Functional insight the role of plasmodium falciparum reticulocyte blinding like protein homologues : RH2B and RH5 during merozoites invasion

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**FUNCTIONAL INSIGHT ON THE ROLE OF *PLASMODIUM*
FALCIPARUM RETICULOCYTE BINDING LIKE PROTEIN
HOMOLOGUES - RH2B AND RH5 DURING MEROZOITES
INVASION**

YAW ANIWEH

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fulfillment of the requirement for the degree of Doctor of Philosophy**

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List of Abbreviations

ACT	Artemisinin Based combination therapy
AMA1	Apical membrane antigen 1
Ca ²⁺ -CaM	Calcium Calmodulin complex
CelTos	Cell-traversal protein for ookinetes and sporozoites
CQ	Chloroquine
CR1	Compliment receptor 1
CT	Chymotrypsin
Cyto D	Cytochalasin D
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
EBA	Erythrocyte binding assay
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescent activated cell sorter
FBS	Fetal bovine serum
GPI	Glycosylphosphatidylinositol
HRP	Horseradish peroxidase
IFA	Immuno fluorescence assay
IP	Immunoprecipitation
kDa	KiloDalton
LLIN	Long lasting Insecticide treated nets
LSA	Liver stage antigen
mAb	monoclonal antibodies

Me-TRAP	Multiple epitope – thrombospondin-related adhesion protein (TRAP)
mg	milligrams
ml	milliliters
MSP	Merozoite surface proteins
MTRAP	merozoite thrombospondin-related anonymous protein
Nm	Neuraminidase
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
Pf	<i>Plasmodium falciparum</i>
PfCSP	<i>Plasmodium falciparum</i> circumsporozoite protein
PfEBA	<i>Plasmodium falciparum</i> Erythrocyte binding antigen
PfROM	<i>Plasmodium falciparum</i> Rhomboid protease
PfSUB	<i>Plasmodium falciparum</i> Subtilisin-like serine proteases
Pk	<i>Plasmodium knowlesi</i>
PV	Parasitophorous vacuole
Pv	<i>Plasmodium vivax</i>
PVM	Parasitophorous vacuole membrane
Py	<i>Plasmodium yoelii</i>
Py235	235 kDa rhoptry protein of <i>Plasmodium yoelii</i>
R	Resistant
RAMA	Rhoptry associated membrane antigen
RAP	Rhoptry associated protein
RBC	Red blood Cells
RBL	Reticulocyte binding ligand
RhoP	High molecular weight Rhoptry protein
RIMA	Ring membrane antigen

RON	Rhoptry neck protein
PfRH	<i>Plasmodium falciparum</i> Reticulocyte binding-like protein homologue
PfRipr	<i>Plasmodium falciparum</i> RH5 interacting protein
rpm	Revolution per minute
S	Sensitive
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERA	Serine repeated proteins
SP	Sulfadoxine- pyrimethamine
SSP	Sporozoite surface protein
SUP	Supernatant
T	trypsin
TEMED	N,N,N',N'-tetramethylethylenediamine
TM	Transmembrane domain
ul	microliters
Un	Untreated
V	volts
WHO	World Health Organisation
O ₂	Oxygen
CO ₂	Carbon dioxide
SSS	Super Sharp Silicon probes
IPTG	Isopropyl β-D-1-thiogalactopyranoside
AFM	Atomic force Microscopy
PHA	Phytohemagglutinin

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ABSTRACT

The process of *Plasmodium* merozoites invasion is a complex multi-step process. In understanding the mechanisms leading to successful merozoites invasion, a group of proteins known as *Plasmodium falciparum* erythrocyte binding ligands as well as *Plasmodium falciparum* reticulocyte binding - like protein homologues (PfRH) have been shown to be necessary. These proteins have been shown to play a role in sensing the erythrocyte. Of the PfRH family, it is becoming clearer of their ability to affect the secretion of microneme proteins which are necessary to establish the tight junction. In this presentation, the role of PfRH2b during merozoite invasion has studied. Like PfRH1, PfRH2b has been shown to regulate the increase in merozoites Ca^{2+} . This, we have shown to affect the release of EBA175 to the erythrocyte surface. Our data also points to the fact that the secretion of EBA175 to the surface is more prominent when the merozoites are engaged with the RBCs. Furthermore we have provided the detailed processing events that occur in the PfRH2b protein before and during merozoites invasion.

Functional insight on RH5-Basigin interaction has been provided. In this, we have shown RH5 to be involved in invasion independent of EBA175 surface expression but functions before tight junction formation. We have provided evidence of RH5-Basigin interaction driving Ca^{2+} in the RBC leading to the remodelling of the RBC cytoskeletal proteins arrangement. We have confirmed that recombinant RH5 has the ability to induce changes in the RBC cytoskeleton upon binding. This mechanism we propose facilitates the insertion of AMA1-RON complex into the RBC membrane leading to tight junction formation. This knowledge we believe will help in advancing our understanding as well as provide avenue to explore the mechanisms of merozoites invasion

1.0 INTRODUCTION

1.1 *Malaria*

Malaria is an infectious disease that has been a burden on the global community. Malaria mortality and morbidity occurs mainly among infants and pregnant women. The World Health Organisation estimates about 200 million malaria cases with about 584, 000 malaria deaths reported in 2013. Of these, about 91% were from predominantly Sub-Saharan Africa, of which children below the age five were the most susceptible group constituting 81% of the cases(Autino *et al.*, 2012, Hay *et al.*, 2010). Overall, 3.2 billion people are estimated to be at risk of malaria infection or manifesting the disease.

1.1.1 Malaria: History

The burden of malaria has been associated with humans for millennia and the parasite has become well adapted to the human system. In ancient times, (850- 400 BC) the Greeks and Empedocles had been aware of the characteristic symptoms associated with malaria. They described observations such as enlarged spleens, and symptoms such as fevers which were found in patients to be as a result of malaria. It was also observed that poor health associated with people living in marshy areas was caused by malaria but the exact causative organism was not known (Cox, 2010, Bynum, 1998). With advances in cell biology and following the initial description of bacteria and microorganisms being associated with infectious diseases, the search for the cause of this disease was staged (Murray *et al.*, 2012). Earlier, it was observed that people that died of the disease had black pigmented granules in their blood samples as well as in their spleen (Manson-Bahr, 1961, Manson-Bahr, 1948). The parasite was eventually discovered in 1880 by Charles Louis Alphonse Laveran (Nye, 2002), which revolutionised the thinking about malaria. The discovery of mosquitoes as the vector for human filarial parasite (Eldridge, 1992) led to the proposal that malaria could also be transmitted by mosquito, hence the mosquito-malaria theory. This theory was later proven by Ronald Ross in 1890 (Nye, 1991). Experimentally, the transmission of malaria from

human to human by mosquitoes was later established in 1897 (Ross, 1897). This led to the development of tools to understand the parasite and strategies were put in place to combat this deadly disease (Carter & Mendis, 2002). The battle against malaria yielded great success following the development of vector control, i.e. using insecticides such as dichloro-diphenyl- trichloro-ethane (DDT) coupled with parasite targeting drugs (chloroquine and amodiaquine) in the 1950s and 1960s (Bruce-Chwatt, 1985b). The combination of these treatments enabled the eradication of malaria from communities with low disease transmission, whilst those in endemic communities were still suffering from the disease (Cox, 2010, Bynum, 1998, Rich *et al.*, 1998, Boyd, 1946). However, soon after this success, resistance of both parasite and vector to the intervention emerged, thereby calling for newer strategies in tackling the disease.

1.1.2 Malaria epidemiology

Malaria, cumulatively over the centuries, is one of the deadliest vector-borne diseases of human kind. It is spread through the bite of an infected female *Anopheles* mosquito. The disease is characterised by fever, chills, fatigue, sweating, headache, nausea as well as vomiting. The disease symptoms usually occur 10 to 15 days after an individual is infected with the parasite. These symptoms can progress from mild to severe forms such as severe anaemia and sometimes coma. Malaria causes multiple complications including cerebral malaria, hypoglycaemia, anaemia, convulsion, renal failure metabolic acidosis and respiratory distress (Gitonga *et al.*, 2012, Perkins *et al.*, 2011, Schumacher & Spinelli, 2012, Weatherall *et al.*, 2002, Abkarian *et al.*, 2011). Children with severe malaria have shown to have multiple episodes of lactic acidosis which result from accumulation of lactic and 3-hydroxybutyric acids upon an increase in parasitaemia (English *et al.*, 1997, Sasi *et al.*, 2007). The pattern of disease morbidity in endemic areas depends on the immune responses and transmission intensities which vary seasonally. Indeed, climatic conditions such as rainfall and drought modulate mosquito breeding and population levels thereby impacting on malaria transmission trends. During the rainy season, more breeding sites are created leading to high mosquito populations and hence increased malaria transmission. The severity of the disease is determined by increased exposure of individuals living in

malaria endemic communities (Marsh & Snow, 1997, Elliott & Beeson, 2008, Guinovart *et al.*, 2012).

1.1.3 Human *Plasmodium*

Malaria is caused by an apicomplexan, obligate parasite of the genus *Plasmodium*. Six members have been shown to cause malaria in humans, namely; *Plasmodium falciparum* (*P. falciparum*), *P. vivax*, *P. ovale*, *P. malariae*, *P. knowlesi* and *P. cynomolgi* (Ta *et al.*, 2014). Different *Plasmodium* species present different degrees of disease burden to patients. *P. falciparum* causes the most severe form of the disease. Indeed, *P. falciparum* has been responsible for the majority of malaria related mortalities worldwide. Whilst *P. ovale* and *P. malariae* cause mild form of the disease (Thera & Plowe, 2012, Vaughan & Kappe, 2012), recent reports indicates that *P. vivax* (Naing *et al.*, 2014, Andrade *et al.*, 2010, Kochar *et al.*, 2009) as well as *P. knowlesi* (Boo *et al.*, 2016, Seilmaier *et al.*, 2014) are able to cause severe disease in patients. Geographically, *P. vivax* has the greatest distribution worldwide (Lover *et al.*, 2014). *P. vivax* and *P. malariae* have been previously described based on the periodic pattern of their disease recurrence as "benign tertian" (every third day) and "quartan" (every fourth day) respectively. *P. malariae* chronic infection has been associated with nephrotic syndrome (Olowu *et al.*, 2010, Hendrickse, 1976), which can be fatal if the condition persists.

All *Plasmodium* species tend to have a geographic dominance. *P. falciparum* is most common in Africa, the Western pacific and South America (Hay *et al.*, 2009). *P. vivax* is common to Asia and South America. *P. vivax* is able to go into dormancy (hypnozoites) in the liver and revive later causing the blood stage infection. It has fast and constant gametocyte induction in asymptomatic individuals facilitating its transmission while in *P. falciparum* gametocytes are induced later in the developmental cycle. Distribution of *P. vivax* is limited to people that have the Duffy receptor on their erythrocyte surface, whilst *P. falciparum* is promiscuous in selection of red blood cells (RBC) types (Campino *et al.*, 2006, Rogerson & Carter, 2008). *P. ovale* and *P. malariae* show a sporadic distribution in

Africa and Asia (Gneme *et al.*, 2013). *P. ovale* is mainly found in Sub-Saharan Africa as well as the Philippines (Lysenko and Beljaev 1969; Collins and Jeffery 2005) but in fewer numbers. *P. knowlesi*, a simian parasite, is reported among humans residing near the forest areas in Southeast Asia with large populations of macaque monkeys (Singh *et al.*, 2004, Cox-Singh *et al.*, 2008). *P. knowlesi* infection is zoonotic. Evidence of human to human disease transmission is yet to be described, even though the number of reported cases are increasing gradually (Barber *et al.*, 2012, Cox-Singh *et al.*, 2008).

P. falciparum (the focus of this study) pathogenesis depends on the efficiency of red blood cell invasion (Weatherall *et al.*, 2002). *P. vivax*, *P. cynomolgi* and *P. ovale* preferentially invade reticulocytes (only 2% of circulating RBCs) while *P. malariae* invades mature RBCs. *P. falciparum* invades RBCs of all ages but prefers reticulocyte to normocytes. *P. malariae* is limited by the number of the infective form produced (Tangpukdee *et al.*, 2009, Schumacher & Spinelli, 2012, Perkins *et al.*, 2011). These impacted on the overall parasite burden in relation to which *Plasmodium* species is causing the disease. *P. falciparum* and *P. vivax* cause the most severe cases of anaemia (Weatherall *et al.*, 2002).

1.2 Malaria parasite Life cycle

Human *Plasmodium spp.* have a complex life cycle involving both mosquito vector (sexual stage) and human host (asexual stage). The different stages of the parasites' life cycle are directed by three invasive forms of the parasite (shown in **Figure 1**); ookinete (invades the endothelium of the mosquitoes' mid-gut), sporozoites (invades hepatocytes), and merozoites (invades erythrocytes (RBC)).

Figure 1. Life cycle of *P. falciparum*.

This indicates the stages of development from the mosquito vector to the human host (comprising liver and blood stages). The parasites (sporozoites) are introduced by an infected female *Anopheles* mosquito. The sporozoites invade hepatocytes and develop through, thereby forming merozoites. Released merozoites invade red blood cells (RBC). In the RBC the parasite undergoes the cyclical order of development ranging from ring, trophozoite and schizont during its 48 hour asexual cycle. Schizont contain merozoites which invades RBC keeping the cycle through, some trophozoites during the developmental cycle differentiate into gametocytes (sexual stage). The gametocytes are taken up by the female *Anopheles* mosquito. In the midgut of the mosquito, zygote is formed. The zygote develops into ookinete which invades the midgut epithelial cells and reside in the epithelial layer of the salivary glands. They develop into oocytes, which contain sporozoites that are released into the salivary glands.

In humans, the parasite's life cycle is initiated by the injection of sporozoites into the blood by an infected female *Anopheles* mosquito during a blood meal. They are carried in the bloodstream until they reach the liver. In the liver, the sporozoites invade hepatocytes (liver cells) and develop into exo-erythrocytic schizonts which harbour many uninucleated merozoites (blood stage infective form of the parasite) (Clyde, 1975, Clyde *et al.*, 1973a). Upon maturation, the hepatocyte membrane ruptures, releasing the merozoites into the bloodstream to invade erythrocytes (RBCs). The RBCs stage is responsible for all the disease symptoms (Clyde, 1975, Bruce-Chwatt, 1985a) associated with malaria. During the RBC stage, the parasite undergoes a trophic period development, ranging from the first establishment of the parasite upon merozoite invasion into the RBC called the rings. The parasite develops into metabolically active stage called trophozoites, which is characterised by the ingestion and digestion of hemoglobin. The trophic period is crowned by a number of rounds of nuclear division leading to the formation of a schizont. The schizogony stage leads to the development of RBC-invasive form of the parasite known as merozoites. In human RBCs, the developmental cycle of the parasites takes from 24, 48, 48, 48 and 72 hour's for *P. knowlesi*, *P. falciparum*, *P. ovale*, *P. vivax* and *P. malariae* respectively (2011, Anthony *et al.*, 2012, Autino *et al.*, 2012, Collins *et al.*, 1990, Cowman *et al.*, 2012, Cowman & Crabb, 2006) as illustrated in **Figure 1**.

During the development of the parasite, some rings differentiate into gametocytes (sexual stage). The gametocytes undergo maturation to the form that can easily be taken up by the female *Anopheles* mosquito during its blood meal. In the midgut of the mosquito, the microgamete and the macrogamete fuse to form a zygote. The zygote develops into ookinete (mosquito invasive form) which invades the midgut epithelial cells and reside in the epithelial layer of the salivary glands. In the midgut epithelial cells, the ookinete develops into oocytes, which harbours 1000 to 5000 sporozoites per cell. The sporozoites are released into the hemocoel of the mosquito upon the rupturing of the oocyt. The sporozoites migrate and invade the salivary glands (Davis *et al.*, 1989) and are released into the bloodstream whenever the mosquito feeds (Mota *et al.*, 2002, Mota *et al.*, 2001, Rosenberg *et al.*, 1990) as shown in **Figure 1**.

During the growth process of the parasite in the RBCs, hemoglobin is broken down and the iron component aggregated into hemozoin (Lamikanra *et al.*, 2009). Hemozoin as well as other parasite metabolites are released into the blood stream when schizonts burst, thereby contributing to the disease symptoms (Lamikanra *et al.*, 2009).

1.3 *Current malaria control strategy*

With the spread of drug resistance in *Plasmodium falciparum* as well as resistance of the mosquito vectors to insecticides, the threat of malaria disease has greatly increased. This has led to a search for compounds and molecules with a strong therapeutic potential (Greenwood & Mutabingwa, 2002, Fairhurst *et al.*, 2012). Hence, there is an urgent need to develop new drugs and possibly a malaria vaccine with long term effect of reducing the disease burden.

Many strategies have been attempted by stakeholders and funding agencies with regards to malaria control and vaccine development. Considering the decrease in the number of malaria death from the year 2000 (1 million) to 2011 (655,000), the goal to achieve complete eradication of malaria may be feasible (Snow *et al.*, 2012, Verma *et al.*, 2013). The strategies used to achieve this range from those that block parasite transmission to those that cure the disease and the symptoms associated with it (Sachs, 2002).

1.3.1 *Vector control strategy*

The key reason for the spread and subsequent impact of malaria has to do with the ability of the parasite to be transmitted by a large numbers of species of the *Anopheles* genus. Of the population of mosquitoes that exist, an estimated 400 species constitute this genus. 70 species have been described as potential vectors for the spread of malaria (Cox, 2010, Dabire *et al.*, 2008, Dabire *et al.*, 2013, Dabire *et al.*, 2012, Diabate *et al.*, 2007). Different

species have different ecological preferences and cues that make their survival possible. To target communities for vector control, special cues have to be drawn from the environmental setting to allow easy targeting (Diabate *et al.*, 2009).

Strategies for vector control include the use of indoor residual insecticide spraying (Ruecker *et al.*), Long- Lasting Insecticidal Nets (LLINs), and insect repellents. This basically works by preventing direct contact to humans at night (high feeding time) while also killing the mosquitoes. These methods are effective and cheap to be adopted by communities (Diabate *et al.*, 2006, Cuzin-Ouattara *et al.*, 1999). Residual spraying targets mosquitoes that reside indoors, known as resting mosquitoes. A number of insecticides have been introduced by the WHO as suitable for residual spraying. These insecticides have been shown to select for mosquitoes that reside indoors. This defeats the effectiveness of controlling the vector population as the feeding habit changes to outdoor feeding (Dabire *et al.*, 2009, Dabire *et al.*, 2006).

A larval control strategy aims at controlling the population of the vector in any community by terminating vector development at the larval stage (source reduction) and employs larvicidal chemicals such as oils. These oils are used to cover mosquito breeding ponds. The use of toxins as well as biological agents (such as bacterium *Bacillus thuringiensis* var. *israelensis* , which is very specific for mosquitoes, blackfly and midges; fungi e.g. *Laegenidium giganteum*) or mermithid nematodes (e.g. *Romanomermis culicivorax*) as well as the use of mosquito fish (including *Gambusia affinis*) which kill the larvae have largely been ineffective except in a few studies (Diabate *et al.*, 2005).

Other strategies such as the release of sterile male into communities with well mapped swamping distribution locations as well as community-based fumigation have been effective in controlling the mosquito populations. Though success has been achieved for some of these strategies, their overall effectiveness has been limited due to the development of resistance to the insecticides and the larvicides used. Also the biological control is limited in

the degree of an area one can tackle at a time (Baldet *et al.*, 2003, Diabate *et al.*, 2005, Dondji *et al.*, 2005, Jones *et al.*, 2012, Roux *et al.*, 2014).

1.3.2 Disease control strategy

Many intervention strategies ranging from long-lasting insecticidal nets (LLIN), indoor residual spraying programmes and artemisinin- based combination therapy (ACT) as well as the use of rapid diagnostic tests to help early detection and to prevent presumptive treatment of malaria have been employed (Fairhurst *et al.*, 2012, Kamal-Yanni *et al.*, 2012, Snow *et al.*, 2012, Abkarian *et al.*, 2011). However, these strategies are gradually being defeated by the emergence of resistant parasite and mosquitoes to the current drugs and insecticides used. Therefore, the need for vaccine has become even more urgent (Verma *et al.*, 2013). The ultimate goal has been to develop an effective malaria vaccine which has high efficacy in protecting the largest number of people against the range of *P. falciparum* and *P. vivax* strains.

Evidence has shown that people living in malaria endemic communities acquire protective immunity which limits the severity of the disease over time (Moorthy *et al.*, 2004, Reiling *et al.*, 2010). With this possibility, a number of antigens expressed at different stages of the parasite life cycle (both sexual and asexual have been marked as key vaccine candidates over the last few decades (Kappe *et al.*, 2010, Vaughan & Kappe, 2012, Wang *et al.*, 2009). This has called for the need to look into the design and delivery methods that will foster strong immunity in patients.

1.3.2.1 Vaccines

Even though the combined efforts aimed at controlling the spread and severity is making an impact, the need for an efficacious vaccine is urgent. The dream for an effective malaria

vaccine has been passed on to successive generations following the failure of the earlier strategies and interventions employed (Sachs, 2002). Following the progress made in understanding the molecular interplay necessary for the survival and development of the parasite, the life cycle of the parasite allows critical control points to be picked out and studied for their potential as vaccine targets (Moorthy et al., 2004, Schwartz *et al.*, 2012).

A vaccine has been developed against the sporozoite stage of development. The RTS,S, a recombinant and subunit vector vaccine, which targets the *Plasmodium falciparum* circumsporozoite proteins (PfCSP) expressed during the sporozoite and early liver stages of the parasite development, had some success and has emphasized the possibility of developing an effective vaccine using parasite antigens (Aide *et al.*, 2011, Campo *et al.*, 2011, Vaughan & Kappe, 2012). It is currently in phase 3 clinical trials. Reports of this phase in eleven sites in seven countries in Africa, indicates that out of the 6537 infants (6-12 weeks) and 8923 children (5-17 months) involved in the study, protection against clinical malaria was 26% and 46% respectively. No protection was reported in infants while in children it is estimated to exhibit 34% efficacy against severe disease. While RTS,S has some impact on transmission, it is more protective in communities with low disease incidence (Duncan & Hill, 2011, Duncan *et al.*, 2011). The combination of vaccination with other control strategies in high incident communities could drastically reduce malaria's burden.

A heterologous prime boosting immunization incorporating *P. falciparum* CSP and *P. falciparum* apical membrane antigen 1 (AMA-1) in a DNA vaccine construct showed initial protective efficacy of 27%, but failed upon subsequent trials (Tamminga *et al.*, 2013). A combination of PfCSP and multi-epitope thrombospondin-related adhesion protein (ME-TRAP) proved to confer sterile immunity in 13% of volunteers while inducing a high T cell response. This indicates the possibility that an optimized immunization protocol could be effective in inducing the required immunity in patients. Other sporozoite proteins such as sporozoite surface protein 2 (SSP2) and liver stage-specific antigen 1 (LSA-1) (Wang *et al.*, 1996, Guerin-Marchand *et al.*, 1987) have all been shown to confer protection that prevents severe disease. Another development which has been shown to be effective in inducing

cross protection in rodent malaria (*P. berghei*) is the cell- traversal protein for ookinetes and sporozoites (CelTOS), which is also currently undergoing clinical trial (Bergmann-Leitner *et al.*, 2010). A new area of focus recently has been to develop a multi-antigen vaccine which incorporates CSP and other sporozoites antigens, as well as those expressed during the liver stage of development.

It has been established for over 40 years now that immunization with radiation attenuated *Plasmodium* sporozoites confers complete protection in patients upon malaria challenge (Clyde *et al.*, 1973a, Clyde *et al.*, 1973b). This earlier observation has motivated great innovations over the years. The use of live attenuated sporozoites has also proven to be promising strategy with high levels of protective efficacy from experimentally challenged naive humans (Epstein *et al.*, 2011, Hoffman *et al.*, 2010, Clyde, 1975). Previously, the major hurdle was introducing sporozoites by mosquito bites. However, this has now been solved with the success of intradermal route of sporozoites introduction (Richie *et al.*, 2012, Seder *et al.*, 2013). The new challenge is how to produce the sporozoites at a high throughput level, attenuated or otherwise, of consistent and acceptable quality. To fully ascertain the immunological impact as well as regulators that play a role in developing sound immune protection, deeper insight to both cellular and humoral immune responses must be understood. This will allow a more targeted approach in facilitating the development of next-generation vaccine strategies that will completely prevent malaria (Sachs, 2002).

Infecting individuals with *P. falciparum* irradiated sporozoites and later treating with drugs showed protection against disease when subsequently challenged (Bijker *et al.*, 2013, Bijker *et al.*, 2014, Behet *et al.*, 2014). Antigens related to the erythrocytic stage such as merozoite surface protein 1 (MSP-1), MSP-3, apical membrane antigen 1 (AMA-1), erythrocyte binding antigen 175 (EBA-175 RII) and serine repeat antigen 5 (SERA 5) have also been considered as vaccine candidates based on their ability to stimulate immune protection or curtail disease severity in patients (Triglia *et al.*, 2000, Verma *et al.*, 2013, Lazarou *et al.*, 2009). All these have failed in field trials as single antigen vaccines, suggesting a need for combinatorial vaccines that target multiple antigens present at different stages of the parasite's development (Schwartz *et al.*, 2012, Tham *et al.*, 2012). ME-TRAP combined

with MSP-1 failed to significantly induce protection (Hodgson *et al.*, 2014), nevertheless, the fused vaccine comprising of *P. falciparum* glutamate- rich protein (PfGLURP) and MSP-3 was able to induce cytophilic antibodies which affected parasite development (Kangoye *et al.*, 2014, Baumann *et al.*, 2012, Tamborrini *et al.*, 2011). With all these parasite ligands, there are still limited numbers of recombinant or vectored vaccine candidates that have been shown to be efficacious in clinical trial (Hill, 2011, Greenwood *et al.*, 2011). To develop interventions that facilitate elimination programs and to foster the development of transmission-blocking vaccines or drugs, a deeper understanding of the complex processes that drive human infectiousness is needed (Bregu *et al.*, 2011).

Many new approaches, such as the use of multiple epitope constructs, (example; polyepitope DNA and recombinant viral subunit vaccines) have emerged and proven to be effective. Also, the advent of more technologies that foster target driven controlled delivery mechanisms such as liposomes, virosomes, microspheres/microparticles, polymeric nanoparticles, and dendrimers are paving way for the development of DNA based vaccines against malaria (Tyagi *et al.*, 2012, Webster *et al.*, 2006). Earlier approaches of developing subunit vaccines had a setback of stimulating non-specific immunity. Whole organism vaccines, for example; genetically attenuated sporozoites, have been shown to induce sterile immunity in naive patients (Schwartz *et al.*, 2012, Vaughan & Kappe, 2013, Vaughan *et al.*, 2010, Forbes *et al.*, 2011, Verma *et al.*, 2013). Also a DNA based vaccine that encodes for five different ligands targeting different stages of development such as exported protein 1, sporozoite surface protein 2, liver stage antigen 1 and 3 as well as CSP has been developed (Biswas *et al.*, 2011, Checkley *et al.*, 2011, Forbes *et al.*, 2011). A more focused approach envisaged to be effective is the use of multi- stage antigens in a single vaccine particle (Forbes *et al.*, 2011). Not only will this impact the disease but also diminish the sexual stage transmission into mosquitoes.

Development of the sexual stage of *Plasmodium* in the gut of the mosquito is another potent target for vaccine development (Vaughan & Kappe, 2012, Wang *et al.*, 2009). Earlier studies have shown that antibodies developed against gametocyte specific antigens and cytokines have the ability to block the fertilization process in the mosquito (Dinglasan &

Jacobs-Lorena, 2008). This observation paved the way for possible transmission blocking vaccines to be studied in depth. These vaccines will aim to terminate sporozoites development thereby preventing subsequent transmission into human. Examples of such ligands studied earlier includes *P. falciparum* ookinete surface proteins Pfs25 and Pfs28 and their *P. vivax* homologues; Pvs25 and Pvs28, Pfs48/45 and Pfs230 (Chowdhury *et al.*, 2009, Wu *et al.*, 2008, Malkin *et al.*, 2005, Bustamante *et al.*, 2000). The advancement in technology is allowing the production of strong and effective adjuvants which will compliment this lapse (Dinglasan & Jacobs-Lorena, 2008).

1.3.2.2 Drugs

Malaria, as an ancient disease, has been handled in different ways throughout the ages. Prior to the development of modern science, malaria had been managed by the use of herbs or their extracts and quinine, which was very effective. As the use of quinine spread, scientists synthesized many more compounds from quinine, such as quinacrine. The treatment of malaria was revolutionised in the 1930's, after the discovery of chloroquine by Hans Andersag. It was used as a strong drug for different campaigns aimed at eliminating malaria or curtailing the disease burden to the barest minimum until the 1990's. Following the failure of chloroquine (CQ) due to the drug resistance that emerged, a number of drugs were introduced; doxycycline, a combination of atovaquone and proguanil hydrochloride and sulfadoxine-pyrimethamine (SP) (reviewed in (White, 2004, Smithuis *et al.*, 2004) to treat the resistant parasites. Together these were inexpensive drugs and had a longer circulating half-life in the body (Trape *et al.*, 1998) making their use effective in disease management. Also they were of the most widely used medications in the tropical areas, hence their misuse lead to resistance over time (Bruce-Chwatt, 1985a). Following the development of resistance to SP, Mefloquine was introduced as an appropriate drug due to its long half life and the fact that it requires a single dose administration (Gonzalez *et al.*, 2014).

The development of resistance by *P. falciparum* lead to global resurgence of malaria possibly doubling malaria related child mortality in the affected communities. As, symptomatic malaria is in most cases uncomplicated a potent drug, would be able to significantly reduce the mortality associated with *P. falciparum* malaria (Dondorp *et al.*, 2010, Duncan *et al.*, 2011).

Artemisinin was another potent first line drug developed for the effective management of malaria. This is a drug is very effective at killing the parasite, and lacks adverse effects, although, it has short half- life (Plowe, 2003). This called for a combination therapy with a slow acting and long half- life drugs to be paired with artemisinin. This was known as the artemisinin-based combination therapy (ACT). ACTs included combinations such as artesunate and amodiaquine, artesunate and primaquine (Pukrittayakamee *et al.*, 2004, Nosten *et al.*, 2000). As potent as artemisinin is, resistance has been reported (Miotto *et al.*, 2015, Dondorp *et al.*, 2010). With the development of resistance, there is the need for new drugs to be developed which is ideally a single- dosed. Over the years, resistance has been associated with mutations or changes in copy number of genes being targeted by the different drugs (Hastings & D'Alessandro, 2000).

In anti-malaria drug development, a major limitation over the years, has been a lack of knowledge of the exact target of action of the drug, which limits effective pharmacodynamic study (White, 1997). An advance in science and technology is thus paving way for identification of targets. This has been possible after the entire complex genome for *P. falciparum* was sequenced. Therefore, parasite growth platforms have been miniaturised, facilitating high throughput screening of targets and chemical libraries (Anthony *et al.*, 2012, Morton *et al.*, 2007, Gardner *et al.*, 2002). Targets such as the *Plasmodium* dihydrofolate reductase, dihydropteroate synthetase, cytochrome bc1 complex and the haemoglobin degradation pathway, are easily validated using strong genetic tools to identify the possible resistance that may

arise, and ways to avert them (Sykes & Avery, 2013, Guiguemde *et al.*, 2012, Gardner *et al.*, 2002). Ideally, elimination efforts require a new class of drugs which clears symptoms as it cures the disease and also prevents transmission (Kamal-Yanni *et al.*, 2012, Diagana, 2015). This new class drugs should have multi species plasmodicidal activity and prevent relapsing stages of both *P. vivax* and *P. ovale*. The drugs should have chemo-protective ability where infants as well as expectant mothers, who suffer the rigor of the disease, are protected (Murray *et al.*, 2012, Griffin *et al.*, 2012, Nambozi *et al.*, 2011)

1.4. *The Merozoite: Blood stage invasive parasite*

1.4.1.0 Merozoite Biology

The merozoite is a 1 to 2 μm sized cell, basically “tear drop” in shape. The cell is polarized such that it has a posterior and an apical end. These are features shared by the invasive form of other apicomplexan parasites. The morphology of the merozoite facilitates its ability to invade erythrocytes. As a eukaryotic cell, merozoites possess all the organelles such as nucleus, endoplasmic reticulum, ribosomes, exonemes, Golgi bodies as well as mitochondrion required for life activities shown in **Figure 2 A**. As a functional adaptation, the apical end is characterised by the presence of organelles which facilitates its ability to invade RBCs (Baum *et al.*, 2005, Cowman *et al.*, 2012, Gaur *et al.*, 2004, Giovannini *et al.*, 2011, Gomez-Escobar *et al.*, 2010, Hadley, 1986).

1.4.1.1 Merozoite surface proteins (MSP)

The merozoite surface coat is made up of glycosylphosphatidylinositol (GPI) (Holder, 2009, Holder *et al.*, 1992, Lin *et al.*, 2014) anchored proteins, mainly, merozoite surface proteins 1 (MSP-1), 2, 4, 5, 10, plasmodium falciparum antigen 12 (Pf12), 38, and 92; which either have EGF or 6- cysteine domains (Cowman *et al.*, 2012, Cowman & Crabb, 2006, Holder,

2009). MSP-1, 2, 5 and 10 have essential roles in the development of the merozoite (Lin *et al.*, 2014). Other proteins such as MSP-3, MSP-6, MSP-7 and MSP-9 are displayed on the surface of the merozoite by interacting with MSP-1 (Holder, 2009, Holder *et al.*, 1992, Lin *et al.*, 2014). MSP7 has been identified to interact with P-selectin (a receptor on the RBC surface), possibly serving an immune-regulatory role in addition to its function during merozoite invasion (Perrin *et al.*, 2015). Merozoite surface protein harbouring Duffy binding like domains (MSPDBL-1 and 2) have been shown to bind to unknown receptor on the RBC (Wickramarachchi *et al.*, 2009). Antibodies to these have been shown to opsonise the merozoite, thereby preventing invasion (Chiu *et al.*, 2015).

Another set of proteins that have been shown to be localized to the merozoite surface in schizonts are the serine repeat antigens (SERA). These SERA proteins contain papain- like protease domain. So far, SERA 3, 4, 5 and 6 have been characterised, of which SERA 4, 5 and 6 have been shown to be essential for the survival of the parasite (Boyle *et al.*, 2014, Huang *et al.*, 2013, Bermudez *et al.*, 2012, Hodder *et al.*, 2003, Safitri *et al.*, 2003). The SERA proteins as well as the S- antigen and GLURP are secreted into the parasitophorous vacuole (PV) where the function to facilitate merozoite egress (Teo *et al.*, 2012, Tanabe *et al.*, 2012).

1.4.1.2 Rhoptry

A common feature of the apicomplexan parasites is the presence of apical secretory organelles such as micronemes, rhoptries and dense granules, that release their content as and when required during the process of invasion (Miller *et al.*, 2002). The rhoptries are prominent, paired organelles that are clubbed shaped (shown in **Figure 2A** and **B**). The rhoptries fuse to the plasma membrane of the merozoites, thereby creating a duct through which its contents are easily secreted out. The rhoptries are sub- compartmentalised into the bulb and the neck (duct). Within these structures, proteins appear to be resident based on when they are required. This is in agreement with the “just in time” proposed order of release of their content (Kats *et al.*, 2008). A number of rhoptry proteins have been shown to

either recognize host cell surface receptors or to be secreted to aid the invasion process (Besteiro *et al.*, 2011, Richard *et al.*, 2010, Srinivasan *et al.*, 2011).

Major proteins in the rhoptry include the rhoptry neck proteins (RONs 2, 3, 4, 5, 6, 8, 12, etc.), reticulocyte binding protein homologues (RHs), rhoptry bulb proteins (rhoptry associated proteins (RAP (RAP-1 and 2) and high molecular weight protein complex (RhopH 1, 2, 3)) and Apical Sushi protein (reviewed (Cowman *et al.*, 2012, Goldberg & Cowman, 2010)). These have all been shown to play a direct or an indirect role during merozoite invasion. During merozoite invasion, the two rhoptries fuse at the rhoptry neck and their content are released (Baum *et al.*, 2005, Baum *et al.*, 2003). A number of studies have shown the role of rhoptry proteins in enabling the merozoite to successfully invade the host RBC. The RON 2 and 4 have been shown to be essential in facilitating the smooth establishment of the tight/moving junction, while members of the RH family have been linked to differentially recognizing host RBC receptors to aid invasion (Giovannini *et al.*, 2011, Richard *et al.*, 2010).

1.4.1.3 Micronemes

The micronemes (**Figure 2A and B**), unlike the rhoptries are small and many in number. They are in close proximity to the duct of the rhoptry (indicated in **Figure 2A**). Typical *Plasmodium falciparum* micronemes are estimated to be ~ 75 x 150 nm (Carruthers & Tomley, 2008). Micronemes, like rhoptries, are thought to originate from the Golgi apparatus through vesicular budding. During invasion, micronemes fuse to the end of the rhoptry neck to facilitate the discharge of its contents (Preiser *et al.*, 2000). These discharged proteins function during invasion to allow the merozoite to select the suitable RBCs, establishing the tight junction upon contact with the RBC (Triglia *et al.*, 2011, Gunalan *et al.*, 2012). Some examples of the proteins resident in the micronemes include apical membrane antigen 1 (AMA1), erythrocyte binding ligands (EBLs; EBA 140, 165, 175, 181, EBL1), those that contain thrombospondin- like binding domains such as merozoite-specific thrombospondin-related anonymous protein (MTRAP), *Plasmodium*

thrombospondin related apical merozoite protein (PTRAMP), surface protein containing an altered thrombospondin repeat domain SPATR (Baum *et al.*, 2006, Cowman *et al.*, 2012) and *P. falciparum* RH5 interacting protein (PfRipr). Contents of the micronemes have been shown to play an important role in the merozoites ability to form the moving junction for which AMA-1 is essential. Members of the EBAs have been linked to the interactions prior to the establishment of the moving junction, thus they are implicated in playing a role in host cell selection.

1.4.1.4 Dense granules

The dense granules (**Figure 2A and B**) are organelles of about 80 nm in diameter as was characterised in *P. knowlesi* (Bannister *et al.*, 1975). They are situated between the nucleus and the rhoptries in a merozoite. It is believed that upon entry into the RBC, the contents of the dense granules are discharged into the parasitophorous vacuole (PV), enabling invagination and the establishment of pores in the PV membrane (PVM) (Culvenor *et al.*, 1991, Bannister *et al.*, 1975). Therefore, the contents of the dense granules are important in the development of the parasite in the RBC, indicating a distinct role to the micronemes and the rhoptries. Dense granules proteins, such as ring infected erythrocyte surface antigen (RESA), have been shown to be secreted into the PV after invasion is completed (Culvenor *et al.*, 1991). Another protein in the dense granules that was identified is a 14 kDa protein which clearly marks the differentiation of invaded merozoites to rings based on its distribution. It is reported to move from sharp punctuated localization in merozoites to a general distribution in the ring stage. Therefore it was called ring membrane antigen (RIMA) (Gardner *et al.*, Trager *et al.*, 1992).

1.4.1.5 Exonemes

Another unique organelle identified recently is the exoneme (Yeoh *et al.*, 2007). The identification of this organelle demonstrates that not only is the merozoite equipped with

specialized organelles for invasion, but also has organelles which harbour proteins necessary for egress. Three subtilisin-like serine proteases (subtilases) genes have been identified to be encoded in *P. falciparum* genome. Of the three, PfSUB3 was identified as being non-essential in *P. falciparum* (Harris *et al.*, 2005b). PfSUB3 expression has been shown not only in asexual stage but also in the gametocyte and sporozoites (Le Roch *et al.*, 2003), while PfSUB2 was related with the shedding of merozoite surface proteins, such as MSP1 (Child *et al.*, 2010), AMA1 (Olivieri *et al.*, 2011) and PTRAMP (Green *et al.*, 2006), during invasion. PfSUB2 has also been shown to be expressed in the gametocyte stage (Florens *et al.*, 2002). A study by Blackman *et al.*, 1998 identified a subtilisin- like serine protease (PfSUB-1), an essential gene (Yeoh *et al.*, 2007), to be maximally expressed during the later stages of schizont development. Contradicting the earlier suggestion of their localisation in the dense granules, Yeoh *et al.*, 2007 showed that these proteases are in another specialised organelle, the exonemes. Furthermore, it has been shown to mediate the maturation of the SERA family of proteins, necessary for egress (Koussis *et al.*, 2009, Arastu-Kapur *et al.*, 2008, Harris *et al.*, 2005b).

1.4.1.6 Mononeme

Invasion-related proteins have been shown to be cleaved by proteases, regulating the different phases of merozoite in the invasion process. These include the family of *P. falciparum* rhomboid proteases (PfROMs). The PfROMs have been described to be distinctly localized to an organelle called mononeme (Singh *et al.*, 2007). The mononeme extends from the anterior end of the merozoite to the posterior, existing singly in each merozoite. Following the release of merozoites, the contents of the mononeme (PfROM1) are trafficked to the surface where they function to cleave micronemes and rhoptry proteins involved in invasion at a position just at the transmembrane region

Figure 2. Three- dimensional (3D) image of the merozoite.

(A) This indicates the different organelles and their organization in the parasite which informs their function. The merozoite surface is covered with a coat on a double membrane. The cytosol houses the nucleus surrounded by the endoplasmic reticulum and Golgi apparatus, the ribosomes. Also prominent is the presence of an apical complex comprising of apical polar rings, micronemes, rhoptries, exonemes, and the dense granules. It also has the sub- pellicular microtubules. (B) The different proteins stored in the different organelles. This shows the merozoite surface coat to anchor the merozoite surface proteins (MSP 1- 10), H101- 103, Pf12, 38, 41, 92, 113, S- antigen, ABRA, GLURP, MSPDBL1, 2, SERA3, 4, 5, 6, MSRP2. The exonemes contain SUB1, while the dense granules house RESA, Exp2, Hsp101, PTEX150. The microneme resident proteins include AMA1, MTRAP, PTRAMP, SPATR, EBA175, 140, 181, EBL1, Ripr. The rhoptry is sub- compartmentalised into the rhoptry tip, which contain PfRh1, 2a, 2b, 4, 5, rhoptry neck containing RON2- 5, ASP and rhoptry bulb which houses the RAP1-3, RAMA, RhoH1-3.(Adapted from Cowman et al., 2012).

1.4.2 Invasion process

The full maturation of schizonts occurs when the individual merozoites have fully budded as individual cells. During this point, the merozoites are ready to be released (egress) to infect new cells (Abkarian et al., 2011, Blackman & Carruthers, 2013). The process of egress is rapid and aided by both parasite and host molecules. It involves the disruption of the parasite plasma membrane, the parasitophorous vacuole membrane and the host surface membrane (Blackman, 2008, Child et al., 2010).

The process of invasion is a complex multi-step sequence of events which occurs within about 21 seconds (Cowman et al., 2012). Successful RBCs invasion by merozoites is essential for the parasite to continue its life cycle. In addition, it is directly linked to patient health as it results in anaemia and many other symptoms associated with the disease (Galinski *et al.*, 1992). Without control, parasite load increases exponentially as infected RBCs burst to release merozoites which invade uninfected RBCs (Autino et al., 2012). A critical aspect of egress is to minimize the opportunity of attack by the immune system (Abkarian et al., 2011) the regulation that leads to the release of the merozoites has been one of the areas currently under extensive study.

Modified from (Cowman et al., 2012)

Figure 3. Host–parasite interactions leading to RBC invasion by merozoite.

Upon egress, the merozoite randomly attaches itself to the surrounding erythrocyte until a suitable one is obtained. The merozoite reorients itself and forms a tight junction with the RBC surface membrane which

commits it to invade. With the help of actin-myosin motor, the parasite is propelled into the erythrocyte followed by the resealing.

Mature merozoites released upon schizont rupture engage in a random interaction with the RBC surface membrane. This is made possible by low-affinity interactions between merozoite surface proteins (MSP1-10) and RBC surface receptors. During this process of primary reversible contact, there are waves of local deformation induced on the RBC (Cowman & Crabb, 2006, Gunalan et al., 2012, Iyer *et al.*, 2007a). Upon identifying the host RBC to invade, the merozoite reorients itself to position its apical end to the RBCs surface. This interaction leads to signalling events, recruiting the appropriate ligands for the formation of a tight junction (Cowman et al., 2012, Duraisingh *et al.*, 2008, Gaur et al., 2004, Hadley, 1986, Tham et al., 2012, Singh *et al.*, 2010).

Key to successful invasion is how efficiently the merozoites are able to form the tight junction. The stages that direct merozoites invasion includes the reversible attachment, tight junction establishment, shedding of merozoite surface proteins, propelling merozoites into the RBC to post-invasion sealing of the parasite within the RBC (Besteiro et al., 2011, Gunalan et al., 2012). The tight junction is an electron dense area (shown by electron microscopy) close to the two cells (the merozoites and the suitable erythrocytes). The tight junction irreversibly commits the merozoite to invade (Baum et al., 2005, Cowman et al., 2012).

Unlike *P. vivax* which is restricted to invading Duffy blood group positive reticulocytes, *P. falciparum* is able to invade all RBCs making use of different receptors. Studies on the restriction by *P. vivax* to erythrocyte invasion led to the discovery of two super-families of proteins which mediate invasion (Tsuboi *et al.*, 1994). These are the *P. vivax* Duffy binding protein (PvDBP) and reticulocyte binding proteins (PvRBP) which are homologous to erythrocyte binding ligands in *P. falciparum* (PfEBLs) and reticulocyte binding homologues (RH) respectively. These have been shown to play key roles in RBCs recognition (Adams *et al.*, 2001, Chitnis & Miller, 1994).

Present at the tight junction is a complex of AMA1 and rhoptry neck proteins (RONs e.g. RON2, 4, 5, 8) which is shown to trigger commitment by merozoite for invasion (Besteiro et al., 2011, Collins & Blackman, 2011, Collins *et al.*, 2009, Giovannini et al., 2011). The full molecular make-up and architecture is not yet known (Besteiro et al., 2011, Duraisingh et al., 2008, Gaur et al., 2004, Gunalan et al., 2012, Hadley, 1986, Tham et al., 2012). Complete entry is completed by resealing at the posterior end of the merozoite (Cowman et al., 2012).

1.4.3 The RBC: Home for *Plasmodium* development

The RBCs are the most abundant cells in the bloodstream. As a key transport tool, it is able to transport oxygen and remove CO₂ to and from tissues. The RBC has an average lifespan of 120 days from the time of differentiation. On average, a typical RBC is 6 to 8 µm in diameter and 2 µm thick, biconcave and spherical in shape. With this, it is extremely flexible enabling passage through small capillaries. Reticulocytes (young RBCs) have been shown to undergo a number of changes as they mature. The receptor density decreases as they mature and they lose about 20% of membrane through vesiculation (Pasini *et al.*, 2010a, Pasini *et al.*, 2010b, Wasserman *et al.*, 1990). This confirms that mechanical changes do occur as the RBC matures, with the membranes of reticulocytes reported to be less stable (Kaestner & Bogdanova, 2014, Wang *et al.*, 2013). These changes are an advantage *Plasmodium* capitalizes in preferentially invading reticulocytes compared to mature normocyte RBC. The cytosol of the RBC consist mainly of haemoglobin while the membrane is well stocked with proteins and protein complexes which are anchored to a networked cytoskeleton (as indicated in **Figure 3A**) (Bogdanova *et al.*, 2013).

The amenability of the cytoskeletal network facilitates the mechanically stable, yet flexible cells. The cytoskeleton is made up of a network of spectrin forming a mesh which is locked up by proteins such as ankyrin, adducin and tropomyosin (Bogdanova et al., 2013). Differential engagements of these have been implicated in regulating different mechanical properties of the cell. Most of the mechanical changes on the RBC have been shown to be

reversible (Shibeshi & Collins, 2005) except in the case of diseased states such as sickle cell anaemia and beta thalassemia (Suwanarusk *et al.*, 2004). A number of proteins have been found to be displayed on the RBC surface, of which some have been the basis for classifying different blood group (ABO, Rhesus, Gerbich, Cromer, Duffy, etc.) (Daniels, 2001). Many of the surface proteins are anchored to the cytoskeleton as a complex or a unit (band 3 complex (4.1RC), ankyrin complex (AC), indicated in **Figure 4A**) (Bogdanova *et al.*, 2013).

Uniquely, Plasmodium has evolved to show preference to some blood types thereby forming the basis for the differential distribution of some species across the globe. *P. vivax* is restricted to Duffy positive blood group while *P. falciparum* is a promiscuous parasite utilising a number of receptors (Lew & Tiffert, 2007, Tiffert & Lew, 2014). Even though promiscuous, the selective usage of receptors by *P. falciparum* has led to different pathways characterised as sialic-acid dependent, sialic acid-independent or the alternative pathway (Triglia *et al.*, 2009) among laboratory parasite strains.

Figure 4. RBC Cytoskeleton and characterised protein complexes.

(A) Components of the cytoskeleton indicating how they are held together by the lipid bi-layer. It indicates the transmembrane proteins; Band 3 dimer, the glycophorins A, B and C, and those that form the spectrin network ($\alpha\beta$ - spectrin filaments). It also indicates the various linker proteins such as adducin, ankyrin, p55, Band 4.1R and Tropomyosin/ actin complex. Adducin binds to the fast growing ends of the actin filament forming a complex with Band 4.1R and held to the membrane at the cytoplasmic tail of glycophorins B creating the Junction complex. When JC interacts with Band 3 anchoring it to the membrane it becomes a multi- protein complex known as 4.1R complex (4.1RC). The involvement of adducin tetramers in docking to band 3 tetramers induces the formation of adducin complex (AC) in the presence of Carbonic anhydrase II (CAII). Upon the activation of NHE1, it complexes with CAII and therefore becomes associated with the adducin complex (AC) forming a super molecular complex. (B) Schematic indicating the different domains of band 4.1R serving as docking hub for band 3, glycophorins C (GPC), p55 and Calcium linked Calmodulin (Ca-CaM). (Image adapted from Bogdanova et al, 2013).

Merozoite invasion is thought to require a certain degree of remodelling (morphological changes) (Lew & Tiffert, 2007) in the RBC to facilitate successful entry and establishment of the infection. However, many of the studies do not delve into the mechanism by which these processes are induced. The signals that aim at remodelling the RBC target either the membrane architecture or the composition of the underlying cytoskeletal proteins complexes are not understood (Bogdanova et al., 2013).

Mechanically, the cytoskeleton imparts great endurance and elasticity of the RBC. The different RBC cytoskeletal complexes are described in **Figure 4A** above. To be able to direct remodelling events to suit a particular purpose, like in the case of merozoite invasion, the alteration of these complexes to induce the different properties conferred on the RBC are required (Iyer et al., 2007a, Lew & Tiffert, 2007). To invade RBCs, merozoites induce rapid localised deformations. The deformation is followed by the insertion of merozoite proteins (AMA1/RON complex) into the RBC membrane. As the merozoite invades, the RBC invaginates leading to the formation of a parasitophorous vacuole and subsequent closure of the RBC membrane post-entry (Bannister et al., 1975). This process requires significant changes to the cytoskeletal make up (Zuccala & Baum, 2011). One process that has been associated with altering membrane properties is an influx of calcium (Ca^{2+}). It has been shown that chelating RBC calcium using EGTA results in annulling the invasion of merozoites, while trophozoite stage parasites were arrested. (Wasserman et al., 1990). This also indicates that any disorder or mutations that affect the shape and mechanics of the RBC will have an effect on merozoite invasion. In addition, cross-linking cytoskeletal protein either by antibodies or chemical agents hindered invasion (Tilley *et al.*, 1990, Dluzewski *et al.*, 1983b).

Another mode of controlling cytoskeletal proteins is through post-translational modification of the cytoskeletal proteins. It has been established that phosphorylation and dephosphorylating events drive engagement or detachment of proteins from the various complexes, thereby altering the mechanical properties of the RBC. One unique example of this is the phosphorylation of band 3. This serves as docking station by which some intracellular complexes can be correctly anchored to the membrane lipid bi-layer (**Figure**

4A) (Berridge *et al.*, 2000, Bogdanova *et al.*, 2013, Lew & Tiffert, 2007). The band 3 protein has been described as a target for many of the serine/threonine, as well as tyrosine kinases. The tyrosine phosphorylation of band 3 protein weakens its affinity of interaction with ankyrin, resulting in a detachment from the spectrin-actin network. This process has been associated with vesiculation as well as lateral movement of the lipid bi-layer (Tiffert & Lew, 2014, Wasserman *et al.*, 1990). Also, phosphorylation of protein 4.1R decreased its ability to complex with the spectrin network and glycophorins C (Wasserman *et al.*, 1999).

Plasmodium infected RBC proteins have been shown to be highly phosphorylated, suggesting that the parasite could activate host tyrosine kinases to compensate for lack of these in the parasite. This process also can be orchestrated by changes in the dynamics of Ca^{2+} in the RBC leading to the activation or deactivation of certain Ca^{2+} sensitive kinases (Cohen and Gascard, 1992). Ca^{2+} functions, as a secondary messenger, either alone or in complex with other proteins. A well characterised interaction between Ca^{2+} and Calmodulin (Ca^{2+} -CaM) (**Figure 4B**) has been implicated in regulating interactions among membrane proteins. For example, upon an increase in RBC intracellular calcium, Ca^{2+} -CaM disrupts band 4.1 protein interactions with the spectrin-actin complex, thereby affecting the membrane stability (Takakuwa & Mohandas, 1988). Also it regulates adducin and ankyrin ability to interact with the spectrin-actin network (Ferru *et al.*, 2011). Kinases such as protein kinase C, transglutaminase, flippase, scramblase, calpain, and channels such as the Ca^{2+} Sensitive K^+ channel and Gardos channel, have been shown to be greatly regulated by the dynamics of Ca^{2+} in the RBC (extensively reviewed by (Bogdanova *et al.*, 2013)). Taken together, RBC deformation and reversible loosening of cytoskeletal protein networks that occur upon passing through blood capillaries are regulated by Ca^{2+} (Muravyov & Tikhomirova, 2013, Muravyov *et al.*, 2010).

During RBC invasion, *Plasmodium* merozoites induce fast, local deformations in the cell subsequently leading to the establishment of high affinity contact, allowing smooth invasion. This leads to efflux and influx of Ca^{2+} (Lew & Tiffert, 2007). Calcium in both the merozoites and RBC has been shown to be essential in the invasion process of merozoites. This was clearly demonstrated by chelating the extra-cellular Ca^{2+} using EGTA. While merozoite intracellular Ca^{2+} is necessary for invasion, extracellular Ca^{2+} is thought to enter

the RBC as the merozoites invade (Lew & Tiffert, 2007). A 100 fold increase in the RBC Ca^{2+} was shown to occur following invasion. Also, a phosphorylation event in beta-spectrin was found to be decrease membrane stability (Manno *et al.*, 1995) while phosphorylation of band 4.1 regulate RBC membrane dynamic properties (Manno *et al.*, 2005). The data presented by both (Wasserman, 1990) and (McCallum-Deighton & Holder, 1992), indicates the need of extracellular Ca^{2+} during invasion. Despite the number of mechanisms being unravelled for the regulation of events as well as protein- protein interactions necessary for invasion, the mechanism by which the parasite is able to initiate and modulate the Ca^{2+} mediated events is still illusive. The differential contribution of the merozoite driven and extracellular Ca^{2+} in the process of invasion are not clear.

Apart from Ca^{2+} mediated invasion events on the RBC, ATP pool regulation has also been shown to have an effect in the invasion efficiency of merozoites. Experiments using RBC ghost membranes supplemented with or without ATP, showed a significant effect of invasion when ATP is present (Rangachari *et al.*, 1986, Tilley *et al.*, 1990, Tiffert & Lew, 2011). This fortified the idea that not only RBC intra- cellular proteins, but also factors such as the nucleotide pool and albumin concentrations had an effect on invasion. It was therefore postulated that the availability of ATP in the host cytosol was necessary in the recognition and subsequent merozoite invasion into RBCs. It was also thought that intracellular ATP regulated phosphorylation events on the cytoskeleton, as cyclic AMP-independent kinase was found to be inseparably associated with the membrane during invasion. Spectrin therefore was considered as a possible substrate to its activity (Dluzewski *et al.*, 1983a, Dluzewski *et al.*, 1983b, Olson & Kilejian, 1982, Rangachari *et al.*, 1986, Tiffert & Lew, 2011). As a key intersection between Ca^{2+} and ATP, it was realised that in the absence of glycolytic substrates, the nucleotide pool was regulated by Ca^{2+} through the interactions of PMCA ATPase, adenylate kinase, and AMP-deaminase enzymes (Tiffert & Lew, 2011).

These all point to a mechanism where the merozoite will have to regulate the dynamics of the RBC cytoskeleton to successfully invade the RBC. Therefore, the process of merozoite invasion is an active tightly controlled process.

1.4.4 Merozoite Invasion Pathways

For successful development of the asexual stage of *Plasmodium spp*, its ability to invade different subsets RBCs as well as be able to evade the immune surveillance is necessary (Adams *et al.*, 1992). The selection of Duffy positive RBCs for invasion by *P. vivax* requires the parasite to be able to distinguish between Duffy positive normocytes from reticulocytes (Horuk *et al.*, 1993, Miller *et al.*, 1976). This implies that the presence of the Duffy antigen on the RBCs is not the only criteria for selection for invasion. This indicates that the parasite is able to utilize alternative pathways in addition to Duffy antigen to effect invasion.

Similarly, *P. falciparum* has the ability to invade all subsets of RBCs but invades reticulocytes better (Mitchell *et al.*, 1986). On the RBC surface, *P. falciparum* has been shown to utilize different receptors, therefore blocking some of the receptors allow the parasite to switch receptor usage during invasion. This is made possible by *P. falciparum* varying the expression of different members of EBAs and RHs enable the use of different receptors (Stubbs *et al.*, 2005). The differential display of these ligands allows *P. falciparum* to cause repeated and chronic infection (Gaur *et al.*, 2006b).

The classification of the possible alternative pathways used in erythrocyte invasion by *P. falciparum* merozoite is based on the nature of erythrocyte receptors involved (Stubbs *et al.*, 2005). Pathways have been defined in the laboratory setting by removing RBC surface receptors with different enzymes. For example, trypsin treated RBCs lack glycophorin A and C, but not glycophorin B and other receptors, chymotrypsin cleaves the different sites of surface proteins including glycophorin B (Gaur *et al.*, 2006b, Gaur *et al.*, 2004). Based on these, the interaction of EBA-175 with glycophorin A has been termed the “Classical invasion pathway” as the receptor is sensitive to neuraminidase and trypsin treatment, but resistant to chymotrypsin (Thompson *et al.*, 2001, Cortes, 2008, Sim *et al.*, 1994, Orlandi *et al.*, 1992). Therefore, all receptors utilized by the parasite in invasion have broadly been classified as either sialic-acid dependent or sialic-acid independent or chymotrypsin

sensitive and resistant (Ogun *et al.*, 2000). Different *P. falciparum* strains have been shown to utilize different pathways. For example, (with the exception of Camp, Dd2 and FCR3) all analyzed lab strains invade neuraminidase treated erythrocytes making them sialic acid independent pathway users (Gaur *et al.*, 2004, Ogun *et al.*, 2000, Sahar *et al.*, 2011). Furthermore, Dolan *et al.*, 1990, showed that parasites are able to switch pathways whereby when Dd2 parasites are grown in neuraminidase treated erythrocytes, the parasite is able to adapt (Dolan *et al.*, 1990).

1.5 Invasion Proteins

The proteins that have been shown to be involved in merozoite invasion recognize erythrocyte surface receptors. Some of the invasion related proteins have been shown to be inserted into the RBC membrane such as the PfRON2/4/5 complex. Others proteins have also been shown to anchor invasion proteins such as the PfRipr/PfCyRPA complex that anchors PfRH5 protein (Dvorin *et al.*, 2010).

As a merozoite is released from a schizont, it engages in weak interactions with the RBCs surface. This is made possible by the merozoite surface proteins which are distributed throughout the surface (not necessarily even) of the merozoites (Gaur *et al.*, 2004). The surface proteins have conserved cysteine-rich domain which mediates adherence. These proteins include ten merozoite surface proteins (MSPs), example MSP1, MSP2, MSP3 etc, the Pf proteins, example Pf38 (Cowman *et al.*, 2012). Of all these, MSP1 is the most abundant surface protein and is thought to be essential for parasite survival. MSP1 has long been considered key vaccine candidates even though its erythrocyte interaction domain can be altered without affecting merozoite invasion (Holder *et al.*, 1992).

Members of the EBLs and RHs specifically interact with receptors on the RBC surface and therefore play an important role in the overall process (Cowman *et al.*, 2012, Gaur *et al.*, 2004). Based on their importance, antibodies against these proteins have the ability to

neutralize merozoites invasion (Gao *et al.*, 2008, Gunalan *et al.*, 2011, Hayton *et al.*, 2008, Jordan *et al.*, 2011, Lobo *et al.*, 2003, Lopaticki *et al.*, 2011, Pandey *et al.*, 2012, Rodriguez *et al.*, 2008, Tham *et al.*, 2009, Triglia *et al.*, 2005).

1.5.1 Erythrocyte Binding-Like (EBL) Proteins

To maintain blood-stage infection and evade the immune system, merozoites must invade rapidly. This depends on the parasite recognizing the suitable RBC and invading (Cowman *et al.*, 2012). Parasite ligands recognizing specific host receptors are crucial for this process. Parasites such as *P. knowlesi* and *P. vivax* recognize single receptor on human reticulocyte surface, that is, Duffy blood group, using their PvDBP (Adams *et al.*, 1990, Chitnis & Miller, 1994). PfEBLs are one of the important families of proteins involved in merozoites invasion. In *P. falciparum*, six members have been studied which includes; erythrocyte binding antigen- 175 (PfEBA-175), PfEBA-140, PfEBA-165 (pseudogene), PfEBA-181, PfEBL-1 and merozoite apical erythrocyte binding ligand (MAEBL) (Adams *et al.*, 2001, Lobo *et al.*, 2003, Mayer *et al.*, 2001, Sim, 1998). Generally, they all have similar multi-exon structures, conserved exon/intron boundaries and amino and carboxyl cysteine-rich domains, also known as Duffy binding like (DBL) domain (Adams *et al.*, 1992, Sim *et al.*, 1994) These have all been localized to the micronemes.

The DBL domains in EBA-175 mediate binding to glycoporphins A by forming a dimer which has been shown to create porches for the binding of the sialic-acid residues. Similar domains in EBA-140 and EBA-181 are thought to mediate receptor binding as well (Duraisingh *et al.*, 2003a, Maier *et al.*, 2009). EBA-140 has been shown to bind to glycoporphin C, of which the sialic acid residues mediate receptor interaction (Lobo *et al.*, 2003). EBL 1 has been shown to bind to glycoporphin B (Mayer *et al.*, 2009, Li *et al.*, 2012).

Members of the EBL family have been shown to be made up of six regions (RI-RVI). Of these regions, region II (RII) and VI (RVI) have been shown to contain conserved cysteine

and aromatic amino acid residues. Region II harbours tandem repeats of the Duffy binding-like (DBL) domains (F1+F2) (Adams et al., 1992). The F1 and F2 sub-domains have been shown to mediate receptor interaction. The cysteines and the aromatic amino acid residues in these domains have been shown to be necessary for the domains function whilst the other amino acids are highly polymorphic (Tolia *et al.*, 2005). In EBA-175, the DBL mediates receptors selection while in EBA-140 and EBA-181 not only does it mediate receptor selection but also the receptor binding specificity (Lopaticki et al., 2011, Baum et al., 2005, Mayer *et al.*, 2002, Gaur et al., 2004). Of the EBLs, MAEBL is the only member that does not contain DBL-like binding domains. MAEBL lack the F1 and F2 sub-domains but have AMA1-like domain known as M1 and M2. The M1 and M2 sub-domains have also been shown to mediate MAEBL receptor binding (Adams et al., 2001, Blair *et al.*, 2002).

Figure 5. Sequence alignment of EBLs.

Sequence alignment of *P. falciparum* and *P. vivax* multi exon structure showing the putative cytoplasmic domain, transmembrane domain, cytoplasmic terminus cysteine -rich domain (c-cys domain), a common tandem duplication of the Duffy binding like domain (DBL) F1 and F2 (region II, in yellow) among all members except EBL1 which has duplication of the MAEBL domains (M1 and M2).

1.5.2 Reticulocyte Binding Like Protein

1.5.2.1 *P. vivax* RH

Reticulocyte binding-like proteins (RBPs) are also key invasion proteins involved in the identification of RBC and the subsequent successful invasion. They were first discovered in *P. vivax* (Ogun & Holder, 1996, Preiser *et al.*, 1999, Adams *et al.*, 1990). As *P. vivax* selectively invades reticulocytes, a merozoite is required to scan through large population of erythrocytes in the blood flow in order to identify a single reticulocyte to invade, as they only make up 1-2% of the circulating red blood cells. This ability is made possible by the *P. vivax* reticulocyte binding protein 1 and 2 (PvRBP 1 and 2) (Galinski *et al.*, 2000, Adams *et al.*, 1992). They have been shown to be associated to each other by a non-covalent interaction, and localized in an apical organelle (Galinski *et al.*, 2000). Following the completion of *P. vivax* genome sequencing, the numbers of PvRBPs have been expanded. The other members are PvRBP1a, 1b, PvRBP2a, 2b, 2c, 2d and PvRBP3. PvRBP2d and PvRBP3 have been described as pseudogenes (Kosaisavee *et al.*, 2012).

1.5.2.2 *P. yoelii* RH

In *P. yoelii*, a family of proteins termed Py235 has been characterized to be involved in parasite virulence and erythrocyte recognition (Ogun & Holder, 1996). This was confirmed as antibodies against these proteins reduced the parasites' ability to invade RBCs (Iyer *et al.*, 2007b, Gruner *et al.*, 2004, Ogun *et al.*, 2000). The Py235 protein was found to be encoded by 14 genes. It has been shown that in a single schizont, the individual merozoites expressed different member of the *py235* genes. The differential expression of the *py235* members has been suggested to facilitate the parasite's ability to evade the host immunity (Preiser *et al.*, 1999, Khan *et al.*, 2001, Gruner *et al.*, 2004, Snounou *et al.*, 2000). This expression pattern of the *py235* genes confers distinct invasion properties to the individual merozoites in a schizont. Work by Ogun and Holder also supports the role of this family in host cell selection (Ogun & Holder, 1996).

1.5.2.3 *P. falciparum* RH

A sequence homology search in *P. falciparum* confirmed the presence of a family which encodes high molecular weight proteins, indicating their possible role in human erythrocyte invasion (Gaur et al., 2004). This family, called the *P. falciparum* reticulocyte binding homologues (PfRHs, in short as RH), have been characterised to have six members as; RH1, 2a, 2b, 3, 4 and 5 (Rayner *et al.*, 2000, Gunalan et al., 2012). RH3 has shown to be a transcribed pseudo- gene (Taylor *et al.*, 2002). RHs have been shown to possess erythrocyte binding and a predicted nucleotide binding regions. Characteristically this family is constituted of proteins which have a large ecto-domain, signal peptide as well as a short cytoplasmic tail making a type-1 transmembrane domain (DeSimone *et al.*, 2009a). The PfRHs have highly acidic cytoplasmic tail ranging from 29- 42% of the amino acids long (Bonifacino & Traub, 2003). Amino acid sequence alignment of all the members of this family, as well as the PY235, identified a conserved motif, KXEXIE (where X is either E or D), except for RH4 (DeSimone et al., 2009a).

Figure 6. RBL family in *P. falciparum*, *P. yoelii* and *P. vivax* and their receptor specificity.

(A) Sequence alignment of PfRH members showed a homology region within the first 500 amino acids (blue shade). Green bars represent homology region based on the alignment of PfRH2a, Py235 and PvRBP2. Nucleotide (ATP/ADP) binding region has been confirmed in PY01365, the dominant member of Py235. Red bars indicate the region of each PfRH member to which antibody has been raised against. These antibodies inhibit parasite invasion *in vitro*. Each member was represented based on the actual size of the protein, as indicated by the scale bar of 250 amino acids (black bar). (B) Receptors for *P. falciparum* reticulocyte-binding protein and their enzyme susceptibility properties. Complement receptor 1 (CR1) and basigin are the erythrocyte receptors for PfRH4 and RH5, respectively. The enzyme sensitivity of RHs to neuraminidase, trypsin and chymotrypsin treated erythrocytes are also listed. S- Sensitive; R- resistance.

RH1, an orthologue of PvRBP 1, is expressed in the merozoites. The RH1 protein has been shown to be located at the apical end of merozoites during invasion. It is present as a single copy gene in laboratory strains of *P. falciparum*, whilst, copy number variation has been reported in different field isolates (Nair *et al.*, 2010). Gene disruption studies have indicated that invasion, as well as the growth rate of the natural parasite population is not affected when the RH1 gene is knocked out (Rayner *et al.*, 2001). RH1 undergoes processing yielding 240 kDa and 120 kDa proteins in solution. Antibodies as well as high-activity binding peptides generated to RH1 protein are able to inhibit merozoites invasion of erythrocytes (Gao *et al.*, 2008). RH1 interacts with a receptor which is sensitive to neuraminidase and chymotrypsin, while resistant to trypsin (Gao *et al.*, 2008, Rayner *et al.*, 2001), suggesting that it is used in the sialic acid dependent pathway.

Evaluating the functions of RH1 during merozoite invasion clearly indicates that antibodies generated against it are able to block invasion and that antibodies targeting different region of the protein have different effects. RH1 has been demonstrated to play a role in merozoite calcium release. A merozoite invasion inhibitory antibody (C41) was shown to block merozoite Ca^{2+} during invasion. Merozoite Ca^{2+} dynamics has been shown to regulate the release of micronemal proteins (such EBA175) leading to the moving junction formation (Singh *et al.*, 2010). In the presence of the C41 antibody, EBA175 surface expression is blocked (Gao *et al.*, 2013), suggesting a sequential release of invasion proteins. Thus, RH1 is thought to be a key signaling molecule necessary for directing the downstream effects leading to tight junction formation (Gunalan *et al.*, 2012).

PfRH2a and RH2b (RH2a/b), the orthologue of *P. vivax* as PvRBP 2 (Rayner *et al.*, 2000) also play a role in merozoites invasion. Both RH2a and RH2b share 8 kb common sequence but are different at the 3' end. They have been predicted to be 370 kDa and 383 kDa weighted proteins respectively (Tham *et al.*, 2012, Rayner *et al.*, 2000). The RH2a and RH2b genes encode for type -1 transmembrane domain proteins. The divergence in these two highly similar proteins occurs at the cytoplasmic region comprising of the C- terminal ecto-domain, the transmembrane region and the cytosolic domain (DeSimone *et al.*, 2009a, Pal-Bhowmick *et al.*, 2012).

Like other members of the PfRHs, PfRH2a and PfRH2b have been identified to have a highly acidic cytoplasmic tails with no apparent canonical protein trafficking or binding motif. These proteins have been shown to be localized to the rhoptry neck and released to the apical end during merozoites invasion (Duraisingh *et al.*, 2003b, Dvorin *et al.*, 2010, Gunalan *et al.*, 2011, Gunalan *et al.*, 2012, Rayner *et al.*, 2000). PfRH2a and PfRH2b expression, in laboratory parasites, has been found to vary from one strain of parasite to the other (Duraisingh *et al.*, 2003b). *P. falciparum* field isolated parasites from Kenya, Senegal and Tanzania, both at the genetic and protein levels, showed expression of these proteins (Jennings *et al.*, 2007, Bei *et al.*, 2007, Nery *et al.*, 2006).

RH2a has been shown to be translocated to the tight junction (Gunalan *et al.*, 2011) and gene disruption studies indicate that both RH2a and 2b are used in sialic acid independent pathways (Reed *et al.*, 2000, Duraisingh *et al.*, 2003b).

RH2a binding has been shown to be neuraminidase (Nm) sensitive, trypsin (T) resistant and chymotrypsin sensitive (Gunalan *et al.*, 2011). A unique observation was made on the processing of RH2a protein. It was shown that the ligand undergo differential processing with the different fragments produced displaying different binding properties to enzyme treated RBC. This phenomenon is proposed to aid in mediating multiple receptor- ligand interaction (Gunalan *et al.*, 2011). This possibly explains an observation in a study of the different pathways used to invade RBC by field isolated parasites in South America (Peru, Colombia and Brazil). It was observed that parasites from Peru and Colombia used an atypical pathway which showed resistance to all enzyme treatment ((Nm-R, T-R and CT-R) with RH2a gene being the dominant expressed gene (Lopez-Perez *et al.*, 2012).

Targeted gene disruption for *RH2b* in *P.falciparum* indicated high invasion in Nm plus T treated and low invasion in T plus CT treated RBCs suggesting sensitivity to CT (Duraisingh *et al.*, 2003b). It was further observed that swapping the cytoplasmic tails of RH2b with RH2a conferred higher activity to RH2a in the 3D7 parasite strain. This

suggested that RH2a and RH2b could be used in different invasion pathways (Dvorin et al., 2010, Duraisingh et al., 2003b). A recent study indicated that RH2b is phosphorylated at the cytoplasmic during late stage of schizont maturation (Engelberg *et al.*, 2013). Furthermore, a study using genetic manipulation to identify and understand the mechanism by which the cytoplasmic tail of RH2b regulates its function in directing pathway usage was conducted. It was identified that, contrary to EBA-175, the cytoplasmic tail of RH2b was functional (DeSimone et al., 2009a). It was observed that the RH2b mutant (without the cytoplasmic tail) was translated and trafficked to the apical complex and did bind to its receptor but signal transduction was abrogated, hence terminating the other events necessary for invasion to occur (DeSimone et al., 2009a). All these findings support the fact that RH2b is highly regulated in its function and may serve as signaling hub to direct trafficking or activating events necessary for merozoite invasion.

PfRH4, a 220 kDa protein, has been shown to be involved in merozoite invasion (Kaneko *et al.*, 2002). RH4 has been observed to vary in expression amongst different isolates (Gomez-Escobar et al., 2010). Being a key invasion protein, antibodies against RH4 have been shown to inhibit merozoites invasion (Tham et al., 2012, Gunalan et al., 2012). RH4 protein interacts with the complement receptor 1 (CR1) to mediate erythrocyte invasion. Thus, antibodies against CR1 have been shown to inhibit merozoites invasion (Tham *et al.*, 2010, Tham et al., 2009). The erythrocyte binding site for RH4 has been identified to be within the N- terminal of the complement control module of CR1. This region harbors the regulatory control site for complement activation (Park *et al.*, 2014).

Acquired human antibodies to *P. falciparum* infection facilitated complement deposition on merozoites, thereby blocking merozoite invasion. This indicates a platform that the parasite could manipulate or will have to escape in order to invade successfully. Also, the ability to fix complement, leading to its deposition, correlated with protection (Boyle *et al.*, 2015). The erythrocyte binding ability of RH4 was shown to be trypsin and chymotrypsin sensitive, as it utilizes sialic acid- independent pathway for erythrocyte invasion (Gaur *et al.*, 2007). Its expression has been associated with switch in merozoite invasion pathway (Stubbs et al., 2005). Gene disruption studies have identified RH4 to be highly expressed in parasite strains

with EBA-175 knocked out (Duraisingh et al., 2003a). Given the relatively high dependence of parasites on RH4, RH4-CR1 is a target for inclusion into a multi- component vaccine and a therapeutic target.

PfRH5, at 63 kDa, is the smallest member of the RH family, as it lacks a transmembrane domain. With the exception of RH5, all the members of the RH family have successfully been knocked-out in parasite strains, showing how important this invasin is to the parasite. As members of this family have been shown to be localized to the rhoptry, RH5 is no exception. It is secreted to the merozoites surface during invasion. (Rodriguez et al., 2008, Cowman et al., 2012). Surprisingly enough, a single amino acid change in the erythrocyte binding domain of this protein prevents receptor binding leading to the usage of an alternate receptor (Hayton et al., 2008, Rodriguez et al., 2008).

RH5 has been shown to form a complex with *P. falciparum* RH5- interacting protein (PfRipr), an epidermal growth factor-like (EGF- like) containing protein, during merozoite invasion. This ligand was shown have a consistent expression pattern with RH5. PfRipr equally was shown to be essential for invasion. Antibodies raised against it inhibited merozoite entry into the RBC (Chen *et al.*, 2011). The RH5-Ripr complex is anchored to the membrane by interacting with a GPI binding protein known as *P. falciparum* cysteine- rich protective antigen (PfCyRPA). Antibodies to PfCyRPA block merozoite invasion (Reddy *et al.*, 2015). Since CyRPA does not bind to the RBC surface directly, the antibodies interferes with its interaction with RH5 or Ripr (Shen *et al.*, 2015, Paul *et al.*, 2015, Weiss *et al.*, 2015, Chen et al., 2011). These findings confirm that RH5/Ripr/CyRPA complex is essential during merozoite invasion.

As a marked vaccine candidate, analysis of possible polymorphisms within the RH5 gene showed 12 non- synonymous single nucleotide polymorphic sites (SNPs). Of the 12, 5 showed a frequency of 10% amongst field isolated parasites indicating a candidate which has less antigenic variation compared to previously identified candidates such as AMA1 (Arevalo-Pinzon *et al.*, 2012, Douglas *et al.*, 2011, Lopez-Perez et al., 2012). Nonetheless,

the polymorphic sites have been shown to mediate host tropism (Wanaguru *et al.*, 2013). Antibodies to RH5 greatly inhibited merozoite invasion and also blocked receptor interactions. Evaluating patient sera for naturally acquired immunity to RH5 showed RH5-specific antibodies to be associated with protection, as well as high density parasitaemia (Patel *et al.*, 2013, Reddy *et al.*, 2014, Shen *et al.*, 2015, Tran *et al.*, 2014, Williams *et al.*, 2012). Immunization of laboratory animals with the full length RH5 induced antibodies which equally blocked merozoites invasion and binding to basigin (RH5 receptor) (Rodriguez *et al.*, 2008, Douglas *et al.*, 2011, Bustamante *et al.*, 2012).

The vaccine potential of RH5 was evaluated in *Aotus* monkeys (an animal model for human disease). The study showed that the monkeys immunized with the protein and challenged with a heterologous and highly virulent parasite strain FVO, the monkeys were protected against disease (Douglas *et al.*, 2015). Regardless of the allelic gene used (for 3D7 or FVO, as these show a four polymorphic amino acid difference), there was cross strain protection. The immunization used Freund's- adjuvant followed by an intravenous challenge. Parasitaemia was reported to be very well controlled compared to other invasion antigens such MSP-1 under the same conditions (Douglas *et al.*, 2015).

Taking advantage of the essentiality of the RH5-basigin interaction, antibodies to Basigin were evaluated for the ability to block merozoite invasion. The result showed that blocking basigin could yield up to 100% invasion blockage. Structural studies aimed at understanding the mode of interaction and the interfaces involved in the process, showed that RH5 assumed a novel folding (Kite- like) which allowed it to interact with basigin (Chen *et al.*, 2014, Drew & Beeson, 2015, Ord *et al.*, 2014). This paved way for the generation of RH5-specific high affinity binding peptides (HABPs) which block merozoite invasion. Amino acid changes in one of the HABPs, was able to induce structural changes in the molecules, thus changing species specific binding of either human and *Aotus* RBCs (Arevalo-Pinzon *et al.*, 2012).

Following the advancement in the therapeutic potential of antibodies over the past decades, a question of whether RH5-Basigin interaction, as essential as it is for merozoite invasion, can be a druggable target was being asked. To address this, humanized antibodies against basigin which showed marked inhibitory effect on an *in vitro* merozoite invasion assay was evaluated for its potential in humanized mouse model, an *in vivo* system. The antibodies were engineered to not recruit undesirable effector functions to the RBC surface. It was clearly shown that the antibodies drastically reduced the parasite population and no recrudescence was observed (Zenonos *et al.*, 2015). Owing to host ligands being less susceptible to antigenic variation and immune pressure, this brings to light a target to focus on in the development of next generation antimalarial therapies (as shown in Figure 7).

Figure 7. Basigin and RH5 complex during merozoite invasion.

Invasion into human erythrocytes by *P. falciparum* merozoites depends on the interactions between erythrocyte surface receptor, basigin and merozoite surface ligand RH5 (left). Antibodies raised against PfRH5 can block merozoite invasion (in the middle) but might be susceptible to polymorphisms and mutations that may arise in the ligand. Antibodies raised against Basigin blocks invasion by merozoites (right).

1.6 *Immunity to invasion ligands*

To further understand the functions of the RH family of proteins, their ability to induce immunity in patients have been shown. Field studies in low transmission communities in Peru (Loreto) showed the presence of antibodies to RH1. Although, the antibodies did not correlate with parasitaemia, it was shown to be high in asymptomatic patients (Villasís *et al.*, 2012). Similarly, antibodies to RH2a were observed in asymptomatic patients in communities in Peru and Indonesia (Sennang *et al.*, 2014, Villasís *et al.*, 2012).

Clinically, antibodies to RH2b have been shown to correlate with protection against symptomatic disease. This was observed in a microarray based study analysing sera collected from Kenya. In the study, antibodies to RH2b were seen to be low in children and gradually increased with the advancement in age (Dent *et al.*, 2015, Crompton *et al.*, 2010). Similar observations were made for sera pooled from Papua New Guinea, where antibodies to the entire length of the protein were found (Reiling *et al.*, 2010).

An antibody evaluation study involving children at different ages in Papua New Guinea showed human antibodies against RH4 were acquired and this correlated with parasitaemia and age. The purified human antibodies against RH4 from patient's sera equally inhibit merozoite invasion (Reiling *et al.*, 2012).

Field studies with individuals living in endemic areas have shown that naturally acquired immunity to RH5 contribute to protection against severity of infection (Guinovart *et al.*, 2012, Tham *et al.*, 2012, Bustamante *et al.*, 2013).

1.7 *Signalling during Merozoites Invasion*

The regulation of merozoites invasion and the proteins involved in the process indicates a regulatory mechanism is in place coordinating every event. One of the mechanisms suggested is an ATP gradient. It has been shown that during merozoites invasion, the RBC intracellular ATP forms a gradient on the surface. This ATP has been shown to be essential for the process of invasion (Olson & Kilejian, 1982). Also, ATP in the RBC has the ability to activate phosphorylation of the RBC cytoskeletal proteins leading to a change in the membrane dynamics (Tiffert & Lew, 2011, Olson & Kilejian, 1982). Importantly, the RH members have been found to have an ATP binding domain (Ramalingam *et al.*, 2008). Though these have not been shown to hydrolyse the ATP, it has been shown that ATP binding to Py235 leads to a conformational change, enhancing erythrocyte binding of the protein (Gruber *et al.*, 2011).

Another key signalling event that has been directly linked to merozoites invasion is the changes in merozoites Ca^{2+} . Ca^{2+} signals have been shown to regulate a number of processes in the eukaryote such as cell division, protein trafficking, transcription and cell motility (Barale *et al.*, 1999, Gaur & Chitnis, 2011). Ca^{2+} has been shown to regulate the development of *P. falciparum*. Also, it was shown that Ca^{2+} is necessary for merozoite invasion (Wasserman *et al.*, 1982, Wasserman *et al.*, 1990). It had been shown that exposing merozoites to the physiological serum level of K^+ drives an increase in the Ca^{2+} in the merozoites. This signal has been shown to drive the release of microneme proteins such as EBA175 and AMA1 (Singh *et al.*, 2010). Recently, it has been evident that merozoite Ca^{2+} is directly or indirectly regulated by RH1, and that this subsequently regulates the release of EBA175 and AMA1 for tight junction formation (Gao *et al.*, 2013). In addition, Ca^{2+} activates kinases such as Ca^{2+} dependent kinases (CDKs) and protein kinase B which activates the phosphorylation or cleavage of proteins in the merozoites during the process of invasion (Chini *et al.*, 2005, Wernimont *et al.*, 2010). In *P. bergeri* and *P. yoelii*, it has been shown that extracellular ATP drives an increase in cytosolic Ca^{2+} and proteolytic processing of MSP1 (Cruz *et al.*, 2012). Taken together, signalling events drives the process of

merozoites invasion. Understanding the key players in the different signalling pathways is necessary in identifying potential targets for drug discovery.

1.8 *Aims for this project*

Malaria continues to be a disease of humanity and of public health importance. Many intervention measures have been instituted in trying to find different ways to reduce the mortalities and morbidities as well as its eradication. Paramount in these intervention strategies is the need to develop vaccine which possibly will reduce the incidence as well as help in the eradication programs against the disease.

Considering the research and the data available in relation to trying to understand the basic science that governs mechanisms of erythrocyte recognition and subsequent invasion, it is established that the family of RH and the EBL proteins play crucial role in this process. *P. falciparum* merozoites express different repertoires of these families of proteins which has been proposed to define the invasion pathways used by the parasite to invade host RBC. PfRH1 has extensively been studied for its role in merozoite invasion as has been reviewed earlier. It is becoming clearer that this protein has multiple domains which help its function being a sialic-acid dependent ligand. The functional role of PfRH 2a and 2b has been studied in relation to merozoites invasion. For the degree of similarities between these two proteins, reagents developed earlier on had targeted both RH2a and 2b conserved regions. With these reagents, it is been shown to undergo differential processing though with antibodies that recognize both RH2a/b. Evident available indicates PfRH2a undergoes differential processing before during and after merozoite invasion using monoclonal antibodies. Also the processed fragments have different susceptibility to enzyme treated RBCs, indicating the possibility of utilizing multiple receptors during invasion. PfRH2b has been studied to be associated with protection against symptomatic malaria disease while the cytoplasmic tails is shown to undergo phosphorylation and also interact with adolase. Thus, we aim to study;

a. the functional role of RH2b plays in the merozoites invasion process,

Much is been studied with PfRH4 and its interaction with Complement receptor 1 and the possibility of use in any multi-component malaria vaccine. PfRH5, as a leading vaccine candidate, has greatly been explored for its role in inducing functional protection against clinical disease in endemic communities. As an essential gene which is less polymorphic, its interaction with Basigin has also been areas of focus in the development of next generation therapeutics which will have the ability block blood stage development of the parasite, hence prevent disease. Therefore, we aim to address the question,

b. what role does PfRH5-Basigin interaction have on merozoite invasion?

2.0 MATERIALS AND METHODS

2.1 *Plasmodium falciparum* parasites and culture

Plasmodium falciparum parasite strains like K1, 3D7, 3D7ΔPfRH2a (RH2a knock out), 3D7ΔPfRH2b (RH2b knock out) T994, T996, W2mef and Dd2 were cultured in fresh erythrocytes from the healthy human donors and RPMI 1640 supplemented with 5% albumax (Trager & Jensen, 1976). Neuraminidase (Sebastian *et al.*) treated erythrocytes used for culturing W2mef parasites for the generation of W2mef/Nm parasite strains. For synchronization, the ring stage parasites were treated with 5% sorbitol, to maintain the developmental stages (Aley *et al.*, 1986). Late schizont stage parasites were purified using 70% Percoll centrifugation as described (Kutner *et al.*, 1985). For tighter synchronization, the purified schizonts stage parasites were allowed to reinvade in fresh erythrocytes. After 5 to 6 h of growth, the parasites were treated with 5% sorbitol, to remove the mature stage parasites.

2.2 *Parasite schizont extract and parasite culture supernatant preparation*

The purified late schizont parasites were washed with incomplete RPMI by centrifugation. The parasite pellet was dissolved in 1 X PBS and stored -20 °C. For western blotting, 2X SDS loading buffer was added to the schizont extract and heated for 5 min at 95 °C.

The parasite culture supernatant was prepared by allowing the percoll-purified schizonts to grow in complete medium in the absence of erythrocytes for 14-16 hrs at 37 °C with constant agitation. After 16 h, the culture was further centrifuged at 35,000 RPM at 4 °C. The supernatant was collected and further centrifuged at 35,000 RPM for 30 min at 4 °C. The supernatant was stored at -80 °C. For western blotting, 6X SDS (sodium dodecyl sulfate) loading buffer was added to the culture supernatants and heated at 95 °C for 5 min whenever there was the need to carry out Western blots.

2.3 *Monoclonal antibody generation*

Hybridoma clones producing monoclonal antibodies specific against RH5 and RH2b were generated by Abmart Inc (China). 13 clones of hybridoma which tested positive by ELISA were obtained for each of the two proteins. Five clones which were validated to specifically recognize native RH5 and RH2b. The hybridoma were grown in media containing RPMI-1640 (Gibco), 20% FBS (Hyclone), 1% glutamine and 30 µg/ml kanamycin. The antibody containing culture supernatants were stored. The purification of the IgG employed 20mM Na₂PO₄ buffered to pH 7.4. The IgG was purified through HiTrap protein G column (GE healthcare) using AKTA FPLC system. Antibodies bound to the column were eluted using glycine at a pH of 2.7. The acid was quickly neutralized Tris base at pH 9.0. Buffer exchange was carried out using PD10 columns according to the manufacturer's protocol.

2.4 *Immunofluorescence Assay*

Thin blood smear containing the late schizont stage of the parasites were prepared and fixed with 100% acetone for 5 min, wrapped in aluminum foil and stored at -20 °C. When required, the slides were taken out and kept at room temperature (RT) for 5-10 min, blocked with 3% BSA in 1X PBS for 30 min, then incubated for 1 hr at 37 °C with primary antibodies: monoclonal antibody (mAbs) anti-PfRH5 clones 7 and 12 (1:200), anti-RH2b A7 (1:200), and rabbit anti RH1(1:500), rabbit anti-PfEBA-175 (1:1000) (MR4) or rabbit anti-PfAMA1 (1:500). The slides were washed for 3 times 5 min with 1X PBS. Then the slides were air-dried, incubated with secondary antibodies: Alexa fluor 488 or 594 goat anti rabbit or goat anti mouse antibodies for 1 h at 37 °C, followed by 3 times 5 min each washing with 1X PBS. The slides were air-dried. Mounting medium containing DAPI (Vectashield) was applied to the slide. Fluorescent images were captured using LSM710 confocal microscopy (Carl Zeiss) and Olympus fluorescent microscopy. The three dimensional (3D) superresolution structured illumination (SR-SIM) images were obtained using the Carl Zeiss Elyra PS.1 with LSM 780 system. The images were processed using the Zen 2012 software and the Z stacks deconvolved. The deconvolved Z stacks were

reconstructed in 3D with interpolation using the Imaris version 7.7.2. The three dimensional (3D) superresolution structured illumination (SR-SIM) images were obtained using the Carl Zeiss Elyra PS.1 with LSM 780 system. The images were processed using the Zen 2012 software and the Z stacks deconvolved. The deconvolved Z stacks were reconstructed in 3D with interpolation using the Imaris version 7.7.2 (Bitplane Scientific). For purposes of clarity of display, the brightness and contrast of the images were adjusted. Co-localization analysis were made using FIJI ImageJ. In evaluating the ability to mAb A7 recognizing PfRH2b ligand, purified merozoites were incubated with 1:500 dilutions of PfRH2b A7 mAbs with complete RPMI and incubated for 1 hour at 37 °C. The merozoites were washed twice using incomplete RPMI. Goat anti-mouse Alexa fluor 488 was diluted in 1: 1000 and incubated for 1 hour at 37 °C. The merozoites were washed twice with incomplete RPMI and fixed using 2% PFA on a glass slide for microcopy using Nikon Eclipse Ti fluorescent microscope. The images were obtained using NIS- element AR 4.20.01 software. Image analysis was carried out using the FIJI ImageJ.

2.5 *Merozoite attachment assay*

Merozoites were arrested in the erythrocyte junction by treating with cytochalasin D (actin polymerization inhibitor). The late stage schizont parasites (3D7, W2mef) were purified by 70% percoll. The bursting schizonts were added to the complete media containing fresh erythrocytes in 96 well plates. Cytochasin D (Sigma) dissolved in dimethyl sulfoxide (Sigma) was added to the final concentration of 0.1µM. After 1-2 h incubation at 37⁰C, thin smears were prepared. Slides were fixed with 100% acetone for 5 min, wrapped in foil and stored at -20⁰C. When required, the slides were allowed to reach the room temperature and IFA was performed

2.6 *Isolation of P. falciparum merozoites.*

Purified *P. falciparum* schizonts were resuspended in warm complete RPMI and allowed to rupture and release merozoites over a period of 2 h at 37°C. Cultures containing unruptured schizonts and released merozoites were centrifuged at 2,200 r.p.m. (942 g) on an Eppendorf 5810R centrifuge for 3 minutes to separate released merozoites from unruptured schizonts and uninfected erythrocytes. Supernatants containing free merozoites were then centrifuged at 5,000 r.p.m. (4,500 g, Sigma 3K15) for 3 minutes to collect merozoites. The merozoites were immediately resuspended in warm complete RPMI medium for further studies.

2.7 *Western Blotting*

Late schizont extracts or culture supernatants in 2X SDS loading buffer was used. Different fractions were separated by running in 6% (for RH1, RH2b and EBA 175) and 12% (for Pf RH5) sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE) and transfer onto nitrocellulose membrane(Bio Rad) for 2 h at 100 V. After the transfer, the membrane was blocked with 5% skimmed milk/PBST (1X PBS/0.5% Tween20) for 1 h and incubated with primary antibodies: mAbs anti-RH5 (5, 7 and 12) (1:500), anti-RH2b (A7) (1:1000), and rabbit ant-EBA175 (1:2000) or anti-actin(1:1000) (Sigma) for overnight at 4 °C. The membrane was washed for 3 times 5 min each with PBST, followed by 1 h incubation with respective horseradish peroxidase (HRP) conjugated secondary antibody at RT. The membrane was washed for 3 times 5 minutes with PBST. Membrane was developed using ECL prime western blotting detection system (GE Healthcare) according to manufacturer's instruction.

2.8 *Erythrocyte binding assay*

The culture supernatants from *P. falciparum* strains (3D7, and W2mef) and erythrocytes (normal, neuraminidase, trypsin, chymotrypsin treated) were used. To 250 µl of parasite culture supernatant 100 µl of packed erythrocytes or enzyme treated erythrocytes were added. To this 50µl of FBS was added and made up to 600 µl with incomplete RPMI, incubated at 37 °C for 2 h with constant rotation. After incubation, the erythrocytes were spun through dibutyl phthalate (Sigma) oil to separate the erythrocytes and the supernatant. The supernatant was removed by aspiration. The proteins bound to the erythrocytes were eluted using 20 µl 0.5M NaCl by incubating at RT for 10 min. Followed by centrifugation at 13,000 rpm for 2 min. The eluted proteins were analyzed by western blotting.

2.9 *Erythrocyte invasion inhibition assay (EIIA)*

Late stage schizonts from W2mef, T994, K1, 3D7, 3D7ΔPfRH2a and 3D7ΔPfRH2b were purified by 70% percoll centrifugation. 30 µl of parasite suspension in complete RPMI was added in quadruplicates in 96 well microtitre plate containing 10 µl of serial dilution from 1 of either anti-RH5 5, 7, 12, RH1 C41 or RH2b A7 monoclonal antibodies depending on the assay. Washed erythrocytes were added to the purified schizonts at 4 % hematocrit with 0.5 % parasitaemia as the starting parasitemia for the assay. The set up was incubated at 37 degree Celsius for 12-16 hours to allow for merozoites invasion. At this time the newly invaded merozoites would have developed into ring stage of the parasite development. The rings in the assay were counted using a FACS machine after they were stained with SYBR-green dyes at 10 nM for 15 minutes. The SYBR-green dyes stains the nuclei making it easy to count using the FITC at 488 nm. The positive stained cells were counted for the presence of rings using FACS LSRII. The control to this assay has been use of isotype control which finally the percentage invasion inhibition is normalized to. % invasion inhibition is calculated as ((set-up without antibodies- set-up with antibodies)/ set-up without antibodies)* 100.

2.10 *Merozoite tight junction Inhibition Assay*

Purified merozoites from 3D7, W2mef and T994 were incubated with RBCs treated with 4 μ M of cytochalasin D in the absence or presence of RH7 or 12 mAbs for 30 minutes at 37°C. After incubation, the cells were washed and the unbound merozoites washed off. As a control purified merozoites were incubated with untreated RBCs. Slides were prepared for all the samples and stained with Giemsa stain. A total of 1000 cells were counted and the percentage those with attached merozoites scored against the cell with no antibodies added. The viability of the merozoites was confirmed upon seeing rings in the untreated RBCs set up.

2.11 *Merozoite EBA175 surface expression was analyzed by Flow Cytometry during the invasion.*

Isolated W2mef merozoites were directly incubated with fresh erythrocytes containing 4 μ M Cyto D in the absence (positive) and presence of mAbs RH1-C41 (0.2mg/ml) and RH2b A7 (0.5mg/ml). Data shown here is a representative. For each experiment about 5,000 DAPI (+) merozoites were captured. The histogram shows the two distinct populations: un-invaded free merozoites (P3 peak with arrow represents Free Mero) and merozoites bound to uninfected erythrocytes (P4 peak with arrow represents RBC bound merozoites (Mero attached to RBCs)). DAPI (+) cells were gated based on size (FSC-A) for all samples. The top right shows the gating hierarchy of the populations. The DAPI (+) population P2 (Cuzin-Ouattara et al.) contains both free merozoites (P3, color peach) and attached merozoites (P4, color pink) of which P5 and P6 (green) represents merozoites positive for both DAPI (+) and EBA175 surface expression (+). Data was presented as number of events (#events) and % of parent population (red circle). The value of % Parent population from different mAbs was compared with that of positive control respectively. The detail of statistical analysis was shown in the bottom Figure.

2.12 *Live video microscopy*

Live imaging of merozoite invasion into RBCs was performed as described (Treeck *et al.*, 2009b, Gao *et al.*, 2013) to analyze the efficacy of the inhibitory antibodies during invasion. To achieve this, we used Axiovert 200M cell observer with a 37°C chamber with continuous supply of 5% CO₂. Late schizont stage parasites in complete RPMI were allowed to settle in 35 mm glass bottom dishes containing well in the presence/absence of inhibitory antibodies RH2b A7, RH1 C41 or RH5(7) at a concentration of 0.5 mg/ml. Live images were captured using Cool SNAP camera and image acquisition were performed using MetaMorph software. Image J software were used for preparing video files.

2.13 *Recombinant RH5 (rRH5) generation and purification*

PfRH5 ecto-domain sequence (aa 26-526) was PCR amplified from 3D7 genomic DNA using primers RH5-Fwd: 5'-ATACCATGGGAAAATGCAATAAAAAAACGAA GAAT CAAG-3', and RH5-Rev, 5'-ATCGAGCTCTCATTGTGTAAAGTGGTTTATTTTTTTTA T AGTTTG -3'. The PCR product encoding full length PfRH5 was digested with NcoI and SacI (New England BioLabs) and inserted into pET9-d1-His6 vector (Gruber *et al.*, 2002). The plasmid was transformed into *E. coli* BL21(DE3) (Stratagene) and cultured in liquid broth (MP Biomedicals) at 37°C and later induced with 0.8 mM isopropyl-β-d-thiogalactopyranoside (IPTG, Sigma) at an optical density at 600 nm (OD₆₀₀) of about 0.6 to 0.8. Cells were harvested by centrifugation at 3,000 ×g after 12 h of induction at 16°C. Cell pellets were lysed by sonication, and rRH5 was found to be slightly soluble though majority was in inclusion bodies. The soluble fraction was purified in 50 mM Tris, 250 mM NaCl (pH 7.5) by using complete His-Tag Purification Resin (Sebastian *et al.*). Further purification was carried out on Superdex 75 Column (GE Healthcare). The purity of rRH5 was verified by separating on a 12% SDS-PAGE and stained with coomassie blue (Bio-Rad). Steady-state CD spectra were measured in far- UV light (194-260 nm) using a CHIRASCAN spectropolarimeter (Applied Photophysics) as described previously (Saw *et al.*, 2013). The spectrum for the buffer was subtracted from the protein. This baseline subtracted spectrum was used for secondary structure prediction on K2D3 web server

(Louis-Jeune *et al.*, 2011). Erythrocyte binding assay was carried out as described earlier using 0, 5, 10, 50 µg/ml of rRH5 and probed using RH5 7.

2.14 *Real-time Ca²⁺ measurement in merozoites during invasion*

Fresh isolated merozoites were preloaded with 6 mM Fluo-4 AM (Invitrogen) in complete RPMI at 37°C for 20 min. Preloaded merozoites were washed twice with warm complete RPMI and then immediately resuspended in fresh RBCs or in the presence of RH5(7) (0.5 mg/ml) and RH1-C41 (0.2 mg/ml), or 10mM Ca²⁺ ionophore A23187 (Calbiochem, USA), or pretreated with 50 mM Ca²⁺ chelator BAPTA-AM (Calbiochem, USA), and cultured in Greiner 96 Black Flat Bottom Polystyrol plates. Changes in cytosolic Ca²⁺ levels during merozoite invasion were timely assessed using a fluorescence plate reader (Infinite M200; Tecan). Fluorescence intensity was measured for 600 s with excitation at 488 nm and emission at 530nm by TECAN-icontrol 1.8.20.0 software. Changes of cytosolic Ca²⁺ levels in the merozoites were timely assessed as described previously(Gao et al., 2013). Data presented were from three biological replicates. Experimental data were shown as the mean±s.e.m. TECAN scanning was performed at 37°C.

2.15 *Real-time Ca²⁺ measurement in RBCs during merozoites invasion*

Fresh RBCs were preloaded with 6 mM Fluo-4 AM (Invitrogen) in complete RPMI at 37°C for 20 min. Preloaded RBCs were washed twice with warm complete RPMI and then immediately resuspended with freshly purified merozoites in the absence (RBC) or presence of RH5(7) (0.15 and 0.4mg/ml, respectively) and cultured in Greiner 96 Black Flat Bottom Polystyrol plates. Changes of cytosolic Ca²⁺ levels inside RBCs during merozoite invasion were timely assessed using a fluorescence plate reader (Infinite M200; Tecan). Fluorescence intensity was measured for 600 s with excitation at 488 nm and emission at 530nm by TECAN-icontrol 1.8.20.0 software. The maximum increase of Ca²⁺ green

fluorescence (F) during a period of 600 s after the start of the stimulation was normalized to the fluorescence intensity before stimulation (F_0). Changes in RBC cytosolic Ca^{2+} levels ($\Delta F(t)$) were expressed as changes in fluorescence intensity as a fraction of the starting fluorescence levels: $\Delta F(t) = (F - F_0)/F_0$ where F = fluorescence intensity(t), F_0 = starting fluorescence signal. The total changes of Ca^{2+} levels ($\sum \Delta F(t)$) in RBC along the 600 s were summed up and plotted against time (s). Data was also analyzed as % decrease ($\sum \Delta F(t)$) = $((F_{max} - F_{RBC}) / F_{max}) \times 100$, where F_{max} represents ($\sum \Delta F(t)$) in RBC at 600 s. Data presented were from three biological replicates. Experimental data were shown as the mean $\pm$ s.e.m. TECAN scanning was performed at 37°C.

2.16 Real-time Ca^{2+} measurement in RBCs

Fresh RBCs were preloaded with 6 mM Fluo-4 AM (Invitrogen) in complete RPMI at 37°C for 20 min. Preloaded RBC were washed twice with warm complete RPMI and then immediately resuspended in the presence of rRH5 at 0.01, 0.06, 0.1 and 0.15 mg/ml respectively and cultured in Greiner 96 Black Flat Bottom Polystyrol plates. A23187 was used as a control. Changes of cytosolic Ca^{2+} levels inside RBCs were timely assessed as described above. Fold increase in RBC cytosolic Ca^{2+} were express as F_{max}/F_{RBC} ; where F_{max} represents ($\sum \Delta F(t)$) in RBC at the 1,200 s. Data presented were from three biological replicates. Experimental data were shown as the mean $\pm$ s.e.m. TECAN scanning was performed at 37°C.

Similar experiments were carried out by using constant rRH5 at 0.2 mg/ml and incubated with RH5(7) at 0.05, 0.15 and 0.25 mg/ml respectively. Changes of cytosolic Ca^{2+} levels inside RBCs were timely assessed as described previously. Data was also analyzed as % Ca^{2+} inhibition = $((F_{max} - F_{RBC}) / F_{max}) \times 100$. Data presented were from three biological replicates. Experimental data were shown as the mean $\pm$ s.e.m.

2.17 *Atomic force microscopy (AFM) analysis of cytoskeletal changes*

Cytoplasmic-surface-exposed samples used for cytoskeletal imaging were prepared as described previously (Shi *et al.*, 2013, Millholland *et al.*, 2011). Briefly, RBCs treated with recombinant RH5 were incubated on the surface of cover slips that were functionalized with Erythro-agglutinating phytohemagglutinin (PHA-E, EY laboratories) prior to incubation. After 3 hours of incubation, cells attached to the coverslips were rinsed with PBS followed by shear washing with excess of 5P8-10 buffer (5 mM Na₂HPO₄/NaH₂PO₄, 10 mM NaCl, pH 8.0). The cytoplasmic-surface-exposed-samples thus obtained were vacuum dried overnight for cytoskeletal imaging and mesh size estimation.

Imaging was performed with a JPK Nanowizard I AFM instrument (JPK Instrument AG, Germany) using super sharp silicon probes (SSS-NCHR probes, Nanosensor, Neuchatel, Switzerland) in intermittent air contact mode. Height images were captured at a resolution of 512 × 512 pixels for 1 µm × 1 µm areas. To quantify the cytoskeletal mesh size, we used Matlab and built skeletonised images from coordinate matrix (withdrawn from JPK data software) and evaluated the average mesh size of each skeletonised image as reported previously (Shi *et al.*, 2013). The AFM as compared to other techniques afforded us the opportunity to evaluate directly the changes in spectrin length as well as mesh volume changes for many membranes even though it requires more time for the sample preparation.

2.18 *Sample preparation, trypsin digestion and phosphopeptide enrichment*

Purified T994 merozoites (Boyle *et al.*, 2010) were incubated with fresh RBCs in the presence of 4 mM Cyto D at 37 °C for 4 hr. The culture was collected by centrifugation at 2,200 rpm (942 g, Eppendorf 5810R) for 3 min and stored at -80 °C. Recombinant RH5-RBC binding was carried out as described above while using non-bound RBC as control. The different treated samples were suspended in 8 M urea/50 mM NH₄HCO₃ with protease inhibitor (Sebastian *et al.*) and phosphatase inhibitor (Sebastian *et al.*) added according to the instructions from the manufacturer, respectively. The suspension was sonicated for 10 s

thrice on ice and centrifuged at 20,000 X g at room temperature (RT) for 20 min. The protein concentration of the supernatant was then determined by the bicinchoninic acid (BCA) assay. Tryptic peptides of MDA-MB-231 cells were prepared as previously described. Phosphopeptides were enriched using titanium dioxide as described (Hao *et al.*, 2011). Phosphopeptides were analyzed using LC-MS/MS as previously described (Thingholm *et al.*, 2006).

2.19 LC MS/MS data analysis

The raw data were processed and searched with MaxQuant 1.3.0.5 with MS tolerance of 6 ppm, and MS/MS tolerance of 0.02 Da. The UniProt human protein database (release 2012_05, 87187 sequences), Uniprot Plasmodium_falciparum_3D7 protein database (release 2014_03, 5542 sequences) and database for proteomics contaminants from MaxQuant were used for database searches. Reversed database searches were used to evaluate false discovery rate (FDR) of peptide and protein identifications. Two missed cleavage sites of trypsin were allowed. Carbamidomethylation (C) was set as a fixed modification. Oxidation (M) and Phospho (STY) were set as variable modifications. The FDR of both peptide identification and protein identification is set to be 1%. The option of “Second peptides” was enabled. Label-free quantification of phosphopeptides between test and control samples was done using MaxQuant (Cox *et al.*, 2011, Cox & Mann, 2008). A paired Student’s t-Test was used to calculate the *p*-value of each comparison. Only phosphopeptides with at least 1.5 time increase ($p \leq 0.05$) and those identified in all replicates of test but not any replicates of control are reported.

Statistical analysis

Statistical comparison was done using one-way ANOVA as appropriate. A *p* value of <0.05 was considered statistically significant. The *p*-value is provided for some individual experiments.

2.20 *Flow-cytometric Method for continuous measurement of RBC intracellular Ca^{2+}*

Fresh RBCs were labelled with 2.5 μM Fluo-4-acetoxymethyl ether (Fluo-4AM, Molecular probes) in 2% dimethylsulfoxide (DMSO, final concentration of 0.0008%) was incubated at 37 C in complete RPMI for 30 minutes. After the incubation, the cells were washed 3 times in 1X PBS by centrifugation at 2000 rpm for 1 minute. For flow cytometry reading, the labelled RBCs were resuspended in complete RPMI. Baseline Ca^{2+} was measured for the RBCs followed by the addition of 2.5 μM A23187 (dissolved in DMSO). The total events were set at 1 million to allow for long time measurement. The experiment was repeated using EGTA, incubation of the labelled RBCs with recombinant RH5 (0.2 mg/ml), recombinant RH1 (0.3 mg/ml) and recombinant STEVOR (0.3 mg/ml). In all the assays, baseline reading was obtained first to ensure consistency. This protocol was adapted from (Vines *et al.*, 2010)

3.0 FUNCTIONAL CHARACTERISATION OF PfRH2b

3.1 *Generation and validation of a PfRH2b specific monoclonal antibodies*

To fully understand the role of PfRH2b during merozoite invasion it is essential to have sensitive and highly specific antibodies. Considering the exceptionally high sequence similarities between RH2a and RH2b at the N-terminal, we raised a panel of monoclonal antibodies (mAbs) against the C-terminal amino acids 2,900 to 3,113 of PfRH2b (**Figure 8A**). Of the antibodies raised, the one that has been used for the characterisation of the RH2b protein is A7.

The ability of the RH2b mAb A7 to recognize the RH2b protein was established by an indirect immunofluorescence assay (Bonifacino & Traub). In IFA staining, the antibody gave a punctuate pattern staining in W2mef schizonts (**Figure 8B**). The RH2b mAb A7 gave punctuated staining pattern at one end of the merozoites in the schizonts. The location of the protein showed co-staining with already characterised merozoite apical proteins such as RH1 (rhoptry neck), AMA1 and EBA175 (microneme proteins). This data confirm an earlier study which showed W2mef parasites to express RH2b protein (Duraisingh et al., 2003a, Triglia et al., 2009). The ability of mAb A7 to identify the RH2b ligand was evaluated in released merozoites (**Figure 9A**). The punctuated staining for RH2b was observed at one end of the merozoite. The mAb A7 staining further showed co-localization with RH1, AMA1 and EBA175. MAb A7 signals co-localized with these apical markers confirming RH2b is at the apical end in free merozoites.

Furthermore, the surface expression of RH2b was evaluated in free non-permeabilized merozoites. To do this, purified merozoites were incubated with mAb A7. The labelled merozoites were washed twice and fixed to a microscope slide for viewing (**Figure 9B**). The results indicated PfRH2b to be localized to the surface of the free merozoites as a punctuated staining. Also the results indicate that ability of the mAb A7 to recognize the ligand on the surface. These all point to PfRH2b being trafficked to the surface of the merozoites most likely at the rhoptry duct where it functions during merozoites invasion.

Figure 8. PfRH2b mAb A7 shows punctuate immuno-staining in both schizonts and merozoites.

(A) The schematic of RH2a and RH2b ligands showing the signal peptide (orange), common region to both RH2a and RH2b (blue rectangle), the variable region to PfRH2a specific region (with circles, blue) and PfRH2b specific region shown in rectangles (orange) and the transmembrane domain (Brown) for both proteins. The region of RH2b used for the antibody generation indicated by the thick bar. (B) The RH2b mAb A7 was evaluated for its ability to recognize the RH2b protein in schizont. The RH2b mAb A7 (green) showed a punctuated pattern of indirect immunofluorescent staining in schizonts. The mAb A7 staining showed co-localization with RH1, AMA1 and EBA175 (all shown in red). DAPI staining shown in blue denotes the nucleus. The co-localization of the all the signals (except the bright field images) indicated as Merge. The bright field images labelled as Bf.

Figure 9. PfrRH2b mAb A7 show punctuated staining in free merozoites.

(A) An IFA was carried out in 3D7 released merozoites. RH2b mAb A7 (red) showed a punctuate staining in free merozoites localized to one end of the merozoite. Co-localization with MSP1, RH1, AMA1, RH2 and EBA175 (all shown in green) showed apical localization of RH2b protein in free merozoites. DAPI staining for nucleus is indicated in blue. The co-localization of all the signals including the bright field images (Bf, in grey) is indicated by Merge. Scale bar indicates 1 μ m. **(B)** Live staining of non- permeabilized W2mef merozoites for PfrRH2b ligand followed by fixation. RH2b staining indicated in green, nuclei staining indicated by blue colour (DAPI staining). Bright field (Bf) images indicated in grey colour. A merge of all the channels is indicated as Merge.

To further confirm the specificity of the RH2b mAb A7 in recognizing the RH2b protein, an IFA was carried out in 3D7, 3D7 Δ RH2a (3D7 RH2a knock out parasites) and 3D7 Δ RH2b (3D7 RH2b knock out parasites). The mAb showed a punctuated pattern of staining of RH2b localized to one end of the merozoite as described **Figures 8 and 9**. In line with apical location, it was observed, RH2b partially co-localizes with RH1, AMA1 and EBA175 whilst, fully co-localizing with RH2 in both 3D7 (**Figure 10A**) and 3D7 Δ RH2a (**Figure 10B**) schizonts. No staining was observed for RH2b in the 3D7 Δ RH2b parasite line (**Figure 10C**).

Figure 10. RH2b mAb A7 is specific in recognizing the protein schizonts.

The specificity of RH2b mAb A7 was evaluated for specificity in recognizing RH2b in 3D7, 3D7ΔPfRH2a and 3D7ΔPfRH2b by indirect immunofluorescence staining. (A) In 3D7 parasites, the RH2b mAb A7 (green) showed a punctuated staining pattern in released merozoites indicating the mAb recognizes the RH2b protein. Co-staining with MSP1, RH1, RH2 (R2A9), EBA175 and AMA1 (known apical markers; red), DAPI for nuclei in 3D7 parasites. Bright field (Bf) images are shown in grey. The co-localization of all the signals including the bright field images (Bf, in gre is indicated by Merge). (B) IFA indicating the RH2b mAb A7 (red) in recognizing RH2b protein in 3D7ΔPfRH2a schizont stage parasites. Co-staining with RH1, RH2 (both RH2a/b), EBA175 and AMA1 (apical markers; red), DAPI (Cuzin-Ouattara et al.) for nuclei (C) IFA indicating the specificity of RH2b mAb A7 not recognizing the RH2b protein in 3D7ΔPfRH2b schizont stage

parasites. Co-staining with RH1, RH2 (both RH2a/b), EBA175 and AMA1 (apical markers; red), DAPI (Cuzin-Ouattara et al.) for nuclei.

To further delineate the proximity of the RH2b protein to the above mentioned apical markers, we imaged the prepared schizont slides using the 3D-SIM platform. The 3D-SIM offers resolution limits (110 to 130 nm) better than that observed from the confocal imaging platform (Schermette *et al.*, 2010, Gustafsson *et al.*, 2008). The RH2b mAb A7 colocalization with AMA1 (**Figure 11A**), EBA175 (**Figure 11B**) and RH1 (**Figure 11C**) was further analysed using this approach. It was observed that, RH2b had higher colocalization with RH1 (60%) compared to AMA1 (30%) and EBA175 (25%) after evaluating ten different schizonts using ImageJ colocalisation analysis tool. Thus RH2b is a rhoptry protein in line with previous studies (Duraisingh et al., 2003b, Triglia et al., 2011). All the data described are consistent with mAb A7 being specific in detecting RH2b, that is, it has no cross reactivity with RH2a shown in **Figure 10C**. RH2b is apically localised in both schizonts and free merozoites. With this evidence, we have shown mAb A7 to be a suitable reagent to study the function of RH2b during merozoite invasion.

Figure 11. A slice from three Dimensional Structured Illumination Microscopy (3D-SIM) imaging of schizont.

3D-SIM imaging of 3D7 schizont immuno-labelled with RH2b mAb A7 (green) the ab indicating the level of co-localization with micronemal proteins AMA1 (A) and EBA175 (B) and rhoptry protein RH1 (C) (All markers shown in red). DAPI (Cuzin-Ouattara et al.) indicates nucleus. Scale bar indicated by heavy white line is 2µm.

Using schizonts extracts from 3D7, 3D7 Δ RH2a and 3D7 Δ RH2b (**Figure 12**), a Western blot using mAb A7 was carried out. Mab A7 recognized the full length RH2b protein of approximately 380 kDa (green arrow) as well as two processed bands of 300 kDa (red arrow) and 250 kDa (black arrow) in 3D7 schizont extracts. Similar bands were recognized in 3D7 Δ RH2a. In contrast to the above observation, no bands were detected by mAb A7 in 3D7 Δ RH2b schizont extracts. In addition to the IFA results, the western blots further confirm the specificity of the mAb A7 in recognizing RH2b protein both native and denatured conditions. Also the western blot shows RH2b to be processed in the schizont stage.

Figure 12. MAb A7 is specific in recognizing RH2b by Western blot.

Western blot evaluation of the ability the monoclonal antibody A7 to recognize the denatured PfRH2b was carried out in 3D7, 3D7 Δ PfRH2a and 3D7 Δ PfRH2b schizont extracts. Protein molecular weight markers (kDa) are indicated on the side with black arrows. The blot was also probed for EBA175. Approximately equal sized bands were obtained for EBA175, indicating the presence of proteins in each of the lanes.

3.2 *PfRH2b is associated with the moving junction during merozoite invasion*

Previous work has shown that RH1 and RH2a associate closely with the moving junction during merozoite invasion of the erythrocyte. To explore whether PfRH2b has a similar location we stained free merozoites at different stages of invasion with mAb A7 and MSP1 or AMA1 or EBA175. RH2b staining imaged with merozoite as well as apical tight junction marker. Co-staining of merozoites with MSP1 showed that free merozoites as well as merozoites that are randomly attached to the RBC have a uniform staining of MSP1 across the surface. Also, PfRH2b was shown to be located as a single or double spot at the apical end (**Figure 13A**). As the merozoite invades the erythrocytes, MSP1 is cleaved and therefore MSP1 is only detected by IFA on the region of the merozoite that has not yet penetrated the RBC. Clearly, proteins that are located in the junction will stain two spots that are at the interphase between the penetrating merozoite and the RBC membrane. Staining with mAb A7 clearly gave two punctuated pattern staining at the merozoite/RBC junction which is consistent with PfRH2b being located at the junction. Using AMA1 as a tight junction marker, rather than MSP1, we showed that in free merozoites, as well as randomly attached merozoites, both AMA1 and RH2b are co-localized at the apical end, although this only occurred partially (**Figure 13B**). Importantly, there is clear co-localization of PfRH2b with AMA1 in the penetrating merozoite, indicated by clear two punctuated staining typical of the tight junction (**Figure 13B**, row 4). This is consistent with PfRH2b being located at the tight junction during merozoite invasion. Similar observations were made with immuno-staining for EBA175. This was shown to co-localize with RH2b staining (**Figure 13C**). RH2b is located at the tight junction. The observations have been summarized in **Figure 13 D** with arrows pointing RH2b localisation during invasion.

Figure 13. PfRH2b is associated with the moving junction during merozoite invasion.

Merozoites were arrested at different stages of invasion into RBC. (A) IFA of merozoites released upon schizont rupture (Burst schizont; row 1). Merozoites were captured at different steps during the invasion process until they are stuck to the RBC. Merozoites were stained for RH2b (red) and MSP1 (green). Nuclei staining with DAPI are shown in blue, while bright field (Bf) images shown in grey. Merozoites randomly interact with the RBC (row 3) until it re-orientates to establish tight junction in order to invade. The junction is indicated with two punctuated stains for RH2b, while MSP1 the part of the merozoite that is not yet into the RBC (row 4). The experiment was repeated using other junction marker as AMA1 (**B**) as well as EBA175 (**C**) whose release is required for junction formation. The different steps typically engaged by merozoites during invasion (**D**) involves random merozoite attachment, re-orientation, tight junction establishment and subsequent invasion (Top-down). For emphasis, merozoites have been marked with a red circle.

To evaluate the proximity between AMA1 and RH2b further, we used the three-dimensional structured illumination microscopy (3D-SIM) imaging platform. The close proximity of RH2b staining with AMA1 at the arrested tight junction showed the RH2b ligand to be at the moving junction (**Figure 14A**). A three dimensional projection of an invading merozoites indicates RH2b has a ring like distribution (**Figure 14B**, red arrow) during merozoite invasion reminiscent of the tight junction (Riglar *et al.*, 2011)

Figure 14. Image of invading merozoite using 3D-SIM.

(A) A slice three-dimensional structural illumination microscopy (3D-SIM) on merozoite at the junction further confirmed the punctuated pattern of PfRH2b (green) to be in close proximity to AMA1 (red). DAPI staining for nucleus indicated in blue. Scale bar indicates 1 μm (B) 3D projection of the localization of PfRH2b during merozoite invasion. PfRH2b formed a ring in the invading merozoite while showing surface distribution in the non-invading merozoite lying close by. Scale bar indicates 0.500 μm . DAPI staining for nucleus indicated in blue

3.3 *mAb A7 inhibits Merozoite invasion before junction formation*

Previous works have shown that antibodies raised to RH1 (Gao et al., 2013, Gao et al., 2008), RH2a (Gunalan et al., 2011), RH4 (Gaur et al., 2007, Tham et al., 2010) and RH5 (Bustamante et al., 2013, Crosnier *et al.*, 2011, Douglas et al., 2011, Williams et al., 2012) inhibit merozoite invasion. To study the invasion inhibitory effect of mAb A7, purified schizonts of 3D7, 3D7 Δ RH2a, 3D7 Δ RH2b and W2mef were incubated with fresh RBCs in the presence or absence of the antibodies (**Figure 15A**). The invasion inhibitory potency of mAb A7 was extrapolated after allowing the merozoites to invade for 16 hours. The mAb A7 inhibited invasion in a dose dependent manner in 3D7, 3D7 Δ RH2a and W2mef. The IC₅₀ for A7 was estimated to be approximately 0.5 mg/ml. In contrast and as was expected no significant inhibition of invasion by mAb A7 was observed for 3D7 Δ RH2b in line with its specificity. It has been shown that inhibition of invasion by monoclonal antibodies against PfRH1 inhibited merozoites before forming the tight junction (Gao et al., 2013). To assess whether this is also the case for antibodies targeting PfRH2a we performed invasion inhibition assays with mAb A7 in the presence of cytochalasin D. Cytochalasin D inhibits merozoite invasion by blocking actin polymerization event, which is necessary in helping propel the parasite during RBC invasion (Srinivasan et al., 2011, Wong *et al.*, 2014). As Cytochalasin D inhibits invasion after junction formation one can rapidly assess whether mAb A7 had an effect on the number of junction arrested merozoites. The data clearly showed that mAb A7 inhibited junction formation in 3D7 parasites, with the inhibitory effect of 27% (**Figure 15B**). In contrast very little inhibitory effect was observed for junction formation in 3D7 Δ RH2b (**Figure 15B**) in line with the specificity of the antibody. Furthermore, in assessing the impact of the antibodies on live merozoite invasion, live video microscopy imaging was carried out using 3D7 purified bursting schizonts. Released merozoites were imaged while invading RBC in the absence (**Figure 15C**) or presence of A7 (**Figure 15D**) to closely monitor the process as previously described for AMA1 (Treeck *et al.*, 2009a) and PfRH1 (Gao et al., 2013). We observed that in the absence of the antibodies, released merozoites rapidly attached and within 20 sec invaded the erythrocytes (**Figure 15C**). The process of invasion ranging from random interaction with the RBC, which is marked by random deformations on the RBC, merozoite induced invagination during entry, echinocytosis of the RBC after entry to the gradual restoration of the RBC to

normal shape occurred within 2 min. Merozoites released in the presence of A7 (**Figure 15D**) were observed to interact with the RBC but were not able to invade. In this Figure many merozoites are seen hovering around the RBC but none was able to invade or establish tight contact with the RBC. The attachment was observed to be short lived (within 2-5 seconds) and then detached. This process was observed for more than 2 minutes clearly indicating the antibodies did block the merozoites from attachment and hence hindered subsequent junction formation. This indicates that the antibodies interrupted a process needed for the recruitment of proteins involved in moving junction formation.

C
Invasion in the absence of mAb A7

D
Invasion in the presence of mAb A7

Figure 15. PfRH2b mAbs A7 inhibits merozoite invasion before junction formation.

(A) Invasion inhibitory effect of mAb A7 was assessed by allowing merozoites from purified schizonts of W2mef, 3D7, 3D7ΔRH2a and 3D7ΔRH2b parasites to invade fresh RBCs. Varied concentration of mAb A7 (a) of up to 2 mg/ml were used, as well as a control (c) (without the mAbs). Invaded cells were counted by FACS. Percentage invasion inhibition was calculated by $\frac{((c-a)/c) \times 100}{}$ and was computed for each of the concentrations. Data represent mean $\pm$ s.e.m (n=3). Merozoite junction inhibition in 3D7 and 3D7ΔRH2b (B) were assessed by incubating merozoite in the presence (s) or absence (control (q)) of mAb A7 (0.5 mg/ml) with cytochalasin D treated erythrocytes. Attached merozoites were counted and the percentage merozoite junction inhibition was calculated as $\frac{((q-s)/q) \times 100}{}$. Experimental results represented as mean $\pm$ std (n=3). Snapshots from time-lapsed live movies shown. These were recorded for merozoite invasion in the absence (C) or presence (D) of mAb A7 to evaluate the live impact on invasion. Starting from schizont rupture in the absence of the antibodies, merozoites invaded RBCs within 20 seconds after they were released from the schizont. The deformed erythrocyte gradually restored shape after 2 minutes. In the presence of A7 merozoites attached to the erythrocytes but were not able to invade after 2 min. Black arrows point to the merozoite of focus.

3.4 *RH2b is processed during invasion*

Most invasion proteins have been shown to be processed during merozoite invasion. RH1, RH2a and RH4 have been shown to undergo different steps of processing before and after invasion (Gao et al., 2013, Gunalan et al., 2011, Tham et al., 2009). RH2b has been shown to undergo processing in different steps using antibodies targeting different regions of RH2a/b common region (DeSimone et al., 2009a, Duraisingh et al., 2003b, Sahar et al., 2011, Triglia et al., 2011). To fully assess the extent of PfRH2b processing in both schizonts and during invasion, extracts were prepared from percoll purified schizonts as well as from parasite supernatant and analysed by Western blot. After probing for RH2b using mAb A7, three distinct bands of approximate sizes; 380, 300 and 250 kDa were consistently detected in the schizont extract. In the parasite supernatant, bands corresponding to those observed in the schizont extract of approximate sizes; 380, 300 and 250 kDa were also consistently detected as well as additional bands around 160 kDa (**Figure 16**). As a unique observation, invasion related protein have been predicted or shown to be cleaved in the transmembrane region by rhomboid proteases. This is believed to regulate the usage of the different invasion proteins in invasion. The cleavage site for RH2a/b had been identified by earlier

works suggesting it occurred towards the N-terminus region (DeSimone et al., 2009a, Duraisingh et al., 2003b, Sahar et al., 2011, Triglia et al., 2011). With these in mind, a model for the sequential processing of RH2b is being proposed here (**Figure 16**). As highlighted in the schematic, the full length RH2b of 384 kDa is processed during schizont maturation to give 300 and 250 kDa bands. During merozoite invasion, an additional processing step occurs to produce ~ 160 kDa bands. These processing are in agreement with an observed ~ 85 kDa bands by Triglia *et al*, 2011. Thus, during invasion, additional bands of ~130 kDa and 220 kDa have been reported to occur (Sahar et al., 2011).

Figure 16. PfrRH2b is processed upon Schizont maturation and during invasion.

The schematic representation of the differential processing of PfrRH2b protein from schizont stage to after merozoite invasion. Upon schizont maturation, the full length protein is processed into ~300- and ~250- kDa proteins (A). During merozoite invasion, a further processing is observed in the parasite yielding a 160 kDa detected after merozoite invasion by mAb A7 in parasite supernatant (A). Processed fragments of 80-, 130-, and 220- kDa (shown in white) were proposed but not detected by A7. The region of the protein which the antibodies were raised against indicated by (A). Transmembrane domains are coloured brown. Rhomboid protease cleavage site is indicated by Protein molecular weight (kDa).

3.5 *mAb A7 inhibits PfRH2b binding to erythrocytes*

To investigate whether the invasion inhibitory effect of mAb A7 was due to its ability to block binding of PfRH2b to its receptor on the RBC, an erythrocyte binding assay was carried out in the presence of increasing concentrations of the A7 (**Figure 17**). In the assay, parasite supernatant was incubated with RBCs without antibodies (NT) as a control for the binding of the different processed fragments. Antibody concentrations of 200 and 500 µg/ml were evaluated. It was observed that in the non- treated lane, the full length RH2b (380 kDa) showed strong binding. The binding was decreased by 60% and 85% for the concentrations of 200- and 500- µg/ml respectively. The 300 kDa processed fragment was observed to decrease with an increasing concentration mAb A7. Also, the 250 kDa fragment showed increased binding at 200 µg/ml of A7, but the binding was restored to similar levels as in the NT at 500 µg/ml. The dynamics of binding observed for the 250 kDa band is unclear, but we believe it could be that the antibody aggregated the protein hence enhancing the binding. Probing for EBA175 on the same membrane confirmed the binding of supernatant proteins (**Figure 17**). This indicates that the C- terminus of the RH2b protein influenced its binding characteristics and the specificity of inhibition by A7.

Figure 17. Effects of mAb A7 on RH2b-receptor interaction.

Effects of mAb A7 (200- and 500- µg/ml) in RBC/ erythrocyte binding assay. W2mef parasite supernatant was bound to RBC in the absence (NT) or presence of antibodies. Three processed fragments of RH2b were observed, 380 (green arrow), 300 (red arrow) and 250 kDa. The membrane was further probed for EBA175 binding as control. We propose that the differential processing allows for receptor interaction and signal transduction. Protein molecular weight (kDa) is indicated on the side with an arrow (Black).

3.6 *RH2b binds to chymotrypsin sensitive receptor*

The erythrocyte binding properties of a number of RHs have been directly studied. Using a genetic approach, disrupting RH2b indirectly showed RH2b utilized a chymotrypsin sensitive receptor during merozoite invasion (Duraisingh et al., 2003b). To evaluate the direct receptor binding properties of RH2b proteins, we carried out erythrocyte binding assays (EBA) using parasite supernatant extract obtained from 3D7. In this assay, RBCs were treated with different concentrations of neuraminidase (Nm), trypsin (T) and chymotrypsin, and incubated with parasite supernatant to assess the binding susceptibility. The blotted membranes were probed for RH2b using mAb A7 and RH2a (Gunalan et al., 2011). The data clearly shows that PfRH2b binds to RBCs (**Figure 18A**). The processed bands of 380, 250 and 160 kDa were detected in the parasite supernatant (SUP) and it was observed that the 380 kDa full length protein showed enriched binding to the erythrocyte. This band was sensitivity to CT; it was drastically reduced by 65% in RBCs pre-treated with 2mg/ml of CT. The binding of the 300 kDa bands, which was not detectable in the SUP, showed some binding to untreated RBCs (UN) while an enrichment of binding was observed in RBCs pre-treated with T or NM. In contrast binding was completely abolished at 2mg/ml CT, indicating sensitivity to CT. The 250 kDa band showed enrichment at 50 U Nm and 2mg/ml T while binding was reduced with increasing concentration of CT from 1 to 2 mg/ml. There was no binding for the 160 kDa processed fragment observed in the SUP as previously described in **Figure 16**. These findings are in agreement with the proposed sensitivity of RH2b binding. For RH2a, the processing of the full length protein releases fragments that have different erythrocyte binding properties. The binding properties observed for RH2a showed that binding of the full length protein (~360 kDa, indicated by the brown arrow) is totally abrogated upon treatment with Trypsin and chymotrypsin, but sensitive to increasing concentrations of neuraminidase (25 U and 50 U). A processed fragment of ~ 270 kDa and 140 kDa appears to bind in a T and CT sensitive fashion (**Figure 18B**) as was described previously by Gunalan et al 2011.

Figure 18. PfrRH2b interacts with chymotrypsin sensitive receptor.

(A) Erythrocyte binding assay was carried out using 3D7 parasite supernatant with untreated RBC (UN), or RBCs treated with neuraminidase (Nm) (25 and 50 U), trypsin (T) (0.1-, 2- mg/ml) or chymotrypsin (CT) (1 and 2 mg/ml). 380, 250 and 160 kDa protein fragments were detected. Upon binding to RBCs, the 300 kDa protein became visible but was highly sensitive to increasing concentrations of CT while the 380 kDa binding was enriched, but was less sensitive to CT. The 250 kDa processed band showed binding but more resistant to CT compared to the 380 and 300 kDa protein bands. The 160 kDa band showed no binding in any of the enzyme treated RBCs. (B) Erythrocyte binding properties of RH2a. The full length (360 kDa, indicated by the brown arrow) protein showed sensitivity to increasing concentrations of Nm but was totally abrogated upon T and CT treatment. The 270 and 140 kDa fragments showed resistance to Nm, but were sensitive to increasing concentration of T and CT. Protein molecular markers (kDa) are indicated on the sides.

Furthermore, we sought to evaluate the differential invasion properties of 3D7 parasites to RBCs treated with different enzymes in the presence of PfRH2b mAb A7 and RH1 C41 (Gao et al., 2013) (**Figure 19**). The data showed that in normal RBCs (untreated), 0.2 mg/ml of RH1 C41 inhibited 20% of invasion while 0.5 mg/ml of mAb A7 resulted in 40% invasion inhibition, indicating a significant difference between the two treatments. This data is consistent with 3D7 parasite being less dependent on PfRH1 mediated invasion pathway. In Nm treated RBC, as expected, RH1 C41 had no effect on invasion (no antibodies (brown bars)). In contrast PfRH2b mAb A7 showed an increased inhibitory effect of 70%, in line with the increased binding of RH2b to Nm-treated RBC. In trypsin treated RBC, the invasion efficiency was decreased by 40% in the control, while the presence of RH1 C41 and A7 showed 50% and 54% decrease, respectively. This suggests that there is an increased accessibility of Nm-sensitive receptors on the RBC that can now be utilized by the PfRH1 ligand. Chymotrypsin treated RBCs decreased merozoite invasion efficiency by 30%, while RH1 C41 and A7 decreased invasion by 60% and 20% respectively. Complementing the effect observed for the trypsin-treated RBCs, the chymotrypsin treatment also enriches the neuraminidase-sensitive receptors; hence the effect of RH1 C41 was high. PfRH2b mAb A7 showed no effect compared to the control. This confirms that PfRH2b engages with a chymotrypsin-sensitive receptor during merozoite invasion.

Figure 19. PfrH2b mAb A7 inhibits merozoites invasion strongly in Nm treated RBCs.

RBCs were treated with neuraminidase (Nm), trypsin (T) and chymotrypsin. 3D7 merozoites invasion of the enzyme treated RBCs were carried out. The percentage (%) inhibition of merozoites invasion was calculated for each of the conditions described above. Brown bars indicate invasion control (without antibodies), green indicates the presence of RH1 C41 mAb (0.2 mg/ml), while Blue indicates the presence of PfrH2b A7 mAb (0.5 mg/ml). Vertical axis indicates the percentage invasion inhibition as normalised with the non-treated RBC control (empty first lane). * $p \leq 0.013$, ** $p \leq 0.00025$. All data presented as mean $\pm$ s.e.m (n=3).

3.7 *mAb A7 inhibits merozoite calcium (Ca²⁺) signalling*

The secretion of calcium has been shown to trigger the release of micronemal protein EBA175 to the merozoite surface facilitating the formation of the moving junction (Singh et al., 2010). Also invasion inhibitory antibodies to PfRH1 were shown to directly affect the release of calcium, thereby blocking merozoite invasion (Gao et al., 2013). To investigate whether the blocking of calcium signalling is a conserved mechanism by which antibodies targeting RH proteins can block merozoite invasion we measured the impact of A7 on the Ca²⁺ within the merozoite during invasion. To study this, purified W2mef merozoites were labelled with Fluo4-AM and incubated with fresh RBCs to evaluate the real time effect of the antibodies on calcium fluxes. The assay was carried out in the absence of antibodies (RBC), presence of Ca²⁺ ionophore A23187 (RBC+A23187), 0.5 mg/ml A7 (RBC+RH2bA7) or 0.2 mg/ml RH1 C41 (RBC+RH1-C41). The effect on calcium dynamics was evaluated in a population cumulative increase over time. We observed that the cumulative intracellular Ca²⁺ released in the merozoites during invasion was significantly increased, as was expected in the presence of Ca²⁺ ionophore A23187 (RBC+A23187). Also during the process of invasion, merozoites intracellular Ca²⁺ signals did increase (RBC). The addition of invasion inhibitory RH2b A7 mAb (RBC+RH2bA7 (0.5mg/ml)) significantly decreased the intracellular Ca²⁺ signals. Similarly to previous observations, we saw a decrease in Ca²⁺ signals in the invading merozoites in the RH1 (RBC+RH1-C41(0.2mg/ml)) treated group (Gao et al., 2013) (**Figure 20**). The overall reduction of Ca²⁺ signals observed in the presence of 0.5 mg/ml mAb A7 was approximately 60%.

Figure 20. PfRH2b A7 mAbs Block merozoites Ca^{2+} increase during invasion.

W2mef merozoites were purified and labelled with Fuo 4- AM. The dynamics of the cytosolic Ca^{2+} levels of invading merozoites were measured using fluorescent plate reader (TECAN) in the absence (RBC) or presence of RH2b A7 (RBC+RH2b A7(0.5mg/ml)) or RH1 C41 (RBC+RH1-C41(0.2mg/ml)). $\Sigma\Delta F(t)$ which denotes the cumulative change in merozoite cytosolic Ca^{2+} was plotted against time (seconds (S)). The total Ca^{2+} in the system was triggered using Ca^{2+} ionophore A23187 (RBC+A23187). The system showed a progressive cumulative Ca^{2+} increase with time (Indicated in red). Upon merozoites invasion, the cumulative Ca^{2+} increase progressively over time. In the presence of RH2b A7 and RH1 C41 mAbs, the cumulative Ca^{2+} decreased significantly. ▲ p value ≤ 0.003729 was determined by one-way ANOVA. Experimental data were reported as mean $\pm$ s.e.m; $n = 3$.

In 3D7 parasites, it was observed that addition of 0.5 mg/ml of mAb A7 reduced the Fluo4-AM signals by 30% (**Figure 21A**). The viability of invading merozoites was confirmed by incubating the free merozoites with fresh RBC and measuring the invasion efficiency. To evaluate the specificity of the RH2b mAb A7 in inhibiting the Ca^{2+} signals, the assay was carried out in 3D7 Δ RH2b (**Figure 21B**) which has the RH2b gene disrupted. As expected, the cumulative Ca^{2+} signal in RBCs invading merozoites (RBC) increased as well as the addition of Ca^{2+} ionophore A23187 (RBC+A23187). Addition of RH2b A7 mAb had no effect on the Ca^{2+} signals in the invading merozoites. These results further buttress the specificity of A7 mAb in detecting RH2b while confirming the participation of this ligand in merozoite Ca^{2+} released as a necessary requirement for merozoite invasion. The degree of inhibition of merozoite invasion and Fluo4 - AM during 3D7 merozoites invasion showed a similar effects of 40 and 30 % (**Figure 21C**). This is indicative of the mechanism by which 3D7 merozoites invaded RBCs, such that blocking the Ca^{2+} during merozoite invasion had a similar effect on inhibiting invasion.

Figure 21. PfRH2b mAb A7 inhibits calcium signalling in merozoites.

Cytosolic calcium (Ca^{2+}) levels in merozoites were measured on a fluorescence plate reader. 3D7 (A) and 3D7 Δ RH2b (B) purified merozoites were preloaded with Fluo-4AM and incubated in the absence (RBC) or presence (RBC + PfRH2b A7 (0.5 mg/ml)) of A7. Calcium ionophore A23187 (RBC + A23187) was used as a positive control. The dynamics of cytosolic calcium during merozoite invasion were recorded over time on a fluorescence plate reader. $\Sigma\Delta F(t)$ which reflects the cumulative change in merozoite cytosolic Ca^{2+} was plotted against time. Experimental data were reported as mean $\pm$ s.e.m; $n=3$. The impact of 0.5 mg/ml of A7 was similar to that of RH1 C41 as these significantly blocked merozoites calcium release in the W2mef parasites. Effects on 3D7 No effects were recorded on the cumulative merozoite Ca^{2+} in 3D7 Δ RH2b parasites. Δp value ≤ 0.00223 were determined by one-way ANOVA. (C) Comparison of the percentage (%) invasion inhibition and the percentage (%) decrease in Fluo-4 AM signals upon the addition of 0.5 mg/ml of PfRH2b mAb A7 in 3D7 parasites.

3.8 Evaluating Merozoite Ca²⁺ using Flow Cytometry

In confirming the effect observed in regulating intracellular Ca²⁺ in invading merozoites, a complementary experimental set up was carried out. In this assay, we employ flow cytometry in measuring the Ca²⁺ levels in RBC invading merozoites in the presence of Cyto D, as well as in the presence or absence of invasion inhibitory RH1 C41 and RH2b A7 mAbs. In this assay, successful invading merozoites will be arrested at the point of tight junction formation, while in the presence of the mAbs; merozoites will either detach from the RBC surface or remain attached but not being able to form any tight junction. Based on this, two populations of merozoites will be observed, those bound to RBC and those that are free. Using DAPI to stain for nuclei, all merozoites were stained, while size gating allowed for the distinction between free merozoites and RBC-bound merozoites (**Figure 22A**, original data). Sorting the different population using DAPI (+) and FITC (+) staining allowed to determine the number of both free and RBC-bound merozoites that also had a Ca²⁺ signal. This showed that on average there were about nine-fold more attached merozoites that had a Ca²⁺ as compared to free merozoites (**Table 1**). This confirms the active involvement of Ca²⁺ in regulating the process of merozoites invasion. It was observed that in the presence of invasion inhibitory antibodies merozoites attachment was inhibited by 41% and 38% for RH1 C41 and RH2b A7 mAbs (**Figure 22B**; red), respectively. The Ca²⁺ signals which were measured with using Fluo4 -AM indicated a signal rate of 19% and 19.5% (**Figure 22B**; red) for RH1 C41 and RH2b A7 mAbs. This shows that, the measurement of Ca²⁺ in the merozoites was devoid of biased signals favouring one condition or the other. The above observations confirm the earlier results, indicating that RH2b is able to regulate merozoite intracellular Ca²⁺ levels during invasion.

A

B

Table 1 Non-attached merozoites with Ca^{2+} signal vs Attached merozoites with Ca^{2+} signals during invasion

Ca^{2+} positive merozoites				
	Non-attached(% Parent)	Attached(% Parent)	Fold	Mean±s.e.m.
Exp1	0.2	3.1	13.4	
Exp2	0.5	2.6	5.1	9.2 ± 2.4
Exp3	0.2	1.8	9.0	

Figure 22. Evaluation of Merozoites Ca²⁺ by Flow Cytometry.

Statistical analysis of the effect of different mAbs on Ca²⁺ signaling during W2mef merozoite invasion by FACS was shown. (A) Representative FACS contour plots were gated by size (FSC-A) vs mean fluorescence of Fluo- 4 AM of W2mef merozoite (DAPI (+)) incubated with erythrocytes in the absence (Positive)/presence of mAbs RH1-C41 (RH1-C41) and RH2b A7 (RH2b A7). Vertical gating is to separate the free merozoites with low FSC from the attached merozoites to RBC with high FSC, whereas horizontal gating is to differentiate intercellular Ca²⁺ levels. Q1 (green) represents free merozoites with Fluo- 4 AM signals. Q2 (green) represents invading merozoites with increasing Fluo- 4 AM signals. The number in Q2 represents % population of attached merozoites. Q3 (peach) represents free merozoites with no signal. Q4 represents attached merozoites with no signal. (B) A bar chart representation indicating PfRH1 C41 mAb and PfRH2b mAb A7 were able to reduce the number of invading merozoites by 40% and 36%, respectively, while the percentage of Fluo4- AM signals were detected to be 18% and 19% for RH1 C41 and RH2b A7 (C), respectively. Data presented in the bar charts represent mean $\pm$ s.e.m, n=3, ns means not significant

3.9 MAb A7 blocks EBA175 surface expression.

To investigate whether the effects the inhibition of Ca²⁺ signalling caused by RH2b mAbs A7, has a downstream effect on the surface expression of EBA175, an assessment of junction arrested merozoites was carried out. The assay was carried in junction arrested W2mef merozoites by flow cytometry. In the assay, purified merozoites were allowed to invade fresh RBCs which had been previously treated with Cyto D in the presence or absence of inhibitory mAbs A7, as well as RH1 C41 for a control (**Figure 23A, B**). For efficient evaluation, labelling of EBA175 expression on the surface of the attached merozoites with anti-EBA175 antibodies allowed classification of different population of cells ranging from free to arrested merozoites. Among these populations, surface expression EBA175 was evaluated. In the assay, ~5,000 DAPI (+) merozoites were analysed. It revealed that, in the absence of inhibitory antibodies, free merozoites constituted 48.8 % of population, with 1% showing positive EBA175 staining. Of the RBC bound merozoites populations, 17% of the 46.3% parent population showed positive EBA175 staining. In the presence of A7 antibodies, free merozoites made up 37.7% of population, which had 1.3% were EBA175 positive, while 10% of 58.7% RBC-bound merozoites were EBA175 positive (**Figure 23A, B**). The data clearly shows that there is an approximately 35% inhibition of

EBA175 surface expression in RBC-bound merozoites in the presence of both RH1 C41 (0.2 mg/ml) and RH2b A7 (0.5 mg/ml), as indicated by the bar chat.

A

B

Figure 23. PfRH2b mAb A7 blocks EBA175 surface expression on merozoites.

Merozoite EBA175 surface expression was analyzed by Flow Cytometry during the invasion. The percentage inhibition of EBA175 surface expression was plotted in a histogram in the absence (positive) or presence of mAbs RH1-C41 (0.2 mg/ml) and RH2b A7 (0.5 mg/ml). For each experiment about 5,000 DAPI (+) merozoites were captured. **(A)** Representative hierarchy for sorting the different populations' of merozoites in the system. This shows the two distinct populations: un-invaded free merozoites (P3 peak) and merozoites bound to uninfected erythrocytes (P4 peak with arrow represents RBC bound merozoites). DAPI (+) cells were gated based on size (FSC-A) for all samples. The top right box shows the gating hierarchy for the different populations. The DAPI (+) population P2 contains both free merozoites (P3, coloured peach) and attached merozoites (P4, coloured pink) of which P5 and P6 (green) represents merozoites positive for both DAPI (+) and EBA175 surface expression (+). Data was presented as number of events (#events) and % of parent population (red circle). **(B)** A histogram showing the percentage (%) of parent population from different mAbs was compared with the positive control respectively. There was no significant statistical (ns) difference RH1-C41 and RH2b A7. The data represented in the bar chart indicates mean $\pm$ s.e.m, n=3, ns means not significant.

3.11 DISCUSSION

Merozoite invasion is a highly complex step involving a number of protein-receptor interactions. The PfRH family of proteins have been shown to play a role in sensing and recognition of the erythrocyte. While the receptor binding characteristics of the various members of the RHs have been characterised, a number of these receptors are yet to be identified. The receptors to RH1, RH2a and RH2b have not yet been identified, even though much progress has been made in characterising the roles of these proteins in RBC invasion by merozoites (Gunalan *et al.*, 2013, Wright & Rayner, 2014). The localization of the members of the RHs to be at the rhoptry and released to the merozoite surface during invasion. Here, we have confirmed that RH2b is apically localised in the merozoite (Wright & Rayner, 2014). Indeed, the high specificity of A7 in recognizing RH2b proteins with no cross reactivity has been established.

Merozoites, as the invasive parasite in the blood stage, have a crucial time limit in invading RBCs in order to keep the life cycle going (Cowman *et al.*, 2012). To achieve this, the parasite initiates different interactions which lead to the establishment of the tight junction, an irreversible commitment to invade. (Weiss *et al.*, 2015, Richard *et al.*, 2010, Cao *et al.*, 2009). RH2b is observed at the tight junction in a ring distributed manner during merozoites invasion. This confirms the observations previously seen using immuno-electron microscopy, which indicated the involvement of PfRH2a/b in the moving junction during the process of merozoite invasion (Triglia *et al.*, 2011).

PfRH2b was observed to bind to RBCs. Of the processing that PfRH2b undergoes during invasion, it was observed that three different fragment of the PfRH2b protein interacted with RBC surface receptors. A 380 kDa protein band, a 300 kDa band and a 250 kDa band were observed to bind to RBC. This observation is consistent with earlier results, in which antibodies raised to PfRH2b specific region recognized 382 kDa as well as 297 kDa processing fragments for PfRH2b (Triglia *et al.*, 2011). The small changes in the estimated molecular weight can thus confirm the identity of these fragments as being consistent. The

additional processed products detected by the antibodies at ~250 kDa and ~160 kDa are complimentary to previously reported 130 kDa and 220 kDa N terminal fragments of RH2b (Triglia et al., 2011, Sahar et al., 2011) making up for the full length protein. The 250 kDa processed fragment harboured the receptor binding region which had been described to be at the N- terminus of the protein (Triglia et al., 2011). The 160 kDa processed fragment, lacked the receptor binding domain, hence showed no binding to RBC, while the 220 kDa fragment bound to RBC (Sahar et al., 2011). These observations intimate the 160 kDa fragment is at the C- terminal region of the RH2b protein. It shows that beyond the receptor binding domain is found a cleavage site. Together with previous studies, we show the direct binding of RH2b protein to a chymotrypsin sensitive RBC surface receptor. Also, we have shown that the N- terminus of RH2b undergo differential processing which, we believe, imparts on the overall function of the protein (DeSimone *et al.*, 2009b, Engelberg et al., 2013).

Based on the processing of RH2a proposed by Gunalan et al, 2011, it was shown that the different processed fragments of RH2a have different receptor binding characteristics to enzyme treated RBCs (Gunalan et al., 2011). Thus, Gunalan et al. 2011 proposed the possibility of the ligand recognizing multiple receptors on the RBC surface. The enzyme sensitive characteristic binding for RH2b- receptor interaction is chymotrypsin sensitive (DeSimone et al., 2009b, Duraisingh et al., 2003b, DeSimone et al., 2009a). With the exception of the initial processed fragments which bound to RBCs surface receptor, the ~ 160 kDa fragments (shown in **Figure 16**) showed no binding to RBCs. This shows the fragment lacked the RBC receptor binding domain.

It was shown that the cytoplasmic tail of RH2b was functional, as it directed downstream events leading to RH2b involvement in merozoite invasion pathway choice (DeSimone et al., 2009a). Also, phosphorylation of the cytoplasmic tail by PfCK2, could direct signal transduction, leading to the trafficking of the protein to the apical end during invasion. Alternatively, the cytoplasmic tail might induce the recruitment of partners necessary for regulating the process of invasion (Engelberg et al., 2013). Overall, the evidence points to signalling events which may include the involvement of RH2b.

The need for Ca^{2+} signals in the secretion of micronemal proteins, such as EBA175 has been established. This leads to the recruitment of proteins, such as AMA1-RON complex, necessary for junction formation (Singh et al., 2010, Singh & Chitnis, 2012, Singh et al., 2007). Supporting this observation, previous report shows that the interaction of RH1 with its receptor on the RBC surface triggers merozoite intracellular Ca^{2+} release, thereby leading to the release of EBA175. Therefore, blocking RH1-receptor interaction interrupted surface expression of EBA175 (Gao et al., 2013). Similarly, blocking RH2b, merozoite Ca^{2+} signalling was during invasion was interrupted as it was further confirmed to affect the trafficking of EBA175 to the merozoite surface. Critically, in this thesis, we have shown that the intracellular Ca^{2+} signals increase in merozoites during invasion is inhibited by the RH2b A7 mAb in a similar fashion as what was previously described for the RH1 C41 mAb. This suggests that members of the RH family have the ability to regulate merozoites Ca^{2+} signal which is essential for invasion.

For successful invasion, the merozoite must engage with RBCs by reversible receptor interactions. Upon active engagement resulting from the re-orientation of its apical end to the membrane, interactions of RH1/RH2b with their cognate receptors triggers an increase in merozoite intracellular Ca^{2+} . This increase facilitates the release of micronemal proteins such as EBA175. The binding of EBA175 leads to the recruitment of proteins necessary for tight junction formation. On the contrary side, with the addition of RH1 or RH2b mAbs, the intracellular Ca^{2+} is abrogated, thereby affecting the surface expression of EBA175 (**Figure 24**). By this mechanism, merozoites will not able to form the tight junction and subsequently will be unable to invade. Our data along with previous published data on RH1 suggests that this might be a conserved function of all the PfRHs in *P. falciparum* and possibly other malaria parasite species. It will now be important to fully investigate how RHs transmit the signal to the merozoite and whether this signalling step might provide a new target for malaria intervention.

Figure 24. Schematic of the effects of RH2b A7 on Ca^{2+} signalling and merozoite invasion.

After the merozoite initially attaches to the erythrocyte (RBC) and reorient itself, RH2b is released from rhoptry neck aiding in the sensing of the RBC by engaging in apical end interaction with the surface of the RBC (a). The interaction between PfrRH2b and its receptor triggers the increase of intracellular Ca^{2+} of the merozoite (b). The increase in cytosolic Ca^{2+} levels lead to a release of microneme proteins such as EBA175 to bind to its receptor glycoprotein A (GPA). This therefore triggers the recruitment of other members leading to moving/ tight junction formation (c). In the presence of RH2b A7 mAb, interactions between RH2b and its receptor is abrogated thereby affecting the intracellular Ca^{2+} increase (d) necessary for the release of EBA175. This hinders recruitment of other proteins needed for merozoite tight junction formation hence No moving junction is formed leading to No invasion.

4.0 RH5-BASIGIN INTERACTION: IMPACT ON RBC CYTOSKELETAL RE-ARRANGEMENT.

PfRH5, is essential for merozoite invasion (Crosnier et al., 2011), and is currently considered as a vaccine candidate. Its interaction with Basigin is also considered as a druggable target (Zenonos et al., 2015). Despite its critical importance, knowledge of the possible regulatory mechanism this interaction has on the entire process of merozoites invasion is lacking. To fully dissect the functional role of RH5 and its interaction with Basigin has on merozoite invasion, we generated a panel of reagents and tools that allowed us to study this process in details.

4.1 *RH5 monoclonal Antibody generation*

Initially we generated range monoclonal antibodies (mAb) against RH5. A total number of 13 clones were obtained proved positive by ELISA in recognizing the peptides used in their generation (Tested by Abmart Inc, China). Of these clones, three hybridoma clones expressing these mAbs were obtained and propagated. The hybridoma clones were cultured and the culture supernatant containing the antibodies was harvested. The IgG in the supernatants were affinity purified as described in section 2.3 using Protein G column. The purified IgG was buffer exchanged and stored in phosphate buffer saline (PBS). The purified mAbs were run under denaturing conditions for the presence of the heavy chain at 50 kDa and the light chain at 25 kDa. The denaturing gel was coomassie blue stained for the band to be visible. As was expected, the mAbs from the different hybridoma clones namely RH5 5, RH5 7 and RH5 12 all showed the 50 kDa band and the 25 kDa bands. No additional bands were observed. This indicated that we had successfully purified the mAbs and it is void of impurities (**Figure 25**).

Figure 25. Coomassie blue stained SDS-PAGE of purified RH5 IgGs.

Purified anti-RH5 IgGs were run under denaturing 12% SDS gel. The gel was stained with coomassie blue to help visualize the protein bands. The gel indicated the heavy chain band at 50 kDa and the light chain band at 25 kDa for RH5 5 (right), RH5 7 (middle) and RH5 12 (left) indicating the purity of the IgGs purified. F(ab)₂ from RH5 7 was generated. As a signature, the F(ab)₂ coomassie stained gel indicated a single band at 50 kDa. No other bands were seen indicating clean F(ab) fragment purification. Protein molecular weight marker (kDa) is indicated on the right by thick black arrows

4.2 Validation of RH5 monoclonal antibodies

RH5 functions basically as an apical protein of the merozoite. Like the other members of the RHs, RH5 is localized in the rhoptry and is trafficked to the surface to engage with basigin during merozoite invasion (Baum *et al.*, 2009b, Hayton *et al.*, 2008). To evaluate the ability of the different mAbs to recognize the RH5 protein, we carried out an indirect immunofluorescent staining for the protein in W2mef schizonts. In confirming the localization of the protein, the IFA was co-stained with other organelle and apical markers such as RH1 for rhoptry neck, EBA175 and AMA1 for the microneme. It was observed that mAb RH5 5 showed punctuate staining pattern in merozoite at the schizont stage of development. The staining pattern localized to one end of the merozoites (**Figure 26A**). The staining for RH5 showed partial co-localization with the apical markers used. It further confirms the apical residence of RH5 in merozoites. Similar observations were made of RH5 7 (**Figure 26B**) and RH5 12 (**Figure 26C**) as they all recognized the ligand to be apically situated as they equally showed co-localization with RH1, while partially co-localizing with EBA175 and AMA1 confirming what had previously been seen (Baum 2009, Rodriguez, 2008).

Figure 26. Validation of RH5 mAb by IFA.

Indirect immunofluorescence assay of a late schizont stage W2mef parasite were stained for RH5 using anti-RH5 5 (**A**), 7 (**B**) and 12 (**C**) (all shown in the green staining). The RH5 labelled schizonts were co-stained with other invasion markers RH1 (Panel I), EBA-175 (Panel II) and AMA1 (Panel III) (all shown with the red colour in the panels above). The bright field images indicated as Bf. Scale bar (white) 2 μ m.

To further characterise the ability of the monoclonal antibodies to recognizing the RH5 protein, Western blot analysis was carried out using W2mef schizont extracts and parasite culture supernatants. The blotted protein was probed using the three PfRH5 mAbs (RH5 5, 7 and 12). A protein band of ~ 63 kDa corresponding to the full-length PfRH5 protein (Stubbs et al., 2005, Gaur *et al.*, 2006a)(Stubbs et al., 2005, Gaur *et al.*, 2006a)(Stubbs et al., 2005, Gaur *et al.*, 2006b) was detected in the schizont extracts (**Figure 27A**) by all the three mAbs. This observation was consistent with what had been shown previously (Rodriguez et al., 2008, Baum *et al.*, 2009a). In the parasite supernatant fractions, the three mAbs recognized the full length RH5 protein. An additional a processed product of ~45 kDa was detected by RH5 7 and 12. This indicates that the RH5 protein undergoes processing during merozoite invasion (**Figure 27B**). The data presented so far indicates good reagents (RH5 5, RH5 7 and RH5 12) that recognize RH5 protein, hence, can be used to dissect the critical role of RH5 during merozoite invasion.

Figure 27. Validation of RH5 monoclonal antibodies by Western blots.

(A) Schizont extracts from W2mef parasites were prepared for Western blots. The samples were run on a 12% SDS-PAGE. The blotted proteins were probed for the RH5 protein using RH5 5, RH5 7 and RH5 12 mAbs. The three mAbs recognized the full length RH5 protein in the schizont extract with no additional bands. (B) Parasite supernatant proteins from W2mef parasites were also blotted and probed with mAbs RH5 5, 7 and 12. The mAbs detected RH5 full length of ~ 63 kDa in the parasite supernatants. Additionally, a processed product of ~ 45 kDa was clearly detected by mAbs RH5 7 and 12 mAb. Protein molecular weight markers (kDa) indicated by arrows.

4.3 *RH5 mAbs inhibits merozoites invasion*

The purified RH5 monoclonal antibodies were tested for their potential to block the merozoite invasion. Late stage W2mef schizonts were purified and allowed to invade RBCs in the absence (control) or presence of PfRH5 5, 7 or 12 mAbs. The parasites were incubated for eighteen hours, after which the parasitaemia for the different conditions were counted by Flow cytometry. The percentage (%) invasion inhibition was computed and normalized with the invasion efficiency of the control set up. It was observed that the three mAbs exhibited a dose dependent inhibitory effect on merozoites invasion (**Figure 28**) The IC₅₀ for the mAbs in blocking merozoites invasion is estimated at 0.12 mg/ml for RH5 5 and RH5 7 and 0.1 mg/ml for RH5 12. The maximum percentage invasion inhibition at 1 mg/ml of the mAbs RH5 5, 7 and 12 were found to be 75%, 84% and 89%, respectively (**Figure 28**), which indicated the saturation (plateau) region for the effects of the antibodies.

Figure 28. PfRH5 mAbs inhibits merozoites invasion.

Invasion inhibition assay was carried out with purified schizont in the absence (control) or presence of RH5 5 (Cuzin-Ouattara et al.), RH5 7 (Red) and RH5 12 mAbs (purple). The percentage invasion inhibition of merozoites invasion was calculated in comparison with the control set up. The various antibodies showed a dose dependent merozoites invasion inhibition with IC₅₀ values of 0.125, 0.120 and 0.1000 mg/ml for RH5 5, RH5 7 and RH5 12, respectively. The invasion inhibitory effects appear to saturate at 1 mg/ml. Data represented indicated mean $\pm$ s.e.m., n=3

With the mAbs being able to inhibit merozoites invasion, we further evaluated their ability to inhibit merozoites invasion in different laboratory strains of *P. falciparum* available to us. Invasion inhibition assays were repeated using 3D7, W2mef, T994 and T996 parasite stains. Purified late schizonts from the different parasites stains were allowed to burst and invade RBCs in the absence (control) or presence of increasing concentrations of RH5 7 mAbs. The merozoite invasion inhibitory effect across the different parasite strains showed a dose dependent effect. The RH5 7 mAbs showed similar IC_{50} of 0.13 mg/ml for all the strains tested. This result indicates that, regardless of the strain used, RH5 protein function retains its essentiality (**Figure 29**).

To further validate whether the invasion inhibition was due to steric interference from the antibodies, we purified the $F(ab)_2$ fragment from RH5 7 mAbs (**Figure 25**). Invasion inhibition assay was carried out using late stage 3D7 purified schizonts in the absence (control) or the presence of RH5 7 (whole antibody) or RH5 7 Fab. It was observed that both RH5 7 (whole antibodies) and $F(ab)_2$ fragments exhibited a dose dependent effect on merozoites invasion (**Figure 29B**). These all showed an IC_{50} value of approximately 0.12 mg/ml in inhibiting merozoites invasion. The inhibitory effect is not due to steric hindrance from the antibodies.

Figure 29. PfrH5 mAbs inhibit merozoites invasion.

(A) Merozoite invasion inhibition in W2mef parasite stain using RH5 7 mAb. In these plots, percentage (%) invasion inhibition is indicated on the vertical axis, while horizontal axis antibody (mg/ml) concentrations used. The invasion inhibition assays were carried out using merozoites from different parasite strains W2mef, 3D7, T996 and T994. (B) Invasion inhibition assay using purified F_{ab} fragments of anti-RH5 7 and intact RH5 7 mAb. A dose dependent inhibition was observed. Data presented as mean $\pm$ s.e.m, n=3, vertical axis- % Invasion inhibition and horizontal- antibody concentrations (mg/ml) (Antibody conc. (mg/ml)).

4.4 *RH5 mAb blocks merozoites attachment to erythrocytes*

Considering the immense inhibitory potentials of RH5 mAbs in merozoite invasion, we evaluated the real time effect of the mAbs. Live video microscopy has emerged as an important imaging technique to study the real time effects of antibodies to protein involved in merozoite invasion (Treeck et al., 2009b). Recently, by this technique, it has been shown that inhibitory antibodies against PfRH1 inhibited merozoites from invading. It was realised that the merozoites did exert deformations on the RBC, yet were not able to invade (Gao et al., 2013). We performed live video microscopy on invading merozoite in the absence (control) or presence of RH5 7 or RH1 C41. From the assay, it was observed that in the control set up, the merozoites were able to invade the RBC within the first twenty seconds. The RBCs echinocytosed and resumed to the original shape after invasion was completed (**Figure 30A**). In the presence of RH5 7 and RH1 C41 mAbs (**Figure 30B** and **C**), the merozoites were not able to invade the RBCs even after two minutes. The merozoites continued exerting efforts to establish tight contact with the RBC but were detached shortly. No physical deformations on the RBCs were observed. These results indicated a possibility of the mAbs blocking merozoites ability to form tight junction with the RBC.

Figure 30. Snapshots of merozoites live invasion.

Live merozoites invasion of RBCs was captured in a video in the absence (**A**) (control) or presence of RH5 7 (**B**) or RH1 C41 (**C**) mAbs. Snapshot images from the time-lapse live video microscopy, showing W2mef merozoites invading under the different conditions. Black arrows point to the merozoites trying to invade erythrocytes post schizont rupture. Time elapsed is indicated in each snapshot in seconds (sec).

To further understand the exact process by which RH5 mAbs block merozoites invasion as observed in the **Figure 30**, we evaluated the ability of the monoclonal antibodies to inhibit merozoite junction formation. The assay was conducted using purified merozoites from 3D7, W2mef and T994 parasite strains. The purified merozoites were incubated with cytochalasin D treated RBCs in the absence or the presence of RH5 7 and RH5 12 mAbs. The ability of the purified merozoites was confirmed by incubating the normal RBCs with the merozoites, for ring stage parasites (**Figure 31A**, control). The merozoites which are able to establish the tight junction with the cytochalasin D treated RBCs were arrested on the membrane (**Figure 31A**, Cyto D treated RBC). Upon comparison of the results obtained without antibodies to that with mAbs, the degree of inhibition of merozoites junction attachment was calculated. It was observed that the two mAbs inhibited merozoites tight junction formation up to 60-70% irrespective of the parasite strain used. The data suggests that the invasion blocking RH5 mAbs possibly act before merozoites tight junction formation.

Figure 31. PfrH5 blocks merozoite tight junction formation.

(A) Representative image of rings stage parasites indicated by the black arrows, and tight junction arrested merozoites indicated by the brown arrows. (B) 3D7, W2mef and T994 purified merozoites were allowed to invade cytochalasin D treated RBC in the absence and presence of RH5 7 and 12 mAbs. Junction arrested merozoites were stained with giemsa and counted microscopically. Percentage (%) tight junction inhibition was computed. Bar chart indicates the percentage inhibition of junction formation in all the three parasite strains. Data indicates mean $\pm$ s.e.m, n=3.

We evaluated if the invasion inhibitory RH5 7 mAbs could block RH5 protein from interacting with its basigin on the RBC surface. To dissect this, erythrocyte-binding assay was carried out using W2mef parasite culture supernatant which tested positive for presence of RH5 protein. The assay was conducted in the absence (0) or presence of varying concentration of RH5 7 mAb (**Figure 32**). Interestingly, it was observed that, full length RH5 protein bound to the receptor on the RBC surface as indicated by the band at ~ 63 kDa. The binding of the RH5 protein showed a dose dependent inhibition in the presence of RH5 7. The binding of the RH5 protein was almost completely abrogated with 300 µg/ml of the antibodies. These observations indicate that, the invasion inhibitory mAbs block merozoites invasion through blocking interactions between the RH5 protein and Basigin on the RBC surface. The data suggest that invasion-blocking antibodies probably act before tight junction formation, by blocking RH5 protein from binding to RBCs and blocking the subsequent steps leading to tight junction formation and finally merozoite invasion.

Figure 32. RH5 7 mAb blocks RH5- Basigin interaction.

Erythrocyte binding assay was performed using parasite culture supernatant in the absence (0) or presence of increasing concentration of RH5 7 monoclonal antibody. This indicates inhibition of RH5 (~60 kDa) binding with increase in mAb concentration. Antibody concentrations are in µg/ml. Protein molecular weights (kDa) are indicated at the right side using the arrows.

4.5 *PfRH5 and merozoite Ca²⁺ signalling*

In *Plasmodium falciparum*, dynamics in Ca²⁺ has been associated with differential phosphorylation of proteins involved in merozoites motility during invasion. Therefore, inhibitors that blocked merozoites Ca²⁺, as well as its interaction with calmodulin, interrupted with invasion (Vaid *et al.*, 2008). In addition, a study indicated that elevation in merozoites cytosolic Ca²⁺ levels lead to the increase surface expression of EBA175 (Singh *et al.*, 2010). It has been shown that invasion inhibitory antibodies against RH1 blocked merozoites calcium signalling, thereby, blocking the secretion of EBA175 to the merozoites surface (Gao *et al.*, 2013). It was also observed that the RH2b inhibitory antibody blocked merozoites invasion by blocking merozoites Ca²⁺, hence blocked EBA175 surface expression. With these evidences, we sought to evaluate the impact of RH5 mAbs on merozoites Ca²⁺ as a requirement for merozoites invasion. In this assay we used both fluorescent plate reader (TECAN) and Flow Cytometry as complimentary methods to establish the dynamics of merozoites Ca²⁺ in the presence of the RH5 inhibitory mAbs.

In the assay involving the plate reader, purified W2mef merozoites were labelled with Fluo - 4 AM. The merozoites were allowed to invade RBCs in the absence (RBC) or presence of RH5 7, RH5 12 or RH1 C41 monoclonal antibodies (**Figure 33**). The total Ca²⁺ signals in the system was triggered using Ca²⁺ ionophore A23187 as a positive control while the addition of Ca²⁺ chelator BAPTA-AM was used as a negative control. The time course of the signal was evaluated cumulatively. The change in fluorescence intensity between the initial reading and the subsequent intensities is computed as $\Delta F(t)$ while the cumulative effect is denoted by $\sum \Delta F(t)$. It was observed that upon merozoites invasion, there was an increase in merozoites Ca²⁺ signals. Whilst, the addition Ca²⁺ chelator BAPTA- AM, the lowest cumulative Ca²⁺ signals were recorded. The addition of RH5 7 and RH5 12 showed not significant inhibitory impact on the merozoites Ca²⁺ signals.

Figure 33. RH5 mAbs do not block merozoites Ca^{2+} signalling.

Cytosolic Ca^{2+} levels of merozoites were measured using fluorescent dye Fluo-4M. Merozoites preloaded with Fluo-4AM were incubated with erythrocytes in the absence (RBC) or presence of RH5 7 (RBC+RH5(7)(0.5mg/ml)) and RH5 12 (RBC+RH5(12)(0.5mg/ml)). The concentration of RH5 mAb used is 0.5 mg/ml each. Preloaded merozoites treated with ionophore A23187 (RBC+A23187) was used as positive control while BAPTA-AM (RBC+BAPTA-AM) as well as RH1 C41 (RBC+RH1-C41(0.2mg/ml)) were used as negative control. The dynamics of cytosolic calcium during merozoite invasion were recorded over time on a fluorescence plate reader. $\Sigma\Delta F(t)$ which reflects the cumulative change in merozoite cytosolic Ca^{2+} was plotted against time. Experimental data were reported as mean $\pm$ s.e.m; n=3. * $p \leq 0.00312$

A complementary evaluation of merozoites Ca^{2+} signals using flow cytometry was also performed. Freshly purified W2mef merozoites labelled with Flou4-AM and allow them to invade fresh RBC in the absence (positive) or presence of RH5 7 or RH1 C41 monoclonal antibodies. On sorting these by FACS, two distinct populations were identified. The populations were those made up of only free merozoites and those that were bound to the RBC. Of these two populations, the free merozoites that showed Ca^{2+} positive staining were gated as Q1 and Ca^{2+} negative merozoites Q3. The attached merozoites with Ca^{2+} signals were gated as Q2 whilst those without the signals gated as Q4. The data showed an 8 fold increase of attached merozoites with a Ca^{2+} signal compared to the free merozoites (**Figure 34A Table 2**). The number of attached merozoites which showed inhibition Ca^{2+} signals upon the addition of RH5 7 was 7% whilst RH1 C41 significantly reduced the signals by 41% (**Figure 34B**). This observation together with that obtained from the plate reader

clearly indicates that, the invasion inhibitory monoclonal antibodies to RH5 protein has little impact on merozoites Ca^{2+} contrary to that observed with monoclonal antibodies to RH1 and RH2b proteins.

Table 2 Free vs Attached merozoites with Ca^{2+} signals

Ca^{2+} (+) cells				
	Free merozoites (% Parent)	Attached merozoites (% Parent)	Fold change	Mean $\pm$ s.e.m.
Expt 1	0.3	3.2	10.9	
Expt 2	0.3	2.3	7.9	7.9 $\pm$ 1.7
Expt 3	0.3	1.7	4.8	

Figure 34. Merozoite Ca²⁺ signaling by FACS.

(A) Representative data shown in FACS contour plots were gated by size (FSC-A) vs mean fluorescence (Mumford *et al.*) of Fluo4-AM of W2mef merozoite (DAPI (+)) incubated with erythrocytes in the absence (Positive)/presence of mAbs RH1-C41 (RH1-C41) and RH5 7. Vertical gating is to separate the free merozoites with low FSC from the attached merozoites to RBC with high FSC, whereas horizontal gating is to differentiate intercellular Ca²⁺ levels. Q1 (green) represents free merozoites with Fluo- 4 AM signals. Q2 (green) represents invading merozoites with increasing Fluo- 4 AM signals. The number in Q2 represents % population of attached merozoites. Q3 (peach) represents free merozoites with no signal. Q4 represents attached merozoites with no signal. (B) Bar chart representing the percentage reduction of Ca²⁺ of attached merozoites upon the addition of RH1 C41 and RH5 7. Experimental data were reported as mean $\pm$ s.e.m; n=3. *p $\leq$ 0.00623

To further understand the sequence of events leading to merozoites invasion and Ca²⁺ signalling, the assay was carried out in the presence of R1 (RBC+R1(125 μ g/ml) peptide and EBA175 R217 mAb (RBC+EBA175-R217(0.5mg/ml), while using RH1 C41 as a control. R1 peptide which is known to interrupt the AMA1 and RON complex needed for the establishment of tight junction was evaluated for its ability to block merozoites invasion. The R1 peptide showed concentration dependent inhibition of merozoite invasion (**Figure 35A**) consistent with previous reports (Lee *et al.*, 2011, Harris *et al.*, 2005a). With this, we evaluated the effects of both R1 peptide and EBA175 R217 for their ability to interrupt merozoite Ca²⁺ signals (**Figure 35B**). Calcium ionophore A23187 (RBC+A23187) showed a cumulative increase in the Ca²⁺ in the system as did invasion into RBCs (RBC). From the data, it was observed that both R1 and EBA175 R217 had no significant effect on the cumulative merozoites Ca²⁺ signals during invasion, whilst, RH1 C41 significantly inhibited merozoites Ca²⁺ signals. This observation supports a sequence of events leading to the release of both microneme and rhoptry proteins during invasion. The effect of the RH5 mAbs suggest it could act after merozoite Ca²⁺ increase but before EBA175 is trafficked to the surface or alternatively acts after EBA175 is released on the surface but before the tight junction is formed. This is possible as PfRipr is micronemal and is required to anchor the RH5 protein for function.

Figure 35. R1 and EBA175 do not affect merozoites Ca^{2+} signals.

(A) Merozoite invasion assay were conducted with increasing concentrations of R1 peptide (7.81, 31.25, 125, 500 $\mu\text{g/ml}$). Data presented as mean $\pm$ s.e.m, $n=3$ (B) Cytosolic Ca^{2+} levels of merozoites were measured using fluorescent dye Fluo-4M. Merozoites preloaded with Fluo-4AM were incubated with erythrocytes in the absence (RBC) or presence of R1 (RBC+R1(125 $\mu\text{g/ml}$)), EBA175-R217 mAb (RBC+EBA175-R217(0.5mg/ml)) and RH5 7 (RBC+RH5(12)(0.5mg/ml)). Preloaded merozoites treated with ionophore A23157 (RBC+A23187) was used as positive control while RH1 C41 mAb (RBC+RH1-C41(0.2mg/ml)) were used as negative control. The dynamics of cytosolic calcium during merozoite invasion were recorded over time on a fluorescence plate reader. $\Sigma\Delta F(t)$ which reflects the cumulative change in merozoite cytosolic Ca^{2+} was plotted against time. Experimental data were reported as mean $\pm$ s.e.m; $n=3$. * $p \leq 0.00423$

4.6 *Effects of RH5 mAbs on EBA175 surface expression*

A test of whether RH5 mAbs block EBA175 surface expression was studied as described by Gao et al. In this assay we employed both the fluorescence plate reader (TECAN) as well as the Flow cytometry (FACS) to evaluate the surface expression of EBA175 in the presence of RH5 mAbs. In the fluorescence plate reader assay, W2mef purified merozoites were incubated with cytochalasin D treated RBCs in the absence or presence of RH5 5 and 7 monoclonal antibodies. The cells were washed twice in incomplete RPMI and probed for the surface expression of EBA175 using EBA175 R217 mAbs and detected using Alexa Fluor 488 secondary antibodies. The results were read on a plate reader for the surface labelled EBA175. The fluorescence signals for the control (absence of antibodies) compared with the antibody treated set ups indicated that both RH5 5 and 7 had no impact on the surface expression of EBA175 (**Figure 36**).

Figure 36. RH5 does not affect EBA175 surface expression.

The ability of RH5 mAbs to block EBA175 surface expression was evaluated using TECAN. RH5 5 and RH5 7 mAbs at 0.5 mg/ml were evaluated for their ability to block merozoite EBA175 surface expression during invasion. Percentage (%) inhibition of EBA175 surface expression was plotted for each of the antibodies. Data presented as mean $\pm$ s.e.m.

To further validate this observation, the assay was repeated and the EBA175 positive cells were sorted by flow cytometry (FACS). The purified merozoites were allowed to invade cytochalasin D treated RBCs in the absence or presence of RH5 7 whilst RH1 C41 mAbs was used as a positive control (Gao et al., 2013). The arrested merozoites on the RBC membrane were stained for EBA175 surface expression by incubating the cell with EBA175 mAbs. These cells were detected by incubating with Alexa 488 secondary antibodies whilst DAPI was used to detect all the merozoites that were bound to the RBCs. Of the number of events recorded (P1), 5,000 DAPI positive cells were recorded as P2. Of the DAPI positive cell population, two distinct cell groups were identified consisting of free merozoites (P3) and merozoites-bound RBCs (P4). The free merozoites (P3) as well as the merozoites-bound RBCs (P4) were further sorted based on EBA175 positive expression as P5 and P6, respectively (**Figure 37A**). It was observed that compared to the control cells, RH1 C41 did block significantly the surface expression of EBA175 by 35% compared to RH5 7 of 7.5%. Generally, it was observed that attached merozoites showed up to 19 fold increase in EBA175 staining compared to the free merozoites. This indicated that EBA175 trafficking to the merozoites surface is enhanced when merozoites are attached to RBCs. Clearly, these observations indicate that RH5 functions after EBA175 surface expression but before merozoites tight junction formation.

Figure 37. EBA175 surface expression analysis by FACS

(A) Representative hierarchical plots showing the different gating and the populations of cells counted for the control set up, RH1 C41 and RH5 7 treatments. (B) Bar chart indicating the percentage inhibition of EBA175 surface expression by RH1 C41 and RH5 7. Experimental data were presented as the mean $\pm$ s.e.m.; $n = 3$. * $p \leq 0.0044$ by one-way ANOVA indicates the significant difference of the effect on EBA175 surface expression between RH1 mAbs RH1-C41 and RH5 mAbs RH5 7.

4.7 *PfRH5 mAbs block Ca²⁺ signals into RBCs*

In understanding the dynamic in RBC Ca²⁺ during merozoites invasion, we carried out an assay measuring the changes that occur during merozoite invasion. In this assay, fresh RBCs were labelled with Fluo-4 AM and the changes in Ca²⁺ that occurred monitored by both by TECAN (fluorescence plate reader) as described by (Gao et al., 2013) and observed for 600 seconds (s). It was observed that during merozoites invasion, the cumulative Ca²⁺ signals ($\sum\Delta F(t)$) in the RBC (RBC) increased. It was further ascertained that, the addition of different concentrations of mAb RH5 7 (RBC+RH5 7 (0.15 mg/ml), red) and (RBC+RH5 7 (0.25 mg/ml), blue), the signals significantly reduced (**Figure 38A**). This indicated that blocking RH5 interaction with Basigin affected RBC Ca²⁺ signals. To further validate this observation, the assay was repeated in the presence of R1 (RBC+R1 (125ug/ml)) peptide, RH1-C41 (RBC+RH1-C41(0.2mg/ml)), RH5(7) (RBC+RH5(7) (0.5mg/ml)), EBA175-R217 (RBC+EBA175-R217 (0.5mg/ml)) mAbs. The assays were carried out for 600 seconds and the effects of the peptide as well as the mAbs evaluated (**Figure 38B**). As expected, invasion by merozoites into the RBC triggered a cumulative increase in RBC Ca²⁺ (RBC). It was observed that interrupting AMA1-RON4 complex interaction did not affect the cumulative Ca²⁺ increase in the RBC. The addition of anti-RH1 and anti-EBA175 had no significant effect on the cumulative RBC Ca²⁺ signals in the RBC, whilst RH5 7 significantly blocked the Ca²⁺ signal in the RBC. This observation, contrary to the effects observed for RH1 and RH2b, indicates an additional function of RH5- Basigin interaction as affecting RBC Ca²⁺ during merozoite invasion. From the data, the inability for R1 to block the signal indicates that RBC Ca²⁺ increase precedes the establishment of the tight junction. This brings to light a sequential order of interaction.

Figure 38. RH5 7 mAb blocks RBC Ca^{2+} signalling.

(A) Preloaded fresh RBC with Fluo-4AM were incubated with merozoites in the absence (RBC; green) or presence of anti-RH5 (RBC+RH5(7)(0.15 mg/ml; red) and (RBC+RH5(7)(0.4 mg/ml; blue)). Experimental data presented as mean $\pm$ s.e.m, $n=3$, $*p \leq 0.0153$, $**p \leq 0.002491$. (B) Preloaded fresh RBCs with Fluo-4AM were incubated with merozoites in the absence (RBC), or presence of RH1-C41 (RBC+RH1-C41(0.2mg/ml)), RH5 7 (RBC+RH5(7)(0.5mg/ml)), EBA175-R217 (RBC+EBA175-R217(0.5mg/ml)) and R1 peptide (RBC+R1(125 μ g/ml)), respectively). $*p \leq 0.022712$ indicates the difference on cumulative Ca^{2+} signal at time of 600 s of RBCs in the presence of RH5 7 compared to that in the absence of RH5 7.

4.8 *Recombinant PfRH5 (rRH5) binding increases RBC Ca²⁺ signals*

The data shown in section 4.7 indicates the possibility of RH5-Basigin interaction having effect on RBC Ca²⁺ signalling during merozoite invasion. Furthermore, it was seen that RH5 7 mAbs block RBC Ca²⁺ signals during merozoite invasion. The ultimate question is, could RH5 binding to basigin be a trigger for the RBC Ca²⁺ signalling during merozoite invasion? To address this question, we generated recombinant RH5 protein (**Figure 39A**) comprising of amino acid 26 to 526. The protein was verified for proper folding. To do this, we made use of the circular dichroism scanner to evaluate the secondary structure of the protein. The secondary structure was determined using 4.0 mg/ml of the purified protein. The α -helix and β -strand content of the protein was estimated from the CD spectrum using the K2D3 web server. The data indicated a 68.8% α -helices, 8.14% β -strands and 23.06% random coils (**Figure 39B**) which agrees with the solved structures of RH5 protein (Chen et al., 2014, Wright *et al.*, 2014). The recombinant protein (buffer exchanged in PBS) was tested for its ability to bind to RBC surface receptor (Basigin) by carrying out an erythrocyte binding assay. Different concentrations of the protein ranging from 5, 10 and 50 μ g/ml were used for the binding assay. The unbound proteins were removed over dibutylphthalate oil, after which the bound proteins were eluted using 0.5M NaCl in PBS. Of the eluted protein, 10 μ l volumes were run on a gel and Western blotted. The membrane was probed using RH5 12 mAbs. It was observed that the binding of the recombinant protein was concentration dependant (**Figure 39C**).

Figure 39. Recombinant RH5 purification and erythrocyte binding.

(A) Coomassie blue staining of purified rRH5. (B) Circular dichroism of purified rRH5 at 4mg/ml. This shows a distribution of 70% helices and 8% β strands. Vertical axis- circular dichroism (mdeg) while horizontal axis- wavelength (nm). (C) Erythrocyte binding assay of recombinant protein in concentration dependent manner (5, 10 and 50- $\mu\text{g/ml}$). Eluted samples were blotted onto the same membrane and probed. Protein molecular weight marker indicated by arrows.

Having successfully purified rRH5 and shown that it binds to the RBC surface receptor, we evaluated the ability of the recombinant protein binding to trigger RBC Ca^{2+} increase. To do this, fresh RBCs were labelled with Fluo-4 AM. The labelled RBCs were washed twice with PBS. Different concentrations of the recombinant protein (0, 0.01, 0.06, 0.1 and 0.15 mg/ml) were incubated at 30°C with the labelled RBCs, to bind. The RBC Ca^{2+} signals upon binding of the rRH5 were read using fluorescence plate reader over 600 seconds. The cumulative Ca^{2+} increase over time was calculated and normalized with the signals from the control (labelled RBC, no rRH5) set up. The fold change in Ca^{2+} signals in comparison with rRH5-free (0) RBC set was calculated. It was observed that different concentration of rRH5 induced an increase in the Ca^{2+} signals in the RBCs significantly (**Figure 40**). This indicated that RH5- Basigin interaction is responsible for this increase in RBC Ca^{2+} signals.

Figure 40. rRH5 specifically triggers RBC Ca^{2+} increase.

RBCs preloaded with Fluo-4-AM were incubated with concentrations of recombinant RH5 (rRH5) (0.01, 0.06, 0.1, 0.15-mg/ml) while using Fluo-4AM loaded RBC as control. The dynamics of Ca^{2+} was monitored for 10 minutes. Fold increase in cytosolic Ca^{2+} signals between the rRH5 bound cells and only Fluo-4 AM loaded RBC (vertical) plotted in bar chart. Each bar on the chart represent mean $\pm$ s.e.m, n=3.

To further our understanding this observation, we tested the ability of RH5 invasion inhibitory mAbs to block this signal. Similar assay as described above was carried out. A fixed 0.2 mg/ml of recombinant RH5 proteins were allowed to bind in the presence of different concentrations of the antibodies. The Ca^{2+} signals were read using the fluorescent plate reader (TECAN). As was expected, without antibodies, the cumulative signals increased upon recombinant RH5 binding (**Figure 41A**). In a concentration dependent fashion, the signals significantly reduced to 36%, 55% and 88% for 0.05, 0.15 and 0.25 mg/ml respectively (**Figure 41B**). This indicates that RH5- Basigin interaction is responsible for triggering an increase in RBC Ca^{2+} .

Figure 41. RH5 7 mAb block rRH5 induced Ca²⁺ signals.

(A) RBC preloaded with Fluo-4AM were incubated with 0.2 mg/ml of rRH5 in the absence (RBC) and in the presence of RH5 7 mAbs (RBC+RH5(7)(0.05mg/ml)), (RBC+RH5(7)(0.15mg/ml)), and (RBC+RH5(7)(0.25mg/ml)). The dynamics of RBC cytosolic calcium induced by the 0.2 mg/ml was recorded over time on a fluorescence plate reader (TECAN). $\sum\Delta F(t)$ which reflects the cumulative change in RBC cytosolic Ca²⁺ was plotted against time. Experimental data were reported as mean $\pm$ s.e.m; n=3. (B) The bar chart indicating the degree of inhibition of Ca²⁺ signals from the RBC by RH5 7 mAbs. Experimental data were reported as mean $\pm$ s.e.m; n=3, * p $\leq$ 0.0239

The specificity of this signal was evaluated with other RBC binding proteins. To this, recombinant RH1 (rRH1) RII3, which focused on the mapped erythrocyte binding region (Gao et al., 2008), as well as the binding region for STEVOR protein (rSTEVOR) which has been shown to interact with glycophorin C (Niang *et al.*, 2014) were evaluated for their erythrocyte binding. The rRH1 (~ 36 kDa) and rSTEVOR (18 kDa) all bound to their receptors on erythrocyte surface and were detected by Western blotting (**Figure 42A**). The ability of the different recombinant proteins to induce increase in RBC Ca²⁺ signal was evaluated. Both rSTEVOR (0.3mg/ml), rRH1 (0.3mg/ml) and rRH5 (0.15 mg/ml) were incubated with Fluo-4 AM labelled RBCs. The dynamic Ca²⁺ signals were measured on a fluorescent plate reader (TECAN). As expected, the addition of Ca²⁺ ionophore A23187 as a positive control activated the highest Ca²⁺ signals while the reading for just the labelled RBC had no impact on the RBC Ca²⁺ signals. Incubating the Fluo-4 AM loaded RBC with rSTEVOR and rRH1 has no effect on RBC Ca²⁺ signalling while rRH5 induced a significant amount of Ca²⁺ signals (**Figure 42B**). These observations all confirm that, the Ca²⁺ triggering in RBC is due to RH5- Basigin interactions.

Figure 42. Recombinant RH1 and STEVR binding does not trigger RBC Ca^{2+} increase.

(A) Western Blot results indicating the binding of rSTEVR and rRH1 RII3. Expected band sizes are indicated by the black arrows while molecular weight indicated in kDa. (B) Cytosolic Ca^{2+} levels of RBC were measured using fluorescent dye Fluo-4M. RBC preloaded with Fluo-4AM were incubated without (RBC) or with rRH5 (0.15mg/ml), rSTEVR (0.3mg/ml) and rRH1 RII3 (0.3mg/ml). Preloaded merozoites treated with ionophore A23157 (A23187) was used as positive control. The dynamics of RBC calcium induced by the binding or the different recombinant proteins were recorded over time on a fluorescence plate reader. $\Sigma\Delta F(t)$ which reflects the cumulative change in RBC cytosolic Ca^{2+} was plotted against time. Experimental data were reported as mean $\pm$ s.e.m; n=3. * $p \leq 0.01435$ for the rRH5 signals, the RBC, STEVR and rRH1 RII3.

4.9 *Evaluation of RBC Ca^{2+} by Flow Cytometry*

Having shown RH5-Basigin interaction to direct an increase in RBC Ca^{2+} by the fluorescent plate reader, we further confirmed the phenomena by employing Flow Cytometry. In this assay, Fluo- 4 AM labelled RBC were sorted to determine the baseline Ca^{2+} levels in the RBC which indicated an average of 0.1% cells as being positive. The addition of Ca^{2+} ionophore A23187 to the system led to the activation of 98% of the cells which confirmed our expectation. Upon RH5 binding, it was observed that about 21.7% of the RBCs showed activation of Ca^{2+} . When the Ca^{2+} in the system had been chelated using EGTA, rRH5 binding had did not trigger Ca^{2+} signals in the RBC. This indicates that the Ca^{2+} ions are from the media. Also the addition of rRH1 did not trigger Ca^{2+} signals the binding of recombinant RH1 had no significant impact on the RBC Ca^{2+} compared to rRH5 (**Figure 44A, B**).

Figure 43. rRH5 specifically triggers RBC Ca^{2+} increase.

(A) Representative Flow cytometry time (s) course experiment evaluating the effects of Fluo-4 AM loaded RBC (Baseline), A23187 treated Fluo-4 AM loaded RBCs (A23187), rRH5 (0.15 mg/ml) treated cells (rRH5), and EGTA treated cells (EGTA). (B) Percentage activation of the different binding conditions under Fluo-4 AM treated cells plotted as a bar chart for the FACS read data. Percentage (%) Calcium (+) cells plotted on vertical axis while treatment conditions on the horizontal axis. All data represented as mean $\pm$ s.e.m, $n=3$ * $p \leq 0.002451$

4.10 *RH5-Basigin interaction leads to RBC cytoskeleton remodeling (in collaboration with Dr. Newman Sze for the Proteomics)*

4.10.1 Recombinant RH5 and Merozoite binding causes differential phosphorylation in cytoskeletal proteins

To understand the effects the changes in RBC Ca^{2+} have on membrane cytoskeletal proteins, an assay was set up to profile the membrane proteins that are differentially phosphorylated upon rRH5 binding or merozoites attachment. This assay involved treating RBCs with rRH5 (0.3 mg/ml) while having untreated RBCs for control under the same conditions. The cells were incubated for 10 minutes, after which the cells were washed to get rid of unbound proteins. The experiment was repeated 3 times independently. To understand the modifications associated with the RBC cytoskeleton during merozoite invasion, a parallel assay was carried out using merozoites. In this assay, purified merozoites were incubated with cytochalasin D treated RBCs whilst maintaining cytochalasin D treated RBCs as control for changes in the cytoskeletal proteins. Proteins were extracted from each of the samples. The proteins were trypsin digested and enriched for phosphopeptides as described in section 2.16 and profiled by LC MS/MS. Together it was observed that merozoite attached or rRH5 bound RBCs showed phosphorylation events on the RBC cytoskeletal proteins.

The total number of phosphorylated proteins identified were 98, 38 (38.78%) showed an up regulation in their phosphorylation with a 1.5 fold cut off. Upon rRH5 binding, 137 proteins were phosphorylated, 82 (59.85%) showed an up regulation in phosphorylation (**Table 3**). Comparing the invading merozoites upregulated phosphoproteins to that the bound rRH5, 24 proteins were common to the two set ups (**Figure 44**).

Table 3 Percentage up regulated phosphoproteins

	% of up - regulated phosphoproteins
Invasion	38.78 (38/98)
rRH5	59.85 (82/137)

Figure 44. Venn diagram of phosphoproteins.

Venn diagram indicating the number of up regulated phosphoproteins upon merozoites attachment (purple) and rRH5 binding (yellow). The phosphoproteins common to these two treatments indicated in the middle.

Of the upregulated phosphoproteins upon merozoites and rRH5 binding, the number of membrane or cytoskeletal associated proteins were ten (10) (**Table 4**) and thirteen (13) (**Table 5**), respectively. Nine of the upregulated membrane proteins were identified for merozoites and rRH5 binding. The unique set of phosphoproteins for the experiments are shown colored. The data shows a common pattern of phosphorylation which is linked to invasion. This suggests that RH5 binding to Basigin on the RBC surface control cytoskeletal proteins modifications. The increase in peptides numbers for the proteins upon rRH5 binding could be accounted for by the fact that the recombinant protein interacted with as many receptors as are on the RBC surface whilst the interaction by the merozoites is to a specific spot on the RBC.

Table 4 Upregulated membrane associated phosphoproteins upon merozoite attachment

Protein name	Number of up regulated phosphopeptides
Protein 4.1	5
Spectrin beta chain, erythrocytic	3
Small integral membrane protein 1	2
Band 3 anion transport protein	2
Ankyrin-1	2
Calpastatin	2
Glycophorin-C	1
Alpha-adducin	1
Beta-adducin	1
Dematin	2

Table 5 Upregulated membrane associated phosphoproteins upon rRH5 binding

Protein name	Number of up regulated phosphopeptides
Ankyrin-1	12
Band 3 anion transport protein	6
Spectrin beta chain, erythrocyte	5
Alpha-adducin	5
Beta-adducin	5
Calpastatin	5
Dematin	5
Glycophorin-A	5
E3 ubiquitin-protein ligase UBR4	4
Glycophorin-C	4
Protein 4.1	3
Spectrin alpha chain, erythrocyte	2
Aquaporin-1	2

4.10.2 Dynamics of RBC membrane proteins

An increase in RBC Ca^{2+} has been shown to regulate the activation of enzymes and recruitment of different proteins to the junction complex or the ankyrin complex (Gauthier *et al.*, 2011). Calmodulin, for example, is activated by Ca^{2+} levels ranging from 100 to 940 nM. Ca^{2+} -calmodulin complex has been proven to disrupt the interactions of proteins such as protein 4.1 and NHE1 to the ankyrin complex. Another unique mechanism described in literature is the differential phosphorylation of proteins upon an increase in RBC Ca^{2+} . Upon phosphorylation, alpha adducin loses its ability to bind to F-actin, implying no capping to the growing actin filament. To evaluate the impact of the induced Ca^{2+} increase upon rRH5 binding on the membrane proteins, we performed erythrocyte binding assay with rRH5. After incubation for 30 minutes, the cells were lysed. The membrane fractions were pelleted by ultra-centrifugation. The membrane proteins were solubilized to obtain the total membrane proteome in solution and quantified. Equal concentrations of the two membrane extracts were separated on SDS-PAGE for Western blotting. The blocked blotted membrane were cut and probed for adducin, dematin, glycophorin C and ankyrin. It was observed that there were no changes in the intensities of both dematin and glycophorin C in the treated and untreated samples. Clearly, the protein intensity for ankyrin was significantly reduced by 10 fold in the treated against the untreated samples. Adducin also showed a decrease by 3 fold in the treated samples. These observations clearly indicate the detachment of both ankyrin and adducin from the complexes which hold the cytoskeletal network firm to the lipid bilayer. This, we believe, is a mechanism which facilitates loosening of the RBC membrane.

Figure 45. Western blot of membrane proteins.

Western blot showing the membrane fractions of recombinant RH5 bound cells (treated) and non-recombinant RH5 bound cells (Untreated). Equal concentrations of membrane extracts were blotted. The blots indicate bands for ankyrin, glycophorin C, dematin and adducin. The protein molecular weight (kDa) is indicated on the left side.

4.10.3 RBC Ca^{2+} increase leads to change in spectrin mesh size (in collaboration with Dr. Rajesh Chandramohanadas).

Assessing the impact of the Ca^{2+} increase, we evaluated membrane cytoskeleton properties such as spectrin length, spectrin mesh size and deformability of the rRH5 bound RBC. From the analysis of the Atomic force images (**Figure 48A**), no significant change on the spectrin length was observed for both control and rRH5 bound membrane fractions (**Figure 48B**). Mesh size for the rRH5 bound membrane fractions significantly increased compared to the control (**Figure 48C**). This indicated an effect of the Ca^{2+} increase on the spectrin network underlying the lipid bi-layer. To evaluate if this effect had any influence the membrane deformation, micropipette aspiration was carried out on both the control cells and the rRH5 bound cells. The results indicated no significant difference in the deformability of the RBCs tested (**Figure 48D**). These all suggest that the effect the Ca^{2+} induced by rRH5 binding was mild on the membrane.

Figure 46. RBC cytoskeletal remodeling upon rRH5 binding.

(A) Representative membrane fraction from fresh RBCs incubated in the absence (Control) or presence of rRH5 was imaged by Atomic force microscopy (AFM). Scale bar = 0.1 μm . (B) Average spectrin filament length as observed from the AFM images. They were quantified using imageJ. (C) Statistic analysis on changes in RBC cytoskeleton mesh size of the rRH5 treated membranes compared to the control was carried out. * $p \leq 0.0324$. Experimental data presented as mean $\pm$ s.e.m, $n=3$. (D) The deformability of the control as well as rRH5 treated cells were measured using Micropipette aspiration measured as shear modulus ($\mu\text{N/m}$). ns indicates not- significant

4.11 Discussion

Merozoite ability to invade RBC has clearly been correlated with the interplay between merozoite surface ligands and RBC surface receptors. Unique among the repertoire of interaction initiated by the merozoite and RBC is RH5- Basigin interaction. This interaction is essential for merozoite invasion (Crosnier et al., 2011, Douglas et al., 2011, Douglas *et al.*, 2014). Antibodies that either recognizes RH5 or Basigin thus have a great effect on merozoite invasion as has been observed with our panel of mAb raised against RH5, both in IFA and Western blotting. The mAbs exhibited high strain transcending merozoite invasion inhibitory effect thus, consistent with the function of RH5 in merozoite invasion.

As merozoite invasion is a complex process involving distinct interactions by the merozoite (Wright & Rayner, 2014), we evaluated the ability of the RH5 mAbs to block merozoite tight junction formation. It was observed that the mAbs exhibited strong tight junction inhibition irrespective of the strain or parasites used. This was further confirmed by the live video microscopy on merozoites invasion. The weak interactions (lasted up to 5 seconds) easily got dislodged, meaning the antibodies blocked the merozoite before forming the tight junction. Although this observation does not indicate the exact point at which the antibodies block invasion, it provides a platform to evaluate the point of action of these antibodies.

Calcium (Ca^{2+}), as a second messenger, has been shown to mediate the release of micronemal proteins, such as EBA175. The release of EBA175 leads to the recruitment of AMA1/RON complex leading to tight junction formation (Singh et al., 2010, Singh & Chitnis, 2012). Therefore, any agent that interrupts with merozoite Ca^{2+} signal will affect the release of EBA175 and block invasion. This mechanism has been described for RH1 invasion inhibitory mAb C41 (Gao et al., 2013). In this regard, we evaluated the RH5 mAbs for their ability to inhibit merozoite Ca^{2+} signals. It turned out that RH5 mAbs has no effect on merozoite Ca^{2+} while RH1 C41 (as a control) blocked the signals. Also the RH5 mAbs had no effect on EBA175 expression. This indicates that the inhibitory effect observed for the RH5 mAbs is independent on EBA175 release. Hence the mAbs are likely to function

after the release of EBA175, thus interrupting with a “check- point” necessary for the insertion of AMA1-RON complex into the RBC membrane.

Pre-invasion events have been suggested to affect Ca^{2+} entry into the RBC during merozoites invasion, hence controlling the merozoite invasion efficiency (Lew & Tiffert, 2007). The Ca^{2+} increase upon merozoites invasion was found to be disrupted in a dose dependent manner by the addition of RH5 7 mAb whilst R1 peptide showed no effect. This indicates the RBC Ca^{2+} is a specific effect resulting from RH5-basigin interaction. To further confirm this observation, we carried an assay with recombinant RH5. It was realized that the binding of the recombinant RH5 to basigin induced an increase Ca^{2+} signals whilst interactions of recombinant STEVOR and recombinant RH1 RII3 to glycophorins C and putative receptor ”Y” showed no effects (Gao et al., 2008, Niang et al., 2014). With the RBC lacking active Ca^{2+} store, our evaluation using EGTA indicated that the observed is from the media. This is consistent with previous observations where chelating the extracellular Ca^{2+} prevented invasion and causing developmental arrest in trophozoites (Wasserman, 1990, Wasserman & Chaparro, 1996).

The modulation in RBC membrane stability has been associated with the dynamics of Ca^{2+} in the RBC (Tiffert & Lew, 2014). The ability of proteins to control the dynamics of RBC membrane revolves around the protein-protein or protein-lipids interactions (Bogdanova et al., 2013, Burris *et al.*, 1980). The dynamism in Ca^{2+} leads to the activation and deactivation of enzymes in the RBC such as protein kinase C, scramblase, flippase, as well as influencing Ca^{2+} dependent phosphorylation of cytoskeletal proteins. The differential phosphorylation of membrane proteins influence their ability to participate in the complex formation (Manno et al., 1995, Pasini et al., 2010a, Wasserman et al., 1990). We show that the phosphorylated proteins upon merozoite invasion and rRH5 binding are similar. Unique among the phosphorylated membrane proteins include adducin, ankyrin, spectrin, protein 4.1, dematin, glycophorins C and band 3. Phosphorylation of adducin and ankyrin prevented their recruitment into the spectrin-actin network, hence causing weakening on the membrane. Also, ankyrin and protein 4.1 associates with band 3 and glycophorins A and C in anchoring the spectrin-actin network to the membrane. Phosphorylation events in these protein leads to

disengagement from the membrane, thereby compromising the integrity of the complex. These influence the stability of the RBC membrane. The increase in phosphorylation events on individual proteins upon rRH5 binding might result from over activation of Ca^{2+} as it binds basigin all over the RBC membrane while merozoites only induce local Ca^{2+} increase.

To evaluate the impact of the Ca^{2+} activation and the corresponding phosphorylation of the membrane cytoskeletal protein, we measure the spectrin length, mesh sizes as well as deformability of the treated cells (Cueff *et al.*, 2010). It was observed that the increase in RBC Ca^{2+} signals had no significant impact on spectrin length. The size of the spectrin mesh significantly increased upon rRH5 binding whilst no significant effect on membrane deformability was recorded. This is a unique observation which we believe is giving us the opportunity to understand into details mechanisms leading to merozoite invasion. Merozoites upon invasion will need to traffic proteins necessary for engagement with the RBC surface receptors maximizing its ability in entering the RBC. Much as the process of trafficking proteins to the surface is important, it has to actively regulate the target RBC allowing it to insert AMA1-RON complex into the RBC membrane. To achieve this, RH5-basigin interaction causes an increase in RBC Ca^{2+} at the site of attachment. This weakens the membrane by loosening the spectrin-actin network, leading to "pore formation" allowing insertion of AMA1-RON complex (Weiss *et al.*, 2015). This also facilitates engagement of the actin-myosin motor thereby leading to invasion (Green *et al.*, 2008). Therefore blocking RH5-basigin interaction, as essential as it is for merozoite invasion, blocks a critical modulation of RBC cytoskeleton allowing merozoite tight junction to be formed.

Mechanistically, we have provided for the first time direct evidence of parasite mediated host cytoskeleton modulation as an important step in allowing merozoites invasion. Even though the downstream interaction making this possible are not yet completely understood, clearly RH5-basigin interaction mediates RBC induces local Ca^{2+} increase. This causes differential phosphorylation events on the cytoskeletal proteins, such as adducin, ankyrin, protein 4.1, dematin, etc. leading their dislocation from the spectrin-actin network. Clearly we have shown that these effects lead to an increase in the mesh size. Thus, we believe the increase in mesh size allows the merozoite to inserting its proteins (AMA1-RON complex)

into the RBC and anchoring firm for invasion. These finding indicates the important of Ca^{2+} increase in RBC during invasion (As described in **Figure 49**). These findings we believe will add other areas to focus in the development of therapeutic and interventions focusing on the family of PfrHs.

Figure 47. Schematic of merozoite invasion showing order of protein interactions.

Upon merozoite contacting RBC in the process of invasion, local deformation in the RBC membrane occurs, rhoptry neck protein PfrH1 is secreted to engage with its putative receptor "Y" on the RBC surface. Upon interaction with "Y", PfrH1 triggers an increase in the merozoite intracellular Ca^{2+} . Merozoite Ca^{2+} increase results in the merozoite trafficking EBA175 to the RBC surface to engage with glycophorin A. To verify the suitability of the RBC for invasion, PfrH5 and its complex is trafficked to engage with basigin. (a) Upon PfrH5-basigin interaction, there is a local Ca^{2+} increase in the RBC causing modifications in the cytoskeletal proteins leading to loosening of the spectrin-actin mesh. The loosening allows the merozoite to securely insert the AMA1-RON complex leading to merozoite tight junction formation as well as actomyosin motor engagement. Thus, propel the merozoite into the RBC. On the other hand, PfrH1 released controls the release of EBA175 to the RBC, hence blocking PfrH1 interferes with the surface expression of EBA175. (b) Contrary to this, blocking PfrH5 had no effect on EBA175 surface expression but blocked merozoite tight junction formation. Hence, in the presence of PfrH5 antibodies, the merozoites were unable to invade. As much as merozoite Ca^{2+} is necessary for trafficking micronemal proteins to the RBC surface, RBC Ca^{2+} is necessary to allow insertion of the tight junction complex.

5.0 Perspectives and Future directions

5.1 *Perspective*

Merozoite invasion is a very complex process. Over the years, great efforts have been made to deconvolute the different interactions making this possible. It had been realised that merozoites once they are released have little time to invade (Cowman & Crabb, 2006). Therefore, for continual survival, invasion must be very fast. Research works over the years has led to the division of the process of merozoites invasion into phases. It begins with an initial random interaction orchestrated by the merozoites surface proteins. It has been characterised to be a weak interaction, which allows the merozoite to engage many times with the RBC before committing to invade (Cowman et al., 2012). The merozoite induces reversible deformations in the RBC during this phase of merozoite-RBC interaction.

During active merozoite invasion, the merozoite, with the help of the EBAs and RHs interacts with RBC surface receptors. To establish the tight junction with the RBC, there is the need for micronemal proteins to be secreted to the surface of the merozoites. These have been shown to be driven by an increase in the merozoites Ca^{2+} (Singh et al., 2010, Singh & Chitnis, 2012). The dynamics of merozoite Ca^{2+} has recently been shown to be influenced by the release of PfRH1. We have further shown that RH2b is also able to modulate the surface expression of EBA175 by affecting merozoites Ca^{2+} increase. This phenomenon we believe is a characteristic of the RH family of proteins. Unique as RH5 is among the PfRHs, it was observed that none of the antibodies to the protein had effect on merozoites Ca^{2+} increase and EBA175 secretion. This observation indicated the function of RH5 relative to EBA175. The function of RH5 is independent of the surface expression EBA175 but as essential as RH5 is to invasion, the effective use the EBA175 mediated pathway will require RH5 for a successful completion. This is evident on the fact that blocking RH5-basigin interaction greatly abrogates merozoites invasion (Crosnier et al., 2011, Douglas et al., 2014).

Differential processing of invasion related proteins is key to merozoite invasion. MSP1 has been shown to undergo different processing event during merozoites invasion (Das *et al.*, 2015, Collins *et al.*, 2009). Blocking MSP1 processing prevents merozoites invasion, thus pointing to the essence of protein processing in the process of merozoites invasion. Similarly, RH1, RH2a, RH2b (Triglia *et al.*, 2005) and RH4 (Triglia *et al.*, 2009) and RH5 have all been shown to undergo different processing events before and during merozoites invasion. Irrespective of the similarity between RH2a and RH2b, they show different processing events which show different RBC binding properties (Duraisingh *et al.*, 2003b, Triglia *et al.*, 2005). In this report, we have shown that a further processing of RH2b does occur after processing in addition to what had previously been proposed. This clearly indicates that, processing of invasion proteins occur, while the key proteases in driving these are not known.

Calcium as a second messenger regulates many activation and deactivation processing the merozoites during invasion. Many of the proteases involved in the processing of the different invasion proteins are either Ca^{2+} dependent or independent. Much as merozoites Ca^{2+} is important in regulating the trafficking of micronemal proteins, it also direct invasion. Calcium form the environment greatly influences the intracellular environment of the host RBCs. It has been shown that chelating the Ca^{2+} in the growth media completely abrogates merozoites invasion in the RBC. This could be a medium of establishing the right equilibrium in facilitating the merozoites engagement with RBC surface proteins. Following the essentiality of RH5 during merozoites invasion, clearly we have added to the fact that RH5-basigin interaction regulates a crucial step in the invasion process which possibly is not dependent on EBA175 release. As the dogma of merozoite invasion is, the release of micronemal proteins leads to the establishment of the tight junction. In blocking RH5 interaction, one is able to block junction formation, pointing to the idea of RH5-basigin interaction acting before final establishment of the tight junction. In this view, the function of RH5-basigin interaction serves as a tight junction establishment "certifier".

In our Ca^{2+} measurements, we evidently show that merozoites invasion requires the extracellular Ca^{2+} pool. Chelating of the extracellular Ca^{2+} with chelators such as EGTA

abrogated the increase during invasion (Wasserman, 1990, Wasserman et al., 1982, Wasserman & Chaparro, 1996). Recombinant RH5 similarly induced the increase in Ca^{2+} in the RBC upon binding. The effect was reversible in the presence of RH5 inhibitory antibodies. As surprising as this observation was to us, it also point to another unique function of RH5-Basigin interaction which has less been explored. For the first time we provided evidence of a merozoite protein modulating RBC Ca^{2+} .

On a deeper mechanistic insight, the data suggests that the increase in RBC Ca^{2+} to regulate differential phosphorylation events on the RBC membrane proteins. Upon modifications, proteins such as adducin and ankyrin showed disengagement with the membrane. It is evident that protein molecule such as calmodulin is activated by a slight increase in the RBC Ca^{2+} from 60 nM. This activation has the ability to membrane protein elements from either interacting or having a weaker interaction (Hoeftlich & Ikura, 2002, Nunomura *et al.*, 2013, Takakuwa & Mohandas, 1988). Enzymes such as PKC when bound by Ca^{2+} is translocated to the membrane interphase where it phosphorylates its target proteins. These effects have the ability to direct a downstream loosening of the junction complex between the membrane and the spectrin network (Gauthier et al., 2011).

The overall impact as we have observed is, that these modifications in the RBC membrane proteins lead to an alteration in the mesh size. This we believe is a mechanism that facilitates the ability of the merozoites to insert the AMA1-RON complex into the RBC during the establishment of the tight junction (Richard et al., 2010). This observation is in agreement with a recent work by Weiss et al, which they observed that RH5-Basigin interaction lead to an opening between the merozoite and the RBC. This opening, to the authors could serve as interphase for molecules as well as invasion components to flow during merozoite invasion (Weiss et al., 2015). Therefore, it becomes clear that the essentiality of RH5-Basigin interaction drives modification in the RBC membrane which favours merozoite invasion. The preferential selection of reticulocytes for invasion over normocytes could be deduced by this mechanism as well (Mons, 1990). In that reticulocytes are enriched for Ca^{2+} storage organelles, making it easy to activate the different Ca^{2+} sensors in the reticulocyte. By this, the process of invasion may become faster. We believe the data

shared in this write up will facilitate an in-depth consideration as we evaluate candidates for their different roles during merozoites invasion.

5.2 *Future directions*

As merozoites invasion is essential for the establishment of the erythrocytic life cycle, understanding the detail molecular interactions facilitating these is very necessary. The dynamics molecular engagement between the merozoites and the RBCs has been the focus of many researches. Notwithstanding the progress made over the year, there is still much to be studied to unravel the mystery surrounding the complex multi-step invasion process. A more comprehensive understanding of the roles of the PfRH family of proteins will help in functionally understanding the interplay leading to sensing of RBCs.

5.2.1 Insight into RH5-Basigin Interaction

- Distribution of Basigin on the RBC surface: From the observation shown in Fig 45 above, the pattern observed for Basigin typifies a possible clustering on the RBC surface. In this work, it will be interesting to go down to know the exact pattern in a high resolution mode.

APPROACH: Using Atomic force microscopy, the probe will be coated with basigin antibodies. The RBC membrane surface will be scanned and the force maps plotted for the regions on the membrane with basigin. From this experiment, it can be deduced the force of interaction between the antibodies and basigin protein. Secondly the distribution per the scanning area can be deduced. It will be worthwhile to understand the pattern of Basigin distribution relative glycophorins A or C as a control. The distribution can be evaluated based on different conditions of the RBC, normal parasite media conditions and when the RBC has been loaded with Ca^{2+} to evaluate if there is differential distribution pattern. With the relative distribution to

any of the glycoporphins, it will be possible to infer the engagements of the ligands on the RBC surface during invasion.

- Basigin interactions on the RBC membrane: Basigin has been implicated in different cell types to play different roles. In melanoma cells, basigin has been shown to interact with cyclophilins A and B. Basigin has been shown to be an essential member of the cyclophilins A- initiated signaling cascade (Yurchenko *et al.*, 2001, Yurchenko *et al.*, 2002). Basigin interaction with cyclophilins B has also been shown to induce Ca^{2+} flux. The interaction of basigin with monocarboxylate transporters (MCTs) facilitates their integration into membrane lipid bi-layers (Kirk *et al.*, 2000). All these points to the multifunctional abilities of Basigin. Though basigin has been shown to be essential for merozoites invasion, it will be worthwhile to understand the different interaction partners in the RBC membrane as well as the cytosol.

APPROACH: Carry out co-immunoprecipitation using anti-basigin on RBC membrane extracts. The RBCs will be treated using different conditions such as incubating the cells in hypotonic buffers, Ca^{2+} rich media and normal parasite growth media. The cells will be lysed and membrane fractions obtained by ultracentrifugation. The membrane proteins will be obtained in their native forms with their interaction partners. The samples will be immunoprecipitated over anti-basigin coated beads. The eluted proteins will be evaluated on an SDS-PAGE and silver stained. Unique interacting bands will be picked out for identified by mass spectrometry to identify the interacting partners. The data that will be obtained will greatly impart on our understanding of the possible roles of basigin on the RBC surface and how different conditions influence different interaction partners in forming complex.

5.2.2 Structural insight into PfrHs

Understanding the structures of the members of the RH family of proteins will further our understanding on how the different regions influence the function of the proteins. The major challenge that is faced on this aim is the high molecular weight of RHs. Key to the development of monoclonal antibodies, identifying unique domains among the different RH members will advance production of direct and specific epitope monoclonal antibodies.

APPROACH: In this aim, we will be making use of the "DIVIDE-and-CONQUER" principle. This will allow the exact interaction domains of the proteins to be narrowed down and the residues that are critical for the receptor ligand interaction to be identified. Using PfrH4 as an example, the globular plots of the protein can be obtained from bioinformatics databases like elm.eu.org. From the plot indicated below, it can be observed that PfrH4 ecto-domain is made up of five globular domains. Each of the domains has coiled-coil regions which have been marked out by the SMART/Pfam domain.

Figure 5.2.2 Globular plot of PfrH4. Using the amino acid sequences of PfrH4 as is available on Plasmodb.org. It indicates the structural predictions made by SMART/Pfam databases for coiled-coil (green), low complexity regions as well as the signal peptide (red) and transmembrane domain indicated in blue. The different globular plots obtained from GlobPlot showed how the protein is sub-divided based on their predicted structural fold. The distribution of ordered and disordered regions of the protein have also been shown spanning the entire length of the RH4 amino acids.

5.2.3 Receptor binding domain mapping

A unique mechanism of the receptor recognition of the various RHs to their respective erythrocyte surface receptor and how this interaction drives the process of merozoites invasion is still undiscovered. Receptors to RH5 and RH4 have been determined to basigin and complement receptor one respectively (Tham et al., 2010, Crosnier et al., 2011). In a

recent work, it had been shown that apart from invasion blocking antibodies against RH4, the authors demonstrated the ability to develop inhibitors that competed for the RH4 binding sites (Lim *et al.*, 2015). The receptor binding properties of RH1, 2a, and 2b have been shown; therefore the need is to identify the exact receptors they interact with on the erythrocyte surface.

APPROACH:

Protein-receptor complex pull down. Using specific monoclonal antibodies generated against RH1, RH2a and RH2b for co-immunoprecipitation. In this approach, purified schizonts will be purified and allowed to egress in the absence of RBCs to generate parasite culture supernatants. The supernatant will be tested for the ability of secreted proteins in it to bind to RBCs and also verified for the presence of RH1, RH2a and RH2b by Western blots. Once shown positive for these proteins, another erythrocyte binding assay will be carried out. All the unbound proteins will be washed out over high density oil. The bound proteins will be cross-linked using EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide), which is a well-known zero length cross linker and bis(sulfosuccinimidyl)suberate (BS3); which is 11.4 Å length, hence any interactions within this length will be captured. With the specific monoclonal antibodies, co-immunoprecipitation will be carried out. The eluted proteins will first be verified by western blot. Isolated complex will then be run in the SDS-PAGE, followed by mass spectrometry to identify the specific receptor.

5.3 *Conclusion*

In this study, the focus has been on obtaining a more functional insight on the roles of RH2b and RH5 during merozoites invasion. We have shown a reagent that specifically recognizes the protein both by IFA and Western blots. RH2b is involved in merozoites tight junction formation during invasion. The A7 mAb exhibited a dose dependent merozoite invasion inhibition. With direct biochemical assays, we show that RH2b protein interacts with a chymotrypsin sensitive, neuraminidase and trypsin resistant receptor on the RBC surface. The binding of RH2b protein to its putative receptor was found to be interrupted in the presence of the mAb. We confirmed that RH2b is processed in the schizont and during merozoites invasion to yield an approximately 160 kDa. With the mAbs, we have shown that RH2b regulates the secretion of micronemal proteins (such as the EBA175). While RH1 and RH2b show similar function during merozoites invasion, they are used in different invasion pathways. In furthering our understanding on RH5-Basigin interaction and its influence during merozoites invasion, we generated RH5 specific mAbs which exhibited strain transcending invasion inhibitory effects. With the antibodies, we have shown that the mAb block tight junction formation as well as RH5 protein interaction to erythrocyte surface receptor. We have shown that RH5 monoclonal antibodies have no effect on merozoites Ca^{2+} signalling as well as the surface expression of EBA175. Evidently, there is a rise in the RBC Ca^{2+} signals during merozoites invasion which is significantly blocked in the presence of the mAbs. For the first time, we have shown that RH5-Basigin interaction leads to increase in RBC Ca^{2+} . This led to the differential phosphorylation of events on the membrane proteins causing a loosening (increase in mesh size) of the membrane junction complexes by decreasing proteins such as ankyrin and adducin at the RBC membrane. With this, we have provided a concrete evidence of a mechanism the parasite uses to manipulate the RBC to facilitate insertion of its proteins leading to tight junction formation and subsequent invasion. Also we propose that members of the PfrH family will be very good candidates for inclusion into multiple component vaccines.

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