sequence for PF3D7_1143500 reveals a partial sequence homology to the RCC1 domain and two nuclear localization signals (NLS) near the N-terminus.

Table 1. Comparative protein sequence analysis. Protein sequence analysis identified PF3D7_1143500 as one of ten RCC1-like proteins in *P. falciparum*. The list of *P. falciparum* proteins with partial sequence homology to human RCC1 protein identified ten proteins, seven of these proteins had a greater sequence alignment similarity to human RCC1 than PFRLP1.

	Gene		Smallest Sum High Probability		
			Score	P(N)	N
1	PF3D7_1137400	Plasmodium_falciparum_3D7	264	1.50E-22	1
2	PF3D7_0711500	Plasmodium_falciparum_3D7	203	2.20E-20	2
3	PF3D7_0508500	Plasmodium_falciparum_3D7	154	2.20E-15	2
4	PF3D7_1356600	Plasmodium_falciparum_3D7	180	1.20E-12	1
5	PF3D7_0403100	Plasmodium_falciparum_3D7	86	2.30E-06	2
6	PF3D7_0919900	Plasmodium_falciparum_3D7	130	4.40E-06	1
7	PF3D7_0418600	Plasmodium_falciparum_3D7	85	0.01	4
8	PF3D7_1143500	Plasmodium_falciparum_3D7	78	3.50E-01	1
9	PF3D7_0930800	Plasmodium_falciparum_3D7	85	7.50E-01	2
10	PF3D7_1451100	Plasmodium_falciparum_3D7	65	9.80E-01	2

PFRLP1 is expressed throughout intraerythrocytic development and localizes to the parasite nuclear periphery.

To obtain insights into the function of PFRLP1 in the wild type parasite the expression level of PFRLP1 was determined during the three stages of the intraerythrocytic cycle by western blot. The sub-cellular location of the protein was also visualized during the different stages of the intraerythrocytic cycle using immunofluorescent microscopy. In order to quantify and visualize the PFRLP1 protein polyclonal antiserum against PFRLP1 was created using recombinant protein techniques. A 177 amino acid sequence was expressed in *E. coli* and column purified to generate antiserum in a rat host (Fig. 5, panel A). The specificity of the rat anti-serum generated against PFRLP1 was confirmed by western blot after production (Harlan Laboratories). The blot showed binding of the serum antibodies to the original bacterial lysates, the purified recombinant protein product, and a high specificity in whole parasite lysate (Fig. 5, panel B). This confirmed the production of antibodies specific for our polypeptide sequence from PFRLP1.

In order to determine the expression level of PFRLP1 during the intraerythrocytic cycle synchronized parasite cultures were used to produce whole parasite lysates of the three different developmental stages in the intraerythrocytic cycle; ring stage (Pre-S phase of cell cycle), trophozoite stage (S-Phase of cell cycle), and schizont stage (S and M phases of cell cycle). Subsequent analysis via western blotting revealed PFRLP1 expression to be at comparable levels throughout the three stages of the intraerythrocytic cycle (Fig. 6, panel A). Immunofluorescent microscopy showed the localization of PFRLP1 to the periphery of the nucleus during the three stages of the intraerythrocytic cycle (Fig. 6, panel B).

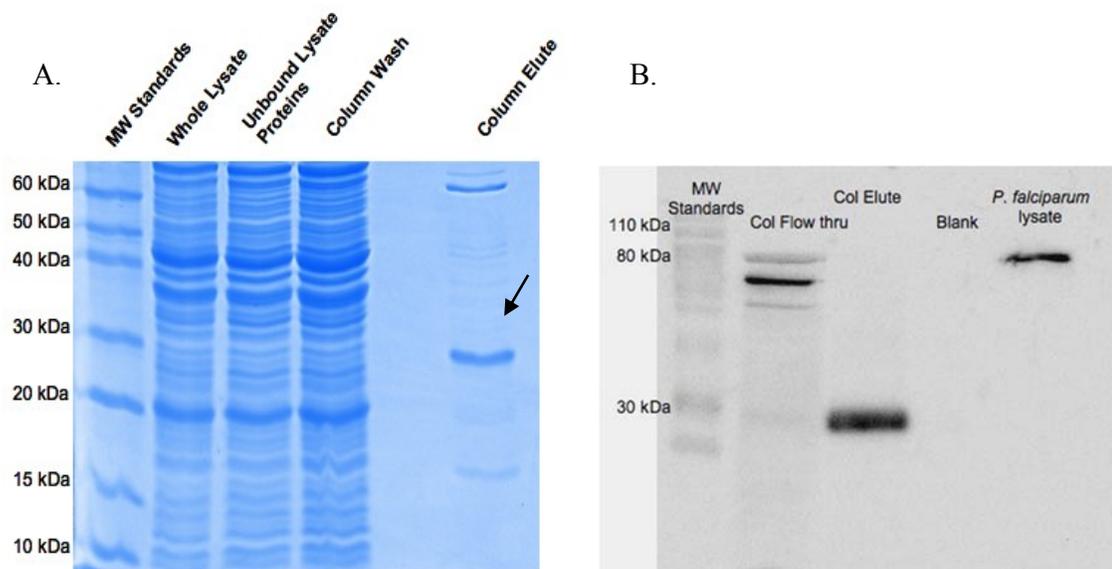


Figure 5. Recombinant protein purification and antiserum specificity A.) The SDS PAGE protein blot shows the isolation of the recombinant protein from the Nickel column. The lanes show protein content from recombinant transformed bacterial culture whole lysate, the unbound lysate proteins, and the protein washed out after loading respectively. The last lane shows the protein eluted from the column at approximately 28 kDa (arrow), which is the predicted size of the 177 amino acid recombinant polypeptide. B) A western blot using the rat sera. The lanes show the bacterial lysate flowed through the column, eluted protein extracted from the recombinant bacterial culture, and the final lane is whole cell lysate from the wild type *P. falciparum* parasite.

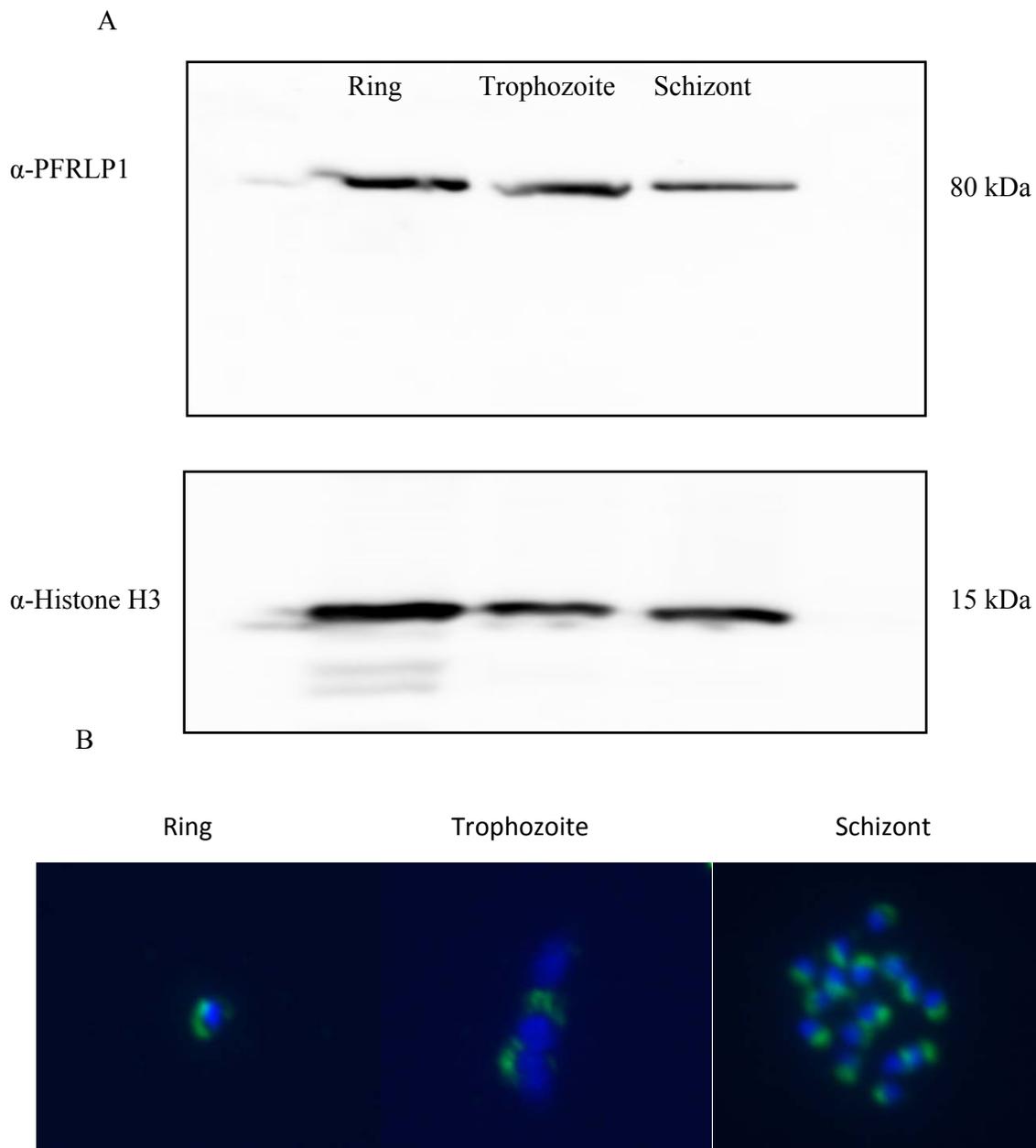


Figure 6. PFRLP1 expression and localization. A) Western blot of the three stages of the intraerythrocytic cycle with the rat antiserum (α -PFRLP1) and anti-histone as a loading control. B) Immunofluorescence assay illustrates localization of PFRLP1 to the nuclear periphery of the three stages of the intraerythrocytic cycle (PFRLP1 in green and nuclei in blue).

Attenuation of the PFRLP1 mutant intraerythrocytic growth rate is due to a reduction in merozoite formation in mature schizonts.

The diminished growth rate observed previously in a forward genetic screen done previously indicated a vital function for PFRLP1 in the intraerythrocytic cycle (Balu et al. 2010). In order to determine the role of PFRLP1 in the intraerythrocytic cycle several important components of the cycle were examined; intraerythrocytic cycle time, parasite proliferation, merozoite formation, RBC invasion, and parasite egress from the RBC. Intraerythrocytic cycle time is defined as the period of time that lapses from one schizont stage to the next. This was determined with flow cytometry analysis and confirmed by microscopy. A time course study was performed over 196 hours with samples taken every four hours. Flow cytometry was used to measure the DNA content of the iRBCs and determine the intraerythrocytic cycle time. The peak schizont time was determined by measuring the DNA content of each iRBC and the cycle time was the time between two peak schizont measurements. The flow cytometry data was confirmed by microscopy, samples from each time point were smeared, stained, and analyzed for schizont content. The microscopy findings confirmed the flow cytometry results. There was no observed difference of the intraerythrocytic cycle time between the wild type and the PFRLP1 mutant, both having a 48 hour intraerythrocytic cycle (Fig. 7).

The proliferation rate in blood culture was examined next. The determination of the proliferation rate was performed by determining the fold increase in parasitemia over eight days in blood culture. The results from analysis of the samples taken was a decrease in percent parasitemia in the PFRLP1 mutant, supporting the previous findings (Balu *et al.* 2010) (Fig. 8).

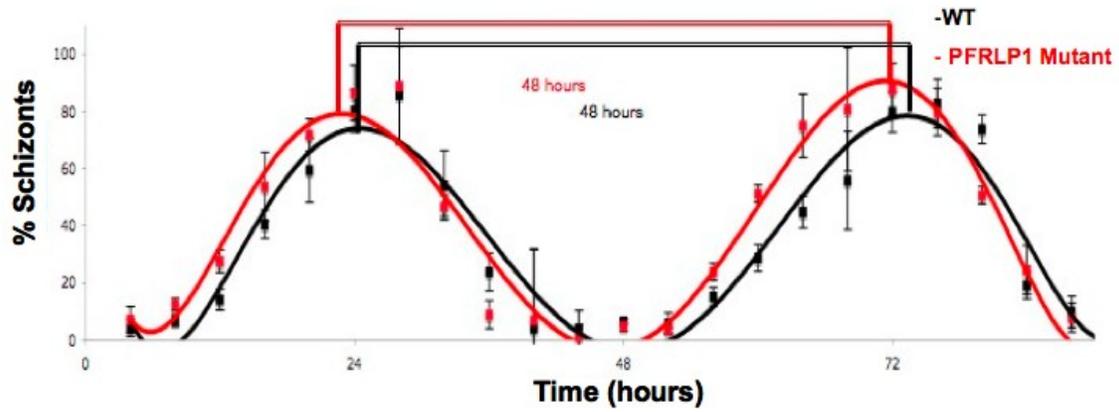


Figure 7. Intraerythrocytic cycle time analysis. Time course samples from triplicate cultures of mutant and wildtype parasites were taken over 96 hours at 4 hour intervals. They were analyzed for the percentage of schizonts by flow cytometry and confirmed by microscopy.

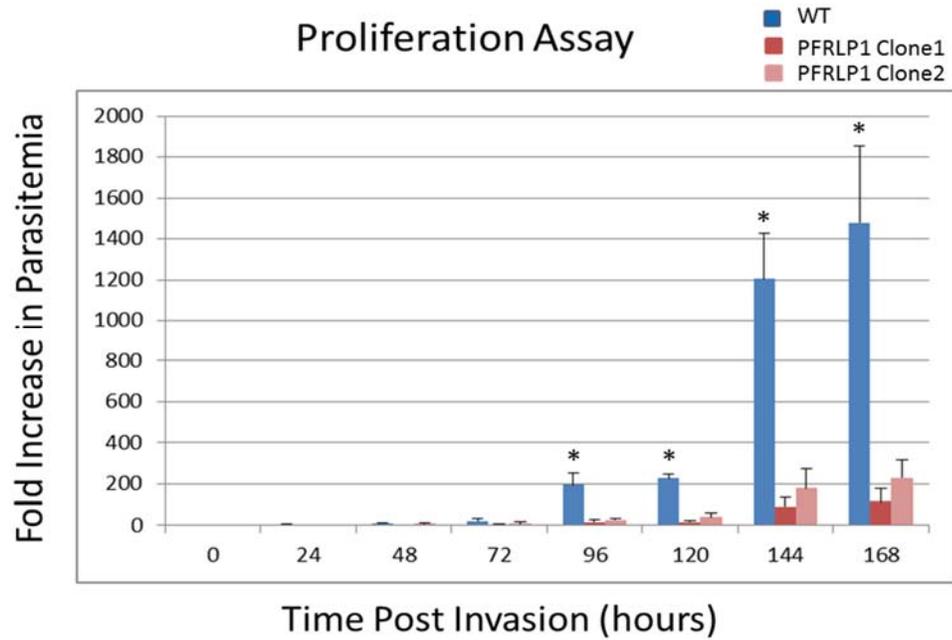


Figure 8. Intraerythrocytic proliferation assay. Fresh blood cultures inoculated with 10^6 late stage iRBCs were sampled every 24 hours over an eight day period. The samples were analyzed via microscopy to calculate the percent parasitemia. The data is represented as fold increase in parasitemia and shows significant difference in proliferation between the WT strain and the two mutants after 96 hours (Means \pm SD are indicated and a two-tailed unpaired t-test was used to determine significance, * = $p < 0.05$).

The next aspect of the intraerythrocytic cycle that was examined to determine the difference in proliferation was merozoite production. Late stage parasites were examined via microscopy and the number of merozoites being formed in mature schizonts was counted (Fig. 9, panel A). The numbers of merozoites formed within 100 schizonts were examined over triplicate cultures. Analysis shows a significant 40% reduction in merozoite formation for the PFRLP1 mutant. (Fig. 9, panel B).

The last facet of intraerythrocytic cycle that was examined was the ability of the parasite to exit the RBC and infiltrate new ones, known as egress and invasion respectively. This was previously examined and there was no difference found between the WT and the PFRLP1 mutant (data not shown)(Balu *et al.* 2010).

Conditional mutagenesis of PFRLP1 validates its role in merozoite production.

A conditional knockout mutant was created using a degradation domain (DD) construct to verify that the observed reduction in merozoite formation was caused by the mutation of PFRLP1. This construct targeted the PFRLP1 protein for proteolysis directly after synthesis, effectively creating a dysfunctional gene (Fig. 10, panel A) The DD is specially designed to work with a specific inhibitor of its degradation, the shield1 compound. Under normal conditions the newly synthesized protein is immediately targeted for degradation, however, when the culture is treated with the shield1 compound, the degradation domain is masked and the protein functions normally. To confirm that the DD construct was incorporated into the genome of *P. falciparum* a southern blot was performed using biotinylated primers designed to span the human dihydrofolate reductase (hDHFR) selection marker and the insertion site. Two different digestion enzymes were used for verification. The bands appeared at the predicted sizes for positive insertion of the DD construct in the

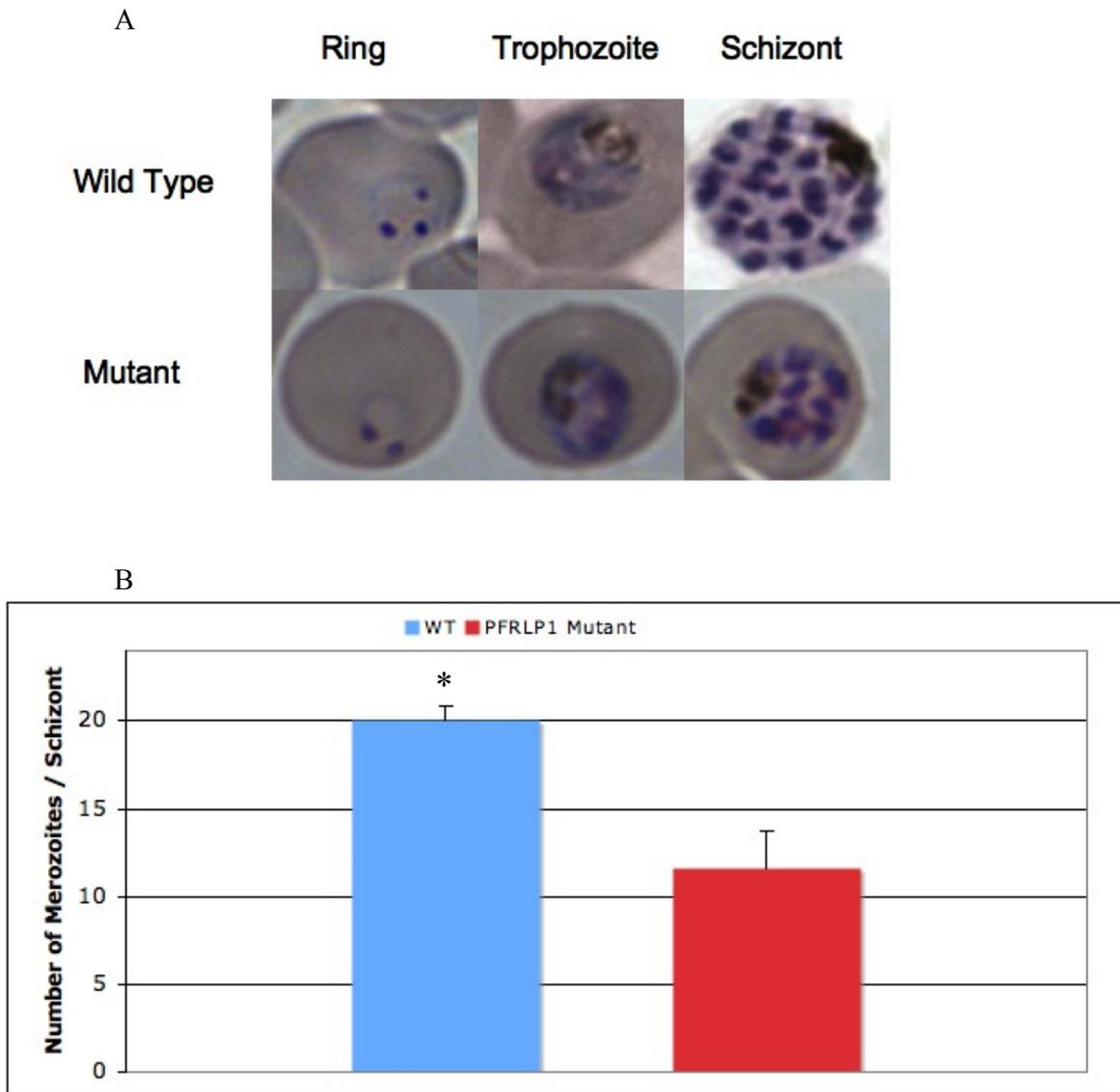


Figure 9. Microscopic analysis of merozoite formation A) Giemsa-stained blood smears of wild-type and mutant parasites. B) The number of merozoites formed in schizonts during the intraerythrocytic cycle was significantly higher in the WT strain (Means \pm SD are indicated and a two-tailed unpaired t-test was used to determine significance, * = $p < 0.005$).

genome, approximately 8 kbp after *EcoRV* digestion and 12 kbp after *BglII* digestion (Fig 10, panel B). Triplicate cultures of shield1 treated and untreated cultures were purified for late stage schizonts and Geimsa stained. Merozoites were counted for 50 schizonts for each slide. The shield1 treated cultures produced approximately the same number of merozoites as the WT parasites (Fig. 10 panel C). DD cultures without shield1 treatment had a 20% decrease in the number of merozoites formed per schizont when compared to the WT strain. The insertion of the DD construct into the PFRLP1 gene, and the conditional restoration of its function under treatment with shield1, confirmed the effect of PFRLP1 on merozoite formation in the schizont stage of the intraerythrocytic cycle.

Determining the initiation of mitosis for insight into the cause of the reduced number of merozoites.

The cell cycle was examined after the identification, and confirmation of, reduced merozoite formation as the defect in the intraerythrocytic cycle of the PFRLP1 mutant. We specifically examined nuclear division. The beginning of nuclear division is indicated by the formation and duplication of MTOCs and their migration to the opposite poles of the nucleus. The presence, division, and migration of these complexes begin the separation of nuclear material and the formation of the separate merozoites (Arnot *et al.* 2011). A time course experiment was conducted to determine if the reduction in the number of merozoites being formed was due to a delay in the initiation of nuclear division. WT and PFRLP1 mutant cultures were synchronized and then sampled every two hours over a period of 16 to 36 hours post synchronization. The time course samples were stained with anti- α tubulin for the detection of the MTOCs (Fig. 11). The appearance of divided MTOCs was used as the characteristic to determine the initiation of nuclear division via microscopy. Subsequent analysis of the mutant showed a significant delay of 6-8 hours in the appearance

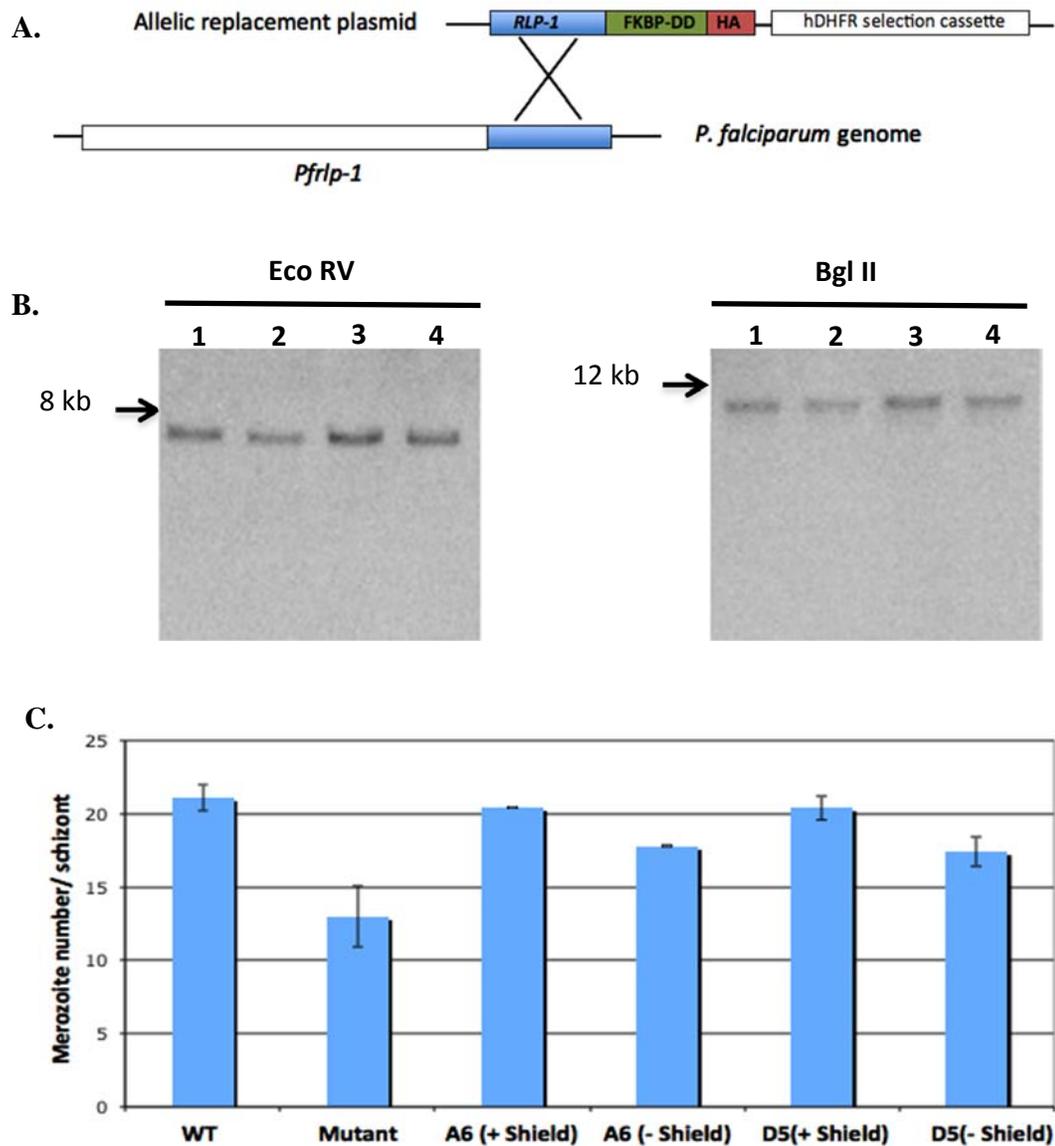


Figure 10. Degradation domain merozoite formation analysis A) Schematic of the degradation domain construct integrated into the WT parasite genome. B) Southern blot of DD clones with two restriction digests. Genomic integration of the construct verified by band size and the presence of the hDHFR selection cassette by PCR primers. C) Graph of the number of merozoites formed per schizont for the degradation domain mutants cultured with and without the inhibitory compound shield1.

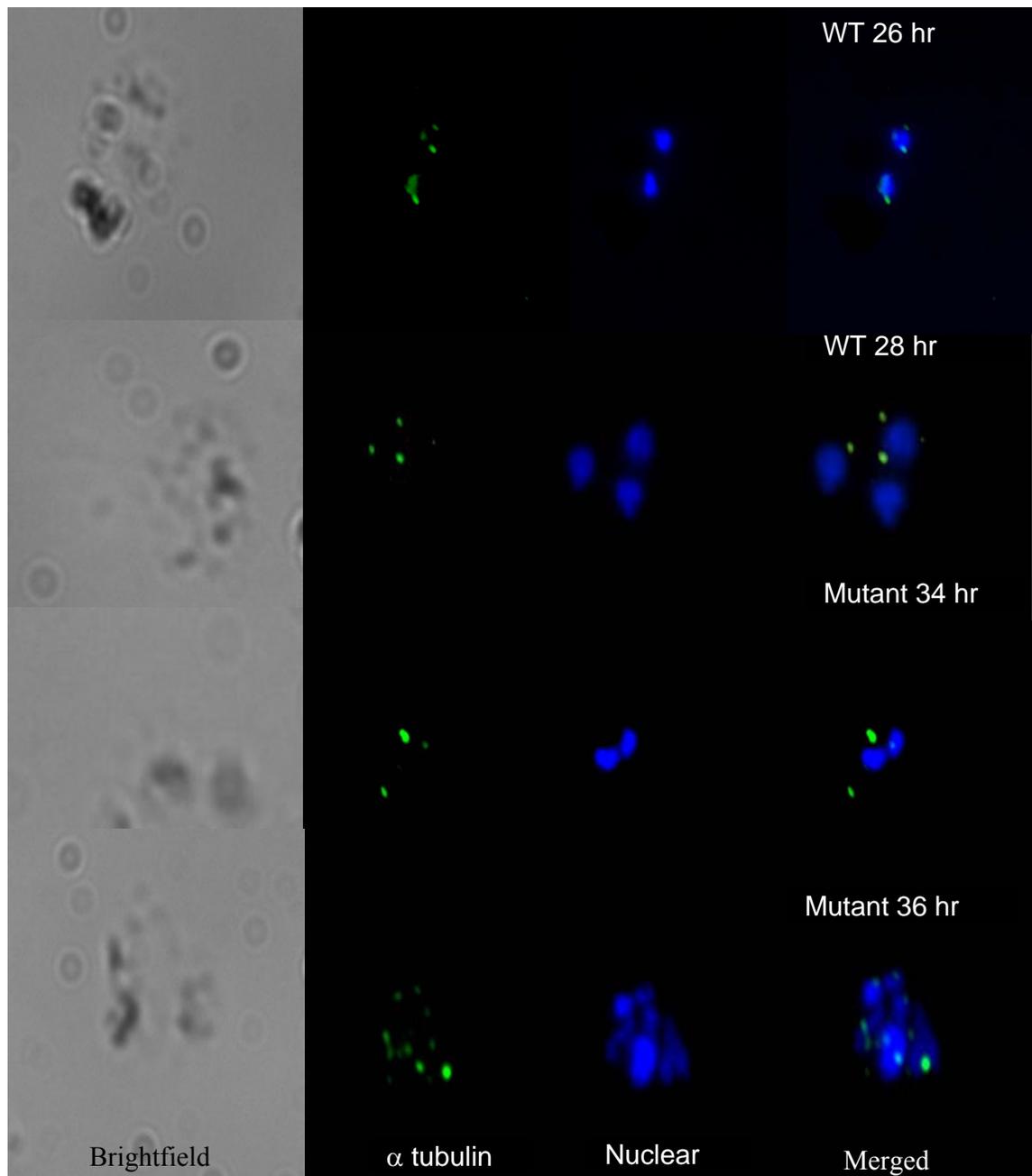


Figure 11. Nuclear division time Course. Indirect immunofluorescence assay on synchronized parasite intraerythrocytic stages over a time course (starting 16 hours post-invasion). Samples were used to determine the appearance and separation of MTOCs represented by the α tubulin staining (green), nuclear staining (blue) and a merged field. Representative images of the time course staining are shown.

of divided MTOCs and their migration along the nuclear envelope (Fig. 12). The identification of the involvement of PFRLP1 in nuclear division led to the investigation of the presence of PFRLP1 in parasite MTOCs. Indirect immunofluorescence assays were used to evaluate the co-localization of PFRLP1 with the previously used MTOC protein, α -tubulin. Microscopic analysis revealed that PFRLP1 co-localized with α -tubulin of the MTOCs during nuclear division, most prevalently during the early stages of nuclear division (Fig. 13).

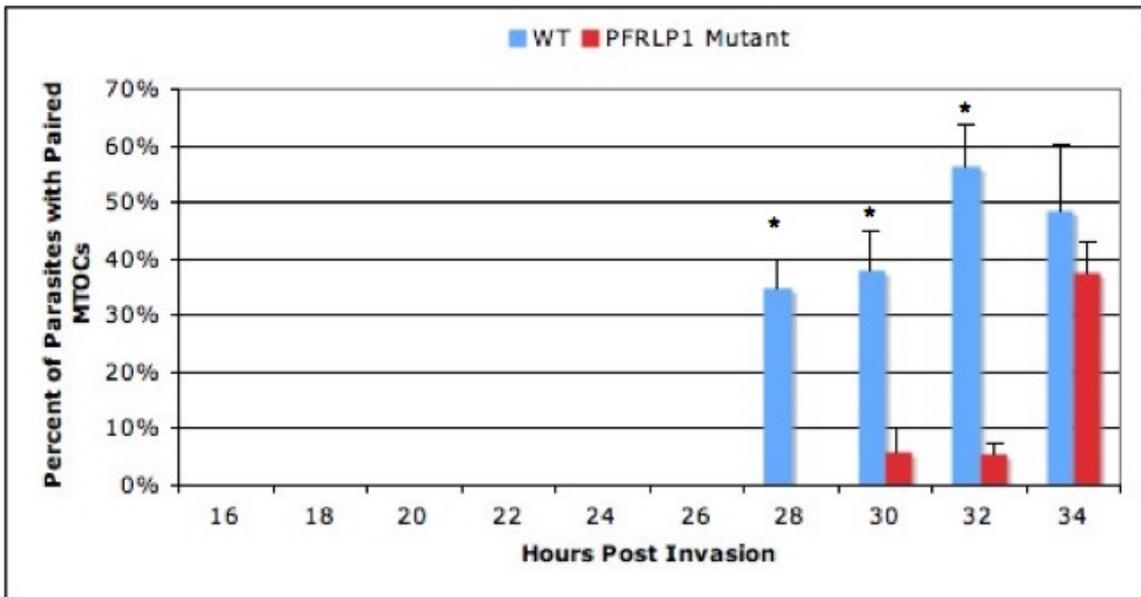


Figure 12. Nuclear division time course analysis. The analysis of the time course samples of both strains over a 20 hour time period was conducted to determine the number of parasites with two MTOCs. The presence of two MTOCs was the indication of the beginning of nuclear division. The WT strain has a six hour advantage with significantly higher numbers of paired MTOCs (Means \pm SD are indicated and a two-tailed unpaired t-test was used to determine significance, * = $p < 0.0005$).

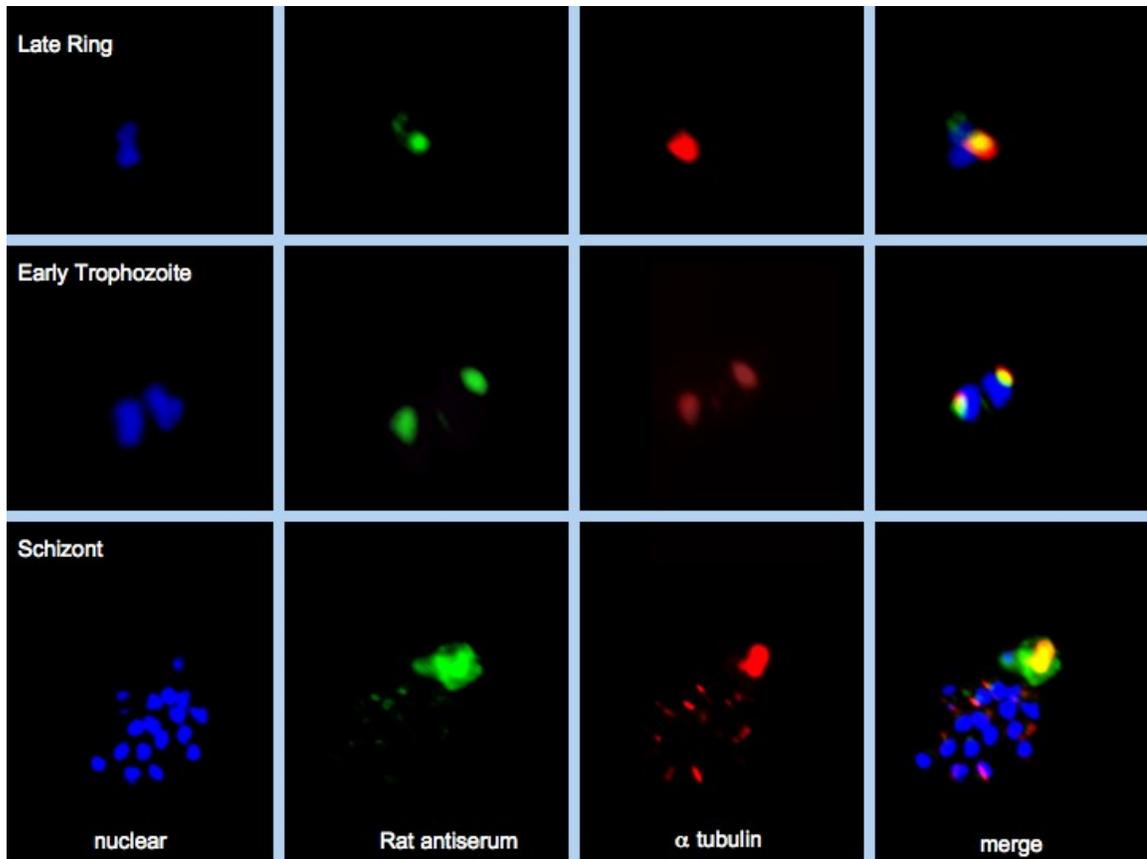


Figure 13. Panel of antiserum and α -tubulin staining of the three stages of intraerythrocytic cycle. A staining panel showing, from left to right; Hoechst staining the nuclei in blue, PFRLP1 antiserum in green, α -tubulin in red, and the merged images. The three stages of the intraerythrocytic cycle are shown by row from top to bottom; ring stage, early trophozoite, and schizont.

Discussion:

Malaria is a deadly disease caused by the single celled protozoan parasite *P. falciparum*. There are more than 100 species of *Plasmodium* that infect a wide range of animal species. There are five strains that infect humans, the deadliest of which is *P. falciparum*. The lethal effects of malarial infection are caused by the intraerythrocytic cycle, known as the blood stage of infection (Fig. 2). During this stage the parasite rapidly multiplies inside of RBCs, creating pathology that results in cyclic fever, anemia, jaundice, splenomegaly, coma, and death (Akinosoglou *et al.* 2012, Chua *et al.* 2013). There are several pharmaceutical interventions available on the market to combat malaria; but the rise of strains resistant to these interventions has become a significant problem. To date, resistant strains have been identified for all current pharmaceutical treatments (Ecker *et al.* 2012, Talisuna *et al.* 2012, White and Pongtavornpinyo 2003).

In order to gain a better understanding of this parasite, scientists around the world combined their efforts to sequence the genome of *P. falciparum*. It has been more than a decade since the genome was sequenced; currently, more than 50% of the genome is still annotated to code for genes of unknown function (Gardner *et al.* 2002). Typical reverse genetic screens have been cumbersome and ineffective due to the majority of the genome being comprised of genes with no significant homologues. The difficulty of inferring function from homologous genes is coupled with the difficulty of efficiently transfecting *P. falciparum*. The development, and advancement, of forward genetic screens has increased the efficiency of genomic experimentation in modern research (Di Girolamo *et al.* 2005). Current research is directed towards identifying critical proteins utilized by *P. falciparum* to regulate the progression through its life cycle (Fu *et al.* 2007, Mahajan *et al.* 2008). These critical proteins are ideal targets for the development of anti-malarial drugs.

The proliferation of *P. falciparum* during the blood stage results in exponential growth of the parasite and a severe pathology in the host (Gerald *et al.* 2011). It is the proliferation stage that results in disease; subsequently making it the stage of greatest interest for identifying critical regulatory proteins. A forward genetic screen for an attenuated proliferation phenotype identified gene PF3D7_1143500 as a critical gene (Balu *et al.* 2010). Bioinformatics analysis of PF3D7_1143500 identified the presence of a nuclear localization signal and two repeats with a partial homology to the human RCC1 protein. RCC1 is known to play a pivotal role in regulating nuclear protein import and export in eukaryotes (Hadjebi *et al.* 2008). The identification of the partial homology to the RCC1 motif led to the labeling of this gene as *P. falciparum* RCC1-like protein 1, or PFRLP1.

Examination of expression and localization during the intraerythrocytic cycle were essential before the role of PFRLP1 could be determined. Antibodies specific for PFRLP1 were produced in order to characterize its expression pattern and localization. First, using EpiC online software, an amino acid sequence with a high antigenicity score and low homology to other *P. falciparum* proteins was identified. Codon optimization technology and an inducible vector construct from the company DNA 2.0 was used to create a plasmid for recombinant protein expression in *E. coli*. The amino acid sequence of interest was expressed, purified, and then inoculated into a rat host (Harlan Labs). The antiserum was tested for specificity to PFRLP1 and used to determine the expression pattern. PFRLP1 was found to be equally expressed over the three stages of the intraerythrocytic cycle. The PFRLP1 protein was found to localizing to the periphery of the nucleus.

The intraerythrocytic cycle is a complex series of events that ultimately leads to exponential parasite growth. One parasite yields approximately 20 new merozoites during the 48 hour intraerythrocytic cycle. The cycle is composed of three basic components; the initial invasion of RBCs, merozoite progression through the cell cycle, and egress from the RBC. A

mutant library of over two hundred strains was screened for reduced proliferation; PFRLP1 was identified during this screen as a gene of interest (Balu *et al.* 2010). This screen for attenuated growth did not identify the cause for the reduction. To further isolate the cause of reduced proliferation a comparative analysis of the intraerythrocytic cycle was conducted.

Any dysfunction within the intraerythrocytic cycle time could have severe impacts on proliferation. *P. falciparum* has been shown to be able to survive while modulating this cycle time (Babbitt *et al.* 2012). An example of this capacity occurs during isoleucine starvation. Under this condition *P. falciparum* has been shown to go into a hibernatory state that can last for up to 72 hours and then successfully continue proliferating after re-supplementation of isoleucine. The lengthening of the cycle time for the PFRLP1 mutant could explain the observed attenuation of proliferation. Synchronized cultures were used for flow cytometry and microscopic analysis to determine the intraerythrocytic cycle time of the PFRLP1 mutant. The analysis revealed no difference between cycle time of the wild type strain and the PFRLP1 mutant, indicating that the dysfunction in the PFRLP1 mutant proliferation was not due to an altered cycle time.

A proliferation assay was conducted and identified an attenuation of the PFRLP1 mutant *in vitro* over an 8 day period in blood culture. A previous study examined RBC invasion and egress of the wild type and the PFRLP1 mutant but no differences were found (Data not shown; Balu *et al.*, unpublished data). The findings of this study confirm previous data (Balu *et al.* 2010). These findings, along with the cycle time data, allowed the scope of the study to be narrowed to the intraerythrocytic cycle.

No differences were found in the different aspects of the intraerythrocytic cycle; cycle time, RBC egress, and RBC invasion. This led to closer examination of the intraerythrocytic cycle stages. The intraerythrocytic cycle is composed of three distinct stages; ring stage, trophozoite stage, and schizont stage. Schizogony is the act of asexual

reproduction by fission that leads to the multiple progeny during the intraerythrocytic cycle and the development of the mature schizont (Hafalla *et al.* 2011). Improper schizogony could have a drastic effect on proliferation and the progression from trophozoite to schizont. Schizogony is known to be regulated by various factors. One example of this is access to proper serum components can inhibit proper schizogony. Under conditions of deprived high density lipoprotein (HDL) the cell cycle of *P. falciparum* can be halted before schizogony (Grellier *et al.* 1990). Another factor known to effect schizogony is proper polypeptide substrates. Each stage is accompanied by specific polypeptide profiles that are essential for the progression of the intraerythrocytic cycle. Blood stage progression of *P. falciparum* can become impaired without the proper substrates for the synthesis of these crucial polypeptides. This sensitivity to the polypeptide substrates is indicative of the essential nature of certain proteins in proper proliferation (Boyle *et al.* 1983). Several aspects have been identified that affect schizogony but there is very little known about the regulation of the cell cycle in *P. falciparum*. There are many different aspects known to be crucial to the proper progression through the intraerythrocytic cycle; the role of epigenetics, the accessibility of genes during the subsequent stages, the function of protein profiles specific to each of these stages, and components of the host serum. Schizogony was examined in order to determine if the attenuation of proliferation was due to a stage specific impairment. This led to the examination of schizogony and mature schizont development. Triplicate cultures of WT and PFRLP1 mutant late stage schizonts were examined via microscopy and the number of merozoites formed during schizogony was counted. Purified late stage parasites in the PFRLP1 mutant showed schizonts with a 40% reduction in the number of merozoites being formed. This is the first insight into a possible cause for the difference found in the proliferation assay.

The discovery of reduced numbers of merozoites formed during intraerythrocytic schizogony in the PFRLP1 mutant led to a conditional knockout study. The conditional knockout study was performed to verify that the restoration of PFRLP1 expression could restore the mutant phenotype to wild type levels. The conditional knockout comes with an ‘on and off’ switch that allows the comparison of a single clone to itself under culture conditions (Balu 2012). A shield1 sensitive DD was inserted into the PFRLP1 gene for the conditional knockout study. The shield1 sensitive DD has been successfully used in *P. falciparum* studies, showing effective degradation without shield1 treatment and phenotypic rescue under shield1 treatment (Russo *et al.* 2009). The conditional knockout model in this study identified a reduction in merozoite formation when clones treated with shield1 were compared to untreated cultures. The untreated DD clones did not reduce the number of merozoites formed per schizont to levels as low as the original PFRLP1 mutant. However, under shield1 treatment, the number of merozoites formed per schizont did return to wild type levels. The DD targets the protein product for autolysis by the proteasome immediately after synthesis. The untreated DD mutants did not have a reduction in merozoite formation as great as the PFRLP1 knockout mutant. A possible explanation for this is incomplete protein degradation. For example, the proteasome may have not been able to degrade all of the PFRLP1 produced. The partial degradation led to a phenotype that was not as severe as the original mutant, but the levels were returned to normal under shield1 treatment, verifying that the phenotype of merozoite formation inhibition is dependent on the level of PFRLP1 expression.

The reduction in the number of merozoites being formed during schizogony, and the subsequent confirmation with the conditional knockout experiment, led to the investigation of the cell cycle. *Plasmodium falciparum* has an atypical mitotic cycle compared to other eukaryotes (Gerald *et al.* 2011). During eukaryotic mitosis the establishment of MTOCs at

polar opposites of the cell occurs before the separation of the duplicated genomes. Unlike mitosis in the typical eukaryote, *Plasmodium* begins to separate the genome before establishing poles (Fig. 14). As the parasite continues the mitotic phase of schizogony there are multiple genomes replicated within one nuclear membrane; and these can be in various stages of the mitotic cycle. Synchronized cultures of iRBCs were used in an immunohistochemistry assay with anti α -tubulin antibodies to identify MTOC formation and duplication as a marker for the initiation of nuclear division. The result of the assay was a delay of 6-8 hours in the initiation of nuclear division in the PFRLP1 mutant. The delayed initiation of nuclear division, while maintaining a normal cycle time, explains the deficiency in the number of merozoites being formed during schizogony.

The identification of a delay in the separation of MTOCs during nuclear division further led to the examination of the relationship between MTOCs and PFRLP1. An immunofluorescence assay was performed to determine the localization of PFRLP1 in respect to the MTOCs during nuclear division. The staining revealed that PFRLP1 localized to the MTOCs, inferring a role in regulating the proper function of MTOCs during nuclear division.

A thorough understanding of the role that PFRLP1 plays in regulating merozoite formation and the initiation of mitosis will require further studies to identify the mechanism of its action. Immunoprecipitation assays would need to be performed to identify any protein partners that interact with PFRLP1. Protein-interaction studies could help identify any biological pathways in which PFRLP1 plays a role, by examining partner protein functions and roles. Since the homologous motif shared between human eukaryotic RCC1 and PFRLP1 is the chromatin-binding motif, chromatin-immunoprecipitation studies using PFRLP1 antibodies could help identify any specific genes or sequences it interacts with in the parasite chromatin.

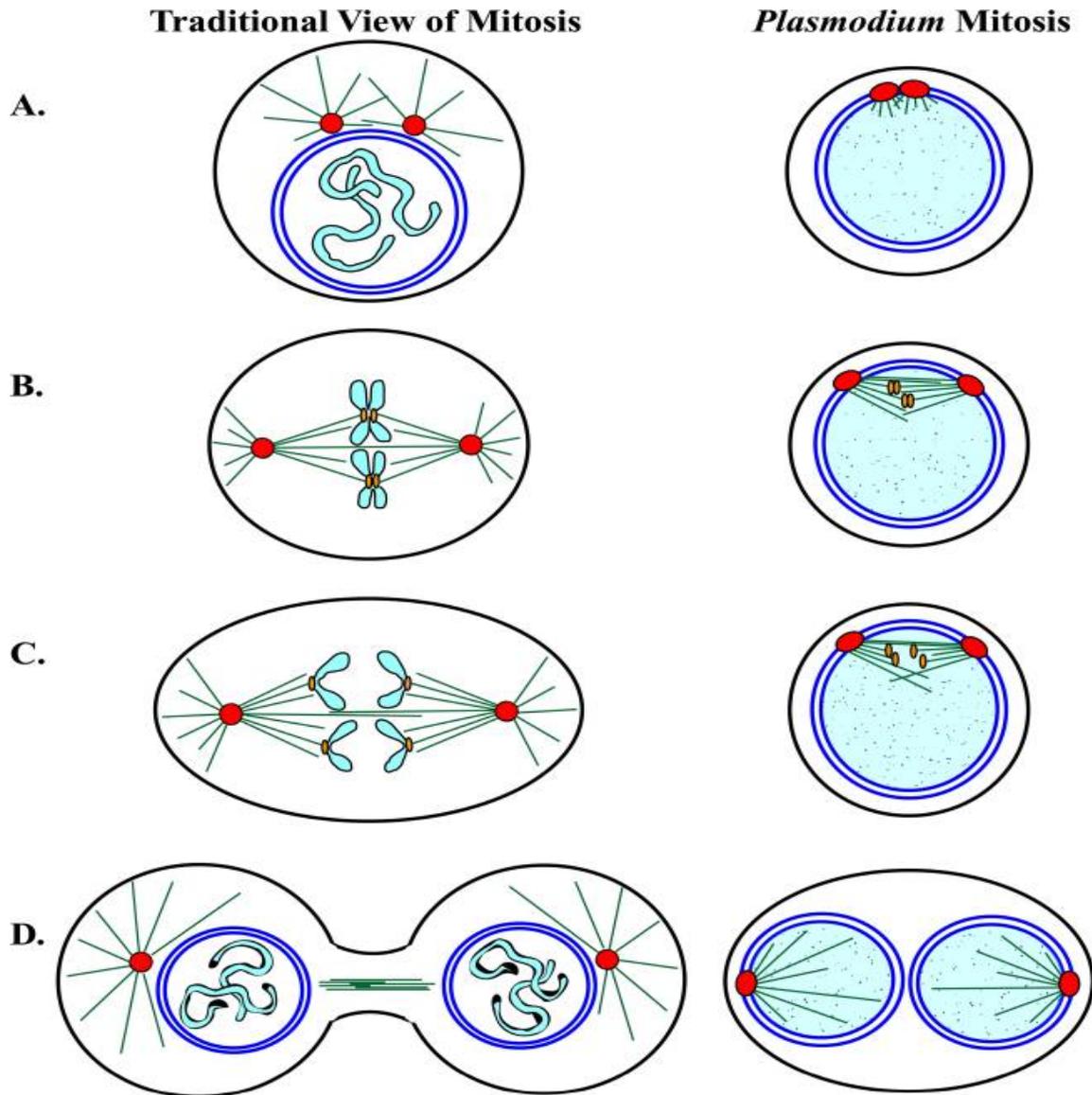


Figure 14. Comparison of mitosis between a typical eukaryotic cell and *Plasmodium*. The division of genomes in *Plasmodium* begins as the MTOCs begin to migrate along the nuclear membrane, while the typical eukaryote does not begin to separate the genomes until after migrating to the poles (Gerald *et al.* 2011).

This study has identified a novel protein in *P. falciparum*. This protein plays a critical role in merozoite formation by regulating the initiation of nuclear division during mitosis. To date there are only two other proteins known to play a role in the regulation of mitosis in *P. falciparum*. One protein is PfCEN1, which is a protein suspected to interact with the centrosome during nuclear division (Mahajan *et al.* 2008). This indicates the importance of proper nuclear division in the proliferation of *P. falciparum*. The second protein is an aurora-like kinase, an enzyme that regulates the proper progression of the cell cycle (Reininger *et al.* 2011). This regulation is achieved by the protein phosphorylating serine/threonine of specific targets essential for initiating the next stage of cellular division. This study has identified a third protein critical for the progression of *P. falciparum* through mitosis. PFRLP1 is therefore a valuable exploratory target for antimalarial drug development, as it offers a crucial biological pathway to target in the intraerythrocytic cycle of *Plasmodium*.

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