UNIVERSITY OF GHANA, LEGON

DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY

ELUCIDATING THE MOLECULAR MECHANISM(S) UNDERLYING THE SUBCELLULAR DISTRIBUTION OF PF3D7_0410600 PROTEIN IN THE MALARIA PARASITE

THIS THESIS/DISSERTATION IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MPHIL IN MOLECULAR AND CELL BIOLOGY OF INFECTIOUS DISEASE DEGREE

BY

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DECLARATION

I, Philip Ilani, hereby declare that the experimental work presented in this thesis was undertaken by me at the West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), Department of Biochemistry, Cell and Molecular Biology under the supervision of Dr. Emmanuel Amlabu, Professor Gordon A. Awandare and Dr. Patrick K. Arthur of the West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), Department of Biochemistry, Cell and Molecular Biology. Dr. Emmanuel Amlabu is also a faculty at the Department of Biochemistry, Kogi State University, Anyigba-Nigeria.

No part of this thesis has been previously submitted for the award of a degree or any other qualification at this or any other institution and I have duly acknowledged all cited references.

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I dedicate this thesis to God almighty for his love, protection and guidance throughout my studies. Also, to my father, Mr. John Aminu Ilani, of blessed memory. I wish you were here to witness this but I hope I have made you proud. To my lovely mother, Mrs. Esther Naomi Ilani, for her unwavering support, prayer and love through this phase of my life. To my siblings Monica, Martha, Matthew and Augustine for their support and good wishes during this period. To my friend, Eleojo Kiteleonele, for her understanding and support during my studies. And to all my friends for their encouragement and support at the time I needed it the most. I love you all.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

2-BMP – 2-Bromopalmitate

ACT – Artemisinin-combination therapy

Acβ – Adenylate cyclase β

AIP – ARO-interacting protein

ARAC – Acyl resin-assisted capture

ARM – Armadillo repeat

BSA – Bovine Serum Albumin

CD – Cluster of Differentiation

CDC – Centres for Disease Control and Prevention

CSS-PALM – Clustering and Scoring Strategy for predicting palmitoylation

CyRPA – Cysteine-rich protective antigen

DAPI – 4', 6'-diamidino-2-phenylindole

DBL – Duffy binding ligand

DHHC-CRD – Asp-His-His-Cys cysteine-rich domain

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

EBL – Erythrocyte binding ligand

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbent assays

ELM – Eucaryotic Linear Motif

ER – Endoplasmic recticulum,

FTI – Farnesyl transferase inhibitor

FV – Food vacuole,

GAC – Glideosome-associated connector

GAP – Glideosome-associated protein

GAPMs – Glideosome-associated protein with multiple membrane spans

GGTI – Geranylgeranyl transferase inhibitor

GPI – Glycosylphosphatidylinositol

GPS – Group-based prediction system

HA – Hydroxylamine

HEAT – Huntingtin, elongation factor 3, protein phosphatase 2A, and the yeast kinase TOR1

HEPES – Piperazineethanesulfonic acid

HRP – Horseradish peroxidase (HRP)

IEX – Ion exchange chromatography

IFA – Immunofluorescence assays

IMC – Inner membrane complex

IPTG – Isopropyl β-D-1-thiogalactopyranoside

ISPs – IMC sub-compartment proteins

LB – Luria-Bertani

MAHRP1 – membrane-associated histidine-rich protein-1

MC – Maurer's cleft,

Ni-NTA – Nickel-nitrilotriacetic acid

NMT – N-myristoyl transferase

OPD – Out Patient Department

PBS – Phosphate buffered saline

PEXEL – Plasmodium Export Element

*Pf*AMA1 – *Plasmodium falciparum* apical membrane antigen 1

*Pf*ARO – *Plasmodium falciparum* Armadillo repeat only protein

*Pf*CDPK1 – *Plasmodium falciparum* calcium-dependent protein kinases

*Pf*MOP – *Plasmodium falciparum* merozoite-organizing protein

*Pf*MSPs – *Plasmodium falciparum* Merozoite surface protein

*Pf*MTIP – *Plasmodium falciparum* myosin tail interacting protein rat antibodies

*Pf*Ripr – *Plasmodium falciparum* Rh5 and its associating protein

PhIL 1 – Photosensitized 5-[125I] iodonaphthalene-1-azide-labeled protein 1

PNEPs – PEXEL negative exported proteins

PPIs – protein-protein interactions

PPM – Parasite plasma membrane,

PTEX – Plasmodium Translocon of Exported proteins

PTMs – Post-translational modifications

PV – Parasitophorous vacuole

PVM – Parasitophorous vacuolar membrane

PVM –Parasitophorous vacuolar membrane,

RBCM – Red blood cell membrane,

REX 1 – ring stage exported protein 1

Rh – reticulocyte-binding-like protein homologue

RON – Rhoptry neck

SBP1 – Skeleton binding protein 1

SDS – Sodium dodecyl sulphate

SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEC – Size exclusion chromatography

SH3 – Src homology 3

SKI – Subtilisin/kesin isoenzyme 1

TAT 1 – Tubulin acetyl transferase 1

TRAP – thrombospondin-related anonymous protein

TVN– Turbo vesicular network

WHO – World Health Organization

β-ME – β-mercaptoethanol

ABSTRACT

Malaria still poses a global threat despite the enormous research and intervention strategies that have been employed to curb the menace of the disease over the years. This has necessitated the characterization of novel drug targets for the development of new intervention against malaria. In this study, I identified two novel *P. falciparum* proteins (PF3D7_0410600 and PF3D7_1459400) and used cellular/biochemical approaches to characterize the proteins. Analysis of the protein sequences revealed structural features that present the novel proteins as key players during the malaria parasite development. I generated rabbit antibodies against the two novel proteins and showed detection of the native parasite proteins in immunoblotting and immunofluorescence assays. The results suggest that PF3D7_1459400 protein may be exported and possibly associates with parasite-induced structures. I also observed that PF3D7_0410600 protein which overlapped with a component of the inner membrane complex (IMC), may not be palmitoylated and only geranylgeranyl transferase inhibitor (GGTI) seemed to have impacted on the localization of the protein. My analysis also suggests that protein-protein interactions may be the probable molecular mechanism governing the recruitment of PF3D7_0410600 protein to the periphery of the parasite. It is therefore conceivable that disruption of the IMC-microtubular interplay may alter the parasite morphology, which may consequently affect its survival, and hence present PF3D7_0410600 protein as a suitable target for such a drug development approach. Similarly, the functional investigation of PF3D7_0410600 protein and its associated complex may provide further understanding on the fascinating biology of the malaria parasite.

CHAPTER ONE

INTRODUCTION

1.0 BACKGROUND

Malaria still poses a persistent global threat. About 219 million cases and more than 435 000 deaths were recently reported for sub-Saharan Africa (WHO, 2018) despite the enormous resources, research and intervention strategies channelled towards reduction and possible elimination of the disease over the past decades. The recent increase in the incidence of malaria disease as opposed to the steady reduction recorded in the preceding years has called for an intensified effort in combating the disease. This shift is thought to be associated with the reduced susceptibility of the parasites to frontline anti-malarial drugs (Blasco *et al.* 2017), resistance to insecticide by mosquito and the poor efficacy of available vaccines which has impaired the progress in eradicating malaria (Trape *et al.* 2011).

The severe form of malaria illness and death results from infection by the *Plasmodium falciparum* parasite with most cases found predominantly in pregnant women and children below 5 years (Snow *et al.* 2005). This vulnerability is associated with low level or immature immunity in children and imbalances in hormones/immune system of pregnant women (Maestre and Carmona-Fonseca, 2014).

Plasmodium exhibits a multifaceted life cycle often involving a vector and a host. For the human species, *P. falciparum*, infected female anopheles mosquitoes inject sporozoites in their saliva into the skin during a blood meal. The sporozoites enter the liver where they asexually replicate to release thousands of merozoites into the blood stream to infect healthy erythrocytes (Cowman *et*

al. 2017). The events surrounding the invasion of erythrocytes is a complicated and poorly understood process (Koch and Baum, 2016). However, the parasites require this crucial step for their survival and this process is therefore very attractive for anti-malarial therapeutics (Wright and Rayner, 2014). Invasion of erythrocytes is mediated by several secretory organelles including rhoptries, micronemes, and the inner membrane complex (IMC) that serve as essential part of the motility machinery required for the process. The IMC does this by acting as a linchpin for the actin-myosin motor that provides the requisite force for the invasion processes (Yeoman *et al.* 2011).

The clinical manifestation of *P. falciparum* malaria occurs during the asexual replication where the parasite undergoes multiple rounds of division to perpetuate the vicious cycle (Kochar *et al.* 2006). In order to ensure continuity of the parasite, some of the released merozoites upon invasion of erythrocytes, undergo gametocytogenesis and differentiate into pre-sexual forms called gametocytes. The mature male and female gametocytes are then picked up during another blood meal by mosquito vectors. The male and female gametocytes fuse and develop in the mid-gut of the mosquito through various stages to produce the infective sporozoites. The signals that trigger the formation of gametocytes is poorly understood, however, gametocytes are thought to be induced under certain environmental conditions (Bruce *et al.* 1990; Baker, 2010)

An exceptional feature of the malaria parasite is its ability to thrive in cells that are metabolically inert and lacking all the necessary protein trafficking machinery (Spielmann and Gilberger, 2015). In order to make themselves comfortable in their new home during the intra-erythrocytic stage, the parasite has devised strategies to successfully traffic several proteins past the parasitophorous vacuolar membrane (PVM) and consequently remodel their host cell (Russo *et al.* 2010; Maier *et*

al. 2009). Protein trafficking in living cells is mediated by several mechanisms including the use of targeting signals, motifs (Haldar, 2016), post translational modifications (Yakubu *et al.* 2018) etc.

Previously, post translational modification of proteins was reported to play key roles in several aspects of *Plasmodium* biology (Yakubu *et al.* 2018). Protein palmitoylation, which is a dynamic process where hexadecanoic acid is covalently added to cysteine residues of proteins has been demonstrated to regulate key cellular processes. This includes; sub-cellular localization (Liao *et al.* 2017; Wetzel *et al.* 2015), protein trafficking (Michaelson *et al.* 2002) gene expression (Park *et al.* 2011; Kostiuk *et al.* 2010), cytoskeletal function (Tremp *et al.* 2017), protein-protein interactions (Blanc *et al.* 2013), host cell invasion and other metabolic processes (Yakubu *et al.* 2018; Caballero *et al.* 2016; Jones *et al.* 2012). Therefore, it is imperative to understand the possible mechanism(s) by which the malaria parasite successfully recruit proteins to different membranous destinations, and this can inform the development of therapeutic intervention to target these pathways using small molecules.

1.1 PROBLEM STATEMENT

The molecular mechanism(s) underlying the distribution of *P. falciparum* proteins to membrane localization is still poorly understood. Therefore, this project sought to shed more insights on the molecular mechanism(s) regulating the targeting of a novel *P. falciparum* protein to membrane localization.

1.2 JUSTIFICATION

It is well-known that the function of a protein is largely determined by its proper folding, stability, localization and other factors. Generally, lipid modifications have been shown to impact on the versatility of protein function and there are several lines of evidence suggesting that protein-protein interactions and/or post-translational modifications may be associated with the membrane distribution of proteins. However, the underlying mechanism(s) regulating the recruitment of *P. falciparum* proteins to different subcellular localization is not well understood. More importantly, there is an increasing need to further dissect the fascinating biology of this parasite and hence provide useful tools in eradicating malaria disease through identification of drug targets with novel modes of action. Therefore, characterizing novel *P. falciparum* proteins that may play a crucial role in the pathogenesis of malaria will be relevant for the proper understanding of the parasite biology.

1.3 HYPOTHESIS

The subcellular localization of PF3D7_0410600 protein in *P. falciparum* is governed by protein-protein interactions (PPIs) and/or post-translational modifications (PTMs).

1.4 AIM

To identify a *P. falciparum* protein that lacks structural characteristics for membrane anchorage but could be localized to the membrane and unravel the likely mechanism(s) governing the membrane localization of the target protein.

1.5 SPECIFIC OBJECTIVES

- To identify a novel *P. falciparum* protein that lack membrane anchoring signatures.
- To determine whether the protein of interest could be targeted to membrane localizations.
- To determine the palmitoylation status of PF3D7_0410600 protein.
- To determine other possible molecular mechanism(s) mediating the subcellular distribution of PF3D7_0410600 protein.

CHAPTER TWO

LITERATURE REVIEW

2.0 GLOBAL BURDEN OF MALARIA

The sub-Saharan Africa region still bears a relatively higher proportion of the global load of malaria. This is because 92% of all malaria cases and 93% of malaria deaths (WHO, 2018) occur in these regions when compared to other regions of the world (Figure 2.1). The WHO world malaria report in 2018 indicated that *P. falciparum* still causes the most malaria cases in sub-Saharan Africa (99.7%) and the majority of cases in South-East Asia (62.8%), Eastern-Mediterranean (69%) and Western Pacific (71.9%) regions. On the other hand, *P. vivax* remain the driver of malaria infection in the American region where it accounts for 74.1% of malaria cases (WHO, 2018).

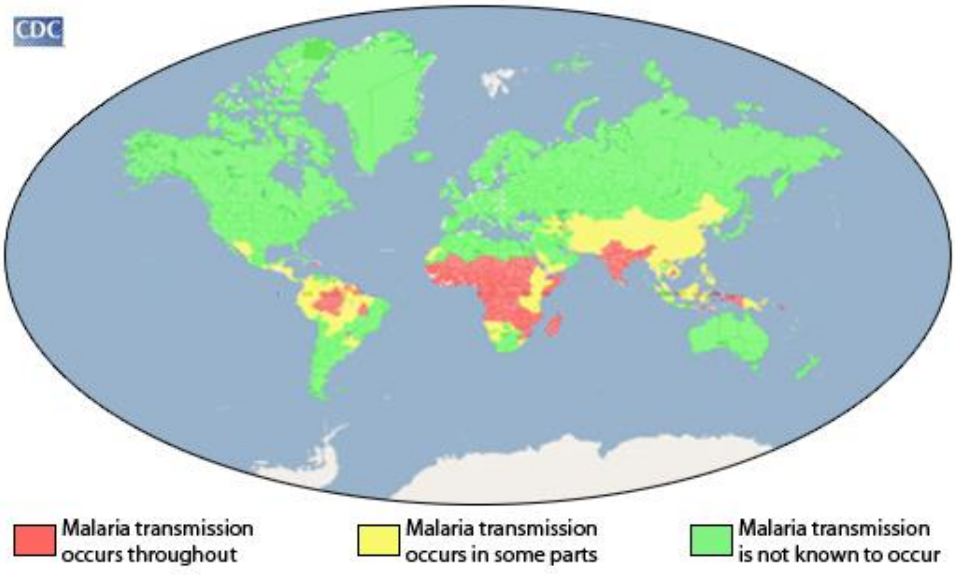


Figure 2.1: Global distribution of malaria. The map shows the sub-Saharan African region, marked in red where malaria transmission is known to persistently occur (CDC, 2020). Other parts marked in yellow have recorded malaria transmission while the parts marked in green have no record of malaria transmission (Adapted from CDC, accessed on 16th Feb., 2020).

Recently, the National Malaria Control Programme of Ghana also reported about 2.3 million suspected malaria cases at the Out Patient Departments (OPDs) of hospitals and clinics in 2017 and this represents a 1.18% rise over cases reported in 2016 (Ghana health service, 2017).

Several strategies such as insecticides and insecticide-treated bed nets, which targets the malaria vector, artemisinin-combination therapy (ACT), which targets the parasite, and the partially effective RTS,S malaria vaccine is currently being employed to curb the menace of malaria. This has led to the reduction of the disease burden in some countries which renewed the hope of eliminating/eradication of malaria in the nearest future. However, the global burden of malaria still persists as consistent exposure to insecticides and anti-malarial drugs have resulted in resistance to these intervention strategies by mosquitoes and *Plasmodium* parasites respectively. More so, a more potent malaria vaccine is yet to be developed even though testing of several candidates is underway.

When compared to some bacterial and viral pathogens that elicit prolonged protection against re-infection often after a one-time infection, malaria infection lacks this memory which is the basis of protective vaccines (Matuschewski, 2020). Hence, the past 3 decades have witnessed different *Plasmodium* antigen combinations which initially composed of the major sporozoite surface proteins (SSPs) circumsporozoite protein (CSP/SSP1) and thrombospondin-related anonymous protein (TRAP/SSP2), the major merozoite surface proteins 1-3 (MSP1-3), and the sexual stage antigens 25 and 230 (Pfs25, Pfs230). Till date, no experimental evidence suggesting any of these antigens as a signature of protective immunity as opposed to parasite exposure is available (Crompton *et al.*, 2010).

The WHO have outlined milestones and specific targets in the global technical strategy for eliminating malaria in 2016-2030. This strategy includes the reduction in mortality/incidence of cases and prevention of re-emergence of the disease in all countries declared as malaria-free (WHO, 2018). One underlying factor in achieving these goals is a proper understanding of the parasite biology and this formed the basis of this thesis.

2.1 LIFE CYCLE AND PATHOGENESIS OF *Plasmodium falciparum*

Malaria is an infectious disease resulting from infection with the obligate intracellular parasite of the *Plasmodium spp*., which belong to the Apicomplexa phylum. Up to six (6) species are known to cause the disease in humans including *P. falciparum, P. vivax, P. knowlesi, P. ovale curtisii, P. ovale wallikeri, and P. malariae* (Cowman *et al.* 2017). *P. falciparum* stands out as the most virulent and widely studied among human *Plasmodium* species. It exhibits a complicated life cycle in the mosquito vector where it undergoes the sexual cycle as well as in the human host where the asexual cycle occurs (Figure 2.2).

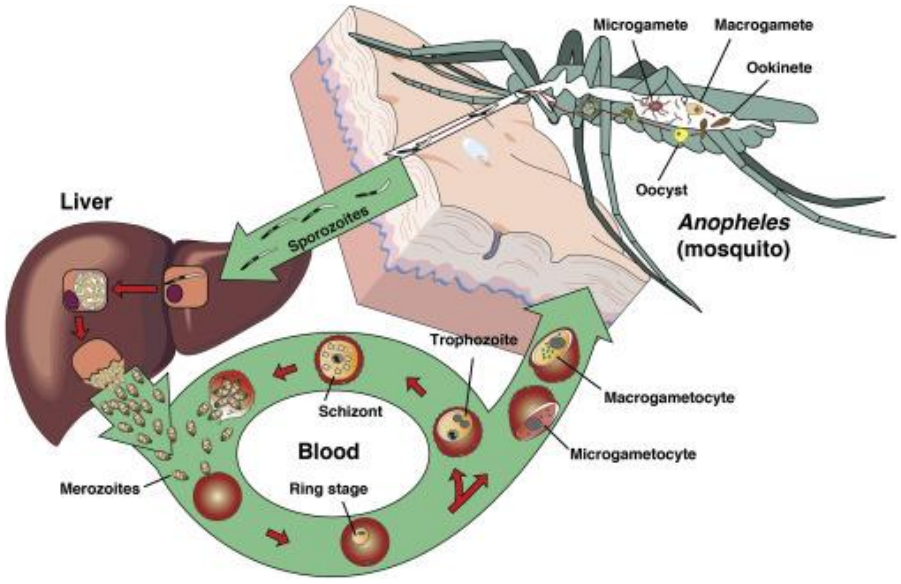


Figure 2.2: Cartoon representation of *P. falciparum* life cycle showing the sexual and asexual cycles involving the mosquito and the human host respectively (Adapted from Cowman *et al.* 2017).

The infection of the human host begins during a blood meal by an infected female *Anopheles* mosquito. The mosquito injects saliva containing the infective forms called sporozoites into the human host. During this process, the invasive sporozoites are inoculated directly into circulation and travel hematogenously to the liver (Yamauchi *et al.* 2007). In the hepatic cells, the sporozoites asexually develops into thousands of merozoites, an invasive form, in a membrane-bound structure known as the merozome (Prudêncio *et al.* 2011). The merozome then ruptures to release invasive merozoites into the circulating blood stream where they invade healthy erythrocytes in a rapid but well-orchestrated manner. To ensure the perpetuity of the parasite, some of these merozoites switch to sexual forms which are picked up by mosquitoes upon subsequent blood meal (Silvestrini *et al.* 2010).

2.1.1 Erythrocyte invasion

Invasion of the erythrocyte is a complex, multi-stage and well-coordinated process that encompasses a myriad of ligand-receptor interactions and different organelles within the parasite. The process of erythrocyte invasion occurs in three sequential phases. The first phase is an initial interaction of the merozoite with the surface of the erythrocyte which is more like a priming step and leads to distortion of the erythrocyte membrane. This is succeeded by a re-orientation of the apex of the merozoite at tangent to the surface of the erythrocyte and subsequent tight junction formation between the apex of the merozoite and the erythrocyte. Finally, echinocytosis occurs and this is characterized by the shrinkage of the invaded erythrocyte and its subsequent recovery after entry of the parasite (Cowman *et al.* 2017).

Evidence exists on the involvement of a group of proteins called the merozoite surface proteins (MSPs) during the early phase of erythrocyte invasion because these proteins form a large complex

with peripheral proteins on the surface of the merozoite. However, no specific ligand-receptor interaction has been described for these proteins to date (Beeson *et al.* 2016). A study indicated that merozoites lacking MSP1 successfully invaded erythrocytes, suggesting that the protein may be dispensable for invasion but important for egress (Das *et al*. 2015).

Apical positioning of the merozoite is a necessary pre-requisite for active invasion since it orients the merozoite for discharge of its rhoptry and micronemal content to initiate binding to the erythrocyte and eventual tight junction formation (Cowman *et al.* 2017). The formation of a tight junction is a very crucial step in erythrocyte invasion because it represents the structural aperture in erythrocytes through which the parasites actively move into the host cell before finally taking residence in the newly formed parasitophorous vacuole. The formation of the tight junction involves two major families of parasite proteins known as the Duffy binding-like (DBL) or erythrocyte-binding like (EBL) protein and the other being reticulocyte-binding-like protein homolog (Rh or RBL) (Duraisingh *et al.* 2003; Mayer *et al.* 2009).

In *P. falciparum*, the EBL family of proteins is made up of erythrocyte binding antigen 140 (EBA-140) for which Glycophorin C has been reported as its receptor (Mayer *et al.* 2006), EBA 175 which interacts with Glycophorin A (Camus and Hadley, 1985), EBL-1 that binds to the Glycophorin B receptor (Mayer *et al.* 2009) and EBA-181 for which the receptor is yet to be identified (Gilberger *et al.* 2003). The Rh family of proteins on the other hand was first identified in *P. vivax* but their orthologues have been characterized in *P. falciparum.* Receptors for most of these Rh proteins such as *Pf*Rh1, *Pf*Rh2a, *Pf*Rh2b remain unknown except for *Pf*Rh4 which has been shown to bind complement receptor 1 (CD35) (Tham *et al.* 2010) and *Pf*Rh5 which binds to its receptor, basigin (CD147) (Crosnier *et al.* 2011).

The deformation of the erythrocytes as a result of the binding of the EBAs/*Pf*Rhs with their respective receptors has been shown to enhance the invasion process since merozoites can securely embed themselves into the deformed surface of the erythrocyte and resist detachment during blood flow (Cowman *et al.* 2017). Merozoites deploy a protein phosphatase complex (calcineurine, which responds to calcium ion signalling) to also fortify the host-parasite association initiated by the binding of EBAs/*Pf*Rhs with their receptors. However, calcineurine has not been shown to play any role during initial interaction of the merozoite and the host cell but rather in signalling events prior to recognition of the host cell (Paul *et al.* 2015).

The binding of *Pf*Rh5 to basigin has also been associated with an influx of calcium ions into the erythrocytes. This has been suggested to originate from the merozoite via a pore that is supposedly formed during the interaction (Volz *et al.* 2016). Even though direct evidence to proof that the Ca^{2+} originates from the merozoite is still lacking, it provides the only tenable explanation that a pore is formed, and that the parasite inserts various antigens into the host cell membrane through this pore (Cowman *et al.* 2017).

The apicomplexan parasites have devised a clever means of deploying their own pair of ligands and receptors to enhance the invasion process. The *P. falciparum* apical membrane antigen 1 (*Pf*AMA1) associates with rhoptry proteins immediately after reorientation and this has been proposed to be crucial for the secretion of the rhoptry content (Richard *et al*. 2010). This association involves rhoptry proteins including RON2, 4 and 5 that were previously demonstrated to play a crucial role in tight junction formation (Triglia *et al.* 2000). Similar studies in *T. gondii* suggested that one of the major components of the RON complex, RON2, is the first protein injected into the erythrocyte cytoskeleton and subsequently serves as a receptor for the AMA1

ligand. This interaction has been shown to be important despite the several debates over the significance of AMA1 to the invasion process (Bargieri *et al.* 2013; Cowman *et al.* 2017).

Upon successful invasion of the erythrocyte, a remodelling event characterized by a different network of protein trafficking creates a suitable niche for the parasite where it can acquire the necessary nutrients required for growth and multiplication in addition to providing an avenue to escape the host immune surveillance (Boddey and Cowman, 2013). The intracellular form of the parasite grows and develops from rings, through trophozoites and finally schizonts containing 16–32 daughter cells. These daughter cells carry on the vicious cycle of infecting healthy erythrocytes leading to the clinical manifestations associated with malaria. Previously, it was unclear whether red blood cells (RBCs) actively participate during the invasion events. But recent evidences suggest that erythrocytes may be playing contributory roles in the invasion process (Dasgupta *et al.* 2014, Koch *et al.* 2017; Sisquella *et al.* 2017).

2.1.2 Invasion-related antigens as target for vaccine development

The most advanced malaria vaccine, RTS,S, adopted an integrated approach involving a virus-like particle that can be produced in large fermenters by recombinant expression of the hepatitis B surface (S) antigen in *Saccharomyces cerevisiae*. A smaller fraction modified with a portion of the *Plasmodium falciparum* circumsporozoite protein and including the repeat region (R) and a T-cell epitope (T), fused to the S antigen was employed for multiple rounds of safety, immunogenicity and efficacy testing and improvements. This resulted in a formulation with a potent liposome-based adjuvant, termed AS01 (Didierlaurent *et al.*, 2017), which was later selected for large-scale studies in at-risk populations (RTS, S Clinical Trials Partnership, 2011). These studies revealed an initial short phase of protection against clinical malaria, in good correlation with exceptionally

high antibody titres resulting from the vaccination scheme but protection disappeared with the concurrent decrease in antibody titres after few months. Since this initial protection was offset in the succeeding years, the overall aim was defeated and hence, the result was no efficacy (Olotu *et al*., 2016). It is pertinent to say that the choice of target antigen is essential, hence, a better molecular understanding of the difference between a protective immune response and immune recognition of parasite exposure is therefore imperative (Matuschewski, 2020).

Investigation of the ligand-receptor interactions that precede the invasion of erythrocytes by the malaria parasite has contributed to the development of inhibitors and antibodies that are capable of blocking parasite invasion. For instance, antibodies against *Pf*Rh5 and its binding partners have been shown to exhibit potent inhibitory effects in both *in vitro* and *in vivo* experiments (Chen *et al.* 2011, 2017; Dreyer *et al*. 2012; Douglas *et al.*, 2011, 2014, 2015; Favuzza *et al.* 2017).

Complement deposition seems to be the most plausible mechanism for most inhibitory antibodies whose mode of action is largely unknown (Cowman *et al.* 2017). *P. falciparum* MSP1 and MSP2 for instance, were previously reported to elicit potent invasion inhibitory antibodies and inhibit erythrocyte invasion by complement deposition (Boyle *et al*. 2015). However, complement deposition is a largely unexplored mechanism of antibody-dependent parasite killing which could be further investigated. On the other hand, antibodies against *P. falciparum* AMA1 has also been reported to inhibit erythrocyte invasion probably by interfering with the association of AMA1 and the RON proteins (Collins *et al.* 2009). Additional reports also reinforced the role of AMA1 in invasion using a binding peptide (R1) where R1 inhibited erythrocyte invasion (Harris *et al*. 2009). However, the polymorphic nature of AMA1 and the MSPs have resulted in their inability to elicit

strain-transcending inhibitory antibodies and hence their failure in clinical trials (Cowman *et al.* 2017).

Notably, among the catalogue of invasion-related antigens that are currently being studied for vaccine development, *Pf*Rh5 appears to be the top blood-stage vaccine candidate largely because it is conserved and seems not to be under immune pressure (Cowman *et al.* 2017). *P. falciparum* Rh5 has been shown to elicit strain-transcending neutralizing antibodies. Also, antibodies against EBA-175 together with *Pf*Rh4 and *Pf*Rh5 have shown synergistic inhibitory effects during parasite invasion. This suggests that the interlinked nature of the invasion process is still poorly understood (Williams *et al.* 2012). Targeting multiple steps of the invasion process has been postulated to likely stimulate better invasion inhibitory antibodies and hence a multivalent vaccine approach promises to be the preferable option in terms of efficacy when compared to single subunit vaccine for malaria (Lopaticki *et al.* 2011; Williams *et al*. 2012).

2.1.3 Key subcellular organelles and their roles in erythrocyte invasion

All the invasive forms of *Plasmodium* possess the secretory apical vesicles that discharges their contents in a serial and well-controlled manner for the priming and invasion of their target host cells (Lal *et al.* 2009). These organelles include; the micronemes, rhoptries and dense granules (Figure 2.3A). Micronemes houses the adhesins that bind erythrocytes and rhoptry contents are necessary for the initial interaction with the host cell. This facilitates the invasion process and the formation of the parasitophorous vacuole (PV) inside which the merozoites undergo replication to form daughter cells (Cowman *et al.* 2017). The inner membrane complex (IMC) is a key organelle that plays a crucial role in the morphology, and rigidity of the cell as well as in erythrocyte invasion

(Kono *et al.* 2012). The dense granules are required for late host cell modification (Kats *et al.* 2008).

Each of these organelles harbour proteins that are important for invasion, growth and development of the parasite. Although a repertoire of these proteins are still hypothetical, many of the proteins secreted by the microneme, including EBA-175 (Pattnaik *et al.* 2007), apical membrane antigen 1 (AMA-1) (Heppner *et al.* 2005) and the thrombospondin-related anonymous protein (TRAP) (Bejon *et al.* 2006), have been characterized and demonstrated to be essential vaccine candidates. Since these organelles form the arsenal of the parasite proteins, it becomes increasingly important to understand how proteins are trafficked to these different subcellular compartments as well as how parasites regulate the secretion of these proteins. This may represent a crucial milestone in dissecting the fascinating parasite biology and also provide new avenues for antimalarial therapeutics.

2.1.4 The inner membrane complex (IMC)

Members of the alveolata super phylum possess a common flattened membranous sac located beneath the plasma membrane. This structure is called alveoli in ciliates, while in dinoflagellates and apicomplexans, it is known as the amphiesmal vesicle and IMC respectively (Morrissette *et al.* 2002). In addition, apicomplexans are bounded by a pellicular structure which comprises the plasma membrane and the proximally adjacent IMC (Foussard *et al*. 1990).

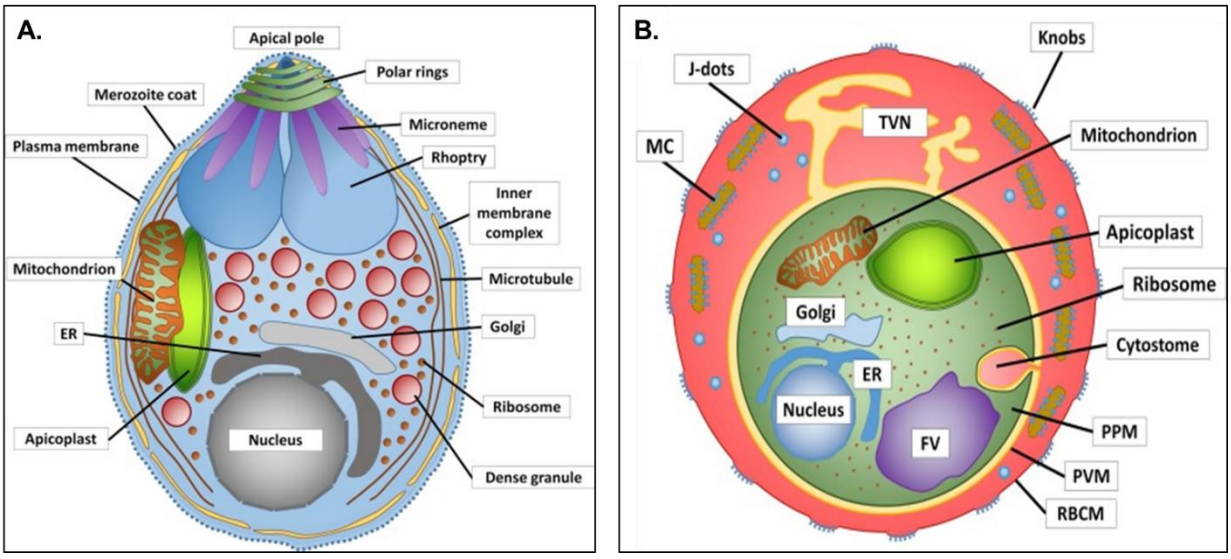


Figure 2.3 Cartoon representation of a merozoite and an infected erythrocyte. (A) The structural architecture of a merozoite is shown with the different organelles involved in invasion. (B) Cartoon of an infected erythrocyte showing the membranes and structures induced by the parasite during infection. MC; Maurer's cleft, PPM; parasite plasma membrane, FV; food vacuole, PVM; parasitophorous vacuolar membrane, RBCM; red blood cell membrane, ER; endoplasmic recticulum, TVN; turbo vesicular network (Adapted from Flammersfeld *et al.* 2018).

The IMC is comprised of flattened disc-like vesicles underneath the plasma membrane and is interwoven with the cytoskeleton (Figures 2.3A and 2.4). This vesicular structure appears to have originated from Golgi-associated vesicles that became flattened to form large enveloping membranous sheets around the parasite during maturation (Bannister *et al.* 2000).

The major role of the IMC is to preserve cell morphology and serve as a scaffolding compartment during cell division (Kono *et al.* 2012; Beck *et al.* 2010). The IMC has also been shown to anchor the actin-myosin motor that constitutes the glideosome machinery which provides the prerequisite force necessary for motility and invasion (Tremp and Dessens, 2011; Yeoman *et al.* 2011).

The glideosome is a mechanical machinery connecting the parasite plasma membrane (PPM) and the IMC which provides structural strength to the parasite. This connection is established through a complex interaction of the actin filaments and the IMC involving a minimum of five (5) parasite-

derived proteins, mainly the MyoA protein belonging to the class XIV myosin which is unique to the apicomplexa, one or more myosin light-chain homologues, and the glideosome-associated proteins; GAP40, GAP45 and GAP50 (Figure 2.4) (Kumpula and Kursula, 2015).

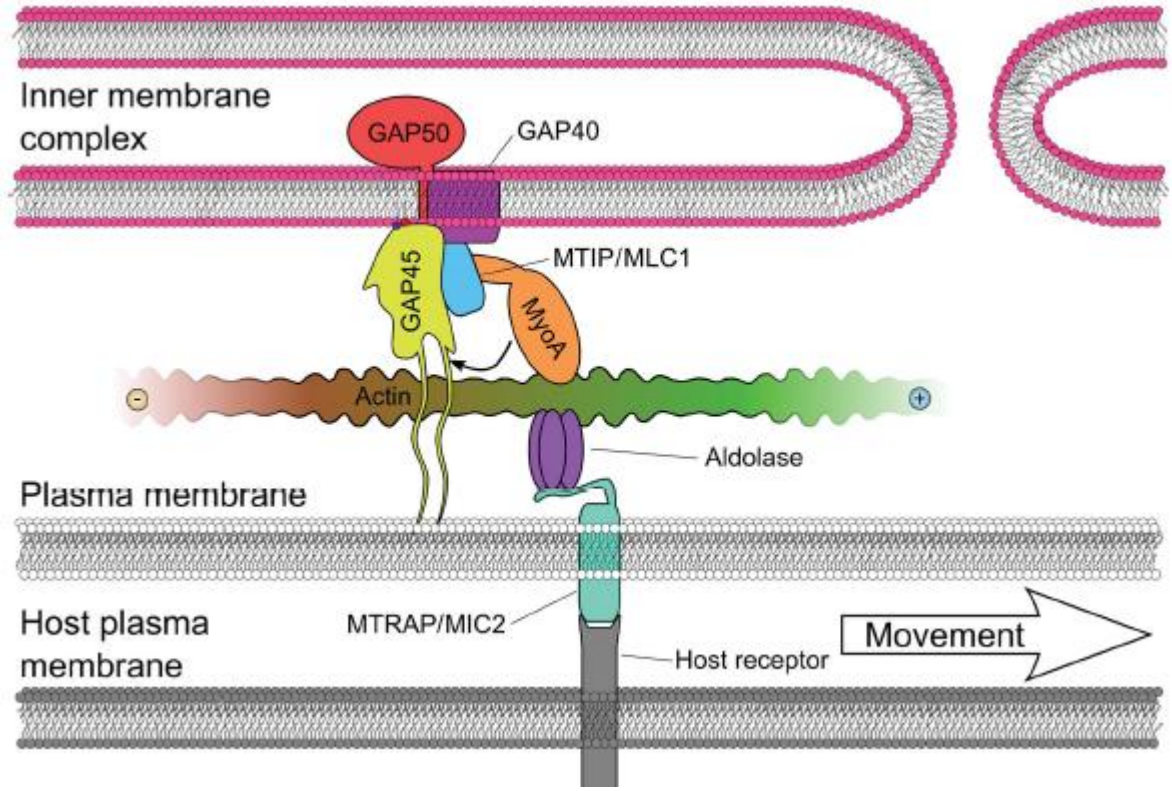


Figure 2.4: Schematic representation of apicomplexan pellicle and proteins that mediate anchorage to the inner membrane complex. The small arrow indicates the direction of the MyoA power stroke, while the large arrow indicates the direction of parasite movement. The directionality of actin polymerization is indicated by + and - signs. (Adapted from Kumpula and Kursula, 2015).

On the other side of the IMC, it is found to be closely linked with a system of intermediate filament-like proteins and sub-pellicular microtubules (Frenal *et al.* 2010). The biogenesis of the IMC has been shown to begin during early schizogony and about 17 IMC proteins were identified in *P. falciparum* (Kono *et al*. 2012). Because of the divergent role of the IMC, it is difficult to classify the member proteins based on a particular feature. However, previous studies have attempted the

grouping of IMC proteins into multi-transmembrane proteins, alveolins and non-alveolins based on distinguishing structural features. The glideosome-associated protein with multiple membrane spans (GAPMs) that has 6 transmembrane domains is an example of a multi-transmembrane protein that is localized to the IMC (Kono *et al*. 2012).

The majority of the well-studied IMC proteins are the non-alveolins, especially those that make up the glideosome (GAP45, GAP50). In a systematic analysis of IMC components, the glideosome associated proteins showed distribution that is restricted to specific compartments within the IMC, hence giving the organelle an apical, central and basal sub-compartments in the developing merozoites of *Plasmodium* spp. (Kono *et al*. 2012; Yeoman *et al.* 2011). This spatial localization pattern has been used to classify IMC proteins into two major groups. The group A proteins (which includes transmembrane proteins, the GAPs and certain non-alveolins) initially showed a dynamic cramp-like structures around the nucleus. They then develop to form ring-like structures that later expand beyond the nucleus to the periphery of the parasite as the schizont matures (Kono *et al.* 2012). Unlike their counterparts, Group B proteins (including the alveolins, and MAL13P1.228-a *Plasmodium* specific protein) displayed a thin ring-like formation during mid-stage schizogony. These structures later expand to the posterior end of individual merozoites in matured schizonts (Kono *et al.* 2012).

Despite this structural stratification of IMC proteins, some protein groups do not fit into any of these categories. Examples of such proteins are the membrane occupation and nexus protein 1 (MORN1), which connects the IMC and the cytoskeleton (Gubbels *et al.* 2006; Lorestani *et al.* 2010) and the IMC sub-compartment proteins (ISPs) which harbours large number of charged amino acid residues and N-terminal modification sites (Beck *et al.* 2010; Fung *et al.* 2012).

Besides involvement in the intraerythrocytic development of the parasite, the IMC was previously shown to be an integral part of the sexual development of *P. falciparum* as it appears to propel the structural transformation of the parasite throughout gametocytogenesis (Kono *et al.* 2012). Hence, disrupting the IMC formation may yet present a convergent multi-target intervention opportunity for malaria.

2.1.5 The IMC and gametocyte development in *P. falciparum*

Current understanding of the different stages of *Plasmodium* biology is still limited despite the extensive study of the developmental stages of the parasite. One of such stage is the gametocyte formation which involves the cellular and morphological transformation of a round-shaped asexual parasite similar to a pigmented trophozoite, into an elongated sexual form (Silvestrini *et al.* 2010). The formation of gametocytes enables the transmission of the parasites from an infected host to a healthy one thereby perpetuating the spread of the disease. The series of events that precede this developmental switch is not clear (Baker, 2010). However, evidence exists supporting the fact that gametocytogenesis might be triggered by the presence of a high parasite load (Bruce *et al.* 1990) while other postulations imply that this switch might actually be the default developmental pathway as seen in related species (Sinden, 2009). Furthermore, it has become increasingly acceptable that the decision to switch from an asexual to a sexual form seems to be made in the preceding asexual cycle (Baker, 2010).

The differentiation of gametocyte from the asexual stage parasites has been reported to be activated by several factors, including human host factors (such as Lysophosphatidylcholine (LPC), haemoglobin level, immunity and presence of anti-malarial drug) as well as parasite factors (such as homocysteine, genetic diversity of infection, mixed infection and density of the asexual stages)

(Brancucci *et al.*, 2017, Beri *et al.*, 2017, Carter *et al.*, 2013; Gbotosho *et al.*, 2011; Peatey *et al.*, 2009; Vardo-Zalik and Schall 2009; Bousema *et al.*, 2008). A recent observation suggested that sexual commitment takes place in the bone marrow, where erythroid progenitor cells are abundant with reduced lysophosphatidylcholine (LPC) concentration which has been associated with the extent of gametocyte production (Brancucci *et al.*, 2017). Afterwards, a contradicting report later showed that committed rings were present in the blood circulation suggesting that commitment perhaps occurs in the bloodstream (Farid *et al.*, 2017). Hence, the actual microenvironment that favours commitment of the parasite to the sexual stage development remains debatable.

However, adaptation to these microenvironments have been shown to be modulated by exported proteins which remodel the host cell membrane (Silvestrini *et al.*, 2010). Recently, proteins localized to osmiophilic bodies have been shown to play a role in the egress of gametocytes (Ishino *et al.* 2020). Also, using gene set enrichment analysis, it was previously shown that exported proteins and those that consequently take part in erythrocyte remodelling are the most abundant protein sets in the early phase of gametocytogenesis (Silvestrini *et al.* 2010). These reports create room for further probing of the parasite transmission events because these proteins would definitely impact the properties of the host cell membrane and hence, the entire transmission biology. Furthermore, functional characterization would be required to decipher the function of such proteins since protein trafficking have been shown to play essential role during the asexual life cycle (Lavazec and Neveu, 2019).

Recently, the activation of the transcription factor AP2-G, has been shown to initiate commitment to gametocytogenesis both in rodent parasites and *P. falciparum* (Kafsack *et al.* 2014; Sinha *et al.* 2014). Interestingly, it was recently observed that the conditional overexpression of AP2-G can be

used to synchronously convert the great majority of the population of parasites into fertile gametocytes (Kent *et al.* 2018). This is an important achievement in the understanding of the biology of *Plasmodium* gametocyte development.

Gametocytes are largely dependent on the IMC as a key structural component for their progressive development which is characterized by morphological changes driven in turn by changes in the IMC and its associated subpellicular microtubule (Kono *et al.* 2012). Unlike the IMC in the 'zoites' form of the parasite, the gametocyte IMC has been shown to have stage-specific functions even though the majority of the proteins involved are yet poorly defined (Schneider *et al.* 2017). It is therefore important to characterize new IMC-resident proteins and determine how these proteins are trafficked to this membranous organelle. More importantly, since the IMC is necessary for parasite division and cell morphology, designing new drugs directed at disrupting the formation of the IMC will provide new intervention strategies against both sexual and asexual stage development in *P. falciparum.*

2.2.0 MECHANISMS OF PROTEIN TRAFFICKING TO SUBCELLULAR LOCATIONS IN THE MALARIA PARASITE

The *Plasmodium* parasite has devised strategies for the successful trafficking of several proteins to membranous destinations and consequently remodel the host cell (Russo *et al.* 2010; Maier *et al.* 2009). Protein export in the malaria parasite facilitates nutrient acquisition, sequesteration in circulation and evasion of host immune responses (Boddey *et al.* 2013). Even more, protein export precedes gametocytogenesis (Silvestrini *et al.* 2010) and this has fostered research interest on how *Plasmodium* parasites target proteins to specific subcellular localizations and the mechanisms that mediate the process.

While there have been significant research efforts towards understanding the parasites' protein trafficking machinery and the characterization of exported proteins (Zhang *et al.* 2018; Rhiel *et al.* 2016; Acharya *et al.* 2012; Grüring *et al.* 2012), some questions remain unanswered. For instance, what mechanism(s) mediate the membrane anchorage of proteins that lack membrane-targeting signals? How are transmembrane domain-containing proteins targeted to the PVM? Answers to these questions will provide clues on targeting specific organelles in *Plasmodium* parasites since many proteins in their organelles have structural and functional differences in comparison with those of the human hosts.

2.2.1 Motifs/Domains involved in protein trafficking

2.2.1.1 PEXEL motifs

Protein export in the malaria parasite is accomplished through a common trafficking machinery known as the ***P****lasmodium* **T**ranslocon of **Ex**ported proteins (PTEX) (de Koning-Ward *et al.* 2009). The export of many *Plasmodium* proteins has been shown to be dependent on important features such as domains and motifs in the protein sequence (Sijwali and Rosenthal, 2010). One of such features is the possession of defined ***P****lasmodium* **Ex**port **El**ement (PEXEL) motif at the N-terminus of proteins (Marti *et al.* 2004; Hiller *et al.* 2004;).

The export of several parasite proteins beyond the parasite confines has been previously demonstrated to be mediated by the PEXEL motif (Hiller *et al.* 2004; Marti *et al.* 2004; MacKenzie *et al.* 2008; Sijwali and Rosenthal, 2010). Proteins that are exported by translocation to the host cytoplasm via the PTEX are reportedly processed (by plasmepsin V) at PEXEL positions 3 and 4 and subsequently N-acetylated to give a mature protein (Sleebs *et al.* 2014; Boddey *et al*. 2013).

The PEXEL motifs can either be canonical or non-canonical. Canonical PEXEL motifs follow a conserved pattern comprised of five (5) amino acids with Arginine (R) at position 1,any charged-neutral amino acid at position 2, Leucine (L) at position 3, any charged-neutral amino acid at position 4 and Glutamic acid (E), Aspartic acid (D) or Glutamine at position 5 (R.L.E/D/Q) (Boddey *et al.* 2009). However, some proteins deviate from this pattern by having a Lysine (K) or Histidine (H) at position 1 (Pick *et al.* 2011) to give non-canonical PEXEL variants (R.I.E/D/Q, K.L.E/D/Q, K.I.E/D/Q, H.L.E/D/Q and H.I.E/D/Q). The functionality of these sets of non-canonical variants has been shown to be dependent on the sequence environment implying that these variants cannot be excluded from the exportome (Schulze *et al.* 2015).

2.2.1.2 PEXEL-Negative Exported Proteins (PNEPs)

On the other hand, there are few parasite proteins that are exported by translocation across the PVM without the PEXEL motifs and these are categorized as PNEPs (Spielmann and Gilberger, 2010). Analysis of the amino acid sequence mediating the export in an identified subset of a group of PNEPs that lack signal peptides at the N terminus indicated that 20 amino acids at the N-terminus was adequate for export of the proteins (Heiber *et al*. 2013). Some of the well-known PNEPs include: skeleton binding protein 1 (SBP1) (Blisnick *et al.* 2000), ring stage exported protein 1 (REX 1) (Hawthorne *et al.* 2004), REX 2 (Spielmann *et* al. 2006), membrane-associated histidine-rich protein-1 (MAHRP1) (Spycher *et al.* 2003), and MAHRP2 (Pachlatko *et al*. 2010) which are all located in the Maurer's cleft and characterized by similar domain architecture. Their localization to the Maurer's cleft is indicative of their connection with protein trafficking since the Maurer's cleft is majorly known for protein trafficking (Figure 2.3B) (Maier *et al.* 2009).

2.2.2 Alveolin repeats

Alveolins were first identified and studied in *Toxoplasma spp* but recent studies have shown their essentiality for motility and maintaining cell shape in *Plasmodium spp.* (Volkmann *et al.* 2012; Tremp *et al.* 2014). Alveolins possess a repetitive amino acid motif that was previously demonstrated to regulate their targeting to the IMC (Gould *et al*. 2010; Fung *et al.* 2012). These motifs were revealed in a systematic analysis of the genomic sequence of all the Alveolata (apicomplexans, dinoflagellates and ciliates). Conserved valine- and proline-rich sub-domains: 'EKIIEVPQ, EKIIEVPK, EKIVEVPH, DKIVEVPQ, EKLIHIPK, ERIKKCSK, ERIIPVPK, EKIVEIPQ, EKVQEIPE and EKIVDRNV' were found to be common to a family of proteins related to IMC1, 3 and 4 (Leander and Keeling, 2003; Gould *et al.*, 2006).

These alveolin repeats were later shown to be sufficient for the localization of two IMC proteins in *T. gondii* (Anderson-White *et al.* 2011). However, it will be exciting to know how proteins lacking any alveolin repeat, acylation motifs or any other recognizable structural motifs such as amphipathic helices could be involved in possible molecular interactions that may facilitate their membrane association.

2.2.3 Armadillo repeats

Armadillo repeat (ARM) proteins are a large protein family characterized by a tandem repeat of a conserved 42-amino acid motif. These repeats have been shown to fold into a super-helix, providing the structural platform for 3-dimensional protein-protein interaction and hence confer versatile function on proteins that harbour the ARM repeats (Coates, 2003; Tewari *et al.* 2010; Berthon and Stratakis, 2014). Previously, ARM repeat-containing proteins were identified and

characterized in other eukaryotes where they play key roles in the biology of these organisms (Coates, 2003; Clevers and Nusse, 2012).

More importantly, ARM proteins have recently gained increasing attention in apicomplexan biology with the identification of proteins with putative ARM repeat in the ApiDB-integrated genome database for apicomplexans (Aurrecoechea *et al.* 2007). This resulted in the identification and characterization of two homologues of ARM proteins, importin-α and PF16 in *P. falciparum* and *P. berghei*, respectively (Mohmmed *et al*. 2003; Straschil *et al*. 2010). The common examples of ARM domain-containing proteins are the β-catenin family of proteins that have been shown to have diverse function as a result of their structure (Xu and Kimelman, 2007). Such diverse functions include; cell signalling, cytoskeletal organization, gene regulation (Coates, 2003; Tewari *et al*. 2010), bridging the cytoplasmic domains of cadherins to α-catenin and the actin cytoskeleton (Hulsken *et al.* 1994; McCrea *et al.* 1991) etc.

The versatility in the function of ARM domain-containing proteins is supported by previous studies which indicated that an armadillo repeats only (ARO) protein is involved in apical orientation of the rhoptry organelle, a necessary step for host cell invasion (Mueller *et al.* 2013). Subsequent findings showed that ARO also functions in the clustering of rhoptry organelles and is involved in a functional interaction with two other rhoptry proteins, ARO-interacting protein (AIP) and adenylate cyclase β (Acβ). The localization of Acβ was shown to be dependent on this molecular interaction as it vanishes from the rhoptries once AIP is absent (Mueller *et al.* 2016). This finding in *T. gondii* represented the identification of a third sub-compartment bridging the rhoptry bulb and neck that was reported previously (Lemgruber *et al.* 2010).

Also, structural analysis on ARO showed that the ARM repeats are arranged in a particular pattern to form a shallow groove thought to be the putative binding site for myosin F (MyoF) that co-immunoprecipitated with ARO (Mueller *et al.* 2013; Jacot *et al.* 2013). This binding groove is flanked with aromatic and acidic residues shown to be important for the binding function as reported previously for human importin-α7 (Pumroy *et al.* 2015). It was also shown that *Pf*ARO exhibits nucleo-cytoplasmic shuttling with a DNA-binding activity during early to late schizogony in *P. falciparum* (Mitra *et al.* 2016). Hence, it might be plausible to consider ARM domains as modules for protein-protein interaction and this in turn could modulate the recruitment of *P. falciparum* proteins that lack membrane targeting signals to membrane destinations.

Interestingly, ARM domain-containing proteins were recently shown to be key players in the biogenesis of the IMC in both sexual and asexual stage parasites (Absalon *et al.* 2016). Therefore, it can be conceived that the possible roles of armadillo repeat in proteins may have implications in tackling the parasite using small molecule inhibitors that can bind such proteins and consequently inhibit their function.

2.2.4 Protein-protein interactions

Protein complexes forms a network of multifaceted interactions in most living organisms. This is especially important in host-parasite interactions that involve intercellular contacts for many biological processes (Paul *et al.* 2017). Several approaches have been employed to study protein-protein interaction in the malaria parasite including a yeast two-hybrid system (LaCount *et al.* 2005) and more recently, by computational methods (Ramaprasad *et al.* 2012).

Protein-protein interactions (PPIs) can undoubtedly mediate the localization of proteins that lack membrane localization signals. This was reported previously for photosensitized 5-[125I] iodonaphthalene-1-azide-labeled protein 1 (PhIL1) that lacks any noticeable transmembrane domains or lipid modification sites, and deleted amphipathic helix at the N-terminus, yet localizes to the peripheral of parasites (Gilk *et al.* 2006; Saini *et al.* 2017). A similar and well-studied interaction has also been reported in peripheral golgi membrane proteins (Ramirez and Lowe 2009).

Another scenario where proteins lacking membrane-anchorage signatures are tethered to the membrane was reported for *Pf*Rh5 and its associating protein *Pf*Ripr. They both lack transmembrane domain or GPI anchor but are tethered to the membrane by interacting with another GPI-anchored protein, CyRPA (Reddy *et al.* 2015).

Previously, it was generally accepted that aldolase acted as the connector of the actin filaments and the cytoplasmic tails of adhesins (Jewett and Sibley, 2003). But, the identification of an armadillo-repeat protein, glideosome-associated connector (GAC) in *T. gondii* has shed more insights on this conundrum (Jacot *et al.* 2016). At one end, GAC binds and stabilizes the F-actin and also binds to phosphatidic acid through a pleckstrin homology domain and this was demonstrated to have crucial implications in motility and invasion (Jacot *et al.* 2016; Cowman *et al.* 2017). This finding provided the experimental evidence to support the invaluable contribution of protein-protein interaction in stabilizing or localizing proteins to different organelles and the subsequent effect on their cellular function.

2.2.5 Post translational modification

Several reports exist on the key roles of post translational modification in various aspects of *Plasmodium* biology. In a broader sense, it involves the covalent processing events where a protein is proteolytically cleaved at a specific site or modifying groups (such as GPI, akyl groups or lipid moieties) are added to one or more amino acids within the protein. These modifications readily change the properties of the modified protein such as its localization, stability, turnover, activity and may enhance possible interaction with other proteins (Mann and Jensen, 2003). Post translational modification has recently gained increasing attention owing to the largely important processes that they modulate in living systems. Many reversible post-translational protein modifications have been reported to affect protein-protein interactions as well as modify diverse functions of such proteins (Stram and Payne, 2016).

Protein phosphorylation for instance, is a reversible process that represents one of the most important ways that cells regulate several physiological and cellular functions including proliferation, differentiation, migration, chromosome condensation, DNA replication, transcriptional regulation and homeostasis (Doerig *et al.* 2015). This process is mediated by protein kinases such as calcium-dependent protein kinases (CDPKs) and protein phosphatases which have no orthologues in mammalian cells or other metazoans making these protein families attractable targets for antimalarial intervention.

Similar reversible protein modification includes acetylation and methylation, which have been widely studied for histone proteins. These modifications have been reported to co-regulate key cellular processes in other living organisms (Kouzarides *et al*. 2007). However, the role of most

methylation and acetylation reactions in the biology of the malaria parasite remain largely unexplored.

Other post translational modifications have also been reported to modulate the function of proteins for growth, development and regular metabolism (Caballero *et al.* 2016; Yakubu *et al.* 2018). For instance, eukaryotes have been shown to widely employ protein lipidation as a mechanism for regulating the recruitment of proteins lacking transmembrane domains to membrane localization (Beck *et al.* 2010). Protein lipidation is characterised by the addition of lipid moieties to specific amino acid residues of proteins. These modifications usually occur post-translation, however certain modifications such as myristoylation can occur co-translational (Doerig *et al.* 2015).

Even though the various lipid modifications may differ in their predictability, frequency and regulatory functions, they share a common function which is to mediate the association of proteins with membranous compartments. Prenylation and myristoylation for instance, are acylation reactions that both occur primarily at predictable, sequence-directed locations, but it is difficult to predict the reversible palmitoylation and glycosylphosphatidylinositol anchor (GPI anchor) sites based on the primary amino acid sequence (Doerig *et al.* 2015).

Several algorithms have been developed over the years to predict lipid modification sites (Zhou *et al.* 2006; Ren *et al.* 2008; Hu *et al.* 2011; Xie *et al.* 2016; Li *et al.* 2017). Some have achieved prediction efficiency close to 100% for the prediction of lipid modification sites (Li *et al*. 2017). However, it is still debatable which of these algorithms can efficiently predict the specific palmitoylation site of proteins. When predictions are insufficient to identify the total lipidated proteome, biochemical characterization or metabolic labelling coupled with mass spectrometry

analysis have been used to purify palmitoylated proteins as reported previously in *P. falciparum* (Jones *et al*. 2012; Doerig *et al.* 2015).

Palmitoylation is a dynamic process characterized by the covalent addition of hexadecanoic acid to cysteine residues of proteins. This process has been shown to regulate key cellular processes including sub-cellular localization (Wetzel *et al.* 2015; Liao *et al.* 2017), protein trafficking (Michaelson *et al.* 2002), gene expression (Kostiuk *et al.* 2010; Park *et al.* 2011), cytoskeletal function (Tremp *et al.* 2017), protein-protein interactions (Blanc *et al.* 2013), host cell invasion and other metabolic processes (Jones *et al.* 2012; Caballero *et al.* 2016; Yakubu *et al.* 2018).

A well-established post translationally-modified protein in *P. falciparum* that is located in the inner leaflet of the IMC is the glideosome-associated protein 45 (GAP45) (Figure 2.5). GAP45 has been shown to be modified by both myristoylation and palmitoylation and its localization was reported to be dependent on these modifications in *P. falciparum* and *T. gondii*, respectively (Rees-Channer *et al*. 2006; Gaskins *et al.* 2004; Gilk *et al*. 2009).

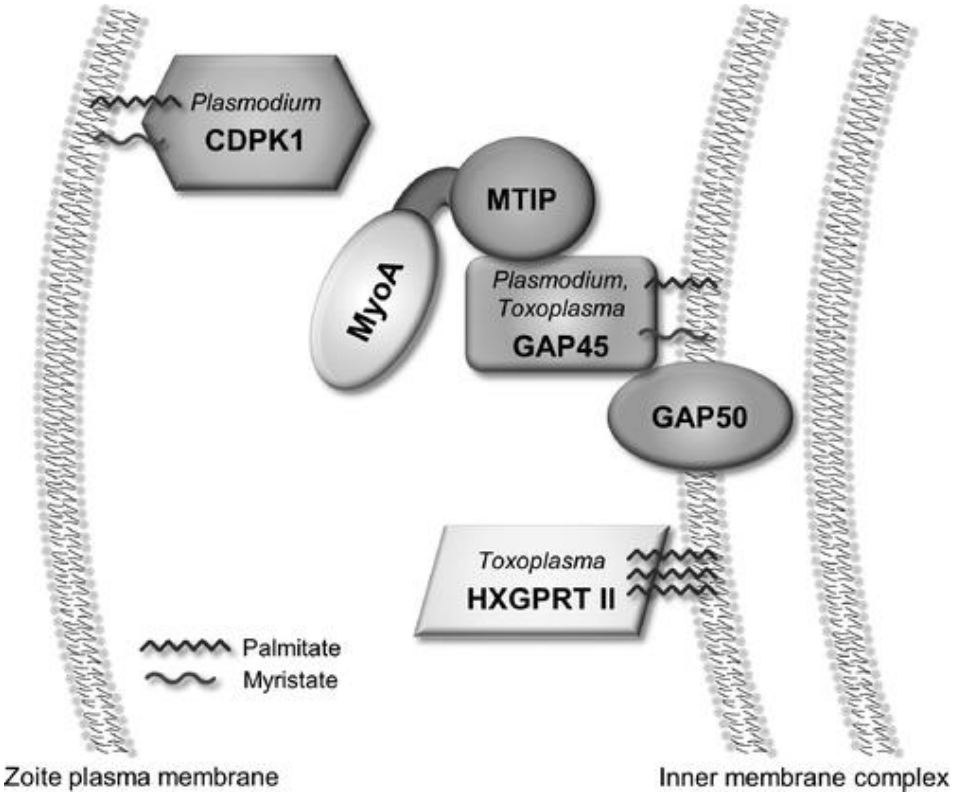


Figure 2.5: Schematics showing modified proteins in *P. falciparum* and *T. gondii*. These proteins have been reported to play a role in gliding motility and invasion. HXGPRTII: Hypoxanthine-xanthine-guanine phosphoribosyl transferase II, CDPK1: calcium-dependent protein kinase 1, GAP45: gliding-associated protein, MTIP: myosin A tail domain interacting protein (Adapted from Jortzik *et al.* 2012).

P. falciparum Calcium-dependent protein kinase 1 (*Pf*CDPK1) has also been shown to localize to the plasma membrane of *Plasmodium* parasites (Figure 2.5) as a result of both myristoylation and palmitoylation of the protein (Moskes *et al.* 2004).

The activity of the palmitoylation enzyme, palmitoyl acyltransferase [denoted by the Asp-His-His-Cys cysteine-rich domain (DHHC-CRD)], has been shown to be residue-specific. For instance, the targeting of three IMC sub-compartment proteins (ISPs) in both *P. falciparum* and *T. gondii* is dependent upon N-terminal modification where mutation of cysteines has been shown to hinder their targeting to the IMC. This suggest the existence of multiple versions of these enzymes in the

IMC and hence dictates its organization since the residues that are predicted to be palmitoylated are also the critical residues for targeting of the proteins to their localizations (Beck *et al.* 2010).

Additionally, an ARM domain-containing protein (*Pf*ARO) was also found to be associated with membranous structures detectable by only markers of the rhoptry membrane and this anchorage of ARO to the membrane was shown to be mediated by acylation (Cabrera *et al*. 2012). Interestingly, 18 DHHC-CRD family of protein acyltransferases have been identified so far in *T. gondii*. Out of this number, only one is localized in the rhoptries and it has been shown that ARO is palmitoylated by this DHHC (Frenal *et al.* 2013; Beck *et al.* 2013) indicating the organelle specificity of these enzymes.

Other lipid modification pathways were previously reported to regulate several processes in the development of *P. falciparum*. Protein prenylation is an irreversible modification of proteins characterized by the addition of hydrophobic isoprenoid moieties (Suazo *et al.* 2016). Prenylation has been shown in many cells to enhance protein-protein interaction and hence, the localization of many proteins involved in cell signalling (Zhang and Casey, 1996; Calero *et al*. 2003; Esher *et al.* 2016). Proteins can be prenylated either via farnesylation (addition of 15-carbon isoprene) or geranylgeranylation (addition of 20-carbon isoprene). These reactions are catalysed by farnesyltransferase or geranylgeranyltransferase I, respectively (Chen *et al.* 2018). Many of the post translational modifications are organelle-specific, playing key regulatory roles in cells and may be very promising for subcellular-targeted therapeutic strategies.

Owing to the emergence of reduced susceptibility to front-line antimalarials (Dondorp *et al.* 2010; Burrows *et al.* 2011), it becomes imperative to identify and develop new drugs with novel modes of action. Among the lipid modification enzymes, N-myristoyl transferase (NMT) that catalyzes

myristoylation is the most widely studied drug target in most parasite species (Bell *et al.* 2012; Goncalves *et al.* 2012; Rackham *et al.* 2013; Wright *et al*. 2014). Previously, the anticancer potential of farnesyl transferase and geranylgeranyl transferase inhibitors have been studied and this has also been shown in different species of *Plasmodium* parasites (Nallan *et al.* 2005)**.** Palmitoylating and de-palmitoylating enzymes have also been proposed for stage-specific drugs since the expression of these enzymes may be stage-specific (Doerig *et al.* 2015). Most of the enzymes responsible for protein modification are crucial for the survival of the blood stage parasites and hence presents them as key targets for the development of antimalarial therapeutics.

CHAPTER THREE

METHODS

3.1.0 GENE IDENTIFICATION

To identify hypothetical genes for this study, published transcriptome (Bozdech *et al.* 2003; Le Roch *et al.* 2003) and palmitome (Jones *et al.* 2012) datasets were used to systematically select the genes of interest. Predicted protein structural features such as possession of transmembrane domain, GPI-anchors and other key motifs were employed in the selection criteria. Also, the timing of expression in sexual and asexual stages were included as part of the selection criteria. This led to the selection of PF3D7_1459400 and PF3D7_0410600 genes for study. The disruptability status of the selected genes was also analyzed using data deposited in the Phenoplasm database (Sanderson and Rayner, 2017). The amino acid sequence of the functional proteins encoded by the selected genes was then probed using Clustering and Scoring Strategy (CSS-PALM) and the Group-based prediction system (GPS) online software for the prediction of the palmitoylation and other lipid modification status of the proteins (Ren *et al*. 2008; Xie *et al*. 2016)**.**

To predict important functional domains of the selected proteins, amino acid sequences were retrieved from the *Plasmodium* genome database (PlasmoDB; Release 44) and submitted to the Eucaryotic Linear Motif (ELM) portal as described previously (Dinkel *et al.* 2016). The ELM platform analyses user-submitted protein sequences by scanning for matches to structural motifs that are already curated in the database. Data obtained from these analyses were used to construct a protein architecture for the two proteins using Microsoft power point (Version 2013).

3.1.1 Codon optimization, gene synthesis and sub-cloning

To enable optimal recombinant protein expression in *Escherichia coli*, the amino acid sequence of the proteins was retrieved from PlasmoDB and codon optimization was performed to supplement for rare codons. The codon-optimized genes encoding the full-length proteins were synthesized with a C-terminal Hexa-histidine (6x-His) tag by BioBasic (Canada). The genes were sub-cloned into a T7 promoter *E. coli* expression vector (pET-24b) with *Nde1* and *Xho1* restriction sites to obtain the plasmids for enhanced expression in the bacteria.

3.2.0 RECOMBINANT PROTEIN PRODUCTION

3.2.1 Transformation of *E. coli* competent cells

Transformation of the competent cells was performed following the manufacturer's protocol (Agilent technologies, UK) with little modifications. Briefly, the BL21-RIPL competent cells were thawed on ice and 100 µL was aliquoted into the required number of pre-chilled BD polypropylene, round-bottom tubes. An additional 100 µL of competent cells were aliquoted for use as a transformation control. Two µL of XL10-Gold β-mercaptoethanol mix (1:10) prepared in sterile double-distilled water was added to each of the competent cells and incubated on ice for 10 minutes with gentle swirling every two minutes. Four µL of the reconstituted plasmid DNA containing each gene of interest was added to each tube of cells. Equal volume of sterile distilled water was added to the control transformation reaction. The reaction mix was incubated on ice for 30 minutes. Afterwards, each transformation mix was heat-pulsed for 20 seconds at 42 °C and incubated on ice for 2 minutes. Then, 0.9 mL of preheated (42°C) terrific broth containing kanamycin (36 mg/ml) was added to each transformation tube and incubated at 37 °C for 1 hour with shaking at 225 rpm. Cells were harvested by centrifuging at 200 ×g for 5 minutes and the

pellets were re-suspended in 100 µL of terrific broth. Using a sterile spreader, 100 µL of the transformed cells were plated onto Luria-Bertani (LB) agar plates containing kanamycin (36 mg/ml). Plates were incubated at 37 °C overnight.

3.2.2 Recombinant protein expression

Recombinant protein production was performed as follows. Single colonies of successfully transformed cells were selected from each agar plate and inoculated into 10 mL of terrific broth containing kanamycin (36 mg/ml) to screen for expression. Cultures were grown at 37 °C with shaking at 225 rpm and induced with a final concentration of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at an optimized optical density of 0.6-0.8. Prior to induction, 1 mL of un-induced samples were aliquoted from the respective cultures for analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Both induced and un-induced cultures were then incubated at an optimized temperature of 37 °C for 4 hours. The cells were harvested by centrifuging at 4 500 ×g for 10 minutes. Cells were then lysed with lysis buffer (Appendix B vi) for 30 minutes at 4 °C and samples were prepared for SDS-PAGE. Upon expression, primary cultures for expressing colonies were used to inoculate 9 L terrific broth for large scale production of the protein following the protocol described above.

3.2.3 Purification of recombinant protein

Purification of the recombinant protein was performed using nickel-nitrilotriacetic acid (Ni-NTA) resin. The large-scale cultures from the above were pelleted by spinning at 4 500 ×g for 10 minutes after which cells were disrupted and lysed with lysis buffer at 4 °C for 30 minutes. After lysis, the bacterial lysate was sonicated on ice at 9 secs on and 9 secs off for 2 hours at 25 % amplitude using

the sonicator (QSONICA, USA). Samples were spun at 4 500 ×g for 10 minutes and the resultant supernatant was applied onto a Ni-NTA pre-packed column. First, the column was washed and equilibrated using lysis buffer and samples were loaded by gravity flow. After several loading steps, the column was washed again with lysis buffer to elute unbound and loosely bound proteins. Bound proteins were then eluted in a multi-step with elution buffer containing different concentration (50, 150, 250 and 500 mM) of imidazole. The eluted samples were analyzed by SDS-PAGE and samples containing protein bands corresponding to the molecular weight of the protein of interest were buffer-exchanged against phosphate buffered saline (PBS) and concentrated using 10 kDa cut-off centrifugal filters. Further purification was performed using size exclusion chromatography (SEC) (GE, Superdex-200 increase 10/300 GL column). The purity of the recombinant proteins was assessed by subjecting 30 µL aliquots of the eluted fractions to SDS-PAGE and subsequently stained with Coomassie brilliant blue dye. The purified proteins devoid of soluble aggregates were stored at -20 °C for antibody generation (BioBasic, Canada).

3.3.0 SDS-PAGE analysis

For SDS-PAGE analysis, twelve percent (12%) polyacrylamide gels were cast according to manufacturer's protocol in the using the BIORAD gel casting assembly (USA). The gels were allowed to polymerize and then mounted in the SDS-PAGE tank with the 1X running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Protein samples or cell lysates were prepared for separation by boiling with appropriate amount of 4X Laemmli buffer (20% β-ME, 40% glycerol, 8% SDS, 0.008% bromophenol blue, 0.25 M Tris HCl, pH 6.8) at 95 °C for 5 minutes and spun for 1 minute at 4500 ×g. Appropriate volume of samples was loaded along with the pre-stained

protein marker and gels were run for 80-100V until samples were completely resolved. The gels were either stained with Coomassie brilliant blue dye for visualization or used for western blotting.

3.3.1 Mass spectrometry

In order to ascertain the identity of the expressed recombinant protein, mass spectrometric analysis was performed. Briefly, the purified protein samples were analysed by SDS-PAGE as described earlier. The resolved protein band corresponding to the expected molecular weight of the protein of interest was excised and digested with trypsin. The samples were then transferred to Oxford Brookes University, UK where the mass spectrometry analysis was performed.

3.4 PEPTIDE SYNTHESIS

Because of the difficulties encountered with the production of soluble PF3D7_1459400 protein, I resorted to generating synthetic peptides which were used to generate the rabbit antibodies. First, B-cell epitope mapping was performed using ABCpred online software as described previously (Saha, and Raghava, 2006). This yielded several peptides from which three were selected based on their antigenicity/surface/hydrophilicity score with the three having the highest score. The selected peptide sequences were sent to GenScript for peptide synthesis and subsequently used for immunization of rabbits to generate the antibodies (GenScript Corporation, USA).

3.5 PROTEIN-G AGAROSE PURIFICATION OF RABBIT ANTIBODIES

Purification of rabbit antibodies using protein G agarose was performed by following the manufacturer's protocol (Thermofisher scientific, USA). The agarose beads and buffers were equilibrated to room temperature and the column was packed with 2 mL of resin slurry. The column was equilibrated by addition of 5 mL of the binding buffer and samples were diluted (1:1)

with the binding buffer and applied to the column. To remove precipitated lipoproteins, the diluted sample was spun at 10,000 ×g for 20 minutes and the supernatant was added to the equilibrated resin. The sample was allowed to flow through the resin and the column was washed with 15 mL of the binding buffer. Antibodies were then eluted with 5 mL of the elution buffer and 0.5 mL fractions were collected. Fifty µL each of the elute was neutralized with the neutralization buffer to adjust the fractions to physiological pH. The fractions were analysed by SDS-PAGE and the remaining samples were preserved at -20 °C for further use. The column was washed with 2 mL of 0.02 % sodium azide and preserved in 3 mL of the solution at 4 °C.

3.6.0 WESTERN BLOT ANALYSIS

For immunoblotting, schizont pellets were lysed with 0.05% saponin in PBS, washed extensively, and lysis buffer was used to extract proteins. After extraction, the appropriate amount of 4X Laemmli buffer was added and proteins were resolved on an SDS-PAGE gel. The proteins were then transferred onto a nitrocellulose membrane by applying a direct current of 180 mA for 2 hours 30 minutes or 20 V overnight in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). The following day, the membrane was blocked with 1.5 % BSA in 0.01 M PBS (NaCl 0.138M; KCl 0.0027M) (pH 7.4) on a rocker for 30 minutes at room temperature (~25 °C) or at 4 °C overnight. The membrane was washed once with PBS containing 0.5% Tween 20 (PBS-T) and twice with PBS at 10 minutes between each washing step. Anti-PF3D7_0410600 or anti-PF3D7_1459400 peptides 1, 2 and 3 rabbit antibodies were diluted (1:1000) in 1.5% BSA prepared in PBS and incubated with the blot on a rocker for 1 hour 30 minutes at room temperature (~25°C). Washing steps were repeated as described earlier. Then goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody was diluted (1:2500) in 1.5 % BSA in PBS and incubated

with the blot for 45 minutes at room temperature (~25 °C). Washing steps were repeated as described earlier and blots were visualized using the enhanced chemiluminescence (ECL) reagents A (2.5 mM luminol, 400 µM p-coumaric acid, 100 mM Tris.HCl pH 8.5) and B (0.018% H_2O_2, 100 mM Tris.HCl pH 8.5) (Pierce, ThermoFisher scientific) mixed in the ratio of 1:1. Images were captured on an Amersham 600 imager (GE Healthcare-Life sciences, Brazil).

3.7.0 PARASITE CULTURE AND SYNCHRONIZATION

The *P. falciparum* strains used for this study included: 3D7, Dd2, W2mef and NF54. Parasites were cultured in normal human O^+ erythrocytes as described previously (Jensen and Trager, 1978). Gametocytes were produced according to published protocols (Brancucci *et al.*, 2015). Complete parasite medium containing RPMI 1640 (Sigma Aldrich) with 5 mg/mL Albumax II (Gibco), 2 mM L-glutamine, 0.2 µg/mL hypoxanthine, 23.8 mM NaHCO3 and 10 µg/mL gentamycin was used for culture maintenance. A mixture of 94% nitrogen, 5% carbon dioxide and 1% oxygen gas were bubbled through the culture for 1-3 minutes at every routine maintenance. The parasites were tightly synchronized by routine sorbitol treatments as described previously (Lambros and Vanderberg, 1979). Blood used in this study for culturing was obtained from healthy donors with informed consent.

3.8.0 IMMUNOFLUORESCENCE ASSAYS

3.8.1 Stage-specific expression analysis

Immunofluorescence assays were employed to screen the selected proteins for membrane association. Smears from tightly synchronous *P. falciparum* cultures were made on glass slides and fixed in methanol (-20 °C pre-chilled). The slides were air-dried and permeabilized with 0.01

% Triton in PBS. After permeabilization, slides were blocked using 1.5 % BSA in PBS for 30 minutes at 4 °C. The slides were incubated at room temperature (~25 °C) for an hour with antibodies against the respective proteins alongside an IMC-marker, anti-*Pf*GAP45 rabbit antibodies, at an optimized 1:100 dilution. After the incubation period, slides were washed and incubated with goat anti-rabbit secondary antibodies conjugated with FITC or Alexa Fluor 488/594. The slides were washed and mounted with VECTASHIELD mounting medium (Burlingame, CA) containing 4', 6'-diamidino-2-phenylindole (DAPI) or incubated with Hoechst for 20 minutes. Slides were then sealed with cover slips using nail polish and viewed on an Olympus (BX-41TF) fluorescence microscope (Japan). The images were captured and processed using the Fiji-Image J software (National Institutes of Health, USA).

3.8.2 Dual immunofluorescence assays for asexual and sexual stage parasites

For dual immunofluorescence assays, smears from synchronized *P. falciparum* parasite cultures were made on glass slides and fixed in pre-chilled methanol (-20 °C). The slides were air-dried and permeabilized with 0.01 % Triton in PBS for 30 minutes on a rocker. After permeabilization, blocking was performed with 1.5 % BSA in PBS for 30 minutes. Slides were then washed once with PBS containing 0.05 % tween-20 (PBS-T) and twice with PBS at 10 minutes intervals. After the washing steps, the slides were probed with the different antibody dilutions: anti-PF3D7_0410600 rabbit antibody (1:100); anti-*P. falciparum* apical merozoite antigen-1 mouse monoclonal antibody (1:100), (anti-*Pf*AMA1); anti-*P. falciparum* gliding-associated protein 45 (anti-*Pf*GAP45) or anti-*P. falciparum* myosin tail interacting protein rat antibodies (anti-*Pf*MTIP), (1:100); anti-*P. falciparum* gametocyte surface protein 48/45 (anti-*Pf*s48/45) mouse monoclonal antibody (1:100), anti-Tubulin acetyl transferase 1 (anti-TAT 1) mouse antibody (1:10). All

incubations were performed at room temperature (~25 °C) for an hour. After the incubation period, the slides were thoroughly washed and incubated with the respective FITC or Alexa Fluor 488/594 conjugated secondary antibodies. The washing steps was repeated as above and slides were mounted with VECTASHIELD mounting medium (Burlingame, CA) with 4', 6'-diamidino-2-phenylindole (DAPI) or incubated with Hoechst for 20 minutes. Slides were then sealed with cover slips using nail polish and viewed on an Olympus BX-41TF fluorescence microscope (Japan). The images were captured and processed using the Fiji-Image J software (National Institutes of Health, USA).

3.9.0 TREATMENT OF PARASITE WITH 2-BROMOPALMITATE (2-BMP)

Since the palmitoylation inhibitor, 2-Bromopalmitate (2-BMP) was previously reported to inhibit erythrocyte invasion (Jones *et al.* 2012), I sought to determine whether the effect of the inhibitor was directly on parasites or on erythrocytes. Healthy erythrocytes were incubated with gradient concentrations of 2-BMP (5 µM- 30 µM), washed off after 4 hours and incubated with untreated rupturing schizonts. Similarly, segmenting stage 3D7 and W2mef schizonts were treated with the same concentrations of 2-BMP as above, washed off after 4 hours and incubated with untreated erythrocytes. The invasion efficiency was measured using a flow cytometer and results were analysed with flowJo. Graphs were plotted using GraphPad prism (Version 6).

3.10.0 ACYL RESIN-ASSISTED CAPTURE (ARAC)

To purify palmitoylated proteins from schizont lysate and determine whether PF3D7_0410600 is palmitoylated, acyl resin-assisted capture assays were performed as described previously (Edmonds *et al.* 2017). First, an aliquot was taken and labelled as “input”. Then, 0.5 % methyl

methanethiosulphonate (MMTS) was added to the sample and incubated at 40 °C for one hour with minimal vortexing at intervals. Three rounds of methanol precipitations were performed as follows; the sample was split into three 15 mL centrifuge tubes and three-times volume of -20 °C pre-chilled methanol (VWR) was added to each tube. The sample was vortexed and spun at 3500 ×g, 4 °C for 2 minutes. The resultant supernatant was discarded and the pellet was re-suspended in 1 mL solubilization buffer (SB; 5 mM EDTA, 4% SDS, 50 mM Tris.HCl, pH 7.4) and incubated for 30 minutes at 37 °C. Lysis buffer (LB; 5 mM EDTA, 50 mM Tris.HCl, 150 mM NaCl, pH 7.4) containing 0.2 % Triton X-100 (LB-T) (Sigma, UK) was added to 4 mL. In the last step of precipitation, the sample was re-suspended in 1 mL binding buffer (1 % SDS, 1 mM EDTA, 100 mM HEPES, pH 7.4) and incubated at 37 °C for 30 minutes with rotation at 180 rpm. Twenty mL of distilled water was used to wash 0.25 g of thiopropyl Sepharose resin (Sigma, UK) for 15 minutes and spun at 3 500 ×g for 2 minutes at 4 °C. The supernatant was discarded, and an equal volume of the binding buffer was added to the resin. The protein sample from above was split into two Falcon tubes and 1 mL of washed resin was added to each tube. Afterwards, equal volumes of 2 M hydroxylamine (HA) pH 7.4 was added to one tube and 2 M Tris.HCl pH 7.4 was added to the other tube along with protease inhibitor. The samples were then incubated overnight with minimal shaking (~25 °C). The following day, both HA-treated and Tris.HCl-treated samples were pelleted at 3, 500 ×g for 2 minutes at 4 °C. The supernatant was recovered as 'unbound fraction' and the bead pellet was washed five times with 5 mL binding buffer with centrifugation at 3, 500 ×g for 2 minutes at 4 °C between each washing step. One % β-mercaptoethanol (β-ME) prepared in 1 mL of LB-T and was used to elute the protein by incubating with the bead pellet at 37 °C with shaking at intervals. After 1 hour of incubation, the samples were spun at 3, 500 ×g for 2 minutes at 4 °C. The supernatant was recovered, split into 300 µL aliquots and 900 µL of pre-chilled (-

20 °C) methanol was used to precipitate the sample by spinning at 9, 000 ×g for 5 minutes at 4 °C. The supernatant was discarded, and the precipitate was resuspended in 200 µL of 4X Laemmli buffer which was transferred to the other two tubes to obtain the 'β-ME elute'. Meanwhile, the bead pellet from the earlier step was treated with 1 mL of 4X Laemmli buffer, boiled and spun at 4 500 ×g for 5 minutes. The supernatant was recovered and labelled as the 'Laemmli elute'. The input, unbound fraction, β-ME elute and the Laemmli elutes were prepared for SDS-PAGE and subsequent mass spectrometry analysis.

3.11.0 SIZE EXCLUSION CHROMATOGRAPHY (SEC)

Analytical size exclusion chromatography (SEC) was performed as described previously (Chen *et al.* 2011). Briefly, NF-54 schizont-infected erythrocytes were lysed with 0.05 % saponin and repeatedly washed with PBS to obtain schizont pellets. The pellets were treated with lysis buffer containing 150 mM NaCl, 1 mM EDTA, 1 % NP-40 25 mM Tris-HCl, and 5 % glycerol, pH 7.4. The clarified lysate (2 mL) obtained was injected into a Superdex^{TM} 200 increase SEC column (10/300 GL, GE Healthcare). The elutes were collected and analysed by immunoblotting.

3.12.0 TREATMENT OF PARASITES WITH PHARMACOLOGICAL INHIBITORS OF OTHER POST TRANSLATIONAL MODIFICATIONS

The 3D7 strain of *P. falciparum* was maintained in culture as described above (Section 3.7.0). Culture was synchronized by treatment with 5 % sorbitol and sub-cultured into 5 T-25 culture flasks. Ten µM each of farnesyl transferase inhibitor and geranylgeranyl transferase inhibitor was used to treat individual culture flasks with DMSO control flask and untreated control. Cultures were maintained over a period of 1 cycle of parasite replication. Thin smears were made on

microscope slides at 12-hour intervals throughout the 48 hours life cycle. Slides were either stained with Giemsa or prepared for IFA. Images from the Giemsa-stained slides of treated cultures were captured and analysed for morphological aberration of the parasite in comparison to the DMSO and untreated controls. In order to determine the effect of the drugs on the localization of PF3D7_0410600 protein, slides were probed with anti-PF3D7_0410600 rabbit antibody (1:100) while goat anti-rabbit secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 (1:100) was used as secondary antibody. All IFA procedures were performed as described above.

CHAPTER FOUR

RESULTS

4.1 PF3D7_0410600 AND PF3D7_1459400 WERE IDENTIFIED FROM THE TRANSCRIPTOME DATA ANALYSIS

I systematically analysed hypothetical proteins in the published transcriptome datasets and identified two hypothetical proteins (PF3D7_0410600 and PF3D7_1459400) for this study. The two proteins were selected based on initial analysis of their amino acid sequences which will enable the probing of the hypothesis stated earlier. PF3D7_0410600 possesses no predicted signal peptide or transmembrane domain but PF3D7_1459400 on the other hand, has a predicted transmembrane domain and an additional PEXEL signature which could be indicative of targeting to the membrane. The latter was used as a control for the immuno-localization studies. In order to predict the possible lipid modification status of the two proteins, I used Clustering and Scoring Strategy (CSS-PALM) and the Group-based prediction system (GPS) online software and the results suggest that only PF3D7_1459400 protein may be palmitoylated (Table 1).

Table 4.1: GPS prediction of PF3D7_0410600 and PF3D7_1459400 proteins

S/N	ID	POSITION	PEPTIDE	SCORE	CUT OFF	TYPE
1	PF3D7_0410600	301	EIELFTDCLKLTKWP	0.831	0	S-Palmitoylation
2	PF3D7_1459400	137	LNIAVINCKSVLPSK	1.114	1.079	S-Palmitoylation

C: Possible palmitoylation site

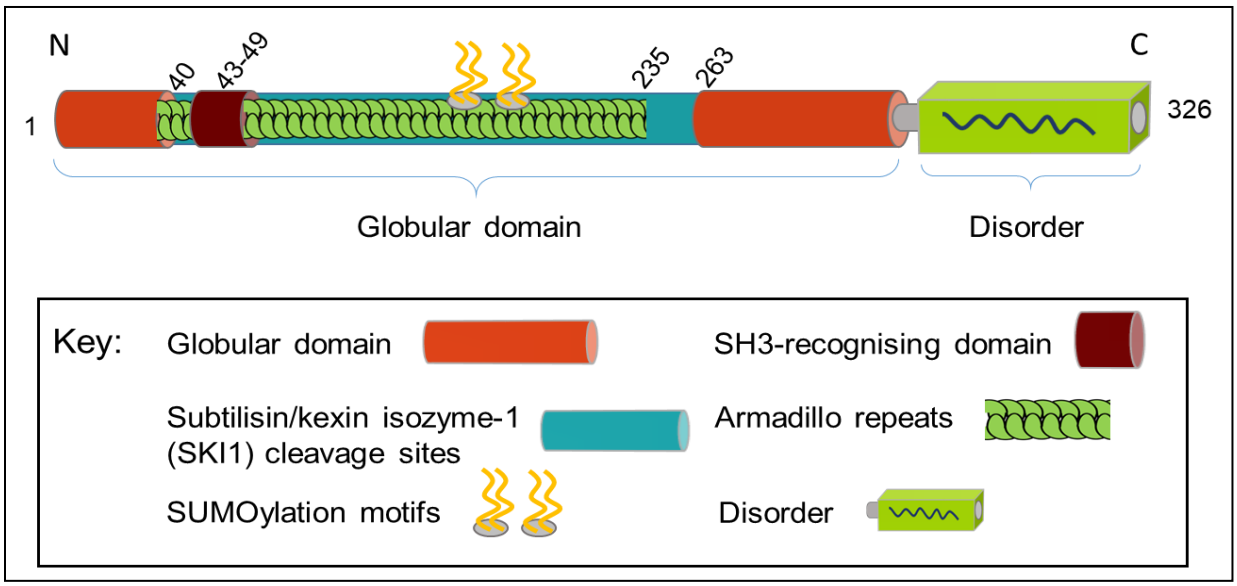
Once all the selection criteria above were determined, I searched the published palmitome and interestingly, only PF3D7_1459400 was reported to be palmitoylated (Jones *et al*. 2012).

4.2 DOMAIN ARCHITECTURE AND SEQUENCE CONSERVATION OF THE TWO NOVEL PROTEINS

In order to study the structural features of PF3D7_0410600 and PF3D7_1459400 proteins, I employed an initial bioinformatics approach to gain insight into the likely domains that may confer important function(s) on the protein. Gene ontology and synteny data documented in the *Plasmodium* database (PlasmoDB) revealed that PF3D7_0410600 is a 3-exon gene that is located on chromosome 4 of the *P. falciparum* genome and encodes a 326-amino acid protein. The protein has a predicted molecular weight of 32 kDa but lacks any recognizable signal peptide, transmembrane domain, myristoylation or acetylation signals.

The amino acid sequence of the protein was interrogated using the ELM online software to predict the important domains within the protein. The analysis showed that PF3D7_0410600 protein is largely globular with a very short disordered region at the C-terminus (Figure 4.1A). PF3D7_0410600 protein harbours motifs that may confer possible diverse roles on the protein during the intraerythrocytic developmental cycle of the malaria parasite. Some of the predicted structural motifs include: the cleavage site for the mammalian subtilisin/kesin isoenzyme 1 (SKI-1) which was reported to process surface glycoproteins in infectious pathogens (Lenz *et al*. 2001; Pullikotil *et al.* 2007), the presence of SUMOylation motifs that has been demonstrated to be key modulators of protein-protein interactions by providing new binding sites for potential interactions with other functional binding partners (Song *et al*. 2004), an Src homology 3 (SH3) recognizing domain that is a docking module for interaction with polyproline motifs associated with several intracellular signalling proteins (Figure 4.1A) (Via *et al.* 2015).

A.



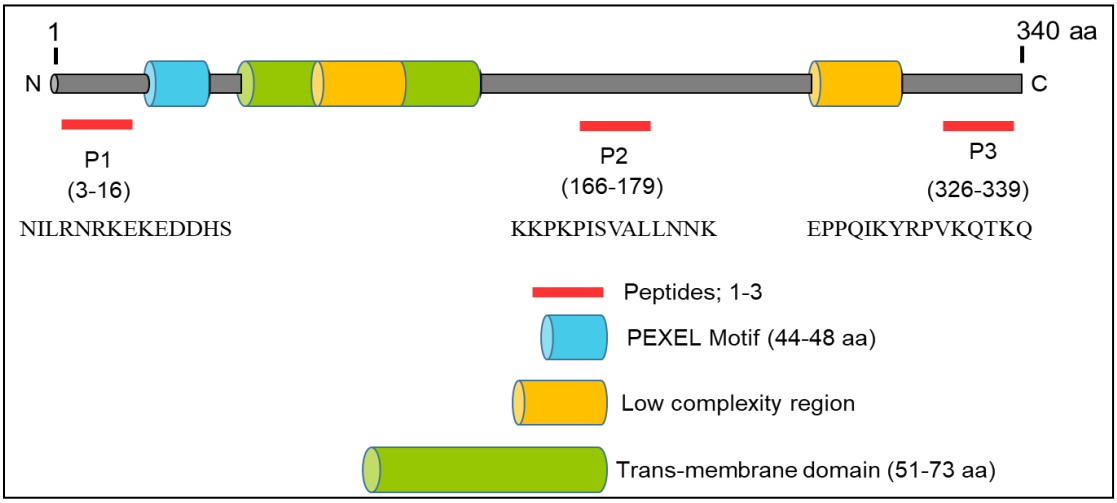
B.

Figure 4.1: Domain architecture and sequence alignment of PF3D7_0410600 protein. (A) Domain architecture of PF3D7_0410600 shows that the protein harbour important domains including an SH3-recognising domain, armadillo repeats, a subtilisin/kexin isozyme-1 (SKI1) cleavage sites and SUMOylation motifs. **(B)** Sequence alignment of PF3D7_0410600 shows a conserved protein sequence across the different species of *Plasmodium.* The blue lines show areas of significant similarity.

PF3D7_0410600 protein also harbours armadillo repeats (ARM), a characteristic feature of the β-catenin family of proteins, suggesting potential docking sites for protein-protein interactions (Coates, 2003; Tewari *et al.* 2010). To determine the conservation of PF3D7_0410600 across the different species of *Plasmodium,* I performed sequence alignment using Clone Manager suite (Version 6) and showed that PF3D7_0410600 protein is evolutionarily conserved across rodent

and primate *Plasmodium* species (Figure 4.1B) and all orthologues have a positionally-conserved cysteine residue at the C-terminal end of the protein.

A



B

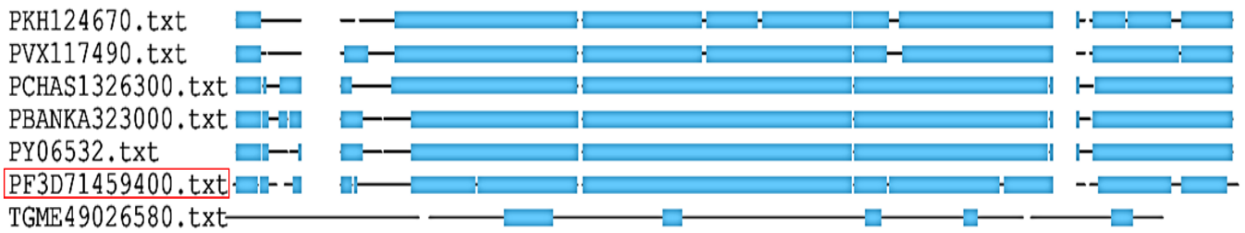


Figure 4.2: Domain architecture and sequence alignment of PF3D7_1459400 protein. (A) Domain architecture of PF3D7_1459400 shows that the protein possesses a transmembrane domain, a non-canonical Plasmodium Export Element (PEXEL) motif at the N-terminus and two low complexity regions. **(B)** Sequence alignment data indicates that PF3D7_1459400 protein is conserved across the different *Plasmodium* species. The blue lines show areas of significant similarity.

Similar approaches were employed for PF3D7_1459400 and the results show that the protein possesses two low complexity regions, a transmembrane domain at the N-terminal end of the protein and a PEXEL motif (Figure 4.2A) which is characteristic of exported proteins (Hiller *et al.* 2004; Marti *et al.* 2004). Sequence alignment results also indicates that PF3D7_1459400 protein is conserved across the species orthologues of *Plasmodium* (Figure 4.2B).

4.3 PF3D7_0410600 WAS EXPRESSED AND PURIFIED FROM BACTERIAL SYSTEM

In order to generate reagents against PF3D7_0410600, I produced the recombinant form of the protein in an *E. coli* expression system under optimized conditions. The expression resulted in the production of a prominent 32 kDa protein band that is consistent with the predicted molecular weight (Figure 4.3A). Once expression was confirmed, large scale cultures were grown, and expression was induced which yielded soluble PF3D7_0410600 protein. The recombinant protein was purified under denaturing conditions using immobilized metal affinity column pre-packed with Ni-NTA beads.

A B

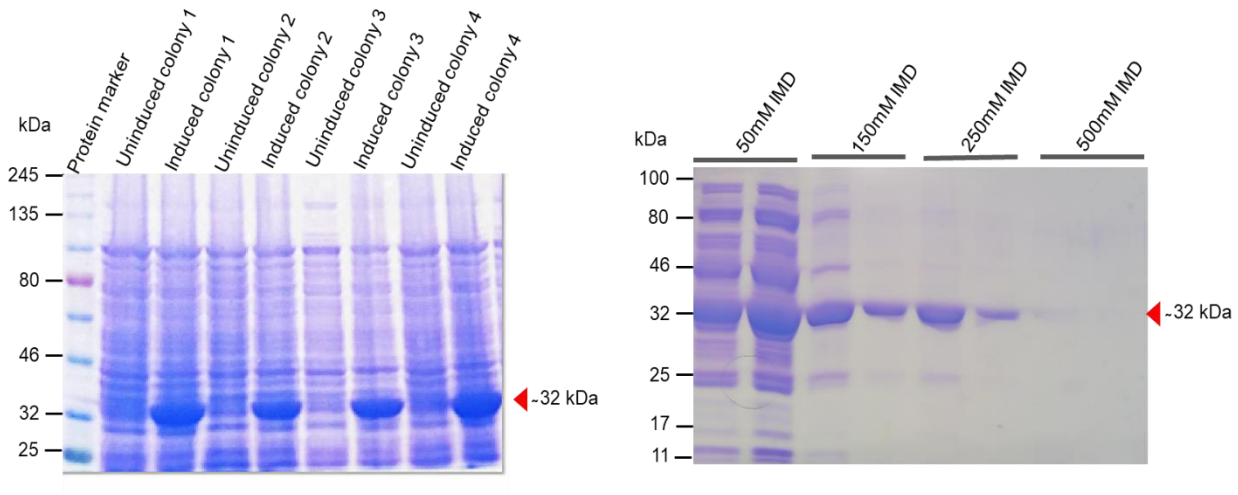


Figure 4.3: SDS-PAGE gels stained with Coomassie brilliant blue dye. (A): Expression gel showing the different colonies that were screened for expression. The recombinant PF3D7_0410600 was found migrating at the expected molecular weight (32kDa). **(B)** Ni-NTA purification gel showing the purified protein band migrating at 32 kDa (red arrow heads). The protein was eluted in a multi-step at different concentrations of imidazole and the double lane for each elute represent two fractions collected at each concentration of imidazole.

This resulted in the enrichment of a protein band that migrated at 32 kDa when analysed on SDS-PAGE gel (Figure 4.3B). The recombinant protein was further purified by size exclusion

chromatography (SEC) in order to remove soluble aggregates. The SEC purified protein produced a monomeric peak on the chromatogram indicating apparent homogeneity of the purified recombinant protein (Figure 4.4). The purified PF3D7_0410600 protein eluted at an elution volume of about 17.5 mL and the fractions collected were analysed by SDS-PAGE alongside the Ni-NTA input. This resulted in a purified PF3D7_0410600 protein which was submitted for mass spectrometric analysis.

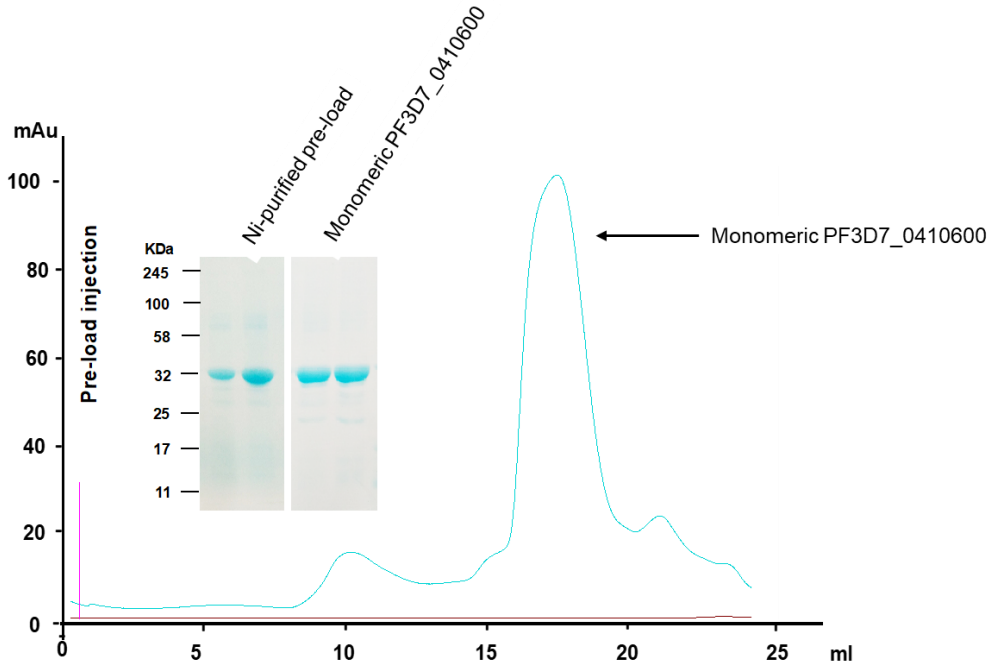


Figure 4.4: Size exclusion chromatogram of the purified recombinant PF3D7_0410600. The protein eluted at an elution volume of about 17.5 mL. The SDS-PAGE gels show the Ni-NTA purified preload that was injected into the column and the monomeric protein after purification by SEC.

4.4 MASS SPECTROMETRY CONFIRMED THE IDENTITY OF THE PURIFIED RECOMBINANT PF3D7_0410600 PROTEIN

In order to ascertain the identity of the recombinant PF3D7_0410600, the SEC-purified protein samples were analysed on SDS-PAGE and bands corresponding to the size of the protein of interest

were trypsinized and analysed by LC-MS analysis. The tryptic peptide sequences obtained were blasted against *P. falciparum* database and the unique peptides, molecular weight, sequence coverage and MS/MS counts confirmed the identity of the purified PF3D7_0410600 recombinant protein (Q9U0I2) (Table 2).

Table 4.2: Mass spectrometry data showing the peptide hits and their abundance for PF3D7_0410600.

Protein IDs	Gene Names	Number of proteins	Peptides	Unique peptides	Mol. Weight (kDa)	Sequence coverage (%)	Intensity	MS/MS count
Q9U0I2	*PFD0525w	2	33	33	37.064	69.6	2.04E+11	197
CON__P01966		1	5	5	15.184	34.5	6.36E+10	48
CON__P04264		1	33	33	66.017	57	3.99E+10	104
CON_P13645;CON....		9	17	17	59.51	30.7	1.65E+10	46
CON__P35527		1	22	22	62.129	51.8	1.63E+10	63
CON__P02768-1;CON__P02769		2	17	17	69.366	24.3	9.41E+09	37
CON_P02070;CON__Q3SX09		2	1	1	15.954	6.9	6.41E+09	7

*PFD0525w: PF3D7_0410600.

4.5 B-CELL EPITOPE MAPPING IDENTIFIED IMMUNOGENIC PEPTIDES FOR WHICH PF3D7_1459400 PEPTIDE ANTIBODIES WERE GENERATED

Efforts to produce the soluble recombinant form of PF3D7_1459400 were quite challenging, therefore I generated synthetic peptides against the protein. B-cell epitope mapping was performed which yielded several peptides (Table 3). Three peptides were selected based on their antigenicity/surface/hydrophilicity score.

Table 4.3: B-cell epitope mapping for PF3D7_1459400.

Start	Antigenic Determinant	Length	Antigenicity/Surface/ Hydrophilicity	Disordered Score	Synthesis	Oryctolagus_cuniculus blast
3	NILRNRKEKEDDHSC*	14	2.78/0.93/1.58	0.2639	Easy	63%
326	CEPPQIKYRPVKQTK*	14	2.75/0.79/0.54	0.2861	Easy	42%
166	CKKPKPISVALLNNK*	14	1.40/0.57/0.20	NONE	Easy	56%
142	CPSKQNIYQRFSLED	14	1.35/0.71/0.09	NONE	Easy	49%
85	IDVSNTEKLKEIFFC	14	1.04/0.57/0.36	NONE	Easy	50%

*Selected peptides for which peptide antibodies were generated

4.6 ANTIBODIES AGAINST PF3D7_0410600 AND PF3D7_1459400 PROTEINS BOTH RECOGNIZED THE NATIVE PARASITE PROTEINS.

Rabbit antibodies generated against the recombinant PF3D7_0410600 protein and the synthetic peptides for PF3D7_1459400 protein both detected the native parasite proteins in immunoblotting using schizont lysates. Anti-PF3D7_0410600 rabbit antibody detected the native protein in both 3D7 and NF54 schizont lysates migrating at the expected molecular weight (Figure 4.5A). Similarly, the peptide antibodies for PF3D7_1459400 protein detected the native parasite proteins (Figure 4.5B). Anti-PF3D7_1459400 peptide-1 antibody detected a truncated fragment of the native parasite protein which could be attributed to possible cleavage of the protein at the PEXEL motif by plasmepsin V as reported previously (Figure 4.5B, i) (Hiller *et al.* 2004; Marti *et al.* 2004). While peptide-2 antibody did not detect any recognizable band (Figure 4.5B, ii), peptide-3 antibody detected the full-length protein migrating at approximately 50 kDa (Figure 4.5B, iii).

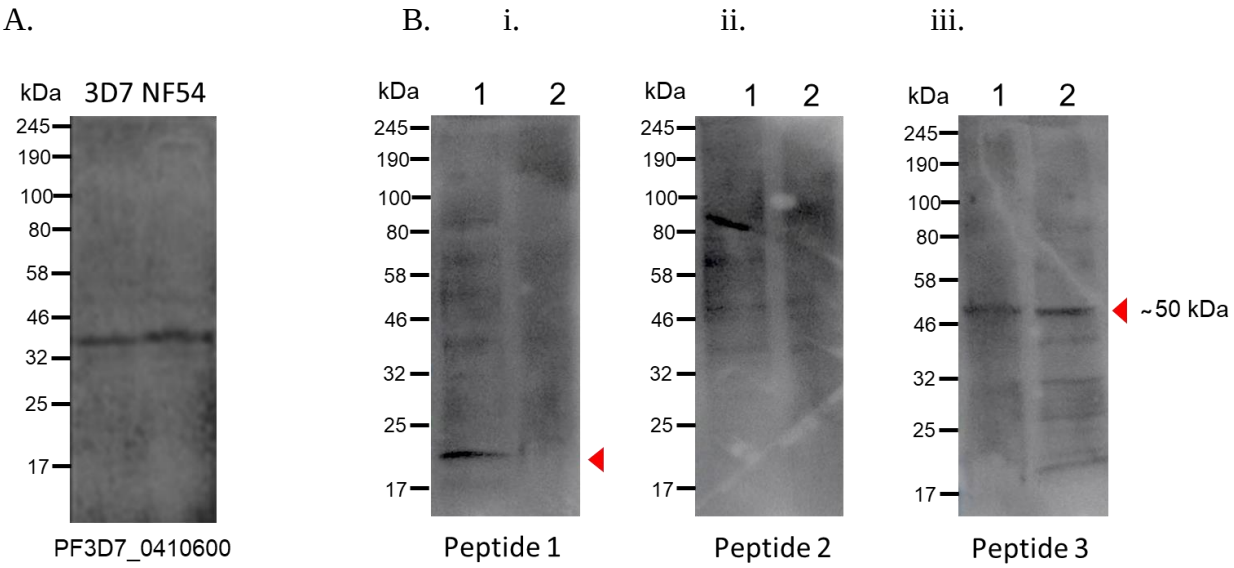


Figure 4.5: Antibodies against the two hypothetical proteins detected the respective native parasite proteins in immunoblotting. (A) Anti-PF3D7_0410600 antibody (1:1000) detected protein bands corresponding to the predicted molecular weight in both 3D7 and NF54 schizont lysates. **(B)** Anti- PF3D7_1459400 peptide antibodies (1:1000) detected the native parasite protein. **(i)** Peptide-1 antibody detected a truncated fragment, **(ii)** Peptide-2 antibody did not detect any noticeable signal; **(iii)** Peptide-3 antibody detected the full-length protein. Lanes 1 and 2 represent 3D7 and Dd2 schizont lysates respectively.

4.7 PF3D7_1459400 AND PF3D7_0410600 PROTEINS EXHIBIT MID-LATE STAGE EXPRESSION PATTERN.

To determine the stage specific expression of the novel hypothetical proteins, I performed immunofluorescence assays and the results show that both PF3D7_1459400 and PF3D7_0410600 proteins exhibit mid-late stage expression pattern (Figure 4.6). Both proteins did not show any detectable expression in early rings similar to *Pf*AMA1 (Figure 4.6A). However, as the parasite progressed to schizonts, I observed deposition of PF3D7_0410600 protein on the infected erythrocyte membrane and a peripheral staining around released merozoites, suggestive of membrane localization (Figure 4.6B). Interestingly, PF3D7_1459400 protein on the other hand

showed heavy deposition on the infected erythrocyte membrane as expected since it possesses the PEXEL motif which could be mediating its export to the membrane (Figure 4.6C).

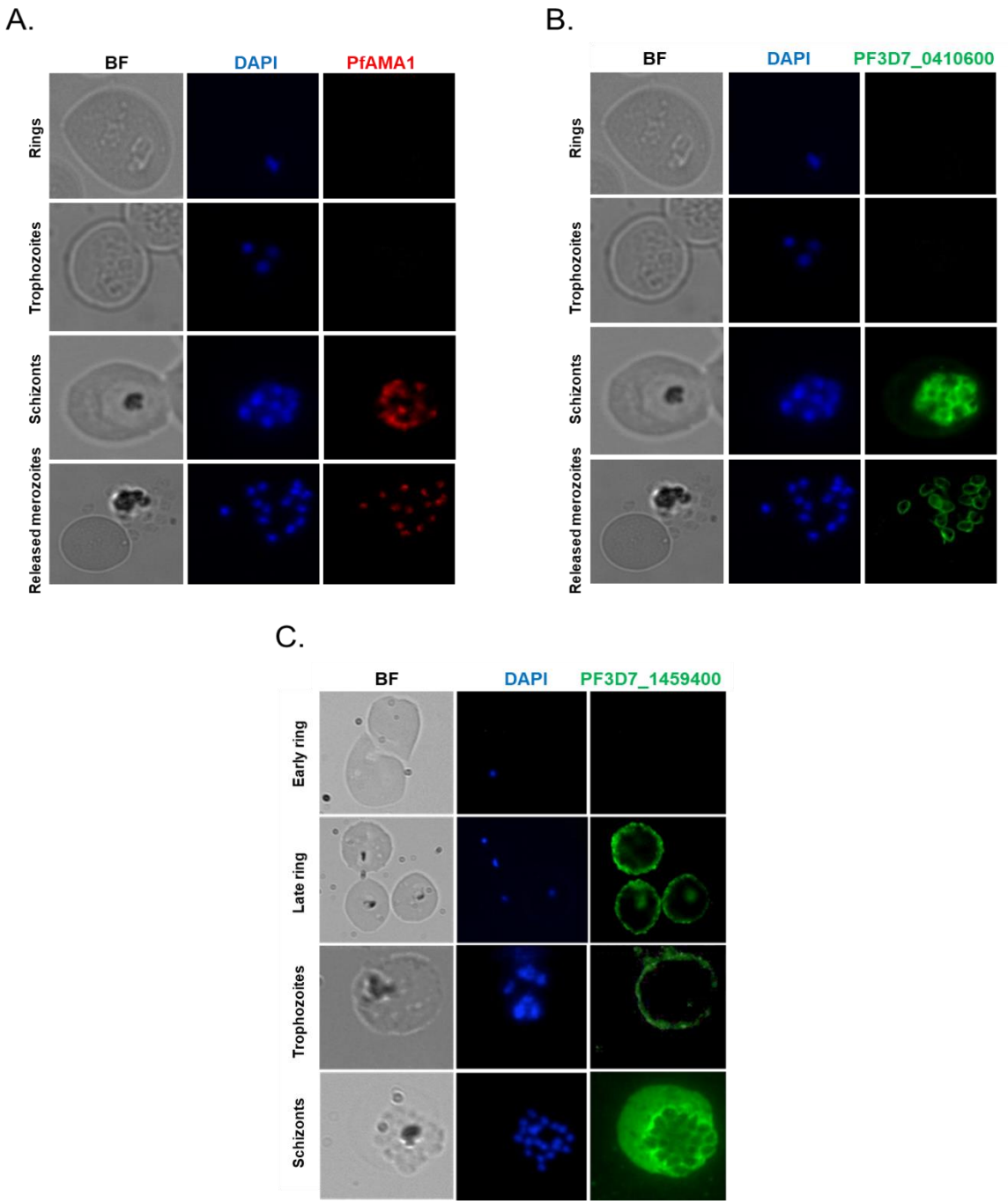


Figure 4.6: PF3D7_0410600 and PF3D7_1459400 proteins are expressed at the mid-late stage of the parasite development. (A) As a control, PfAMA1 expression was analysed alongside the two hypothetical proteins. Staining was observed in schizonts and released merozoites. **(B)** PF3D7_0410600 protein showed staining on the infected erythrocyte in developing schizonts and a peripheral staining pattern around released merozoites. **(C)** PF3D7_1459400 protein showed heavy deposition on infected erythrocyte membrane in late rings, trophozoites and schizont stages. Slides were probed anti-PfAMA1 mouse antibody (red) (1:100), anti-PF3D7_0410600 rabbit antibody (green) (1:100) and anti-PF3D7_1459400 peptide-3 antibody (green) (1:100). Nuclei were stained with DAPI.

4.8 PF3D7_1459400 AND PF3D7_0410600 PROTEINS ACCUMULATE NEAR THE NUCLEAR AREA UPON BREFELDIN A TREATMENT

Since both PF3D7_1459400 and PF3D7_0410600 novel proteins appear to be deposited on erythrocyte membrane and on the periphery of merozoites respectively, I tested the sensitivity of both proteins to Brefeldin-A, a fungal metabolite that blocks anterograde transport of proteins (Benedetti *et al.* 1995). Upon treatment with Brefeldin A, both proteins attained a restricted localization near the proximity of the nucleus when compared to the untreated controls (Figure 4.7 A and B) indicating that both proteins may follow the secretory pathway. ER-tracker was used as a positive control and a partial colocalization was observed when overlaid with the Brefeldin-A treated group (Figure 4.7 B, bottom panel).

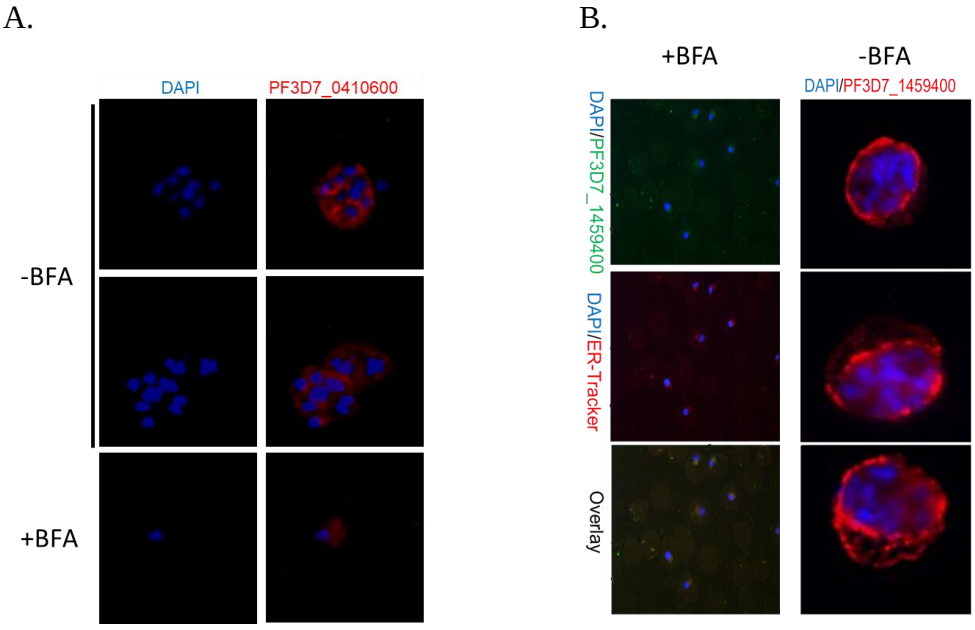


Figure 4.7: PF3D7_0410600 and PF3D7_1459400 proteins are sensitive to Brefeldin-A treatment. (A) The localization of PF3D7_0410600 is sensitive to Brefeldin-A treatment as it accumulates at the nuclear area as compared to the untreated controls **(B)** PF3D7_1459400 protein is also sensitive to Brefeldin-A treatment as it shows a partial merge with the ER-tracker which is localized around the proximity of the nucleus. Slides were probed anti-PF3D7_0410600 rabbit antibody (red) (1:100) and anti-PF3D7_1459400 peptide-3 antibody (red) (1:100). Nuclei were stained with DAPI.

4.9 PF3D7_0410600 AND PF3D7_1459400 PROTEINS ARE EXPRESSED IN GAMETOCYTES

To determine the stage specific expression of the two novel proteins in sexual stage parasites, I performed immunofluorescence assays with gametocyte specific markers. The staining of both PF3D7_0410600 and PF3D7_1459400 proteins appeared cytoplasmic (Figure 4.8 A and B) and results show that both novel proteins are expressed across the different stages of gametocyte development as compared to the gametocyte surface marker *Pf*s48/45. This is important because it provides an avenue to develop an intervention strategy targeting both sexual and asexual development of the parasite.

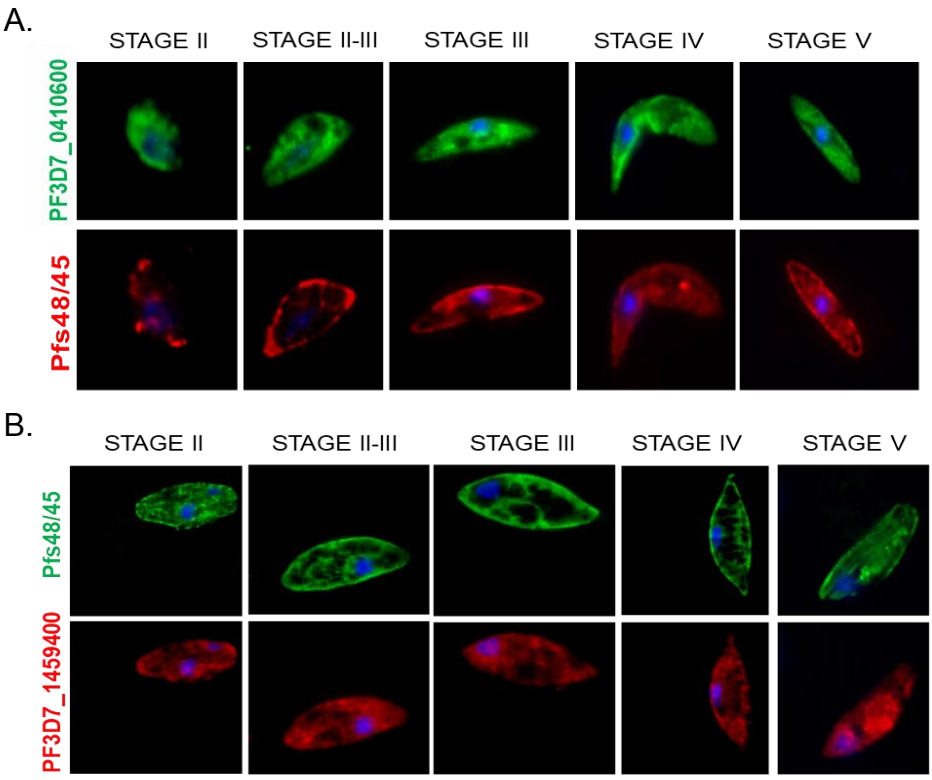


Figure 4.8: PF3D7_0410600 and PF3D7_1459400 proteins are expressed in gametocytes. (A) PF3D7_0410600 showed a cytoplasmic staining pattern as compared to the gametocyte-specific surface marker. **(B)** PF3D7_1459400 showed a similar cytoplasmic staining across the stages of gametocyte development. Slides were probed anti-PF3D7_0410600 rabbit antibody (green) (1:100), anti-PF3D7_1459400 peptide-3 antibody (red) (1:100) and Pfs48/45 (red and green in A and B respectively). Nuclei were stained with DAPI.

4.10 PF3D7_0410600 LOCALIZES TO THE PERIPHERY OF ASEXUAL PARASITES AND APPEAR CYTOPLASMIC IN SEXUAL FORMS

The initial IFA results above showed that PF3D7_0410600 protein which lacks any membrane anchoring signatures localized to the periphery of parasites. Also, PF3D7_1459400 which possess a transmembrane domain and a PEXEL motif that could possibly mediate its export or association with the membrane was localized to the membrane of erythrocytes as expected. This is consistent with previous reports which suggested PF3D7_1459400 to be an exported protein (Zhang *et al*. 2017). Another report identified its orthologue in *T. gondii* (Huynh and Carruthers, 2016) and Jones *et al.* also reported the protein to be palmitoylated (Jones *et al.* 2012). Therefore, I focused on PF3D7_0410600 that lacks membrane anchoring signature but is localized to a membrane destination. I performed dual immunofluorescence assays for intact schizonts and released merozoites with markers of the inner membrane complex (IMC), *P. falciparum* myosin A-tail interacting protein (*Pf*MTIP). I observed colocalization of PF3D7_0410600 with *Pf*MTIP in schizonts and free merozoites (Figure 4.9A) which suggest possible IMC localization in asexual stages. The expression of PF3D7_0410600 was previously reported in gametocytes and based on annotated gene ontology component and predicted gene ontology function, the protein has been linked with microtubule motor activity that is associated with the dynein complex (Tao *et al.* 2014). I therefore performed dual immunofluorescence assays for gametocytes with tubulin acetyl transferase 1 (TAT1) that recognizes both α and β forms of tubulin and observed a partial colocalization (Figure 4.9B) suggesting an IMC-microtubular interplay.

A. B.

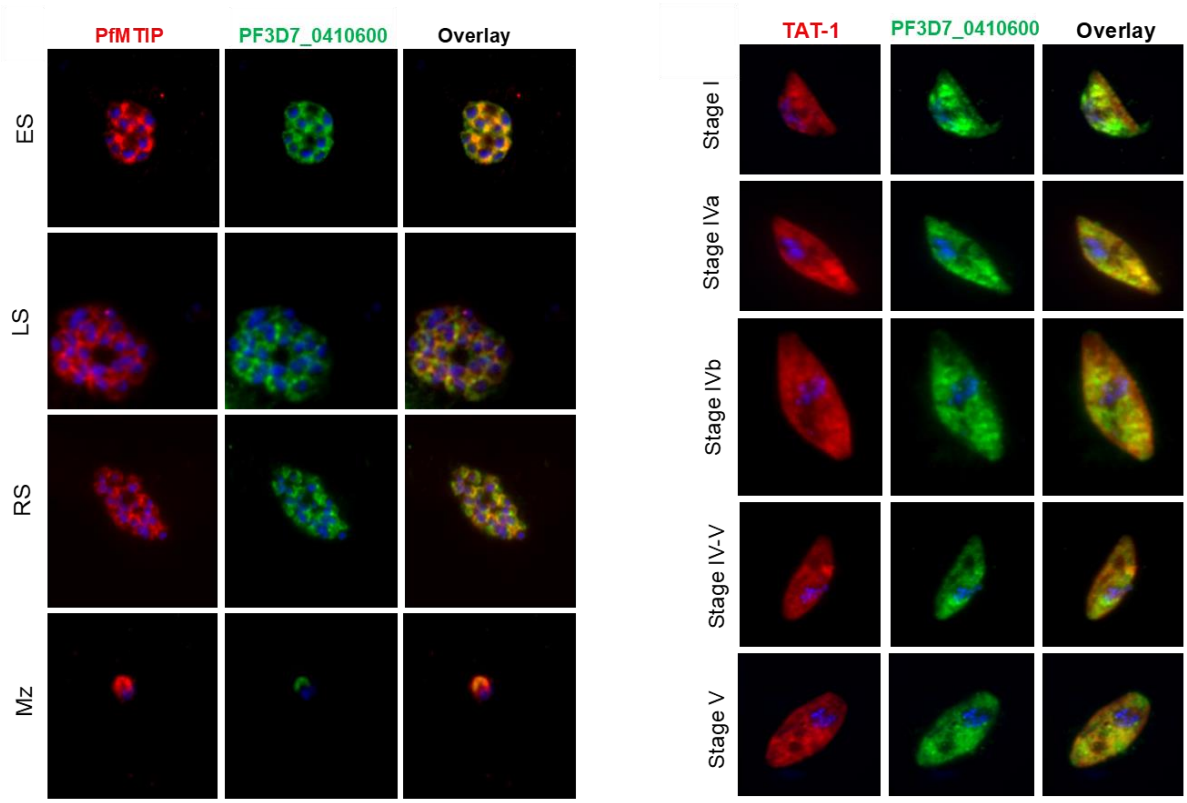


Figure 4.9: PF3D7_0410600 localizes to the periphery of parasites in asexual forms and appears cytoplasmic in sexual forms. (A) Dual immunofluorescence assays were performed for released merozoites and intact schizonts using a marker of the inner membrane complex, *Pf*MTIP. PF3D7_0410600 showed colocalization with *Pf*MTIP. Slides were incubated with anti-PF3D7_0410600 rabbit antibody (green) (1:100) and anti-*Pf*MTIP mouse **a**ntibody (red) (1:100) antibody. **(B)** Dual staining was performed for gametocytes using anti-TAT1 antibody (red) (1:10) and anti-PF3D7_0410600 rabbit antibody (green) (1:100). (**ES**, Early schizonts; **LS**, Late schizonts; **RS**, Rupturing schizonts; **Mz**, Merozoite). Nuclei were stained with DAPI.

4.11 2-BROMOPALMITATE IMPACTS SCHIZONT DEVELOPMENT

2-Bromopalmitate (2-BMP) was reported previously to inhibit erythrocyte invasion (Jones *et al.* 2012). Hence, I sought to investigate whether the invasion inhibitory effect was directly on schizonts or resulted from morphological perturbations in the erythrocyte cytoskeleton that consequently resulted in reduced invasion efficiency. I incubated healthy erythrocytes with gradient concentrations of 2-BMP, washed off after 4 hours and incubated with untreated rupturing

schizonts to test the invasion efficiency. I observed that invasion efficiency was fairly normal with parasites invading efficiently at 30 µM concentration of 2-BMP as compared to the mock treated group (Figure 4.10). However, when I repeated the experiment with 2-BMP-treated schizonts, I observed a marked effect on invasion efficiency that appears to be concentration dependent for both 3D7 and W2mef parasite strains (Figure 4.10 A and B). This is indicative of the fact that the invasion inhibitory effect of 2-BMP is directly on the parasite and not on erythrocytes.

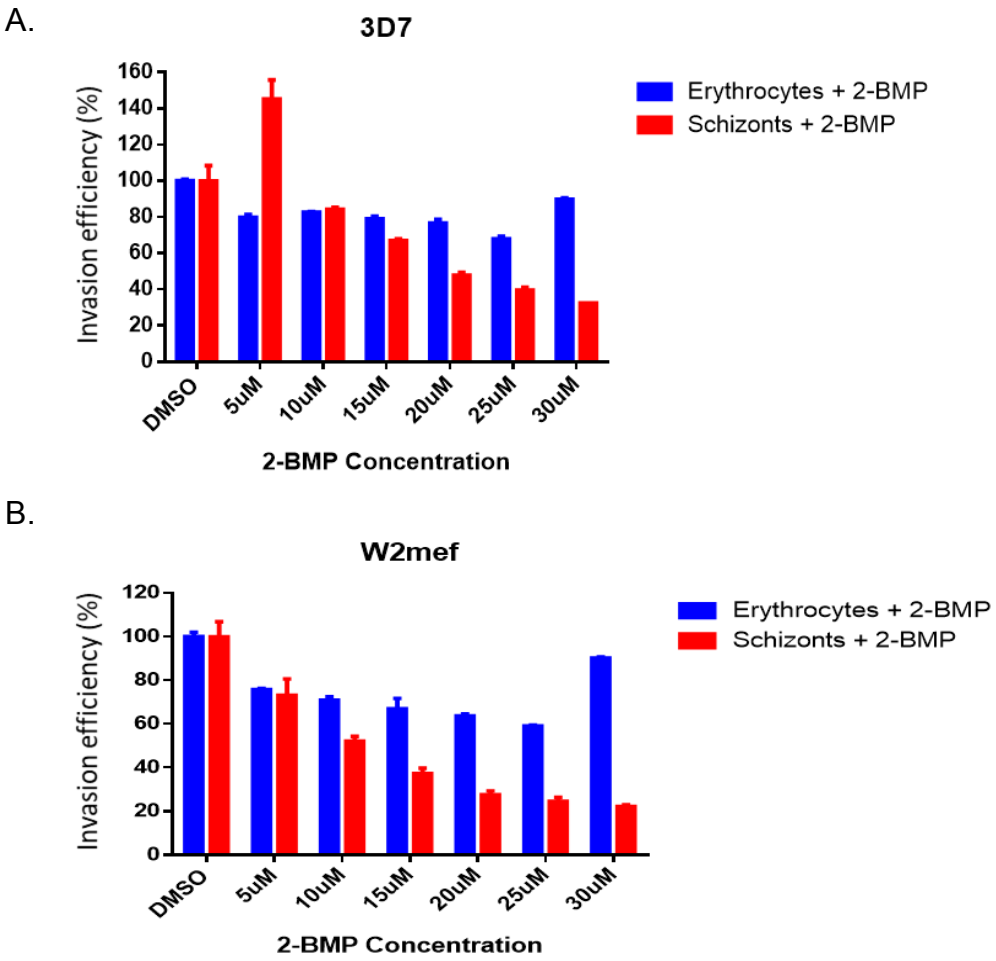


Figure 4.10: 2-Bromopalmitate impacts schizont development. (A-B) Erythrocytes and schizonts were treated with gradient concentration of 2-BMP and washed off, then invasion efficiency was monitored using the flow cytometer. 2-BMP showed a direct effect on schizont development and not erythrocyte when compared to the DMSO treated controls. Data were analysed using GraphPad Prism v.6.01 and presented as bar graphs with mean and standard error of the mean.

4.12 NATIVE PF3D7_0410600 PROTEIN MAY NOT BE PALMITOYLATED

Among the lipid modification pathways that have been reported to govern membrane attachment of proteins, palmitoylation represents the most common and widely studied (Jones *et al.* 2012; Wetzel *et al*. 2015). This may be due to the reversibility of the reaction as a result of the labile nature of the thiol bonds that are formed. This process allows proteins to be dynamically recruited to membrane localization, such as the IMC as has been reported for PfGAP45 and HXGPRTII (Rees-Channer *et al*. 2006; Gaskins *et al.* 2004; Gilk *et al*. 2009; Jortzik *et al.* 2012). Therefore, I sought to determine if PF3D7_0410600 protein is palmitoylated and whether its membrane association is dependent on this modification. I performed Acyl Resin-Assisted Capture (ARAC) to purify palmitoylated proteins from *P. falciparum* schizont lysate (Figure appendix 1.1A). Elutes from the resin were probed with anti-PF3D7_0410600 antibody (Figure appendix 1.1B). In keeping with previous report (Jones *et al.* 2012), anti-PF3D7_0410600 antibody did not detect any protein band indicating that PF3D7_0410600 protein is not palmitoylated.

4.13 GERANYLGERANYL TRANSFERASE INHIBITOR MAY HAVE AN IMPACT ON THE LOCALIZATION OF PF3D7_0410600

I investigated the possibility that other lipid modifications could be responsible for the membrane association of PF3D7_0410600 protein. I monitored the localization of the protein in parasites that were treated with farnesyl transferase inhibitor (FTI) and geranylgeranyl transferase inhibitor (GGTI) alongside the untreated and mock treated controls at a 12-hour time-point throughout the 48-hour life cycle of the parasite. The immunofluorescence assays showed that among the pharmacological inhibitors of lipid modification, only GGTI appeared to mis-localize the protein (Figure 4.11). This is in contrast to the FTI, untreated and mock treated controls that showed

similar staining patterns. These inhibitors have been shown to have pleiotropic effects often associated with non-specific or off-target effects as reported previously (Davda *et al.* 2013). Hence, further experimentation is required to validate this lead.

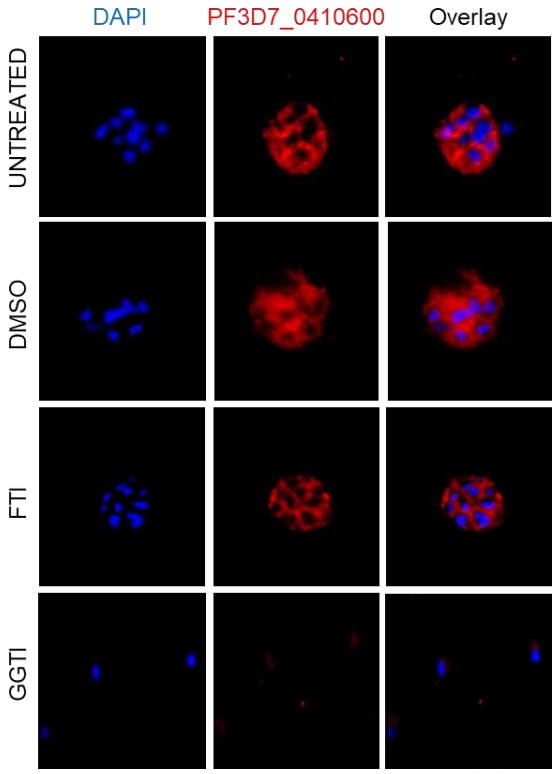


Figure 4.11: GGTI impacts on the localization of PF3D7_0410600. Immunofluorescence assays show the effect of treatment with other pharmacological inhibitors of lipid modification. Only GGTI appear to have an impact on the localization of the protein after 48 hours**.** GGTI, Geranylgeranyl transferase inhibitor; FTI, Farnesyl transferase inhibitor; DMSO: Dimethyl sulfoxide. Images were captured for 40-48-hour time point. Slides were probed with anti-PF3D7_0410600 rabbit antibody (green) (1:100) and the nuclei were stained with DAPI.

4.14 PF3D7_0410600 PROTEIN MAY EXIST AS A MULTIPROTEIN COMPLEX

PF3D7_0410600 protein harbours armadillo repeats and SUMOylation motifs as shown in the protein architecture (Figure 4.1A). This necessitated the assessment of the possibility that the protein could exist in a functional interaction with other binding partners which may be facilitating its association with the membrane. To investigate this hypothesis, I employed analytical size

exclusion chromatography (SEC) which has been previously used to demonstrate similar interaction of *Pf*MTRAP with semaphorin-7A (Bartholdson *et al.* 2012). First, I resolved NF54 schizont lysate on a native PAGE gel and probed with anti-PF3D7_0410600 antibody in immunoblotting. Interestingly, I observed that monomeric PF3D7_0410600 protein which was initially migrating at 32 kDa on a denaturing gel migrated at a high molecular weight of >200 kDa indicating its possible existence in a complex (red arrow head) (Figure 4.12A). I then sought to resolve this complex using size exclusion chromatography as described previously (Bartholdson *et al.* 2012). The void volume of the column was determined using blue dextran (2000 kDa) on an analytical Superdex 200 increase column (10/300 GL) and the elution volume was observed to be 7.5 mL (Figure 4.12B, i). Next, the column was calibrated using SEC markers of known molecular weights (Figure 4.12 B, ii). The schizont lysates were resolved and fractions corresponding to the elution peaks on the chromatogram (Figure 4.12 B, iii) were collected. The fractions were analysed on a denaturing PAGE and probed in immunoblotting using anti-PF3D7_0410600 rabbit antibody. Since I observed the elution volumes and their corresponding peaks on the chromatogram, it was possible to deduce the fraction that contained PF3D7_0410600 protein from the immunoblot. Surprisingly, monomeric PF3D7_0410600 protein which eluted at an elution volume of 17.5 mL (Figure 9) corresponding to elute 10 (E10) (Figure 4.12C) was observed to be eluting at an earlier volume (12.5 mL) (Figure 4.12 B, iii, red stroke; C, red arrow head) which corresponds to the elution volume of a 440 kDa marker protein-apoferritin (Figure 4.12 B, ii). This is indicative of the fact that PF3D7_0410600 protein may be involved in a molecular interaction. Besides, yeast-2-hybrid system have been previously used to identify two interacting partners of PF3D7_0410600 protein (LaCount *et al.* 2005). The interacting partners proteins identified previously are PF3D7_0818200 (*Pf*14-3-3) and another hypothetical protein (PF3D7_1207000).

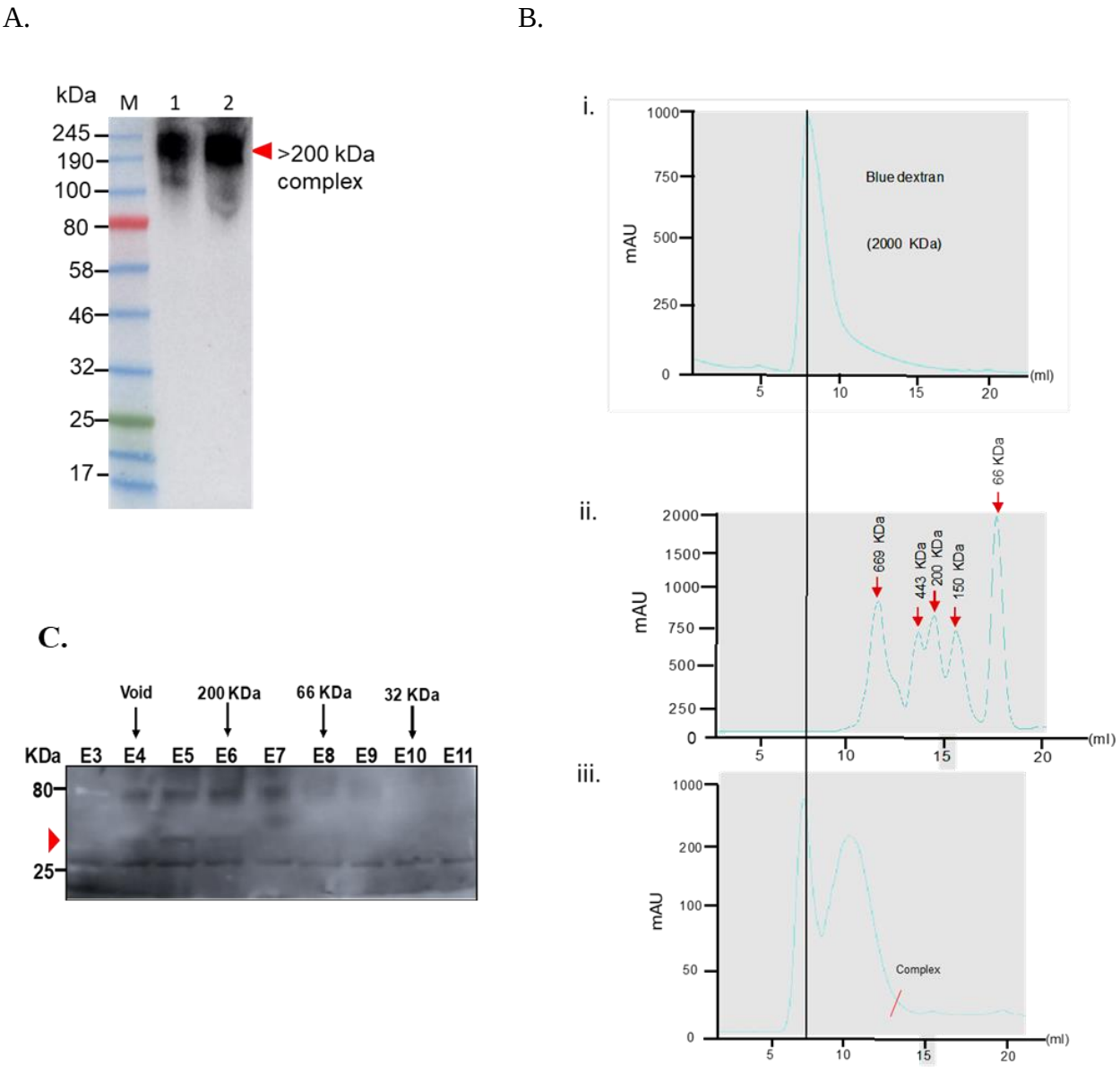


Figure 4.12: PF3D7_0410600 protein may exist as a multiprotein complex: (A) Immunoblot of native PAGE of schizont lysate shows a high molecular weight complex when blots were probed with anti-PF3D7_0410600 rabbit antibody (1:1000). Lane 1 and 2 corresponds to different preparations of schizont lysate. **(B)** Chromatogram showing the (i) void volume determination using blue dextran, (ii) elution profiles for the calibration of the column using markers with known molecular weights (iii) profile of schizont lysate indicating the elution peak of PF3D7_0410600 (red stroke). **(C)** Immunoblot of the fractions (#E3-E11) probed with anti-PF3D7_0410600 rabbit antibody (1:1000). Native PF3D7_0410600 (32 kDa) co-eluted (#E5, red arrow head) with apoferritin, a 440 kDa marker protein.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

The *P. falciparum* genome still has about 60 % of genes/proteins without a known function. However, efforts from published reports such as the genome (Gardner *et al*. 2002), transcriptome (Bozdech *et al*. 2003), proteomic datasets (Bowyer *et al.* 2011) and the more recent genetic screens (PlasmoGEM and piggyBac) have made it possible to identify and functionally characterize novel parasite proteins. Even though these reports represented landmark findings in malaria research, detailed molecular characterization of individual *P. falciparum* proteins is important. This will form a critical component in the selection and prioritization of antigens (Richards and Beeson 2009) as potential targets that could be exploited in the development of novel intervention strategies (drugs or vaccines) against malaria.

In this thesis work, I have identified two novel *P. falciparum* proteins (PF3D7_0410600 and PF3D7_1459400) and used cellular/biochemical approaches to characterize the proteins. The gene encoding PF3D7_0410600 protein was previously reported to be crucial for blood-stage parasite growth in a recent piggyBac transposon saturation-level mutagenesis screen (Zhang *et al.* 2018). Analysis of protein sequence using bioinformatics portals revealed various predicted structural characteristics which indicates that the novel proteins could be playing diverse roles during the malaria parasite development.

In the analysis of published transcription profiles, PF3D7_1459400 was also shown to co-express with other genes involved in the intra-erythrocytic and gametocyte development stages as reported

previously (Pelle *et al*. 2015). Also, a recent systematic screen for uncharacterized *P. falciparum* proteins (Amlabu *et al*. 2018) revealed that PF3D7_1459400 intercepts with other merozoite proteins reported to be temporally expressed in the invasion cluster (Le Roch *et al.* 2003). More interestingly, transcriptomic data of all proteins expressed in male and female gametocytes of *P. falciparum* identified PF3D7_1459400 to be enriched in both sexual forms of the parasite (Lasonder *et al.* 2016) and was also shown to be a conserved, apicomplexan-specific, putative invasion protein (Huynh and Carruthers, 2016). These necessitated the characterization of this protein.

To study these novel proteins, I expressed soluble recombinant PF3D7_0410600 protein in bacterial system and generated rabbit antibodies against the protein. I also generated peptide antibodies against PF3D7_1459400 protein due to challenges with producing the soluble form of the protein. I tested the antibodies generated against the proteins and both rabbit antibodies reacted with the native parasite protein in immunoblotting. I used these antibodies to study the cellular location of both novel proteins by immunofluorescence assays (IFA) which revealed that PF3D7_0410600 protein is localized to the periphery of parasites. But PF3D7_1459400 protein on the other hand appeared to be exported and is deposited on the erythrocyte membrane. This is expected since the protein harbours a transmembrane domain and a PEXEL motif at the N-terminus which has been shown to mediate export of proteins beyond the parasite boundary (Hiller *et al.* 2004; Marti *et al.* 2004). However, further experimentation will be required to substantiate the possible association of PF3D7_1459400 protein with parasite-induced structures such as the Maurer's cleft, J-dots or the knobs.

Analysis of PF3D7_0410600 protein sequence revealed that the protein harbours armadillo repeats (ARM) amongst other functional domains. Proteins that harbour the ARM domains are known to have versatile functions and the classification of ARM proteins has been quite challenging given that some of the current annotations of armadillo repeats are incomplete or may be incorrect (Gul *et al.* 2017). This could be as a result of the difficulties in distinguishing between armadillo repeat types and the high similarity with Huntingtin, elongation factor 3, protein phosphatase 2A, and the yeast kinase TOR1 (HEAT) repeats (Kippert and Gerloff, 2009). Therefore, a detailed characterization of individual ARM proteins is important in order to understand the roles of these proteins in the development of the malaria parasite.

Apicomplexan ARM proteins that have been functionally characterized so far include *T. gondii* glideosome-associated connector (TgGAC) (Jacot *et al.* 2016), *P. falciparum* armadillo repeat only protein (*Pf*ARO) (Mitra *et al.* 2016), and *P. falciparum* merozoite-organizing protein (*Pf*MOP) (Absalon *et al.* 2016) were all apically localized. Surprisingly, PF3D7_0410600 protein showed peripheral localization around merozoites. Although the exact localization of *Plasmodium* proteins is determined by immuno-electron microscopy, when I co-stained the PF3D7_0410600 protein with an IMC marker-*Pf*MTIP, I observed some colocalization which indicates a possible localization of the protein to the IMC. I also observed partial colocalization of PF3D7_0410600 protein with tubulin acetyl transferase 1 (TAT1) in sexual stages. This is consistent with the crucial role of another ARM protein (PF16) which is expressed in male gamete flagellum, where it maintains the correct microtubule structure in the central apparatus of the axoneme (Straschil *et al.* 2010).

Despite the partial colocalization of PF3D7_0410600 protein with TAT1 in sexual stages, it remains to be established if there exists, a likely shuttling of the protein between the IMC and its associated microtubules, which could represent the poorly described interplay between the microtubular network and the IMC. This is because the observed colocalization of PF3D7_0410600 protein and TAT1 may have resulted during the lateral expansion of the IMC around the girth of the parasite where it associates with the microtubules (Schneider *et al.* 2017). However, PF3D7_0410600 protein including other ARM proteins belongs to the β-catenin family of proteins and the interaction of this protein family with dynein appears to tether microtubules at adherens junctions in epithelial cells (Ligon *et al.* 2001).

The IMC is a cisternal organelle that is assembled beneath the plasma membrane of merozoites, sporozoites, ookinetes and gametocytes (Schneider *et al.* 2017). The important structural role of this organelle in the cellular remodelling events that is associated with gametocyte elongation (Dearnley *et al*. 2012) and the role of specific IMC proteins in cell morphology has been described previously (Tremp *et al*. 2011). During gametocyte development for instance, elongation is powered by a network of microtubules that assemble under the IMC (Schneider *et al.* 2017). Importantly, the gametocyte IMC has a stage-specific function that may involve a poorly defined set of proteins (Schneider *et al.* 2017). Also, a member of the glideosome assembly, *Pf*GAP50 is known to be recruited to the periphery of gametocytes and appears to be coordinated with the laying down of microtubules (Dearnley *et al.* 2012).

Several IMC proteins have been classified into alveolins, non-alveolins and multi-transmembrane proteins based on their structural features (Kono *et al.* 2012). While a number of IMC proteins are recruited to the IMC via protein-protein interactions (Kono *et al.* 2012; Schneider *et al.* 2017),

others deploy lipid modifications for membrane attachment (Wetzel *et al*. 2015). *P. falciparum* GAP45 and HXGPRTII for instance, are recruited to the IMC solely via lipid modification (Gaskins *et al.* 2004; Rees-Channer *et al*. 2006, Gilk *et al*. 2009 Jortzik *et al.* 2012).

Since, my interest was on understanding how PF3D7_0410600 protein that lacks membrane attachment motifs could localize to the periphery of parasites, I tested the possible mechanisms that could be mediating this membrane localization. Considering the fact that lipid modification has been reported to regulate membrane association of proteins, I investigated whether this mechanism was responsible for the association of PF3D7_0410600 protein with the membrane. Even though, the results from the acyl resin-assisted capture are yet to be confirmed by mass spectrometry, the low prediction score for palmitoylation status and the data documented in the published palmitome (Jones *et al*. 2012) points to the fact that PF3D7_0410600 protein may not be palmitoylated.

I also investigated the possibility that other lipid modification pathways may be responsible for the membrane association of PF3D7_0410600 protein. Only geranylgeranyl transferase inhibitor (GGTI) seemed to have impacted the localization of the protein when parasites were treated with different inhibitors of lipid modification. Although these inhibitors are known to have pleiotropic effects and the observed phenotype may have resulted from an off-target effect as reported previously for 2-bromopalmitate which is generally used as the reference for palmitoylation inhibition (Davda *et al.* 2013). However, further investigation will shed more insight on the role of geranylgeranylation on the localization of PF3D7_0410600 protein.

Several parasite proteins that lack structural characteristics for membrane anchorage like PF3D7_0410600 protein have been recruited to membranous compartments via specific protein-

protein interactions (Gilk *et al.* 2006; Reddy *et al.* 2015; Saini *et al.* 2017). I therefore investigated the possibility that PF3D7_0410600 protein may be part of a molecular complex that could mediate its recruitment to the membrane. Using analytical SEC, I have reported that PF3D7_0410600 protein appears to exist as part of a larger-order protein complex, which could potentially play a role in its recruitment to the periphery of the parasite.

Previously, high-throughput versions of a yeast two-hybrid system have been used to show that PF3D7_0410600 protein interacts with PF3D7_0818200 (*Pf*14-3-3 protein) and PF3D7_1207000 proteins (LaCount *et al.* 2005). The potential interactors of PF3D7_0410600 protein that was identified by yeast two-hybrid screens have not been validated independently, and there are known false positive issues with yeast two-hybrid systems (Huang *et al.* 2007; Stellberger *et al.* 2010). However, *Pf*14-3-3 is a 30 kDa protein that lacks a signal peptide and a transmembrane domain. The protein has been reported to play key roles in several biological processes and also interacts with other functional binding proteins (Via *et al.* 2015). *Pf*14-3-3 is associated with regulation of subcellular localization and it was previously localized in both nuclear and cytoplasmic compartments (Dastidar *et al.* 2013). Thus, the interaction of PF3D7_0410600 protein with *Pf*14-3-3 protein may not necessarily be the basis for the peripheral localization of PF3D7_0410600 protein because *Pf*14-3-3 protein was not localized to the surface, IMC or microtubules. On the other hand, PF3D7_1207000 is a 311 kDa hypothetical protein that also lacks a signal peptide and a transmembrane domain. Although it remains to be established whether the peripheral localization of PF3D7_0410600 protein is as a result of this reported interaction with PF3D7_1207000 protein, this interaction may be responsible for the membrane association of PF3D7_0410600 protein.

5.2 CONCLUSION

In summary, this work presents for the first time, the characterization of PF3D7_0410600 and PF3D7_1459400 proteins. My findings suggest that PF3D7_1459400 protein may be exported and possibly associates with parasite-induced structures. On the other hand, lipid modification and protein-protein interaction may be the probable molecular mechanisms governing the recruitment of PF3D7_0410600 protein to the periphery of the parasite. It is therefore conceivable that disruption of the IMC-microtubular interplay may alter the parasite morphology, which may consequently affect its survival, and hence the apparent essentiality of the PF3D7_0410600 gene.

5.3 RECOMMENDATIONS

Further functional characterization of PF3D7_1459400 protein is required to elucidate possible association of the protein with parasite induced structures such as the Maurer's cleft, J dots or the knobs. This will involve membrane solubility/topology assays, co-immunoprecipitation and mass spectrometric analysis. It will be interesting to study the phenotypes of PF3D7_1459400 knock down parasite lines to determine the function of this protein in the parasite. Also, parasites with mutated PEXEL motif can be studied to assess the impact of mutating the PEXEL signature on the observed export of the protein. Put together, this may shed more insight on the significance of the protein and overall present it as a suitable target for drug development.

Also, the functional investigation of PF3D7_0410600 protein and its associated complex may also provide avenues for new therapeutic intervention strategies against malaria. Thus, co-immunoprecipitation and mass spectrometry using schizont lysates is required to probe the possible interactors of PF3D7_0410600 protein. In order to avoid the detection of an artefactual

interaction in the co-immunoprecipitation experiments, an endogenously-tagged PF3D7_0410600 parasite line will be required to validate the identities of the PF3D7_0410600 binding partner proteins. This is because, the anti-PF3D7_0410600 rabbit antibodies that was used detected both processed fragments and dimeric forms of the protein.

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APPENDIX

A. **B.**

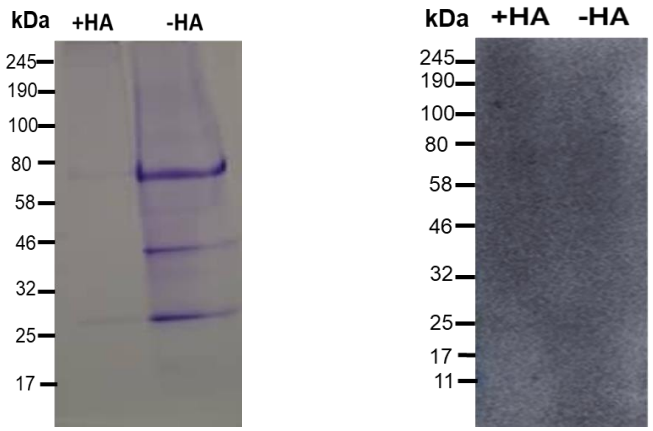

Figure appendix 1.1: Palmitoylation status of PF3D7_0410600 protein. (A) SDS-PAGE gel showing the elutes from the Acyl resin assisted capture. **(B)** Immunoblot probed with anti- PF3D7_0410600 rabbit antibody (1:1000) did not detect any protein band. (HA: Hydroxylamine).

A. **B.**

ATGGTAAACATAAATTGGCCAGGGCTTCTTAAGTGGTCTACGAAATATGCAGACGGGACTATTGATA
CAAATAAACGTTTAAGCAAAGAAGACATTGAATTTTTACAAGGGGCTATAAAAGATGCCTTAAGTCA
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TAAAATTAAAGCTATAGACCCATTATTAAAATTATTAGATAATTCTAATAATCATATTTTAGAATCAG
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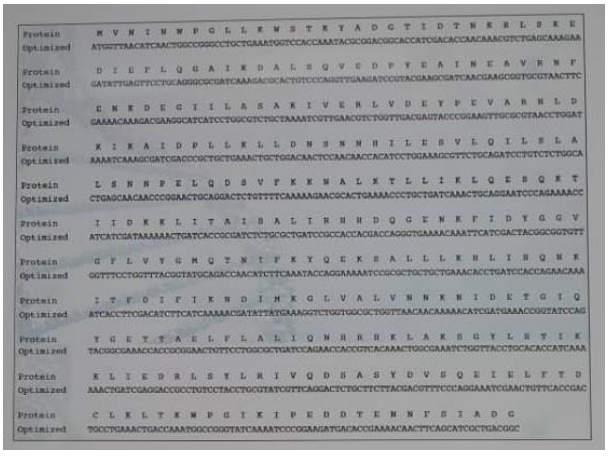

Figure appendix 1.2: Sequence map of PF3D7_0410600 gene (A) before and after **(B)** codon optimization.

A.

ATGAAAAACATATTGAGGAATAGAAAAGAAAAAGAAGATGATCATAGTTCTAG
TAATGTAACGAATGAAGTAAAAAAGGCTAGTTTTATAAATTATATATGTACAAGT
CTTAAGAATGTAGTAAAAAACAAAATTCAAAGTAAACATAAATATTCCTTACTAA
TAGGATTATTTATTACATTTTTAATATTTTGTGGTTTTTTTAGATATATGACAGAAT
TATATCATACTCATTTTTATAATTTCAAATATATTGATGTATCTAATACAGAAAAAT
TAAAAGAAATATTTTTTAGTAATAATCCATATTTAGTATATTGCAAGAATGATAAA
AATACAAGTATCCACCCTGTGATTAATGCTAGTCGATCTAACTTACCGGATATTCT
TAATATTGCTGTAATTAATTGTAAAAGTGTTTTACCAAGTAAACAAAATATATATC
AAAGATTTAGTTTAGAAGACAATACACCTGCTTTTGTAATTTGTTATGGCAAGA
AACCAAAACCAATAAGTGTAGCTTTATTAAATAATAAAAAGAAATTTATAACTTA
TGTACGTGAAGCTTTAGTATTCAATGTTCCTTTTTTTAGTAAGTTCCCACAATTC
CAAACCAAGTGTCTAAATAAAAATAAAAAATGTGTACTCTTTATTTACAACTTAG
AATTAACAACTAAAAATATCCAATATAATTATATTAATGATTTATTTGTTACAAATA
AATATTTCAATATAAGCCCATTAATTATTGATAATAAAAGATTCTTAATAAAATTAA
ATAATGAATTAAATAAATCCTATTTTAAAAAAAATGATGTACATGTTTTATGCCTT
TTTAATGATTCAGTTAAAAATGTGGAGACGTTCTATGGATTCTTTTATAATGATA
ACTTTGATGACTTTAAAAAATTATCCACTTTTGTTTCTACATGTATAAATGCAAAT
AACCAAACCAAACAAGCTATAAAATTGTCGGAGCCTCCACAAATTAAATATAGA
CCAGTCAAGCAAACCAAACAATGA

B.

AACCCGTACCTGGTGTATTGCAAAAACGACAAGAACAC
CAGCATTCACCCGGTTATCAACGCGAGCCGTAGCAAC
CTGCCGGACATCCTGAACATTGCGGTGATCAACTGCAA
AAGCGTTCTGCCGAGCAAGCAGAACATTTACCAACGT
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GAACAAAAGCTATTTTAAAAAGAACGACGTGCACGTT
CTGTGCCTGTTCAACGATAGCGTGAAGAACGTTGAAAC
CTTTTACGGCTTCTTTTATAACGAT

Figure appendix 1.3: Sequence map of PF3D7_1459400 gene (A) before and after **(B)** codon optimization

Table A1: List of synthetic gene and antibodies

S/N	Gene ID	Order ID	Lot No.	Company
1.	PF3D7_0410600 Gene	20010878KOGI1001-4	161207Q8954-1R12	Biobasic
2.	PF3D7_0410600 Antibody	AB-UG001-2	20180124	Biobasic
3.	PF3D7_1459400-P1 Antibody	U4739CD070-2	A417040252	GenScript
4.	PF3D7_1459400-P2 Antibody	U4739CD070-5	A417040250	GenScript
5.	PF3D7_1459400-P3 Antibody	U4739CD070-8	A417040248	GenScript

Ab: Antibody, P: peptide

Table A2: List of reagents

Reagent	Source
Glycerol	VWR, UK
Kanamycin	Sigma- Aldrich
Albumax II	Invitrogen
Gentamicin	Invitrogen
L-Glutamine	Sigma-Aldrich
L-Alanine	Sigma-Aldrich
Phosphate Buffered Saline, pH 7.4	Sigma-Aldrich
RPMI 1640 medium (with L-Glutamine, $NaHCO_3$)	Sigma-Aldrich
Bovine Serum Albumin (BSA)	VWR, UK
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich
Tris glycine	VWR, UK
Methanol	VWR, UK
Sodium Chloride	Sigma-Aldrich
Triton X-100	Thermofisher scientific

A. Materials and wares

- Sterile syringe and needles
- Sterile Syringe filtering units (0.2 μm)
- 1.5-2 mL Eppendorf tubes
- FALCON centrifuge tubes (15 mL and 50 mL)
- Sample racks
- Petri dish
- Latex gloves
- Tissue paper
- Alcohol swabs
- Permanent markers
- Microscope slides
- Cover slips
- 1 L culture flasks
- 250 mL culture flasks
- 1 mL, 20 and 200 μL pipette tips
- BD polypropylene, round-bottom tubes.

B. Buffer preparation

i. 4X Laemmli buffer

- 20% β-ME
- 40% glycerol
- 8% SDS

- 0.008% bromophenol blue
- 0.25 M Tris.HCl
- pH 6.8

ii. SDS-PAGE running buffer

- 25 mM Tris
- 192 mM glycine
- 0.1% SDS

iii. Transfer buffer

- 25 mM Tris
- 192 mM glycine
- 20% methanol

iv. PBS

- NaCl 0.138M
- KCl 0.0027M
- pH 7.4

v. Solubilization buffer (SB)

- 5 mM EDTA
- 4% SDS
- 50 mM Tris.HCl
- pH 7.4

vi. Lysis buffer (LB)

- 5 mM EDTA

- 50 mM Tris.HCl
- 150 mM NaCl
- 0.2 % Triton X-100 (for LB-T)
- pH 7.4

vii. Binding buffer

- 1 % SDS
- 1 mM EDTA
- 100 mM HEPES
- pH 7.4

C. Parasite culture media

i. Albumax

- 1 L RPMI 1640
- 50 g Albumax II
- 200 mg Hypoxanthine

ii. Incomplete Medium (PWM)

- 1 L RPMI 1640
- 5 mL Gentamicin (10 mg/mL)
- 100 µL L-Glutamine (200 mM)

iii. Complete Parasite Medium (CPM)

- 1 L RPMI 1640
- 5 mL Gentamicin (10 mg/mL)
- 100 µL L-Glutamine (200 mM)
- 1000 mL Albumax