# Piecing Together the *Plasmodium falciparum* Genome Puzzle: Characterization of Genes/Proteins, PFE0565w and PF11_0394

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A Dissertation

presented to

the Faculty of the Graduate School

at the University of Missouri

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In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

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DECEMBER 2011

The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

## Piecing Together the *Plasmodium falciparum* Genome Puzzle: Characterization of Genes/Proteins, PFE0565w and PF11_0394

presented by MAGGIE S. SCHLARMAN,

a candidate for the degree of DOCTOR OF PHILOSOPHY,

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I dedicate this dissertation to all of my family and friends for always standing by my side, during the good times and the bad. I could not have achieved this goal without all of you.

# ACKNOWLEDGEMENTS

First, I would like to thank my advisor Dr. Brenda Beerntsen for her patience, encouragement, and guidance. In addition, thanks to Drs. Karen Bennett, William Stich, Qisheng Song, and Cynthia Besch-Williford for the time to serve as my committee and for all the advice.

Next, I would like to thank my lab members (past and present). To Alexis LaCrue, Michael Kariuki, Songie Wang, and Erjun Ling, your knowledge, advice, and help really aided me in the process of conducting my own research. To Ruguang Ou, thank you for always assisting me in my experiments and keeping our lab in order. To Renee Roberts, thank you for being my lab partner-in-crime. Your friendship, assistance, and hard-work made the lab fun and research easier to understand. Lastly, I would like to thank all of our undergraduates we have had over the years for all their hard work.

Also, I would to acknowledge all of the MMI/VPB faculty, staff, and graduate students for help and support throughout the years. I need to personally thank Sherrie Neff for aiding us in our mouse work and the staff at the research core facilities on the Mizzou campus that helped make our experiments possible.

In addition, I want to thank my family. To my parents, Jim and Brenda Christ, I thank you for raising me to be a hard worker, never giving up on my dreams, and always being there to lend a hand. Your love and support mean everything to me. To my brother, Benjamin Christ, I want to thank you for always

being someone I can count on to talk to and understand everything I am going through. I could not imagine having a better brother. To my mother- and father-in-law, Dana Albertearnst and Dave Schlarman, I thank you for being like actual parents to me and always being there for me. Dad S, we lost you far too soon and I miss you more than I can explain. I want you to know that I think about you every day, try to live up to the amazing person you were while on Earth, and I will always love you. To my brother- and sister-in-law, Ryan and Tiffany Schlarman, I want to thank you for always lending and extra hand. Tiffany, I need to especially thank you for taking the time to watch JD for his first two years of life while I was doing my research for this dissertation.

Last, but definitely not least, I want to thank my husband, Dan Schlarman, and son, JD Schlarman. Dan, I could not have done this without you; you are my constant. Thank you for celebrating in my successes and being there for me when things did not go well. You are a wonderful husband and father. JD, I could not have done this without you either. Even though you were too little to understand everything going on during this time, you helped me get through the tough times by just being you; you are such a joy! Dan and JD, I love you more than words can describe.

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**PIECING TOGETHER THE *PLASMODIUM FALCIPARUM* GENOME PUZZLE: CHARACTERIZATION OF GENES/PROTEINS PFE0565W AND PF11_0394**

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ABSTRACT

Malaria is still a significant problem around the world and, thus, better control methods are in great need. A key stage in the *Plasmodium* life cycle is the sporozoite because it exhibits dual infectivity in both the mosquito vector and vertebrate host and, therefore, is a promising target for discovering effective ways of controlling malaria. The *P. falciparum* genes, *PFE0565w* and *PF11_0394*, were chosen as candidates for study based on data available on PlasmoDB, the *Plasmodium* database, indicating that they are expressed both at the transcriptional and protein levels in sporozoites and likely encode putative surface proteins. Transcripts of both *PFE0565w* and *PF11_0394* are present in both mosquito and vertebrate host life cycle stages, but both of their proteins are specific to salivary gland sporozoites as shown by immunofluorescent assays and/or GFP-trafficking studies. Functional studies for PFE0565w are currently in progress to determine if it may play a role in parasite development and/or invasion of host tissues. Because PFE0565w and PF11_0394 do not have homology with any human proteins, they could be targets for new drugs and/or vaccines. Lastly, in addition to studies conducted with *P. falciparum*, a preliminary comparative study between the *P. berghei* orthologs of PFE0565w and PF11_0394, PBANKA_111090 and PBANKA_091050, respectively, was conducted.

# Chapter 1: Introduction

## 1. Background and Significance

### *1.1. General overview*.

Malaria is a mosquito-borne disease that is caused by protozoan parasites of the genus *Plasmodium*. For humans, the species of mosquitoes that are responsible for transmitting the parasites are of the genus *Anopheles*. There are more than 100 species of *Plasmodium*, but only five cause human disease, *P. vivax*, *P. malariae*, *P. ovale*, *P. knowlesi* and *P. falciparum*. Of the five human malaria parasites, *P. falciparum* is the most pathogenic (Lee *et al.*, 2008; Tuteja, 2007; van Hellemond *et al.*, 2009; Yegneswaran *et al.*, 2009). It is estimated that up to 300 million cases of malaria occur annually and, of those, up to one million are fatal with nearly 95% of the fatalities occurring in African children under the age of five and pregnant women (Moorthy *et al.*, 2004; WHO, 2010). Malaria has become more of a burden recently due to the development of insecticide-resistant mosquitoes, drug-resistant *Plasmodium* parasites, and economic/political instability in areas of the world where malaria is a significant problem (Porter, 2006). Because of the devastation that malaria causes around the world and the resistance to common control techniques that is spreading in both parasites and mosquitoes, it is critical that improved and/or new control methods (e.g., new drugs and the creation of a vaccine) be developed.

*1.2. Plasmodium parasites*.

In 1880, *Plasmodium* parasites were discovered by C.L.A. Laveran when he noticed the parasites in the blood of a patient suffering from the disease malaria. Later in 1897 and 1898, R. Ross and G. Grassi discovered that these parasites were transmitted via the bite of an infected mosquito and that only *Anopheles* transmit human malaria, respectively (Gilles, 2002).

*Plasmodium* are single celled protozoans that belong to the phylum Apicomplexa (Roberts *et al.*, 2009). Several other organisms of human and veterinary importance belong to this phylum, including organisms in the genera *Toxoplasma*, *Cryptosporidium*, *Theileria*, *Babesia*, and *Eimeria*. These organisms are distinguished from other parasites because they possess a plastid organelle, called an apicoplast, most likely derived from endosymbiosis of a cyanobacterium, specifically an algal cell (Roos *et al.*, 2002; Yeh & DeRisi, 2011). In addition, apicomplexans move and invade their hosts using an apical complex (Figure 1.1). Apical complexes are composed of organelles called polar rings, rhoptries, micronemes, and dense granules. It is thought that polar rings serve as structural elements that aid in locomotion and that rhoptries, micronemes, and dense granules help in penetration of the host cells by releasing enzymes (Roberts *et al.*, 2009). The invasion process of apicomplexan parasites is well conserved across species and can be broken down into four main steps: initial contact between the parasite and host cell, host cell entry by the apex of the parasite, internalization of the parasite into the host cell, and

formation of the parasitophorous vacuole (PV) (Dubremetz *et al.*, 1998; Santos *et al.*, 2009). Specific locomotion and invasion details of *Plasmodium* will be discussed in subsequent sections.

*1.3. Malaria, the disease*.

Malaria is the disease caused by *Plasmodium* and its name is derived from the term “mal aria”, which means “bad air” in Italian. It was so named because people that lived near bodies of stagnant water tended to contract the disease (Tuteja, 2007). Malaria was considered eradicated from the United States in the early 1950s by eliminating the *Anopheles* vector; however, it is still a major disease around the world today. Currently, it is estimated that half the world’s population is still at risk for contracting malaria (CDC, 2010).

Symptoms of malaria do not manifest themselves until the parasite invades the red blood cells (RBCs) of humans, where eventually 10% of the cells can be infected with *Plasmodium* (Wirth, 2002). The onset and severity of malaria depend on several factors, including what species of the parasite one is infected with, one’s age/sex, and if one is immunocompromised (Figure 1.2). Humans infected with *P. falciparum, P. malariae,* and *P. knowlesi* will present with disease symptoms within a couple weeks of becoming infected with the parasites. On the other hand, it could be months (or even years) until people initially infected with *P. vivax* or *P. ovale* display disease symptoms because these parasites can remain “hidden” as hypnozoites in the liver before invading the RBCs. This characteristic may also lead to a relapse of infection following

the initial infection. The same can happen with *P. malariae* and *P. knowlesi*, as they can remain "hidden" from the human immune system within RBCs by staying at a low parasitemia (parasite density below the level of detection, leading to minimal/no disease symptoms present) a process called recrudescence (Collins & Jeffery, 2007; Kantele & Jokiranta, 2010; Miller *et al.*, 2002; Roberts *et al.*, 2009). In addition, an interesting trait about *P. knowlesi* is that it is zoonotic, utilizing simians as reservoirs and is the most recent emerging human malaria parasite (Kantele & Jokiranta, 2010). In all cases of malaria, the most common symptom that occurs is a fever/chill cycle that mimics the release and reinvasion of parasites into the red blood cells. These cycles occur every 48 hours for *P. falciparum*, *P. vivax*, and *P. ovale* and every 72 hours for *P. malariae* and *P. knowlesi*. In addition, many people experience anemia (the most severe cases occur with both *P. falciparum* and *P. vivax*), malaise, and acidosis, which can lead to respiratory distress (Gilles, 2002; Tuteja, 2007). The human immune response to a malaria infection initiates a release of proinflammatory cytokines that can cause splenomegaly, hepatomegaly, inflammation of many other tissues, and filtration of both infected and non-infected RBCs by the spleen, significantly adding to the problem of anemia (Collins & Jeffery, 2007; Miller *et al.*, 2002).

*P. falciparum* is the most pathogenic of the human malaria parasites. These parasites can secrete proteins (such as erythrocyte membrane protein-1 (PfEMP-1) that lead to the formation of knobs on human RBCs, causing them to adhere to endothelial linings. The sequestration of the RBCs can lead to poor

circulation and/or blockage of the microvasculature. Depending on where these blockages occur, the results can cause cerebral malaria (hemorrhaging, coma, and death can occur), placental malaria (the fetus can be harmed), and respiratory distress. Other organs can be affected as well (Miller *et al.*, 2002). In addition to the mechanical blockage theory described above, there is another theory regarding the causes of severe malaria symptoms: the cytokine theory. According to this theory, the parasites cause an increase in cytokines, specifically tumor necrosis factor-alpha (TNF-α), leading to inflammation of the vessels and organs. Most likely, the symptoms of severe malaria are caused by a combination of both mechanical blockage of the vessels by the parasites themselves and a release of human cytokines (Idro *et al.*, 2005).

Early diagnosis of malaria is very important so that treatment can start as soon as possible, preventing not only severe disease, but death. People traveling to countries with endemic malaria can take prophylactic medications to prevent the parasite from invading RBCs, protecting them from disease. In malaria endemic regions, premunition (natural immunity) can be developed over years of repetitive exposure to the parasite. This immunity does not fully protect from the disease, but does significantly decrease the risk of developing severe malaria (WHO, 2010). Besides medication and premunition, there are other highly effective methods to prevent malaria. These include using insecticide-treated mosquito nets and/or indoor residual spraying to protect people against infected mosquitoes (Gilles, 2002; Miller *et al.*, 2002).

Malaria is a huge burden to society. It tends to greatly impact areas that are already poor and, unfortunately, is a large contributor to keeping the poverty level high. In areas where the malaria burden is significant, the gross domestic product can be decreased by 1.3% (WHO, 2010). In addition, in some heavily burdened countries, the disease accounts for up to 40% of public health expenditures, 30-50% of inpatient hospital admissions, and up to 60% of outpatient health clinic visits (WHO, 2010).

## 2. *Plasmodium falciparum* Life Cycle, Development, Host Cell Invasion, and Key Proteins.

### *2.1. General overview.*

*Plasmodium* parasites have a complex life cycle (Figure 1.3) that requires both a mosquito vector and vertebrate host for survival in nature; without one of these factors, the life cycle will be interrupted (Kumar, 2005; Wirth, 2002). The *Anopheles* mosquito becomes infected when it ingests a blood meal containing a mixed population of *Plasmodium* blood stages. A stage called the gametocyte, which is a sexual stage of the parasite and also the infectious form to the mosquito, differentiates into male and female gametes after ingestion and sexually reproduces to form a diploid zygote. The zygote then elongates into a motile ookinete that traverses the mosquito midgut epithelium and eventually resides between the basal lamina and midgut epithelium. This is a major bottle-neck in the parasite life cycle, as the number of parasites drastically decreases

due to the immune system of the mosquito (Wirth, 2002). Next, the ookinete becomes an oocyst full of parasites that have asexually reproduced to form sporozoites. The sporozoites egress from the oocyst and freely move throughout the hemolymph of the mosquito. Sporozoites then invade the salivary glands of the mosquito, where they can remain infectious for weeks (Tuteja, 2007). It is when the infected mosquito takes another blood meal that the sporozoites are injected into the skin of the human. Some of the sporozoites will make their way to the blood stream and eventually reside in the liver where they invade hepatocytes. Within the hepatocytes, the sporozoites undergo several rounds of asexual reproduction to form tens of thousands of merorozites. These merozoites burst from the hepatocytes and invade human RBCs, where the life cycle continues (CDC, 2010; Roberts *et al.*, 2009; Wirth, 2002).

*2.2. Infection of the Anopheles vector and sexual reproduction of Plasmodium.*

Sexual reproduction of *Plasmodium* parasites begins in the human host by gametocyte formation and ends in the mosquito midgut with the formation of a motile ookinete (Figure 1.4). The transmission of *Plasmodium* from the human host to the mosquito vector is one of the most critical stages when it comes to possible transmission-blocking mechanisms; however, it is currently one of least understood stages of the life cycle. Inside the human host, a process called gametocytogenesis occurs. This process results in the formation of male and female gametocytes (classified as stage I-V depending on their maturity) approximately 10 days after a blood infection is established. The exact

mechanism(s) that leads to the formation of gametocytes is not known, but factors such as the human immune system, hormones, anemia caused from the parasites, and drug therapies may all play a role (Alano, 2007; Baker, 2010). It is known that sexual commitment is predetermined during the asexual blood stages and that maturation of gametocytes predominately happens within the bone marrow and spleen (Paul *et al.*, 2002; Smalley & Sinden, 1977; Smith *et al.*, 2000; Thomson & Robertson, 1935). Eventually, mature stage V gametocytes are released and freely circulate in human blood. Gender bias is typical in *Plasmodium* gametocytes, where generally a 1:5 male to female ratio occurs (Kuehn & Pradel, 2010).

When parasites are transmitted from their human host back to a mosquito host, they are subjected to external stimuli that instigate activation of the sexual cycle, meaning female macrogametes and male microgametes are produced. These stimuli include a drop in temperature of 5ºC, exposure to the mosquito metabolite xanthurenic acid (XA), and an increase in pH from 7.4 to 8.0, although pH is thought to be an indirect factor in stimulating gametes (Billker *et al.*, 1998; Garcia *et al.*, 1998; Nijhout & Carter, 1978; Sinden, 1983; Sinden *et al.*, 1996). Once the mature gametocytes encounter these stimuli, parasites of both sexes round up and begin to egress from human RBCs. Parasite egress occurs via an inside-out model, where first the PV ruptures, followed by the erythrocyte membrane (EM) and this process takes approximately 10 minutes to complete once inside the mosquito host (Quakyi *et al.*, 1989; Sologub *et al.*, 2011). As gametocytes mature, the presence of osmiophilic bodies occurs, especially in

females. These are gametocyte-specific secretory organelles that appear to be linked to parasite egress (Kuehn & Pradel, 2010). Two proteins associated with these organelles have been identified, Pfg377 and male development gene-1/protein of early gametocyte-3 (MDV-1/PEG3). Gene disruption studies in *P. falciparum* indicate that parasites lacking Pfg377 have reduced numbers of osmiophilic bodies and fail to properly egress from human RBCs (de Koning-Ward *et al.*, 2008). Gene disruption studies in *P. berghei* (a rodent malaria parasite) demonstrate that parasites lacking MDV-1/PEG3 fail to disrupt the PV, resulting in failed egress from mouse RBCs and, thus, reduction in zygote formation (Ponzi *et al.*, 2009). The PV of gametocytes ruptures in multiple spots and it is likely that *Plasmodium* perforin-like proteins (PPLP1-5 and PF08_0058) play a role. These proteins have high homology with membrane attack complexes that are important in pore formation in membranes of other Apicomplexans (Kafsack & Carruthers, 2010). To date, the exact mechanism of *Plasmodium* egress from human RBCs inside the mosquito has not been determined. It is likely that in addition to perforin-like proteins, proteases also help in the process (Pradel, 2007; Sologub *et al.*, 2011).

While the gametocytes are exiting the human RBCs, male gametocytes replicate their genome three times, differentiating into eight flagellated microgametes (exflagellation). The external stimuli mentioned above also trigger a biochemical cascade in the parasites that lead to *Plasmodium* kinases helping in this process of gamete differentiation and fertilization: a *P. berghei* calcium-dependent protein kinase, CDPK4, allows males to enter into the cell cycle and a

MAP kinase, map2, causes cytokinesis of the male gametocyte, resulting in the eight microgametes within the vector, *An. stephensi* (Billker *et al.*, 2004; Khan *et al.*, 2005; Rangarajan *et al.*, 2005). The male microgametes bind with other non-infected RBCs to remain hidden, a process known as rosetting. Exflagellation, specifically the number of exflagellation centers, is used to determine a successful gametocyte culture *in vitro.* Eventually, the female macrogametes and male microgametes are completely free from the human RBCs and the males are able to freely move and fuse with a female macrogamete (fertilization). There are proteins that have been shown to be critical for fertilization to occur correctly. A *Plasmodium* protein, P48/45, is stored in the PV and eventually exported to the surface of the gamete, anchored to the plasma membrane. This protein directly interacts with another surface protein, P230. When using monoclonal antibodies to block P48/45 and P230, there is a block in transmission from human to mosquito (Targett *et al.*, 1990). In addition, when P48/45 was disrupted in both *P. berghei* and *P. falciparum*, it was shown that male gametes cannot successfully penetrate fertile, female gametes, leading to a significant reduction in zygote formation (van Dijk *et al.*, 2001). In another study when P230 was disrupted in *P. falciparum*, male gametes were unable to bind RBCs, decreasing the number of exflagellating centers. This resulted in poor mosquito transmission and female fertilization (Eksi *et al.*, 2006). Ultimately, male and female gametes bind with one another and it has been shown that a group of proteins with adhesive domains, the *P. falciparum Limulus* coagulation factor C domain-containing proteins (PfCCp), are critical for gamete-gamete interaction

(Baker, 2010). The nucleus of the male gametocyte enters the cytoplasm of the female and eventually fuses with the nucleus of the female, resulting in a diploid zygote (Janse *et al.*, 1986; Sinden, 1983). Within 24 hours of the formation of the zygote, it elongates to form a motile ookinete. This marks the end of sexual reproduction of *Plasmodium*.

*2.3. Ookinete formation and the traversal of the Anopheles midgut.*

In order for *Plasmodium* to complete sexual reproduction, the parasites undergo meiosis while maturing into ookinetes. A *Plasmodium* protein kinase called NEK4, NIMA [never in mitosis *Aspergillus*]-related kinase 4, regulates this process as shown in *P. berghei* NEK4 disruption studies (Reininger *et al.*, 2005). The ookinetes traverse the mosquito peritrophic matrix, using a “grab-and-push”-style forward motion, and develop into an oocyst between the basal lamina and midgut epithelium of the *Anopheles* midgut (Figure 1.5). To help in this process, ookinetes possess organelles called micronemes, which secrete proteins needed for motility, cell traversal, and invasion of host tissues (Vlachou *et al.*, 2006).

From the mosquito midgut lumen, the ookinetes must first penetrate the peritrophic matrix within the midgut (which is composed of 3-13% chitin by weight and needed as a scaffold). *Plasmodium* parasites utilize secreted chitinases to aid in this process (Langer & Vinetz, 2001). The usage of parasite-derived chitinase (CHT1/2) appears to be critical for both *P. falciparum* and *P. gallinaceum,* an avian malaria parasite, but not for *P. berghei*. In studies with *P. falciparum* and *P. gallinaceum*, allosamidin (a chitinase inhibitor) was added to

the *An. stephensi* and *Aedes aegypti* blood meal, respectively, which resulted in ookinetes not being able to penetrate the mosquito midgut (Vinetz *et al.*, 1999; Vinetz *et al.*, 2000). In *P. berghei*, PbCHT1 was deleted, creating a mutant parasite strain lacking CHT1. Studies demonstrated that the mutant parasites had a reduction in oocyst numbers in *An. stephensi*, but were still produced. These data demonstrate that *Plasmodium* chitinases are important for obtaining an optimal infection, but are not essential. Thus, results suggest that mosquito chitinases are most likely contributing to infection (Dessens *et al.*, 2001).

One of the key features of ookinetes is their ability to move. Two proteins that help in parasite locomotion are calcium-dependent protein kinase 3 (CDPK3) and circumsporozoite and TRAP (thrombospondin-related anonymous protein)-related protein (CTRP). In *P. berghei*, the CDPK3 gene was either disrupted or deleted in two independent studies (Ishino *et al.*, 2006; Siden-Kiamos *et al.*, 2006). In the gene disruption study, it was shown that parasites lacking CDPK3 could not access the midgut epithelium. In the gene deletion studies, the CDPK3 mutant parasites appeared morphologically normal, but did not glide (move) effectively. This led to their inability to successfully infect *An. stephensi* (Ishino *et al.*, 2006; Siden-Kiamos *et al.*, 2006). The other protein, CTRP, is a membrane-bound microneme protein that has been shown to be essential in mosquito infection in *P. berghei*. Using targeted CTRP disruption studies, mutant parasites had reduced motility, failed to traverse *An. stephensi* midgut epithelial cells, and thus, were not able to form oocysts (Dessens *et al.*, 1999; Yuda *et al.*, 1999).

Once ookinetes penetrate the peritrophic matrix, they come into contact with the apical end of the mosquito midgut epithelium. Although it has not been fully identified yet, it is thought that this contact and invasion is mediated via a receptor-ligand interaction; however, it has been shown that a protein called the membrane attack ookinete protein (MAOP) may serve as an initial contact point between *Plasmodium* and the mosquito midgut epithelium (Kadota *et al.*, 2004). MAOP is a microneme protein that contains a membrane attack complex. When this gene was disrupted in *P. berghei*, results indicated that parasites lacking this protein fail to infect the *An. stephensi* midgut. Further electron microscopy data revealed that this protein acts on the plasma membrane of epithelial cells, forming pores for the parasite to penetrate (Kadota *et al.*, 2004). Once the ookinetes are inside the epithelial cells, they have to cross the cytoplasm and travel though many cells before exiting at the basal lamina. During the process of cell traversal, the mosquito epithelial cells that the parasites invade become apoptotic and are removed from the cell layer. An important *Plasmodium* microneme protein, called cell-traversal protein for ookinetes and sporozoites (CelTOS) is partly responsible for the success of the parasites to penetrate the epithelial layer of the mosquito midgut. Using targeted CelTOS gene disruptions in *P. berghei*, mutant parasites showed a 200-fold decrease in *An. stephensi* midgut infectivity (Kariu *et al.*, 2006).

Eventually, the ookinetes will traverse their final epithelial cell and take up residence between the epithelial cells and basal lamina of the mosquito midgut. Two glycosylphosphatidylinositol (GPI)-anchored *Plasmodium* proteins, P25 and

P28, play an important role in the ookinetes embedding themselves within the basal lamina. In *P. berghei*, Tomas *et al.* (2001) demonstrated that P25 and P28 proteins have partially redundant functions. When each protein was individually deleted, there was only a small decrease in the number of parasite oocysts formed. On the other hand, when both proteins were deleted, a reduced number of ookinetes were produced and it completely abolished oocyst formation (Tomas *et al.*, 2001). It was then shown with a yeast two-hybrid system that both *P. berghei* P25 and P28 interact with *An. gambiae* laminin, with P28 interacting weakly, indicating that they are a receptor-ligand complex (Vlachou *et al.*, 2001). Another protein that is important for ookinetes to adhere to the basal lamina is the secreted ookinete adhesive protein (SOAP). Using *P. berghei* and *An. gambiae*, a yeast two-hybrid system showed that SOAP also interacts with mosquito laminin. In the same studies, mutant *P. berghei* parasites in which SOAP was deleted were unable to successfully invade *An. stephensi* midguts and did not produce oocysts (Dessens *et al.*, 2003; Vlachou *et al.*, 2001). Lastly, the previously mentioned CTRP protein has also been shown, using a yeast two-hybrid system, to interact with mosquito laminins, indicating that it has a potential role in helping parasites bind the mosquito basal lamina (Mahairaki *et al.*, 2005).

*2.4. Oocyst maturation and sporozoite development.*

When ookinetes make contact with the basal lamina of the mosquito midgut epithelium, it allows the parasite to switch to the mode of oocyst formation. It takes 10-14 days for the oocyst to fully mature depending on the

*Plasmodium* species (reaching a size of 50-60 µm, utilizing nutrients from the mosquito hemolymph for growth) and produce sporozoites. The process of sporozoite formation begins by the parasite undergoing several rounds of nuclear division without cytokinesis, which is called schizogony (Matuschewski, 2006). The oocyst is composed of two layers: the outer layer containing mainly laminin from the basal lamina of the mosquito (this is thought to help protect it from the immune system of the mosquito) and an inner plasma membrane (Kappe *et al.*, 2004).

The process of sporogony, formation of sporozoites (Figure 1.6), begins when the oocyst plasma membrane forms invaginations. The lobes that are formed are called sporoblasts and they express a *Plasmodium* protein on their surface, the circumsporozoite (CS) protein. The formation of the sporozoite intermembrane complex (IMC), under the plasma membrane, is the first sign of budding sporozoites. The IMC is closely associated with a network of microtubules (MTs) and it is the continuous expansion of the plasma membrane, IMC, and MTs that allows the sporozoite to grow/elongate into its triple-pellicle structure (Kappe *et al.*, 2004). By the time sporogony is complete, there will be thousands of sporozoites in each oocyst.

To date, there are a handful of proteins that have been shown to be necessary for sporozoite production and proper maturation. The CS protein, mentioned above, is the most abundant sporozoite surface protein. It is anchored to the oocyst plasma membrane and surface of sporozoites by a GPI-

anchor. CS gene disruptions in *P. berghei* demonstrated that parasites lacking CS produce oocysts, but sporozoites never form in them (Menard *et al.*, 1997). Additional studies in *P. berghei* led to the discovery that the CS protein is needed for proper membrane development, which is essential for sporozoite budding. Ultimately, the amount of CS protein present is directly correlated with the number of sporozoites that are produced by the oocyst (Thathy *et al.*, 2002). Another protein that has been found to be important in the formation of sporozoites is called scavenger receptor (PxSR). Using *P. berghei* gene disruption studies, it was shown that parasites lacking PxSR develop normal numbers of oocysts compared to wild-type parasites, but the oocysts failed to produce sporozoites in *An. stephensi* (Claudianos *et al.*, 2002). Lastly, the formation of the IMC of the sporozoite is critical and it is composed of a protein called inner membrane complex protein 1 (IMC1). Once again, using gene disruption strategies in *P. berghei*, data generated revealed that IMC1 is needed for proper sporozoite structure, development, and motility in *An. stephensi* (Khater *et al.*, 2004).

*2.5. Sporozoite egress and invasion of Anopheles salivary glands.*

In order for sporozoites to successfully egress from oocysts and invade mosquito salivary glands, they have to become motile. Gliding movement of the parasites occurs by use of an actin-myosin motor (Figure 1.7). Parasite IMC proteins interact with myosin (Myo A). Myo A binds actin (F-Actin), which indirectly binds a *Plasmodium* surface protein called TRAP (thrombospondin-

related anonymous protein) via aldolase. TRAP and CS proteins interact with mosquito substrates, pushing the parasite forward (Kappe *et al.*, 2004). Once sporozoites are mature and are able to glide, exiting of oocysts occurs. In the past, it was thought that rupturing of the oocyst was a passive process that occurred due to the sporozoites growing in size; however, this is not the case. It was found that parasite cysteine proteases are a necessity for proper oocyst egress (Aly & Matuschewski, 2005). Specifically, a cysteine protease called egress cysteine protease 1/serine repeat antigen 8 (ECP1/SERA8) seems to play a role. Using disruption studies in *P. berghei*, parasites that lacked ECP1 produced sporozoites, but they were not able to leave the oocyst (Aly & Matuschewski, 2005). The question then became, what protein does ECP1/SERA8 proteolytically process? To answer this question, additional studies in *P. berghei* were conducted and demonstrated that mutations made to the CS protein caused sporozoites to never leave the oocysts. Thus, data suggest that ECP1/SERA8 proteolytically cleaves the CS protein (Matuschewski, 2006; Wang *et al.*, 2005).

After the sporozoites leave the oocyst, they move freely about the mosquito hemolymph, with 20% of them making it to their destination of the salivary glands and the remaining 80% being eliminated from the hemolymph by the immune system of the mosquito (Mueller *et al.*, 2010). The sporozoites first come into contact with the basal lamina of the mosquito salivary gland epithelial cell, bind via a receptor-ligand interaction, and penetrate the salivary gland cells. When the parasites penetrate the glands, they are temporarily enveloped in a

PV. Eventually, the sporozoites leave the PV and reside in the secretory cavity of the mosquito salivary gland where they clump together in bundles, with a few of them entering the salivary duct of the glands (Figure 1.8). In contrast to mosquito midgut invasion, sporozoite invasion of the salivary glands does not cause apoptosis of the epithelial cells (Ghosh & Jacobs-Lorena, 2009). Interestingly, not all salivary gland sporozoites that reside in the glands of mosquitoes are infectious, as proven by studies by Noden *et al*. (1995). In these studies, they found that mice intravenously inoculated with 24,000 *P. yoelii* sporozoites obtained from *An. albimanus* salivary glands did not always yield an infection in the mice; however, when mice were intravenously inoculated with only 300 *P. yoelii* sporozoites obtained from either *An. stephensi* or *An. freeborni*, mice always developed an infection (Noden *et al.*, 1995). This discovery led to the conclusion that some mosquito vectors may either contain a factor that leads to a lack of sporozoite infectiousness or may be missing a factor needed by the sporozoite to become infectious (Noden *et al.*, 1995).

As previously mentioned, when sporozoites are travelling through the hemolymph of the mosquito, some of them will end up invading the salivary glands of the mosquito. In 1985, Rosenberg demonstrated that sporozoites most likely bind to salivary glands in a receptor-ligand mediated fashion. Using *P. knowlesi* and two *Anopheles* species, *An. dirus* (susceptible to *P. knowlesi* infection) and *An. freeborni* (not susceptible to *P. knowlesi* infections), they switched the midguts of the two mosquito species that had heavy oocyst infections. This resulted in *An. freeborni* midguts being placed inside *An. dirus*

and vice versa. Controls were also done such that infected midguts were dissected from mosquitoes and placed into new mosquitoes of the same species. The results were interesting: *An. freeborni* midguts placed into *An. dirus* did not develop a *P. knowlesi* infection in the salivary glands and *An. dirus* midguts put into *An. freeborni* did not yield successful sporozoite invasion of salivary glands either. These data demonstrate that *Plasmodium* parasites specifically recognize salivary glands in a species dependent manner, hinting at the potential of a receptor-ligand interaction (Rosenberg, 1985).

As sporozoite research progressed, two *Plasmodium* proteins have been found to act as ligands and two mosquito proteins have been found to serve as their receptors on mosquito salivary glands. The CS protein, mentioned above, is one *Plasmodium* protein that appears to serve in binding to the mosquito salivary glands. Using *P. gallinaceum*, it was shown that infection of *Aedes aegypti* salivary glands was completely inhibited when anti-CS monoclonal antibodies (which were used to bind to the CS protein and, thus, block the ability of the salivary gland sporozoites to interact with the mosquito salivary glands) were either co-injected with sporozoites or injected during sporozoite release during naturally acquired infections (Warburg *et al.*, 1992). Further studies with various sequence mutations in the CS protein revealed that a specific region (region II) is needed for motility and binding of *Plasmodium* to mosquito salivary glands (Sinnis *et al.*, 2007). The most likely salivary gland receptors for the CS protein are heparan sulfates found on the salivary glands, as the CS protein is known to bind heparin sulfates found on hepatocytes for invasion of liver cells

(Ghosh & Jacobs-Lorena, 2009; Sinnis *et al.*, 2007). Another protein mentioned above, TRAP, is also needed for both proper parasite gliding and binding of the mosquito salivary glands. Using gene disruption techniques in *P. berghei*, it was shown that parasites lacking a functional TRAP protein did not have proper gliding motility and could not infect salivary glands of *An. stephensi* (Sultan *et al.*, 1997). Scientists then began to investigate if TRAP had a receptor. Using a phage library, a mosquito peptide called salivary gland and midgut peptide 1 (SM1) was found to bind to mosquito salivary glands (Ghosh *et al.*, 2001). Later, the same group used UV-crosslinking experiments to determine that the SM1 peptide binds a mosquito salivary gland protein called saglin. Using antibodies, they found that the SM1 peptide is a mimotope of the *Plasmodium* TRAP protein. Additional competition studies and various mutations in the *P. berghei* TRAP protein revealed that TRAP utilizes saglin as a receptor on *An. stephensi* salivary glands and competes with the SM1 peptide for binding (Ghosh & Jacobs-Lorena, 2009).

In addition to the CS protein and TRAP, there are other *Plasmodium* proteins that are also important in invasion of tissues, but their potential receptors have not yet been identified. One of these proteins is called apical membrane antigen/erythrocyte binding-like protein (MAEBL) (Kappe *et al.*, 1998). Using targeted gene disruption studies in *P. berghei*, it was shown that MAEBL is not critical for sporozoite motility, but that it is needed for invasion of *An. stephensi* salivary glands (Kariu *et al.*, 2002). Besides the previously mentioned proteins, there are other proteins that have been found to be important in sporozoite

invasion of salivary glands. These proteins are called upregulated in oocyst sporozoites 3 (USO3) and TRAP-related protein/sporozoite-specific gene 6 (TREP/S6). USO3 studies done in *P. yoelii*, another rodent malaria model, demonstrated that parasites lacking this protein fail at invading *An. stephensi* salivary glands (Mikolajczak *et al.*, 2008). For TREP/S6, deletion studies in *P. berghei* show that parasites lacking this protein are partially inhibited from invading *An. stephensi* salivary glands and also have a partial loss in motility (Combe *et al.*, 2009; Steinbuechel & Matuschewski, 2009). Next, another group of proteins that appear to be essential in invasion of the mosquito salivary gland is the cysteine repeat modular proteins 1-4 (PCRMP1-4). Gene disruption studies of these proteins in *P. berghei* result in sporozoites that are unable to invade *An. stephensi* salivary glands (Thompson *et al.*, 2007). Lastly, a protein discovered in *P. berghei* called the sporozoite invasion-associated protein 1 (SIAP-1) is also critical for invasion of salivary glands. Gene disruption of SIAP-1 in *P. berghei* revealed that this protein is necessary for proper gliding motility of sporozoites, causes a partial defect in sporozoite egress from oocysts on *An. stephensi* midguts, and abolishes parasite invasion of the *An. stephensi* salivary glands (Engelmann *et al.*, 2009).

*2.6. Infection of the human host and exoerythrocytic/pre-erythrocytic (liver) stage development.*

When an infectious mosquito is probing for a blood meal on a human host, sporozoites can be injected into the dermis. Based on experimental transmission

studies using *An. freeborni* and *An. gambiae* and a membrane feeding system, it was determined that each infectious mosquito that bites a person is, on average, capable of injecting 5-25 sporozoites (Beier *et al.*, 1992; Ejigiri & Sinnis, 2009). Studies using rodent malaria models demonstrate that sporozoites in the skin are in a migratory mode for travel to the liver (Figure 1.9). Recent data indicate that at least 50% of *P. berghei* sporozoites remain in the skin for multiple hours (typically 1-6 hours) (Yamauchi *et al.*, 2007). Of these parasites that stay in the dermis, 10% end up developing in the epidermis, dermis, and hair follicles into merosomes (PVs full of parasites) that can be infectious to human RBCs and the other 40% are cleared by the immune system (Gueirard *et al.*, 2010). Of the remaining 50% of sporozoites, approximately 70% enter the blood stream, utilizing the circulatory system as a transport system to the liver and the other 30% go to the draining lymph nodes (Ejigiri & Sinnis, 2009). Sporozoites leave the dermis and travel to the blood stream by cell traversal, allowing the parasite to actually penetrate cells to help the parasites evade the immune system. There are multiple proteins with similar functions that have been found to be important in this process; these proteins include sporozoite microneme protein essential for cell traversal (SPECT), SPECT2/perforin-like protein 1 (PLP1), CelTOS (previously mentioned), phospholipase (PL), and TRAP-like protein (TLP) (Bhanot *et al.*, 2005; Ishino *et al.*, 2005; Ishino *et al.*, 2004; Kaiser *et al.*, 2004; Kariu *et al.*, 2006; Moreira *et al.*, 2008). When using *P. berghei* and *P. yoelii*, parasites lacking these proteins as a result of gene disruption techniques all displayed a failure in cell traversal, resulting in parasites not able to invade the

livers of mice (Bhanot *et al.*, 2005; Ishino *et al.*, 2005; Ishino *et al.*, 2004; Kaiser *et al.*, 2004; Kariu *et al.*, 2006; Moreira *et al.*, 2008).

Once sporozoites effectively exit the dermis and enter the blood stream, they travel to the liver and efficiently arrest there. To penetrate the liver, the sporozoites have to traverse the sinusoidal layer, which is partially composed of Kupffer cells (resident macrophages of the liver) (Ejigiri & Sinnis, 2009). Many of the proteins mentioned above for aiding the parasite in traversing the dermis also are needed for the parasite to traverse this barrier to the hepatocytes. The exact mechanism by which sporozoites gain access to the hepatocytes is not known; however, it is hypothesized that most of the parasites go directly through the Kupffer cells to gain access (Sinnis & Coppi, 2007).

When sporozoites finally reach hepatocytes, they switch from a migratory mode to an invasive mode. Current research has given insight into how this happens. In addition to its importance in development and invasion of tissues in the mosquito, the CS protein is also needed for invasion/development in the liver stages (Ejigiri & Sinnis, 2009). It is also known that heparan-sulfate proteoglycans (HSPGs) are important in serving as a binding partner for the CS protein. Current *P. berghei* data now show that the high levels of HSPGs in the liver are what trigger sporozoites to convert from migratory mode to invasion mode and that processing of the CS protein is needed as well (Coppi *et al.*, 2011; Coppi *et al.*, 2007). When in the dermis, there are low levels of HSPGs and the CS protein is full-length. When *P. berghei* sporozoites come into contact with

rodent hepatocytes, they are exposed to high levels of HSPGs, which result in activation of calcium-dependent protein kinase 6 (CDPK6). The activation of CDPK6 then leads to the production of a cysteine protease that is responsible for cleavage of the CS protein (Coppi *et al.*, 2007). The subsequent cleavage of the CS protein produces a truncated form of the protein, exposing the region needed for invading hepatocytes (Coppi *et al.*, 2011).

There are other parasite proteins and human hepatocyte proteins that have been found to be important for sporozoites to successfully invade hepatocytes. In brief, TRAP, apical membrane antigen-1 (AMA-1), P36p, and thrombospondin-related sporozoite protein (TRSP) have all been shown to play a part in hepatocyte invasion using antibody blocking studies and/or disruption studies (Robson *et al.*, 1995; Silvie *et al.*, 2004; Sinnis & Coppi, 2007; van Dijk *et al.*, 2005). A *Plasmodium* serine protease called Rhomboid 1 (ROM1) appears to be important for early development of exoerythrocytic stages, but not essential (other *Plasmodium* proteases most likely have partially redundant functions) as found by gene deletion studies in *P. berghei* (Srinivasan *et al.*, 2009). Next, studies in *P. berghei* have shown that sporozoites secrete cysteine protease inhibitors to control both parasite and host-produced proteases. Without these protease inhibitors, infective invasion of hepatocytes does not occur (Rennenberg *et al.*, 2010). Lastly, there are two host hepatocyte proteins that are known to be important for *Plasmodium* sporozoites to invade the liver, scavenger receptor BI (SR-BI), which is the major receptor for uptake of cholesterol by hepatocytes, and tetraspanin CD81 (tetraspanins interact with one

another to help create membrane microdomains). To confirm the role of SR-BI in sporozoite invasion of hepatocytes, studies were done such that sporozoites were given the opportunity to invade either wild-type hepatocytes or hepatocytes lacking the SR-BI protein. Results showed that lower numbers of sporozoites entered hepatocytes with deleted SR-BI compared to wild-type hepatocytes. Currently, it is hypothesized that SR-BI may aid in the formation of microdomains, or PVs, that are important for parasite entry into the hepatocytes (Rodrigues *et al.*, 2008; Yalaoui *et al.*, 2008). Next, to demonstrate the importance of tetraspanin CD81 in sporozoite invasion of the liver, anti-CD81 human and mouse antibodies were used to block the proteins on human and mouse hepatocytes, respectively. The results demonstrated that *P. falciparum* and *P. yoelii* sporozoites are not able to infect human and rodent hepatocytes in the presence of the antibodies (Silvie *et al.*, 2003).

Sporozoites that enter hepatocytes reside within a PV. Over a period of two weeks, the parasites become round, form a schizont, remain virtually hidden from the human immune system, and undergo multiple rounds of asexual reproduction. One sporozoite can result in a schizont containing up to 30,000 merozoites. Eventually, the schizonts rupture such that the schizonts separate from the hepatocytes (merosomes) and the merozoites are released into the blood stream where they invade human RBCs (Ambion, 2011; Gilles, 2002).

*2.7. Merozoite invasion of human red blood cells.*

The next step in the *Plasmodium* life cycle is for the merozoites to invade the human RBCs, which is an intricate process like the other invasion processes previously mentioned. Merozoite invasion can be broken down into several steps (see Figure 1.10). In general, merozoites make an initial contact with the human RBC on any region of the parasite. These initial contacts are termed long distance because they are of low-affinity. Once this happens, the parasite has to reposition itself, a process called reorientation, such that its apical side comes into contact with the human RBC. When reorientation is complete, the interaction of the merozoite with the RBC is of high-affinity and binding occurs via a receptor-ligand interaction (Cowman & Crabb, 2006). Next, a tight junction is formed between the parasite and the human RBC and the invasion process begins. The parasite utilizes its actin-mysoin motor system (previously described in section 2.4 for sporozoite invasion of salivary glands), allowing movement from the apical end to the posterior end (Keeley & Soldati, 2004). While this movement is occurring, the outer proteins on the parasite surface are being removed by proteases and rhoptries are depleted. At the end of the invasion process, the merozoite resides inside the RBC, fully enclosed within a PV. All of this takes place very rapidly, with full invasion completed in approximately one minute (Bannister & Mitchell, 2009; Cowman & Crabb, 2006).

Because the erythrocytic stages of some *Plasmodium* species can be cultured *in vitro*, researchers have been able to thoroughly study this life cycle

stage. Even with the vast data that have been gathered, many of the exact functions of the *Plasmodium* proteins that most likely make initial contact with the host cells are not completely elucidated. The most abundant (and well-characterized) protein groups on the surface of the merozoite are the merozoite surface proteins (MSPs), specifically MSP-1. MSP-1 is thought to be essential in invasion and/or survival of this parasite stage because parasites with MSP-1 deletions have not been able to be created. It is hypothesized to interact with RBCs via a sialic acid-dependent manner (Perkins & Rocco, 1988). More recent studies with *P. falciparum* have demonstrated that MSP-1 and MSP-9 form a co-ligand complex that binds a RBC transport glycoprotein receptor called band 3 (Goel *et al.*, 2003; Li *et al.*, 2004). In addition, *in vitro* antibody blocking studies with MSP-1 inhibit parasites from invading RBCs and injection of the recombinant protein leads to protection from the disease in monkeys and mice (Daly & Long, 1993; Kumar *et al.*, 1995; Ling *et al.*, 1994). Overall, even though the MSPs are well-studied and appear to play a significant role in parasite invasion, more research needs to be conducted to determine their exact functions/mechanisms. This could be done by using “rescue” techniques recently developed by two research groups. For example, Armstrong and Goldberg (2007) developed a method to control the level of protein production in *P. falciparum* using a ligand-regulatable FK506 binding protein (FKBP) destabilization domain controlled by the presence/absence of Shld1, an analog of rapamycin. This allows a protein of interest to be expressed even in a deletion mutant, which can help prove it is essential during the erythrocytic stages (Armstrong & Goldberg, 2007). In

addition to this strategy, the use of tetracycline-regulated transactivators also can be used to rescue the expression of essential proteins in gene disruption/deletion studies in *P. falciparum* erythrocytic stages (Meissner *et al.*, 2005). Even though these two approaches are promising to try and prove that certain genes are essential, there are limitations to them and, thus, they are not always feasible to use. The Shld system is "leaky" and may not cause a phenotype in some proteins and making fusion proteins can cause proteins-of-interest to not function properly. Next, the chemicals used to regulate these gene expression systems can be toxic to *Plasmodium*. Lastly, these two systems are more beneficial for analyzing proteins during certain stages of the life cycle of the parasite. For example, the promoter used in the Shld system has peak expression during the trophozoite and schizont stages (Armstrong & Goldberg, 2007; Meissner *et al.*, 2005). However, recently, Muralidharan *et al.* (2011) developed a gene regulation system using a degradation domain from *Escherichia coli* dihydrofolate reductase together with green fluorescent protein (GFP). Regulation of protein expression with this system is controlled using trimethoprim, a folate analog that is not toxic to *Plasmodium*, and the GFP allows both protein localization patterns and protein-protein interactions to be determined (Muralidharan *et al.*, 2011). Therefore, this new gene regulation technology may be a better system for use in the future.

The next step in merozoite invasion is apical reorientation and formation of the tight junction. Even though this process is not well understood, it is thought that two *Plasmodium* proteins work together to aid in this process: AMA-1

(previously mentioned during the liver stage section) and rhoptry neck protein 2 (RON2). Multiple research groups have data that support the importance of AMA-1 in the RBC invasion process (Deans *et al.*, 1982; Triglia *et al.*, 2000). Using anti-AMA-1 antibodies in *in vitro* blocking studies, they showed that these antibodies could inhibit merozoite invasion of both *P. falciparum* and *P. knowlesi* (Deans *et al.*, 1982; Triglia *et al.*, 2000). Recent data demonstrate the importance of AMA-1 interacting with RON2. Antibodies against both AMA-1 and R1 (the AMA-1 peptide that binds to RON2) block the interaction of these two proteins and inhibit merozoite invasion of RBCs (Richard *et al.*, 2010). Like MSP-1, gene deletions have not been possible for AMA-1, indicating that it likely has an essential role during this life cycle stage, but more research will have to be done to determine its exact function.

The binding of *Plasmodium* merozoites to the RBCs once the tight junction begins to form is of high-affinity and irreversible, thus suggesting receptor-ligand interactions. Currently, it appears that there are many parasite ligands that bind many different RBC receptors. Several of the initial merozoite invasion studies were done using the human parasite, *P. vivax*, because it was found that certain cohorts of people in Africa could not become infected with *P. vivax,* a trait called Duffy negativity. It was found that in order for *P. vixax* to successfully invade human RBCs, the Duffy Antigen Receptor for Chemokines (DARC) had to be present on the RBCs (Gaur *et al.*, 2004). Eventually, it was determined that *P. knowlesi* uses this receptor for invasion into human RBCs as well (Gaur *et al.*, 2004). For *P. falciparum*, studies showed that glycophorins A, B, and C (GPA,

GPB, and GPC) are used for invasion. Unlike *P. vivax*, eliminating these receptors does not completely abolish merozoite invasion, proving that redundancy occurs for ligands binding to receptors in *P. falciparum* (Gaur & Chitnis, 2011). Shortly after these host receptors were found, their Duffy-binding-like domains (parasite ligands) were discovered via erythrocyte binding assays. These included the Duffy binding proteins (DBPs) of *P. vivax* and *P. knowlesi* and the erythrocyte binding antigens (EBAs) of *P. falciparum* (Gaur et al., 2004). Other *P. falciparum* ligands, called the PfRH proteins, were also discovered and erythrocyte binding assays with these have determined that these ligands bind with various RBC receptors: PfRH1 binds Receptor Y, PfRH2 binds Receptor Z, PfRH4 binds Complement Receptor 1, and PfRH5 binds Receptor W (Gaur & Chitnis, 2011).

Once the tight junctions are formed, signals from the environment and parasite trigger activation of CDPKs, which in turn lead to a release of additional apical organelle proteins. The parasite uses its actin-myosin motor system to move completely into the RBC and reside within a PV, while using sheddases to remove its surface proteins (Cowman & Crabb, 2006). Within the PV, the merozoite develops into a feeding trophozoite stage, and eventually undergoes asexual reproduction, resulting in the formation of more merozoites that reside in a schizont. Eventually, these schizonts rupture, and each merozoite is capable of invading an additional RBC. After some time, a group of these parasites will differentiate into male and female gametocytes, entering the stage which is once

again infectious to the mosquito and the life cycle begins again (Gilles, 2002; Wirth, 2002).

## 3. Malaria Vaccine Development

### *3.1. Overview of vaccine development and its challenges.*

Despite the many types of malaria control efforts, such as better distribution of insecticide treated bed nets, use of combination drug therapy (especially artemisinin-based therapies), and other mosquito control methods, malaria is still a significant problem around the world. These control attempts have not been enough due to several reasons, including *Plasmodium* becoming resistant to common drug treatments, mosquitoes becoming resistant to certain insecticides, and political/economic strife causing groups of people to not have access to treatment and/or protective bed nets. Thus, the creation of a malaria vaccine is of great interest and importance (Crompton *et al.*, 2010). Unfortunately, the development of a safe, affordable, and effective malaria vaccine has been a great challenge. To date, there are vaccines that have progressed to clinical trials, but there is no vaccine available to the general public (Vanderberg, 2009). One positive aspect is that there have been some discoveries that point to the conclusion that a vaccine against malaria should be feasible, but how effective it will be is of concern. First, it has been found that people can acquire natural immunity to malaria when exposed to *Plasmodium* parasites over a long period of time. Second, immunization of mice, monkeys,

and humans with radiation-attenuated sporozoites (RAS) can lead to sterile, protective immunity against the liver stages of the disease (Hafalla *et al.*, 2011; McCarthy & Clyde, 1977; Nussenzweig *et al.*, 1967).

Development of a malaria vaccine has been difficult because of the complexity of the mosquito life cycle, the antigenic variation (switching of different proteins expressed on the surface of the parasite) in *P. falciparum*, and a lack of full understanding of how *Plasmodium* interacts with the human immune system. Major antigenic variation of *P. falciparum* occurs during the erythrocytic stages, when disease symptoms are present. The parasite can encode proteins, called variant surface antigens (VSAs), that are expressed on the surface of infected human RBCs (iRBCs), resulting in the formation of knobs (Smith *et al.*, 1995). These knobs cause the iRBCs to adhere tightly to endothelial linings, helping to protect the parasites from the immune system and also leading to severe forms of disease. The exact proteins that are expressed can change and also adapt when they are exposed to environmental factors that impede their survival, leading to drug resistance and a difficultly in vaccine design (Hviid, 2010).

There are various strategies being employed to generate a malaria vaccine. First, there are vaccines being made with the goal to prevent infection of *Plasmodium* (targeting the sporozoite and liver stages of the parasite). Next, there are vaccines that aim to prevent disease symptoms from developing (targeting erythrocytic stage parasites). Lastly, there are vaccines being developed to prevent transmission of the parasite (targeting parasite stages that

would inhibit infections in *Anopheles*). Because of all the approaches to successfully create a malaria vaccine, there are many candidates and trials that have occurred or are currently underway. Thus, there are too many vaccine candidates to discuss in this dissertation; however, some of the most promising will be highlighted. For more information, a recent review that was published by Crompton *et al*. (2010) has a series of tables that list vaccine candidates by category and is a good resource for additional information.

*3.2. Sporozoite/pre-erythrocytic stage vaccines.*

Sporozoite/pre-erythrocytic stage vaccines are the most promising candidates so far in clinical trials (e.g., the RTS, S vaccine) and are considered the "gold standard" of malaria vaccines aiming to prevent an infection from developing in humans (Vanderberg, 2009). Vaccines created with sporozoites/sporozoite proteins are useful in that they can target the parasite before large numbers of them are produced and before they cause an onset of disease symptoms (because parasites will never get the chance to invade RBCs). One of the first demonstrations of this type of vaccine was the use of RAS (these parasites are live, but attenuated via radiation). In 1967, Nussenzweig and colleagues demonstrated that mice that were immunized with RAS (*P. berghei*) were protected when challenged with fully infectious sporozoites (Nussenzweig *et al.*, 1967). Throughout the 1970s, multiple research groups moved into trials in humans and showed that both *P. falciparum* and *P. vivax* RAS also protected humans against infection with those species

(Clyde *et al.*, 1973; McCarthy & Clyde, 1977; Rieckmann *et al.*, 1974). Eventually, Dr. Stephan Hoffman and colleagues tested a *P. falciparum* RAS vaccine in larger clinical trials performed between the years 1989-1999 and continued to show how effective this type of vaccine is, as volunteers that received the vaccine that were subsequently challenged with *P. falciparum* showed a 90% protection from disease development (Hoffman *et al.*, 2002). In order for individuals to obtain a high level of protection from the RAS vaccine, each person had to be exposed to 1,000 RAS. Thus, the largest challenge of this vaccine was to successfully produce large enough numbers of sporozoites because producing this many sporozoites via an *in vitro* lab setting is very difficult. However, Hoffman and colleagues were able to overcome this challenge by utilizing knowledge gained over Hoffman's years of malaria research as the Director of the Malaria Program at the Naval Medical Research Center and later as the Vice President of Biologics for Celera Genomics (the company that helped with the sequencing of the *An. gambiae* genome). In 2002, he left Celera and founded Sanaria, a company that focuses on malaria eradication through vaccine development (www.sanaria.com). With the help of a large number of staff members, Sanaria is able to massively produce sporozoites for a *P. falciaparum* sporozoite (PfSPZ) vaccine using irradiated sporozoites. Currently, they are preparing to enter Phase 3 clinical studies with this vaccine (Hoffman *et al.*, 2010). Overall, the approach to using RAS as a vaccine against malaria is promising, but it is not a perfect one, as protection against the disease is lost after 42 weeks (Hoffman *et al.*, 2010; Hoffman *et al.*, 2002).

Another vaccine in this category is the RTS, S vaccine. This vaccine utilizes the CS protein of *Plasmodium* sporozoites, along with the hepatitis B viral antigen (HBsAg) used in Hepatitis B vaccines. The company Glaxo-Smith-Kline and the Walter Reed Army Institute of Research collaborated to generate this combination vaccine. The vaccine is made up of a large central portion of the CS protein and its entire C-terminal flanking regions (specifically amino acids 207-395) of *P. falciparum*. This, along with wild-type HBsAg, was co-expressed in yeast cells for stabilization of the viral particles. The end result was the RTS, S product, comprised of 25% fusion protein RTS (B-cell Repeats + T-cell epitopes + HBsAg antigen) and 75% wild-type HBsAg (S) antigen (Gordon *et al.*, 1995). In several early clinical trials, the RTS, S vaccine had an efficacy of 30-70% in disease prevention in volunteers observed six months after they had been vaccinated. Due to these results, this vaccine is currently in Phase 3 clinical trials in sub-Saharan Africa (Casares *et al.*, 2010).

There are other sporozoite proteins that are currently used as antigens for pre-erythrocytic stage vaccines, including TRAP and liver stage antigen-1 (LSA-1). Many of these are still in Phase 1/2a trials (Crompton *et al.*, 2010). In addition, researchers are also utilizing genetically attenuated parasites (GAPs) as vaccines. The best GAP model is with the deletion of the *P52* and *P36* genes in *P. falciparum*, which encode proteins important for development of the liver stages. Currently, this GAP vaccine is in Phase 1/2a trials in the United States (Crompton *et al.*, 2010; VanBuskirk *et al.*, 2009).

*3.3. Erythrocytic stage vaccines.*

Targeting the blood stages of the parasite life cycle is another approach to produce malaria vaccines and the goal of this type of vaccine is to prevent disease symptoms from developing by inhibiting the development of an infection in the RBCs. Sporozoite/pre-erythrocytic stage vaccines are clearly very promising and important; however, even if one sporozoite is successful at invading the human liver, a person could develop a blood stage infection and display disease symptoms. Thus, the creation of an erythrocytic stage vaccine is an important approach for disease prevention. Currently, there are many candidates that are in Phase 1/2 clinical trials. The most targeted antigens used in these vaccines are MSPs, AMA-1, and EBA-175. There is also a vaccine called Combination B (which is a mixture of four recombinant erythrocytic stage proteins and one synthetic peptide) that is currently in Phase 2b trials in Papau New Guinea. This vaccine has demonstrated a 62% reduction of parasite density in children that have not been pre-treated with other malaria prophylactic medication (Crompton *et al.*, 2010; Ellis *et al.*, 2010).

*3.4. Transmission-blocking vaccines.*

The next category of malaria vaccines is one that would prevent/limit transmission of *Plasmodium*, the transmission-blocking vaccine (TBV). The basis of this type of vaccine is to target antigens expressed during the mosquito stages (mainly gametes, zygotes, and ookinetes). In theory, when the mosquito ingests blood from a human treated with this type of vaccine, the antibodies

produced would also be ingested and prevent development of the parasites in the mosquito, thereby preventing transmission. The major controversy to a TBV is that it will not prevent disease in newly-infected people and is thought to potentially be harmful based on a hypothesis that it could delay/prevent people from acquiring natural immunity (Sauerwein, 2007). *Plasmodium* antigens that have been and are currently being used for these types of vaccines include gametocyte/gamete proteins P48/45 and P230 and ookinete proteins P25 and P28 (Crompton *et al.*, 2010; Saul, 2007).

*3.5. Multi-stage vaccines.*

The last major category of malaria vaccines and, in my opinion the most logical, is multi-stage vaccines. These vaccines utilize antigens that are present during multiple parasite life cycle stages. Thus, if one target area is "leaky" (e.g., if a vaccine targeting a sporozoite stage is used and one sporozoite still reaches the liver, a blood stage infection could develop and disease could occur), the vaccine will target other stages. Most of the multi-stage vaccines combine a pre-erythrocytic stage antigen with a blood stage antigen. Two current examples in Phase1/2 trails are CS coupled with AMA-1 and a cocktail of AMA-1, MSP-1 and TRAP (Crompton *et al.*, 2010).

## 4. Laboratory Research Focus

Malaria research has advanced tremendously over the last decade due, in part, to the genome sequencing of both *P. falciparum*, the parasite that causes

the most pathogenic form of human malaria, and *An. gambiae*, the primary vector of *P. falciparum* in Africa (Gardner *et al.*, 2002; Holt *et al.*, 2002). With this sequencing came the production of two databases containing the sequence information of the two organisms, PlasmoDB, the *P. falciparum* database, and AnoBase, the *An. gambiae* database (Bahl *et al.*, 2003; Topalis *et al.*, 2005). In addition to sequence information, tools (such as BLAST analysis, proteomic programs, and links to other related organisms) and other information (such as microarray, mass spectrometry, and proteomics data) are available on the sites. PlasmoDB has made it possible for researchers to conduct *in silico* searches of *P. falciparum* genes/proteins to study further in hopes of learning as much as possible about transcript/protein expression patterns, the functions of the parasite proteins, and how the parasite interacts with its hosts. In addition, data generated from these experiments will allow for the discovery of new drug and vaccine candidates.

Thus, the focus of this dissertation was to further characterize two *P. falciparum* genes, *PFE0565w* and *PF11_0394*, and their proteins. These two genes/proteins were identified using an *in silico* data mining process based on the criteria that they were predicted to be sporozoite surface proteins. This was done by first using PlasmoDB and searching for proteins predicted to only be expressed by the sporozoite stage via mass spectrometry (Florens *et al.*, 2002). Next, several sequence analysis programs found on the ExPASy Bioinformatics Resource Portal (www.expasy.org) and Softberry (www.softberry.com) were used to narrow the candidates down to those predicted to be surface proteins.

We set these specific standards for two reasons: first, not many sporozoite proteins are well characterized based on the difficulty of working with *P. falciparum* mosquito stages in a laboratory setting and, second, many *Plasmodium* proteins that have been shown to be critical for invasion and/or development of the parasite are surface proteins.

Overall, data collected in these characterization studies have allowed us to elucidate both the transcript and protein expression patterns of two novel *P. falciparum* genes/proteins. While functional studies have been attempted, current efforts have not been successful, and ongoing experiments are still in progress. Thus, although more work will have to be done to determine the function of PFE0565w and PF11_0394 in parasite development and/or invasion of tissues, the expression profiles provide valuable insight as to where and when these proteins may be important in the life cycle of *Plasmodium*.

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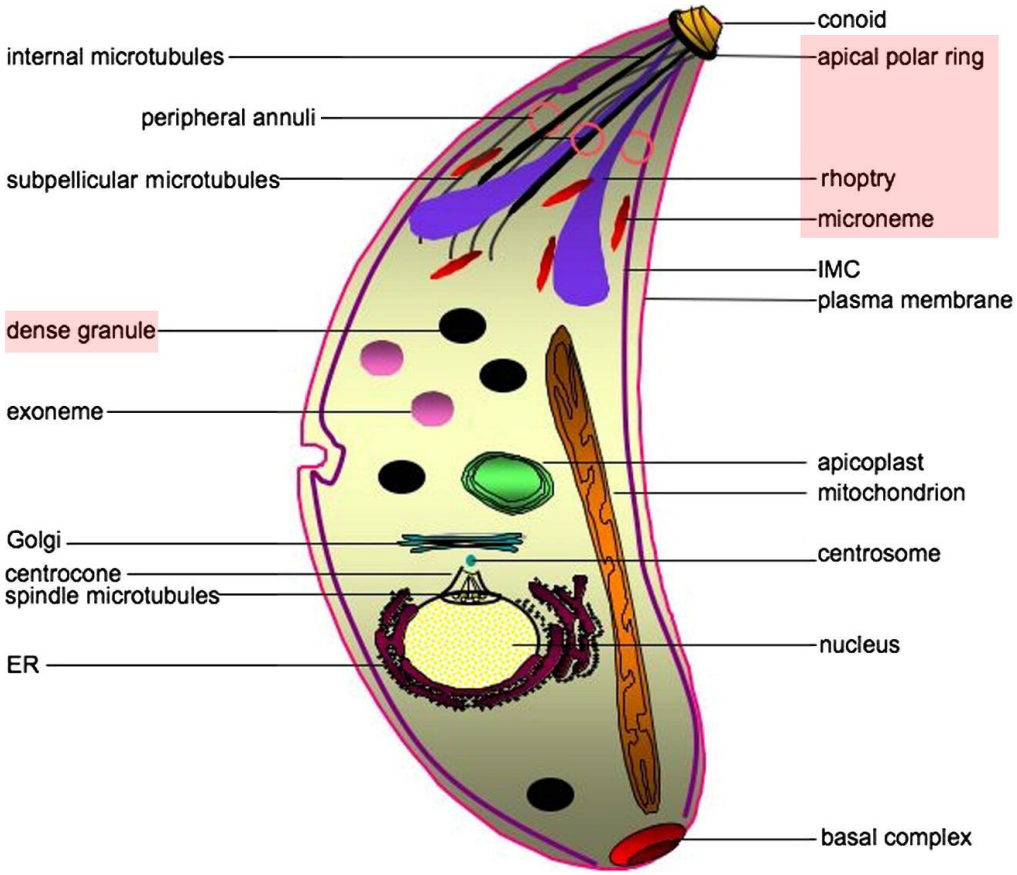


**Figure 1.1. Characteristic model of Apicomplexans.** *Plasmodium* parasites belong to the phylum, Apicomplexa. These parasites contain an apicoplast and move via an apical complex. The apical complex region contains four main organelles (see shaded boxes): polar rings, rhoptries, micronemes, and dense granules. ER = endoplasmic reticulum and IMC = inner membrane complex. This figure was modified from Santos *et al*., 2009.

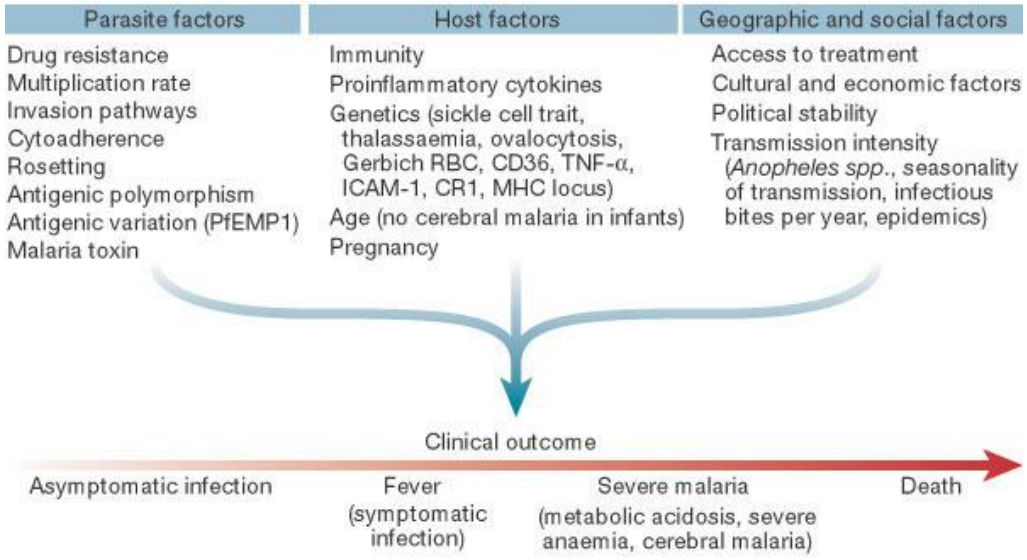


**Figure 1.2. The severity of malaria is dependent on a variety of complex features.** Malaria can range in its severity based on many reasons, including the human immune system, human genetics, the age of the host, geographical location, and many parasite factors. This figure was obtained from Miller *et al*., 2002.

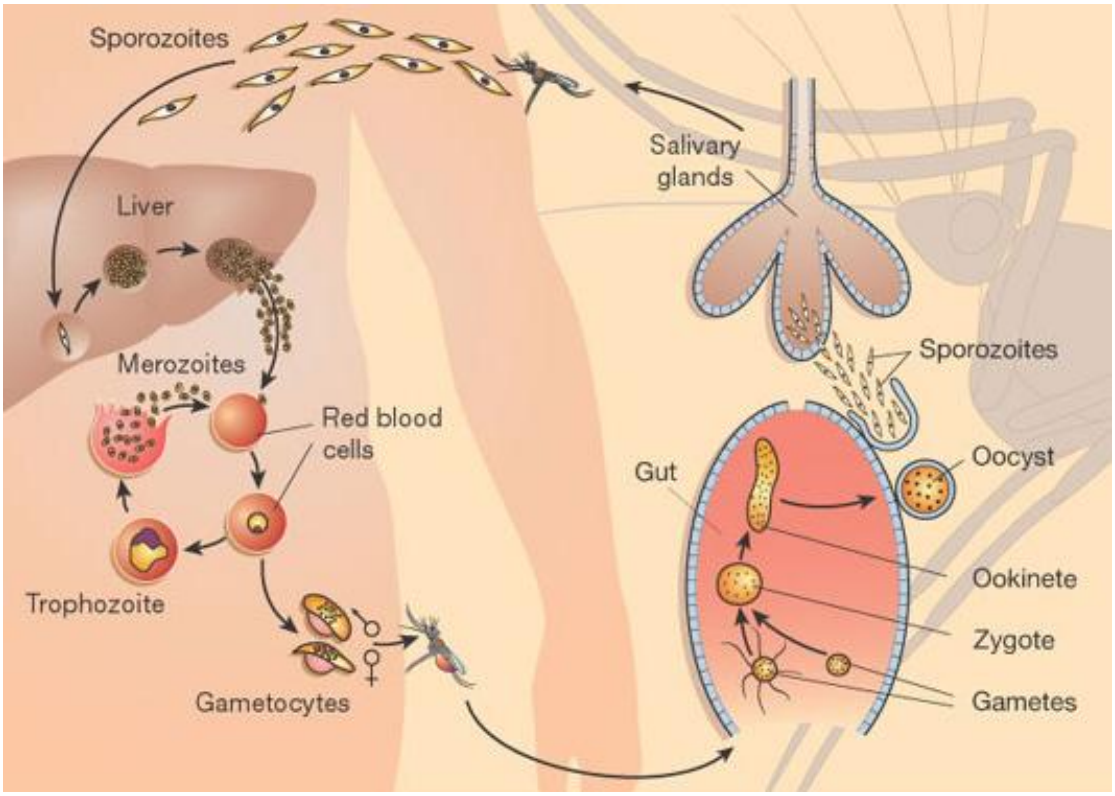


**Figure 1.3. The *Plasmodium falciparum* life cycle.** *Plasmodium* parasites require both a vertebrate host and a mosquito host to survive. When a naïve mosquito feeds on a human, she ingests human red blood cells (RBCs) containing gametocytes, the infective parasite stage for the mosquito. In the mosquito host, the parasites differentiate to gametes and sexually reproduce to form zygotes, which become motile ookinetes. The ookinetes traverse the mosquito midgut and eventually form sessile oocysts. Within oocysts, parasites asexually reproduce to form sporozoites. The sporozoites migrate to the mosquito salivary glands and are transmitted to the human host when the mosquito probes for a blood meal. The sporozoites proceed to the liver, asexually reproduce to form merozoites, and then burst from hepatocytes and

invade human RBCs. The life cycle then starts again. This figure was modified from Wirth 2002.

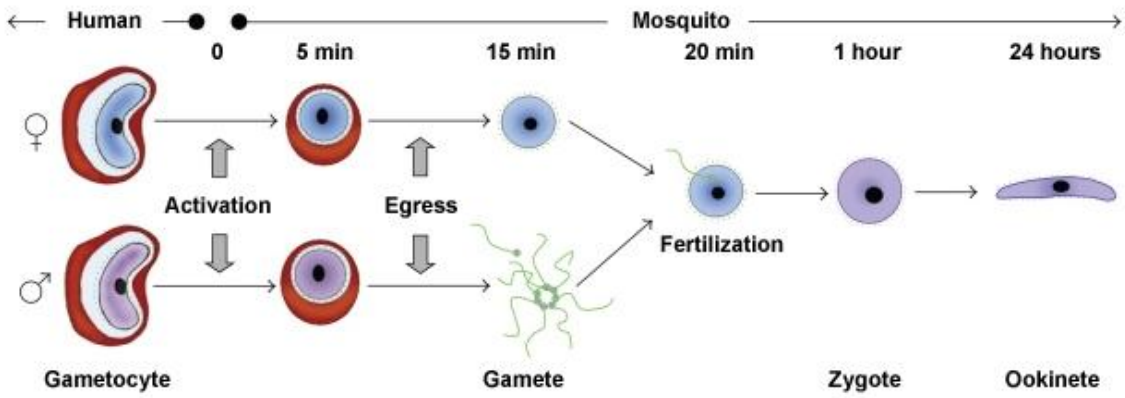


**Figure 1.4. The sexual stages of the *Plasmodium* life cycle, transitioning from human to mosquito.** Female and male gametocytes are ingested by a mosquito, where environmental changes trigger females to become macrogametes and males to become microgametes within five minutes of the blood meal. Fertilization occurs within 20 minutes after ingestion of gametocytes. This fusion of the male and female gametes results in a diploid zygote (one hour after ingestion of the blood meal), which elongates into a motile ookinete within 24 hours. This figure was modified from Kuehn and Pradel 2010.

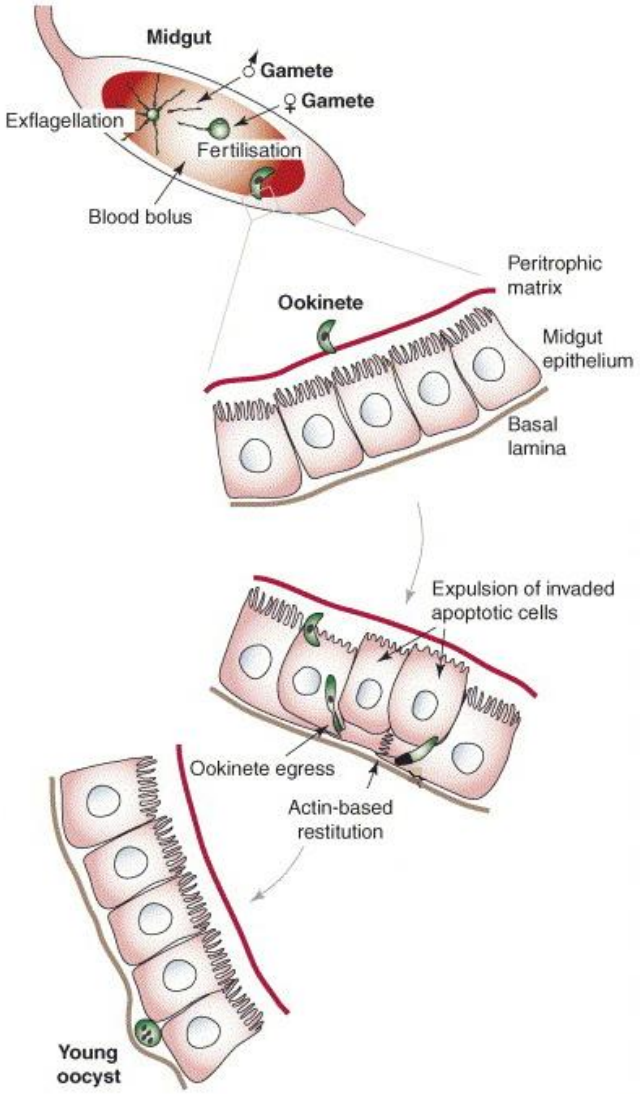


**Figure 1.5. Traversal of the *Anopheles* midgut by ookinetes.** Once fertilization is complete, the *Plasmodium* zygote elongates to form a motile ookinete. The ookinetes penetrate the midgut peritrophic matrix and traverse several midgut epithelial cells. The midgut cells that were invaded by parasites are removed from the cell layer via apoptosis. Eventually, the ookinete resides between the midgut epithelial cells and basal lamina where it develops into an oocyst. This figure was modified from Vlachou *et al*., 2006.

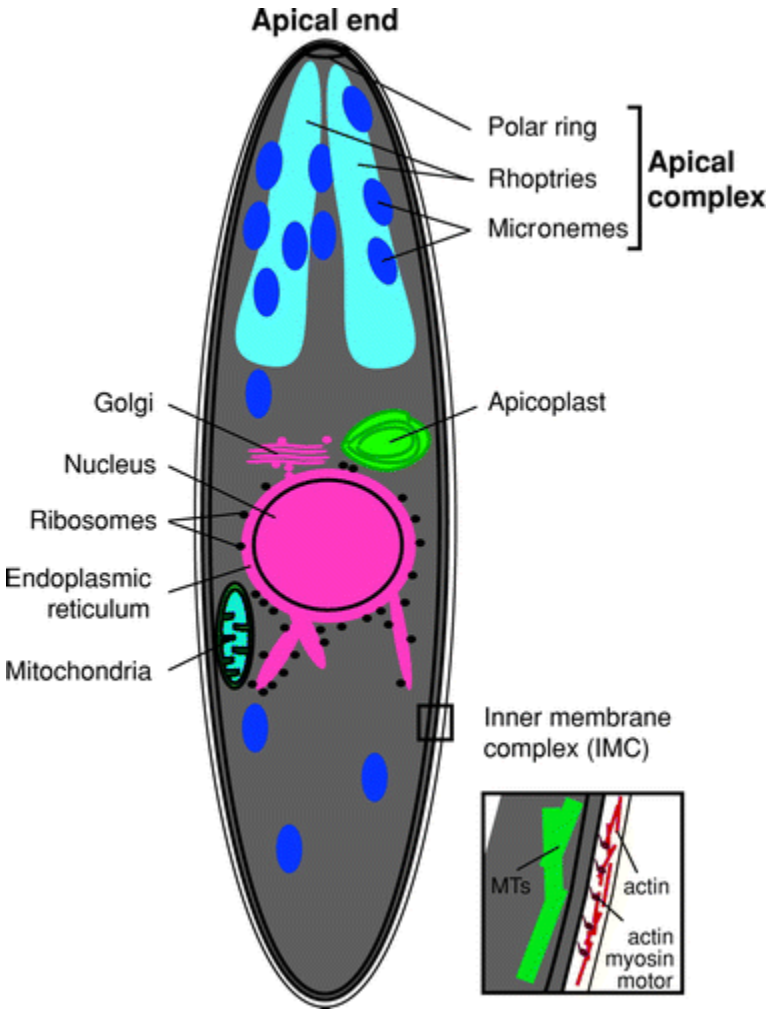


**Figure 1.6. *Plasmodium* sporozoite morphology.** Mature sporozoites are produced from sporoblasts in maturing oocysts. They have a triple-pellicle structure composed of the plasma membrane, intermembrane complex (IMC) and a network of microtubules for support. Sporozoites also contain an apical complex that produces proteins needed for motility and host cell invasion. This figure was modified from Kappe *et al.*, 2004.

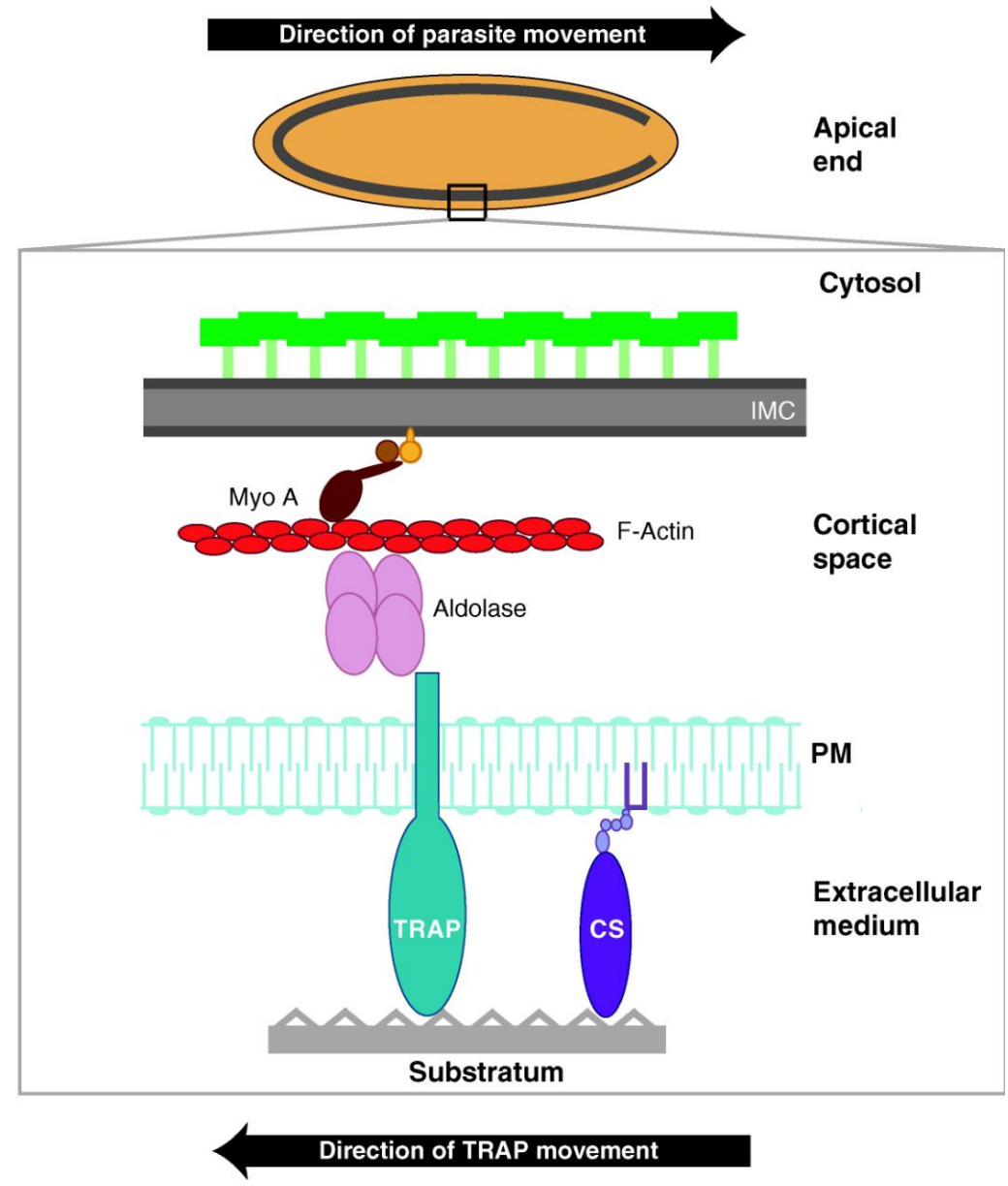


**Figure 1.7.** ***Plasmodium*** **motility machinery.** In order for sporozoites to egress from oocysts and invade mosquito salivary glands, they have to become motile and use an actin-myosin system for their gliding. Intermembrane complex (IMC) proteins interact with myosin (Myo A). Myo A binds actin (F-Actin), which indirectly binds the plasma membrane (PM) protein TRAP (thrombospondin-

related anonymous protein) via aldolase. TRAP and CS (circumsporozoite) proteins interact with mosquito substrates, pushing the parasite forward. This figure was modified from Kappe *et al.*, 2004.

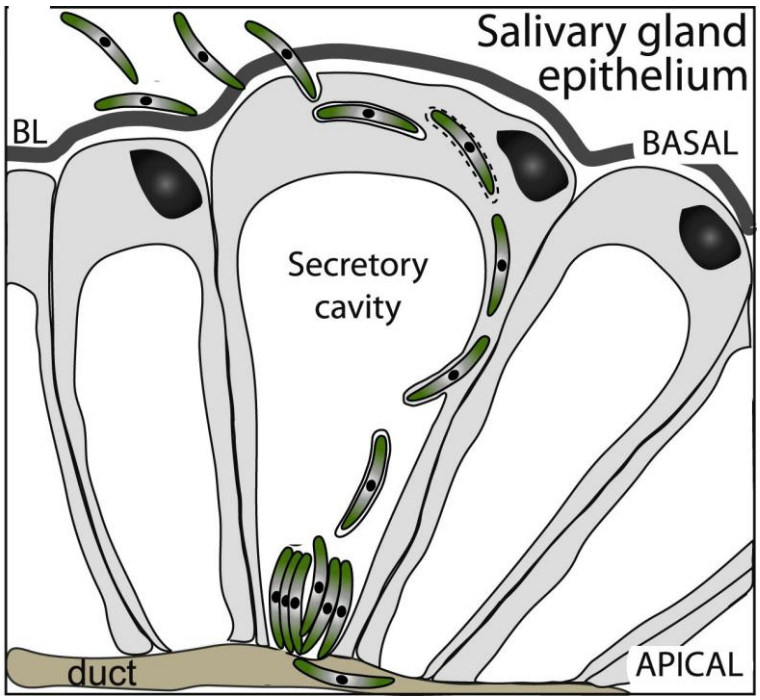


**Figure 1.8. Sporozoite invasion of the mosquito salivary glands.** Sporozoites first come into contact with the basal lamina of the mosquito salivary glands and enter via a receptor-ligand interaction. The sporozoites penetrate the salivary gland epithelial cells, without damaging them, and migrate to the secretory cavity. The sporozoites bundle together and some of them enter the gland duct, allowing them to be transmitted to the vertebrate host when the mosquito takes a blood meal. This figure was obtained from Mueller *et al.*, 2010.

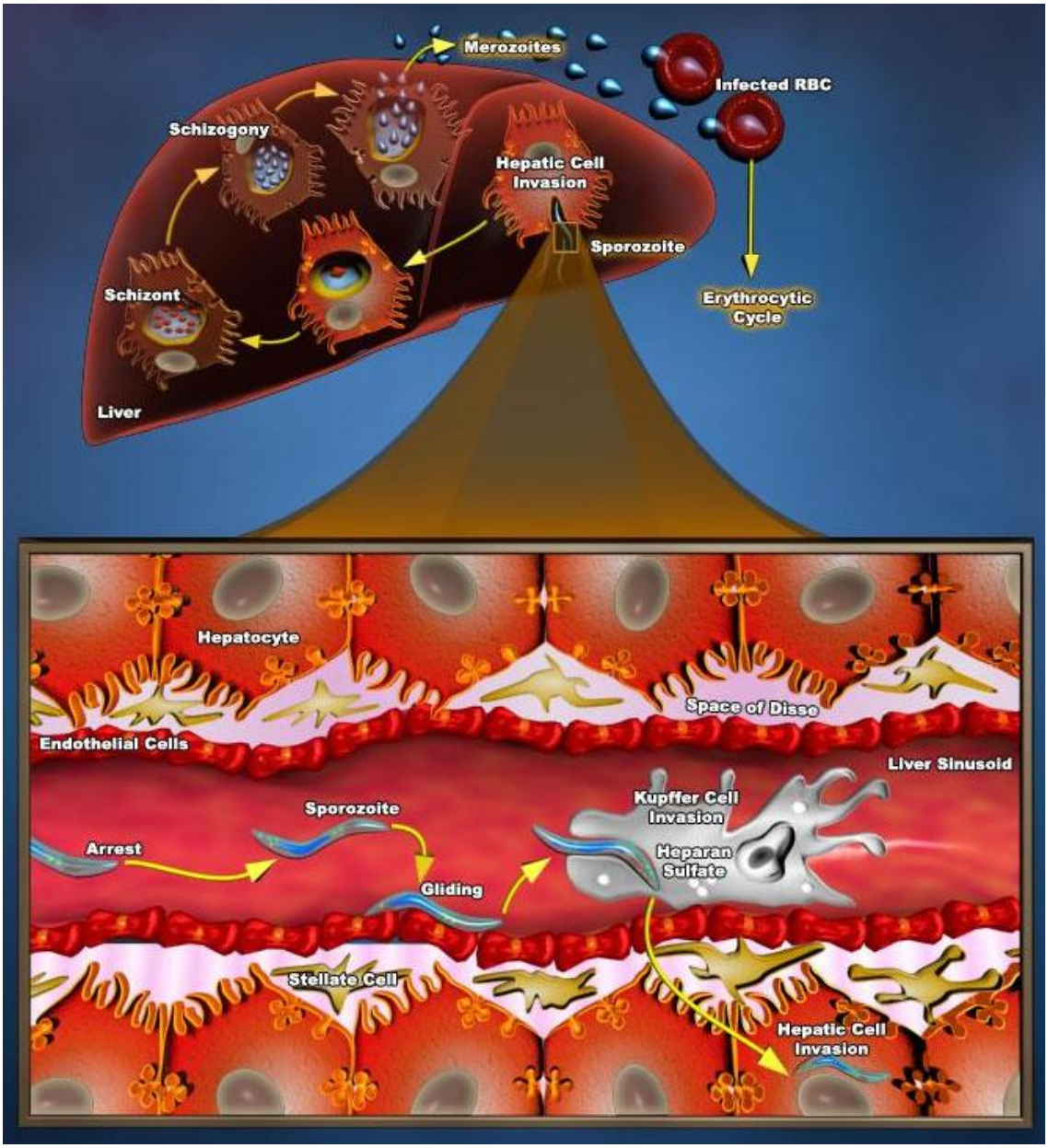


**Figure 1.9. Sporozoite invasion of the human liver.** Once sporozoites are injected into the human dermis, some of them make their way to the liver via the circulatory system. The sporozoites have to traverse the liver sinusoid, which is partly composed of Kupffer cells. When the parasites come into contact with the liver, they are exposed to high levels of heparan-sulfate proteoglycans (HSPGs)

which trigger the parasite to become invasive. When the parasite invades the hepatocytes, they reside within a parasitophorous vacuole (PV) and asexually reproduce to form tens of thousands of merozoites. These merozoites eventually rupture the human hepatoycte and proceed to infect human red blood cells (RBCs). This figure is from Ambion 2011.

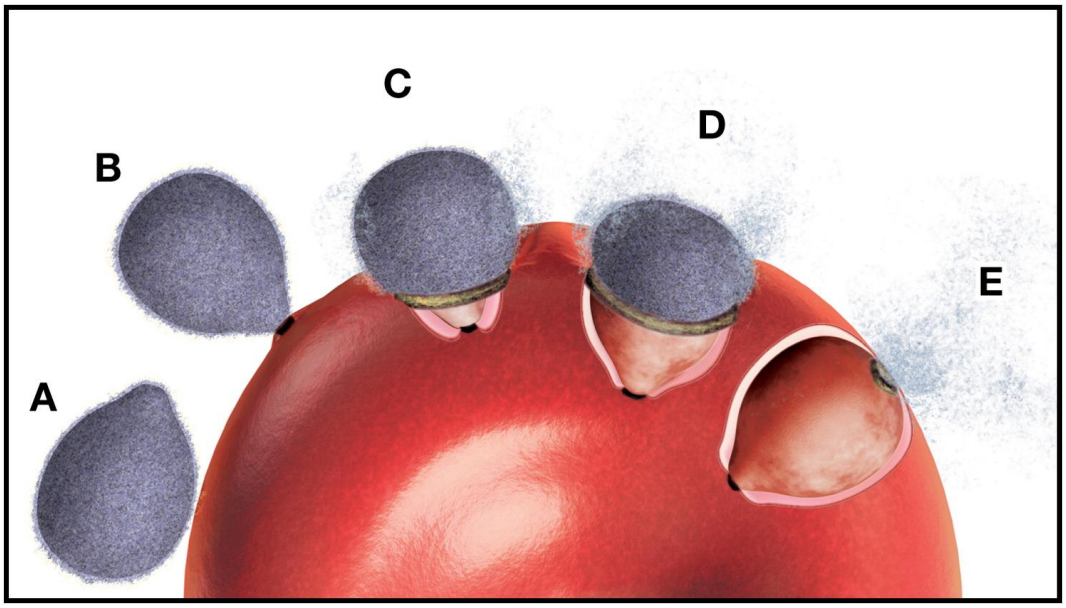


**Figure 1.10. Merozoite invasion of human red blood cells (RBCs).** A) Merozoites make an initial, low-affinity contact with the human RBC. B) Reorientation occurs such that the apical end of the parasite makes contact with the RBC. C) Eventually, high-affinity receptor-ligand interactions occur, a tight junction is formed, and the parasite begins invasion. D) The merozoites utilize actin-myosin motors (described in section 2.4) allowing movement from the apical end to the posterior end of the parasite. While this movement is occurring, the outer proteins on the parasite are being removed by a serine protease called sheddase. E) By the end of the RBC invasion, the adhesive proteins of the parasite also are removed by proteases, which ultimately allow the merozoite to become fully enclosed within a parasitophorous vacuole. This figure was modified from Cowman *et al.*, 2006.

# Chapter 2: Characterization of PFE0565w, a *Plasmodium falciparum* Protein Expressed by Salivary Gland Sporozoites


## ABSTRACT

The *Plasmodium falciparum* gene, *PFE0565w*, was chosen as a candidate for study due to its potential role in the invasion of host tissues. This gene was selected based on data from PlasmoDB, indicating that it is expressed both at the transcriptional and protein levels in sporozoites and likely encodes a putative surface protein. Additional sequence analysis shows that the PFE0565w protein has orthologs in other *Plasmodium* species, but none outside of the genus *Plasmodium*. PFE0565w expresses transcript during both the sporozoite and erythrocytic stages of the parasite life cycle, but does not appear to express it during exoerythrocytic stages. Despite transcript being present in several life cycle stages, the PFE0565w protein is present only during the salivary gland sporozoite stage. Furthermore, a gene disruption construct has been successfully created for PFE0565w and studies are in progress to assess a potential function of the protein in parasite development and/or invasion of host tissues.



**Keywords:** *Plasmodium falciparum*, sporozoite, surface protein

## 1. Introduction

Malaria is a devastating disease caused by *Plasmodium* parasites that are transmitted via the bite of an infected mosquito. The disease is still a significant problem around the world due, in part, to the development of insecticide-resistant mosquitoes and drug-resistant *Plasmodium* parasites. It is estimated that up to 300 million cases of malaria occur annually and, of those, up to one million are fatal (WHO, 2010). Because of malaria's significant impact on human health and welfare, it is critical for improved and/or new control methods to be discovered, such as drugs and the development of vaccines. Therefore, because *Plasmodium* sporozoites have dual infectivity for tissues of both the mosquito vector and vertebrate host, they are a promising target for efforts designed to find effective ways of controlling malaria.

Using data mining techniques, the *P. falciparum* gene, *PFE0565w*, was chosen as a candidate for study due to its potential role in the invasion of host tissues. This gene was selected based on data collected from PlasmoDB, the *P. falciparum* database (www.plasmodb.org), indicating that this gene likely encodes a putative surface protein, which is expressed in the sporozoite both at the transcriptional and protein levels (Bahl *et al.*, 2002; Florens *et al.*, 2002). The experiments proposed herein are to further characterize the gene, *PFE0565w*, and the protein it produces to determine its expression profile, localization, and potential function. Data gathered from this research will allow further understanding of transcript and protein expression patterns of another novel *P.*

*falciparum* gene/protein, understand more about parasite and host interactions, and may also provide the field with a new drug and/or vaccine target.

## 2. Materials and Methods

### *2.1. Parasite maintenance, parasite transmission, and cell cultures.*

*Plasmodium falciparum* strain NF54 was used for the following experiments (a gift from Dr. Shirley Luckhart at the University of California-Davis) and was maintained according to procedures described by Carter *et al.*, 1993. Briefly, *P. falciparum* cultures were maintained in human blood (O+ male, BioChemed Services) at a 6% hematocrit in complete culture medium, RPMI 1640 (Gibco) supplemented with 25mM HEPES (Gibco), 0.5% Albumax (Invitrogen), and 0.005% hypoxanthine (Sigma). The medium was changed every 48 hours and the parasites were kept in a gas mixture (3% $O_2$, 3% $CO_2$, and 94% $N_2$) at 37ºC (Carter *et al.*, 1993). Cultures were diluted/split to prevent the parasitemia from becoming too high by addition of fresh, washed 50% blood (blood washed three times with sterile RPMI and diluted 50:50 with RPMI), maintaining a 6% hematocrit level. The use of human blood was in compliance with federal guidelines and institutional policies. All experiments described throughout this chapter were approved by the Institutional Biosafety Committee (IBC), the Institutional Review Board (IRB), and the University of Missouri Institutional Animal Care and Use Committee (IACUC).

In order to obtain infected mosquitoes to study the parasite stages within the vector host (e.g., zygotes, ookinetes, oocyst sporozoites, hemolymph sporozoites, and salivary gland sporozoites), four-five day old, female *An. stephensi* (details in section 2.2) were exposed to *P. falciparum*-infected blood (1:1 ratio of infected blood and human serum), using induced gametocyte cultures. Gametocyte cultures were produced by setting a standard culture (described above) at a parasitemia of between 0.5-1.0% and maintaining them in complete culture media supplemented with 10% human serum (A+ male, Interstate Blood Bank); however, instead of splitting the parasites with fresh RBCs, the culture was left undiluted such that a high parasitemia developed and the parasites became stressed. This was done for 16 days and resulted in a mixture of male and female gametocyte stages (I-V), with a majority of them being mature stages. The mosquitoes were fed the infected blood for approximately 30 minutes, using a 37°C water-jacketed membrane feeding system (Figure 2.1). After the blood feed, the percentage of females that fed was recorded and any feeds where less than 75% of the females did not feed were not used for any experiments. The infected mosquitoes were kept in an incubator (Low Temperature Illuminated Incubator 818, Precision) at 26-27ºC with 82-88% humidity.

To obtain exoerythrocytic stages (liver stages), two approaches were used: infection of primary human hepatocytes and axenic cultures (*i.e.*, cultures without hepatocytes). For the former, *in vitro* cultures of the primary human hepatocytes (Clonetics ™ normal human hepatocytes, Lonza) were seeded 48

hours before infection with *P. falciparum* sporozoites in CellBind® 48-well plates (Corning), which were coated with 60 µg/cm$^2$ of rat tail collagen (BD Biosciences), at a density of 150,000 cells/cm$^2$. The hepatocytes were maintained according to the manufacturer's instructions using their hepatocyte maintenance medium supplemented with growth factors (insulin, GA-1000, and dexamethasone) in a 37ºC incubator with 5% $CO_2$ (Cui *et al.*, 2009; Talman *et al.*, 2010). To infect the hepatocytes with *P. falciparum*, approximately 5 X 10$^4$ salivary gland sporozoites were added to each well and allowed to incubate in invasion medium (hepatocyte maintenance medium supplemented with 10% human serum and 200 units/ml and 100 µg/ml penicillin and streptomycin, respectively) for four hours in a 37ºC incubator with 5% $CO_2$. The medium was then removed and fresh invasion medium was added daily, until the cells were harvested for experimental use after 72 hours (Cui *et al.*, 2009).

For axenic exoerythrocytic stage cultures, an online protocol by Kappe *et al*. (2010) was followed. In brief, approximately 5 X 10$^4$ salivary gland sporozoites were added to a well of a 48-well plate (Corning) and allowed to incubate in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and 500 units/ml and 500 µg/ml penicillin and streptomycin, respectively. These cultures were kept in a 37ºC incubator with 5% $CO_2$ for 24 hours before collection for transcript expression studies (Kappe *et al.*, 2010).

*2.2. Mosquito maintenance.*

*Anopheles stephensi* mosquitoes were used for all studies because they can maintain the life cycle of *P. falciparum*. Mosquitoes were reared using protocols available from the Malaria Research and Reference Reagent Resource Center (MR4). In brief, larvae and adults were maintained in an insectary with 78-85% humidity at approximately 80ºF on a 12 hour light/dark cycle. Larvae were fed both a mixture of 0.33 g yeast (Fleischmann's) and 0.66g micron (Sera) per 50 ml water and game fish chow (Purina). Adults were fed sucrose (0.3 M) *ad libitum*.

*2.3. Selection of candidate gene, PFE0565w.*

An *in silico* data mining procedure was used to select *PFE0565w* as a gene of interest. Briefly, the *Plasmodium* database, PlasmoDB, was utilized to search for *P. falicparum* proteins predicted to be expressed only by the sporozoite and contain a signal peptide, increasing its probability of being a surface and/or secreted protein (Bahl *et al.*, 2002; Kissinger *et al.*, 2002). This process narrowed gene numbers down to 34 candidate genes. Next, additional sequence analysis programs available on the ExPASy Bioinformatics Resource Portal (www.expasy.org) and SoftBerry (www.softberry.com), such as PSORT and ProtComp, were used to verify that the proteins encoded by the genes were predicted to either be located on the surface and/ or secreted by the parasite. Those proteins that were verified by these two programs to meet the required

criteria (and were not proteins that had been studied or were currently being studied) became our proteins of interest, including PFE0565w.

*2.4 PFE0565w sequence analysis.*

Using PlasmoDB, the full genomic DNA (gDNA), complimentary DNA (cDNA), and protein sequence of PFE0565w was obtained. A list of PFE0565w's orthologs was compiled using both PlasmoDB and the National Center for Biotechnology Information's (NCBI) BLAST analysis program (Altschul *et al.*, 1997). The ortholog sequences were then aligned using Vector NTI (Explorer or Contig Express, Invitrogen). Additional sequence information for PFE0565w was obtained by using software programs such as TargetP, SignalP, PSORT II, WoLF PSORT, and PROSITE, all found via the ExPASy Bioinformatics Resource Portal (www.expasy.org).

*2.5 Isolation of P. falciparum-infected tissues for transcript and protein expression studies.*

Oocyst sporozoites. *An. stephensi* were infected with *P. falciparum* as described previously. Ten days post-infection (PI), when oocyst sporozoites were mature using our laboratory conditions, 50 *An. stephensi* midguts were dissected from mosquito abdomens that had fed on a *P. falciparum*-infected blood meal. The tissues were placed in 50 µl 1X phosphate buffered saline (10X PBS, 0.2 M phosphate buffer and 1.5 M NaCl pH 7.0, diluted 1:10 with Millipore

water) in microcentrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80°C until needed for RNA isolation.

Salivary gland sporozoites. Fifty sets of *An. stephensi* salivary glands were dissected from mosquitoes that had fed on a *P. falciparum*-infected blood meal fourteen days PI because sporozoites reside in the glands at this time under our laboratory conditions. The tissues were put in 50 µl 1X PBS in microcentrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80°C until needed for RNA isolation.

Exo-erythrocytic stages. Primary human hepatocytes were prepared, maintained, and infected with *P. falciparum* sporozoites as previously described. Infected hepatocytes were collected at three days PI by digestion with trypsin-EDTA (0.25%, Invitrogen) at 37°C for five minutes. The cells were collected by centrifugation at 100 x g for five minutes, washed with 1X PBS, and used for RNA isolation via Trizol (Invitrogen). For axenic exoerythrocytic stages, Trizol was directly added to the cultures to begin the process of RNA isolation.

Mixed erythrocytic stages and gametocytes. *P. falciparum* cultures were maintained as previously described. Either mixed erythrocytic stage (ES) cultures (containing a mixture of rings, trophozoites, and schizonts) or 16 day old mixed gametocyte cultures (containing a mixture of stage I-V gametocytes, but with more mature forms present) were collected by centrifugation at 2,650 x g for five minutes. The infected RBCs were lysed with 0.05% saponin (Invitrogen) in complete culture medium for three minutes at room temperature (RT) and

parasites collected by centrifugation for five minutes at 2,650 x g. Purified parasites were then washed once with RPMI 1640 medium and collected again by centrifugation as previously described. The parasite pellets were stored at -80°C until needed for either RNA isolation or Western blot analysis.

*2.6 RNA/DNA isolation and transcriptional analysis by reverse transcription-polymerase chain reaction (RT-PCR).*

Total RNA was isolated from *Plasmodium*-infected tissues using a Trizol reagent-based protocol, following the manufacturer's instructions (Invitrogen). The samples were all DNase-treated (Promega), according to the manufacturer's instructions, to remove any contaminating gDNA. Approximately 2-3 µg of the DNase-treated RNA was used to synthesize cDNA using OligoDT primers from a SuperScript™ III First-Strand Synthesis System (Invitrogen), following the manufacturer's instructions.

Genomic DNA was isolated following the manufacturer's instructions using a DNeasy® Blood and Tissue Kit (Qiagen) and was used as a positive control for all RT-PCR experiments. *PFE0565w* full-length gene specific primers (5'-atgaagatgattaatattgg-3' forward and 5'-tcacataaattcctgttgaattttg-3' reverse) were used to amplify a 1,146 base pair (bp) DNA fragment in a polymerase chain reaction (PCR) using 2.0 µl gDNA (~100 ng total)/cDNA (~1/10 total volume synthesized from above), 1.25 units GoTaq® DNA Polymerase (Promega), 1X GoTaq® Flexi Buffer, 1 mM $MgCl_2$, 0.2 mM di-nucleotide tri-phosphate mix, and 0.5 µM primers. PCR conditions were as follows: an initial denaturing step of

95ºC for three minutes, 35 repetitive cycles of denaturing at 95°C for 30 seconds, primer annealing at 53°C for 30 seconds and an extension at 62°C for three minutes, and then a final extension at 62°C for 10 minutes (Su *et al.*, 1996). For RT-PCR positive controls used during the exoerythrocytic stages, primers specific for *P. falciparum liver stage antigen-1* (5'-aatctaacttgagaagtgg-3' forward and 5'-ctgcatcgtcatttattatg-3' reverse), *P. falciparum heat shock protein-70* (5'-aggtatagaaactgtgggtgg-3' forward and 5'-gattggttggcatacagcttc-3' reverse), and *human glyceraldehyde 3-phosphate dehydrogenase* (5'-accacagtccatgccatcac-3' forward and 5'-tccaccaccctgttgctgta-3' reverse) were used as described above. After PCR amplification, all samples were separated on a 1% agarose gel and stained with ethidium bromide (EtBr) for UV detection. The experiment with exoerythrocytic stages was done once, the experiments done with oocyst sporozoites were done in biological duplicates, and the experiments with salivary gland sporozoites, mixed ES, and mixed gametocytes were done in biological triplicates.

*2.7 Production of recombinant protein for antibody production and purification.*

Recombinant protein for PFE0565w was produced to use in generating polyclonal antibodies by cloning cDNA, representing the protein coding region, into the pET32a expression vector containing both thioredoxin and His-tags (Novagen). Due to the size of the PFE0565w protein, it was divided into halves with the first half designated as recombinant PFE0565wB, representing amino acids 25-212 (eliminating the hydrophobic signal peptide sequence), and the

second half designated as PFE0565wC, representing amino acids 187-381. The primers used to amplify PFE0565wB were 5'-gatcggatccagtgatacctttagagtatttg-3' forward and 5'-gatcctcgagtcacataaattcctcttgaattttg-3' reverse. The primers used to amplify PFE0565wC were 5'-gatcggatccaataagtatcttaacttttttactcg-3' forward and 5'-gatcctcgagttatttcataggcaccataaagg-3' reverse. The restriction enzymes, BamHI and XhoI (New England Biolabs) are underlined and were used for cloning into the pET32a vector. The mentioned primers were used to amplify products of the proper size via PCR using 2.0 µl DNA (~100 ng), 1.0 µl FastStart High Fidelity Taq Polymerase (5U/µl, Roche), 1X FastStart Buffer, 1 mM $MgCl_2$, 0.2 mM di-nucleotide tri-phosphate mix, and 0.5 µM primers. PCR conditions were as follows: an initial denaturing step of 95ºC for three minutes, 35 repetitive cycles of denaturing at 95ºC for 30 seconds, primer annealing at 56°C for 30 seconds, and an extension at 62°C for three minutes and 30 seconds, and then a final extension at 62°C for 10 minutes.

The products were double-digested with BamHI and XhoI, along with the pET32a vector, separated via gel electrophoresis, gel-purified according to the manufacturer's instructions using a QIAquick® Gel Extraction Kit (Qiagen), and ligated with T4 DNA Ligase (Promega) following the manufacturer's instructions. Two microliters of the ligation products were transformed into DH10B electrocompetant cells via electroporation and streaked on antibiotic resistant plates. Using colonies that grew on the plates, gDNA was isolated as previously described. The DNA was sequenced at the DNA Core Facility at the University of Missouri and aligned with the PFE0565w sequence available on PlasmoDB

using Vector NTI (Invitrogen) to confirm that the correct protein coding sequences were obtained. Once correct sequences/constructs were found, the DNA was transformed into Rosetta-gami DE3 pLysS electrocompetent cells (Novagen). The recombinant proteins were produced via induction with 1.0 mM IPTG and then purified using a nickel column (Novagen), utilizing the His-tag incorporated into the proteins. Because the purified PFE0565wB was insoluble, one milligram of protein was separated using 10% dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the gel stained with Coomassie blue, and the protein excised from the gel for injection into rabbits using a commercial company (Sigma). The purified PFE0565wC protein was soluble and supernatant containing one milligram of the protein was used for polyclonal antibody production in rabbits at a commercial company (Sigma). For each protein, rabbits were injected with a total of 0.7 mg of recombinant protein. The rabbits were administered a total of six injections containing the proteins over a period of 77 days, with the first injection containing 200 µg of protein in Complete Freund's Adjuvant and the subsequent injections containing 100 µg of protein in Incomplete Freund's Adjuvant. PFE0565wB and PFE0565wC preimmune sera were obtained from the rabbits before recombinant protein injection began.

*2.8. Purification of antibodies.*

Pre-absorption of antibodies. To minimize binding of the PFE0565w antibodies (immune and preimmune) to both mosquito tissues and pET32a bacterial lysate, a pre-absorption protocol was developed. Fifty pairs of salivary

glands or midguts were dissected from naïve *An. stephensi*, placed in 1X PBS (1.0 µl per pair of glands or midgut), and the tissues snap-frozen in liquid nitrogen and stored at -80°C until use. pET32a lysate was induced with IPTG, as previously described, and lysate stored at -20ºC until further use. Salivary glands and midguts were homogenized using a motorized homogenizer with a pestle and placed, along with 20 µl of the pET32a lysate, at 95°C for five minutes to denature the proteins. Ten microliters of each sample were separated via SDS-PAGE using a short, 10% mini-gel (approximately one inch long) and were then transferred to a nitrocellulose membrane (Lacrue *et al.*, 2005). The membranes were stained with Ponceau S (0.1% in 10% acetic acid) for one-two minutes to observe the proteins and mark the lanes of the gel for cutting into strips. The stain was removed by washing the membrane three times with 1X Tris-buffered saline (TBS: 50 mM Tris base and 150 mM NaCl, pH 7.4). For pre-absorption of the PFE0565w antibodies (including preimmune serum), a 1:2 dilution of the antibodies was made using 1X PBS. One strip was placed into a 2.0 ml microcentrifuge tube containing 1.0 ml of the antibodies, with the protein side facing inwards (making full contact with the antibodies). The tube was placed on a shaker and allowed to rotate at 4°C overnight. The next day, the strip was switched out for a fresh one and allowed to incubate an additional 30 minutes, shaking at 4°C. This step was repeated two more times, for a total of four strips each.

Purification of antibodies using pET32a-bound beads. To further eliminate non-specific binding of anti-PFE0565w antibodies (immune and and preimmune)

during experiments with the erythrocytic stages and gametocytes, an additional purification protocol was used. Briefly, the pET32a expression vector was induced using 1.0 mM IPTG and the protein lysed with urea buffer (8 M urea, 2 M thiourea, 1% CHAPS, and 20 mM DTT). The pET32a protein lysate was then bound to nickel beads (Novagen) following the manufacturer's instructions. The beads were washed twice with 10 ml of urea buffer and washed three times with 10 ml of 1X PBS. The beads were then aliquoted into 1.5 ml centrifuge tubes at a concentration of 50% beads in 1X PBS (200 µl each) and stored at 4°C until use. To purify preimmune and immune PFE0565w serum, a 1:5 dilution (1.0 ml total) of antibodies was made in 1X PBS and added to a tube of pET32a-bound beads, which then rotated for one hour at 4°C. After a brief centrifugation, the antibody supernatant was removed and placed into another aliquot of beads to rock one additional hour. Antibodies were then removed from the tube as described above and aliquoted into clean microcentrifuge tubes for storage at -20°C until use (50 µl each).

*2.9 Western blot.*

Recombinant protein verification. PFE0565wB or C recombinant proteins and induced pET32a lysate (positive control) were dissolved in either reducing sample buffer (0.5% bromophenol blue, 4% SDS, and 10% 2-β-mercaptoethanol) or non-reducing sample buffer (same as reducing buffer minus the 10% 2-β-mercaptoethanol) and separated via 10% SDS-PAGE. The proteins were then transferred to an Optitran nitrocellulose membrane (Schleicher and Schuell) at 15

volts for 50 minutes using a Transblot, SD-semi-dry transfer cell (BioRad). The membranes were incubated overnight at 4ºC in blocking solution (5% non-fat, dried milk in 1X TBS) to prevent non-specific binding of antibodies. The next day, the membranes were washed three times for 10 minutes each with 1X TBS and incubated with anti-polyhistidine primary antibody (Sigma, 1:500 dilution) for three hours at RT. The membranes were washed three times for 10 minutes each with 1X TBST (1X TBS with 0.05% Tween20) and then incubated in goat anti-mouse secondary antibody conjugated with horseradish peroxidase (Cell Signaling Technologies, 1:5,000 dilution) for two hours at RT. Following additional washes, an enhanced chemiluminescent detection method (GE Healthcare) was used, following the manufacturer's instructions, to visualize proteins of interest via autoradiography.

Erythrocytic stage expression studies. PFE0565wB recombinant protein (50 ng, positive control), *P. falciparum* mixed ES lysate and mixed gametocyte lysate (10 µl of a 5% parasitemia and 2% gametocytemia, respectively), and non-infected red blood cells (10 µl of 50% red blood cells, used as a negative control) were resuspended in reducing or non-reducing sample buffer and separated on a 10% gel via SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane as previously described. The membranes were incubated at RT for two hours in blocking solution (5% non-fat, dried milk in 1X TBS). The membranes were washed three times for 10 minutes with 1X TBS and incubated at 4°C overnight in either anti-PFE0565wB primary antibody or PFE0565wB preimmune serum (1:500 dilution of the antibodies purified with pET32a-bound

beads). The next day, the membranes were washed three times for 10 minutes with 1X TBST and then incubated in goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Cell Signaling Technologies, 1:10,000 dilution) for three hours at RT. The membranes were then washed and visualized as previously described. Following protein detection, the membranes were stripped in 0.1M NaOH for five minutes at RT, washed three times for 10 minutes with 1X TBS, and blocked at 4°C overnight (5% milk in 1X TBS). The membranes were washed as mentioned above and incubated for three hours in RT with either anti-MSP1-19 or anti-Pfs48/45 primary antibodies (obtained from the Malaria Research and Reference Reagent Resource Center, MR4, 1:1,000 dilution), used as a positive control to detect the presence of mixed erythrocytic stage parasites or gametocytes, respectively (Blackman *et al.*, 1991; Rener *et al.*, 1983). After washing, the membranes were incubated in goat anti-mouse secondary antibody conjugated with horseradish peroxidase (Cell Signaling Technologies, 1:5,000 dilution) for three hours at RT. The proteins of interest were detected as previously described. These experiments were done in biological triplicates.

*2.10. Parasite collection and fixation.*

Oocyst and salivary gland sporozoites. To obtain oocyst sporozoites, midguts were dissected from *P. falciparum*-infected *An. stephensi* abdomens ten days PI and placed on a slide in 1X PBS. A coverslip then was placed on top of the collected midguts and tapped to allow the mature sporozoites to be released

from the oocyst (LaCrue *et al.*, 2006). Using a finely drawn siliconized pipette, the sporozoites were collected and placed in a microcentrifuge tube. To obtain salivary gland sporozoites, salivary glands were dissected out of *P. falciparum*-infected *An. stephensi* 14 days PI and collected in a microcentrifuge tube containing 50 µl 1X PBS.

Both oocyst and salivary gland sporozoites were homogenized via pipetting and washed via a series of centrifugations at 18,000 x g for five minutes with 1X PBS rinses in between. Sporozoite numbers were determined by counting a portion of the population using a hemocytometer. The sporozoites were fixed in methanol-free 4% paraformaldehyde (EM Sciences) in 1X PBS containing 0.075% TritonX and spotted (6,000-10,000 sporozoites per spot) onto slides coated with 3-aminopropyl-triethoxysilane (Sigma) to increase sporozoite adherence to the slides and then allowed to air dry overnight (LaCrue *et al.*, 2006). Slides were either used immediately when dried or stored at -20ºC until further use.

Mixed erythrocytic stages and day 16 gametocytes. Using slides, blood smears of *P. falciparum* mixed ES parasites (smears containing a mixture of rings, trophozoites, and schizonts) or day 16 mixed gametocytes (smears containing a mixture of stage I-V gametocytes) were made. The slides were fixed in 100% methanol for 20 minutes at -20°C and in 100% acetone for five minutes at RT. Slides were either used immediately after drying or stored at -20ºC until further use.

*2.11. Immunofluorescence assays and confocal microscopy.*

Oocyst and salivary gland sporozoites. The fixed slides, described above, were washed three times for five minutes with 1X PBS and blocked in 1X PBS containing 10% goat serum and 2% bovine serum albumin (BSA) for four hours at RT to inhibit non-specific binding of antibodies. The sporozoites were washed another three times for five minutes with 1X PBS and incubated overnight at 4°C in either anti-PFE0565wB immune or preimmune antibodies (1:25 dilution using preabsorbed antibodies). The next day, the sporozoites were washed three times for five minutes with 1X PBS and incubated for four hours at RT with a second primary antibody, anti-circumsporozoite (CS) monoclonal antibody (deposited by Dr. John Adams to MR4, 1:100 dilution for a positive control). The sporozoites were again washed three times for five minutes in 1X PBS and incubated for four hours at RT with both Alexa-488 (goat anti-rabbit) and Alexa-546 (goat anti-mouse)-conjugated secondary antibodies (1:200 dilution, Invitrogen) and TO-PRO-3 (1:50 dilution, Invitrogen), a nuclear stain. The sporozoites were washed a final time for five minutes in 1X PBS and 20 µl of Prolong Gold (Invitrogen) was added to the slide and a coverslip placed on top to help preserve fluorescence. To visualize the results, either a Radiance 2000 confocal system (BioRad) coupled to an Olympus IX70 inverted microscope or a Zeiss LSM 510 Meta NLO confocal microscope was utilized. The experiments analyzing oocyst sporozoites were done in biological duplicates and the experiments analyzing salivary gland sporozoites were done in biological triplicates.

Mixed erythrocytic stages and day 16 gametocytes. The fixed slides, mentioned above, were washed and blocked as previously described for the sporozoite stages. They were then incubated overnight at 4°C with either anti-PFE0565wB immune or preimmune antibodies (1:50 dilution using antibodies purified with pET32a-bound beads). The next day, the slides were washed as described above and incubated for four hours at RT with a second primary antibody, anti-MSP1-19 (for mixed ES) or anti-Pfs230 (for gametocytes) antibodies (MR4, 1:100 dilution for a positive control) (Blackman *et al.*, 1991; Quakyi *et al.*, 1987). The slides were washed and incubated with secondary antibodies as previously described. A nuclear stain, DAPI (Invitrogen), was then added to the slides for five minutes at RT. The slides were washed a final time and visualized as mentioned above for the sporozoite stages. The experiments analyzing mixed ES and mixed gametocytes were each done in biological triplicates.

*2.12. Creation of GFP-trafficking, gene disruption, and gene deletion constructs.*

GFP-trafficking construct. To analyze the protein expression pattern of PFE0565w throughout the entire life cycle of the parasite and verify other data obtained, a PFE0565w GFP-trafficking construct was made by cloning base pairs 253-1143 (excluding the stop codon) of the open reading frame of the gene into the pPM2GT vector (obtained from MR4) (Klemba *et al.*, 2004). The primers used to amplify the region were 5'-ccgctcgaggataatatcataaccagaaag-3' forward and 5'-ccgcctaggcataaattcctcttgaatttg-3' reverse. The restriction enzymes, XhoI

and AvrII (New England Biolabs) are underlined and were used for cloning into the pPM2GT vector. These primers were used to amplify an 890 base pair product via PCR using 2.0 µl DNA (~100 ng total), 1.0 µl FastStart High Fidelity Taq Polymerase (5U/µl, Roche), 1X FastStart Buffer, 1 mM $MgCl_2$, 0.2 mM di-nucleotide tri-phosphate mix, and 0.5 µM primers. PCR conditions were as follows: an initial denaturing step of 95ºC for three minutes, 35 repetitive cycles of denaturing at 95ºC for 30 seconds, primer annealing at 54°C for 30 seconds, and an extension at 62°C for three minutes, and then a final extension at 62°C for 10 minutes. The product and vector were double-digested with XhoI and AvrII. Purification, ligation, transformation and sequencing of the trafficking construct were done as previously described in section 2.7.

Gene disruption construct. To assess a potential function for PFE0565w, a gene disruption construct was produced by cloning base pairs 68-800 of the open reading frame of the gene into the pHD22y *P. falciparum* disruption plasmid (obtained from MR4) (Fidock & Wellems, 1997). The primers used to amplify the region were 5'-gatcggatcctatggtagtgatacctttagagt-3' forward and 5'-gatcctcgagtccttctacttctccttctc-3' reverse. The restriction enzymes BamHI and NotI (New England Biolabs) are underlined and were used for cloning into the pHD22y vector. These primers were used to amplify a 732 base pair product via PCR using 2.0 µl DNA (~100 ng total), 1.0 µl FastStart High Fidelity Taq Polymerase (5U/µl, Roche), 1X FastStart Buffer, 1 mM $MgCl_2$, 0.2 mM di-nucleotide tri-phosphate mix, and 0.5 µM primers. PCR conditions were as follows: an initial denaturing step of 95ºC for three minutes, 35 repetitive cycles of

denaturing for 30 seconds at 95ºC, primer annealing at 56°C for 30 seconds, and an extension at 62°C for three minutes, and then a final extension at 62°C for 10 minutes. The product and vector were double-digested with BamHI and NotI. Purification, ligation, transformation and sequencing of the final disruption construct were done as previously described in section 2.7.

Gene deletion constructs. To assess a potential function of PFE0565w, two gene deletion constructs were produced by cloning 629 and 616 base pairs of the 5' and 3' untranslated regions (UTRs) and open reading frames of the gene into both the pHHT-TK (obtained from MR4) and pCC-1 (a gift from D.A. Fidock) *P. falciparum* deletion vectors (Duraisingh *et al.*, 2002; Maier *et al.*, 2006). The primers used to amplify the 5' region were 5'-catgccatggcataatctcgcccacatc-3' forward and 5'-ccggaattccatcaaaatcttcaaatactc-3' reverse. The primers used to amplify the 3' region were 5'-ggactagtggacaagatgaaaacactg-3' forward and 5'-tcccgcggccacctttaagaaacaatc-3' reverse. The restriction enzymes, NcoI and EcoRI (New England Biolabs) are underlined and were used for cloning the 5' region of PFE0565w into both deletion vectors. The restriction enzymes, SpeI and SacII (New England Biolabs) are underlined and were used for cloning the 3' region of PFE0565w into both deletion vectors. These primers were used to amplify the 629 base pair and 616 base pair products via using 2.0 µl DNA (~100 ng total), 1.0 µl FastStart High Fidelity Taq Polymerase (5U/µl, Roche), 1X FastStart Buffer, 1 mM $MgCl_2$, 0.2 mM di-nucleotide tri-phosphate mix, and 0.5 µM primers. PCR conditions were as follows: an initial denaturing step of 95ºC for three minutes, 35 repetitive

cycles of denaturing at 95ºC for 30 seconds, primer annealing at 52°C for 30 seconds, and an extension at 62°C for three minutes, and then a final extension at 62°C for 10 minutes. The products and vectors were double-digested with the appropriate restriction enzymes mentioned above and the final gene deletion constructs were created as described in section 2.7.

*2.13. Transfection of parasites with trafficking, disruption, and deletion constructs.*

Transfections of *P. falciparum* were carried out according to Crabb *et al*., 2004. Before performing the transfections, mixed ES parasite cultures were synchronized with 5% D-sorbitol (Sigma) for 10 minutes followed by two washes with RPMI 1640 (Gibco) at 1,600 x g for five minutes two days before transfection. In addition, plasmid DNA was isolated using a Plasmid Maxi Kit (Qiagen) and equilibrated in CytoMix (120 mM KCl, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM $K_2HPO_4$ pH 7.6, and 25 mM HEPES pH 7.6). The synchronized *P. falciparum* ring stage NF54 parasites were electroporated (BTX 600, BTX Harvard Apparatus; 0.2 cm cuvette, 0.31 kV, 950 µF, maximum resistance) with 50 µg of the plasmid DNA in cytomix. Transfected *P. falciparum* cultures were maintained as previously described.

Two days following electroporation, media containing WR99210 (2.5 nM, Sigma) was added to the cultures to begin the process of selecting transfected parasites utilizing the human dihydrofolate reductase gene drug cassette present in all constructs used (Crabb *et al.*, 2004). To enrich for GFP-trafficking and

gene disruption recombinants (PFE0565w/GFP and PFE0565w/pHD22y) and eliminate episomal plasmids, parasites were subjected to at least three rounds of drug selection (three weeks on drug and three weeks off drug for each round). For gene deletion constructs (PFE0565w/pHHT-TK and PFE0565w/pCC-1), parasites were first positively selected with WR99210 (2.5 nM) until parasites were detected (roughly five weeks post-transfection) and then placed under negative selection (WR99210 is still used as well) with ganciclovir (4 µM Sigma, utilizing the thymidine kinase drug cassette) or 5-fluorocytosine (1 µM, gift from D.A. Fidock, utilizing the cytosine deaminase drug cassette), respectively (Crabb *et al.*, 2004; Duraisingh *et al.*, 2002; Maier *et al.*, 2006).

To obtain a clonal population of parasites with no presence of wild-type parasites carrying episomes, a limiting dilution was performed on the transfected parasites. Parasites were seeded in 96-well plates (200 µl volume) at two concentrations, 0.5 and 0.25 infected red blood cells per well, and maintained in a gassed modular incubator chamber (Billups-Rothenberg, Inc., 3% $O_2$, 3% $CO_2$, and 94% $N_2$) at 37°C. Cultures were gassed every other day for 20 days. On days 7, 14, and 17, 0.4% fresh red blood cells were added. On day 20, 150 µl of the parasite cultures were transferred to a 96-well plate to begin gDNA isolation for use in PCR and Southern blot analysis to determine if clonal populations of parasites had been successfully created. To isolate the gDNA, 50 µl of 6% saponin (Sigma) was added to the 150 µl of cultures in the 96-well plate and incubated for five minutes at RT. The plate was centrifuged for 15 minutes at 2,650 x g and supernatant removed. One hundred microliters of 1X PBS was

added to each well to wash the parasites and the plate was centrifuged as previously described. The 1X PBS was removed and 40 µl of down scale prep buffer (DSP, 1M Tris-Cl pH 8.0, 1M KCl, and 1M $MgCl_2$) working stock (985 µl DSP stock, 10 µl proteinase K, and 5 µl Tween 20) was added to each well and parasite pellets resuspended in the DSP solution (Adjalley *et al.*, 2010). The plate was incubated for 30 minutes at 50ºC and then for 10 minutes at 95ºC. The resulting gDNA was stored at 4ºC until further use. The remaining 50 µl of parasites were used for expansion and cryopreservation of promising clonal parasite populations.

*2.14. PCR and Southern blot verification of GFP-trafficking and disruption constructs.*

Integration of the transfected DNA at the correct location was verified for each PFE0565w/GFP and PFE0565w/pHD22y clone by PCR and Southern blot analysis. To verify integration at the *PFE0565w* locus by PCR, the primers 5'-atgaagatgattaatattgg-3' *PFE0565w* gene specific forward primer and 5'-tccgtatgttgcatcacc-3' GFP reverse primer were used for the GFP-trafficking construct and the primers 5'-atgaagatgattaatattgg-3' *PFE0565w* gene specific forward primer and 5'-ggaaacagctatgaccatg-3' pHD22y vector reverse primer were used for the pHD22y disruption construct. In both cases, 4.0 µl gDNA (isolated from the 96-well plate described above), 1.25 units GoTaq® DNA Polymerase (Promega), 1X GoTaq® Flexi Buffer, 1 mM $MgCl_2$, 0.2 mM di-nucleotide tri-phosphate mix, and 0.5 µM primers were used. PCR conditions for

both were as follows: an initial denaturing step of 95ºC for three minutes, 35 repetitive cycles of denaturing at 95ºC for 30 seconds, primer annealing at 52°C for 30 seconds and an extension at 62°C for three minutes and 30 seconds, and then a final extension at 62°C for 10 minutes. The samples were all separated via gel electrophoresis (1% gel) and visualized via UV detection using EtBr.

Southern blotting was performed with gDNA isolated as previously described from ES parasites and the DIG Nonradioactive Nucleic Acid Labeling technology (Roche) was used for visualization of the DNA. For the GFP-trafficking constructs, 2-3 µg of gDNA digested with BamHI and BssI were hybridized with an 891-bp fragment of *PFE0565w* created with the PCR DIG Probe Synthesis Kit following the manufacturer's instructions (Roche). For the pHD22y disruption constructs, 2-3 ug of gDNA digested with EcoRI and SacII were hybridized with a 732-bp fragment of *PFE0565w* created as previously described. Before hybridization, the DNA was separated on a 0.5% agarose gel and transferred to a positively charged nylon membrane (Osmonics) overnight via an upward transfer method. Following the manufacturer's instructions, the membrane was washed, hybridized with the above probes, and DNA products detected by autoradiography using the DIG Nucleic Acid Detection Kit (Roche).

*2.15. Preliminary PCR verification of parasites transfected with the PFE0565w/pHHT-TK gene deletion construct.*

PCR was used to determine if integration of the PFE0565w/pHHT-TK construct had occurred in the appropriate location after drug selection, but before

limiting dilution. To verify integration at the 5' region of the *PFE0565w* locus by PCR, the primers 5'-caaaaacgagacatgcatgc-3' *PFE0565w* chromosome specific forward and 5'-ataaagtacaacattaatatatagc-3' pHHT-TK vector reverse were used. To confirm integration at the 3' region of the *PFE0565w* locus, the primers 5'-ataataaattcaaatttgttttcc-3' *PFE0565w* chromosome specific reverse and 5'-caaaatggttaacaaagaagaag-3' pHHT-TK vector forward were utilized. For the PCR reaction, 2.0 µl gDNA (~100 ng total), 1.25 units GoTaq® DNA Polymerase (Promega), 1X GoTaq® Flexi Buffer, 1 mM $MgCl_2$, 0.2 mM di-nucleotide tri-phosphate mix, and 0.5 µM primers were used. PCR conditions for both were as follows: an initial denaturing step of 95ºC for three minutes, 35 repetitive cycles of denaturing at 95ºC for 30 seconds, primer annealing at 52°C for 30 seconds and an extension at 62°C for three minutes and 30 seconds, and then a final extension at 62°C for 10 minutes. The samples were all separated via gel electrophoresis (1% gel) and visualized via UV detection using EtBr.

*2.16. GFP-trafficking studies.*

The GFP-trafficking studies described below were done in biological duplicates, using two independent PFE0565w/GFP clones obtained via the limiting dilution process previously described.

Mixed erythrocytic stages and gametocytes. Both *P. falciparum* mixed ES cultures and day 16 mixed gametocyte cultures were obtained by collecting 200 µl of infected blood from culture flasks. This protocol was done for all experimental groups: PFE0565w/GFP, NF54 WT negative control parasites, and

3D7HT-GFP (obtained from MR4) positive control parasites (Talman *et al.*, 2010). The collected, infected blood was centrifuged for five minutes at 2,650 x g and the supernatant removed. The infected RBCs (iRBCs) were resuspended in 200 µl 1X PBS containing DAPI nuclear stain (1:1,000 dilution, Invitrogen) and incubated in the dark for five minutes at RT. The iRBCs were centrifuged again for five minutes at 2,650 x g, washed once with 200 µl 1X PBS, centrifuged a final time for five minutes at 2,650 x g, and a small drop of the blood was placed on a slide. Coverslips were placed on the slides and they were viewed with a 100X objective using an Olympus BX51 inverted fluorescent microscope coupled with a X-Cite® Series 120 fluorescent light source. The entire slide was scanned, with at least 100 iRBCs and 50 gametocytes observed for each group.

Zygotes and ookinetes. For all experimental groups listed above, six midguts were dissected from *P. falciparum*-infected *An. stephensi* 24-30 hours PI. The midguts were placed in 1X PBS containing DAPI nuclear stain (1:1,000 dilution) and incubated at RT for five minutes. The midguts were then placed (three each) into 15 µl of Matrigel™ (BD Biosciences) and coverslips placed on top. The infected midguts were then viewed as previously described. Due to limited numbers, at least five zygotes and three ookinetes were observed for each group.

Oocyst sporozoites. For all experimental groups listed above, six midguts were dissected from *P. falciparum*-infected *An. stephensi* 10 days PI. The midguts were placed in 1X PBS containing DAPI nuclear stain (1:1,000 dilution)

and incubated at RT for five minutes. Three midguts were then placed into 15 µl of 1X PBS on a slide and coverslips placed on the slides. The infected midguts were then viewed as previously described. At least half of the midguts had infections with 1-7 oocysts per midgut for each group.

Hemolymph sporozoites. Hemolymph sporozoites were collected by perfusing the body cavity of 10 *P. falciparum*-infected *An. stephensi* 12 days PI with 1X PBS. Hemolymph was collected for all experimental groups described above by collecting it in microcentrifuge tubes containing 40 µl 1XPBS with DAPI nuclear stain (1:1,000 dilution). The sporozoites were concentrated by centrifugation at 18,000 x g for five minutes, supernatant removed, and 10 µl of sporozoites spotted on slides containing 10 µl of Matrigel™. Coverslips were placed on top of the slides and they were viewed as previously described. Due to the difficulty of isolating hemolymph sporozoites from *An. stephensi*, 2-5 hemolymph sporozoites were observed for each group.

Salivary gland sporozoites. For all three experimental groups, six pairs of salivary glands were dissected from *P.falciparum*-infected *An. stephensi* 13-20 days PI. The glands were placed in 1X PBS containing DAPI nuclear stain (1:1,000 dilution) and incubated at RT for five minutes. The glands were then placed into 15 µl of 1X PBS on a slide and coverslips placed on top. The infected salivary glands were then viewed as previously described. For each experimental condition, at least 50% of the salivary glands were infected with hundreds of sporozoites observed per set of infected glands.

## 3. Results and Discussion

### *3.1. PFE0565w sequence analysis.*

*PFE0565w* is a 1,146 base pair gene (containing no intron) located on chromosome five that encodes a 381 amino acid *P. falciparum* protein with an estimated molecular weight of 45,583 Daltons (Table 2.1). Initial PlasmoDB data, based upon mass spectrometry results and sequence analysis, suggested that the PFE0565w protein was expressed in salivary gland sporozoites, has a signal peptide, and one transmembrane domain (Bahl *et al.*, 2003; Florens *et al.*, 2002). To confirm these data and obtain more information about PFE0565w, additional sequence analysis programs were utilized. SignalP revealed that PFE0565w is predicted to have a cleavable signal peptide from amino acids 1-25 (Bendtsen *et al.*, 2004). Analysis using TargetP predicted that the protein enters the secretory pathway and, more specifically, is either predicted to be a membrane protein located on the surface of the parasite (plasma membrane) or in the endoplasmic reticulum (ER) according to both PSORTII and WoLF PSORT (Emanuelsson *et al.*, 2000; Nakai & Horton, 1999). Additional sequence analysis using PROSITE, PROTCOMP, Profam, and NCBI (BLASTp) sites predicted the protein has no GPI-anchor, has multiple glycosylation and phosphorylation sites and has no functional identity with other known proteins (Gattiker *et al.*, 2002).

Next, PlasmoDB indicated that PFE0565w has orthologs in other *Plasmodium* species. The PFE0565w protein has homology with proteins in *P. vivax* (Pv = PVX_080665 in PlasmoDB), *P. knowlesi* (Pk = PKH_102160 in

PlasmoDB), *P. berghei* (Pb = PBANKA_111090 in PlasmoDB), *P. yoelii* (Py = PY00913 in PlasmoDB), and *P. chabaudi* (Pc = PCHAS_111060 in PlasmoDB). Using a BLAST analysis, the PFE0565w protein does not appear to have any orthologs with other proteins from members outside of the genus *Plasmodium* (Figure 2.2) (Altschul *et al.*, 1997). No orthologs were found in *P. gallinaceum* or *P. reichenowi*, which could be due to the low coverage of the genome sequencing for these two *Plasmodium* species. According to the Sanger Institute, *P. gallinacium* sequencing stands at a three-fold coverage and sequenceing for *P. reichenowi* is preliminary, with host DNA contamination still present (www.sanger.ac.uk/). Even though the proteins listed above are considered orthologs of PFE0565w, their sequences do not have high homology with PFE0565w (ranging from 15-18.1% identity). Like PFE0565w, the proteins listed here are all predicted to have signal peptides and enter the secretory pathway, but not all are predicted to be surface proteins or located in the ER; some are predicted to be located within the nucleus as well. In addition, the proteins do not have any known function and/or identity with known proteins with the exception of PY00913, which is predicted to be a CCAAT-box DNA binding protein subunit B protein according to data available on PlasmoDB. Therefore, since PFE0565w does not have homology with any known human protein, it could be a good candidate gene for a new drug and/or vaccine.

*3.2. Production of PFE0565w recombinant protein and antibody production.*

Recombinant protein for PFE0565w was produced to use in generating polyclonal antibodies by cloning cDNA, representing the protein coding region,

into the pET32a expression vector (Novagen). Due to the size of the protein, it was divided into halves with the first half designated as recombinant PFE0565wB, representing amino acids 25-212, and the second half designated as PFE0565wC, representing amino acids 187-381. The PFE0565wB protein and subsequent antibodies directed against it were previously produced by technical staff in the lab and verified by Western blot analysis to confirm they recognize PFE0565wB and to determine the concentration needed for subsequent experiments (Figure 2.3A). The purified PFE0565wC protein was soluble and supernatant containing it was sent to a commercial source (Sigma) for polyclonal antibody production in a rabbit. The resulting antibodies also were used in a Western blot to confirm that they recognized the bacterially-expressed PFE0565wC and also to determine the primary antibody concentration to use in further experiments (Figure 2.3B).

*3.3. PFE0565w transcript is present in both mixed erythrocytic stages (ES) and mixed gametocyte stages, but its protein is not produced by either stage.*

*PFE0565w* transcript is present in both mixed ES (culture containing a mixture of rings, trophozoites, and schizonts) and mixed gametocyte stages (culture containing a mixture of stage I-V gametocytes), as demonstrated via RT-PCR (1,146 bp product - Figure 2.4). The gDNA positive control amplified a product and the negative control (no reverse transcriptase) did not. Transcript expression results for *PFE0565w* obtained during the mixed erythrocytic stages and mixed gametocyte stages are different than data available on PlasmoDB and

found while conducting literature searches for *PFE0565w* and its predicted rodent malaria orthologs in both *P. berghei* and *P. yoelii* (Table 2.2). These data demonstrate that its transcript is not present during these stages (free merozoites, rings, trophozoites, schizonts, and gametocytes) (Bozdech *et al.*, 2003; Le Roch *et al.*, 2003; Young *et al.*, 2005). The difference in data obtained from these studies and what is found in the literature may be the result of different experimental techniques used to generate results. All of the data from the literature came from oligonucleotide microarrays and these data were obtained via RT-PCR, where RNA was isolated from large numbers of parasites and used for detecting one gene of interest. If *PFE0565w* is present at low levels compared to other genes, it may be undetectable using a microarray approach.

Another interesting discovery concerning the transcript presence of *PFE0565w* during the erythrocytic stages is that a ~450 base pair alternative transcript (see asterisks on Figure 2.4) is produced during the ES and during the gametocyte stage, where only this alternative transcript is detected. To confirm that this RT-PCR product was *PFE0565w*, gene specific primers were used for sequencing the RT-PCR product generated in the PCR analysis. DNA sequencing using PFE0565w full length gene specific primers confirmed it was a 448 base pair *PFE0565w* product, with base pairs 291-988 missing from the sequence (Figure 2.5A). Based on the obtained sequence data, this transcript would not be capable of producing a full-length protein, as *in silico* translation of the sequence using the Translate program from the ExPASy Bioinformatics Resource Portal (www.expasy.org) demonstrates that the truncated transcript

does not produce a full protein (Figure 2.5B). Published data support the hypothesis that this truncated transcript could represent the product of an uncharacterized antisense RNA that the parasite produces. As the transcriptome of *P. falciparum* continues to be studied, new and/or alternative splice junctions are being discovered and some of these appear to allow for the splicing of antisense transcripts, which have been shown to make up 12% of the transcriptome and that the parasite may need for either transcriptional or translational regulation (Gunasekera *et al.*, 2004; Hughes *et al.*, 2010; Lu *et al.*, 2007; Sorber *et al.*, 2011). For example, Sorber *et al.* (2011) used an in-house developed splice site detection algorithm (HMMSplicer) and found a total of 982 new splice junctions absent from current *Plasmodium* models. They also found 310 alternative splicing events that occurred in 254 genes during the erythrocytic stages. Many of these alternative splicing events resulted in an antisense transcript that had a truncated open reading frame, just as was discovered for *PFE0565w* (Sorber et al., 2011). Future studies will need to be conducted to determine if the 449 base pair *PFE0565w* transcript is an antisense RNA product.

Despite the presence of *PFE0565w* transcript, Western blot analysis demonstrated that the PFE0565w protein (~43 kDa without a signal peptide) is not present in either mixed ES or mixed gametocytes (Figure 2.6A). Anti-MSP1-19 (19 kDa) or anti-Pfs48/45 (45 kDa) antibodies were used as a positive control to show that parasite protein was present in mixed ES and mixed gametocytes,

respectively. PFE0565w preimmune serum was used as a negative control with no proteins detected in any sample.

Results obtained via Western blot were confirmed using immunofluorescent assays. The PFE0565w protein was not detected during mixed ES (11 / 945 = 1.1%) or gametocyte stages (8 / 604 = 1.3%, parasites would appear green, Figure 2.6B and Table 2.3). Overall, hundreds of parasites were observed (from three biological replicates) on an individual basis, examining multiple parasite stages (rings, trophozoites, schizonts, and stage I-V gametocytes). The labeling of the few parasites which appeared to express PFE0565w was most likely the result of non-specific binding of the anti-PFE0565w antibodies. PfMSP1-19 and Pfs230 (used as positive controls for mixed ES and gametocytes, respectively) are shown in red, and were expressed by all parasites observed. DAPI was used as a nuclear stain and appears as blue. PFE0565w preimmune serum was used as a negative control and no protein labeling occurred. Using data available from PlasmoDB and the literature search described above (Table 2.2), proteomics data acquired during these studies confirm previous findings for PFE0565w, that the protein is not present during erythrocytic stages of the parasite life cycle (Florens *et al.*, 2002; Hall *et al.*, 2005; Khan *et al.*, 2005; Lasonder *et al.*, 2002).

*3.4. PFE0565w transcript is present in both oocyst and salivary gland sporozoites, but the protein is only produced by salivary gland sporozoites.*

*PFE0565w* transcript is present in both oocyst (Figure 2.7A) and salivary gland (Figure 2.7B) sporozoite stages, as demonstrated via RT-PCR (1,146 bp transcript). In both stages, the gDNA positive control amplified a product and the negative control (no reverse transcriptase, no RT) did not. According to PlasmoDB and transcript expression studies for *PFE0565w* in the literature, these data confirm transcript expression by salivary gland sporozoites. The expression of *PFE0565w* transcript in oocyst sporozoites has not been described elsewhere and represents new knowledge about the *P. falciparum* transcriptome.

Even though transcript is present in oocyst and salivary gland sporozoites, the PFE0565w protein (visualized in green) is not present during the oocyst sporozoite stage (0 / 213 = 0.0%); however, it is present during the salivary gland sporozoite stage as demonstrated by immunofluorescent assays coupled with confocal microscopy (507 / 516 = 98.2%, Figure 2.8 and Table 2.4). The CS protein (visualized in red), which is a sporozoite surface protein, was used as a positive control for both oocyst and salivary gland sporozoites and was detected by all parasites (213 / 213 = 100% for oocyst sporozoites and 516 / 516 = 100% for salivary gland sporozoites). A merged image is displayed and co-localization of PFE0565w and the CS protein appears as yellow during the salivary gland sporozoite stage, indicating potential surface localization of PFE0565w during the salivary gland sporozoite stage. Preimmune serum was used as a negative control and no protein was detected. Using data available from PlasmoDB and the literature described above (Table 2.2), proteomics data obtained during these

studies confirm previous findings for PFE0565w: that the protein is present during the salivary gland sporozoite stage (Florens *et al.*, 2002; Hall *et al.*, 2005).

*3.5. Preliminary data reveal that PFE0565w transcript is not present during exoerythrocytic stages, suggesting that the PFE0565w protein is not present during exoerythrocytic stages.*

To determine if *PFE0565w* transcript is present during exoerythrocytic (liver) stages of the life cycle, *P. falciparum* liver cell invasion studies were originally attempted using the human HC-04 hepatoctye line (obtained via MR4) (Sattabongkot *et al.*, 2006); however, this cell line was eventually recalled by MR4 because it had been found to actually be a derivative of HepG2 cells, which do not support *P. falciparum* infections well. Consequently, primary human hepatocytes were utilized (Lonza). Unfortunately, when assessing if *PFE0565w* transcript was present during this stage of the life cycle, not only did *PFE0565w* primers not amplify a product, but the positive control (the *circumsporozoite protein* gene) did not amplify a RT-PCR product either. Therefore, additional parasite control primers, the *P. falciparum heat shock protein-70* (*PfHsp70*) gene and *P. falciparum liver stage antigen-1* (*PfLSA*-1) gene, were used because these genes are known to be expressed during exoerythrocytic stages, but not sporozoite stages of the *Plasmodium* life cycle. The absence of RT-PCR products representing the liver stage parasites suggested that no exoerythrocytic stage parasites were produced, even though the human hepatocytes were viable as shown by the presence of the *human glyceraldehyde 3-phosphate*

*dehydrogenase* gene transcript (Figure 2.9). Currently, more liver stage invasion experiments are in progress to try and generate exoerythrocytic stage parasites for transcriptional and protein analysis.

Since generating exoerythrocytic stage parasites using primary human hepatocytes was not successful, a method to produce exoerythrocytic stages using axenic cultures (production of liver stage parasites without the presence of liver cells) also was utilized (Kappe *et al.*, 2010). Preliminary data using axenic exoerythrocytic stages and RT-PCR suggest that *PFE0565w* transcript is not present during exoerythrocytic stages (Figure 2.10). However, a transcript for the *PfHsp70* gene, used as a positive control, was detected, suggesting that exoerythrocytic stage parasites were indeed produced by the axenic cultures. For both *PFE0565w* and *PfHsp70*, gDNA was used as a positive control and a no reverse transcriptase reaction was used as a negative control. Exoerythrocytic transcript expression data in the literature for *PFE0565w* (and its predicted rodent orthologs in *P. berghei* and *P. yoelii*) have conflicting results. Some literature found expression during these stages, while others did not (Table 2.2) (Siau *et al.*, 2008; Tarun *et al.*, 2008; Williams & Azad, 2010). Additional data will have to be obtained to confirm which results are accurate.

Due to the difficulty of generating liver stage parasites *in vitro*, protein expression studies were not able to be conducted for PFE0565w during this particular life cycle stage. Nevertheless, based on initial data suggesting transcript is not present during the exoerythrocytic stages, it is predicted that the PFE0565w protein would not be present during the liver stages.

*3.6. GFP-trafficking studies confirm that the PFE0565w protein is only present during the salivary gland sporozoite stage.*

To assess the PFE0565w protein expression profile throughout the entire life cycle of the parasite and confirm data found in the literature, a PFE0565w/GFP trafficking construct was created using the pPM2GT vector (obtained from MR4) and was transfected into the genome of the parasite via a homologous recombination technology (Figure 2.11A) (Klemba *et al.*, 2004). After a limiting dilution, two PFE0565w/GFP clones were isolated after initial PCR analysis (Figure 2.11B) confirming that they had integrated into the genome of the parasite and that these parasite populations lacked expression of WT parasites carrying episomes. These data were confirmed by Southern blot analysis (Figure 2.11C), as the predicted integration products of 12,134 base pairs and 9,762 base pairs were detected along with no presence of WT parasites carrying episomes. Using the two PFE0565w/GFP clones, two independent GFP-trafficking studies were completed. WT parasites were used as a negative control and 3D7HT-GFP parasites (obtained from MR4) constitutively expressing GFP throughout the life cycle of the parasite were used as a positive control (Talman *et al.*, 2010). The various developmental stages of the parasite were examined using fluorescent microscopy (Figure 2.12). The stages observed were: mixed ES (contained a mixture of rings, trophozoites, and schizonts), mixed gametocytes (induced for 16 days and contained a mixture of stages I-V male and female gametocytes), zygotes (24 hours post infection, PI), ookinetes (24-30 hours PI), oocyst sporozoites (8-10 days PI), hemolymph

sporozoites (12 days PI), and salivary gland sporozoites (13-20 days PI). The PFE0565w protein was only detected in salivary gland sporozoites (200 / 200 = 100% expression by salivary gland sporozoites counted, with hundreds of additional parasites observed as well). For all experiments, all of the 3D7HT-GFP parasites were positive for GFP expression and all of the WT parasites were negative (the parasites did not express GFP). These data confirm previous results obtained via Western blot analysis and by immunofluorescent assays for the mixed erythrocytic stages, mixed gametocyte stages, oocyst sporozoites, and salivary gland sporozoites that the PFE0565w protein is present only during the salivary gland sporozoite stage. In addition, these data confirm previous mass spectrometry results found Florens *et al*. (2002) that the PFE0565w protein is only expressed by salivary gland sporozoites (Table 2.2) and now provides additional protein expression information (*i.e.* no expression observed) for *P. falciparum* stages not previously studied, such as the zygote, ookinetes, and hemolymph sporozoite (Florens *et al.*, 2002; Hall *et al.*, 2005; Khan *et al.*, 2005; Lasonder *et al.*, 2002; Tarun *et al.*, 2008).

*3.7. A PFE0565w gene disruption construct was created for functional analysis.*

In an attempt to assess the potential function of PFE0565w, gene disruption techniques were employed using the pHD22y vector (Figure 2.13A) (Fidock & Wellems, 1997). After a limiting dilution, one PFE0565w/pHD22y clone was isolated after initial PCR analysis (Figure 2.13B) confirmed that it had properly integrated into the genome of the parasite and the population lacked the

presence of WT parasites carrying episomes. These data were confirmed by Southern blot analysis (Figure 2.13C), as the predicted integration products of 9,210 base pairs and 5,987 base pairs were detected along with no presence of WT parasites with episomes. Before gametocyte induction, the PFE0565w/pHD22y mixed erythrocytic stage cultures propagated well and appeared to be identical to WT parasite cultures. However, when trying to induce gametocyte formation with the PFE0565w/pHD22y parasites for infection of the *Anopheles* host, the cultures never produced gametocytes. Instead, these cultures became over-populated with other erythrocytic stages and the parasites died; this occurred during three different attempts. Since the PFE0565w protein is not expressed by mixed erythrocytic stage parasites or gametocytes, it is hypothesized that the PFE0565w protein is not essential during this stage of the life cycle. One explanation for the lack of gametocyte development in the mutant parasites is that the parasites were maintained in culture too long (six months) before a disruption clone was able to be obtained and the parasites lost their ability to produce gametocytes, which has been previously documented (Schuster, 2002; Trager & Jensen, 1997).

*3.8. Creation of a PFE0565w gene deletion parasite population is in progress.*

Because of the issues with the gene disruption construct for *PFE0565w* described above, creation of a *PFE0565w* gene deletion population is in progress. Using the pHHT-TK deletion vector (obtained from MR4) and double cross-over homologous recombination technology (Figure 2.14A), transfection of

the PFE0565w/pHHT-TK construct into the genome of the parasite was done (Duraisingh *et al.*, 2002). PCR analysis was used to determine if the PFE0565w/pHHT-TK deletion construct had integrated at both the 5' and 3' ends of the gene before limiting dilution was done (Figure 2.14B). Results demonstrate that the construct has integrated into the genome of the parasite at both the 5' untranslated region of the *PFE0565w* locus and 3' untranslated region of *PFE0565w* locus. The presence of wild-type parasites carrying episomes was also detected. These PFE0565w/pHHT-TK parasites have only been in continuous culture for nine weeks, compared to six months for the gene disruption construct, so it is anticipated that these parasites will be able to produce gametocytes. The next step in obtaining a clonal *PFE0565w* gene deletion population is to conduct a limiting dilution with the parasites.

## 4. Conclusions

In summary, data obtained from these studies demonstrate that PFE0565w is a *P. falciparum* protein that has orthologs in other *Plasmodium* species, but does not have orthologs with any protein outside this genus. The PFE0565w protein shares no functional identity with other known proteins, making it a novel protein to study in *P. falciparum* biology. Transcript expression studies determined that the *PFE0565w* transcript is present throughout a majority of the life cycle of the parasite, including mixed ES, mixed gametocyte stages, oocyst sporozoites, and salivary gland sporozoites, but preliminary data suggest it is not present during axenic exoerythrocytic stages. In addition, an alternative

*PFE0565w* transcript was discovered to be produced during the erythrocytic stages, primarily by gametocytes, and may represent an anti-sense RNA made during this stage of the life cycle. This anti-sense RNA could be important for transcriptional/translational regulation of the parasite (Hughes *et al.*, 2010; Sorber *et al.*, 2011). Protein expression studies demonstrated that the PFE0565w protein is present during the salivary gland sporozoite stage, but not in other stages that were examined. Protein expression studies for PFE0565w during the exoerythrocytic stages will have to be conducted in order to determine whether the protein is present during this stage of the life cycle.

These transcript and protein expression profiles for PFE0565w produced data demonstrating that the protein is only detected by salivary gland sporozoites, despite a broader transcript presence. Since the PFE0565w protein is present in salivary gland sporozoites, the protein may be important for survival/development within mosquito salivary glands and/or may be needed for either development within or invasion of human host tissues. There is evidence in the literature that supports this hypothesis, as PFE0565w has been placed into a category of genes that is highly up-regulated in salivary gland sporozoites compared to other life cycle stages (called a Sporozoite Conserved Orthologous Transcript, SCOT) and their gene products are predicted to be important in early establishment of exoerythrocytic stages (Westenberger *et al.*, 2010). Taken together, PFE0565w could be another candidate for a new drug and/or pre-erythrocytic stage vaccine since it does not share identity with any known human protein.

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**Figure 2.1. Membrane feeding apparatus used for infecting *Anopheles stephensi* with *Plasmodium falciparum*.** Mixed gametocyte cultures that had been induced for 16 days were fed to *An. stephensi* for 30 minutes using a water-jacketed membrane feeding system set to 37°C.

| | |
|---|---|
| **Molecular Weight and Gene/Protein Size** | 45,583 Daltons<br>1,146 base pairs<br>381amino acids |
| **Intron(s)** | None |
| **SignalP** | Signal Peptide<br>(Amino Acids 1-25) |
| **Transmembrane Domain(s)** | Yes: One<br>(Amino Acids 330-352) |
| **TargetP** | Secretory Pathway |
| **PSORTII** | Membrane: Plasma or ER |
| **WoLF PSORT** | Membrane: Plasma or ER |
| **PROSITE** | 1. N-glycosylation site<br>2. Protein kinase C Phos Site<br>3. Casein kinase II Phos Site<br>4. cAMP/cGTP Dependent Protein kinase Phos Site<br>5. Tyrosine kinase Phos Site<br>6. N-myristoylation |

**Table 2.1. PFE0565w is predicted to be a sporozoite surface protein based upon PlasmoDB data and additional sequence analysis.** Initial gene characterization for *PFE0565w* was performed by gathering information from PlasmoDB (Aurrecoechea *et al.*, 2009; Bahl *et al.*, 2002). In order to further characterize the gene and verify data from PlasmoDB, additional programs found on the ExPASy Bioinformatics Resource Portal (www.expasy.org) and Softberry website (www.softberry.com) were utilized. ER = endoplasmic reticulum and Phos = phosphorylation.

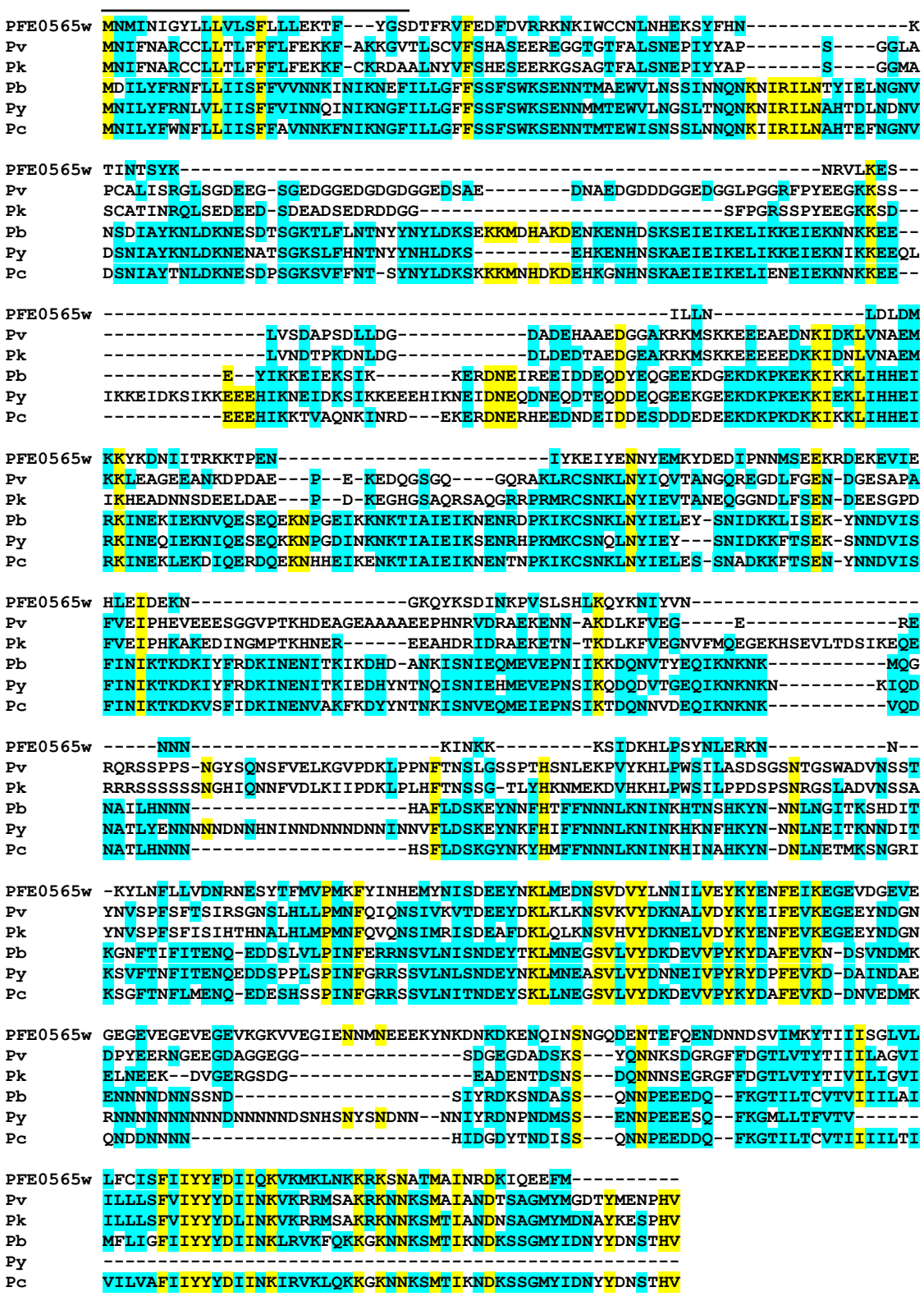


**Figure 2.2. *Plasmodium falciparum* protein, PFE0565w, has orthologs in other *Plasmodium* species.** Data obtained from PlasmoDB and via a BLAST analysis of the PFE0565w sequence showed that the protein has orthologs in other *Plasmodium* species; however, PFE0565w does not have orthologs with any proteins from members outside of the group Apicomplexa. Amino acids highlighted in yellow are identical in all proteins and amino acids highlighted in blue are similar in a majority of the proteins. The PFE0565w protein has orthologs in *P. vivax* (Pv = PVX_080665 in PlasmoDB), *P. knowlesi* (Pk = PKH_102160 in PlasmoDB), *P. berghei* (Pb = PBANKA_111090 in PlasmoDB), *P. yoelii* (Py = PY00913 in PlasmoDB), and *P. chabaudi* (PCHAS_111060 in PlasmoDB). The solid black line indicates the signal peptide sequence of PFE0565w.

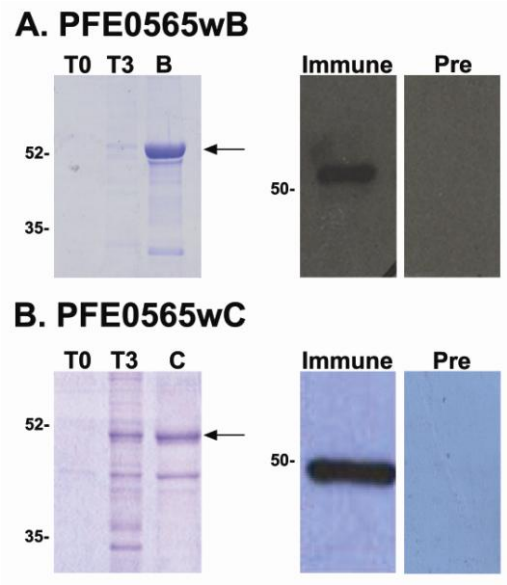



**Figure 2.3. Recombinant protein representing the N-terminal (A, PFE0565wB) and carboxy (B, PFE0565wC) halves of the PFE0565w protein was produced and used to generate polyclonal antibodies.** SDS-PAGE gels stained with Coomassie blue (images on left) were used to separate and visualize both induced PFE0565wB and PFE0565wC. The produced proteins were used to make polyclonal antibodies which recognized the recombinant proteins, as shown by Western blot analysis (images on right). Preimmune serum did not react with the recombinant protein. $T_0$ = non-induced protein lysate, $T_3$ = IPTG-induced protein lysate at 3 hours following induction, B = PFE0565wB purified recombinant protein, C = PFE0565wC purified recombinant protein, Immune = PFE0565w immune serum and Pre = PFE0565w preimmune serum. The arrows denote purified PFE0565wB and C recombinant proteins.

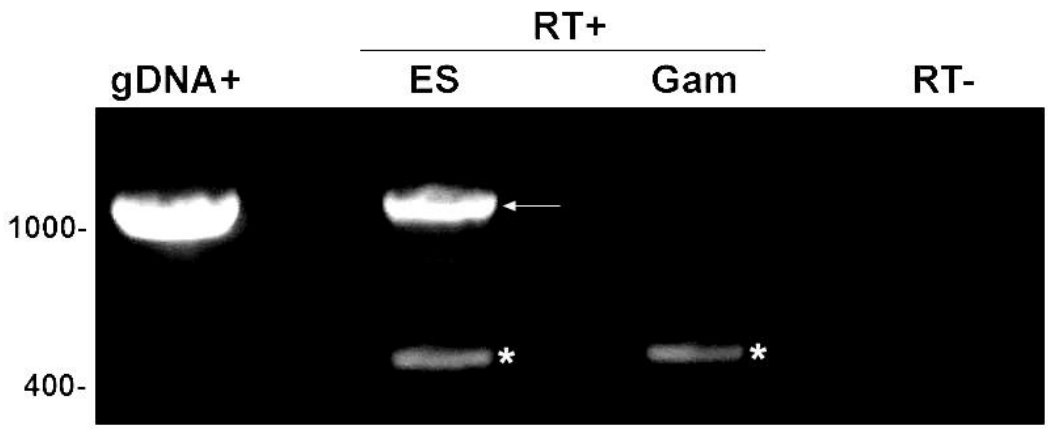



**Figure 2.4. *PFE0565w* transcripts are present in the *P. falciparum* erythrocytic stages, including gametocytes.** Primers specific for *PFE0565w* were used to amplify cDNA products (RT+), indicating the expression of the gene in both mixed erythrocytic stages (ES) and gametocyte stages (Gam) of infection. A 1,146 base pair product was the expected size and was amplified in mixed ES; however there also was an unpredicted product amplified in both the mixed ES and Gam. This product was sequenced and was confirmed to be *PFE0565w*, missing base pairs 291-988 (the resulting sequence does not translate into a protein). Genomic DNA (gDNA) was used as a positive control and a no reverse-transcriptase (RT-) reaction was used as a negative control to show that the RNA was not contaminated with gDNA. *PFE0565w* does not contain an intron and the arrow indicates the predicted *PFE0565w* RT-PCR product and the asterisks indicate the alternative *PFE0565w* RT-PCR products. This figure is a representative image of three biological replicates of both mixed erythrocytic stages and day 16 gametocytes.

| PFE0565w | | | |
|---|---|---|---|
| | ***P. falciparum*** | ***P. berghei*** | ***P. yoelii*** |
| **Gametes** | **No-P**<br>Lasonder *et al*., 2002 | **No Data Available** | **No Data Available** |
| **Zygotes** | **No Data Available** | **No Data Available** | **No Data Available** |
| **Ookinetes** | **No Data Available** | **No-P**<br>Hall *et al*., 2005 | **No-P**<br>Hall *et al*., 2005 |
| **Oocyst Sporozoites** | **No Data Available** | **No-P**<br>Hall *et al*., 2005 | **No-P**<br>Hall *et al*., 2005 |
| **Salivary Gland Sporozoites** | **Yes-T and P**<br>Florens *et al*., 2002<br>Le Roch *et al*., 2003 | **No-P**<br>Hall *et al*., 2005 | **No-P**<br>Hall *et al*., 2005 |
| **Liver Stages** | **No-T**<br>Siau *et al*., 2008 | **No Data Available** | **No-T and P**<br>Tarun *et al*., 2008<br>**Yes-T**<br>Williams *et al*., 2010 |
| **Free Merozoites** | **No-T and P**<br>Florens *et al*., 2002<br>Le Roch *et al*., 2003<br>Bozdech *et al*., 2003 | **No Data Available** | **No Data Available** |
| **Rings** | **No-T**<br>Le Roch *et al*., 2003<br>Bozdech *et al*., 2003 | **No-P**<br>Hall *et al*., 2005 | **No-P**<br>Hall *et al*., 2005 |

| | | | |
|---|---|---|---|
| **Trophozoites** | **No-T and P**<br><br>Florens *et al*., 2002<br>Lasonder *et al*., 2002<br>Le Roch *et al*., 2003<br>Bozdech *et al*., 2003 | **No-P**<br><br>Hall *et al*., 2005 | **No-P**<br><br>Hall *et al*., 2005 |
| **Schizonts** | **No-T**<br><br>Lasonder *et al*., 2002<br>Le Roch *et al*., 2003<br>Bozdech *et al*., 2003 | **No-P**<br><br>Hall *et al*., 2005 | **No-P**<br><br>Hall *et al*., 2005 |
| **Gametocytes** | **No-T and P**<br><br>Florens *et al*., 2002<br>Lasonder *et al*., 2002<br>Le Roch *et al*., 2003<br>Young *et al*., 2005 | **No-P**<br><br>Khan *et al.*, 2005<br>Hall *et al*., 2005 | **No-P**<br><br>Hall *et al*., 2005 |

**T = transcript and P = protein**

**Table 2.2. Transcript and protein expression patterns of PFE0565w throughout the life cycle of *Plasmodium* based on data obtained from the literature.** Initial literature searches for PFE0565w and its orthologs in *P. berghei* and *P. yoelii* (two rodent malaria parasites) indicate that PFE0565w transcript is present in salivary gland sporozoites and liver stages, with its protein only expressed by salivary gland sporozoites (Bozdech *et al.*, 2003a; Bozdech *et al.*, 2003b; Florens *et al.*, 2002; Hall *et al.*, 2005; Khan *et al.*, 2005; Lasonder *et al.*, 2002; Le Roch *et al.*, 2003; Siau *et al.*, 2008; Tarun *et al.*, 2008; Williams & Azad, 2010; Young *et al.*, 2005).

## A. *PFE0565w* full length and truncated sequence alignment

```
FL     ATGAACATGATTAATATTGGATATTTGCTTTTAGTTTTATCTTTTTTGTTATTGGAAAAGACATTTTATGGTAGTGATACC
Trunc  ATGAACATGATTAATATTGGATATTTGCTTTTAGTTTTATCTTTTTTGTTATTGGAAAAGACATTTTATGGTAGTGATACC

FL     TTTAGAGTATTTGAAGATTTTGATGTTAGAAGAAAAAACAAAATATGGTGTTGTAATTTAAATCATGAAAAAAGTTATTTT
Trunc  TTTAGAGTATTTGAAGATTTTGATGTTAGAAGAAAAAACAAAATATGGTGTTGTAATTTAAATCATGAAAAAAGTTATTTT

FL     CATAACAAAACTATAAATACGTCATACAAAAATAGAGTTTTAAAAGAAAGCATTTTACTTAATTTAGATTTAGATATGAAG
Trunc  CATAACAAAACTATAAATACGTCATACAAAAATAGAGTTTTAAAAGAAAGCATTTTACTTAATTTAGATTTAGATATGAAG

FL     AAATATAAGGATAATATCATAACCAGAAAGAAAACACCTGAAAATATTTATAAAGAAATATATGAAAATAATTATGAAATG
Trunc  AAATATAAGGATAATATCATAACCAGAAAGAAAACACCTGAAAATAT----------------------------------

FL     AAATATGATGAAGATATTCCTAATAATATGAGTGAAGAAAAAAGAGATGAAAAGGAAGTGATTGAACATCTAGAAATAGAC
Trunc  ----------------------------------------------------------------------------------

FL     GAAAAAAACGGAAAACAATACAAAAGTGATATAAATAAACCAGTTAGTTTATCACATCTTAAACAATATAAAAATATTTAT
Trunc  ----------------------------------------------------------------------------------

FL     GTTAATAACAATAATAAAATAAATAAAAAAAAAAAGTATAGACAAACATTTACCTTCATATAATTTAGAAAGGAAAAATAAT
Trunc  ----------------------------------------------------------------------------------

FL     AAGTATCTTAACTTTTTACTCGTAGATAATAGGAATGAATCTTATACCTTTATGGTGCCTATGAAATTTTATATAAATCAT
Trunc  ----------------------------------------------------------------------------------

FL     GAAATGTATAATATATCAGATGAGGAATATAATAAATTAATGGAAGATAATAGTGTAGATGTTTATTTAAATAATATATTG
Trunc  ----------------------------------------------------------------------------------

FL     GTTGAATACAAATATGAAAATTTCGAAATAAAAGAAGGAGAAGTTGATGGAGAAGTTGAAGGAGAAGGAGAAGTAGAAGGA
Trunc  ----------------------------------------------------------------------------------
FL     GAAGTAGAAGGAGAAGTAAAAGGAAAAGTGGTAGAAGGAATAGAAAATAACATGAATGAGGAAGAAAAATATAATAAAGAT
Trunc  ----------------------------------------------------------------------------------

FL     AATAAAGATAAGGAAAATCAAATAAATTCAAACGGACAAGATGAAAACACTGAATTTCAAGAAAATGATAACAATGATAGT
Trunc  ----------------------------------------------------------------------------------

FL     GTAATTATGAAATATACCATTATTATTTCAGGATTAGTTCTCTTATTTTGTATCAGTTTTATTATTTATTATTTTGATATT
Trunc  ----------------CCATTATTATTTCAGGATTAGTTCTCTTATTTTGTATCAGTTTTATTATTTATTATTTTGATATT

FL     ATACAAAAGGTAAAAATGAAGCTAAATAAAAAAAAGAAAATCTAATGCAACCATGGCAATAAATAGAGACAAAATTCAAGAG
Trunc  ATACAAAAGGTAAAAATGAAGCTAAATAAAAAAAGAAAATCTAATGCAACCATGGCAATAAATAGAGACAAAATTCAAGAG

FL     GAATTTATGTGA
Trunc  GAATTTATGTGA
```

## B. Translation of *PFE0565w* truncated sequence

```
Met Asn Met Ile Asn Ile Gly Tyr Leu Leu Leu Val Leu Ser Phe Leu Leu Leu Glu Lys Thr Phe
Tyr Gly Ser Asp Thr Phe Arg Val Phe Glu Asp Phe Asp Val Arg Arg Lys Asn Lys Ile Trp Cys
Cys Asn Leu Asn His Glu Lys Ser Tyr Phe His Asn Lys Thr Ile Asn Thr Ser Tyr Lys Asn Arg
Val Leu Lys Glu Ser Ile Leu Leu Asn Leu Asp Leu Asp Met Lys Lys Tyr Lys Asp Asn Ile Ile
Thr Arg Lys Lys Thr Pro Glu Asn Ile His Tyr Tyr Phe Arg Ile Ser Ser Leu Ile Leu Tyr Gln
Phe Tyr Tyr Leu Leu Phe *** Tyr Tyr Thr Lys Gly Lys Asn Glu Ala Lys *** Lys Lys Lys Ile
*** Cys Asn His Gly Asn Lys *** Arg Gln Asn Ser Arg Gly Ile Tyr Val
```

**Figure 2.5. An alternative *PFE0565w* transcript is present in the *P. falciparum* erythrocytic stages, with only this alternative transcript present**

**in gametocytes; however, it does not translate into a full-length protein.** DNA sequencing using *PFE0565w* gene specific primers was conducted to verify that the alternative transcript amplified during the RT-PCR experiments using mixed erythrocytic and mixed gametocyte stages was *PFE0565w*. A) DNA sequencing results were aligned with the full-length *PFE0565w* sequence obtained from PlasmoDB using VectorNTI Contig Express (Invitrogen). B) The truncated *PFE0565w* sequence obtained does not translate into a protein. FL = full length *PFE0565w* transcript and Trunc = truncated *PFE0565w* transcript.

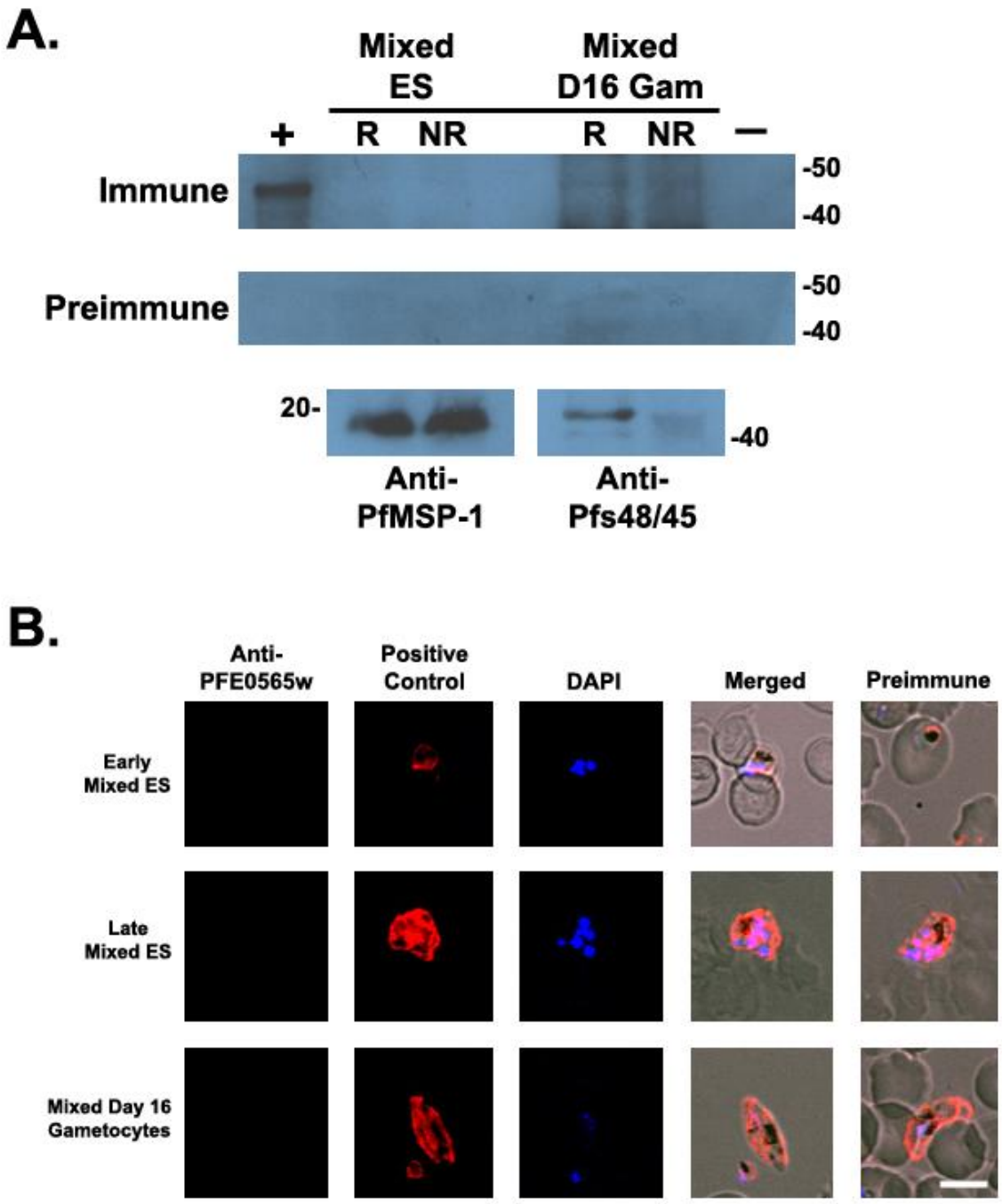



**Figure 2.6. The PFE0565w protein is not present during the erythrocytic stages as demonstrated by Western blot analysis (A) and confocal microscopy (B).** The PFE0565w protein is not present during the erythrocytic stage parasites, including gametocytes, when compared to preimmune controls

(PFE0565w would be a 43-kDa product in the Western blot and appear in green in the confocal image). Anti-PfMSP-1-19 and anti-Pfs48/45 or anti-Pfs230 antibodies were used as positive controls for mixed erythrocytic stages (cultures containing a mixture of rings, trophozoites, and schizonts) and mixed gametocytes (cultures containing a mixture of Stage I-V gametocytes), respectively (red) to demonstrate the presence of parasites. Preimmune serum was used as a negative control and no labeling of parasites occurred. This figure is a representative image from three biological replicates for both mixed erythrocytic stages and day 16 gametocytes. Early = ring or early trophozoite stage parasites, Late = late trophozoite or schizont stage parasites, ES = erythrocytic stages, Gam = gametocytes, R = reduced, NR = non-reduced, + = rPFE0565wB protein (45 kDa, N-terminal portion of protein plus the His-tag, S-tag, and thioredoxin) used as a positive control, and - = non-infected red blood cells used as a negative control. The scale bar represents 5.0 µm.

## A. Erythrocytic Stages

| Bio Rep | Immune | Positive Control | Preimmune |
|---|---|---|---|
| 1 | 4 / 324 = 1.2%<br>Scanned 40 fields<br>of 4-12 iRBCs | 324 / 324 = 100%<br>Scanned 40 fields<br>of 4-12 iRBCs | 0 / 318 = 0.0%<br>Scanned 40 fields<br>of 4-12 iRBCs |
| 2 | 4 / 311 = 1.3%<br>Scanned 23 fields<br>of 7-19 iRBCs | 311 / 311 = 100%<br>Scanned 23 fields<br>of 7-19 iRBCs | 0 / 314 = 0.0%<br>Scanned 23 fields<br>of 7-19 iRBCs |
| 3 | 3 / 310 = 0.97%<br>Scanned 18 fields<br>of 15-24 iRBCs | 310 / 310 = 100%<br>Scanned 18 fields<br>of 15-24 iRBCs | 0 / 319 = 0.0%<br>Scanned 18 fields<br>of 15-24 iRBCs |

## B. Gametocyte Stages

| Bio Rep | Immune | Positive Control | Preimmune |
|---|---|---|---|
| 1 | 3 / 200 = 1.5% | 200 / 200 = 100% | 0 / 188 = 0.0% |
| 2 | 2 / 200 = 1.0% | 200 / 200 = 100% | 0 / 200 = 0.0% |
| 3 | 3 / 204 = 1.5% | 204 / 204 = 100% | 0 / 203 = 0.0% |

**Table 2.3. *P. falciparum* mixed erythrocytic stage parasites (A) and day 16 mixed gametocytes (B) were counted and the PFE0565w protein pattern was observed for each parasite during immunofluorescent assays.** To verify PFE0565w protein presence in all erythrocytic stages, protein patterns were examined for each parasite counted. This was done for both PFE0565w immune and preimmune serum. All parasites counted labeled with the positive controls (parasites were triple-labeled - PfMSP1-19 for mixed erythrocytic stages and Pfs230 for gametocyte stages). The very limited labeling of parasites with immune serum likely reflects non-specific binding of the anti-PFE0565w antibodies.

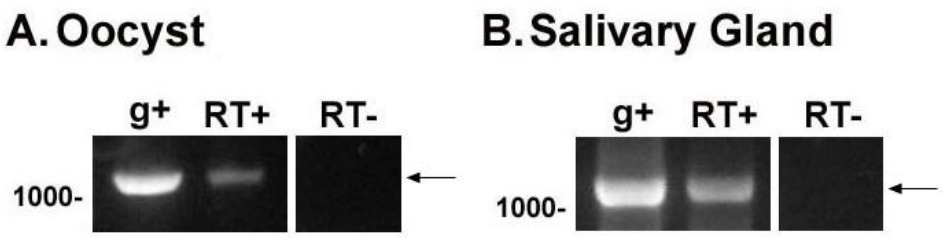



**Figure 2.7. *PFE0565w* transcript is present in both the *P. falciparum* oocyst sporozoite (A) and salivary gland sporozoite (B) stages, as shown by RT-PCR.** Primers specific for *PFE0565w* were used to amplify cDNA fragments of the correct product size (RT+), indicating the expression of the gene during the sporozoite stages of infection. Genomic DNA (gDNA) was used as a positive control and a no reverse-transcriptase (RT-) reaction was used as a negative control to show that the RNA was not contaminated with gDNA. PFE0565w does not contain an intron and the arrows indicate the *PFE0565w* RT-PCR products. This figure is a representative image from two biological replicates using oocyst sporozoites and three biological replicates using salivary gland sporozoites.

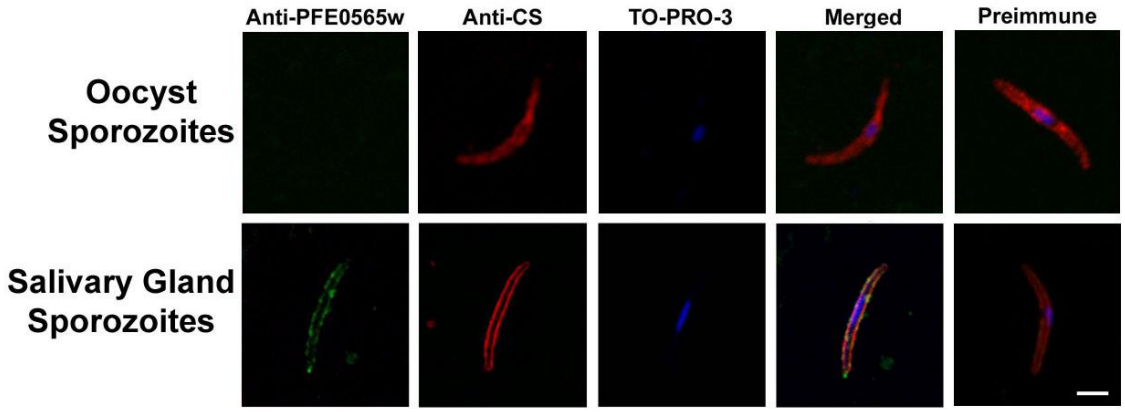



**Figure 2.8. The PFE0565w protein is present on the surface of *P. falciparum* salivary gland sporozoites, but not oocyst sporozoites, as indicated by confocal microscopy.** Sporozoites isolated from both mosquito midguts and salivary glands were triple-labeled using anti-PFE0565wB antibodies, anti-circumsporozoite (CS) mAb, and TO-PRO-3, a nuclear stain. Alexa-488 (goat anti-rabbit) and Alexa-546 (goat anti-mouse)-conjugated secondary antibodies were used to observe fluorescence. Sporozoites were visualized using either a Radiance 2000 confocal system coupled to an Olympus IX70 inverted microscope or a Zeiss LSM 510 Meta NLO confocal microscope. PFE0565w expression is shown in green and CS, used as a positive control, is shown in red with TO-PRO-3 staining the nucleus blue. The merged image for salivary gland sporozoites shows co-localization of PFE0565w with the CS protein, which appears yellow, indicating probable surface expression of PFE0565w. Preimmune serum was used as a negative control and did not label the sporozoites. The scale bar represents 2.5 µm. This figure is a representative

image from two biological replicates for oocyst sporozoites and three biological replicates for salivary gland sporozoites.

## A. Oocyst Sporozoites

| Bio Rep | Immune | Positive Control | Preimmune |
|---|---|---|---|
| 1 | 0 / 30 = 0.0% | 30 / 30 = 100% | 0 / 30 = 0.0% |
| 2 | 0 / 183 = 0.0% | 183 / 183 = 100% | 0 / 175 = 0.0% |

## B. Salivary Gland Sporozoites

| Bio Rep | Immune | Positive Control | Preimmune |
|---|---|---|---|
| 1 | 158 / 160 = 98.7% | 160 / 160 = 100% | 0 / 160 = 0.0% |
| 2 | 160 / 163 = 98.1% | 163 / 163 = 100% | 0 / 159 = 0.0% |
| 3 | 189 / 193 = 97.9% | 193 / 193 = 100% | 0 / 188 = 0.0% |

**Table 2.4. The numbers of *P. falciparum* oocyst (A) and salivary gland (B) sporozoites were determined and the PFE0565w protein pattern was observed for each sporozoite during immunofluorescent assays.** To verify PFE0565w protein presence in the sporozoite stages, protein patterns were observed for each sporozoite that was examined. All sporozoites were labeled with anti-circumsporozoite protein antibodies, which was used as a positive control (parasites were triple-labeled).

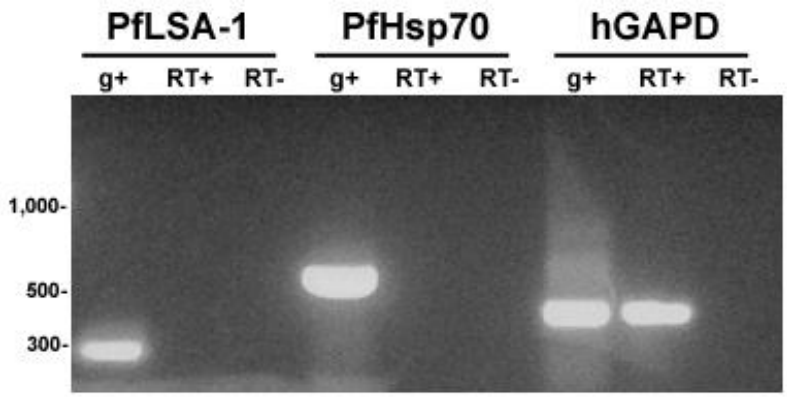



**Figure 2.9. Initial attempts to generate exoerythrocytic stage parasites using primary human hepatocytes were not successful.** RT-PCR with gene specific primers did not detect transcripts for either *P. falciparum liver stage antigen-1* (PfLSA-1) or the *P. falciparum heat shock protein-70* (PfHsp70) gene, suggesting that exoerythrocytic stage parasites were not present following a liver stage invasion experiment. To verify that intact hepatocyte RNA was isolated from the primary human hepatocytes, primers specific to *human glyceraldehyde 3-phosphate dehydrogenase* (hGAPD) were utilized and amplified transcript of the proper size indicating that at least the liver cells used were viable at the time of isolation. Genomic DNA (g+) was used as a positive control for all primer sets and reactions without reverse transcriptase (RT-) were used as negative controls, confirming there was no genomic DNA contamination of the RNA. RT+ = cDNA generated from isolated RNA.

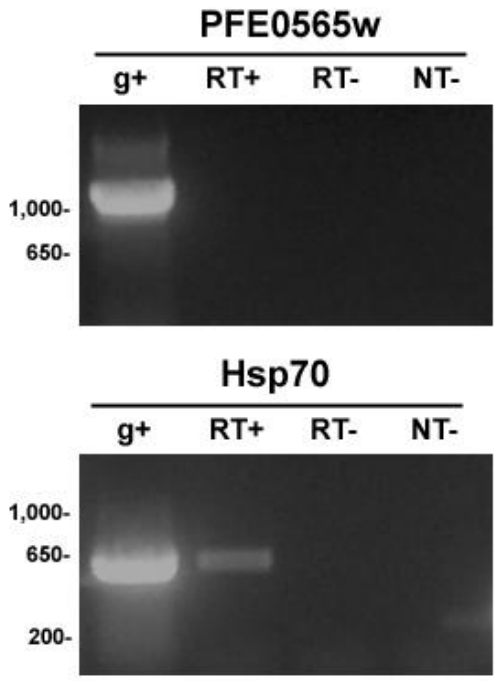



**Figure 2.10. Preliminary RT-PCR data suggest that *PFE0565w* transcript is not present during axenic *P. falciparum* exoerythrocytic stages as compared to parasite control transcript that was detected.** Primers specific for *PFE0565w* were used to amplify cDNA fragments (RT+) and no product was amplified, indicating that *PFE0565w* transcript is not present during the exoerythrocytic stage of infection produced from axenic cultures (*i.e.*, exoerythrocytic parasites generated without the presence of hepatocytes). Primers specific to the *P. falciparum heat shock protein-70* (Hsp70) gene were used as a positive control to verify the presence of *P. falciparum* exoerythrocytic stages. Genomic DNA (gDNA) was used as a positive control for both primer sets and a no reverse-transcriptase (RT-) reaction was used as a negative control to show that the RNA was not contaminated with gDNA.

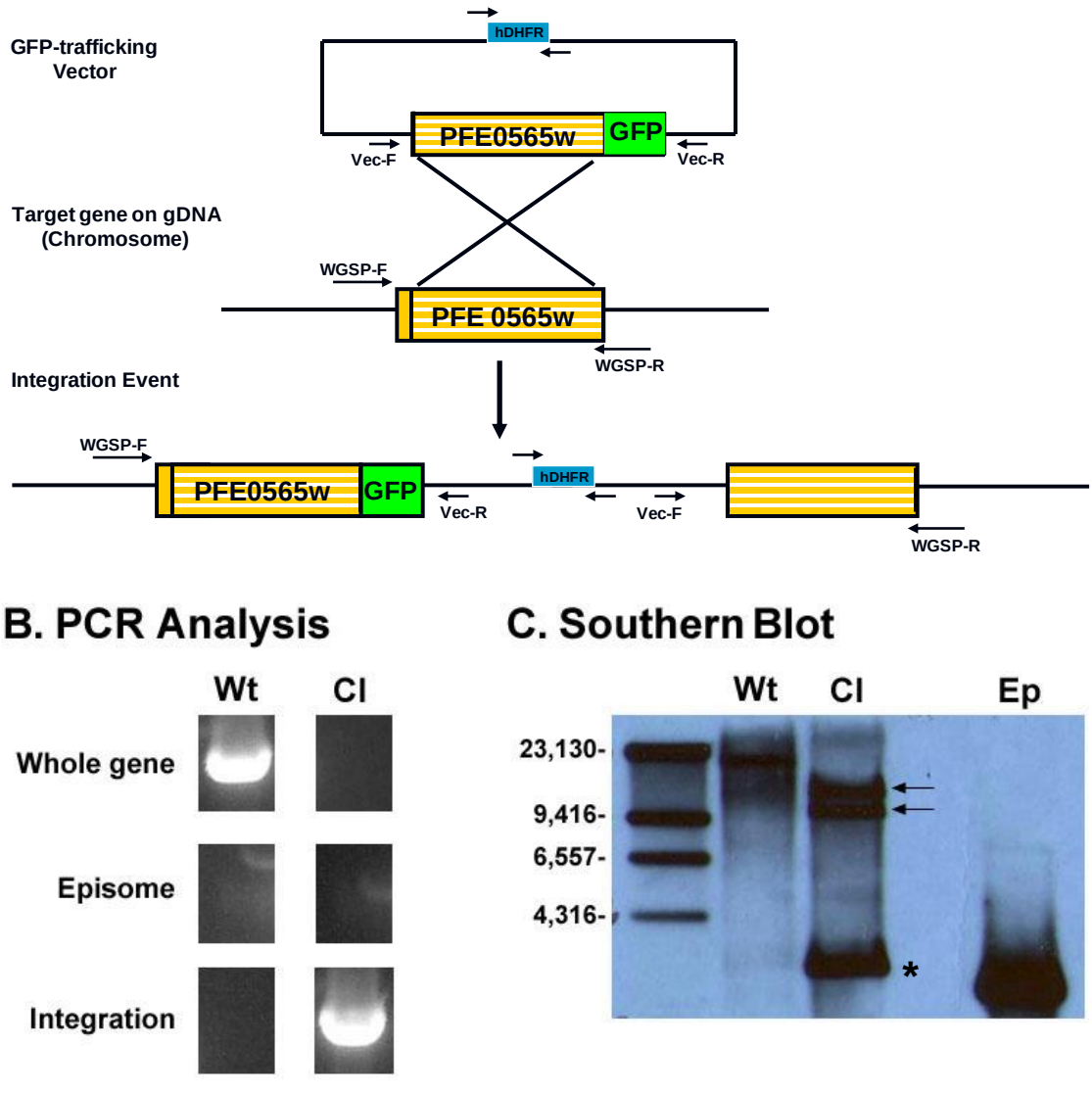



**Figure 2.11. A PFE0565w/GFP clonal parasite population was successfully created for trafficking studies throughout the entire life cycle of the parasite.** A) Transfection schematic demonstrating how the PFE0565w/GFP construct was created using the pPM2GT vector (obtained from MR4) and successfully incorporated into the genome of the parasite via homologous

recombination (Klemba *et al.*, 2004). Expression of GFP is driven by the endogenous promoter of *PFE0565w*. Generation of a PFE0565w/GFP clonal parasite population was verified by both PCR analysis (B) and Southern blot analysis using digoxigenin (DIG) technology coupled with autoradiography (C). Arrows indicate the predicted integration products of 12,134 base pairs and 9,762 base pairs using a PFE0565w specific probe after restriction digestion with BamHI and BssI. The product indicated by an asterisk is of unknown origin and is likely a rearrangement of the plasmid that occurred after transfection. The drug cassette within the vector used for positive selection is human dihydrofolate reductase (hDHFR). This figure is a representative image of the two clonal PFE0565w/GFP parasite populations obtained after limiting dilution. GFP = Green fluorescent protein, Wt = Wild-type parasite genomic DNA, Cl = Clonal PFE0565w/GFP parasite genomic DNA, and Ep = PFE0565w/GFP plasmid DNA representing the episome. The arrows on the transfection schematic represent primers used for PCR analysis.

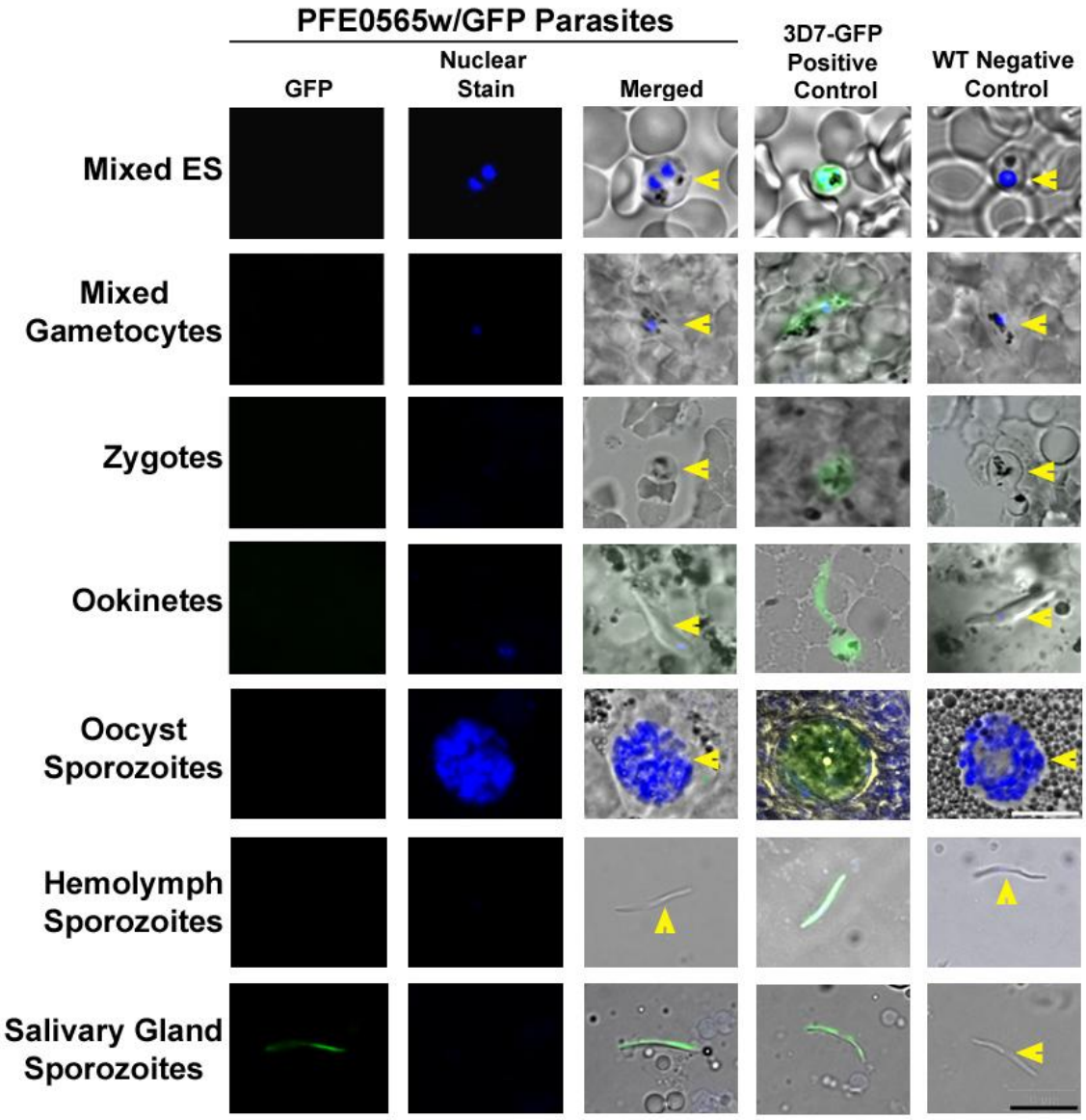



**Figure 2.12. PFE0565w/GFP-trafficking studies confirm that the PFE0565w protein is only present in salivary gland sporozoites.** The PFE0565w protein is not present during mixed erythrocytic stages (ES, culture representing a mixture of rings, trophozoites, and schizonts), mixed gametocytes (culture representing a mixture of stage I-V gametocytes), zygotes, ookinetes, oocyst sporozoites, and hemolymph sporozoites. The PFE0565w protein is present in

salivary gland sporozoites. 3D7HT-GFP (3D7-GFP) constitutively expressing parasites were used as a positive control (Talman *et al.*, 2010) and wild-type (WT) parasites were used as a negative control. This figure is a representative image from two biological replicates (one with each independent clone created). Yellow arrowheads depict the presence of parasites that lack GFP expression (merged images). GFP = green fluorescent protein and nuclear stain = DAPI. The black scale bar in the lower right represents all of the images (except for the oocyst sporozoite stage) and is 10 µm. The white scale bar for the oocysts is 40 µm.

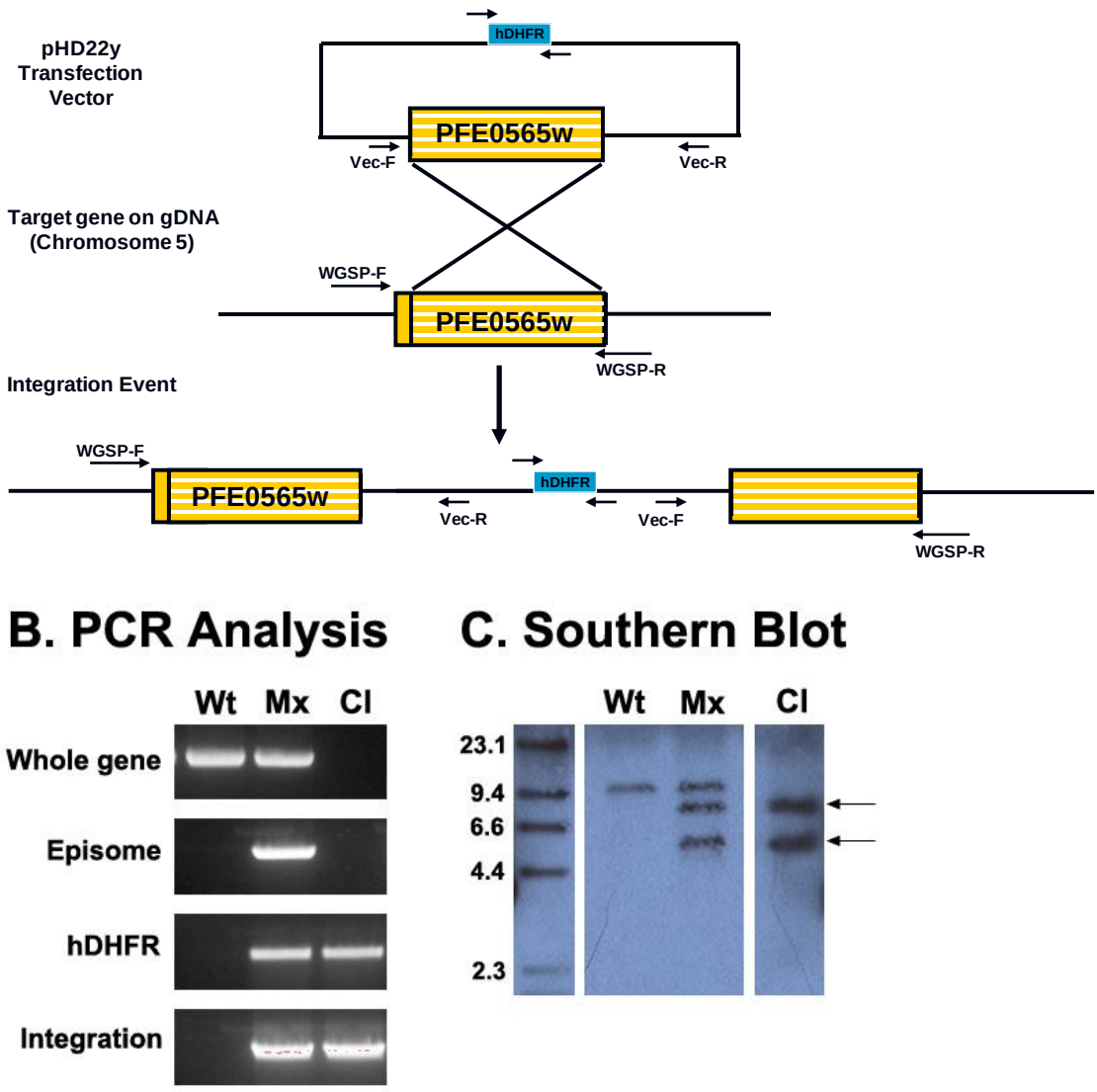
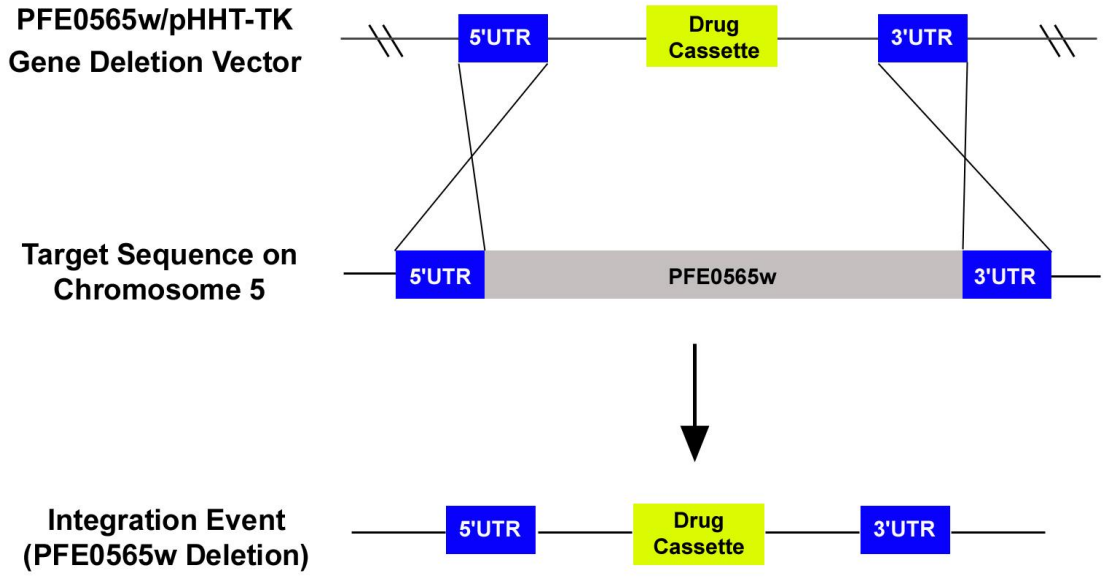



**Figure 2.13. A PFE0565w mutant parasite strain has been created and is being used to assess the potential function of PFE0565w in the invasion of host tissues and/or parasite development.** A) Transfection schematic demonstrating how the *PFE0565w* gene was successfully disrupted via homologous recombination using the pHD22y disruption construct (Fidock & Wellems, 1997). Creation of a PFE0565w/pHD22y clonal parasite population

was verified by both PCR analysis (B) and Southern blot analysis using DIG technology coupled with autoradiography (C). Arrows indicate the predicted integration products of 9,210 base pairs and 5,987 base pairs using a PFE0565w specific probe after restriction digestion with EcoRI and SacII. Wt = Wild-type parasite genomic DNA, Mx = population of transfected parasites before limiting dilution, Cl = Clonal PFE0565w/pHD22y parasite genomic DNA, and hDHFR = human dihydrofolate reductase (vector drug cassette). Arrows represent primers used in PCR analysis.

## A. Double Cross-Over Transfection Schematic




## B. PFE0565w/pHHT-TK Deletion Construct Analysis

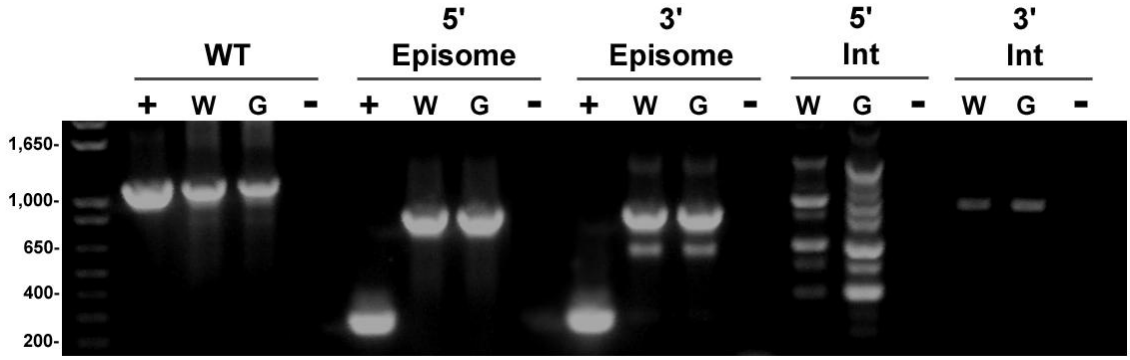



**Figure 2.14. Creation of a *PFE0565w* deletion parasite population is in progress for use in a functional analysis of PFE0565w.** A) Transfection schematic of the double cross-over, homologous recombination event to obtain mutant *PFE0565w* gene deletion parasites using the pHHT-TK deletion vector (Duraisingh *et al.*, 2002). B) PCR analysis was used to assess the progress in

achieving integration events at both the 5’ and 3’ ends of *PFE0565w* with the PFE0565w/pHHT-TK deletion construct before limiting dilution. 5’UTR = the 5’ untranslated region of *PFE0565w*, 3’UTR = the 3’ untranslated region of *PFE0565w*, drug cassette = thymidine kinase, WT = wild-type, Int = integration, + = positive control, W = parasites under WR99210 drug selection only, G = parasites under both WR9920 and gancyclovir drug selection, and - = no template negative control.

# Chapter 3: A Transcript and Protein Expression Study of PF11_0394, a *Plasmodium falciparum* Gene/Protein


## ABSTRACT

The *Plasmodium falciparum* gene, *PF11_0394*, was chosen as a candidate for study due to its potential role in the invasion of host tissues. This gene was selected based on data from PlasmoDB, indicating that it is expressed both at the transcriptional and protein levels in sporozoites and likely encodes a putative surface protein. Additional sequence analysis showed that the PF11_0394 protein has orthologs in other *Plasmodium* species and Apicomplexans, but none outside of the group Apicomplexa. *PF11_0394* transcript is present during both the sporozoite and erythrocytic stages of the parasite life cycle, but no transcript is detected during axenic exoerythrocytic stages. Despite the presence of transcript throughout several life cycle stages, the PF11_0394 protein only appears in salivary gland sporozoites.



**Keywords:** *Plasmodium falciparum*, sporozoite, surface protein

## 1. Introduction

Malaria is a mosquito-borne disease that is caused by protozoan parasites of the genus *Plasmodium*. It is estimated that up to 300 million cases of malaria occur annually and, of those, up to one million are fatal with nearly 95% of the fatalities occurring in African children under the age of five and pregnant women (Moorthy *et al.*, 2004; WHO, 2010). Malaria is still a major problem around the world due to the development of insecticide-resistant mosquitoes, drug-resistant *Plasmodium* parasites, and economic/political instability in areas of the world where malaria is a significant problem (Porter, 2006). Because of these issues, it is critical for improved and/or new malaria control methods to be developed.

The *P. falciparum* gene, *PF11_0394*, was chosen as a candidate for study due to its potential role in the invasion of host tissues based on an *in silico* data mining procedure. This gene was selected based on data collected from PlasmoDB, the *P. falciparum* database (www.plasmodb.org), indicating that this gene likely encodes a putative surface protein, which is found in the sporozoite both at the mRNA transcript and protein levels (Bahl *et al.*, 2002; Florens *et al.*, 2002). The experiments proposed herein are to further characterize the gene, *PF11_0394*, and the protein it produces to determine its detection profile and localization. Data gathered from this research will allow us to further understand transcript and protein patterns of another novel *P. falciparum* gene/protein and may also provide the field with a new drug and/or vaccine target.

## 2. Materials and Methods

### *2.1. Parasite maintenance, parasite transmission, and cell cultures.*

*Plasmodium falciparum* strain NF54 was used for the following experiments (a gift from Dr. Shirley Luckhart at the University of California-Davis) and were maintained according to procedures described by Carter *et al.*, 1993. Briefly, *P. falciparum* cultures were maintained in human blood (O+ male, BioChemed Services) at a 6% hematocrit in complete culture medium, RPMI 1640 (Gibco) supplemented with 25mM HEPES (Gibco), 0.5% Albumax (Invitrogen), and 0.005% hypoxanthine (Sigma). The medium was changed every 48 hours and the parasites were kept in a gas mixture (3% $O_2$, 3% $CO_2$, and 94% $N_2$) at 37ºC (Carter *et al.*, 1993). Cultures were diluted/split to prevent the parasitemia from becoming too high by addition of fresh, washed 50% blood (blood washed three times with sterile RPMI and diluted 50:50 with RPMI), maintaining a 6% hematocrit level. The use of human blood was in compliance with federal guidelines and institutional policies. All experiments described in this chapter were approved by the Institutional Biosafety Committee (IBC), the Institutional Review Board (IRB), and the University of Missouri Institutional Animal Care and Use Committee (IACUC).

In order to obtain infected mosquitoes to study the parasite stages within the vector host (e.g., zygotes, ookinetes, oocyst sporozoites, hemolymph sporozoites, and salivary gland sporozoites), four-five day old, female *An. stephensi* (details in section 2.2) were exposed to *P. falciparum*-infected blood

(1:1 ratio of infected blood and human serum), using induced gametocyte cultures. Gametocyte cultures were produced by setting a standard culture (described above) at a parasitemia of between 0.5-1.0% and maintaining them in complete culture media supplemented with 10% human serum (A+ male, Interstate Blood Bank); however, instead of splitting the parasites with fresh RBCs, the culture was left undiluted such that a high parasitemia developed and the parasites became stressed. This was done for 16 days and resulted in a mixture of male and female gametocyte stages (I-V), with a majority of them being mature stages. The mosquitoes were fed the infected blood for approximately 30 minutes, using a 37°C water-jacketed membrane feeding system (Figure 2.1). After the blood feed, the percentage of females that fed was recorded and any feeds where less than 75% of the females did not feed were not used for any experiments. The infected mosquitoes were kept in an incubator (Low Temperature Illuminated Incubator 818, Precision) at 26-27ºC with 82-88% humidity.

To obtain exoerythrocytic stages (liver stages), two approaches were used: infection of primary human hepatocytes and axenic cultures (*i.e.*, cultures without hepatocytes). For the former, *in vitro* cultures of the primary human hepatocytes (Clonetics ™ normal human hepatocytes, Lonza) were seeded 48 hours before infection with *P. falciparum* sporozoites in CellBind® 48-well plates (Corning), which were coated with 60 µg/cm$^2$ of rat tail collagen (BD Biosciences), at a density of 150,000 cells/cm$^2$. The hepatocytes were maintained according to the manufacturer's instructions using their hepatocyte

maintenance medium supplemented with growth factors (insulin, GA-1000, and dexamethasone) in a 37ºC incubator with 5% $CO_2$ (Cui *et al.*, 2009; Talman *et al.*, 2010). To infect the hepatocytes with *P. falciparum*, approximately 5 X $10^4$ salivary gland sporozoites were added to each well and allowed to incubate in invasion medium (hepatocyte maintenance medium supplemented with 10% human serum and 200 units/ml and 100 µg/ml penicillin and streptomycin, respectively) for four hours in a 37ºC incubator with 5% $CO_2$. The medium was then removed and fresh invasion medium was added daily, until the cells were harvested for experimental use (Cui *et al.*, 2009).

For axenic exoerythrocytic stage cultures, an online protocol by Kappe *et al*. (2010) was followed. In brief, approximately 5 X $10^4$ salivary gland sporozoites were added to a well of a 48-well plate (Corning) and allowed to incubate in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and 500 units/ml and 500 µg/ml penicillin and streptomycin, respectively. These cultures were kept in a 37ºC incubator with 5% $CO_2$ for 24 hours before collection for transcript expression studies (Kappe *et al.*, 2010).

*2.2. Mosquito maintenance.*

*Anopheles stephensi* mosquitoes were used for all studies because they can maintain the life cycle of *P. falciparum*. Mosquitoes were reared using protocols available from the Malaria Research and Reference Reagent Resource Center (MR4). In brief, larvae and adults were maintained in an insectary with 78-85% humidity at approximately 80ºF on a 12 hour light/dark cycle. Larvae

were fed both a mixture of 0.33 g yeast (Fleischmann's) and 0.66g micron (Sera) per 50 ml water and game fish chow (Purina). Adults were fed sucrose (0.3 M) *ad libitum*.

*2.3. Selection of candidate gene, PF11_0394.*

An *in silico* data mining procedure was used to select *PF11_0394* as a gene of interest. Briefly, the *Plasmodium* database, PlasmoDB, was utilized to search for *P. falciparum* proteins predicted to be expressed only by the sporozoite and contain a signal peptide, increasing its probability of being a surface and/or secreted protein (Bahl *et al.*, 2002; Kissinger *et al.*, 2002). This process narrowed gene numbers down to 34 candidate genes. Next, additional sequence analysis programs available on the ExPASy Bioinformatics Resource Portal (www.expasy.org) and SoftBerry (www.softberry.com), such as PSORT and ProtComp, were used to verify that the proteins encoded by the genes were predicted to either be located on the surface and/or secreted by the parasite. Those proteins that were verified by these two programs to meet the required criteria (and were not proteins that had been studied or were currently being studied) became our proteins of interest, including PF11_0394.

*2.4 PF11_0394 sequence analysis.*

Using PlasmoDB, the full genomic DNA (gDNA), complimentary DNA (cDNA), and protein sequence of PF11_0394 was obtained. A list of orthologs of PF11_0394 was compiled using both PlasmoDB and the National Center for

Biotechnology Information's (NCBI) BLAST analysis program (Altschul *et al.*, 1997). The ortholog sequences were then aligned using Vector NTI (Explorer or Contig Express, Invitrogen). Additional sequence information for PF11_0394 was obtained by using software programs such as TargetP, SignalP, PSORT II, WoLF PSORT, and PROSITE, all found via the ExPASy Bioinformatics Resource Portal (www.expasy.org).

*2.5 Isolation of P. falciparum-infected tissues for transcript and protein expression studies.*

Oocyst sporozoites. *An. stephensi* were infected with *P. falciparum* as described previously. Ten days post-infection (PI), when oocyst sporozoites were mature using our laboratory conditions, 50 *An. stephensi* midguts were dissected from mosquito abdomens that had fed on a *P. falciparum*-infected blood meal. The tissues were placed in 50 µl 1X phosphate buffered saline (10X PBS, 0.2 M phosphate buffer and 1.5 M NaCl pH 7.0, diluted 1:10 with Millipore water) in microcentrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80°C until needed for RNA isolation.

Salivary gland sporozoites. Fifty sets of *An. stephensi* salivary glands were dissected from mosquitoes that had fed on a *P. falciparum*-infected blood meal fourteen days PI because sporozoites reside in the glands at this time under our laboratory conditions. The tissues were put in 50 µl 1X PBS in microcentrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80°C until needed for RNA isolation.

Exo-erythrocytic stages. Primary human hepatocytes were prepared, maintained, and infected with *P. falciparum* sporozoites as previously described. Infected hepatocytes were collected at three days PI by digestion with trypsin-EDTA (0.25%, Invitrogen) at 37°C for five minutes. The cells were collected by centrifugation at 100 x g for five minutes, washed with 1X PBS, snap-frozen in liquid nitrogen, and stored at -80°C until needed for RNA isolation. For axenic exoerythrocytic stages, Trizol was directly added to the cultures to begin the process of RNA isolation.

Mixed erythrocytic stages and gametocytes. *P. falciparum* cultures were maintained as previously described. Either mixed erythrocytic stage (ES) cultures (containing a mixture of rings, trophozoites, and schizonts) or 16 day old mixed gametocyte cultures (containing a mixture of stage I-V gametocytes, but with more mature forms present) were collected by centrifugation at 2,650 x g for five minutes. The infected RBCs were lysed with 0.05% saponin (Invitrogen) in complete culture medium for three minutes at room temperature (RT) and parasites collected by centrifugation for five minutes at 2,650 x g. Purified parasites were then washed once with RPMI 1640 medium and collected again by centrifugation as previously described. The parasite pellets were stored at -80°C until needed for RNA isolation.

*2.6 RNA/DNA isolation and transcriptional analysis by reverse transcription-polymerase chain reaction (RT-PCR).*

Total RNA was isolated from *Plasmodium*-infected tissues using a Trizol reagent-based protocol, following the manufacturer's instructions (Invitrogen). The samples were all DNase-treated (Promega), according to the manufacturer's instructions, to remove any contaminating gDNA. Approximately 2-3 µg of the DNase-treated RNA was used to synthesize cDNA using OligoDT primers from a SuperScript™ III First-Strand Synthesis System (Invitrogen), following the manufacturer's instructions.

Genomic DNA was isolated following the manufacturer's instructions using a DNeasy® Blood and Tissue Kit (Qiagen) and was used as a positive control for all RT-PCR experiments. *PF11_0394* full-length gene specific primers (5'-atgaaaatttttaattacatatgtg-3' forward and 5'-ttatataatatttctattatcttcc-3' reverse) were used to amplify a 762 base pair (bp) gDNA fragment and a 561 bp cDNA fragment in a polymerase chain reaction (PCR) using 2.0 µl gDNA (~100 ng total)/cDNA (~1/10 the volume synthesized from above), 1.25 units GoTaq® DNA Polymerase (Promega), 1X GoTaq® Flexi Buffer, 1 mM $MgCl_2$, 0.2 mM di-nucleotide tri-phosphate mix, and 0.5 µM primers. PCR conditions were as follows: an initial denaturing step of 95ºC for three minutes, 35 repetitive cycles of denaturing at 95°C for 30 seconds, primer annealing at 56°C for 30 seconds and an extension at 62°C for three minutes, and then a final extension at 62°C for 10 minutes (Su *et al.*, 1996). For RT-PCR positive controls used during the exoerythrocytic stages, primers specific for *P. falciparum liver stage antigen-1* (5'-aatctaacttgagaagtgg-3' forward and 5'-ctgcatcgtcatttattatg-3' reverse), *P. falciparum heat shock protein-70* (5'-aggtatagaaactgtgggtgg-3' forward and 5'-

gattggttggcatacagcttc-3' reverse), and *human glyceraldehyde 3-phosphate dehydrogenase* (5'-accacagtccatgccatcac-3' forward and 5'-tccaccaccctgttgctgta-3' reverse) were used as described above. After PCR amplification, all samples were separated on a 1% agarose gel and stained with ethidium bromide (EtBr) for UV detection. The experiment with exoerythrocytic stages was done once, the experiments using oocyst sporozoites were done in biological duplicates, and the experiments with salivary gland sporozoites, mixed ES, and mixed gametocytes were done in biological triplicates.

*2.7. Creation of GFP-trafficking, gene disruption, and gene deletion constructs.*

GFP-trafficking construct. To detect the protein presence of PF11_0394 throughout the entire life cycle of the parasite, a PF11_0394/GFP-trafficking construct was made by cloning base pairs 28-759 (excluding the stop codon) of the open reading frame of the gene into the pPM2GT vector (obtained from MR4) (Klemba *et al.*, 2004). The primers used to amplify the region were 5'-ccgctcgagcgtcctttaagaaatggtg-3' forward and 5'-ccgcctaggtataatatttctattatcttcc-3' reverse. The restriction enzymes XhoI and AvrII (New England Biolabs) are underlined and were used for cloning into the pPM2GT vector. These primers were used to amplify a 732 bp product via PCR using 2.0 µl DNA (~100ng total), 1.0 µl FastStart High Fidelity Taq Polymerase (5U/µl Roche), 1X FastStart Buffer, 1 mM $MgCl_2$, 0.2 mM di-nucleotide tri-phosphate mix, and 0.5 µM primers. PCR conditions were as follows: an initial denaturing step of 95ºC for three minutes, 35 repetitive cycles of denaturing at 95ºC for 30 seconds, primer annealing at

54°C for 30 seconds, and an extension at 62°C for three minutes, and then a final extension at 62°C for 10 minutes.

The product was double-digested with XhoI and AvrII, along with the pPM2GT vector, separated via gel electrophoresis, gel-purified according to the manufacturer's instructions using QIAquick® Gel Extraction Kit (Qiagen), and ligated with T4 DNA Ligase (Promega) following the manufacturer's instructions. Two microliters of the ligation products were transformed into DH10B electrocompetant cells via electroporation and streaked on antibiotic resistant plates. Using colonies that grew on the plates, gDNA was isolated as previously described. The DNA was sequenced at the DNA Core Facility at the University of Missouri and aligned with the PF11_0394 sequence available from PlasmoDB using Vector NTI (Invitrogen) to confirm that the correct protein coding sequence was obtained.

Gene disruption construct. To assess a potential function for PF11_0394, a gene disruption construct was produced by cloning base pairs 38-700 of the open reading frame of the gene into the pHD22y *P. falciparum* disruption plasmid (obtained from MR4) (Fidock & Wellems, 1997). The primers used to amplify the region were 5'-gatcggatccgaaatggtggtactgctccact-3' forward and 5'-gatcctcgagcacggtagaaactgtagcaac-3' reverse. The restriction enzymes, BamHI and NotI (New England Biolabs) are underlined and were used for cloning into the pHD22y vector. These primers were used to amplify a 663 bp product via PCR using 2.0 µl DNA (~100ng total), 1.0 µl FastStart High Fidelity Taq

Polymerase (5U/µl, Roche), 1X FastStart Buffer, 1 mM $MgCl_2$, 0.2 mM di-nucleotide tri-phosphate mix, and 0.5 µM primers. PCR conditions were as follows: an initial denaturing step of 95ºC for three minutes, 35 repetitive cycles of denaturing at 95ºC for 30 seconds, primer annealing at 56°C for 30 seconds, and an extension at 62°C for three minutes, and then a final extension at 62°C for 10 minutes. The product and vector were double-digested with BamHI and NotI. Purification, ligation, transformation and sequencing of the final disruption construct were done as previously described for the GFP-trafficking construct.

Gene deletion constructs. To assess a potential function of PF11_0394, two gene deletion constructs were produced by cloning two 612 base pair sets of the 5' and 3' untranslated regions (UTRs) and open reading frames of the gene into both the pHHT-TK (obtained from MR4) and pCC-1 (a gift from D.A. Fidock) *P. falciparum* deletion vectors (Duraisingh *et al.*, 2002; Maier *et al.*, 2006). The primers used to amplify the 5' region were 5'-catgccatggggttatatattaagtaatgta-3' forward and 5'-ccggaattcggggaataaaaaaataagatat-3' reverse. The primers used to amplify the 3' region were 5'-ggactagtccgccaattcaattttagtt-3' forward and 5'-tcccccgcgggactttccacatttatata-3' reverse. The restriction enzymes NcoI and EcoRI (New England Biolabs) are underlined and were used for cloning the 5' region of PF11_0394 into both deletion vectors. The restriction enzymes SpeI and SacII (New England Biolabs) are underlined and were used for cloning the 3' region of PF11_0394 into both deletion vectors. These primers were used to amplify the two 612 bp products via PCR using 2.0 µl DNA (~100ng total), 1.0 µl FastStart High Fidelity Taq Polymerase (5U/µl, Roche), 1X FastStart Buffer, 1

mM $MgCl_2$, 0.2 mM di-nucleotide tri-phosphate mix, and 0.5 µM primers. PCR conditions were as follows: an initial denaturing step of 95ºC for three minutes, 35 repetitive cycles of denaturing at 95ºC for 30 seconds, primer annealing at 52°C for 30 seconds, and an extension at 62°C for three minutes, and then a final extension at 62°C for 10 minutes. The products and vectors were double-digested with the appropriate restriction enzymes mentioned above and the final gene deletion constructs were created as previously described.

*2.8. Transfection of parasites with trafficking, disruption, and deletion constructs.*

Transfections of *P. falciparum* were carried out according to Crabb *et al*., 2004. Before performing the transfections, mixed ES parasite cultures were synchronized with 5% D-sorbitol (Sigma) for 10 minutes followed by two washes with RPMI 1640 (Gibco) at 1,600 x g for five minutes two days before transfection. In addition, plasmid DNA was isolated using a Plasmid Maxi Kit (Qiagen) and equilibrated in CytoMix (120 mM KCl, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM $K_2HPO_4$ pH 7.6, and 25 mM HEPES pH 7.6). The synchronized *P. falciparum* ring stage NF54 parasites were electroporated (BTX 600, BTX Harvard Apparatus; 0.2 cm cuvette, 0.31 kV, 950 µF, maximum resistance) with 50 µg of the plasmid DNA in cytomix. Transfected *P. falciparum* cultures were maintained as previously described.

Two days following electroporation, media containing WR99210 (2.5 nM, Sigma) was added to the cultures to begin the process of selecting transfected parasites utilizing the human dihydrofolate reductase gene drug cassette present

in all constructs used (Crabb *et al.*, 2004). To enrich for GFP-trafficking and gene disruption recombinants (PF11_0394/GFP and PF11_0394/pHD22y) and eliminate episomal plasmids, parasites were subjected to at least three rounds of drug selection (three weeks on drug and three weeks off drug for each round). For gene deletion constructs (PF11_0394/pHHT-TK and PF11_0394/pCC-1), parasites were first positively selected with WR99210 (2.5 nM) until parasites were detected (roughly five weeks post-transfection) and then placed under negative selection (WR99210 is still used as well) with ganciclovir (4 µM Sigma, utilizing the thymidine kinase drug cassette) or 5-fluorocytosine (1 µM, gift from D.A. Fidock, utilizing the cytosine deaminase drug cassette), respectively (Crabb *et al.*, 2004; Duraisingh *et al.*, 2002; Maier *et al.*, 2006).

To obtain a clonal population of parasites with no presence of wild-type parasites carrying episomes, a limiting dilution was performed on the transfected parasites. Parasites were seeded in 96-well plates (200 µl volume) at two concentrations, 0.5 and 0.25 infected red blood cells per well, and maintained in a gassed modular incubator chamber (Billups-Rothenberg, Inc., 3% $O_2$, 3% $CO_2$, and 94% $N_2$) at 37°C. Cultures were gassed every other day for 20 days. On days 7, 14, and 17, 0.4% fresh red blood cells were added. On day 20, 150 µl of the parasite culture were transferred to a 96-well plate to begin gDNA isolation for use in PCR and Southern blot analysis to determine if clonal populations of parasites had been successfully created. To isolate the gDNA, 50 µl of 6% saponin (Sigma) was added to the 150 µl of cultures in the 96-well plate and incubated for five minutes at RT. The plate was centrifuged for 15 minutes at

2,650 x g and supernatant removed. One hundred microliters of 1X PBS was added to each well to wash the parasites and the plate was centrifuged as previously described. The 1X PBS was removed and 40 µl of down scale prep buffer (DSP, 1M Tris-Cl pH 8.0, 1M KCl, and 1M $MgCl_2$) working stock (985 µl DSP stock, 10 µl proteinase K, and 5 µl Tween 20) was added to each well and parasite pellets resuspended in the DSP solution (Adjalley *et al.*, 2010). The plate was incubated for 30 minutes at 50ºC and then for 10 minutes at 95ºC. The resulting gDNA was stored at 4ºC until further use. The remaining 50 µl of parasites were used for expansion and cryopreservation of promising clonal parasite populations.

*2.9. PCR and Southern blot verification of the GFP-trafficking construct.*

Integration of the transfected DNA at the correct location was verified for the PF11_0394/GFP clones by PCR and Southern blot analysis. To verify integration at the *PF11_0394* locus by PCR, the primers 5'-atgaaaatttttaattacatatgtg-3' *PF11_0394* gene specific forward primer and 5'-tccgtatgttgcatcacc-3' GFP reverse primer were used for the GFP-trafficking construct and 4.0 µl gDNA (isolated from the 96-well plates above), 1.25 units GoTaq® DNA Polymerase (Promega), 1X GoTaq® Flexi Buffer, 1 mM $MgCl_2$, 0.2 mM di-nucleotide tri-phosphate mix, and 0.5 µM primers were used. PCR conditions were as follows: an initial denaturing step of 95ºC for three minutes, 35 repetitive cycles of denaturing at 95ºC for 30 seconds, primer annealing at 52°C for 30 seconds and an extension at 62°C for three minutes and 30 seconds,

and then a final extension at 62°C for 10 minutes. The samples were all separated via gel electrophoresis (1% gel) and visualized via UV detection using EtBr.

Southern blotting was performed with gDNA isolated as previously described from ES parasites and the DIG Nonradioactive Nucleic Acid Labeling technology (Roche) was used for visualization of the DNA. Genomic DNA (2-3 µg) that was digested with SapI and KpnI were hybridized with a 732 bp fragment of *PF11_0394* created with the PCR DIG Probe Synthesis Kit following the manufacturer's instructions (Roche). Before hybridization, the DNA was separated on a 0.7% agarose gel and transferred to a positively charged nylon membrane (Osmonics) overnight via an upward transfer method. Following the manufacturer's instructions, the membrane was washed, hybridized with the above probes, and DNA products detected by autoradiography using the DIG Nucleic Acid Detection Kit (Roche).

*2.10. GFP-trafficking studies.*

The GFP-trafficking studies described below were done using two independent PF11_0394/GFP clones obtained via the limiting dilution process previously described. Each independent clone was used in a biological replicate, with a technical replicate conducted for each as well.

Mixed erythrocytic stages and gametocytes. Both *P. falciparum* mixed ES cultures and day 16 mixed gametocyte cultures were obtained by collecting 200 µl of infected blood from culture flasks. This protocol was done for all

experimental groups: PF11_0394/GFP, NF54 WT negative control parasites, and 3D7HT-GFP (obtained from MR4) positive control parasites (Talman *et al.*, 2010). The collected, infected blood was centrifuged for five minutes at 2,650 x g and the supernatant removed. The infected red blood cells (iRBCs) were resuspended in 200 µl 1X PBS containing DAPI nuclear stain (1:1,000 dilution, Invitrogen) and incubated in the dark for five minutes at RT. The iRBCs were centrifuged again for five minutes at 2,650 x g, washed once with 200 µl 1X PBS, centrifuged a final time for five minutes at 2,650 x g, and a small drop of the blood was placed on a slide. Coverslips were placed on the slides and they were viewed with a 100X objective using an Olympus BX51 inverted fluorescent microscope coupled with a X-Cite® Series 120 fluorescent light source. The entire slide was scanned, with at least 100 iRBCs and 50 gametocytes observed for each group.

Zygotes and ookinetes. For all experimental groups listed above, six midguts were dissected from *P. falciparum*-infected *An. stephensi* 24-30 hours PI. The midguts were placed in 1X PBS containing DAPI nuclear stain (1:1,000 dilution) and incubated at RT for five minutes. The midguts were then placed (three each) into 15 µl of Matrigel™ (BD Biosciences) and coverslips placed on top. The infected midguts were then viewed as previously described. Due to limited numbers, at least nine zygotes and five ookinetes were observed for each group.

Oocyst sporozoites. For all experimental groups listed above, six midguts were dissected from *P. falciparum*-infected *An. stephensi* 10 days PI. The midguts were placed in 1X PBS containing DAPI nuclear stain (1:1,000 dilution) and incubated at RT for five minutes. Three midguts were then placed into 15 µl of 1X PBS on a slide and coverslips placed on the slides. The infected midguts were then viewed as previously described. At least 75% of the midguts had infections with 3-18 oocysts per midgut for each group.

Hemolymph sporozoites. Hemolymph sporozoites were collected by perfusing the body cavity of 10 *P. falciparum*-infected *An. stephensi* 12 days PI with 1X PBS. Hemolymph was collected for all experimental groups described above by collecting it in microcentrifuge tubes containing 40 µl 1XPBS with DAPI nuclear stain (1:1,000 dilution). The sporozoites were concentrated by centrifugation at 18,000 x g for five minutes, supernatant removed, and 10 µl of sporozoites spotted on slides containing 10 µl of Matrigel™. Coverslips were placed on top of the slides and they were viewed as previously described. Due to the difficulty of isolating hemolymph sporozoites from *An. stephensi*, only 3-5 hemolymph sporozoites were observed for each group.

Salivary gland sporozoites. For all three experimental groups, six pairs of salivary glands were dissected from *P. falciparum*-infected *An. stephensi* 13-20 days PI. The glands were placed in 1X PBS containing DAPI nuclear stain (1:1,000 dilution) and incubated at RT for five minutes. The glands were then placed into 15 µl of 1X PBS on a slide and coverslips placed on top. The

infected salivary glands were then viewed as previously described. For each experimental condition, at least 75% of the salivary glands were infected with hundreds of sporozoites observed per sets of infected glands.

## 3. Results and Discussion

### *3.1. PF11_0394 sequence analysis.*

*PF11_0394* is a 762 bp gene on chromosome eleven, containing one intron, resulting in a 561bp cDNA product. The cDNA product encodes a 186 amino acid *Plasmodium falciparum* protein with an estimated molecular weight of 21,026 Daltons (Table 3.1). Initial PlasmoDB data, based upon mass spectrometry results and sequence analysis, suggested that the PF11_0394 protein was expressed by salivary gland sporozoites, has a signal anchor, and four transmembrane domains (amino acids 28-50, 65-84, 97-119, and 149-171) (Bahl *et al.*, 2003; Florens *et al.*, 2002). To confirm these data and obtain more information about PF11_0394, additional sequence analysis programs were utilized. SignalP revealed that PF11_0394 is predicted to have a non-cleavable signal anchor (Bendtsen *et al.*, 2004). Analysis using TargetP predicted that the protein enters the secretory pathway and, more specifically, is predicted to be a plasma membrane protein (located on the surface of the parasite) according to PSORTII and WoLF PSORT (Emanuelsson *et al.*, 2000; Nakai & Horton, 1999). Additional sequence analysis using PROSITE, PROTCOMP, Profam, and NCBI (BLASTp) sites predicted the protein has no GPI-anchor, has multiple

glycosylation and phosphorylation sites and has no functional identity with other known proteins (Altschul *et al.*, 1997; Gattiker *et al.*, 2002).

Next, PlasmoDB and results from a BLAST analysis identified orthologs of PF11_0394 in other *Plasmodium* species (Figure 3.1A). The PF11_0394 protein has homology with proteins in *P. vivax* (Pv = PVX_092525 in PlasmoDB), *P. knowlesi* (Pk = PKH_093600 in PlasmoDB), *P. reichenowi* (Pr = c000130608.contig1 in Sanger), *P. berghei* (Pb = PBANKA_091050 in PlasmoDB), *P. yoelii* (Py = PY06419 in PlasmoDB), *P. chabaudi* (Pc = PCHAS_071180 in PlasmoDB), and *P. gallinaceum* (Pg = c000315856.contig1 in Sanger). The PF11_0394 protein also has orthologs with other Apicomplexans (Figure 3.1B), including *Babesia bovis* (Bb = BBOV_I000760 in GenBank), *Theilaria parva* (Tp = XP_765419 in GenBank), *Cryptosporidium hominus* (Ch = Chro.30131 in CryptoDB), and *Toxoplasma gondii* (Tg = 38.m02365 in ToxoDB). The PF11_0394 protein does not appear to have any orthologs with other proteins from members outside of the Apicomplexan group. PF11_0394 appears to be a highly conserved protein within the genus *Plasmodium* (79.6-98% conserved when PF11_0394 is compared to its orthologs) and also conserved in several other Apicomplexans. Like PF11_0394, the proteins listed here are all predicted to have signal anchors (minus *P. yoelii*, whose sequence is not complete), enter the secretory pathway, and are predicted to be surface proteins or located in the endoplasmic reticulum. In addition, the proteins do not have any known function and/or known identity with known proteins, but based on their conserved identity it is predicted that they would have similar functions in

Apicomplexan biology. Since PF11_0394 does not have homology with any known human protein, it could be a good target candidate for a new drug and/or vaccine.

*3.2. PF11_0394 transcript is present throughout a majority of the P. falciparum life cycle.*

*PF11_0394* transcript is present in oocyst sporozoites (Figure 3.2A), salivary gland sporozoites (Figure 3.2B), mixed erythrocytic stages (ES, cultures containing a mixture of rings, trophozoites, and schizonts) (Figure 3.2C), and mixed gametocyte stages (Figure 3.2D, cultures containing a mixture of stage I-V gametocytes), as demonstrated via RT-PCR (561 bp transcript). For all experiments, the gDNA positive control amplified the correct intron-containing product (762 bp) and the negative control (no reverse transcriptase) did not amplify any products.

To determine if *PF11_0394* transcript is present during exoerythrocytic (liver) stages of the life cycle, *P. falciparum* liver cell invasion studies were originally attempted using the human HC-04 hepatoctye line (obtained via MR4) (Sattabongkot *et al.*, 2006); however, this cell line was eventually recalled by MR4 because it had been found to actually be a derivative of HepG2 cells, which do not support *P. falciparum* infections well. Consequently, primary human hepatocytes were utilized (Lonza). Unfortunately, when assessing if *PF11_0394* transcript was present during this stage of the life cycle, not only did *PF11_0394* primers not amplify a product, but the positive control (the *circumsporozoite*

*protein* gene) did not amplify a RT-PCR product either. Therefore, additional parasite control primers, the *P. falciparum heat shock protein-70* (*PfHsp70*) gene and *P. falciparum liver stage antigen-1* (*PfLSA*-1) gene, were used because these genes are known to be expressed during exoerythrocytic stages, but not sporozoite stages of the *Plasmodium* life cycle. The absence of RT-PCR products representing the liver stage parasites suggested that no exoerythrocytic stage parasites were produced, even though the human hepatocytes were viable as shown by the presence of the *human glyceraldehyde 3-phosphate dehydrogenase* gene transcript (Figure 3.3). Currently, more liver stage invasion experiments are in progress to try and generate exoerythrocytic stage parasites for transcriptional and protein analysis.

Since generating exoerythrocytic stage parasites using primary human hepatocytes was not successful, a method to produce exoerythrocytic stages using axenic cultures (without the presence of liver cells) also was utilized (Kappe *et al.*, 2010). Preliminary data using axenic exoerythrocytic stages and RT-PCR suggest that *PF11_0394* transcript is not present during exoerythrocytic stages (Figure 3.4). However, a transcript for the *PfHsp70* gene, used as a positive control, was detected, suggesting that exoerythrocytic stage parasites were indeed produced in the axenic cultures. For both *PF11_0394* and *PfHsp70*, gDNA was used as a positive control and a no reverse transcriptase reaction was used as a negative control.

In addition to data available on PlasmoDB, a literature search was conducted for PF11_0394 and its predicted orthologs in two rodent malaria

models, *P. berghei* and *P. yoelii* (Table 3.2), to assess previous transcript and protein detection data generated throughout the life cycle of the parasite. According to the literature and PlasmoDB, *PF11_0394* transcript is present during the salivary gland sporozoite stage and erythrocytic stages (specifically free merozoites, rings, trophozoites, schizonts, and gametocytes). Transcript results for exoerythrocytic stages are unresolved, with a study in *P. falciparum* indicating no transcript expression and two studies done in *P. berghei* confirming transcript expression (Bozdech *et al.*, 2003a; Bozdech *et al.*, 2003b; Florens *et al.*, 2002; Hall *et al.*, 2005; Isokpehi & Hide, 2003; Kappe *et al.*, 2001; Khan *et al.*, 2005; Lasonder *et al.*, 2002; Le Roch *et al.*, 2003; Llinas *et al.*, 2006; Rosinski-Chupin *et al.*, 2007; Siau *et al.*, 2008; Silvestrini *et al.*, 2005; Tarun *et al.*, 2008; Williams & Azad, 2010; Young *et al.*, 2005; Zhou *et al.*, 2008). Data obtained during these studies in *P. falciparum* for the salivary gland sporozoite stage, mixed erythrocytic stages, and mixed gametocyte stages confirms results in the literature. Preliminary results obtained for exoerythrocytic stage expression supports the *P. falciparum* literature that transcript is not present during this stage. The presence of *PF11_0394* transcript in oocyst sporozoites has not been described elsewhere and represents new knowledge about the *P. falciparum* transcriptome.

*3.3. The PF11_0394 protein is present in salivary gland sporozoites, as demonstrated by GFP-trafficking studies.*

Several attempts to produce PF11_0394 recombinant protein using a variety of approaches and generate antibodies against PF11_0394 were

employed, but were never successful (see Appendix 1 for a summary). Thus, PF11_0394/GFP clonal populations were generated for use in protein studies.

To assess the PF11_0394 protein detection profile throughout the entire life cycle of *P. falciparum* and confirm data found in the literature, a PF11_0394/GFP trafficking construct was created using the pPM2GT vector (obtained from MR4) and transfected into the genome of the parasite via homologous recombination technology (Figure 3.5A) (Klemba *et al.*, 2004). After a limiting dilution, two PF11_0394/GFP clones were isolated after initial PCR analysis (Figure 3.5B) confirmed that they had properly integrated into the genome of the parasite and the populations lacked the presence of WT parasites carrying episomes. These data were confirmed by Southern blot analysis (Figure 3.5C), as the predicted integration products of 10,454 base pairs and 6,602 base pairs were detected along with no presence of WT parasites carrying episomes. Using the two PF11_0394/GFP clones, two independent GFP-trafficking studies were completed (with two technical replicates each). WT parasites were used as a negative control and 3D7HT-GFP parasites (obtained from MR4) constitutively expressing GFP throughout the life cycle of the parasite were used as a positive control (Talman *et al.*, 2010). The various developmental stages of the parasite were examined using fluorescent microscopy (Figure 3.6). The stages observed were: mixed ES (contained a mixture of rings, trophozoites, and schizonts), mixed gametocytes (induced for 16 days and contained a mixture of stages I-V male and female gametocytes), zygotes (24 hours post infection, PI), ookinetes (24-30 hours PI), oocyst sporozoites (8-10 days PI), hemolymph sporozoites (12

days PI), and salivary gland sporozoites (13-20 days PI). The PF11_0394 protein was only present in salivary gland sporozoites (200 / 200 = 100% detection by salivary gland sporozoites counted, with hundreds of additional parasites observed as well). For all experiments, all of the 3D7HT-GFP parasites were positive for GFP expression and all of the WT parasites were negative (the parasites did not express GFP). These data confirm previous mass spectrometry results found by Florens *et al.* (2002) that the PF11_0394 protein is present in salivary gland sporozoites (Table 3.2) and now provide additional protein information for *P. falciparum* stages not previously studied, such as the zygote, ookinete, and hemolymph sporozoite (Florens *et al.*, 2002; Hall *et al.*, 2005; Khan *et al.*, 2005; Lasonder *et al.*, 2002; Tarun *et al.*, 2008).

Due to the difficulty of generating liver stage parasites *in vitro*, protein detection studies were not able to be conducted for PF11_0394 during this particular life cycle stage. Nevertheless, based on preliminary data suggesting transcript is not present during the exoerythrocytic stages, it is predicted that the PF11_0394 protein is not present during the liver stages.

## 4. Conclusions

In summary, data obtained from these studies demonstrate that PF11_0394 is a *P. falciparum* protein that has orthologs in other *Plasmodium* species and also has orthologs with other Apicomplexans. PF11_0394 does not have orthologs with any protein outside of the Apicomplexan group and shares no functional identity with other known proteins, making it a novel protein to study

in *P. falciparum* biology. Transcript detection studies determined that *PF11_0394* transcript is present throughout a majority of the life cycle of the parasite, including mixed ES, mixed gametocyte stages, oocyst sporozoites, and salivary gland sporozoites, but based upon preliminary data, it does not appear to be present during the exoerythrocytic stages. Protein detection studies demonstrated that the PF11_0394 protein is present in salivary gland sporozoites and not in other stages examined. Protein studies for PF11_0394 during the exoerythrocytic stages will have to be conducted in order to determine whether the protein is present during this stage of the life cycle.

These data collected for PF11_0394 have allowed us to determine that the PF11_0394 protein is present in salivary gland sporozoites and, thus, may be a protein needed for development/survival in the mosquito salivary glands and/or development/invasion within human host tissues. Based on its protein detection profile, PF11_0394 could be another candidate gene for a new drug and/or pre-erythrocytic stage vaccine since it does not share identity with any known human protein.

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| | |
|---|---|
| **Molecular Weight and Gene/Protein Size** | 21,062 Daltons<br>762 base pairs gDNA<br>561 base pairs cDNA<br>186 amino acids |
| **Intron(s)** | One<br>(Base pairs 216-416) |
| **SignalP** | Signal Anchor |
| **Transmembrane Domain(s)** | Yes: Four<br>(Amino Acids 28-50, 65-84, 97-119, and 149-171) |
| **TargetP** | Secretory Pathway |
| **PSORTII** | Membrane: Plasma |
| **WoLF PSORT** | Membrane: Plasma |
| **PROSITE** | 1. N-glycosylation site<br>2. Protein kinase C Phos Site<br>3. Casein kinase II Phos Site |

**Table 3.1. PF11_0394 is predicted to be a sporozoite surface protein based upon PlasmoDB data and additional sequence analysis.** Initial gene characterization for *PF11_0394* was performed by gathering information from PlasmoDB (Aurrecoechea *et al.*, 2009; Bahl *et al.*, 2002). In order to further characterize the gene and verify data from PlasmoDB, additional programs found on the ExPASy Bioinformatics Resource Portal (www.expasy.org) and SoftBerry (www.softberry.com) were utilized. Phos = phosphorylation.

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## A. Orthologs in Other *Plasmodium* Species

```
PF11_0394    (1) MKIFNYICGRPLRNGGTAPLIYNPVRKWLIILMILYICLSILSYLIFLFPKASDLQCLAL
Pv           (1) MKILNYICGRPLRSGGTAPLIYNPIRKWLIILMILYMCLSLLSYGVFLYPKASDLHCSPL
Pk           (1) MKILNYICGRPLRSAGTAPLIYNPIRKWLIILMTLYICLSLLSYGVFLYPKASDLHCSPL
Pb           (1) MRILNYICGRSLRSSGAAPLIYNPIQKLLIILTLLYICLSALSCSIFLFPKVSDLHCSPL
Py           (1) ------------------------------------------------------------
Pc           (1) MRILNYICGRSLRSSGAAPLIYNPIQKLLIILTLLYICLSVLSCCIFLFPKVSDLHCSPL
Pr           (1) MKIFNYICGRPLRNGGTAPLIYNPVRKWLIILMILYICLSILSYLIFLFPKASDLQCLAL
Pg           (1) MKIFNYICGRPLS-GGAAPLIYNPVRKWLIILMMLYICLSILSYGIFLFPKASDLQCSAL

PF11_0394   (61) IDSLFNFSLSIG------------VSYVMAPYYSIISCREWGTEYEWGIVAVVSAVMAIV
Pv          (61) IDSLFGFSLSIG------------ASNLMAPYYSVISCREWGTENEWSLVAIVSAVMAIV
Pk          (61) IDSLFSFSVSIG------------ASNLMAPYYSVISCREWGTENEWSVVAVVSAVMAII
Pb          (61) VDSLFNFYLSMG------------ASNVMAPYYSMISCREWGTEHEWVVVAIVSAVMAII
Py           (1) ----------------------------MAPYYSMISCREWGTENEWVVVAIVSAVMAII
Pc          (61) VDSLFSFYLSMG------------GSNVMAPYYSIISCREWGTETEWVIVATVTAVMAII
Pr          (61) IDSLFNFSLSIG------------VSYVMAPYYSIISCREWGTEYEWSIVAVVSAVMAIV
Pg          (60) INSLFNFSLSIGEKNVILLFFFQGVSNIMAPYFSIISCREWGTEHEWALVALVSAVMAII

PF11_0394  (109) DVLSSCYGIYVLYTITSVVFNKRIGMNNDCNSYNAVLFFSANSILVFLHLTVATVSTVVY
Pv         (109) DLSSSFYGIYVLCTIIDVVFTNISGMP-DCNCYKAIVFFFANATLVFLHLVVAIVSIVAY
Pk         (109) DLSSSFYGIYVLCTIIDVVFTNITGMP-DCTCYKAIVFFFANATLVFLHLVVAIVSIVVY
Pb         (109) DVSSSFYGIYVLYTIIDIVFTNITDMN-ECNCYKSIIFFSANAFLVVLHLIVAITSIVVY
Py          (33) DVSSSFYGIYVFKCFFSRSSFYCCYYKHSCVLYAYEKY----------------------
Pc         (109) DVTSSFYGIYLLYTIIDIVFTNITGMT-ECTCYKSIIFFSSNAFLVALHLIVAITSIVVY
Pr         (109) DVLSSCYGIYVLYTITSVVFNKRIGMNNDCNSYNAVLFFSANSILVFLHLTVATVSTVVY
Pg         (120) DVSSSCYGIYVLYTIIDVIFVNISGIHGDCNSYKAVLFFSANSFLVVLHLIVAIVCTVVF

PF11_0394  (169) FLLMKGIDKQLEDNRNII
Pv         (168) FLLMASIERQLEDNRNVI
Pk         (168) FVLMANIDKQLEDNRNVI
Pb         (168) YMLMKNIDKQLEDNRNII
Py          (71) ------------------
Pc         (168) YMLMKNIDKQLENNRNII
Pr         (169) FLLMKGIDKQWEDNRNII
Pg         (180) FLLMAGIDKQLEDNRNII
```

## B. Orthologs in Other Apicomplexans

```
PF11_0394    (1) MKIFNYICGRPLRNGGTAPLIYNPVRKWLIILMILYICLSILSYLIFLFPKASDLQCLAL
  B.bovis    (1) MKYNFVLWQRVKKRGGPVPLLRNPVKGWLQLLIFLHLFLLTLSCVTISFPYIYDLHCSIL
 T.parvum    (1) -MLILKSFKRPKG-GGPCPLIQNPVKKWLKYLSIFHIILLVMTCVSISFPFIHDLHCSIL
C.hominus    (1) -MFLNVLKLTPLG-GGPAPLLKNQIVRQCYSLMFLNIFIAIWSISSILYSKFTNANFGSE
 T.gondii    (1) -MMFKYLWSKPAG-GGPAPLISNPVKHWMVTLVALHLFLFAASCFTLAFPSITDMSCQML

PF11_0394   (61) IDSLFNFSLSIGVSYVMAPYYSIISCREWGTEYEWGIVAVVSAVMAIVDVLSSCYGIYVL
  B.bovis   (61) FNSMANIIASCVGSFVMAAYYTGIAAKDWGTETEWRITKGITVSMVVVDIAVSAWGIYAL
 T.parvum   (59) FNSLANLIASAICSTLMASYYIFVSNKDLGTENEWAITAIITITVVIIDISISGWGIYTF
C.hominus   (59) ISTYFFIVFLIG--SVNSIFFSKLGSLRYCSDSEWSNRLILILIINLVQYGAILWGCWIS
 T.gondii   (59) MVNSAYCAACGGVAFIMLFYFSVLSCQTWGTEQYWTIAAVVTLSMAFVDIVAAGWGIYVF

PF11_0394  (121) YTITSVVFNKRI---GMNNDCNSYNAVLFFSANSILVFLHLTVATVSTVVYFLLMKGIDK
  B.bovis  (121) TNASVKIYKALHNSLELEPECLQFKAALFYFAAFGVIFLHLIVAATCAVVAALLGKGIKR
 T.parvum  (119) TNSSFKIYKALHDEFQVEPDCLQTKARVFYISGFIVICLHIFVAGTGLATAHTDLSS---
C.hominus  (117) YKVFMSRYLAGTKMYYYFIFSRWMYNTLFGMTAFFSFSVIYTSFIYSKLLVSLTHQRVL-
 T.gondii  (119) IEATTNLHEVDQ---ETQVV----------------------------------------

PF11_0394  (178) QLEDNRNII
  B.bovis  (181) QLIDIRDMY
 T.parvum  (176) ---------
C.hominus  (176) ---------
 T.gondii  (136) ---------
```

**Figure 3.1. *Plasmodium falciparum* protein, PF11_0394, has orthologs in other *Plasmodium* species (A) and other Apicomplexans (B).** BLAST analysis of PF11_0394 showed that the protein has orthologs in other *Plasmodium* species (A), as well as other Apicomplexans (B); however, PF11_0394 does not have orthologs with any proteins from members outside of the group Apicomplexa. Amino acids highlighted in yellow are identical in all proteins and amino acids highlighted in blue are similar in a majority of the proteins. The PF11_0394 protein has homology with proteins in *P. vivax* (Pv = PVX_092525 in PlasmoDB), *P. knowlesi* (Pk = PKH_093600 in PlasmoDB), *P. berghei* (Pb = PBANKA_091050 in PlasmoDB), *P. yoelii* (Py = PY06419 in PlasmoDB), *P. chabaudi* (Pc = PCHAS_071180 in PlasmoDB), *P. reichenowi (*Pr = reich166f05 in Sanger), and *P. gallinaceum* (Pg = Pgal0546c06 in Sanger). The PF11_0394 protein also has orthologs with other Apicomplexans, including *Babesia bovis* (Bb = BBOV_I000760 in GenBank), *Theilaria parva* (Tp = XP_765419 in GenBank), *Cryptosporidium hominus* (Ch = Chro.30131 in CryptoDB), and *Toxoplasma gondii* (Tg = 38.m02365 in ToxoDB). The solid black line indicates the predicted signal anchor sequence of PF11_0394.

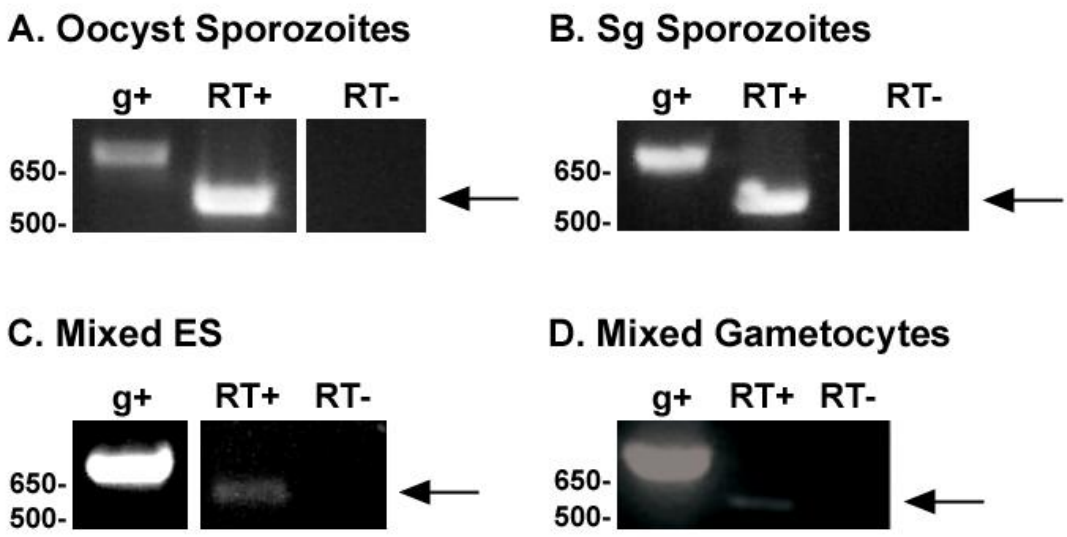


**Figure 3.2. *PF11_0394* transcript is present in *P. falciparum* oocyst sporozoites (A), salivary gland sporozoites (B), mixed erythrocytic stages (C), and mixed gametocyte stages (D) as shown by RT-PCR.** Primers specific for *PF11_0394* were used to amplify cDNA fragments of the correct product size (RT+), indicating the expression of the gene during the oocyst sporozoite stage, salivary gland sporozoite stage, mixed erythrocytic stages (containing a mixture of rings, trophozoites, and schizonts), and mixed gametocyte stages (containing a mixture of stage I-V gametocytes). Genomic DNA (g+) was used as a positive control and a no reverse-transcriptase (RT-) reaction was used as a negative control to show that the RNA was not contaminated with gDNA. *PF11_0394* contains an intron, resulting in the size difference between gDNA and cDNA, and the arrows indicate the *PF11_0394* RT-PCR products. Sg = salivary gland and ES = erythrocytic stages.

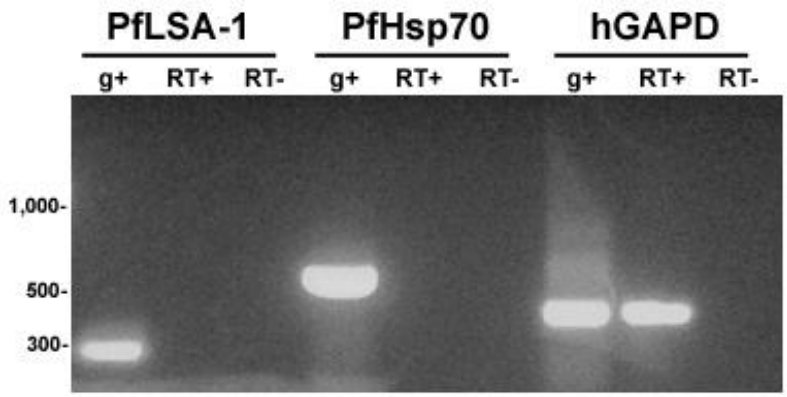


**Figure 3.3. Initial attempts to generate exoerythrocytic stage parasites using primary human hepatocytes were not successful.** RT-PCR with gene specific primers did not detect transcripts for either *P. falciparum liver stage antigen-1* (PfLSA-1) or the *P. falciparum heat shock protein-70* (PfHsp70) gene, suggesting that exoerythrocytic stage parasites were not present following a liver stage invasion experiment. To verify that intact hepatocyte RNA was isolated from the primary human hepatocytes, primers specific to *human glyceraldehyde 3-phosphate dehydrogenase* (hGAPD) were utilized and amplified transcript of the proper size indicating that at least the liver cells used were viable at the time of isolation. Genomic DNA (g+) was used as a positive control for all primer sets and reactions without reverse transcriptase (RT-) were used as negative controls, confirming there was no genomic DNA contamination of the RNA. RT+ = cDNA generated from isolated RNA.

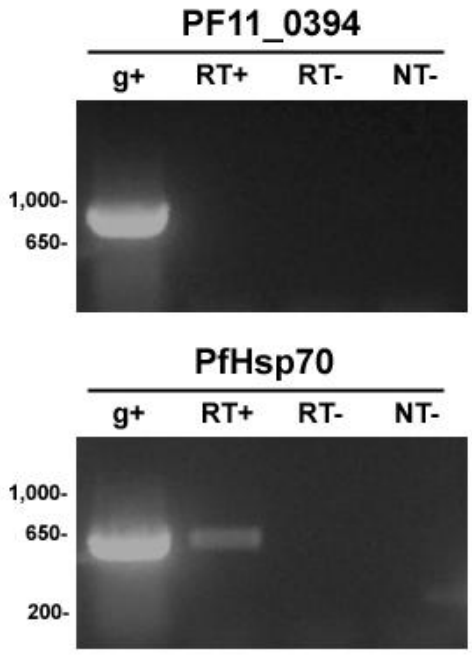


**Figure 3.4. Preliminary RT-PCR data suggest that *PF11_0394* transcript is not present during axenic *P. falciparum* exoerythrocytic stages as compared to parasite control transcript that was detected.** Primers specific for *PF11_0394* were used to amplify cDNA fragments (RT+) and no product was amplified, indicating that *PF11_0394* transcript is not present during the exoerythrocytic stage of infection produced from axenic cultures (*i.e*., exoerythrocytic parasites generated without the presence of hepatocytes). Primers specific to the *P. falciparum heat shock protein-70* (Hsp70) gene were used as a positive control to verify the presence of *P. falciparum* exoerythrocytic stages. Genomic DNA (gDNA) was used as a positive control for both primer sets and a no reverse-transcriptase (RT-) reaction was used as a negative control to show that the RNA was not contaminated with gDNA.

| PF11_0394 | | | |
|---|---|---|---|
| | ***P. falciparum*** | ***P. berghei*** | ***P. yoelii*** |
| **Gametes** | **No-P**<br>Lasonder *et al*., 2002 | **No Data Available** | **No Data Available** |
| **Zygotes** | **No Data Available** | **No Data Available** | **No Data Available** |
| **Ookinetes** | **No Data Available** | **No-P**<br>Hall *et al*., 2005 | **No-P**<br>Hall *et al*., 2005 |
| **Oocyst Sporozoites** | **No Data Available** | **No-P**<br>Hall *et al*., 2005 | **No-P**<br>Hall *et al*., 2005 |
| **Salivary Gland Sporozoites** | **Yes-T and P**<br>Florens *et al*., 2002<br>Le Roch *et al*., 2003 | **No-P**<br>Hall *et al*., 2005 | **No-P**<br>Hall *et al*., 2005 |
| **Liver Stages** | **No-T**<br>Siau *et al*., 2008 | **No Data Available** | **Yes-T / No-P**<br>Tarun *et al*., 2008<br>**Yes-T**<br>Williams *et al*., 2010 |
| **Free Merozoites** | **Yes-T / No-P**<br>Florens *et al*., 2002<br>Le Roch *et al*., 2003<br>Bozdech *et al*., 2003 | **No Data Available** | **No Data Available** |
| **Rings** | **Yes-T**<br>Le Roch *et al*., 2003<br>Bozdech *et al*., 2003 | **No-P**<br>Hall *et al*., 2005 | **No-P**<br>Hall *et al*., 2005 |

| | | | |
|---|---|---|---|
| **Trophozoites** | **Yes-T / No-P**<br><br>Florens *et al*., 2002<br>Lasonder *et al*., 2002<br>Le Roch *et al*., 2003<br>Bozdech *et al*., 2003 | **No-P**<br><br>Hall *et al*., 2005 | **No-P**<br><br>Hall *et al*., 2005 |
| **Schizonts** | **Yes-T**<br><br>Lasonder *et al*., 2002<br>Le Roch *et al*., 2003<br>Bozdech *et al*., 2003 | **No-P**<br><br>Hall *et al*., 2005 | **No-P**<br><br>Hall *et al*., 2005 |
| **Gametocytes** | **Yes-T / No-P**<br><br>Florens *et al*., 2002<br>Lasonder *et al*., 2002<br>Le Roch *et al*., 2003<br><br>**No-T**<br><br>Young *et al*., 2005 | **No-P**<br><br>Khan *et al.*, 2005<br>Hall *et al*., 2005 | **No-P**<br><br>Hall *et al*., 2005 |

**T = transcript and P = protein**

**Table 3.2. Transcript and protein detection patterns of PF11_0394 throughout the life cycle of *Plasmodium* based on data obtained from the literature.** Initial literature searches for PF11_0394 and its orthologs in *P. berghei* and *P. yoelii* (two rodent malaria parasites) indicate that *PF11_0394* transcript is present in salivary gland sporozoites, exoerythrocytic stages, and erythrocytic stages (including gametocytes), with its protein only present in salivary gland sporozoites (Bozdech *et al.*, 2003a; Bozdech *et al.*, 2003b; Florens *et al.*, 2002; Hall *et al.*, 2005; Khan *et al.*, 2005; Lasonder *et al.*, 2002; Le Roch *et al.*, 2003; Siau *et al.*, 2008; Tarun *et al.*, 2008; Williams & Azad, 2010; Young *et al.*, 2005).

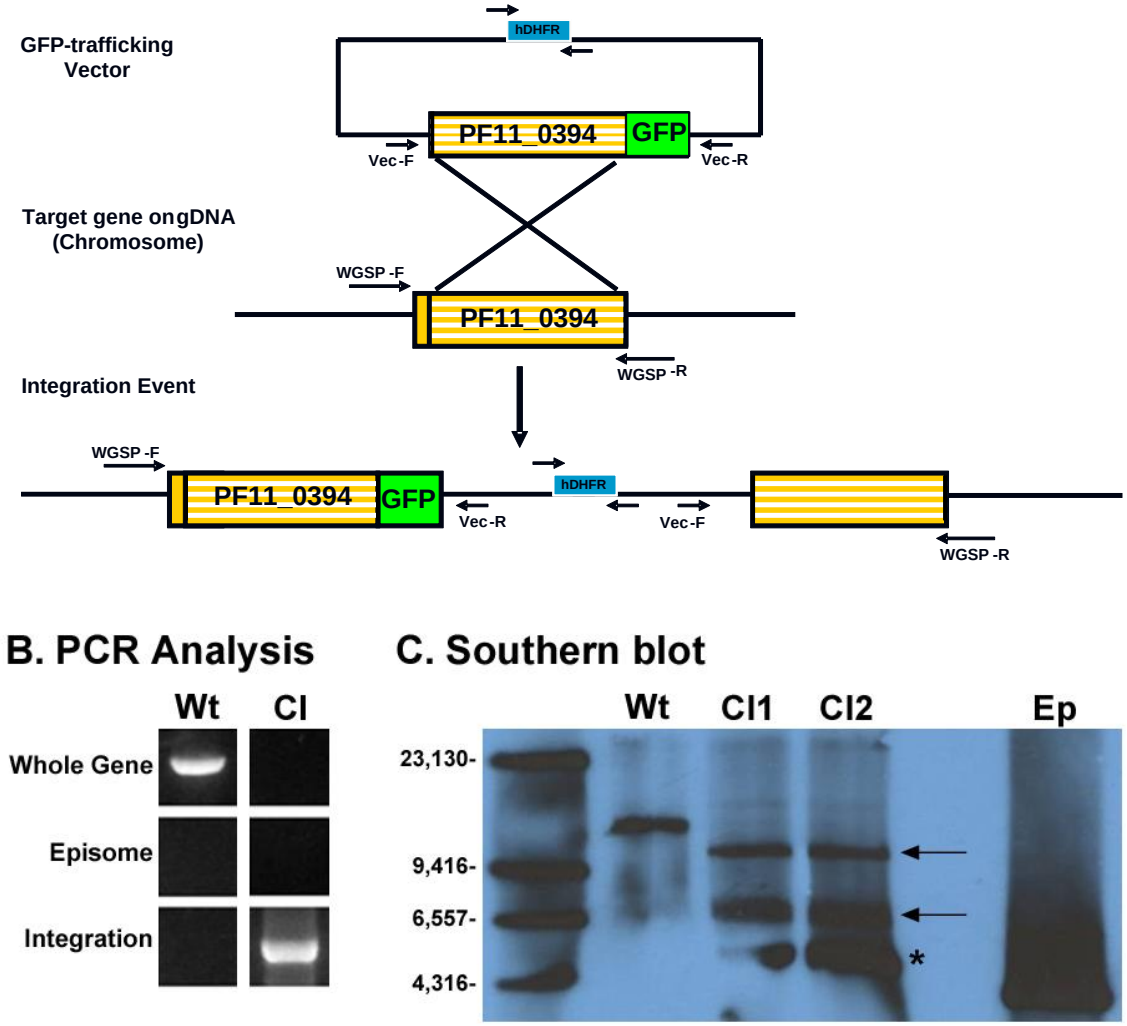


**Figure 3.5. Two PF11_0394/GFP clonal parasite populations have been successfully created for trafficking studies throughout the entire life cycle of the parasite.** A) Transfection schematic demonstrating how the PF11_0394/GFP construct was created using the pPM2GT vector (obtained from MR4) and successfully incorporated into the genome of the parasite via homologous recombination (Klemba *et al.*, 2004). Expression of GFP is driven

by the endogenous promoter of *PF11_0394*. Generation of two PF11_0394/GFP clonal parasite populations were verified by both PCR analysis (B) and Southern blot analysis using digoxigenin (DIG) technology coupled with autoradiography (C). Arrows indicate the predicted integration products of 10,454 base pairs and 6,602 base pairs using a PF11_0394 specific probe after restriction digestion with SapI and KpnI. The product indicated by an asterisk is of unknown origin and is likely a rearrangement of the plasmid that occurred after transfection. The drug cassette within the vector used for positive selection is human dihydrofolate reductase (hDHFR). GFP = Green fluorescent protein, Wt = Wild-type parasite genomic DNA, Cl = Clonal PF11_0494/GFP parasite genomic DNA, and Ep = PF11_0394/GFP plasmid DNA representing the episome. The arrows on the transfection schematic represent primers used for PCR analysis.

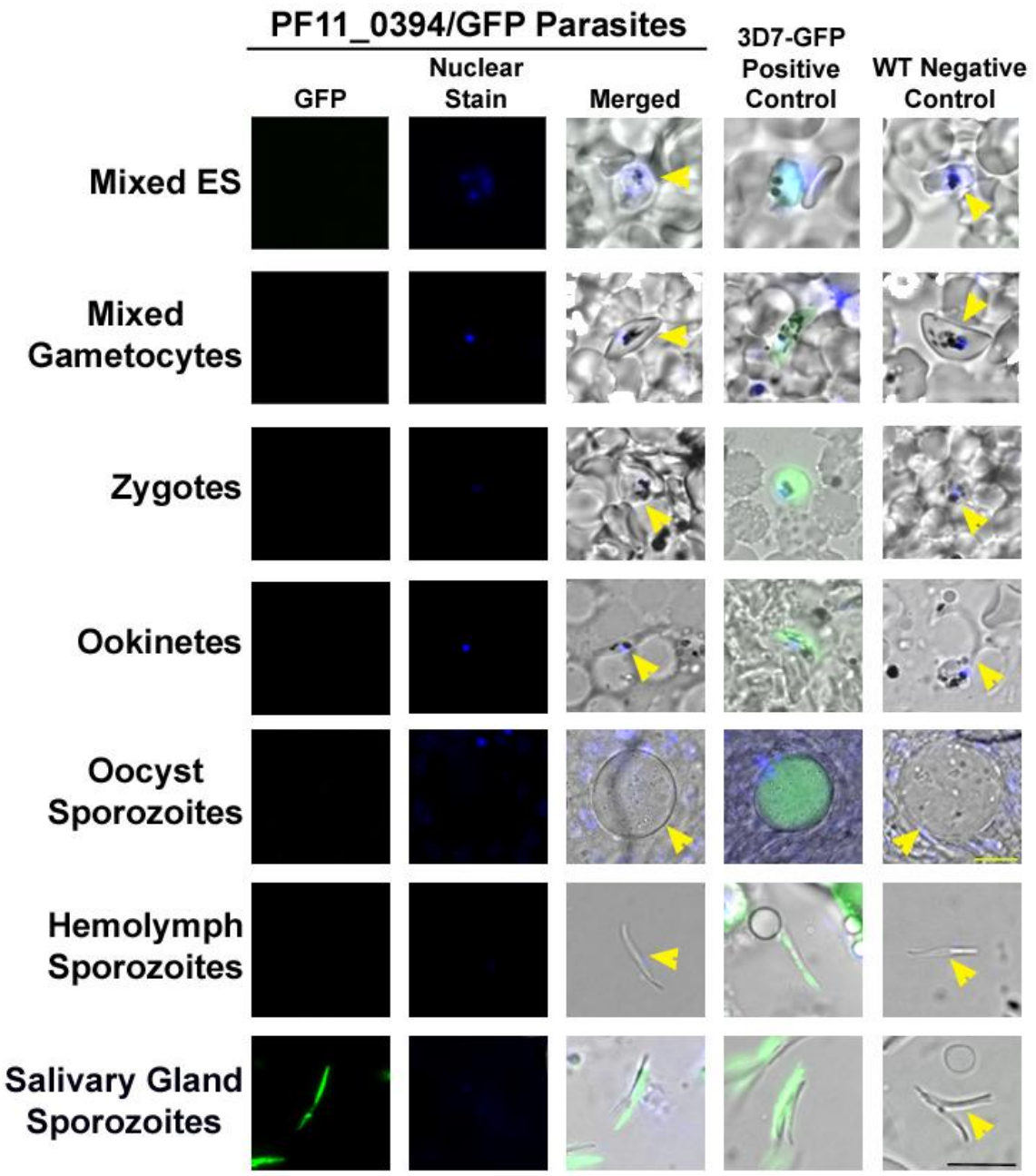


**Figure 3.6. PF11_0394/GFP-trafficking studies demonstrate that the PF11_0394 protein is present in salivary gland sporozoites.** The PF11_0394 protein is not detected during mixed erythrocytic stages (ES, culture representing a mixture of rings, trophozoites, and schizonts), mixed gametocytes (culture representing a mixture of stage I-V gametocytes), zygotes, ookinetes, oocyst

sporozoites, and hemolymph sporozoites. The PF11_0394 protein is detected by salivary gland sporozoites. 3D7HT-GFP (3D7-GFP) constitutively expressing parasites were used as a positive control (Talman *et al.*, 2010) and wild-type (WT) parasites were used as a negative control. This figure is a representative image from two biological replicates (one with each independent clone created) with two technical replicates each. Yellow arrowheads depict the presence of parasites that lack GFP expression (merged images). GFP = green fluorescent protein and nuclear stain = DAPI. Yellow arrowheads depict the presence of parasites that lack GFP expression (merged images). GFP = green fluorescent protein and the nuclear stain used was DAPI. The black scale bar in the lower right represents all of the images (except for the oocyst sporozoite stage) and is 10 µm. The yellow scale bar for the oocysts is 20 µm.

# Chapter 4: Initial Characterization of PBANKA_111090 and PBANKA_091050, *Plasmodium berghei* orthologs of PFE0565w and PF11_0394


## ABSTRACT

Using data mining techniques, the *P. falciparum* genes, PFE0565w and PF11_0394, were chosen as candidates for study due to their potential role in the invasion of host tissues. These genes were selected based on data from PlasmoDB, the *Plasmodium* database, indicating that these genes likely encode putative surface proteins and are expressed both at the transcriptional and protein levels in sporozoites. *P. falciparum* is a difficult organism to work with under laboratory conditions compared to its rodent malaria counterpart, *P. berghei*. Therefore, as a complementary study, the *P. berghei* orthologs of PFE0565w and PF11_0394, PBANKA_111090 and PBANKA_091050, respectively, were characterized. Results obtained for *P. falciparum* studies (Chapter 2 and Chapter 3) were compared/contrasted to preliminary data generated for their *P. berghei* orthologs.



**Keywords:** *Plasmodium berghei*, ortholog, comparative study

## 1. Introduction

*P. falciparum* is a difficult organism to work with in laboratory settings because experimental techniques, such as gene disruption/deletion processes, are very time consuming and the establishment of infections in mosquitoes is difficult and inconsistent. On the other hand, *P. berghei* is a rodent malaria model that is much easier to use under lab settings since the techniques used with this species are better established and faster to complete. Thus, *P. berghei* parasites are a good complementary system to use along with working with *P. falciparum.* Therefore, in addition to characterizing the *P. falciparum* genes described earlier in this dissertation, *PFE0565w* and *PF11_0394*, their orthologs in *P. berghei*, *PBANKA_111090* and *PBANKA_091050*, were characterized. Data obtained from these studies have allowed for the preliminary comparison of data generated from their *P. falciparum* orthologs.

## 2. Materials and Methods

### *2.1. Parasite and mouse strains used.*

*Plasmodium berghei* strain ANKA clone cl15cy1 (obtained from the Malaria Research and Reference Reagent Resource Center, MR4) and male Swiss Webster mice (Charles River) were used for the following experiments (Janse *et al.*, 2006; Janse *et al.*, 1986). The mice were maintained on a 12 hour light/dark cycle in an animal facility at the University of Missouri. All mice had access to food and water *ad libitum*. All experiments described throughout this

chapter were approved by the Institutional Biosafety Committee (IBC), the Institutional Review Board (IRB), and the University of Missouri Institutional Animal Care and Use Committee (IACUC).

*2.2. Selection of candidate genes, PBANKA_111090 and PBANKA_091050.*

An *in silico* data mining procedure was used to select the *P. falciparum* orthologs of *PBANKA_111090* and *PBANKA_091050, PFE0565w* and *PF11_0394*, respectively, as previously described in Chapters 2 and 3. After those genes were identified, the *Plasmodium* database, PlasmoDB, was used to identify their *P. berghei* orthologs, *PBANKA_111090* and *PBANKA_091050* (Aurrecoechea *et al.*, 2009; Bahl *et al.*, 2002).

*2.3. PBANKA_111090 and PBANKA_091050 sequence analyses.*

Using PlasmoDB, the full genomic DNA (gDNA), complimentary DNA (cDNA), and protein sequence of both *PBANKA_111090* and *PBANKA_091050* were obtained. Their protein sequences were aligned with their *P. falciparum* orthologs using Vector NTI (Explorer or Contig Express, Invitrogen). Additional sequence information for both *PBANKA_111090* and *PBANKA_091050* was obtained by using software programs such as TargetP, SignalP, PSORT II, WoLF PSORT, and PROSITE, all found via the ExPASy Bioinformatics Resource Portal (www.expasy.org).

*2.4. Isolation of P. berghei-infected tissues for transcript detection studies.*

Oocyst sporozoites. *An. stephensi* infected with *P. berghei* (obtained from MR4) were used 10 days post-infection (PI), when oocyst sporozoites were mature using our laboratory conditions. Fifty *An. stephensi* midguts were dissected from the mosquitoes, placed in 50 µl 1X phosphate buffered saline (10X PBS, 0.2 M phosphate buffer and 1.5 M NaCl pH 7.0, diluted 1:10 with Millipore water) in microcentrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80°C until needed for RNA isolation.

Salivary gland sporozoites. *An. stephensi* infected with *P. berghei* (obtained from MR4) were used 21 days post-infection (PI), when salivary gland sporozoites reside in the glands using our laboratory conditions. Fifty sets of *An. stephensi* salivary glands were dissected from the mosquitoes, placed in 50 µl 1X PBS in microcentrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80°C until needed for RNA isolation.

Mixed erythrocytic stages. Five naïve Swiss Webster mice were intraperitoneally (IP) injected with 0.1 ml of thawed *P. berghei* strain ANKA clone cl15cy1. On day five PI, mice were anesthetized and parasites collected by cardiac puncture into centrifuge tubes containing ~200 µl heparin (1,000 USP Units/ml, APP Pharmaceuticals). The infected blood was centrifuged at 2,650 x g for five minutes, supernatant removed, and the infected red blood cells were lysed with 0.05% saponin (Invitrogen) in 1X PBS for three minutes at room temperature (RT). The parasites were collected by an additional centrifugation

step for five minutes at 2,650 x g. Purified parasites were then washed once with 1X PBS and collected again by centrifugation as previously described. The parasite pellets were stored at -80°C until needed for RNA isolation.

*2.5. RNA/DNA isolation and transcriptional analysis by reverse transcription-polymerase chain reaction (RT-PCR).*

Total RNA was isolated from *Plasmodium*-infected tissues using a Trizol reagent-based protocol, following the manufacturer's instructions (Invitrogen). The samples were all DNase-treated (Promega), according to the manufacturer's instructions, to remove any contaminating gDNA. Approximately 2-3 µg of the DNase-treated RNA was used to synthesize cDNA using OligoDT primers from a SuperScript™ III First-Strand Synthesis System (Invitrogen), using manufacturer's instructions.

Genomic DNA was isolated following the manufacturer's instructions using a DNeasy® Blood and Tissue Kit (Qiagen) and was used as a positive control for all RT-PCR experiments. *PBANKA_111090* full-length gene specific primers (5'-atggatatattatattttaggaatttc-3' forward and 5'-ttatacatgagtactattatcatag-3' reverse) were used to amplify a 1,710 base pair (bp) fragment and *PBANKA_091050* full-length gene specific primers (5'-atgagaattcttaattatatatgtgg-3' forward and 5'-tcatataatgtttctattatcttc-3' reverse) were used to amplify a 701 bp gDNA fragment and a 558 bp cDNA fragment in a polymerase chain reaction (PCR) using 2.0 µl gDNA (~100ng total)/cDNA (~1/10 the volume synthesized above), 1.25 units GoTaq® DNA Polymerase (Promega), 1X GoTaq® Flexi Buffer, 1 mM $MgCl_2$, 0.2

mM di-nucleotide tri-phosphate mix, and 0.5 µM primers. PCR conditions were as follows: an initial denaturing step of 95ºC for three minutes, 35 repetitive cycles of denaturing at 95°C for 30 seconds, primer annealing at 54°C for 30 seconds and an extension at 62°C for three minutes, and then a final extension at 62°C for 10 minutes (Su *et al.*, 1996). After PCR amplification, all samples were separated on a 1% agarose gel and stained with ethidium bromide (EtBr) for UV detection. The experiment with oocyst sporozoites and salivary gland sporozoites was done one time and the experiments with mixed erythrocytic stages were done in biological duplicates.

*2.6. Creation of GFP-trafficking and gene deletion constructs.*

GFP-trafficking constructs. To analyze the protein expression pattern of both PBANKA_111090 and PBANKA_091050 throughout the entire life cycle of the parasite, both PBANKA_111090/GFP and PBANKA_091050/GFP-trafficking constructs were made by cloning base pairs 727-1,707 of PBANKA_111090 and the entire open reading frame of *PBANKA_091050* (excluding the stop codons) into the pL0031 vector (obtained from MR4) (Kooij *et al.*, 2005). The primers used to amplify the region of *PBANKA_111090* were 5'-agtgcggccgccctaaaattaaatgctcaaataa-3' forward and 5'-cgggatcctacatgagtactattatcatag-3' reverse and the primers used to amplify the region of PBANKA_091050 were 5'-agtgcggccgcatgagaattcttaattatatatgtgg-3' forward and 5'-cgggatcctataatgtttctattatcttctaattg-3' reverse. The restriction enzymes NotI and BamHI (New England Biolabs) are underlined and were used

for cloning into the pL0031 vector. These primers were used to amplify a 981 bp product and 701 bp product via PCR, respectively, using 2.0 µl DNA (~100ng total), 1.0 µl FastStart High Fidelity Taq Polymerase (5U/µl, Roche), 1X FastStart Buffer, 1 mM $MgCl_2$, 0.2 mM di-nucleotide tri-phosphate mix, and 0.5 µM primers. PCR conditions were as follows: an initial denaturing step of 95ºC for three minutes, 35 repetitive cycles of denaturing at 95ºC for 30 seconds, primer annealing at 54°C for 30 seconds, and an extension at 62°C for three minutes, and then a final extension at 62°C for 10 minutes.

The products were double-digested with NotI and BamHI, along with the pL0031 vector, separated via gel electrophoresis, gel-purified according to the manufacturer's instructions using QIAquick® Gel Extraction Kit (Qiagen), and ligated with T4 DNA Ligase (Promega) following the manufacturer's instructions. Two microliters of the ligation products were transformed into DH10B electrocompetant cells via electroporation and streaked on antibiotic resistant plates. Using colonies that grew on the plates, gDNA was isolated as previously described. The DNA was sequenced at the DNA Core Facility at the University of Missouri and aligned with either the PBANKA_111090 or PBANKA_091050 sequences available on PlasmoDB using Vector NTI (Invitrogen) to confirm that the correct protein coding sequences were obtained.

Gene deletion constructs. To assess potential functions of PBANKA_111090 and PBANKA_091050, gene deletion constructs were produced by cloning portions of the 5' and 3' untranslated regions (UTRs) and

open reading frames of the genes into pL0001 (the following reagent was obtained through the MR4 as part of the BEI Resources Repository, NIAID, NIH: *Plasmodium berghei* pL0001, MRA-770, deposited by AP Waters). The *PBANKA_111090* primers used to amplify the 788 bp 5' region were 5'-tcccgcggttcttttttcttcagaactttgc-3' forward and 5'-ggactagttttcttcaccttgttcataatc-3' reverse. The primers used to amplify the 839 bp 3' region were 5'-cccaagctttatgaaggaaaataacaataacg-3' forward and 5'-ggggtaccggataattatataatcaaatttgg-3' reverse. The *PBANKA_091050* primers used to amplify the 728 bp 5' region were 5'-tcccgcggtaattataatttaaaagataaagcc-3' forward and 5'-ggactagtaaaaatatactacatgatagagc-3' reverse. The primers used to amplify the 730 bp 3' region were 5'-cccaagcttatatagtatttacaaatattacgg-3' forward and 5'-ggggtaccttctaactgctatatttggaac-3' reverse. The restriction enzymes SacII and SpeI (New England Biolabs) are underlined and were used for cloning the 5' regions into the deletion constructs. The restriction enzymes HindIII and KpnI (New England Biolabs) are underlined and were used for cloning the 3' regions into the deletion constructs. These primers were used to amplify the DNA products via PCR using 2.0 µl DNA (~100ng total), 1.0 µl FastStart High Fidelity Taq Polymerase (5U/µl, Roche), 1X FastStart Buffer, 1 mM $MgCl_2$, 0.2 mM di-nucleotide tri-phosphate mix, and 0.5 µM primers. PCR conditions were as follows: an initial denaturing step of 95ºC for three minutes, 35 repetitive cycles of denaturing at 95ºC for 30 seconds, primer annealing at 52°C for 30 seconds, and an extension at 62°C for three minutes, and then a final extension at 62°C for 10

minutes. The products and vectors were double-digested with the appropriate restriction enzymes mentioned above and the final gene deletion constructs were created as previously described for the GFP-trafficking constructs.

*2.7. Transfection of parasites with GFP-trafficking and gene deletion constructs.*

Transfections of *P. berghei* strain ANKA clone cl15cy1 were carried out according to Janse *et al*. (2006) using Swiss Webster male mice age six weeks. Briefly, prior to transfections, ~10 µg each of PBANKA_111090/GFP, PBANKA_111090/pL0001, PBANKA_091050/GFP, and PBANKA_091050/pL0001 plasmid DNA was isolated using a Plasmid Maxi Kit (Qiagen), ethanol precipitated into a 10 µl volume in Millipore water, and stored at -20ºC until use. To begin the transfection process, naïve mice were infected with *P. berghei* and purified schizonts were collected after approximately six days using a 50% Nycodenz-PBS solution (Janse *et al.*, 2006). While the schizonts were being collected, 100 µl Nucleofector 88A6 solution (Amaxa™ Basic Parasite Kit 2, Lonza) was added to each of the 10 µg plasmid DNA samples and then resuspended with the collected schizonts. These mixtures were transfered to electroporation cuvettes and constructs transfected into the parasites using an Amaxa Nucleofector (Lonza) device using program U33 following the manufacturer's instructions. Fifty microliters of warm medium was immediately mixed with the transfection mixtures (150 µl volume) and injected into the tail veins of naïve mice. One day after infection, the mice were provided drinking water containing pyrimethamine (7mg/ml in DMSO diluted 100 times with tap

water, pH 3.5-5.0) to begin the drug selection process of successfully transfected parasites. The water was provided until the collection of the parasites (generally after 7-9 days PI) via cardiac puncture (Janse *et al.*, 2006).

To obtain a clonal population of parasites lacking the presence of wild-type parasites carrying an episome, a limiting dilution was performed on the transfected parasites. The limiting dilutions were conducted according to an online protocol provided by Janse *et al*. (2004). Briefly, naïve mice were IP-injected with each drug-selectable construct (PBANKA_111090/GFP, PBANKA_111090/pL0001, PBANKA_091050/GFP, and PBANKA_091050/pL0001). When parasites developed, the parasitemia was determined via Giemsa (Sigma)-stained blood films. Using mice with a 0.3-1.0% parasitemia, 5 µl of infected blood was collected to determine the number of red blood cells using a hemocytometer. Once both the parasitemias and red blood cell counts on the mice were complete, the blood was diluted such that two parasites were present per 200 µl of medium. This mixture was then injected into the tail veins of 15-20 naïve mice. At day nine PI, the parasitemias of these mice were determined and parasites collected from those with detectable parasites using cardiac puncture.

*2.8. PCR verification of the GFP-trafficking and gene deletion constructs.*

Integration of the transfected DNA at the correct location was verified for PBANKA_111090/GFP and PBANKA_091050/GFP by PCR analysis. To confirm integration at the *PBANKA_111090* locus by PCR, the primers 5'-

atggatatattatattttaggaatttc-3' *PBANKA_111090* gene specific forward primer and 5'-tagtggaagtgggagagg-3' pL0031 reverse primer were used. To verify integration at the *PBANKA_091050* locus by PCR, the primers 5'-gttacctttagctaagtttcc-3' *PBANKA_091050* chromosome specific forward and 5'-tagtggaagtgggagagg-3' pL0031 reverse were utilized. PCR was conducted for the GFP-trafficking construct using 2.0 µl gDNA (~100ng total), 1.25 units GoTaq® DNA Polymerase (Promega), 1X GoTaq® Flexi Buffer, 1 mM $MgCl_2$, 0.2 mM di-nucleotide tri-phosphate mix, and 0.5 µM primers. PCR conditions were as follows: an initial denaturing step of 95ºC for three minutes, 35 repetitive cycles of denaturing at 95ºC for 30 seconds, primer annealing at 52°C for 30 seconds and an extension at 62°C for three minutes and 30 seconds, and then a final extension at 62°C for 10 minutes. The samples were all separated via gel electrophoresis (1% gel) and visualized via UV detection using EtBr.

Integration of the transfected DNA at the correct location was verified for the PBANKA_111090/pL0001 and PBANKA_091050/pL0001 gene deletion populations by PCR. To verify integration at the 5' region of the *PBANKA_111090* locus by PCR, the primers 5'-catgtgttattctattaaaaacg-3' *PBANKA_111090* chromosome specific forward and 5'-aagttactaagtatttatcaacc-3' pL0001 vector reverse were used. To confirm integration at the 3' region of the *PBANKA_111090* locus, the primers 5'-gtatatattaatataatagcacc-3' *PBANKA_111090* chromosome specific reverse and 5'-attgatataaaaatatataataataac-3' pL0001 vector forward were utilized. For the 5' region of the *PBANKA_091050* locus, the primers 5'-gttacctttagctaagtttcc-3'

*PBANKA_091050* chromosome specific forward and 5'-aagttactaagtatttatcaacc-3' pL0001 vector reverse were used. To confirm integration at the 3' region of the *PBANKA_091050* locus, the primers 5'-tttcatttaataaatatcacatgg-3' *PBANKA_091050* chromosome specific reverse and 5'-attgatataaaaatatataataataac-3' pL0001 vector forward were utilized.

For all gene deletion analyses, a PCR was conducted utilizing 2.0 µl gDNA (~100ng total), 1.25 units GoTaq® DNA Polymerase (Promega), 1X GoTaq® Flexi Buffer, 1 mM $MgCl_2$, 0.2 mM di-nucleotide tri-phosphate mix, and 0.5 µM primers. PCR conditions were as follows: an initial denaturing step of 95ºC for three minutes, 35 repetitive cycles of denaturing at 95ºC for 30 seconds, primer annealing at 52°C for 30 seconds and an extension at 62°C for three minutes and 30 seconds, and then a final extension at 62°C for 10 minutes. The samples were all separated via gel electrophoresis (1% gel) and visualized via UV detection using EtBr.

## 3. Results and Discussion

### *3.1. PBANKA_111090 and PBANKA_091050 sequence analysis and their comparison/contrast to their P. falciparum orthologs.*

*PBANKA_111090* is a 1,710 bp gene (containing no intron) that encodes a 569 amino acid *Plasmodium berghei* protein with an estimated molecular weight of 67,159 Daltons (Table 4.1). Sequence analysis using SignalP revealed that PBANKA_111090 is predicted to have a cleavable signal peptide (Bendtsen *et*

*al.*, 2004). Analysis using TargetP predicted the protein enters the secretory pathway and, more specifically, is either predicted to be located on the surface of the parasite or in the endoplasmic reticulum according to both PSORTII and WoLF PSORT (Emanuelsson *et al.*, 2000; Nakai & Horton, 1999). Additional sequence analysis using PROSITE, PROTCOMP, Profam, and NCBI (BLASTp) sites predicted the protein has multiple glycosylation and phosphorylation sites and has no functional identity with other known proteins (Gattiker *et al.*, 2002).

*PBANKA_091050* is a 701 bp gene, containing one intron, resulting in a 558 bp cDNA product. The cDNA product encodes a 185 amino acid *Plasmodium berghei* protein that has four transmembrane domains, with an estimated molecular weight of 20,933 Daltons (Table 4.2). Sequence analysis using SignalP revealed that PBANKA_091050 is predicted to have a non-cleavable signal anchor (Bendtsen *et al.*, 2004). Analysis using TargetP predicted the protein enters the secretory pathway and, more specifically, is predicted to be located on the surface of the parasite according to both PSORTII and WoLF PSORT (Emanuelsson *et al.*, 2000; Nakai & Horton, 1999). Additional sequence analysis using PROSITE, PROTCOMP, Profam, and NCBI (BLASTp) sites predicted the protein has multiple glycosylation and phosphorylation sites and has no functional identity with other known proteins (Gattiker *et al.*, 2002).

Next, protein sequence alignments were done using VectorNTI Explorer (Invitrogen) to determine the homology between PBANKA_111090 and PFE0565w (Figure 4.1A) and PBANKA_091050 and PF11_0394 (Figure 4.1B). Even though PBANKA_111090 and PFE0565w are considered orthologs on

PlasmoDB, they do not have significant homology with one another (18.1% identical). This leads to the question, are these two proteins truly orthologs (are the proteins expressed at similar life cycle stages and do these proteins function in similar ways)? In contrast, PBANKA_091050 and PF11_0394 (also considered orthologs on PlasmoDB) are highly homologous, with 79.6% of their amino acids being identical. These data suggest that these two proteins are truly orthologs and would most likely be expressed during the same life cycle stages and function in similar ways.

*3.2. PBANKA_111090 and PBANKA_091050 transcript detection studies.*

*PBANKA_111090* transcript is only present in salivary gland sporozoites, as demonstrated via RT-PCR (1,710 bp transcript). It is not present during oocyst sporozoite and mixed erythrocytic stages (ES) (Figure 4.2A). Therefore, transcript data obtained for *PBANKA_111090* differs from that of its ortholog *PFE0565w*, whose transcript is present in salivary gland sporozoites, oocyst sporozoites, and mixed ES. *PBANKA_091050* transcript is present in both salivary gland sporozoites and mixed ES, as demonstrated via RT-PCR (558 bp transcript). It is not present during the oocyst sporozoite stage (Figure 4.2B). Thus, transcript data obtained for *PBANKA_091050* differs from that of *PF11_0394*, whose transcript is present in salivary gland sporozoites, oocyst sporozoites, and mixed ES. It is interesting that their transcription patterns differ since their protein sequences are so similar. In all experiments, the gDNA positive control amplified a product and the negative control (no reverse

transcriptase, no RT) did not. Primers specific to the *circumsporozoite protein* (CS) gene were used as a positive control to verify the presence of *P. berghei* for the oocyst sporozoite and salivary gland sporozoite stages (Figure 4.2C). Primers specific to the *merozoite surface protein-1* (MSP-1) gene were used as a positive control to confirm the presence of *P. berghei* in mixed ES (Figure 4.2C).

*3.3. PBANKA_111090 and PBANKA_091050 GFP-trafficking constructs.*

In an attempt to analyze the protein detection profiles of PBANKA_111090 and PBANKA_091050 throughout the entire *P. berghei* life cycle of the parasite, PBANKA_111090/GFP and PBANKA_091050/GFP-trafficking constructs were made and transfected into parasites via single cross-over homologous recombination technology as described in Chapter 2 (see Figure 2.11). The entire open reading frames of both genes (excluding the stop codon) were cloned into the pL0031 vector (obtained from MR4) and transfected into mice (Kooij *et al.*, 2005). After transfections were complete, PCR analysis was used to determine if integration of the GFP constructs occurred at the proper gene loci. Both PBANKA_111090/GFP and PBANKA_091050/GFP-trafficking constructs displayed integration into the genomes of the parasites (Figure 4.3). In addition, wild-type parasites carrying an episome were present as well. After two rounds of limiting dilutions (30-40 mice total for each group) for both GFP-trafficking constructs, the population of transfected parasites was enriched for integration, but still contained wild-type parasites carrying episomes. Thus, even after multiple limiting dilutions into a number of mice that likely should have yielded

GFP-expressing PBANKA_111090 and PBANKA_091050 clonal parasite populations, obtaining them was not successful.

*3.4. PBANKA_111090 and PBANKA_091050 gene deletion constructs.*

In an attempt to determine if the PBANKA_111090 and PBANKA_091050 proteins are important for parasite development and/or invasion of host tissues, *PBANKA_111090* and *PBANKA_091050* gene deletion constructs were created using double cross-over homologous recombination technology as described in Chapter 2 (see Figure 2.14). Portions of the 5' and 3' untranslated regions and open reading frames of both genes were cloned into the pL0001 vector (the following reagent was obtained through the MR4 as part of the BEI Resources Repository, NIAID, NIH: *Plasmodium berghei* pL0001, MRA-770, deposited by AP Waters) and transfected into mice. After transfections were complete, PCR analysis was used to determine if integration of the gene deletion constructs had occurred at the proper gene loci. Both *PBANKA_111090* and *PBANKA_091050* gene deletion constructs displayed integration into the genomes of the parasites (Figure 4.4); however, integration was only detected at the 5' region of the parasite locus for *PBANKA_111090* and only detected at the 3' region of the parasite locus for *PBANKA_091050*. These results suggest that homologous recombination did occur, but only via a single cross-over event. In addition, both populations displayed the presence wild-type parasites carrying episomes. After two rounds of limiting dilutions (30-40 mice total for each group) for both gene deletion constructs, the population of transfected parasites was enriched for

integration, but still contained wild-type parasites carrying episomes. Thus, even after multiple limiting dilutions into a number of mice that should have yielded PBANKA_111090 and PBANKA_091050 gene deletion clonal parasite populations, obtaining them was not successful.

## 4. Conclusions

Since *P. falciparum* is difficult to work with under laboratory conditions and does not allow full *in vivo* experimentation, rodent malaria models are commonly employed instead. In addition, gene modification technology is well-established in rodent malaria models and generally takes only weeks to obtain results versus months compared to human malaria transfections technology (Janse *et al.*, 2006). Thus, as complementary experiments to those conducted for the *P. falciparum* genes/proteins (Chapter 2 and Chapter 3), their *P. berghei* orthologs were also studies.

According to data available on PlasmoDB, both PFE0565w and PF11_0394 have orthologs in *P. berghei*, PBANKA_111090 and PBANKA_091050, respectively. Using sequence analysis programs previously described, the gene and protein sequences were analyzed for each gene/protein and compared to their *P. falciparum* counterparts. Data obtained were interesting because the PFE0565w/PBANKA_111090 ortholog set appears different from one another. PBANKA_111090 is much larger than PFE0565w and their proteins have very limited homology (only 18.1% identical). In contrast,

PF11_0394 and PBANKA_091050 appear to be orthologs as their sequences (DNA and protein) are highly conserved (79.6% identical on a protein level).

The next step in further characterizing PBANKA_111090 and PBANKA_091050 was to determine their transcription profiles and then compare the results with their *P. falciparum* orthologs. It was found that PBANKA_111090 had transcript present only in salivary gland sporozoites. This is different from PFE0565w, whose transcript was not only present in oocyst sporozoites and mixed ES, but also in salivary gland sporozoites; however, according to the literature, PFE0565w transcript was only expressed by salivary gland sporozoites, so the data obtained from PBANKA_111090 matches the data found in the literature of its *P. falciparum* ortholog (Bozdech *et al.*, 2003a; Bozdech *et al.*, 2003b; Florens *et al.*, 2002; Hall *et al.*, 2005; Khan *et al.*, 2005; Lasonder *et al.*, 2002; Le Roch *et al.*, 2003; Siau *et al.*, 2008; Tarun *et al.*, 2008; Williams & Azad, 2010; Young *et al.*, 2005). Next, it was determined that PBANKA_091050 had transcript present in both salivary gland sporozoite stages and mixed ES. Even though their sequences are nearly identical, this transcription profile is different from PF11_0394, whose transcript was present not only in salivary gland sporozoites and mixed ES, but also in oocyst sporozoites; however, according to literature, PF11_0394 transcript was only present in salivary gland sporozoites and mixed ES, so the data obtained for PBANKA_091050 matches results found in the literature of its *P. falciparum* ortholog (Bozdech *et al.*, 2003a; Bozdech *et al.*, 2003b; Florens *et al.*, 2002; Hall *et al.*, 2005; Khan *et al.*, 2005; Lasonder *et al.*, 2002; Le Roch *et al.*, 2003; Siau *et al.*, 2008; Tarun *et al.*, 2008;

Williams & Azad, 2010; Young *et al.*, 2005). Transcription studies for mixed gameotocyte stages and exoerythrocytic stages were not conducted for PBANKA_111090 and PBANKA_091050, so comparisons cannot be made between *P. berghei* and the data obtained for their *P. falciparum* orthologs for these specific life cycle stages.

Unfortunately, since clonal parasite populations were not able to be generated for both GFP-trafficking constructs and gene deletion constructs for either protein, protein detection profiles and functional analyses of the proteins were not able to be completed. Based on transcription studies, it is hypothesized that PBANKA111_090 would have protein present during the salivary gland sporozoite stages, just like its PFE0565w ortholog. Thus, even though their sequences are very different, they may indeed be orthologs based on preliminary studies. Further experiments would have to be conducted and data obtained to determine if that is true. In addition, initial data gathered for PBANKA_091050 also leads to the hypothesis that PBANKA_091050 would have protein present during the salivary gland sporozoite stage since it has transcript present at this time and its protein sequence shares high homology with PF11_0394, which is present in salivary gland sporozoites. As mentioned above, more experiments would have to be completed to determine the accuracy of these statements.

Overall, data acquired from these experiments have allowed for a preliminary comparison of two ortholog sets from *P. berghei* and *P. falciparum*: PBANKA111_090/PFE0565w and PBANKA_091050/PF11_0394. Additional PBANKA_111090 and PBANKA091050 studies will have to be done to determine

their full transcription profiles, protein detection profiles, and potential functions in parasite development and/or invasion of host tissues. Once these studies are finished, a full comparison with *P. falciparum* can be completed to assess if these ortholog sets are truly orthologs. Depending on results obtained, these experiments could validate or refute the approach of using rodents as malaria models for what happens with the disease in humans.

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| | PFE0565w | PBANKA_111090 |
|---|---|---|
| **Molecular Weight and Gene/Protein Size** | 45,583 Daltons<br>1146 base pairs<br>381amino acids | 67,159 Daltons<br>1710 base pairs<br>569 amino acids |
| **Intron(s)** | None | None |
| **SignalP** | Signal Peptide | Signal Peptide |
| **Transmembrane Domain(s)** | Yes, 1 | Yes, 1 |
| **TargetP** | Secretory Pathway | Secretory Pathway |
| **PSORTII** | Membrane: Plasma | Membrane: Plasma |
| **WoLF PSORT** | Membrane: Plasma | Membrane: ER or Plasma |
| **PROSITE** | N-glycosylation site<br>Protein kinase C Phos Site<br>Casein kinase II Phos Site<br>cAMP/cGTP Dependent Protein kinase Phos Site<br>Tyrosine kinase Phos Site<br>N-myristoylation | N-glycosylation site<br>Protein kinase C Phos Site<br>Casein kinase II Phos Site |

**Table 4.1. Sequence comparison/contrast of PBANKA_111090 with its predicted *P. falciparum* ortholog, PFE0565w.** Initial gene characterization for both *PBANKA_111090* and *PFE0565w* were performed by gathering information from PlasmoDB. In order to further characterize the genes and verify data from PlasmoDB, additional programs found on the ExPASy Bioinformatics Resource Portal (www.expasy.org) and SoftBerry (www.softberry.com) were utilized. ER = endoplasmic reticulum and Phos = phosphorylation.

| | PF11_0394 | PBANKA_091050 |
|---|---|---|
| **Molecular Weight and Gene/Protein Size** | 21,026 Daltons<br>762 base pairs<br>186 amino acids | 20,933 Daltons<br>701 base pairs<br>185 amino acids |
| **Intron(s)** | Yes, 1 intron | Yes, 1 intron |
| **SignalP** | Signal Anchor | Signal Anchor |
| **Transmembrane Domain(s)** | Yes, 4 | Yes, 4 |
| **TargetP** | Secretory Pathway | Secretory Pathway |
| **PSORTII** | Membrane: Plasma | Membrane: Plasma |
| **WoLF PSORT** | Membrane: Plasma | Membrane: Plasma |
| **PROSITE** | N-glycosylation site<br>Protein kinase C Phos Site<br>Casein kinase II Phos Site | N-glycosylation site<br>Protein kinase C Phos Site<br>Casein kinase II Phos Site |

**Table 4.2. Sequence comparison/contrast of PBANKA_091050 with its *predicted P. falciparum* ortholog, PF11_0394.** Initial gene characterization for both *PBANKA_091050* and *PF11_0394* were performed by gathering information from PlasmoDB. In order to further characterize the genes and verify data from PlasmoDB, additional programs found on the ExPASy Bioinformatics Resource Portal (www.expasy.org) and SoftBerry (www.softberry.com) were utilized. Phos = phosphorylation.

## A. PBANKA_111090 and PFE0565w Protein Alignment

```
PB   (1) MDILYFRNFLLIISFFVVNNKINIKNEFILLGFFSSFSWKSENNTMAEWVLNSSINNQNKNIRIL
PF   (1) MNMINIGYLLLVLSFLLLEKTFYGSDTFRVFEDFDVRRK------------------NK-----

PB  (66) NTYIELNGNVNSDIAYKNLDKNESDTSGKTLFLNTNYYNYLDKSEKKMDHAKDENKENHDSKSEI
PF  (42) -------------IWCCNLNHEKSYFHNKT--INTSYKNRVLKESILLNLDLDMKKYKDNIITRK

PB (131) EIKELIKKEIEKNNKKEEEYIKKEIEKSIKKERDNEIREEIDDEQDYEQGEEKDGEKDKPKEKKI
PF  (92) KTPENIYKEIYENN----------YEMKYDEDIPNNMSEEKRDEKEVIEHLEIDEKNGK----QY

PB  196) KKLIHHEIRKINEKIEKNVQESEQEKNPGEIKKNKTIAIEIKNENRDPKIKCSNKLNYIELEYSN
PF (143) KSDINKPVSLSHLKQYKNIYVNNNNK----INKKKSIDKHLPSYNLE--RKNNKYLNFLLVDNR-

PB (261) IDKKLISEKYNNDVISFINIKTKDKIYFRDKINENITKIKDHDANKISNIEQMEVEPNIIKKDQN
PF (201) ------------------NESYTFMVPMKFYINHEMYNISDEEYNKLMEDNSVDVYLNNILVEYK

PB (326) VTYEQIKNKNKMQGNAILHNNNHAFLDSKEYNNFHTFFNNNLKNINKHTNSHKYNNNLNGITKSH
PF (248) YENFEIK-EGEVDGEVEGEGEVEGEVEGEVKGKVVEGIENNMNEEEKYNKDNKDK----------

PB (391) DITKGNFTIFITENQEDDSLVLPINFERRNSVLNISNDEYTKLMNEGSVLVYDKDEVVPYKYDAF
PF (302) ------------ENQ--------INSNGQ-------------------------------------

PB (456) EVKNDSVNDMKENNNNDNNSSNDSIYRDKSNDASSQNNPEEEDQFKGTILTCVTVIIILAIMFLI
PF (311) ----DENTEFQENDNNDS----------------------------VIMKYTIIISGLVLLFCI

PB (521) GFIIYYYDIINKLRVKFQKKGKNNKSMTIKNDKSSGMYIDNYYDNSTHV
PF (343) SFIIYYFDIIQKVKMKLNKKRKSNATMAINRDKIQEEFM----------
```

## B. PBANKA_091050 and PF11_0394 Protein Alignment

```
PB   (1) MRILNYICGRSLRSSGAAPLIYNPIQKLLIILTLLYICLSALSCSIFLFPKVSDLHCSPLVDSLF
PF   (1) MKIFNYICGRPLRNGGTAPLIYNPVRKWLIILMILYICLSILSYLIFLFPKASDLQCLALIDSLF

PB  (66) NFYLSMGASNVMAPYYSMISCREWGTEHEWVVVAIVSAVMAIIDVSSSFYGIYVLYTIIDIVFTN
PF  (66) NFSLSIGVSYVMAPYYSIISCREWGTEYEWGIVAVVSAVMAIVDVLSSCYGIYVLYTITSVVFNK

PB (131) ITDMN-ECNCYKSIIFFSANAFLVVLHLIVAITSIVVYYMLMKNIDKQLEDNRNII
PF (131) RIGMNNDCNSYNAVLFFSANSILVFLHLTVATVSTVVYFLLMKGIDKQLEDNRNII
```

**Figure 4.1. PBANKA_111090 and PFE0565w (A) and PBANKA_091050 and PF11_0394 (B) protein sequence alignments to assess homology.** Using protein sequences obtained from PlasmoDB, an alignment using VectorNTI Explorer (Invitrogen) was conducted on the two ortholog sets to determine their

homologies with one another. PBANKA_111090 and PFE0565w are considered to be orthologs according to PlasmoDB, but share little homology with one another. On the other hand, PBANKA_091050 and PF11_0394, which are also predicted to be orthologs according to PlasmoDB, are highly homologous. Amino acids highlighted in yellow are identical in both proteins and amino acids highlighted in blue are similar. For part A, PB = *P. berghei* PBANKA_111090 in PlasmoDB and PF = *P. falciparum* PFE0565w in PlasmoDB. For part B, PB = *P. berghei* PBANKA_091050 in PlasmoDB and PF = *P. falciparum* PF11_0394 in PlasmoDB.

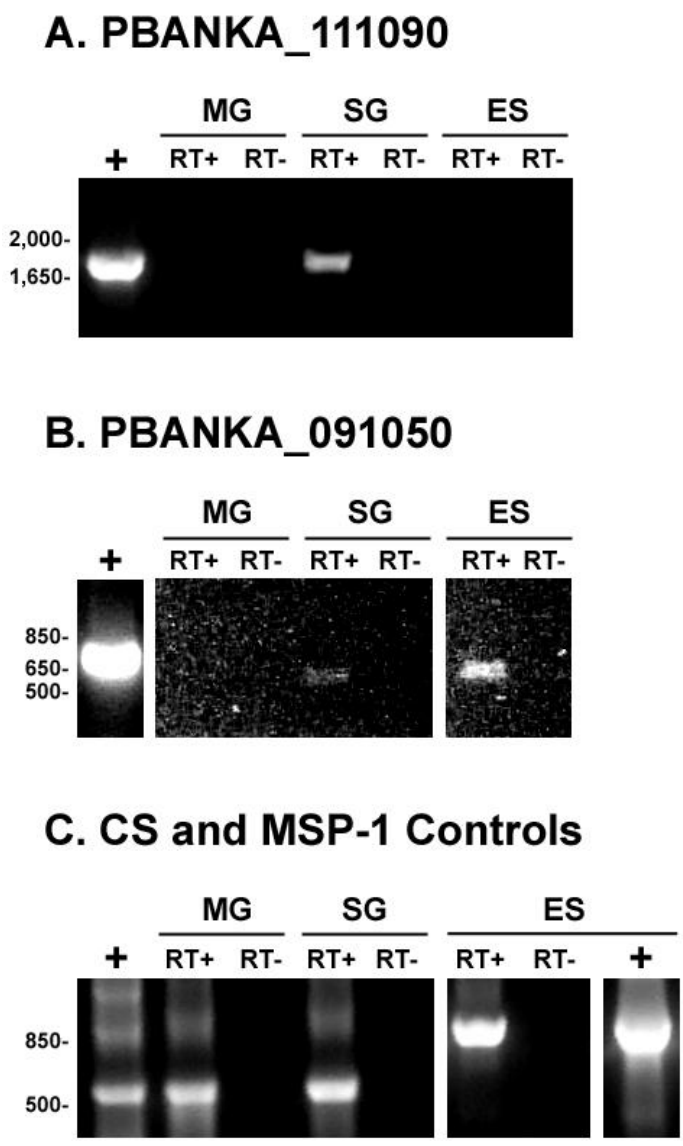


**Figure 4.2. Transcription analysis of *PBANKA_111090* and *PBANKA_091050* in midgut oocyst sporozoites (MG), salivary gland sporozoites (SG), and mixed erythrocytic (ES) stages via RT-PCR.** Primers specific for *PBANKA_111090* and *PBANKA_091050* were used to amplify cDNA fragments of the correct product size (RT+), indicating the presence of the genes during certain stages of the *P. berghei* life cycle. Genomic DNA (+) was used as a positive control and a no reverse-transcriptase (RT-) reaction was used as a

negative control to show that the RNA was not contaminated with gDNA. Primers specific to the *circumsporozoite protein* (CS) gene were used as a positive control to verify the presence of *P. berghei* for the oocyst sporozoite and salivary gland sporozoite stages. Primers specific to the *merozoite surface protein-1* (MSP-1) gene were used as a positive control to confirm the presence of *P. berghei* in mixed ES. *PBANKA_111090* transcript is present in salivary gland sporozoites, whereas *PBANKA_091050* transcript is present during both salivary gland sporozoite stages and mixed ES.

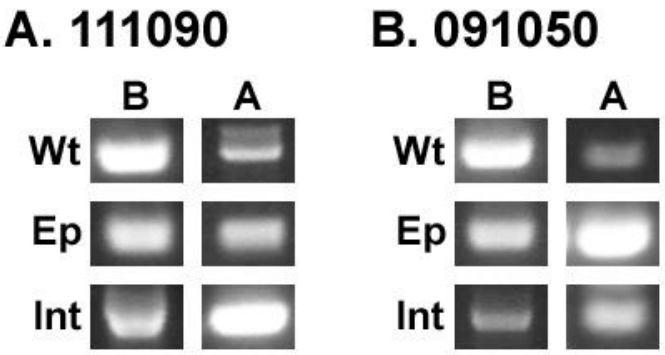


**Figure 4.3. GFP-trafficking constructs for both PBANKA_111090 (A) and PBANKA_091050 (B) were made, but clonal parasite populations were never obtained.** Using single cross-over homologous recombination technology (described in section 2.12 and Figure 2.11 in Chapter 2), both PBANKA_111090/GFP and PBANKA_091050/GFP constructs were created using the pL0031 vector (obtained from MR4) in an attempt to analyze their protein expression throughout the entire life cycle of the parasite (Kooij *et al.*, 2005). After transfections into mice, both constructs displayed integration (Int) into the genomes of the parasites, but also had wild-type (Wt) parasites carrying the episome (Ep). After multiple limiting dilutions, parasites transfected with both constructs had enriched integration, as demonstrated by an increase in band intensity, but Wt parasites carrying an Ep were still present. Thus, generating GFP-expressing clonal populations of parasites was not successful. 111090 = PBANKA_111090, 091050 = PBANKA_091050, B = parasite population before limiting dilution, and A = parasite population after limiting dilution.

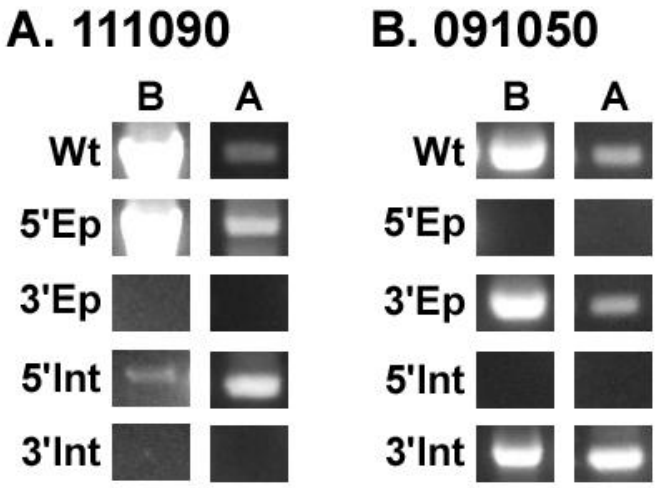


**Figure 4.4. Gene deletion constructs for both PBANKA_111090 (A) and PBANKA_091050 (B) were created, but clonal parasite populations were never obtained.** Using double cross-over homologous recombination technology (described in section 2.12 and Figure 2.14 in Chapter 2), both PBANKA_111090 and PBANKA_091050 gene deletion constructs were made using the pL0001 vector (this reagent was obtained through the MR4 as part of the BEI Resources Repository, NIAID, NIH: *Plasmodium berghei* pL0001, MRA-770, deposited by AP Waters) to analyze their potential functions in parasite development and/or invasion of host tissues. After transfections into mice, both constructs displayed integration (Int) into only one region of the genomes of the parasites and the populations also had wild-type (Wt) parasites carrying an episome (Ep) present. These results suggested that homologous recombination did occur, but only via a single cross-over event. After multiple limiting dilutions, parasites transfected with both constructs had enriched integration, as demonstrated by an increase in band intensity, but Wt parasites carrying an Ep

were still present. Thus, generating clonal populations of the gene deletion parasites was not successful. 111090 = PBANKA_111090, 091050 = PBANKA_091050, B = parasite population before limiting dilution, and A = parasite population after limiting dilution.

## Chapter 5: Discussion and Future Studies

Studies described within this dissertation have generated new *P. falciparum* genome data through the characterization of two genes, *PFE0565w* and *PF11_0394*, and their proteins produced. As new information is obtained about the expression and localization patterns of *P. falciparum* proteins, a better foundation of how the parasite interacts with and develops within its hosts, and what specific proteins may be involved with these processes, is achieved. A stage of the *Plasmodium* life cycle, called the sporozoite, is unique since it has dual infectivity: it is the stage that both invades the salivary glands of the mosquito and eventually invades the human liver (Gilles, 2002). In addition, several *Plasmodium* proteins that have been found to be critical for development and/or invasion of host tissues, including during the sporozoite stage of the life cycle, are surface proteins. For example, both the circumsporozoites (CS) protein and thrombospondin-related anonymous protein (TRAP) are surface proteins that are essential for proper development and invasion of both mosquito and mammalian host tissues (Ejigiri & Sinnis, 2009; Ghosh *et al.*, 2001; Kappe *et al.*, 2004; Menard & Janse, 1997; Sinnis *et al.*, 2007; Sultan *et al.*, 1997; Warburg *et al.*, 1992). Therefore, elucidating the transcript and protein expression profiles of *P. falciparum* genes/proteins PFE0565w and PF11_0394 has allowed for additional knowledge to be gained about two apparent surface proteins that appear to be uniquely expressed by salivary gland sporozoites. Ultimately, these

two genes could represent new candidates for the development of drugs and vaccines.

*PFE0565w* is a gene found on chromosome five of the *P. falciparum* genome (Chapter 2). In the stages of the life cycle that were examined, the PFE0565w protein is only expressed by salivary gland sporozoites. Despite this limited protein expression, the *PFE0565w* transcript was found in oocyst sporozoites, salivary gland sporozoites, mixed erythrocytic stages, and gametocytes. The presence of transcript in several life cycle stages is not necessarily surprising since protein production/levels in *P. falciparum* are controlled through mRNA processing and translation, as opposed to gene transcription (Dechering *et al.*, 1999; Horrocks *et al.*, 1998; Moreira *et al.*, 2008). Additionally, preliminary data suggest that *PFE0565w* does not have transcript present during exoerythrocytic stages.

During the erythrocytic stages, *PFE0565w* produces an alternative transcript, which appears mainly in gametocytes. This product could be an antisense RNA that may be needed for transcriptional/translational regulation as a result of an alternative splicing event. Along with standard pre-mRNA splicing, many organisms use alternative splicing as a means of manipulating their transcripts for additional gene regulation, including *P. falciparum* (Hughes *et al.*, 2010). Studies in the literature suggest that antisense RNAs are common in *P. falciparum* and make up at least 12% of the erythrocytic stage RNA (Gunasekera *et al.*, 2004). In addition, a study conducted by Lu *et al*. (2007) reported that the

*P. falciparum* genome databases had predicted gene products incorrectly in 24% of the genes during the time of their research and that some of these inaccuracies were the result of alternatively spliced transcripts produced by the parasite (Lu *et al.*, 2007). As the *Plasmodium* transcriptome is further studied, several alternative splice junctions, in addition to the standard intronic sequence boundaries of GU at the 5' end and AG at the 3' end, have been identified (Hughes *et al.*, 2010; Sorber *et al.*, 2011). In a recent study by Sorber *et al*. (2011), five GC-AG splice junctions were discovered along with 977 standard GU-AG splice sites that previous experimentation had failed to detect (Sorber *et al.*, 2011). In addition to these data, 310 alternative splicing events, many of which resulted in a truncated open reading frame, like discovered for *PFE0565w*, were found (Sorber *et al.*, 2011). All of these data support the hypothesis that the alternative *PFE0565w* transcript found during the mixed erythrocytic stages and gametocytes could be another antisense RNA product not previously identified in *P. falciparum*. Further studies would need to be conducted in order to confirm that the product is an antisense RNA. This could be done by exposing *P. falciparum* mixed erythrocytic stage/gametocyte cultures to the RNA polymerase II inhibitor α-amanitin. Previous studies have demonstrated that *P. falciparum* antisense transcript synthesis is sensitive to this inhibitor and could prove that the *PFE0565w* alternative transcript is an antisense RNA if experimentation shows it does not get produced in the presence of α-amanitin (Militello *et al.*, 2005).

In an effort to determine if the PFE0565w protein is important in parasite development and/or invasion of host tissues, a PFE0565w/pHD227 disruption clone was successfully created. Unfortunately, when setting cultures to induce gametocytes, the cultures always resulted in “unhealthy” parasites. Compared to wild-type cultures set for gametocytes at the same time, the mutant parasites tended to keep replicating without forming gametocytes. This resulted in over-populated cultures that eventually died and were not able to be used for *Anopheles* infections. There are several possibilities as to why the mutant parasites were not able to produce gametocytes. First, the mutant parasites could have been kept in continuous culture too long while trying to obtain the mutant parasite population. The entire process to create the PFE0565w/pHD227 clonal population resulted in at least six months of continuous culturing of the parasites. Previous studies have demonstrated that the longer *P. falciparum* parasites are kept in continuous culture, the more likely that those cultures are not able to produce gametocytes well (Schuster, 2002; Trager & Jensen, 1997). Data generated by Janse *et al*. (1992) using *P. berghei* demonstrated that the lack of gametocyte production in prolonged cultures likely resulted from the deletion of genes essential for gametocyte formation. A large proportion of these genes are located on the telomeres of the parasites and sub-telomeric regions have been shown to be depleted from the chromosomes during continuous *in vitro* replication (Janse *et al.*, 1992). Another possibility of why PFE0565w/pHD22y parasites never produced healthy gametocytes is because the gene disruption could cause the *PFE0565w* alternative transcript to not be

made during the erythrocytic and gametocyte stages. If this transcript is an antisense RNA that is important for transcriptional and/or translational regulation, it could affect gene regulation during the gametocyte stages that results in poor gametocyte formation (Hughes *et al.*, 2010). Lastly, following transfection and having a disruption construct integrate into the genome of the parasite via homologous recombination, there is a chance that this process could disrupt genes upstream and downstream of the target gene (personal communication, Dr. Michael Kariuki). Thus, if genes located upstream and downstream of *PFE0565w* are needed for the parasite to optimally function during the gametocyte stage, an unintentional disruption to their gene sequences could yield poor gametocyte production.

*PF11_0394* is a gene found on chromosome eleven of the *P. falciparum* genome (Chapter 3). The PF11_0394 protein was found to only be expressed during the salivary gland sporozoite stage of the life cycle in stages examined during these studies. Despite its protein being expressed in this one life cycle stage, its transcript was found to be expressed in multiple mosquito and human host life cycle stages. Preliminary data suggest that *PF11_0394* does not have transcript present during exoerythrocytic stages.

Extensive measures were taken to produce recombinant PF11_0394 protein for antibody generation, including *E. coli* expression plasmids (with and without codon optimization), a baculovirus expression system, and synthetic peptide synthesis (see Appendix 1 for details); however, none of these

approaches were successful. PF11_0394 sequence analysis revealed that this protein has a signal anchor and four transmembrane domains, resulting in a largely hydrophobic protein embedded within the membrane of the parasite (Aurrecoechea *et al.*, 2009). Thus, this feature of the PF11_0394 protein likely caused the protein production techniques used to be unsuccessful. Fortunately, PF11_0394 GFP-trafficking clonal populations were able to be generated, allowing for the protein expression profile of PF11_0394 to be elucidated in all life cycle stages of the parasite except for exoerythrocytic stages.

A PF11_0394/pHD22y gene disruption construct was made for PF11_0394 for functional analysis. Unfortunately, once the construct was transfected into *P. falciparum*, integration of the construct was never observed via PCR analysis, even after four rounds of drug selection. The PF11_0394 protein is not expressed by mixed erythrocytic stage parasites (the stage of the life cycle when transfections are conducted), so the failure to obtain a mutant PF11_0394 parasite population was not likely due to its being essential during this life cycle stage. The genome of *P. falciparum* is highly AT-rich (~80%) with greater than 90% of it occurring in the intronic regions of the genome (Hughes *et al.*, 2010). Because *PF11_0394* has one intron, it is a possibility that when the PF11_0394/pHD22y construct was transfected into the *P. falciparum* genome, the highly AT-rich intron of PF11_0394 may have caused the construct to homologously recombine with the wrong *P. falciparum* gene, most likely another gene that has an intron. Future studies would have to be conducted to verify if this is what happened.

Because there were issues with the gene disruption constructs for both *PFE0565w* and *PF11_0394*, gene deletion constructs also were made for each of the genes. Separate *P. falciparum* cultures were transfected with all the gene deletion constructs and drug selection for obtaining integrants began. After seven weeks, none of the *P. falciparum* cultures appeared to have parasites growing in the cultures. The technique and reagents used in the transfection and drug selection processes were reexamined. Disappointingly, an error was made in the drug concentration for the mutant parasite selection; twice the amount of drug was used than suggested in the literature (5 µm instead of 2.5 µm of WR99210 was used). Once this flaw was discovered, the drug concentrations were adjusted, but it was too late for a majority of the parasites. Only the deletion population, PFE0565w/pHHT-TK, recovered and was able to continue surviving in culture.

Even though functional analyses of PFE0565w and PF11_0394 were not able to be completed, both of the proteins of interest appear to only be present during the salivary gland sporozoite stage of the parasite life cycle. Experiments do have to be conducted to determine whether these proteins are present during the exoerythrocytic stage of the parasite life cycle; however, since RT-PCR was used to generate preliminary data suggesting that both *PFE0565w* and *PF11_0394* transcripts are not present during the liver stage, it is predicted their proteins are not expressed during this stage either. Once sporozoites reach the salivary glands of the mosquitoes, the next step in the life cycle includes being injected into the vertebrate host (Roberts *et al.*, 2009). Therefore, since

PFE0565w and PF11_0394 proteins are present in salivary gland sporozoites, it is hypothesized that if the PFE0565w and/or PF11_0394 proteins are needed for either parasite development or invasion of host tissues, it would be for survival within the salivary glands of the mosquito and/or during the early stages in the human host (e.g. migration to the liver, binding human hepatocytes for entry into the liver, and/or early exoerythrocytic stage development). A recent study supports the hypothesis that PFE0565w may be needed for invasion and/or early development in the liver, as it has been placed in a group of genes called the Sporozoite Conserved Orthologous Transcripts (SCOT) (Westenberger *et al.*, 2010). These genes were identified because their transcripts were highly upregulated in salivary gland sporozoites relative to blood stages in three species of *Plasmodium*: *P. falciparum*, *P. vivax*, and *P. yoelii*. The prediction is that these genes are likely to be essential to sporozoite cell biology for hepatocyte cell invasion or early liver stage development (Aurrecoechea *et al.*, 2009; Westenberger *et al.*, 2010). Despite not knowing the functions of PFE0565w and PF11_0394, they could still be good targets for new drugs and/or pre-erythrocytic stage vaccines since their proteins have no homology with any known human protein.

Since human malaria parasite species can be difficult to work with under laboratory conditions and do not allow full *in vivo* experimentation, rodent malaria models, such as *P. berghei*, are commonly used on their own for experimentation or in conjunction with *P. falciparum*. Therefore, as a complement to the experiments conducted for the *P. falciparum* genes/proteins, PFE0565w and

PF11_0394, their *P. berghei* orthologs, PBANKA_111090 and PBANKA091050, were characterized as well (Chapter 4). Results obtained with these studies have allowed for a preliminary comparison to be conducted between the two malaria species ortholog sets.

According to PlasmoDB, PFE0565w and PBANKA_111090 are considered orthologs, even though their protein sequences are not highly conserved (18.1% identical) (Aurrecoechea *et al.*, 2009). Preliminary transcript expression studies of the stages that were examined for *PBANKA_111090* generated via RT-PCR show that its transcript is only present in salivary gland sporozoites. This is different from *PFE0565w*, whose transcript is present not only in salivary gland sporozoites, but also in mixed erythrocytic stages and oocyst sporozoites. PF11_0394 and PBANKA_091050 are also considered orthologs according to data available on PlasmoDB (Aurrecoechea *et al.*, 2009). In contrast to the PFE0565w/PBANKA_111090 ortholog set, the PF11_0394 and PBANKA_091050 proteins are highly conserved between the two species (79.6% identical). Preliminary transcript expression studies for *PBANKA_091050* generated via RT-PCR demonstrate that its transcript is present in salivary gland sporozoites and mixed erythrocytic stages. This is different from *PF11_0394*, whose transcript is present not only in salivary gland sporozoites and mixed erythrocytic stages, but also in oocyst sporozoites.

To determine the protein expression profiles of both PBANKA_111090 and PBANKA_091050, GFP-trafficking constructs were produced and transfected into *P. berghei*; however, attempts to isolate clonal parasite

populations were not successful for either gene despite *P. berghei* being considered an “easier” model to use for transfections (Janse *et al.*, 2006). The same results occurred when trying to produce gene deletion populations for *PBANKA_111090 and PBANKA_091050* to determine if these proteins play a role in parasite development and/or invasion of host tissues. Gene deletion constructs were made, but after transfections into mice, *PBANKA_111090 and PBANKA_091050* gene deletion populations were never able to be isolated, despite using numbers of mice that should have yielded a clonal population.

There are several experiments that need to be conducted for the *P. berghei* work to complete these projects, but are beyond the scope of this dissertation research. Some of these studies include repeating transcript expression experiments and also obtaining transcript data for exoerythrocytic stages since rodent models are easier for *in vivo* analysis. In addition, protein expression studies need to be conducted for both PBANKA_111090 and PBANKA_091050 proteins. Next, if gene deletion populations could be isolated, experiments could be completed to determine the function of both PBANKA_111090 and PBANKA_091050. Overall, *P. berghei* research is in its early stages. It would be interesting to finish the comparative studies between the two *Plasmodium* species to verify that these two ortholog sets are really orthologs. Rodent malaria models are commonly used for studying what happens during malaria parasite infection in humans. However, there have been cases reported, especially in drug and vaccine studies, where there is success within the mouse model, but then the drug/vaccine it is not successful in humans

(Langhorne *et al.*, 2011; Workshop, 2010). Therefore, any data generated by studying putative orthologs of each *Plasmodium* species could provide information to either support or challenge the use of mice as human disease models for malaria.

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# Appendix 1: Attempts of PF11_0394 Recombinant Protein Production and Antibody Generation

## 1. Attempts at PF11_0394 Recombinant Protein and Antibody Production

### *1.1. Production of recombinant protein with expression plasmids and E. coli.*

Recombinant protein production for PF11_0394 was not successful. Several types of *E. coli* (*e.g*., Rosetta-gami, BL21, BL21(DE3)pLysS, and DH10B cells) and expression plasmids (*e.g*., pET32a, pGEX, and pAKSS) were used in attempts to generate protein, but all of them failed at inducing PF11_0394 (full-length, full-length minus the signal anchor, and truncated forms).

### *1.2. Production of recombinant protein using a baculovirus expression system.*

Since induction of PF11_0394 protein did not work in *E. coli*, the Bac-to-Bac Baculovirus Expression System (Invitrogen) also was used to try and produce the protein. In brief, the entire PF11_0394 gene (excluding the signal anchor) was cloned into the pFastBac-HTb vector (containing a His-tag) and transformed into DH10Bac cells that contained the baculovirus (bacmid) DNA following the manufacturer's instructions. The bacmid DNA was isolated and used to infect insect cell cultures (both High Five and Sf9) to induce PF11_0394 protein production. Three different viral titers were used to infect the cells and collections were made 24, 48, and 72 hours post-infection. Using the insect cell cultures, Western blot analysis was utilized to determine if PF11_0394 was produced and it appeared to be successful because a protein product was

detected at the predicted size for PF11_0394. Since a His-tag was incorporated in the sequence, protein purification was conducted using a nickel column (Novagen). Although several types of elution strategies were employed, Western blot analysis failed to detect the purified PF11_0394 protein. This result led to the conclusion that the baculovirus system was not successful in producing sufficient quantities of the PF11_0394 protein and/or the PF11_0394 protein was not stable once purified and eluted.

*1.3. Commercial synthesis of PF11_0394 protein.*

The DNA sequences of *Plasmodium* genes are very AT-rich and this particular gene has many "standard" codon biases that likely made it difficult for both *E. coli* and baculovirus to overcome (Xiao *et al.*, 2007). Thus, the *PF11_0394* gene was commercially synthesized by Genscript such that its sequence would encode the same protein, but used codons that were common in *E. coli.* Once the optimization was complete, the company cloned the gene into an expression plasmid and tried to express the PF11_0394 protein. They were able to minimally express the protein, but when the PF11_0394 recombinant protein was purified, it appeared to rapidly degrade (even with addition of protease inhibitors). These data may prove why the effort to previously recover PF11_0394 from insect cells and *E. coli* expression systems were not successful.

*1.4. PF11_0394 peptide production and antibody generation.*

Since the production of any type of PF11_0394 recombinant protein was unsuccessful, Genscript was used again for production of two synthetic

PF11_0394 peptides. The two peptide regions chosen were areas that were the least hydrophobic and were predicted to be located on the extracellular side of the plasma membrane of the parasite. These peptides were combined together and sent to Avian Immunology for injection into chickens for IgY antibody production. After isolation of both PF11_0394 preimmune and immune sera from eggs, the antibodies were tested via Western blot analysis (using peptide dot blots) and immunofluorescent assays to determine their quality. The results proved they were not immunogenic, as the antibodies did not strongly recognize the PF11_0394 peptides used to make the antibodies via dot blot and did not label any sporozoites (the positive control did). After this, two peptide boosts were given to the chickens and more eggs and antibodies were collected and isolated. After antibody purification, dot blots and immunofluorescent assays were once again conducted and similar results were obtained; the PF11_0394 antibodies did not appear to recognize the PF11_0394 protein.

After all these methods were attempted and all proved ineffective, efforts to create recombinant PF11_0394 protein and antibodies were discontinued. PF11_0394 is a highly hydrophobic protein since a majority of it is predicted to be embedded within the plasma membrane of *P. falciparum*. As a consequence of this characteristic, PF11_0394 is most likely just a poor candidate for protein production. As a result, GFP-trafficking studies were used to assess the protein expression profile of PF11_0394 throughout the life cycle of the *Plasmodium* parasite.

## 2. References

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## VITA

Maggie (Christ) Schlarman was born on July 14, 1982 in Kirkwood, Missouri to the parents of James and Brenda Christ. She has one younger sibling, Benjamin Christ. She graduated from Fox High School in May of 2000. After high school, Maggie began her education at the University of Missouri and earned a Bachelors of Science degree in Biology with departmental honors in May of 2004. In November of 2004, she married Daniel Schlarman of Imperial, Missouri. Maggie decided to continue her education and began her doctorate training in the lab of Dr. Brenda Beerntsen at the University of Missouri in the Molecular Microbiology and Immunology and Veterinary Pathobiology Joint Graduate Program in June of 2005. In July of 2009, she and her husband had their first child, James David Schlarman. In December of 2011, Maggie graduated with her Doctor of Philosophy degree from the University of Missouri.