DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOOGY

COLLEGE OF BASIC AND APPLIED SCIENCES

UNIVERSITY OF GHANA

DECONSTRUCTING INVASION PHENOTYPE SWITCHING IN *PLASMODIUM FALCIPARUM*

THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF

MPHIL MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES DEGREE

BY

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JULY 2018.

DECLARATION

I, Prince Berko Nyarko, declare that the work reported in this thesis was undertaken by me at the West African Centre for Cell Biology of Infectious Pathogens, Department of Biochemistry, Cell and Molecular Biology, under the supervision of Professor Gordon Akanzuwine Awandare and Dr. Yaw Aniweh. All references used are duly cited.

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(SUPERVISOR) (CO-SUPERVISOR)

DATE: …… DATE……..

ACKNOWLEDGEMENTS

I am eternally grateful to the Almighty God for seeing me through this phase of my education. My heartfelt appreciation goes to Professor Gordon A. Awandare for the opportunity to work in his laboratory and the mentorship and support he provided me during my studies at the University of Ghana and the course of this project. To my co-supervisor Dr. Yaw Aniweh, thank you very much for your support, guidance and encouragement throughout this project. A big thank you to Reuben Ayivor-Djanie for introducing me to transcriptomic analyses and for being a wonderful lab partner and colleague. I am also thankful to Professor José A. Stoute for his insight during the drafting of the manuscript on this project. My appreciation would not be complete without acknowledging the wonderful members of the Cell Biology and Immunology Laboratory for their constructive inputs in bringing this project into perspective. I would like to extend my profound gratitude to our collaborators at the London School Hygiene and Tropical Medicine; Professor David Conway, Dr. Sarah Jayne Tar and Lindsay Stewart for assisting me to plan and successfully execute the whole transcriptome studies which is an extended part of this study. Finally, I would like to say a big thank you to WACCBIP for funding this project.

DEDICATIONS

Without mentioning names, I dedicate this work to all the wonderful people who contributed in diverse ways to making sure I received the best education at the highest level possible.

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AMA1 – Apical membrane antigen 1

cAMP – Cyclic adenosine monophosphate

CD – Cluster of differentiation

CLAG – Cytoadherence linked antigen

CR1 – Complement receptor 1

CSA – Chondroitin sulfate A

CSP – Circumsporozoite protein

CT – Chymotrypsin treated

DAF – Decay accelerating factor

DBL – Duffy binding ligand

EBA – Erythrocyte binding antigen

EBL – Erythrocyte binding ligand

EPCR - Endothelial protein C receptor

GAG – Glycosaminoglycans

GDV1 – Gametocyte development 1

GLURP – Glutamine rich protein

GPI – Glycosylphosphatidylinositol

HRP – Histidine rich protein

ICAM1 – Intercellular adhesion molecule 1

IRS – Indoor residual spray

ITN – Insecticide treated nets

MM – Molecular marker

MSP – Merozoite surface protein

Nm - Neuraminidase

NT – Neuraminidase treated

NTC – Non-template control

PCR – Polymerase chain reaction

PECAM-1 - Platelet endothelial cell adhesion molecule-1

Pf – Plasmodium falciparum

PfEMP1 – Plasmodium falciparum erythrocyte membrane protein 1

PIR - Plasmodium interspersed repeat

RAP – Rhoptry associated protein

RBC – Red blood cell

RDT – Rapid diagnostic test kit

RH – Reticulocyte binding-like homologue

RhopH – Rhoptry bulb heavy proteins

RIF - Repetitive interspersed family

RIFIN – repetitive interspersed family protein

RON – Rhoptry neck

RPM – Revolutions per minute

R-ST – Returned to static culture

RT – Reverse transcription

RT-qPCR – Reverse transcription quantitative polymerase chain reaction

SA – Sialic acid

SP – Suspended

ST – Static

STEVOR – Subtelomeric variant open reading frame

TT – Trypsin treated

US - Unstained

UT – Untreated

VCAM - vascular cell adhesion molecule 1

VIR - *Plasmodium vivax* interspersed repeat

WHO – World Health Organization

ABSTRACT

The extensive redundancy in the use of invasion ligands by *Plasmodium falciparum*, and its unique ability to switch between invasion pathways have hampered vaccine development. The Dd2 and W2mef strains of *P. falciparum* have been shown to change from sialic acid (SA)-dependent to SA-independent phenotypes when selected on neuraminidase-treated erythrocytes. Following an observation of increasing ability of Dd2 to invade neuraminidase-treated cells when cultured for several weeks, a systematic investigation of this phenomenon was conducted by comparing invasion phenotypes of Dd2, W2mef and 3D7 strains of *P. falciparum* that were cultured with gentle shaking (*Suspended*) or under static (*Static*) conditions. While *Static* Dd2 and W2mef remained SA-dependent for the entire duration of the investigation, *Suspended* parasites spontaneously and progressively switched to SA-independent phenotype from week 2 onwards. Furthermore, returning *Suspended* cultures to *Static* conditions led to a gradual reversal to SA-dependent phenotype. The switch to SA-independent phenotype was accompanied by upregulation of the key invasion ligand, reticulocyte-binding homologue 4 (RH4), and the increased invasion was inhibited by antibodies to the RH4 receptor, CR1. The data demonstrates a novel mechanism for inducing the switching of invasion pathways in *P. falciparum* parasites and may provide clues for understanding the mechanisms involved.

CHAPTER ONE

1.0 INTRODUCTION

Malaria remains a major public health menace, accounting for an estimated 445,000 deaths globally in 2016 (WHO, 2017). Despite the gains made since the inception of the millennium development goals in the control of infectious diseases, malaria continues to persist largely due to the persistent and continuous emergence of drug resistant parasites and insecticide resistant mosquitoes. Given the history of drug resistance, chemotherapeutic measures in controlling malaria appear to be temporal. An effective vaccine remains the most viable malaria control and elimination strategy. However, no such effective vaccine is currently available.

As an obligate intracellular parasite, *Plasmodium falciparum* actively invades and establishes successful infection in human erythrocytes. This process is critical for the cyclical development, thus, making erythrocyte invasion an attractive target for malaria vaccine development (Wright and Rayner, 2014). The invasion process requires interactions between erythrocyte surface receptors and parasite ligands. A major class of erythrocyte surface molecules exploited by *P. falciparum* for invasion are the receptors that have sialic acid (SA) moieties present such as the erythrocyte surface glycophorins (Deas and Lee, 1981; Perkins, 1981; Ahouidi *et al.*, 2016; Satchwell, 2016). However, a large proportion of both laboratory-adapted strains and clinical isolates of *P. falciparum* successfully invade SA-deficient erythrocytes (Mitchell *et al.*, 1986; Hadley *et al.*, 1987; Lobo *et al.*, 2004; Deans *et al.*, 2007; Gomez-Escobar *et al.*, 2010; Mensah-Brown *et al.*, 2015). Therefore, depending on the requirement of SA for erythrocyte invasion, *P. falciparum* parasites are broadly classified as either SA-dependent or SA-independent. Other erythrocyte receptors that have been shown to be used by the parasite include complement receptor 1 (CR1) (Spadafora *et al.*, 2010; Tham *et al.*, 2010; Awandare *et al.*, 2011b), basigin (Crosnier *et*

al., 2011), band 3 (Goel *et al.*, 2003; Li *et al.*, 2004; Baldwin *et al.*, 2014), decay-accelerating factor (DAF, CD55) (Egan *et al.*, 2015), as well as others yet to be identified (Bei and Duraisingh, 2012; Bartholdson *et al.*, 2013; Egan *et al.*, 2015; Kanjee *et al.*, 2017).

The mechanism of invasion is complicated. *P. falciparum* deploys a wide repertoire of proteins (ligands) for interaction with the erythrocyte receptors in a sequence of steps (Srinivasan *et al.*, 2011; Weiss *et al.*, 2015; Cowman *et al.*, 2017). These invasion related ligands generally belong to two major protein families namely the *P. falciparum* erythrocyte binding antigens (PfEBAs), which include, EBA175, EBA140, EBL1 and EBA181, and the *P. falciparum* reticulocyte binding-like homologues (PfRHs) comprising, RH1, RH2a, RH2b, RH4 and RH5 (Cowman *et al.*, 2017). Redundancy in the functions of erythrocyte invasion related ligands allows the parasite to use the differential expression of these ligands to continuously switch pathways to evade immune recognition and ensure its survival (Persson *et al.*, 2008; Wright and Rayner, 2014). The mechanisms responsible for the switching in gene expression of invasion ligands remain unclear; however, they are thought to be epigenetic, possibly involving histone methylation (Jiang *et al.*, 2010). Furthermore, the signals that trigger the changes in ligand gene expression are not well-understood, but immune pressure and limiting receptor availability are logical candidates.

Much of the current understanding of the ligand switching mechanisms has come from studying two parasite strains, Dd2 and W2mef, which can be induced to switch invasion phenotypes from SA-dependent to SA-independent *in vitro* (Dolan *et al.*, 1990; Reed *et al.*, 2000; Duraisingh *et al.*, 2003a; Stubbs *et al.*, 2005). This switch is normally accomplished by growing the parasites in neuraminidase-treated erythrocytes, which lack SA, and thus induce the parasites to deploy alternative SA-independent pathways (Dolan *et al.*, 1990; Stubbs *et al.*, 2005); in support of receptor availability accounting for phenotypic variation. Currently, there are no *in-vitro* systems

to assay the contribution of immunological factors to invasion phenotype variation. However, empirical evidence from clinical studies show that acquired antibodies differentially inhibit erythrocyte invasion by *P. falciparum* strains utilizing different invasion pathways (Persson *et al.*, 2008), thus supporting immune response as a contributing factor to phenotypic switching.

Despite the overwhelming and convincing evidence supporting receptor availability and immune surveillance as contributing factors to phenotypic variation, less is known about the possible contributions of other host physiological conditions and parasite properties that may account for invasion phenotype variation, as has been observed in other parasite phenotypes such as differentiation and proliferation (Hotta *et al.*, 2000; Mideo *et al.*, 2013; Lima *et al.*, 2016; Mancio-Silva *et al.*, 2017; Hirako *et al.*, 2018; Prior *et al.*, 2018), as well as disease outcome (Murray *et al.*, 1975; Zijlmans *et al.*, 2008; Danquah *et al.*, 2010; Mejia *et al.*, 2015; Mancio-Silva *et al.*, 2017).

While cultivating Dd2 as a reference for routine invasion phenotyping of clinical parasite isolates, it was observed that the ability of the parasite to invade neuraminidase-treated erythrocytes gradually increased over time. This phenomenon appeared to coincide with a change to the use of a shaking incubator for keeping parasite cultures. Therefore, this study sought to comprehensively investigate the switching phenomenon by temporally monitoring invasion phenotypes of Dd2, together with its closely related strain W2mef, and a commonly used SA-independent strain, 3D7. Understanding the molecular mechanism involved in the invasion phenotype variation will add a new twist to the complexity of *P. falciparum* invasion mechanisms, and also demonstrate the physiological relevance of methods used for parasite cultivation *in vitro* and the study of invasion phenotypes in culture-adapted clinical isolates. Furthermore, delineating this observation hold the potential of identifying the contribution of non-immune physiological conditions to invasion

phenotype variations, in addition to the tractability of the parasite to changes in host physiological conditions.

1.0.1 Hypotheses

Based on preliminary findings, it is proposed that:

1. Dd2 and other sialic acid-dependent strains are capable of switching invasion phenotype when cultivated under flow conditions as compared to static cultures.
2. The switch in invasion phenotype is dependent on the differential expression of invasion related genes.

1.0.2 Aim

To elucidate the dynamics of invasion phenotype switching by *Plasmodium falciparum* under different culturing conditions.

1.0.3 Specific objectives

1. To determine the ability of Dd2, W2mef and 3D7 to switch invasion phenotype in static and suspension cultures.
2. To identify the molecular mediators responsible for the phenotype switching.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.0.1 The malaria burden

Approximately one-half of the world's population is at risk of malaria, with pregnant women and children under five years being the greatest casualties. According to the World Health Organization (WHO), about 216 million malaria cases were recorded worldwide in 2016, with an estimated 445000 mortalities occurring (WHO, 2017). Sub-Saharan Africa accounted for about 90 % of all cases and mortalities recorded. Malaria incidence and mortality rates have decreased by 37 % and 60 %, respectively, from 2000 to 2015 (Cibulskis *et al.*, 2016). The incidence rate has decreased by 18 % globally between 2010 and 2015, with only 15 out of the 91 malaria-endemic countries accounting for approximately 80 % of the malaria burden in 2016 (WHO, 2017). The enormous gains have been made through the combined effects of control measures such as the use of insecticide-treated bed net (ITNs), indoor residual spraying (IRS), intermittent preventive treatment in vulnerable groups such as pregnant women and children under five years, improved and accessible diagnostic tools, and the availability of efficacious chemotherapies such as the artemisinin derivatives and their partner drugs (WHO, 2017).

Despite the prospects made since the inception of the Millennium Development Goals, malaria remains a public health menace (Cibulskis *et al.*, 2016). Currently, there are reports of resistance against the artemisinin derivatives-based drugs which are used as the first line chemotherapies (Noedl *et al.*, 2008; Dondorp *et al.*, 2010; Dondorp *et al.*, 2011; Hott *et al.*, 2015; Laurent *et al.*, 2015; White *et al.*, 2015). Similarly, there are reported cases of vector resistance against the insecticides (pyrethroids) used for ITNs and IRS (Ranson *et al.*, 2011; Ranson and Lissenden,

2016). Although microscopy remains the major, and most reliable means of diagnosing malaria, rapid diagnostic tests kits (RDTs) are commonly used for malaria diagnosis, particularly in remote areas deprived of microscopes or microscopists, and in emergency situations. The currently available RDT kits are based on the identification of the parasite's histidine-rich protein 2 (HRP2) in the peripheral blood of patients. Malaria diagnosis with these RDTs are increasingly becoming unreliable due to reported cases of deletions in the HRP2 gene, leading to false negative results (Amoah *et al.*, 2016; Beshir *et al.*, 2017; Gupta *et al.*, 2017). These observations require that new strategies be developed towards malaria control either through new rapid diagnostic tools based on a more stable antigen/analyte, or new drugs against both the malaria parasite and the mosquito vector. Nonetheless, given the history of anti-malaria drug resistance it appears that such interventions are only temporal. A more efficacious and lasting approach is required to overcome the malaria menace. A number of viral and bacterial diseases such as small pox, polio, hepatitis, etc. have either been eradicated or controlled through the use of effective vaccines. An effective vaccine, therefore, remains the viable antidote to malaria control and possible eradication.

2.0.2 Malaria pathogenesis and parasite biology

Plasmodium parasites of the protozoan phylum apicomplexa are the causative agents of malaria. Five species of *Plasmodium* namely, *P. falciparum*, *P. ovale*, *P. vivax*, *P. knowlesi* and *P. malariae* are responsible for malaria in humans. Of these, *P. vivax* is the most widely distributed in terms of the affected populations (Gething *et al.*, 2012). *Plasmodium falciparum* is, however, the most virulent and most prevalent species in Africa (Snow *et al.*, 1999; Miller *et al.*, 2002).

P. falciparum malaria is characterized by periodic fever, chills, sweating, headache and vomiting. The severity of *P. falciparum* malaria results from unique virulent properties of the parasite. For instance, *P. falciparum* can infect erythrocytes of all ages, thus enabling it to establish high

parasitemias (Iyer *et al.*, 2007; Gunalan *et al.*, 2013). Unlike *P. falciparum*, the other species of *Plasmodium* are selective on the type of erythrocyte they infect. While *P. vivax* and *P. ovale* infect immature erythrocytes (reticulocytes) only, *P. malariae* infects mature erythrocytes (normocytes) only (Iyer *et al.*, 2007; Gunalan *et al.*, 2013). Establishing high parasitimic infections leads to increased host erythrocyte destruction during parasite egress, which may result in anaemia. Because the parasite mainly undergoes glycolysis, high parasitemias may lead to hypoglycemia due to depletion of serum glucose and lactic acidosis as a result of the lactate generated as by-product of glycolysis. In addition, parasite egress leads to the release of toxins, which promotes an excessive inflammatory response to the infection leading to immune-pathologies (Coban *et al.*, 2018). Again, the release of the parasite pigment, hemozoin into the blood and its subsequent uptake by phagocytes has been implicated in the suppression of phagocytic activity of these cells and their pathogen killing abilities (Awandare *et al.*, 2007). The accumulation of hemozoin in the bone marrow also suppresses erythropoiesis, leading to anaemia in severe cases (Lamikanra *et al.*, 2009; Awandare *et al.*, 2011a; Perkins *et al.*, 2011).

Apart from its ability to establish higher parasitimic infections, *P. falciparum* expresses adhesive molecules on the surface of the erythrocyte it infects, enabling it to evade splenic clearance by adhering to endothelial cells. The *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is the best studied parasite adhesion molecule (Hviid, 2011; Smith, 2014; Hviid and Jensen, 2015; Cutts *et al.*, 2017; Shabani *et al.*, 2017; Ofori *et al.*, 2018; Zanghì *et al.*, 2018). This protein is coded by the *var* multi gene family. Each *P. falciparum* has about 60 var genes of which it expresses one at a time; a phenomenon which ensures that the parasite evades the host immune defense mechanism (Chen *et al.*, 1998; Scherf *et al.*, 1998; Cortés, 2008; Scherf *et al.*, 2008). Erythrocyte surface-expressed PfEMP-1 form knobs which facilitate the adhesion of infected erythrocytes to molecules

such as intercellular adhesion molecule 1 (ICAM-1; CD54), CD36, endothelial protein C receptor (EPCR), vascular cell adhesion molecule 1 (VCAM-1; CD106), chondroitin sulfate A (CSA), platelet endothelial cell adhesion molecule-1 (PECAM-1; CD31), glycosaminoglycans (GAGs), E-selectin, on blood vessel walls; a phenomenon referred to as cytoadherence (Rowe *et al.*, 2009; Smith *et al.*, 2013; Turner *et al.*, 2013; Sampath *et al.*, 2015). Erythrocyte surface expressed PfEMP-1 also binds to CR1 on the surfaces of uninfected erythrocytes to form a cluster of uninfected erythrocytes with a centrally-placed infected erythrocyte; a phenomenon called rosette formation (Rowe *et al.*, 1997; Rowe *et al.*, 2000). In addition to the *var* genes, other multi-gene families such as the repetitive interspersed family (*rif*) and subtelomeric variant open reading frame (*stevor*) which encodes the RIFINs and STEVORs, respectively, have also been implicated in cytoadherence and rosette formation (Kyes *et al.*, 1999; Niang *et al.*, 2014; Goel *et al.*, 2015; Wang and Hviid, 2015). Both cytoadherence and rosetting result in complications such as cerebral malaria, respiratory dysfunctions, multiple organ failure, coma and in extreme cases, death. *Plasmodium vivax* also possesses cytoadherent properties through the *P. vivax* interspersed repeat (VIR) proteins (Carvalho *et al.*, 2010), coded by the *Plasmodium* interspersed repeat (*pir*) multigene superfamily (Cunningham *et al.*, 2010). However, due to lower parasitemia of *P. vivax* infections relative to *P. falciparum* infections, the pathologies associated with cytoadherence in *P. vivax* is less severe compared to *P. falciparum*.

A virulent property which appears to be unique to *P. vivax* and *P. ovale*, but not *P. falciparum* and *P. malariae* is the ability of the liver stages to establish dormant forms called hypnozoites (Richter *et al.*, 2010; Markus, 2011; Mikolajczak *et al.*, 2015). These hypnozoites do not undergo vegetative growth and are resistant to most of the first line anti-malaria drugs, except primaquine in some cases (Dembele *et al.*, 2011; Fernando *et al.*, 2011; Gural *et al.*, 2018). Formation of hypnozoites

allow for relapse of the infection even after months of treatment without reinfection (White, 2011). This form of the parasite is also inert to cellular immune response (Rénia and Goh, 2016), thus thwarting control measures in areas with *P. vivax* and *P. ovale* infections. It is somewhat a relief that *P. falciparum* does not have the ability to exist as hypnozoites. However, a worrying observation made in the studies of artemisinin resistance in *P. falciparum* shows that resistant parasites have delayed ring stage development which exists as inactive form during drug pressure (Hott *et al.*, 2015). This observation suggests that under selective pressure *P. falciparum* is capable of existing in hypnozoite-like forms, a situation that threatens current malaria control methods. Nonetheless, the recent identification of small molecular inhibitors of histone modifying enzymes capable of reactivating quiescent hypnozoites may provide an antidote to recrudescence (Malmquist *et al.*, 2012; Dembélé *et al.*, 2014).

2.0.3 The *Plasmodium falciparum* Life cycle

Plasmodium parasites have a complex life cycle which alternates between a vertebrate host and a mosquito vector (Figure 2.1). Mosquitoes are generally herbivores, feeding on nectar and pollen (Gu *et al.*, 2011). However, during reproduction the female mosquitoes take in blood meal to nourish and develop their eggs (Gu *et al.*, 2011). When a mosquito feeds on a *Plasmodium*-infected person, it ingests the sexual stage of the parasite (gametocytes), thus becoming infected. Upon reaching the midgut of the mosquito, the gametocytes differentiate to form male (micro) and female (macro) gametes. Formation of female gamete is characterized by the enlargement and rounding up of the gametocyte while male gametes undergo exflagellation leading to the formation of eight gametes. Gametogenesis is stimulated by conditions such as low temperature, high pH and xanthurenic acid within the gut of the mosquito (Billker *et al.*, 1997; Ghosh *et al.*, 2000; Ghosh *et al.*, 2010). A zygote, formed by the fusion of a micro and a macro gamete develops into an

ookinete, which is the invasive form at the mosquito stage. Ookinetes migrate from the mid-gut to the basal lamina where they form oocysts. The oocyst then divides to form multiple haploid sporozoites which subsequently migrate to the salivary gland of the mosquito through the haemolymph (Ghosh *et al.*, 2000; Nikolaeva *et al.*, 2015). While feeding, an infected mosquito inoculates sporozoites into the skin of the vertebrate host. Upon entering the circulatory system through damaged vesicles, the sporozoites migrate to the liver, invade hepatocytes and develop into schizonts containing merozoites. Mature merozoites bud off as vesicles called merosomes that move through the sinusoid lumen into the lung vasculature before releasing the merozoites into the blood (Coquelin *et al.*, 1999; Baer *et al.*, 2007). The merozoites infect erythrocytes and develops through three stages. The newly invaded merozoites are referred to as rings due to their characteristic ring-like shape. Rings feed and grow into pigmented forms called trophozoites. These trophozoites subsequently develop into schizonts which then undergo schizogony to asexually generate daughter merozoites. On maturation, merozoites break out of the erythrocyte and are released into circulation. Each merozoite then invades a new erythrocyte to establish another infection. This asexual cycle takes approximately 48 hours (Miller *et al.*, 2002). Erythrocyte destruction and parasite toxin release as a result of merozoite egress is responsible for the fever and anaemia associated with malaria. Occasionally, a limited proportion of parasites commit to the formation of sexual stages called gametocytes which can be picked up by a mosquito during a blood meal to initiate the mosquito stage of the parasite (Miller *et al.*, 2002; Cowman *et al.*, 2012).

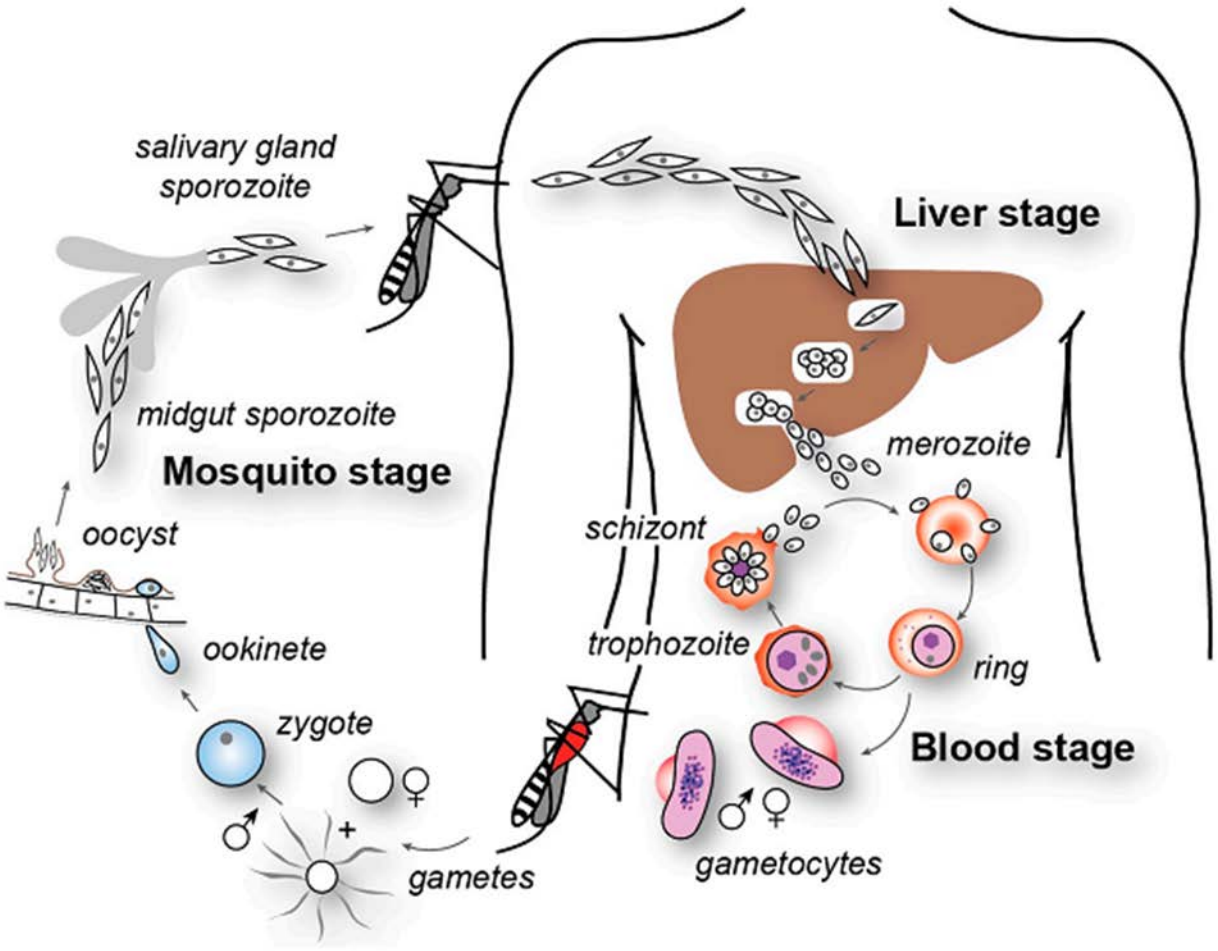

Figure 2. 1: The life cycle of *Plasmodium falciparum* (Cowman *et al*., 2012).
During blood meal, an infected mosquito inoculates the vertebrate host with sporozoites which migrate to and infect liver cells. The sporozoites develop into liver-stage schizonts which produces thousands of merozoites that are released into circulation to infect erythrocyte. Upon entry into erythrocytes, the parasite grows and undergo three cyclical developmental stages namely rings, trophozoites and schizonts. Occasionally, some parasites develop into gametocytes which can be picked up by a feeding mosquito to start the mosquito stage of the parasites life cycle. In the mosquito, the gametes fuse and develop into zygotes. These develop into ookinetes which invade the gut epithelial cells and form oocysts. The oocysts develop into sporozoites which migrate to the salivary gland awaiting transmission to a vertebrate host during a blood meal.

2.0.4 Current vaccine strategies

Efforts to elucidate viable vaccine candidates have exploited the various stages of the parasite's life cycle. These include transmission, liver-stage (pre-erythrocytic) and erythrocyte invasion-blocking vaccine candidates which target the gametocytes, sporozoites and merozoites, respectively.

The goal of earlier malaria vaccine strategies was to achieve sterile immunity. Various studies explored ways to interrupt parasite development at the sporozoite stage so that the blood stage infection accounting for clinical pathologies does not occur. The first attempt relied on the induction of immune responses against the sporozoite by inoculating susceptible hosts with attenuated forms of the parasite (Nussenzweig *et al.*, 1967; Rieckmann *et al.*, 1974; Clyde *et al.*, 1975; Rénia and Goh, 2016; Draper *et al.*, 2018). Though successful, upscaling the production of attenuated sporozoites was a major challenge, thus ushering in sporozoite sub-unit vaccines which lead to the development of the current malaria vaccine, RTS,S (Stoute *et al.*, 1997; Birkett, 2010; Crompton *et al.*, 2010). This approach targets the major antigen on the sporozoite surface, the circumsporozoite proteins (CSP) (Draper *et al.*, 2018). However, with the low efficacy of RTS,S, (Rts, 2015) attenuated whole sporozoite vaccines have been revisited. Irradiated sporozoites capable of infecting liver cells and maturing into schizonts but incapable of releasing merozoites have been developed lately, with efficacy of approximately 90 % (Hoffman *et al.*, 2002; Hoffman *et al.*, 2010). This vaccine, however, faces logistical challenges largely due to the need for liquid nitrogen-based cryopreservation of sporozoites to maintain viability (Hill, 2011).

Central to malaria elimination and subsequent eradication is total abrogation of transmission. With limited chemotherapies against the transmissive form of the parasite in humans, coupled with failed vector control strategies, transmission blocking vaccines have emerged as the alternative.

The ability of gametocyte immunized sera to thwart gamete development in the mosquito was reported in the mid twentieth century, first in avian, and later in mammalian malaria parasites (Huff *et al.*, 1958; Carter and Chen, 1976; Rener *et al.*, 1983; Carter and Stowers, 2001; Nikolaeva *et al.*, 2015). Transmission blocking vaccines target the surface antigens of gametes, preventing their fusion and/or development in the mosquito. Three major gamete surface proteins; Pfs25, Pfs48/45 and Pfs230 have been identified as targets for transmission blocking vaccines (Rener *et al.*, 1983; Nikolaeva *et al.*, 2015). Although these antigens are less polymorphic and antibodies against them are capable of blocking transmission to approximately 90 %, expression of the proteins in their native conformations has proven difficult due to the presence of a six cysteine disulfide bonding pattern present in the protein structure (Carter *et al.*, 1995; Nikolaeva *et al.*, 2015). However, co-expression of Pfs48/45 with periplasmic folding catalysts, as well as codon harmonization in *E. coli* expression systems has yielded correctly folded proteins with transmission blocking activity (Outchkourov *et al.*, 2008; Chowdhury *et al.*, 2009). Recently, a recombinant chimera of Pfs48/45 and the blood stage antigen, glutamine-rich protein (GLURP) has been produced successfully (Theisen *et al.*, 2014). Antibodies against this chimera exhibit multi-stage inhibitory activity, thus providing evidence of the feasibility of a multistage malaria vaccine. The progress made thus far in respect of the expression of immunogenic transmission blocking antigens hold promise towards a possible transmission blocking malaria vaccine development.

Disease pathology occurs during the erythrocytic cycle of the parasite, thus making this stage another critical point for vaccine interventions. Passive transfer of immunoglobulin gamma (IgG) from malaria exposed adults to malaria-infected children showed massive clearance of parasites (Cohen *et al.*, 1961; Sabchareon *et al.*, 1991; Ademolue and Awandare, 2018). These studies

provided evidence that humoral response against the blood stage of the infection is capable of parasite killing and gave the first indication that a blood stage malaria vaccine is feasible.

Adherence of *Plasmodium*-infected erythrocyte to tissue endothelium (cytoadherence) and uninfected erythrocyte (rosetting) cause most of the clinical manifestations of malaria such as cerebral malaria, and pregnancy associated malaria which may lead to underweight babies, stillbirths or even maternal deaths (Duffy and Fried, 2005; Rogerson *et al.*, 2007). The parasite antigens PfEMP1, STEVORs and RIFINs are the major culprits in the adhesive properties of *P. falciparum*-infected erythrocytes (Rowe *et al.*, 1997; Rowe *et al.*, 2009; Niang *et al.*, 2014; Goel *et al.*, 2015; Wang and Hviid, 2015). Thus, vaccines against these antigens have the potential to reduce disease severity (Hviid *et al.*, 2018). However, an effective vaccine against these antigens has not materialized due to the large number of genes encoding each antigen as well as the presence of high sequence polymorphisms. Furthermore, some of these antigens evade immune detection by inhibiting the proper functioning of host immune cells (Saito *et al.*, 2017). Nonetheless, surface associated antigens are targets of acquired immunity and antibodies against them correlate with protection against malaria (Abdel-Latif *et al.*, 2003; Turner *et al.*, 2011; Chan *et al.*, 2012; Beeson *et al.*, 2013; Chan *et al.*, 2014), thus making them vaccine targets.

Destruction of erythrocytes coupled with the release of toxins into circulation during parasite egress is another cause of malaria pathology. Interrupting the cycle by preventing the parasite from entering erythrocytes would alleviate malaria morbidity and also block transmission. Erythrocyte invasion is thus another target for malaria vaccine development. Though invasion related antigens are targets of acquired immunity (Reiling *et al.*, 2012; Badiane *et al.*, 2013; Persson *et al.*, 2013; Beeson *et al.*, 2016; Partey *et al.*, 2018), the redundancy of these ligands coupled with polymorphisms in their sequences make it impossible to target a single ligand since the importance

of a particular ligand is dependent on the genetic background of the parasite strain. Thus, efforts are geared toward making a multicomponent vaccine that would target a number of high priority invasion related ligands (Hutchings *et al.*, 2007; Williams *et al.*, 2012; Draper *et al.*, 2015).

2.0.5 The *P. falciparum* erythrocyte invasion process.

Invasion of erythrocytes by *P. falciparum* is a multi-stage process involving several parasite ligand-erythrocyte receptor interactions (Figure 2.2). The process involves an initial attachment of the merozoite to the erythrocyte surface, re-orientation to juxtapose the apical end of the parasite to the erythrocyte plasma membrane, formation of a tight junction, and finally, entry into the erythrocyte with concomitant shedding of merozoite surface proteins (Cowman and Crabb, 2006; Srinivasan *et al.*, 2011; Paul *et al.*, 2015a; Cowman *et al.*, 2017).

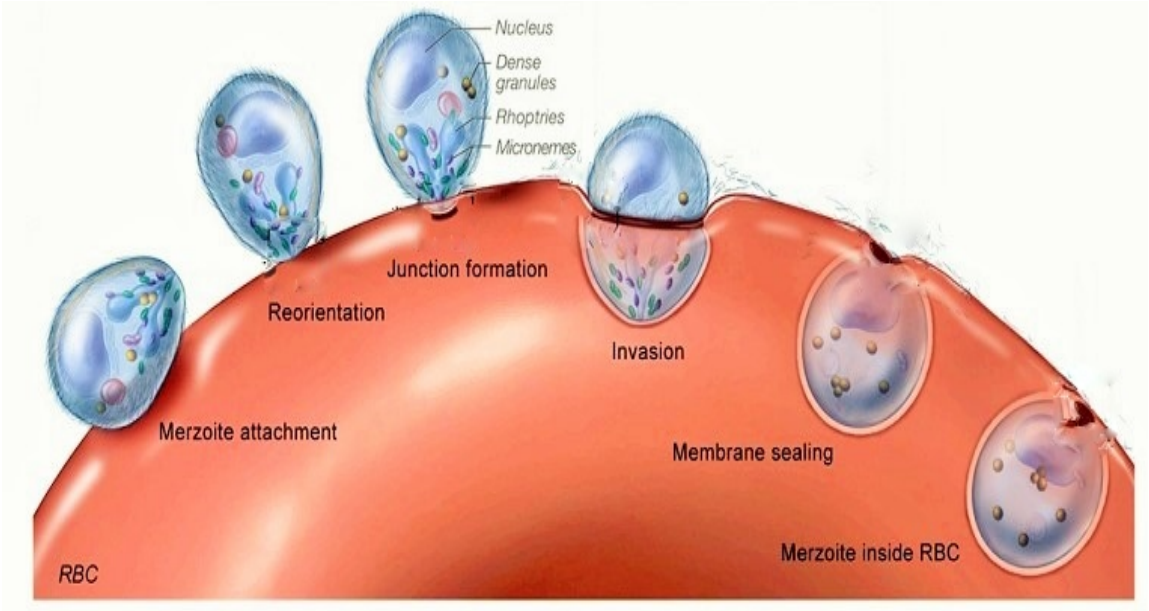

Figure 2. 2: Multi-step events of erythrocyte invasion by *P. falciparum* (Srinivasan *et al.*, 2011).
Upon initial attachment to erythrocytes the merozoite re-orients itself to bring its apical end in close proximity with the erythrocyte. The re-oriented parasite engages in strong interaction with erythrocyte surface receptors through micronemal and rhoptry secreted ligands leading to the formation of tight and moving junctions which drive the parasite into the erythrocyte aided by the parasites actinomyosin motor. Invasion is accompanied by the shedding of merozoite surface proteins and the subsequent sealing of the erythrocyte membrane to enclose the parasite.

The merozoite erythrocyte invasion machinery is consigned to organelles at the apical end of the parasite with notable ones being the micronemes and rhoptries (Blackman and Bannister, 2001; Cowman *et al.*, 2012; Weiss *et al.*, 2015). The micronemes contain proteins belonging to the erythrocyte binding antigens/ligands (EBAs/EBLs), made up of EBA-175, EBA-140, EBA-181, EBA-165 and EBL-1, which are orthologues of the *P. vivax* Duffy Binding Ligands (DBLs), as well as apical membrane antigen-1 (AMA-1) among other invasion related ligands (Bannister *et al.*, 2003; Alexander *et al.*, 2006; Collins *et al.*, 2009; Srinivasan *et al.*, 2011). On the other hand, the rhoptries contain proteins of the *P. falciparum* reticulocyte binding-like homologues (PfRHs) which include RH1, RH2a, RH2b, RH4 and RH5 (Counihan *et al.*, 2013; Weiss *et al.*, 2015). The RH proteins are also orthologues of the *P. vivax* reticulocyte binding proteins. Other invasins in the rhoptry are the rhoptry neck protein (RON) complex (Cao *et al.*, 2009; Proellocks *et al.*, 2010; Richard *et al.*, 2010; Counihan *et al.*, 2013) and the rhoptry bulb heavy proteins (RhopH) (Counihan *et al.*, 2013; Ito *et al.*, 2017; Sherling *et al.*, 2017). The merozoite surface proteins (MSPs) which form a coat around the parasite are the other major parasite ligands involved in host receptor engagement during invasion (Weiss *et al.*, 2015).

Upon initial attachment the parasite engages in strong receptor-ligand interactions which allows the merozoite to reorient itself and also deform the host erythrocyte. Micronemal discharge leads to engagement of the EBAs with their cognate receptors and subsequently signaling rhoptry content discharge. In addition to the RHs, the rhoptries contain the RON complex which include RON 2, 4 and 5. While RON4 and RON5 are discharged into the host erythrocyte, RON2 is inserted into the plasma membrane and serves as a parasite-derived receptor for AMA1 to form the tight junction (Cao *et al.*, 2009; Collins and Blackman, 2011; Riglar *et al.*, 2011; Srinivasan *et al.*, 2011; Bargieri *et al.*, 2014). The tight junction is anchored at the cytoplasmic end of AMA1

by the parasite's actinomyosin motor which provided the traction force for entry into the host erythrocyte (Perrin *et al.*, 2018).

2.0.6 Receptor-ligand interactions during erythrocyte invasion

The sequential steps leading to invasion of erythrocyte requires specific ligand-receptor interactions (Figure 2.3). The MSPs, chiefly dominated by the glycosylphosphatidylinositol (GPI)-anchored MSP1 are involved in the initial attachment of the parasite to erythrocytes (Harvey *et al.*, 2012). Though the receptor for MSP1 is unknown, it is believed to be a glycoprotein with heparin-like properties since the processed MSP1 fragments MSP1-42 and MSP1-33 bind heparin; which blocks initial stages of erythrocyte invasion (Boyle *et al.*, 2010). However, heparin-mediated invasion inhibition occurs at a step later than initial attachment, suggesting that either MSP1 may be involved at a later stage (Weiss *et al.*, 2015) or heparin may be interacting with other invasion related ligands involved in re-orientation and tight junction formation (Kobayashi *et al.*, 2013).

The reticulocyte binding-like homologues and erythrocyte binding antigens are implicated in re-orientation and strong interactions leading to junction formation. The most studied member of the EBAs, EBA175, binds to glycophorin A (Orlandi *et al.*, 1992; Sim *et al.*, 1994; Tolia *et al.*, 2005; Bei and Duraisingh, 2012; Tham *et al.*, 2012) while EBA140 binds glycophorin C (Maier *et al.*, 2003). Although glycophorin B has been identified as the receptor for EBL1 (Mayer *et al.*, 2009), glycophorin B usage is observed in studies with *P. falciparum* strains that have mutated or deleted EBL1 (Gaur *et al.*, 2003; Dankwa *et al.*, 2017), suggesting that an alternative receptor with similar properties as the EBAs may be involved. The receptor for EBA181 has been described as neuraminidase and chymotrypsin sensitive but trypsin resistant (Gilberger *et al.*, 2003), hypothetically named W. With the exception of RH4 and RH5 which bind CR1 (Spadafora *et al.*, 2010; Tham *et al.*, 2010), and basigin (Crosnier *et al.*, 2011), respectively, receptors for the other

members of the RH family have remained elusive; despite the receptor for RH1 (receptor Y) being described as neuraminidase sensitive but trypsin and chymotrypsin resistant (Rayner *et al.*, 2001; Triglia *et al.*, 2005; Gao *et al.*, 2008) while that of RH2b (receptor Z) is neuraminidase and trypsin resistant but chymotrypsin sensitive (Duraisingh *et al.*, 2003b; Aniweh *et al.*, 2016). The receptor for RH2a is sensitive to chymotrypsin treatment but different fragments exhibit differential sensitivities to neuraminidase and trypsin (Gunalan *et al.*, 2011).

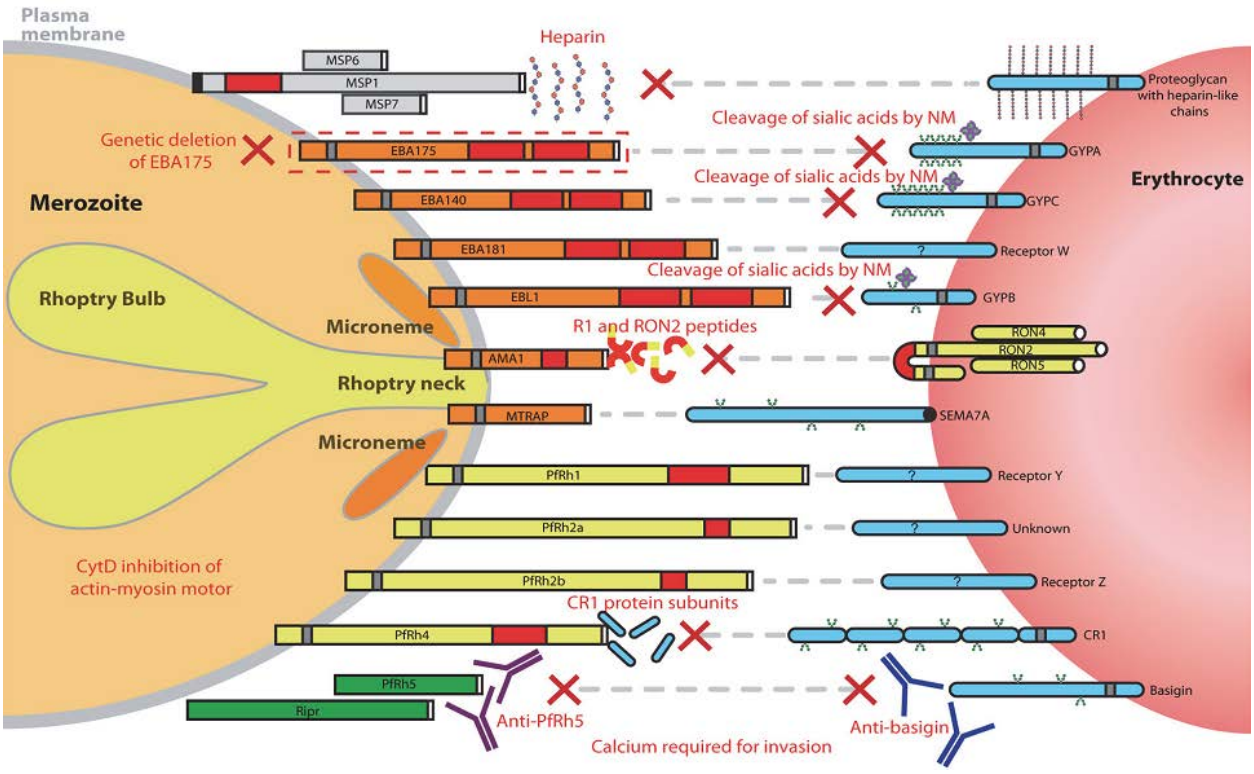

Figure 2. 3: Receptor-ligand interactions during erythrocyte invasion (Weiss *et al.*, 2015). The merozoite actively invade erythrocytes by interactions with host surface receptors. While the MSPs form a complex (shown in grey) involving MSP6, MSP1 and MSP7, and bind to heparin-like receptors, the EBAs (shown in orange) resident in the micronemes (orange) interact with sialated glycoproteins on the erythrocytes. AMA1 forms complex with RON4, RON2 and RON5 which is crucial for invasion. The RHs reside in the rhoptry neck of the matured merozoite and bind to varied receptors, with RH4 binding to CR1 and RH5 binding to basigin, while the receptors for RH1 and RH2 remain elusive. The AMA1-RON2 interaction is a unique case where the parasite provides its own ligand and receptor, respectively, and thus is independent of host determinants. Each of these interactions can be interrupted using techniques such as enzyme treatment, chemical inhibition and competitive antibody or peptide inhibition.

Considering our knowledge of the receptor-ligand engagements mediating invasion, the intricacies of the entire process in respect of the specific functions of individual interactions are unclear. Furthermore, the functions of these ligands appear to be dependent on the genetic background of the strain studied (Duraisingh *et al.*, 2003a; Triglia *et al.*, 2005). Studies aimed at delineating the temporal molecular interactions leading to invasion revealed that interactions of the EBAs and RH4 with their cognate receptors are required for strong anchorage of the parasite and deformation of the erythrocytes, whereas the RH5-basigin interaction is required for rhoptry discharge, leading to tight junction formation through AMA1-RON2 interactions (Figure 2.4) (Weiss *et al.*, 2015). The RH5-basigin interaction has also been shown to alter the cytoskeletal architecture of the erythrocyte and thus making it amenable to parasite entry (Aniweh *et al.*, 2017) .

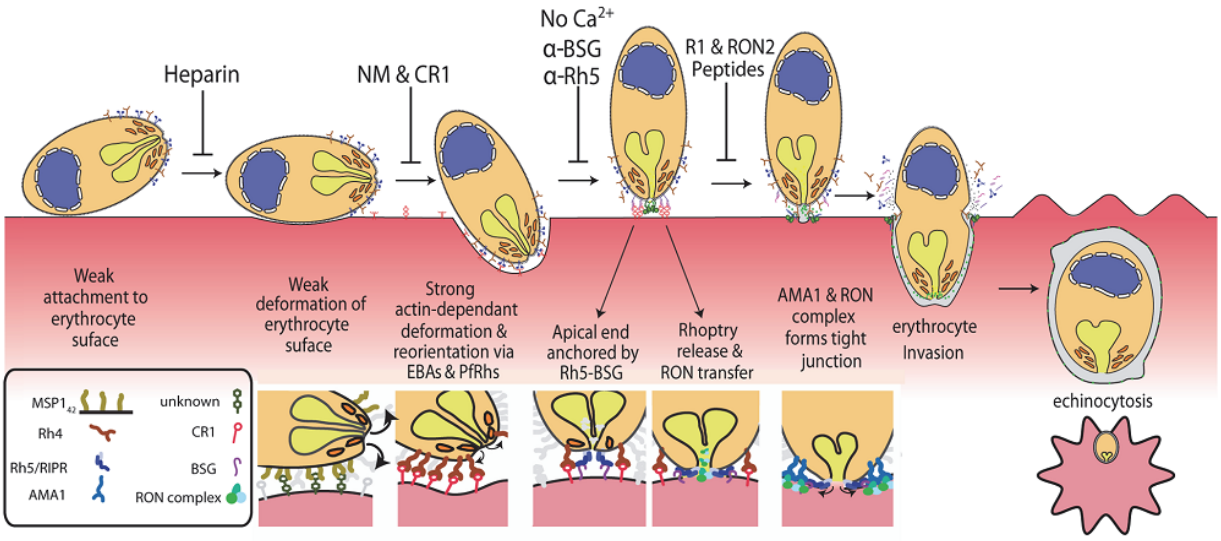

Figure 2. 4: Sequential interaction during erythrocyte invasion (Weiss *et al*., 2015). Erythrocyte invasion is a coordinated process which occurs in a sequence orchestrated by ligand-receptor interactions. Weak erythrocyte deformation after initial attachment precedes strong attachment and deformations mediated by the EBAs and RHs during reorientation. Erythrocyte deformation is abrogated by the absence of SA or the presence of anti-CR1 antibodies; confirming the role of the EBAs and RH4 in the process. Reorientation and deformation is followed by interaction of RH5 with basigin leading to the release of Ca^{2+} ions and rhoptry content discharge; confirmed by the absence of these processes in the presence of anti-RH5 and anti-basigin antibodies. AMA1 then engages the RON complex discharged into the erythrocyte to form the tight junction prior to invasion. Interaction of the cytoplasmic tail of AMA1 with the parasite's actinomyosin motor provides the traction force for entry into the erythrocyte, with concurrent shedding of merozoite surface antigens. Discharge of rhoptry content into the erythrocyte leads to echinocytosis (spiking of the erythrocyte membrane). The rhoptry components released into the erythrocyte also form part of the parasitophorous vacuole which encloses the parasite within the erythrocyte.

In relation to mechanism, it was previously proposed that interaction between the EBAs and their respective receptors initiate a series of signaling cascades leading to rhoptry discharge (Singh *et al.*, 2010), a phenomenon likely mediated by the parasite calcineurin (Singh *et al.*, 2014). However, other studies have opposed these assertions (Gao *et al.*, 2013b; Paul *et al.*, 2015b; Aniweh *et al.*, 2016). The counter argument is that the interaction of RH1 (Gao *et al.*, 2013a) and RH2b (Aniweh *et al.*, 2016) play a sensing role leading to an increase in intracellular calcium ion (Ca^{2+}) concentrations and the subsequent release of EBA175 and other components of the invasion

machinery. While RH1 functions specifically prior to the formation of the tight junction (Gao *et al.*, 2008; Gao *et al.*, 2013b), both RH2a and RH2b have been observed as part of the moving junction together with RH4 and RH5 (Gunalan *et al.*, 2011; Weiss *et al.*, 2015; Aniweh *et al.*, 2016). Both RH4 and RH5 have been shown to play signaling roles (Tham *et al.*, 2015; Weiss *et al.*, 2015), thus, supporting the hypothesis that the RHs likely serve as sensors of erythrocytes prior to stronger ligand-receptor interactions (Gunalan *et al.*, 2013).

2.0.7 Erythrocytes invasion mechanisms

As an obligate intracellular parasite, erythrocyte invasion is central to the survival, propagation and transmission of the *Plasmodium* parasite. Erythrocyte invasion is an active process requiring ligand-receptor interactions. Earlier invasion studies with different donor blood showed that erythrocytes of the En(a-), Tn and M^kM^k phenotypes, all of which lack sialic acids, were resistant to *P. falciparum* invasion (Miller *et al.*, 1977; Pasvol *et al.*, 1982a; Pasvol *et al.*, 1982b; Mitchell *et al.*, 1986; Hadley *et al.*, 1987; Bei and Duraisingh, 2012; Tham *et al.*, 2012; Bartholdson *et al.*, 2013). Likewise soluble membrane fractions containing GPA inhibited erythrocyte invasion (Deas and Lee, 1981; Perkins, 1981; Baldwin *et al.*, 2015). Neuraminidase and/or trypsin treatment of erythrocytes resulted in a similar phenotype (Dolan *et al.*, 1990; Dolan *et al.*, 1994; Thompson *et al.*, 2001; Baum *et al.*, 2003). Together, these studies led to the identification of the glycoproteins and sialic acids as requirements for erythrocyte invasion. However, a large body of evidence shows that both laboratory and clinical isolate of *P. falciparum* are capable of invading erythrocytes independent of sialic acids (Mitchell *et al.*, 1986; Hadley *et al.*, 1987; Okoyeh *et al.*, 1999; Lobo *et al.*, 2004; Deans *et al.*, 2007; Gomez-Escobar *et al.*, 2010; Mensah-Brown *et al.*, 2015). Thus, depending on the sialic acid requirement, *P. falciparum* erythrocyte invasion strategies are broadly classified into two phenotypes; sialic acid dependent and sialic acid independent.

Members of the EBA family of proteins mainly bind to sialic acid moieties on host erythrocyte surface receptors, evidenced by their inability to bind neuraminidase-treated erythrocytes (Bei and Duraisingh, 2012; Tham *et al.*, 2012; Weiss *et al.*, 2015). Similarly, binding of RH1 to host erythrocytes is SA-dependent (Rayner *et al.*, 2001; Triglia *et al.*, 2005; Gao *et al.*, 2008). Thus, the EBAs and RH1 mediate the SA-dependent invasion pathway. Conversely, the other members of the RH family of proteins largely mediate the SA-independent pathway as they bind host erythrocytes in the absence of sialic acids (Triglia *et al.*, 2009; Crosnier *et al.*, 2011; Gunalan *et al.*, 2011; Tham *et al.*, 2012; Aniweh *et al.*, 2016).

The invasion pathways utilized by *P. falciparum* strains are elucidated by assessing their ability to invade erythrocytes lacking specific surface receptors. Thus, erythrocytes null for specific surface receptors either by natural occurrence or genetic modification, as well as enzyme treatment are used for invasion phenotype identification *in vitro* (Mitchell *et al.*, 1986; Hadley *et al.*, 1987; Bei *et al.*, 2010; Gomez-Escobar *et al.*, 2010; Egan *et al.*, 2015; Mensah-Brown *et al.*, 2015; Dankwa *et al.*, 2017; Awandare *et al.*, 2018). Various erythrocyte surface receptors have been identified to be sensitive to particular enzymes (Figure 2.5). For instance, neuraminidase removes sialic acid residues on glycosylated proteins, trypsin cleaves off the peptide backbone of glycophorins A, C and CR1, while chymotrypsin cleaves glycophorin B, band 3 and CR1 on the surfaces of erythrocytes (Bei and Duraisingh, 2012; Tham *et al.*, 2012). The selective sensitivity of invasion related receptors to these enzymes has provided a tractable tool for the study of receptor-ligand interactions required by *P. falciparum* strains/isolates for erythrocyte invasion.

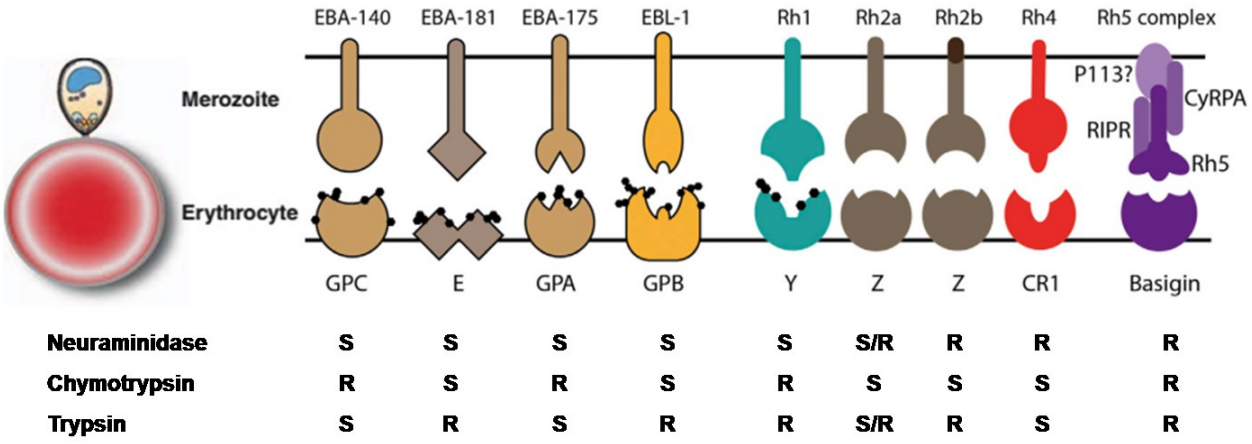

Figure 2. 5: Sensitivity of receptor-ligand interaction to enzyme treatment.
With the exception of the RH5-basigin binding, the various receptor-ligand interactions can be disrupted by enzyme treatments. Interaction of all the EBAs and RH1 are sensitive to neuraminidase treatment. Chymotrypsin cleaves glycophorin B and the receptors for EBA181, RH2a and RH2b, while trypsin cleaves glycophorins A and C. In addition, both enzymes cleave CR1. Sensitivity of RH2a to neuraminidase and trypsin is dependent on the proteolytic processing of the ligand, with different fragments showing differential sensitivity to the two enzymes.

2.0.8 Host immune evasion mechanisms by *Plasmodium falciparum* merozoites

The merozoite is the only extracellular form of the parasite during the erythrocytic stage and is thus liable to recognition by the immune system of the host. At this stage, the parasite is at risk of recognition by the host's parasite-specific antibodies that could opsonize it for easy clearance by phagocytic cells, sterically block ligand-receptor interactions required for erythrocyte invasion or activate the complement system (Wright and Rayner, 2014). To survive, the parasite deploys strategies to counteract the potential threat posed by the host's immunological response to the infection (Figure 2.6). Understanding these immune evasion mechanisms is key to the quest for identifying vaccine targets.

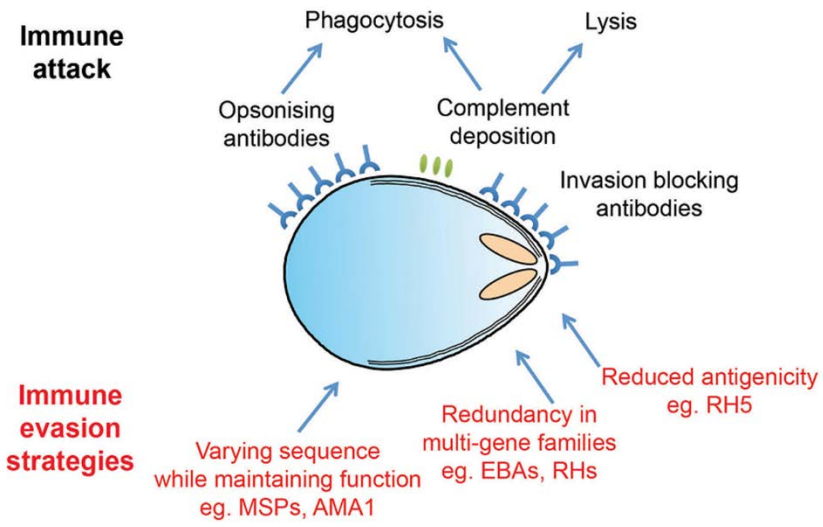

Figure 2. 6: Immune evasion mechanisms by *P. falciparum* (Wright and Rayner, 2014). Release merozoites are exposed to immunological attack by the vertebrate host. Host immunological responses include invasion inhibition by antibodies against invasion related proteins and opsonization by complement and parasite specific antibodies. The parasite counteracts host immune responses by varying the sequences of antigens used for invasion, presenting multiple ligands with complementary and overlapping functions for erythrocyte invasion and possessing antigens that are less immunogenic.

A major challenge towards the development of an invasion-blocking vaccine is the presence of multiple-member protein families in the genome of *Plasmodium falciparum*; the EBAs and RHs. With the exception of RH5 (Baum *et al.*, 2009; Crosnier *et al.*, 2011; Mensah-Brown *et al.*, 2015) and its interacting partners, RH5-interacting protein (RIPR) (Chen *et al.*, 2011), Cysteine-rich protective antigen (CyRPA) (Reddy *et al.*, 2015), and P113 (Galaway *et al.*, 2017), all other members of the EBAs and RHs have been shown to be non-essential for invasion (Duraisingh *et al.*, 2003a; Duraisingh *et al.*, 2003b; Stubbs *et al.*, 2005; Triglia *et al.*, 2005; Lopaticki *et al.*, 2011). Likewise, although AMA1 is thought to be required for erythrocyte invasion (Srinivasan *et al.*, 2011; Srinivasan *et al.*, 2013; Yap *et al.*, 2014), other studies have shown it to be dispensable (Bargieri *et al.*, 2013). Another level of complexity is the observation that mutations in some of these ligands leads to alterations in their receptor specificities (Maier *et al.*, 2009). Having multiple

copies of invasion related ligands thus offers a competitive advantage to the parasite in terms of overcoming either humoral and cell-mediated immunological responses to particular ligands, or differences in receptor availability on host erythrocytes.

The presence of multiple members of the EBAs and RHs presents the parasite with an array of invasion ligands to depend on. To date, all members of the EBAs have been shown to be sensitive to neuraminidase treatment, suggesting a conserved function. The RHs on the contrary are resistant to neuraminidase treatment, except for RH1. A plethora of studies with laboratory strains of *P. falciparum* have shown that increased expression of the RHs are observed when either the receptors or ligands for SA-dependent invasion are absent (Stubbs *et al.*, 2005; Gaur *et al.*, 2006; Lopaticki *et al.*, 2011). Clinically, it has been observed that immunity to the EBAs precede that of the RHs (Persson *et al.*, 2008). These observations suggest the emergence of the RHs to compensate for the loss of the SA-dependent invasion pathway either due to immune response to the ligands mediating the pathway or the variability in host erythrocyte receptors.

Apical membrane antigen 1 (AMA1) has been shown to be a crucial component of the invasion machinery, forming the tight junction by interacting with RON2 (Srinivasan *et al.*, 2011; Weiss *et al.*, 2015). Since formation of the tight junction is the committed step for invasion, inhibiting the AMA1-RON2 interaction is likely to abrogate invasion. Luckily, clinical studies have shown robust antibody responses to AMA1 (Polley *et al.*, 2004; Chelimo *et al.*, 2005; Cortés *et al.*, 2005). However, an AMA1-based vaccine has not been successful largely due to the high polymorphic nature of the gene (Remarque *et al.*, 2008). Thus, antibodies against AMA1 from one *P. falciparum* strain is unlikely to offer protection against infections with another strain. Attempts to mitigate the challenge with polymorphisms in AMA1 by immunization with AMA1 from different strains have been promising but unsuccessful thus far (Kusi *et al.*, 2009; Kusi *et al.*, 2011). Again, although

immunization of mice with *P. yeolii* AMA1-RON2 complex showed protection against severe disease, it was not strain transcending (Srinivasan *et al.*, 2014). The merozoite surface proteins (MSPs) which are the most abundant merozoite antigens and most prominently exposed to immune detection also show sequence polymorphisms and present similar challenges as AMA1 (Crompton *et al.*, 2010). The polymorphisms exhibited by these antigens depict the classical strategy deployed by the parasite to overcome host immune response.

Despite RH5 and its interacting partners RIPR, CyRPA and P113 being essential for invasion (Baum *et al.*, 2009; Chen *et al.*, 2011; Crosnier *et al.*, 2011; Mensah-Brown *et al.*, 2015; Reddy *et al.*, 2015; Galaway *et al.*, 2017; Awandare *et al.*, 2018) and that antibodies against these antigens correlate positively with protection against malaria (Tran *et al.*, 2013; Chiu *et al.*, 2014; Partey *et al.*, 2018), humoral responses to them are low in natural infections (Payne *et al.*, 2017; Partey *et al.*, 2018), possibly due to the transient exposure of the antigens. Additionally, humoral immune responses against RH5 and its interacting partners are short-lived (Partey *et al.*, 2018). Also, studies with *P. falciparum* clinical isolates showed that blocking the RH5-basigin interaction is not enough to completely ablate erythrocyte invasion (Mensah-Brown *et al.*, 2015). Again, affinity purified RH5-specific IgG from natural infections could not completely inhibit erythrocyte invasion at concentration in excess of 500 µg/ml (Patel *et al.*, 2013). Together, these observations show that RH5 is not an excellent immunogen, and that a sub-unit vaccine based on RH5 and its interacting partners alone may not provide adequate protection.

In addition to adaptive immunity the innate component of the vertebrate immune system also responds to parasite presence. The exposed merozoite is liable to complement-mediated lysis or opsonization for clearance by phagocytes (Boyle *et al.*, 2015). However, the parasite appears to exploit the complement system for its survival. The most prominent of these is the parasite's

dependence on interaction with CR1 (Spadafora *et al.*, 2010; Tham *et al.*, 2010; Awandare *et al.*, 2011b) and DAF (Egan *et al.*, 2015) for erythrocyte invasion. Furthermore, the presence of an active complement aids erythrocyte invasion and parasite growth (Biryukov *et al.*, 2016). The working theory is that the complement component of serum aids the parasite to interact with CR1, thus enhancing invasion. Consequently, a vaccine strategy targeting complement activation would likely enhance parasite growth and therefore disease severity.

2.0.9 Invasion phenotype variation by *Plasmodium falciparum*

In vitro studies of invasion phenotype switching mechanisms require either modification of erythrocyte surface with enzymes or genetically manipulating parasites to elucidate compensatory mechanisms in the absence of preferred invasion pathways. For instance, cultivation of Dd2, W2mef and CSL2 (SA-dependent strains) in neuraminidase-treated erythrocytes results in the selection of clones which can effectively invade through the SA-independent route (Dolan *et al.*, 1990; Stubbs *et al.*, 2005). Likewise, knocking out EBA-175 in these parasite lines result in a SA-independent invasion phenotype (Reed *et al.*, 2000; Duraisingh *et al.*, 2003a; Stubbs *et al.*, 2005). In both scenarios, there is increased expression of RH4 and EBA-165 (Stubbs *et al.*, 2005; Gaur *et al.*, 2006; Desimone *et al.*, 2009). However, the invasion phenotype of 3D7 (an SA-independent strain) is not affected by either culturing with SA deficient erythrocytes or knocking out EBA-175 (Duraisingh *et al.*, 2003a). Systematically knocking out members of the EBAs leads to increased expression of other members of the EBAs or RHs, suggesting the deployment of a compensatory mechanism to account for the lost ligand (Lopaticki *et al.*, 2011). Nonetheless, knocking out the RH2b gene of 3D7 results in a switch in invasion phenotype to a pathway insensitive to chymotrypsin treatment but with no change in the expression of any member of either the EBAs or RHs except the transcribed pseudogene RH3 (Baum *et al.*, 2005); suggesting the availability of

compensatory ligands that are less dominant in the presence of RH2b. Cumulatively, these evidences unambiguously show that different strains of *P. falciparum* have different ligand requirements for erythrocyte invasion and phenotypic switching, and highlight the presence of compensatory invasion mechanisms and thus the plasticity of the parasite in overcoming receptor heterogeneity among potential host erythrocytes as well as immune responses against invasion related ligands.

2.0.10 The *Plasmodium* genome.

Plasmodium falciparum has an estimated 23 mega-bases (Mb) genome, organized into 14 linear chromosomes containing approximately 5400 open reading frames (ORFs) (Gardner *et al.*, 2002; Liew *et al.*, 2010; Otto *et al.*, 2014), of which about 88 % and 60 % are expressed during the entire life (Le Roch *et al.*, 2003) and intra-erythrocytic cycles (Bozdech *et al.*, 2003; Llinas *et al.*, 2006; Foth *et al.*, 2011), respectively. In addition to the nuclear DNA is a 35 kilo-base (kb) circular plastid (apicoplast) DNA and a 6 kb linear mitochondrial DNA (Gardner *et al.*, 2002; Narayan *et al.*, 2012). The nucleotide bases of the *P. falciparum* genome are approximately 80% adenine (A) and thymidine (T), making it one of the genomes with the highest A-T content. Comparatively, *P. knowlesi* has a relatively lower A-T content of 61% while *P. vivax*, *P. berghei* and *P. chabaudi* have approximately 62%, 76% and 75% A-T contents, respectively (Carlton *et al.*, 2004). Of the over 5000 *P. falciparum* genes approximately 60 % are hypothetical; unique to this parasite and thus have no orthologues in other organisms to which functions could be inferred (Gardner *et al.*, 2002). Structurally, the genomes of the *Plasmodia* are highly conserved across closely related species (Hall *et al.*, 2005; Kooij *et al.*, 2005). However, high levels of variability occur at the telomeric and sub-telomeric regions which usually contain virulence genes such as *var*, *stevor* and *rifin*. Similarly, genomic mapping studies show high degrees of conservation and synteny of

centrally placed genes between different species of *Plasmodium*, with major differences occurring at the telomeres and sub-telomeres (Hall *et al.*, 2005; Kooij *et al.*, 2005; Liew *et al.*, 2010). A genome scale functional screen of the rodent model *P. berghei* showed that at least two thirds of the genome is required for survival (Bushell *et al.*, 2017). Similarly, a mutagenic screen of *P. falciparum* identified approximately 50 % of genes to be essential for growth at the blood stage (Zhang *et al.*, 2018). The large proportions of the Plasmodium genome being required for normal growth could possibly be due to genomic shrinkage as a result of evolution and adaptation to parasitic life (Woo *et al.*, 2015). Knowledge of the essential parts of the genome is a critical step towards the identification of viable therapeutic targets (White and Rathod, 2018).

2.0.11 Mechanisms for phenotypic variation.

The complexity of the *Plasmodium* life cycle requires the parasite to adapt to life in different niches, traverse different cells and adopt different morphologies. In both hosts, the parasite must persist long enough to ensure successful transmission. In addition, the parasite needs to deal with the immune systems of both hosts. To survive the parasite ought to accurately sense its environment and control the set of genes to express at a particular time.

A common strategy deployed by parasites in overcoming dangers from the host is by varying the antigens they express on their surfaces, thus adopting new phenotypes on successive host colonization (Duraisingh and Skillman, 2018). In *Plasmodium*, antigenic variation is achieved by various strategies. The most prominent is the presence of multi-gene families within the parasite genome. For instance, there are an estimated 60, 36 and 200 genes belonging to the *var*, *STEVOR*, and *RIFIN* gene families, respectively (Kyes *et al.*, 1999; Gardner *et al.*, 2002; Blythe *et al.*, 2009; Cunningham *et al.*, 2010). Likewise, there are 5 members each of the EBAs and RHs, respectively, and approximately 10 members of the MSPs in each parasite genome (Cowman *et al.*, 2017). The

presence of multigene families arose possibly due to gene copy number amplification coupled with ectopic recombination and accumulated genetic mutation (Hinterberg *et al.*, 1994; Freitas-Junior *et al.*, 2000; Barry *et al.*, 2003; Kooij *et al.*, 2005; Dvorin *et al.*, 2010). Over time some of the genes acquire functions that may be complementary or slightly different from the ancestral gene (paralogues). Cumulatively, possessing multigene families and polymorphic antigens allows the parasite to vary its antigens during successive generations to overcome host immune responses and variations in the host receptors that the antigens interact with, thus ensuring survival of the parasite.

Having multi gene families requires gene regulation mechanisms to ensure only a subset of these genes are expressed temporally. A key mechanism for gene regulation in *Plasmodium* is epigenetics (Figure 2.7). Modification of histone components of the chromatin ensures the accessibility of DNA to the transcription machinery or otherwise. Methylation and acetylation are the best studied histone modifications governing gene expression in *Plasmodium*. At the centre of epigenetic gene regulation is the heterochromatin protein 1 (HP1). HP1 binds to di and tri-methylated lysine 9 residue on the histone 3 (H3K9me3) and recruits histone methyl-transferases which modifies nearby nucleosomes; thus, forming a feedforward loop of heterochromatin formation and gene silencing (Jiang *et al.*, 2010; Coleman *et al.*, 2012; Volz *et al.*, 2012; Jiang *et al.*, 2013). Histone methylation is preceded by the removal of acetyl groups by histone modifying enzymes such as the *P. falciparum* homologues of the yeast silent information regulator 2, PfSir2 (Duraisingh *et al.*, 2005; Freitas-Junior *et al.*, 2005; Tonkin *et al.*, 2009) and the *P. falciparum* histone deacetylase 2 (PfHda2) (Coleman *et al.*, 2014). Contrary to gene silencing by H3K9me3, other histone methylations such as H3K4me3, H4K20me1, and acetylation of specific histone

residues such as H3K9ac, H4K8ac, H4K16ac leads to transcriptional activation (Jiang *et al.*, 2010; Gupta *et al.*, 2013).

In a recent study, the *Plasmodium falciparum* gametocyte development 1 (PfGDV1) protein was shown to alleviate HP1-dependent gene silencing by evicting HP1 from the heterochromatin, thus making the underlying DNA sequence transcriptionally accessible (Filarsky *et al.*, 2018). Clonally variant expression of the virulent var genes, the *clag* multigene family responsible for nutrient acquisition from the host, the invasion related ligand RH4 and the master regulator of sexual commitment AP2-G are all regulated by HP1-mediated gene silencing (Brancucci *et al.*, 2014; Coleman *et al.*, 2014; Sinha *et al.*, 2014; Duraisingh and Skillman, 2018). Co-regulation of parasite virulence gene expression and sexual commitment is likely an evolutionary mechanism to streamline antigenic variation with sexual reproduction to ensure that the next generation of parasites express antigens different from those expressed by the parent; a biological phenomenon that will lead to immune evasion by the progeny.

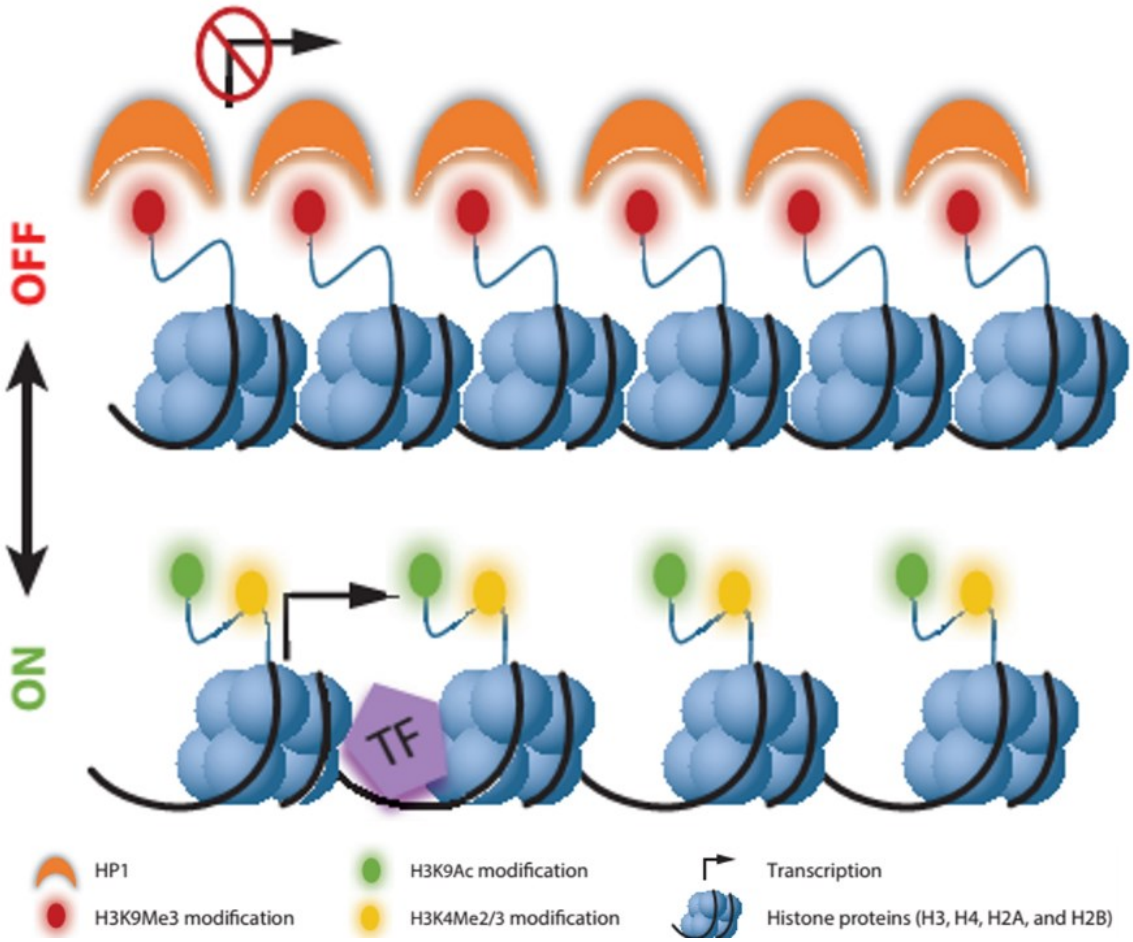

Figure 2. 7: Epigenetic gene regulation in *P. falciparum* (Duraisingh and Skillman, 2018). Histone modifications at the nucleosome defines the transcriptional status of a gene. Trimethylation of the lysine 9 residue on histone 3 leads to the formation of heterochromatin through the binding of the heterochromatin protein 1, thus silencing genes whose promoters are within modified nucleosome. Histone demethylation and acetylation leads to the formation of euchromatin, thus making the promotor region of genes accessible to the transcription machinery.

Another level of gene regulation which complements epigenetics is the nuclear positioning of the gene's locus within the nucleus (Figure 2.8). The nucleus of the parasite appears to form micro-domains, with repressive and active domains localized to the nuclear periphery (Duraisingh *et al.*, 2005; Coleman *et al.*, 2012; Duraisingh and Horn, 2016; Duraisingh and Skillman, 2018). While the active domain contains the transcription machinery, silent domain appears to lack the transcription machinery required for successful gene expression. Thus, gene expression only

occurs when an epigenetic and transcriptionally active gene is positioned in the activation domain of the nucleus. This level of gene regulation has been described for the var genes, *RIFINs* and *RH4* (Duraisingh *et al.*, 2005; Coleman *et al.*, 2012). In the instance of the var genes, only one out of the approximately 60 members is positioned at the active domain during a single growth cycle. Repositioning to this domain occurs after the var gene has been transcriptionally activated by the epigenetic modifications at its locus. When another gene gets activated it moves into the activation domain while the previously active gene moves to the silent domain; thus leading to antigenic variation (Duraisingh and Horn, 2016; Duraisingh and Skillman, 2018).

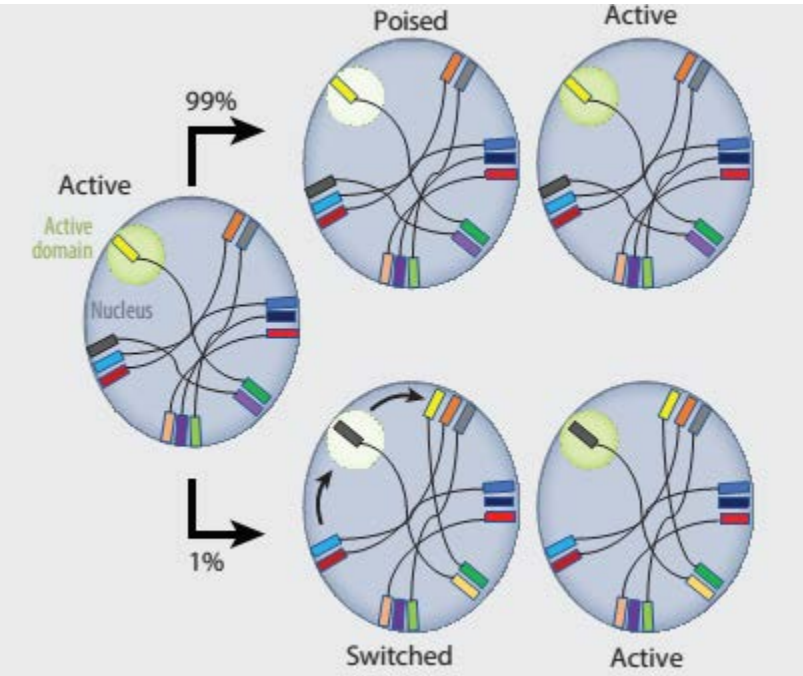

Figure 2. 8: Gene regulation by nuclear positioning (Duraisingh and Skillman, 2018).
The nuclear periphery forms activation and silencing domains which regulate gene expression. The activation domain provides the optimal environment for the transcription of genes whose promoters are accessible to the transcription machinery. Transcriptionally active genes are switched on and off by their positioning either within the activation domain or silencing domains, respectively. During proliferation, genes within the active domain remain in a poised state ready for transcription at the onset of the next cycle.

2.0.12 The evolutionary arms race between parasite and host

The malaria parasite is one of the most successful human parasites, and arguably the most selective force on the evolution of the human genome. Conditions such as sickle cell anaemia, thalassemia and glucose-6-phosphate dehydrogenase deficiency have direct correlations with the incidence of malaria (Fortin *et al.*, 2002; Kwiatkowski, 2005; Parikh and Rosenthal, 2008; Ndila *et al.*, 2018). Notwithstanding, the presence of the malaria parasite impacts the expression and extent of sequence variation of erythrocyte surface receptor genes (Mayer *et al.*, 2004; Kwiatkowski, 2005; Ko *et al.*, 2011; Idaghdour *et al.*, 2012; Network, 2015; Dankwa *et al.*, 2017; Leffler *et al.*, 2017; Ndila *et al.*, 2018). Notably, the absence of the chemokine receptor *FY* in most Africans is believed to be an evolutionary response to *P. vivax* infection (Chitnis and Miller, 1994; Kwiatkowski, 2005; Egan, 2017). Similarly, the high rate of polymorphisms in the glycophorins, and other invasion-mediating erythrocyte surface receptors in people living in malaria endemic regions is thought to be an adaptation against parasite assault on the erythrocytes (Mayer *et al.*, 2004; Kwiatkowski, 2005; Egan, 2017; Leffler *et al.*, 2017; Ndila *et al.*, 2018).

The evolutionary arms race between parasite and the vertebrate host extends beyond genomics to physiology. Classical example is studies with *P. chabaudi* which showed that parasite differentiation and proliferation depends on the circadian cycle of its host (Hirako *et al.*, 2018). It was realized that parasites differentiate from rings to trophozoites when the host is in a hypoglycemic state while schizogony and egress occurs during hyperglycemic state. Likewise, in both clinical human studies and animal models, disease severity appears to correlate positively with the nutrition status of the host (Murray *et al.*, 1975; Hunt *et al.*, 1993; Mejia *et al.*, 2015). These observations are corroborated by the observation that risk of infection and disease severity are associated with diabetes (Danquah *et al.*, 2010) and insulin resistance (Zijlmans *et al.*, 2008),

respectively. A recent study showed that a parasite-specific nutrient sensor KIN is responsible for the reduced parasite proliferation during host starvation (Mancio-Silva *et al.*, 2017); a likely parasitic evolutionary trait, given that the parasite depends more on the host for its nutritional needs as it commits most of its resources to parasitism. Another study with a mouse model showed that melatonin increases the levels of the secondary messenger cyclic adenosine monophosphate (cAMP), leading to the expression of genes which regulate the synchronous development of the parasite (Lima *et al.*, 2016). Given that melatonin is a sleep hormone produced at night, and that mice are nocturnal organisms, observations which associate melatonin with increased parasite proliferation in mice models (Hotta *et al.*, 2000; Gazarini *et al.*, 2011) suggest an evolutionary trait which synchronizes parasite proliferation with the host's nutritional status. These observations demonstrate that the parasite has co-evolved with its host such that its development is partly regulated by the physiological state of the host.

2.0.13 Conditions for *in vitro* phenotyping of *P. falciparum*

Signals accounting for invasion phenotype switching in *P. falciparum* are not well understood. Host immune response to invasion ligands and variation in receptor availability among susceptible host erythrocytes are logical candidates; demonstrated by the ability of some *P. falciparum* strains to switch invasion phenotypes by selection on enzyme treated erythrocytes or genetic deletions of invasion related ligands (Dolan *et al.*, 1990; Reed *et al.*, 2000; Duraisingh *et al.*, 2003a; Duraisingh *et al.*, 2003b; Baum *et al.*, 2005; Stubbs *et al.*, 2005). Although genetic manipulation of parasites and erythrocyte surface modification with enzymes have proven to be great tools for the study of phenotypic switching, they do not reflect the natural conditions the parasite is exposed to, given that *in vivo*, both parasite proteins and erythrocyte surface receptors are readily available for interactions leading to invasion. Considering the deficiencies associated with the current modules

for studying phenotypic switching, a more robust module which reflects *in-vivo* growth conditions is required.

Studies in yeast showed that almost the entire genome is required for growth under different conditions, although approximately 80 % of the genome is deemed dispensable in rich culture media (Hillenmeyer *et al.*, 2008); suggesting that different genes are required for growth under different culturing conditions. Culturing conditions thus have implications during *in vitro* studies of cellular biology.

The most widely used methods for culturing *P. falciparum in vitro* is based on the method described by Trager and Jensen (Trager and Jensen, 1976; Duffy and Avery, 2018), requiring that parasites are kept in micro-aerophilic environment under static conditions. However, given that the parasite spends majority of its life time circulating, variations in the culturing method has included agitation of the cells (Butcher, 1981; Dalton *et al.*, 2012; Ribacke *et al.*, 2013). Determinants of *P. falciparum* virulence such as parasite growth, multiplication rate, knob formation, cytoadherence and rosette formation have been shown to be prominent in suspended compared to static cultures (Dalton *et al.*, 2012; Ribacke *et al.*, 2013). However, no studies have exploited the impact of agitation on erythrocyte invasion. The only report thus far linking culture agitation and invasion is a study which showed that higher levels of parasite calcineurin are required for successful invasion in suspended cultures (Paul *et al.*, 2015b). Given that parasite growth is dependent on effective erythrocyte invasion, the increased growth observed in suspended compared to static cultures may be related to differences in invasion capabilities. Additionally, suspended cultures provide the rheological and micro-aerophilic conditions similar to those in the human vasculature, and thus represent a better tool for studying *P. falciparum* cell biology *in vitro*.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.0.1 Ethical approval

Ethical approval was obtained from the Institutional Review Committee of the Noguchi Memorial Institute for Medical Research, University of Ghana, and all the methods used in the study were in accordance with the guidelines and regulations provided by the ethical committee. All human erythrocytes used in this study were obtained with the consent of the donors.

3.0.2 Parasite culturing

Plasmodium falciparum strains Dd2, W2mef and 3D7 were cultured in RPMI-1640 (Sigma) supplemented with 0.5 % Albumax II (Gibco), 20 mg hypoxanthine, 2 g sodium bicarbonate (Sigma) and 0.05 mg/ml gentamicin sulfate (Sigma) using human group O^+ erythrocytes at 4 % hematocrit in a mixed gas environment of 93 % nitrogen, 5 % CO_2, and 2 % oxygen (Air Liquide, Birmingham, United Kingdom). Cultures were incubated at 37 ^{0}C in either a static incubator (*Static* culture), or with gentle shaking on an orbital shaker rotating at 44 rpm (*Suspended* cultures).

3.0.3 Enzyme treatment of erythrocyte and invasion assays

Treatment of erythrocytes with enzymes and invasion assays were done as previously described (Mensah-Brown *et al.*, 2015), with few modifications. Briefly, O^+ erythrocytes were treated with 1 mg/ml trypsin from bovine pancreas (Sigma Aldrich), 1 mg/ml chymotrypsin from bovine pancreas (trypsin activity removed by pretreatment with 1-chloro-3-tosylamido-7-amino-2-heptanone)(Sigma Aldrich) or 250 mU/mL of neuraminidase (Nm) from *vibrio cholera* (Sigma Aldrich, St. Louis, Missouri) by an hour incubation at 37 ^{0}C while agitating gently. Enzyme-treated

cells were subsequently washed with RPMI, and stained with (5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (5(6)CFDA-SE; 20 µM, Invitrogen), a cytoplasmic fluorescent stain, prior to assay plating to differentiate them (acceptor cells) from residual erythrocytes present in the parasite inoculum. Late stage parasites were mixed with acceptor cells in a 1:1 ratio at 2 % hematocrit in 96-well titre plates and incubated overnight at 37 ^{0}C in a mixed gas environment.

To determine the contributions of CR1 (CD35) and basigin (CD147) as host determinants for the invasion phenotype switching, experiments were conducted using chicken anti-human CR1 antibodies or control chicken immunoglobulin Y (IgY) (12 µg/mL, Gallus Immunotech, Fergus, Canada), and mouse monoclonal anti-human CD147 antibodies (clone MEM-M6/6; Abcam, USA) or mouse monoclonal IgG1 control (10 µg/mL; Abcam, USA) to block the two receptors, respectively. All experiments were setup in triplicate in 96-well plates under static conditions and repeated at least once. Plates were incubated overnight (~14 hours) at 37 ^{0}C with mixed gas. The cultures were then stained with Hoechst 33342 (5 µM, Sigma Aldrich) after incubation to differentiate infected erythrocytes from uninfected ones, and invasion levels determined by flow cytometry (LSR Fortessa X-20, BD) as previously described (Theron *et al.*, 2010; Mensah-Brown *et al.*, 2015). The percentage of erythrocytes that were dual positive for CFDA and Hoechst 33342 was recorded as the invasion rate.

3.0.4 Genotypic identification of parasite strains.

At week 6 when the gain of SA-independent phenotype by Suspended Dd2 and W2mef was exponential, genomic DNA was extracted from both Static and Suspended Dd2, W2mef and 3D7 using the QIAamp blood mini kit (QIAGEN, Hilden, Germany) by following the manufacturer's recommendations. The full length of the highly polymorphic Merozoite Surface Protein 2 (MSP2) gene was amplified by polymerase chain reaction using the One Taq DNA polymerase kit (New

England Biolabs Inc.) and a previously published primer pair (Frank *et al.*, 2011). Cycling conditions were as recommended by the kit manufacturer. Amplicons were resolved on an ethidium bromide-stained 1 % agarose gel and images documented with the Amersham Imager 600 (General Electric Healthcare Life Sciences, USA).

3.0.5 Estimation of erythrocyte surface receptor expression.

Freshly washed O^+ erythrocytes were inoculated with *P. falciparum* and split into two cultures, one kept static and the other shaking at 44 rpm. Both cultures were kept at 37 ^{0}C continuously for 1 week. The parasitized erythrocytes were pelleted and aliquots re-suspended at 25 % hematocrit in incomplete media. As controls, erythrocytes from the same batch kept at 4 ^{0}C were treated with phosphate buffered saline (PBS; untreated) neuraminidase (250 mU), trypsin (1 mg/ml) and chymotrypsin (1 mg/ml) at 37 ^{0}C for 1 hour. All cells were re-suspended at 25 % hematocrit with incomplete media. Primary monoclonal mouse anti-human glycophorin A (GPA, clone E4; 0.2 mg/ml; Santa Cruz biotechnology, Inc.) and mouse anti-human CR1 (clone J3D3; 0.1 mg/ml; Santa Cruz biotechnology, Inc.) were diluted in a 1:50 ratio with staining buffer (3 % BSA in PBS). A total of 10 µl each of untreated controls (UT), Nm-treated (NT), static (ST) and suspended (SP) erythrocytes were co-incubated with anti-GPA antibodies in a 96 well U-bottom plate at 37 ^{0}C for 1 hour. For CR1, same volumes of UT, ST, SP, trypsin-treated (TT), and chymotrypsin-treated (CT) erythrocytes were incubated with anti-CR1 antibodies. As negative controls, wells containing UT erythrocytes were incubated without primary antibodies and designated as unstained (US). After incubation, the cells were washed thrice with PBS and subsequently incubated for a further 1 hour with a 1:100 dilutions of phycoerythrin (PE)-conjugated polyclonal goat anti-mouse IgG (0.2 mg/ml; ThermoFisher Scientific). All cells were washed three times

afterwards and the results acquired by flow cytometry. Data were analysed with Flowjo software (V 10.0.4).

3.0.6 Determination of parasite growth rate

Parasite cultures were enriched for ring stages by pre-treatment with sorbitol solution (5 %) as previously described (Lambros and Vanderberg, 1979; Miao and Cui, 2011). For growth in untreated erythrocytes, parasitemia levels were adjusted to approximately 1 % with untreated erythrocytes. For growth in Nm-treated erythrocytes, parasitized erythrocytes were treated with neuraminidase (250 mU) for 1 hour at 37 ^{0}C. This was done to prevent SA-dependent reinvasion of uninfected erythrocytes present in parasite inoculum. In addition, uninfected erythrocytes required to sub-culture parasites were also treated with neuraminidase. The parasitemia levels were subsequently adjusted to approximately 1 % with the uninfected Nm-treated erythrocytes. All parasitemia levels were further confirmed by flow cytometry. The parasites were then cultured for two complete asexual cycles (96 hours). At the end of each cycle, parasitemia were determined by flow cytometry and growth rates calculated by dividing the parasitemia at the end of the cycle by the starting parasitemia.

3.0.7 Expression analyses of invasion related genes

Schizont stage parasites were purified from cultures using the percoll-alanine density gradient centrifugation method. Briefly, parasitized erythrocytes suspended at 50 % hematocrit were layered over a percoll-alanine density gradient comprising of 90 %, 70 % and 40 % peroll-alanine (from bottom to top, respectively) in a 15 mL centrifuge tube. Tubes were centrifuged at 2400 rpm for 20 minutes. Late stage parasites at the 70 % / 90 % interface were harvested and washed with RPMI. The Schizonts were homogenized with TRIzol Reagent (Ambion/Life Technologies,

Carlsbad, California) and total RNA was extracted using the Direct-Zol RNA MiniPrep Plus kit (Zymo Research, USA) according to manufacturer's protocol. Expression of mRNA transcripts for selected invasion ligands was determined using the Luna Universal One-Step RT-qPCR Kit (New England Biolabs, Inc.), on a QuantStudio 5 Real-Time PCR System (Applied Biosystems), using manufacturer's recommendations. Data were analyzed with Microsoft Excel and GraphPad software (v.7). Gene expression levels in *Suspended* cultures were expressed as fold-change relative to levels in *Static* cultures, after normalization to the expression of the 60S ribosomal protein L18 as an endogenous control and apical membrane antigen 1 (AMA1) as a parasite maturation marker. Fold-change in gene expression of ≥2 was considered significant (Stubbs *et al.*, 2005; Gaur *et al.*, 2006).

3.0.8 Estimation of the copy numbers of up-regulated genes

Genomic DNA was extracted from both Static and Suspended Dd2, W2mef and 3D7 using the QIAamp blood mini kit (QIAGEN, Hilden, Germany) according to manufacturer's protocol. Gene copy numbers were determined using the Luna Universal One-Step RT-qPCR Kit (New England Biolabs, Inc.), on a QuantStudio 5 Real-Time PCR System (Applied Biosystems), using manufacturer's recommendations, with slight modifications; the RT step was skipped. Data were analyzed with Microsoft Excel and GraphPad software (v.7). Gene copy numbers were estimated by comparing them to the copy number of the 3D7 seryl-tRNA synthetase (Nery *et al.*, 2006). The single copy gene AMA1 was included as an internal control for all strains assayed.

3.0.9 Data analyses

All data were analysed with Microsoft Excel and GraphPad software (v.7). All statistical analyses were based on student t-test with 95 % confidence.

CHAPTER 4

4.0 RESULTS

4.0.1 *P. falciparum* Dd2 and W2mef spontaneously switch invasion phenotype in suspension cultures

The *P. falciparum* strains Dd2 and W2mef are SA-dependent and therefore their invasion of erythrocytes is ablated upon neuraminidase treatment of erythrocytes (Dolan *et al.*, 1990; Duraisingh *et al.*, 2003a; Stubbs *et al.*, 2005; Gaur *et al.*, 2006). However, both parasite strains are capable of switching invasion phenotype when continuously selected on neuraminidase (Nm)-treated erythrocytes (Dolan *et al.*, 1990; Stubbs *et al.*, 2005).

To investigate the switching of invasion phenotype by Dd2 parasites cultured in suspended conditions, aliquots of Dd2, W2mef, and a commonly used SA-independent strain 3D7, were thawed and split equally into two flasks, one of which was kept in a static incubator (*Static* culture, ST), and the other placed in a gently shaking incubator (*Suspended* culture, SP). All parasites were then maintained in long, continuous cultures for approximately six months (25 weeks) to select for variant phenotypes, which was monitored by determining the invasion phenotypes of the respective cultures weekly.

After 25 weeks of continuous culture, Dd2 ST and W2mef ST maintained SA-dependent invasion phenotype, with less than 10 % invasion efficiency in neuraminidase-treated erythrocytes (Figures 4.1A and B). On the other hand, a dramatic increase in invasion of neuraminidase-treated erythrocytes by Dd2 SP and W2mef SP was observed, beginning from about 20 % efficiency at week 3 and peaking at > 60 % after 6-9 weeks (Figures 4.1A and B), indicating a switch to SA-independent invasion phenotype. In contrast, invasion efficiency of the 3D7 strain remained essentially unchanged in both ST and SP cultures over the entire duration of the experiment,

although fluctuating within 10 % variation (Figure 4.1C). Remarkably, when the Dd2 and W2mef SP cultures were taken off the shaker and returned to static conditions (R-ST), the parasites gradually lost their ability to invade neuraminidase-treated erythrocytes and appeared to revert to a SA-dependent phenotype (Figures 4.1A and B).

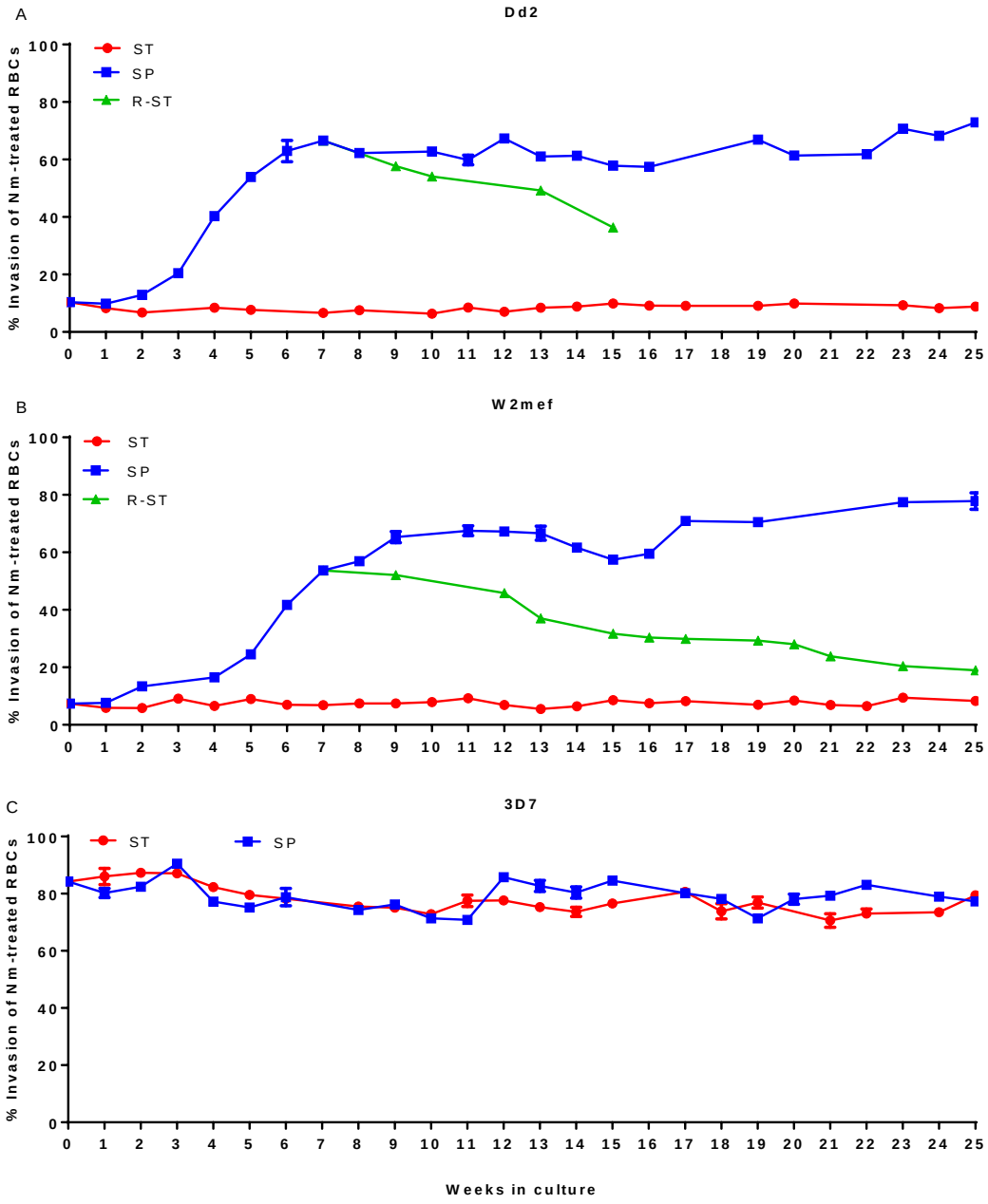

Figure 4. 1: Dd2 and W2mef, but not 3D7, spontaneously switch invasion phenotype in suspension culture.

The *P. falciparum* isolates were adapted to parallel *Static* (ST) and *Suspended* (SP) cultures (44rpm) and their invasion phenotypes assessed weekly using untreated and neuraminidase (Nm)-treated erythrocytes as target cells. Returning SP cultures to ST conditions (R-ST), led to a gradual loss of their ability to invade Nm-treated erythrocytes. Schizonts were co-incubated with target cells for approximately 14 hours. Flow cytometry was used to determine invasion rates as percentage of infected CFDA-positive erythrocytes. Invasion efficiency in Nm-treated erythrocytes were expressed as percentages relative to invasion of untreated erythrocytes. Data are presented as mean ± standard error of the means of triplicate biological experiments**.**

4.0.2 Static and Suspended parasites are genetically identical.

To determine if the Dd2 and W2mef cultures were contaminated with 3D7 or another SA-independent parasite strain, the genetic identities of both *Static* and *Suspended* cultures of all three strains were ascertained. To this effect, variations in the highly polymorphic merozoites surface protein 2 (MSP2) gene was analysed. The polymorphic nature of MSP1 and 2 allows for the identification of different parasite strains and is thus used as a tool for malaria epidemiological surveillance (Viriyakosol *et al.*, 1995; Soulama *et al.*, 2009; Frank *et al.*, 2011; Yavo *et al.*, 2016; Amoah *et al.*, 2017). The data shows that both *Static* and *Suspended* cultures of Dd2, W2mef and 3D7 yield a single PCR product, indicating that all parasite strains are monoclonal, in respect of the MSP2 locus. For Dd2 and W2mef, both *Static* and *Suspended* parasites showed a product of approximately 900 base pairs (bp) (Figure 4.2). Similarly, both *Static* and *Suspended* 3D7 had identical products of approximately 800 bp in size. A mixture of amplicons from *Static* Dd2, W2mef and 3D7 results in two distinct bands with sizes corresponding to that of 3D7 (800 bp) and Dd2 and W2mef (900 bp), respectively (Figure 4.2). The product sizes observed are as expected for 3D7, and the Indochina strains which includes both Dd2 and W2mef (Smythe *et al.*, 1990; Wong and Davis, 2012). The similarity in the genotypes of Dd2 and W2mef is unsurprising because Dd2 is a sub-clone of W2mef (Guinet *et al.*, 1996; Uhlemann *et al.*, 2005). Thus, it is logical that both strains have identical sequences at the target region. Together, these data show that *Suspended* cultures of Dd2, W2mef and 3D7, were genetically identical to their respective *Static* cultures.

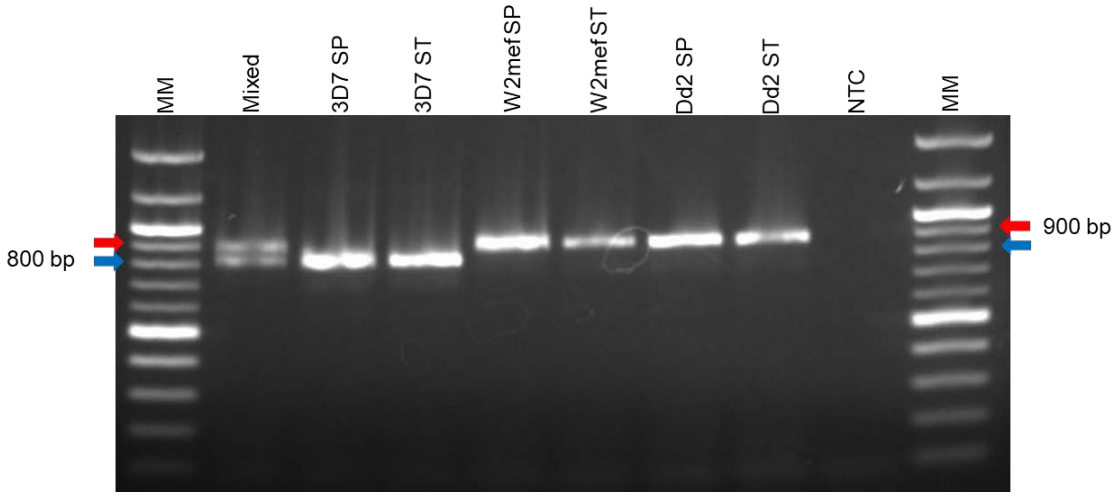

Figure 4. 2: Static and Suspended parasites are genetically identical.
A section of the MSP2 gene was amplified from genomic DNA of week 6 parasites. Amplicons were resolved on an ethidium bromide-stained 1 % agarose gel. The primer pair generated amplicons of approximately 800 bp (blue arrow) for 3D7, and 900 bp (red arrow) for Dd2 and W2mef. A combination of all three strains (mixed) showed only two distinct bands of sizes equivalent to 800 bp (3D7) and 900 bp (Dd2/W2mef), respectively. Product sizes were estimated with a 100 bp molecular weight marker (MM). NTC = non-template control, ST = *Static*, and SP = *Suspended***.**

4.0.3 Shaking does not affect the expression of SA and CR1 on the surface of erythrocytes.

To ascertain whether the switch in invasion phenotype by *Suspended* Dd2 and W2mef were due to the loss of SA on the surface of *Suspended* erythrocytes, the relative quantities of SA on the surface of both *Static* and *Suspended* cells were estimated. In addition, the relative amount of CR1 on the surface of both cell populations were determined. Per the data, there is no difference in the expression of SA (Figure 4.3A) and CR1 (Figure 4.3B) on the surfaces of *Static* (ST) and *Suspended* (SP) cells, with levels comparable to untreated (UT) controls. Nm-treated erythrocytes (NT) showed SA expression levels similar to unstained control (US), thus showing that the enzyme is active, and the antibodies are specific to SA (Figure 4.3A). Similarly, chymotrypsin-treated (CT) and trypsin-treated (TT) erythrocytes exhibited CR1 expression levels comparative to US controls, again, indicating that the enzymes are active and also that the antibodies are specific to CR1

(Figure 4.3B). These observations are indications that the switch in invasion phenotype by *Suspended* Dd2 and W2mef is modulated by parasite-specific factors as opposed to host factors.

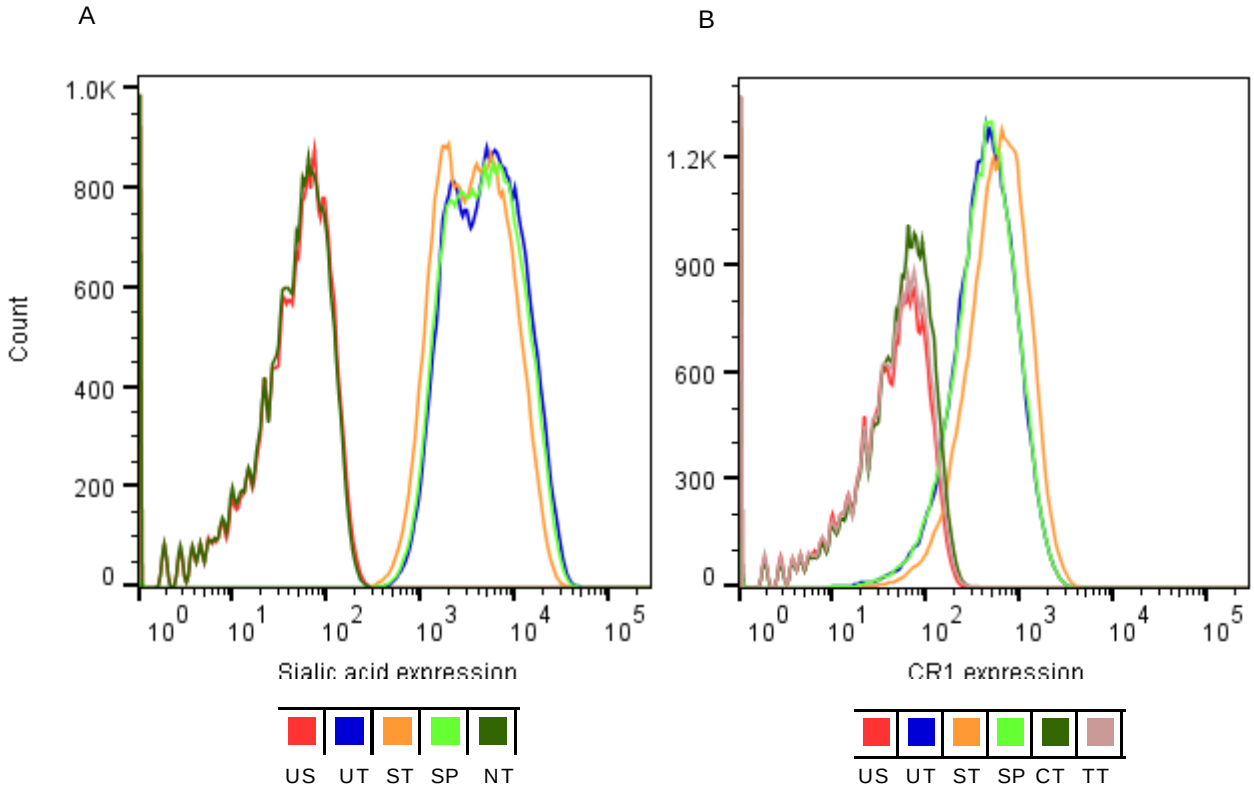

Figure 4. 3: Shaking does not affect the levels of sialic acid and complement receptor 1 on erythrocyte surfaces.
Erythrocyte surface expression of the invasion receptors (A) sialic acid (SA) and (B) complement receptor 1 (CR1) was determined in *Static* and *Suspended* cultures after two weeks of incubation using flow cytometry. Untreated control (UT), Nm-treated (NT), Chymotrypsin-treated (CT), and trypsin-treated (TT) erythrocytes were stained with mouse monoclonal anti-human GPA antibodies specific to SA or mouse monoclonal anti-human CR1 antibodies. Unstained control erythrocytes (US) were included for comparison. Phycoerythrin (PE)-conjugated goat polyclonal anti-mouse antibodies were used as secondary antibodies.

4.0.4 Growth rates of *Static* and *Suspended* parasites in neuraminidase-treated erythrocytes

High multiplication rates, and thus high parasite densities, are virulence features of *P. falciparum* infections (Miller *et al.*, 2002). Cultivation of *P. falciparum* parasites in suspended cultures is known to favour a higher rate of multiplication (Butcher, 1981; Allen and Kirk, 2010; Ribacke *et al.*, 2013). Therefore, it was of interest to determine if the change in invasion phenotypes observed when Dd2 and W2mef were maintained in a shaking incubator was also associated with an increase in growth rate. Furthermore, we sought to establish that *Suspended* Dd2 and W2mef can thrive in Nm-treated erythrocytes, in a similar manner as Dd2NM (Dolan *et al.*, 1990; Gaur *et al.*, 2006). Thus, ring stage parasites from 12-week *Static* and *Suspended* cultures were incubated with Nm-treated erythrocytes for two complete erythrocytic parasite cycles, under *Static* and *Suspended* conditions, respectively. Parallel cultures with normal untreated erythrocytes were established as references. Generally, *suspended* parasites grew better than *Static* parasites in untreated erythrocytes (Figures 4.4A-C). However, statistical significance was only achieved at the end of cycle 1 for Dd2 and W2mef (cycle 1: P=0.007 and P=0.045 for Dd2 and W2mef, respectively; cycle 2: P=0.1302 and P=0.0965 for Dd2 and W2mef, respectively; Figures 4.4A and B). In 3D7, however, the growth rate in *Suspended* parasites was higher than in *Static* cultures at the end of both cycles (P=0.012 and 0.007, respectively; Figure 4.4C). In the Nm-treated erythrocytes (Figures 4.4D-F), *Static* Dd2 and W2mef seemed incapable of establishing successful infections, with multiplication rates of about 1.0. (PMR=1.01 and PMR=0.96 at the end of cycle 2 for Dd2 and W2mef, respectively; Figures 4D and E). In contrast, *Suspended* Dd2 and W2mef successfully thrived and established efficient infections (PMR=8.31 and PMR=12.93 at the end of cycle 2 for Dd2 and W2mef, respectively), with growth rates well above those in *Static* cultures (P<0.006 and 0.002 for Dd2 and W2mef, respectively; Figures 4.4D and E). Both *Static* and *Suspended* 3D7

grew normally in Nm-treated erythrocyte (PMR=12.10 and 12.89 for ST and SP, respectively, at the end of cycle 2), with no statistically significant differences between ST and SP (*P*=0.403; Figure 4.4F). The results clearly demonstrate that *Suspended* parasites are capable of establishing successful infections in SA-deficient erythrocytes, confirming that these parasites are SA-independent.

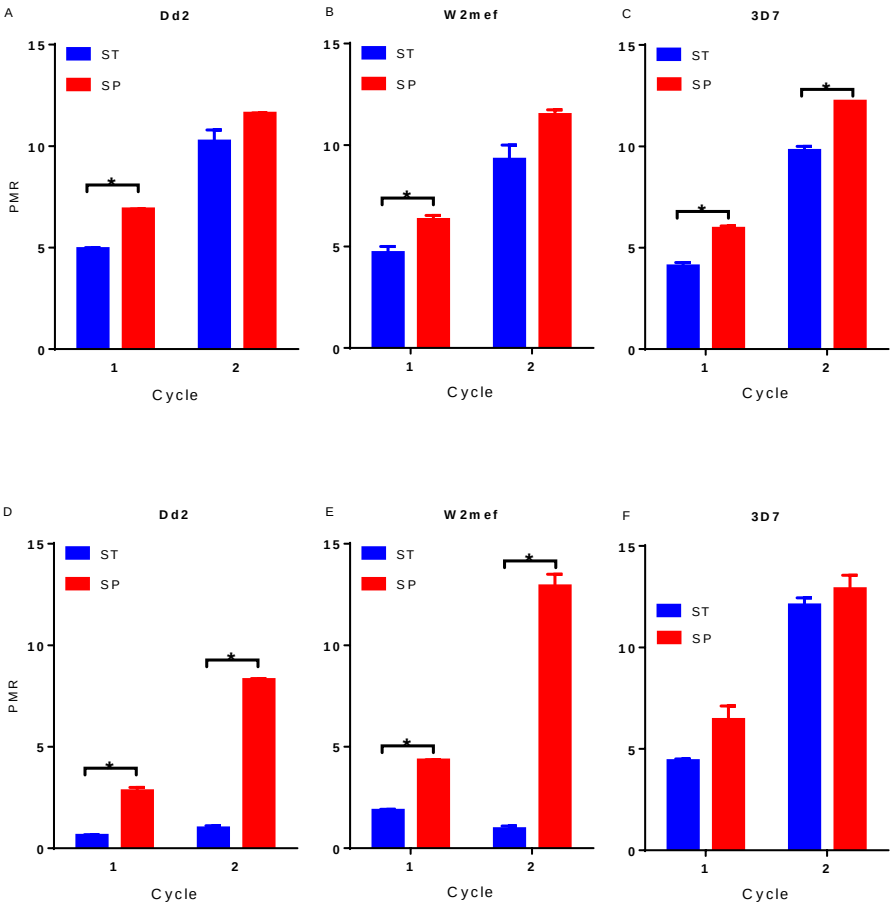

Figure 4. 4: Suspended, but not Static Dd2 and W2mef, establish successful infection in Nm-treated erythrocytes.
Static (ST) and *Suspended* (SP) parasites were cultivated in normal (A) and Nm-treated (B) erythrocytes for 2 asexual cycles. Flow cytometry was used to determine parasitemias as percentage of ring-infected erythrocytes at the end of each cycle. Parasite multiplication rates (PMR) were estimated by dividing the parasitemia at the end of each cycle by the starting parasitemia. Data are presented as mean ± standard error of the means of 2 independent experiments. * *P*-value < 0.05 at 95 % CI; Student t-test.

4.0.5 Sensitivity of *Static* and *Suspended* parasites to neuraminidase, chymotrypsin and trypsin treatment of erythrocytes.

To determine the relative contributions of the major receptors to the invasion of *Suspended* cultures, parasites were incubated with erythrocytes treated with Nm, chymotrypsin or trypsin, which are enzymes that selectively remove receptors from the cell surfaces. Trypsin usually cleaves glycophorins A and C, but not B, while chymotrypsin cleaves glycophorin B and band 3 (Thompson *et al.*, 2001; Duraisingh *et al.*, 2003b; Tham *et al.*, 2012). CR1 is sensitive to both trypsin and chymotrypsin (Tham *et al.*, 2012), while basigin is resistant to both enzymes (Crosnier *et al.*, 2011; Tham *et al.*, 2012). *Static* Dd2 and W2mef parasites were highly sensitive to Nm treatment, with invasion of enzyme-treated erythrocytes reduced to less than 10 % relative to untreated erythrocytes (Figure 4.5A). Consistent with the switch in phenotype, *suspended* parasite cultures of Dd2 and W2mef were resistant to Nm treatment (Figure 4.5A), with invasion reduced to only 75% relative to untreated erythrocytes (Figure 4.5A). In the 3D7 cultures, there were no differences in the invasion of Nm-treated erythrocytes by both *Static* and *Suspended* parasites (Figure 4.5A). Of interest, invasion by *Suspended* W2mef and Dd2 parasites were significantly more sensitive to chymotrypsin treatment compared to their corresponding *Static* cultures, (P=0.004 for W2mef and P<0.001 for Dd2; Figure 4.5B). Conversely, invasion by *Suspended* W2mef and Dd2 cultures were slightly more resistant to trypsin treatment compared to the respective *Static* parasites (P=0.046 for W2mef and P=0.034 for Dd2; Figure 4.5C). Sensitivities of *Suspended* 3D7 cultures to chymotrypsin and trypsin were not significantly different from the corresponding *Static* cultures (P=0.288 for chymotrypsin and P=0.526 for trypsin; Figures 4.5B and C).

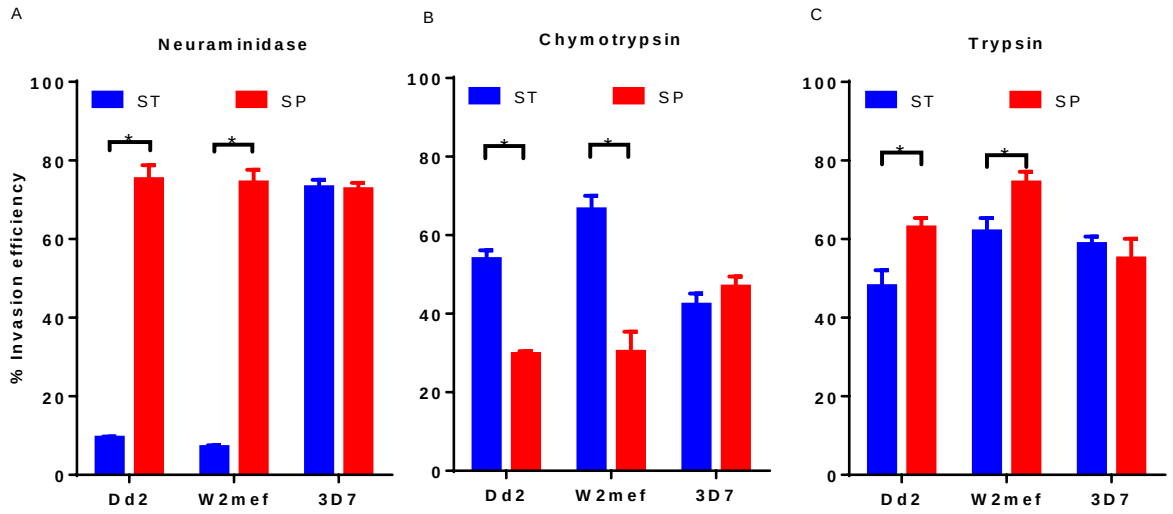

Figure 4. 5: Differential sensitivities of Static and Suspended parasites to enzyme treatment of erythrocytes.
Invasion efficiencies of *Static* (ST) and *Suspended* (SP) into erythrocytes treated with neuraminidase (250mU), chymotrypsin (1mg/ml) and trypsin (1mg/ml) were compared for *P. falciparum* Dd2, W2mef and 3D7 strains. Schizonts were co-incubated with target cells for approximately 14 hours. Flow cytometry was used to determine invasion rates as percentage of infected CFDA-positive erythrocytes. Invasion efficiencies were expressed as percentages relative to invasion of untreated erythrocytes. Data are presented as mean ± standard error of the means of three independent experiments performed in triplicates. *P-value < 0.05 at 95 % CI; Student t-test.

4.0.6 Basigin is essential for invasion but not involved in invasion phenotype switching

The role of basigin in the switching of invasion phenotype in *Suspended* cultures was also investigated. Relative to IgG control, antibodies against basigin significantly inhibited invasion of untreated erythrocytes by both *Static* and *Suspended* cultures for all three parasites (P<0.001 for all comparisons; Figure 4.6). Since *Static* Dd2 and W2mef invade Nm-treated erythrocytes poorly, anti-basigin antibodies did not seem to have any significant effect on the invasion of these parasites when compared to IgG control (P=0.069 for Dd2 and P=0.128 for W2mef; Figures 4.6A and C). However, invasion of *Suspended* Dd2 and W2mef parasites into Nm-treated erythrocytes was markedly abrogated by anti-basigin antibodies, relative to IgG control (P<0.001 for both

comparisons; Figures 4.6B and D). Similarly, anti-basigin antibodies significantly inhibited invasion of both *Static* and *Suspended* 3D7 into Nm-treated erythrocytes (*P*<0.0001 for all comparisons; Figures 4.6E and F). Altogether, the data demonstrate that basigin is essential for invasion of *Suspended* cultures into both untreated and Nm-treated erythrocytes, thus not associated with the phenotypic switch.

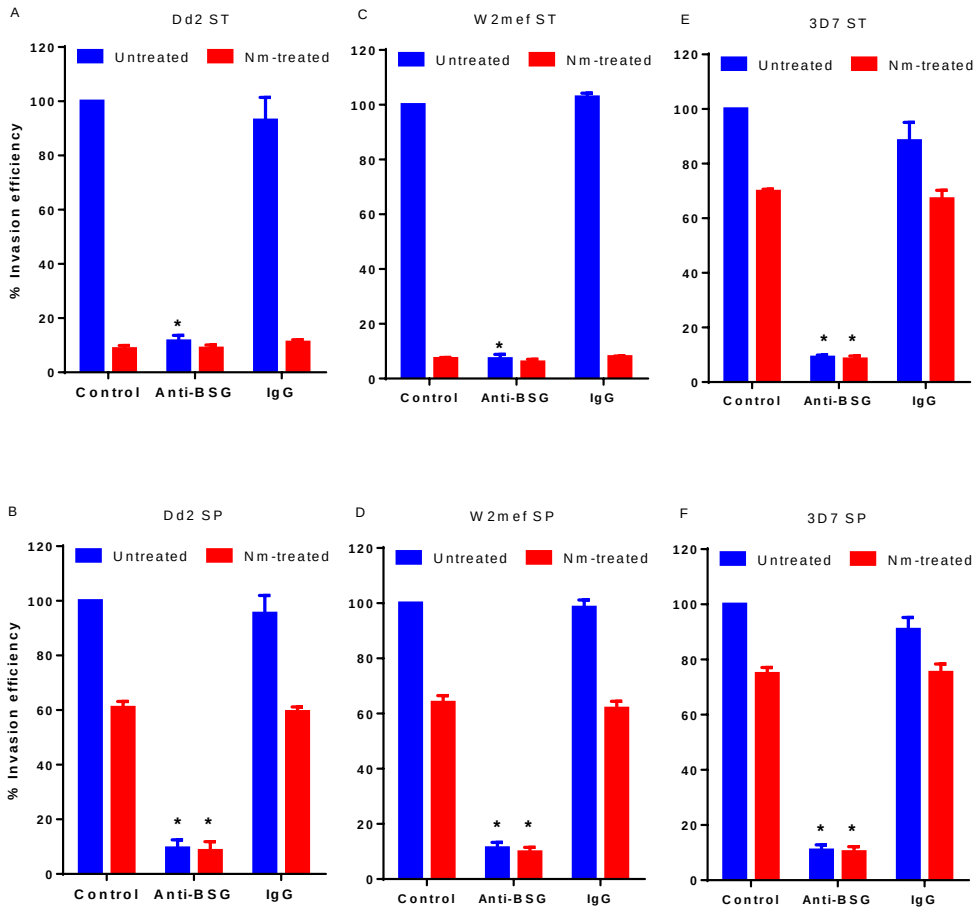

Figure 4. 6: Phenotypic switching of Suspended cultures not basigin-dependent.
Anti-basigin antibodies (10 µg/mL) ablated invasion of both *Static* (ST) *Suspended* (SP) parasites into untreated and Nm-treated erythrocytes. Schizonts were co-incubated with target cells for approximately 14 hours. Flow cytometry was used to determine invasion rates as percentage of infected CFDA-positive erythrocytes. Invasion efficiencies in Nm-treated erythrocytes were expressed as percentages relative to invasion of untreated erythrocytes. Data are presented as mean ± standard error of the means of three independent experiments performed in triplicates. * *P*-value < 0.05 at 95 % CI compared to IgG control; student t-test.

4.0.7 Sialic acid-independent invasion in switched *Suspended* parasites is CR1-dependent

The switch in Dd2 and W2mef when they are cultured on neuraminidase-treated erythrocytes is known to be associated with an increased expression of RH4 (Stubbs *et al.*, 2005; Gaur *et al.*, 2006), an invasion ligand whose receptor is CR1 (Spadafora *et al.*, 2010; Tham *et al.*, 2010; Awandare *et al.*, 2011b). Therefore, the effect of anti-CR1 antibodies on invasion efficiency of *Static* and *Suspended* cultures into untreated or Nm-treated erythrocytes was examined. Anti-CR1 did not significantly inhibit invasion of any of the parasite strains into untreated erythrocytes compared to IgY isotype control antibodies (P>0.100 for all comparisons; Figure 4.7). Furthermore, since *Static* Dd2 and W2mef cultures invade Nm-treated erythrocytes poorly, anti-CR1 antibodies had no significant impact on invasion relative to control IgY (P>0.100 for all comparisons; Figures 4.7A and C). However, for *Suspended* cultures, invasion of both Dd2 and W2mef parasites into Nm-treated erythrocytes was substantially abrogated by anti-CR1 antibodies compared to control IgY (P=0.004 and P=0.003 for Dd2 and W2mef, respectively; Figures 4.7B and D). For 3D7 parasites, invasion into Nm-treated erythrocytes was significantly inhibited by anti-CR1 antibodies compared to IgY for both *Static* and *Suspended* cultures (P=0.001 and P<0.001 respectively; Figures 4.7E and F).

These observations were specific to CR1, given that anti-basigin antibodies abrogated invasion of both *Static* and *Suspended* Dd2 and W2mef parasites, and the inhibition was similar in both untreated and Nm-treated erythrocytes. Taken together, these data suggest that SA-independent invasion in *Suspended* Dd2 and W2mef cultures is significantly dependent on CR1 receptor usage.

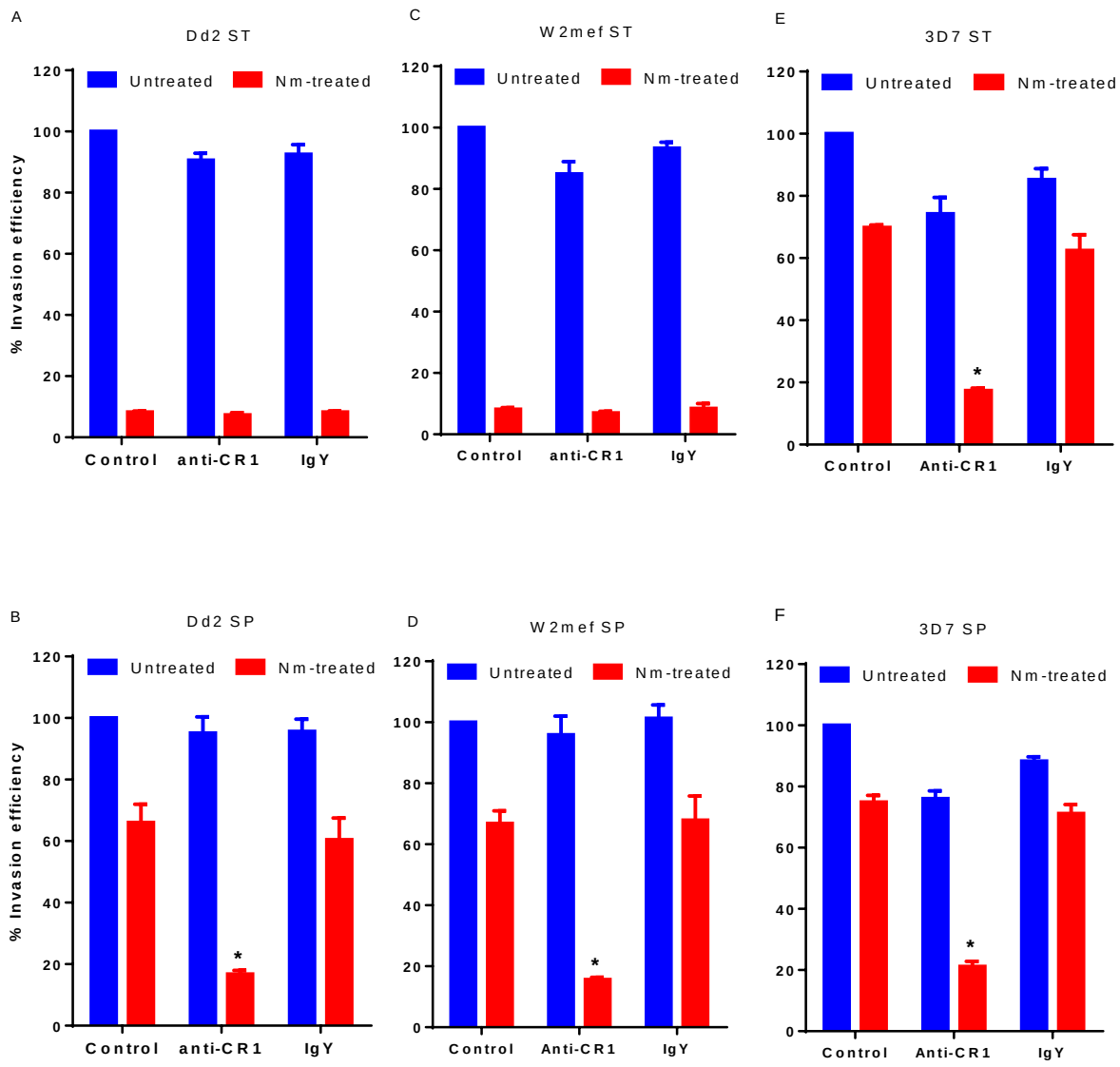

Figure 4. 7: Phenotypic switching of Suspended cultures highly dependent on complement receptor 1 (CR1).

Anti-CR1 antibodies (12µg/mL) inhibited invasion of *Suspended* (SP) parasites into Nm-treated erythrocytes but not untreated erythrocytes. Schizonts were co-incubated with target cells for approximately 14 hours. Flow cytometry was used to determine invasion rates as percentage of infected CFDA-positive erythrocytes. Invasion efficiencies were expressed as percentages relative to invasion of untreated erythrocytes. Data are presented as mean ± standard error of the means of three independent experiments performed in triplicates. **P*-value < 0.05 at 95 % CI compared to IgY control; student t-test.

4.0.8 Specific invasion related genes are upregulated in *Suspended* parasites.

To investigate the possible mechanisms responsible for the switch in phenotypes, gene expression levels in *Suspended* vs *Static* cultures were examined for a panel of invasion related genes for which primers were available. Comparison of gene expression levels in *Suspended* Dd2 or W2mef to levels in *Static* cultures after 12 weeks of culture revealed an upregulation and down-regulation of many genes, including RH4, PEBL RH1, RH2b, RH5, actin-1, calcineurin B (CnB), the transcription factor AP2-I and two cyclophilins (PF3D7_1202400 and PF3D7_1215200; Figure 4.8A). However, genes with approximately two-fold change or greater, in *Suspended* vs *Static* parasites of both strains were considered significant (Stubbs *et al.*, 2005; Gaur *et al.*, 2006), and additional temporal expression patterns for these genes (RH4, PEBL, RH1 and PF3D7_1202400) were determined (Figure 4.8B). Expression of all four genes seemed to be upregulated from weeks 2 to 12, particularly for RH4 and PEBL, whose expressions remained high at week 12.

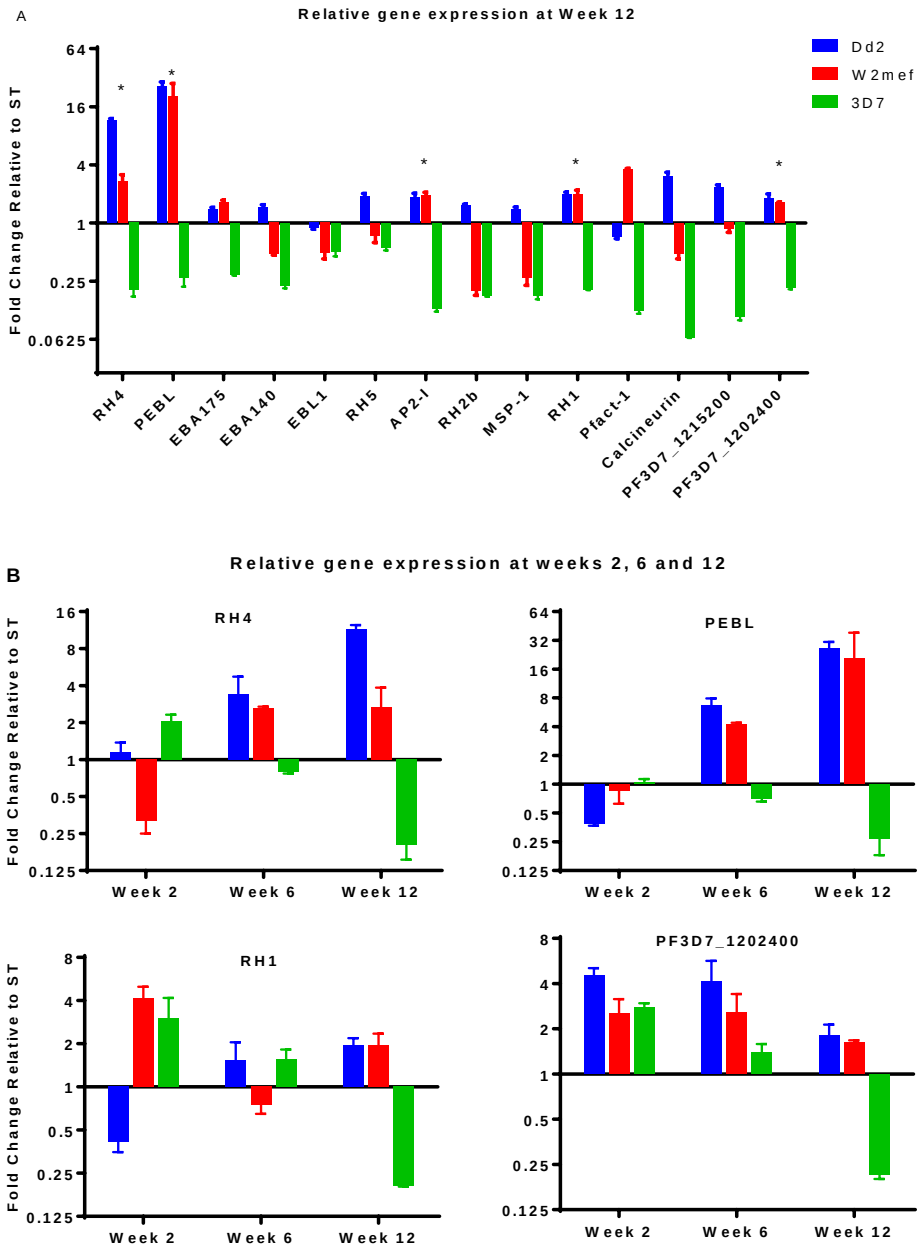

Figure 4. 8: RH4 and PEBL are upregulated in suspended Dd2 and W2mef.
Transcript levels of selected *P. falciparum* invasion related genes were determined by reverse transcription quantitative polymerase chain reaction analyses in *Static* (ST) and *Suspended* (SP) Dd2, W2mef and 3D7. The expression level of each gene was normalized to 60S ribosomal L18 protein as an endogenous control and then to AMA1 as a late stage marker. Relative fold changes were determined by normalizing the expression levels in *Suspended* parasites to *Static* parasites. (A) represent transcript levels of all genes at week 12. The transcript level of RH4, PEBL, RH1 and PF3D7_1202400 at weeks (W) 2, 6 and 12 are shown in (B). All experiments were run in triplicates. Error bars represent standard errors. * Genes with ≥ 2-fold change in expression at week 12 in both Dd2 and W2mef.

4.0.9 Copy numbers of upregulated genes are similar in *Suspended* and *Static* parasites

In addition to gene regulation mechanisms such as epigenetics, promoter sequence duplication, gene copy number amplification is another mechanism used by cells to increase the expression of a particular gene. Increasing the number of copies of a gene ensures that with similar expression rate, the product of a gene is significantly amplified. To ascertain the underlying mechanism(s) involved in the increased expression of gene in *Suspended* parasites, copy numbers of the genes were determined with quantitative real time PCR by comparison to the 3D7 seryl-tRNA synthetase. Except for RH1 and PF3D7_1202400, all genes assayed had a single copy in both *Static* and *Suspended* cultures (Figure 4.9). While Dd2 had a single copy of RH1, W2mef had two copies; consistent with previous reports (Nair *et al.*, 2010). The gene PF3D7_1202400 had five and four copies in Dd2 and W2mef, respectively, with no differences observed for both *Static* and *Suspended* cultures. Together, these observations show that gene copy number variation is not the mechanism accounting for the increased expression of the upregulated genes in the suspended parasites.

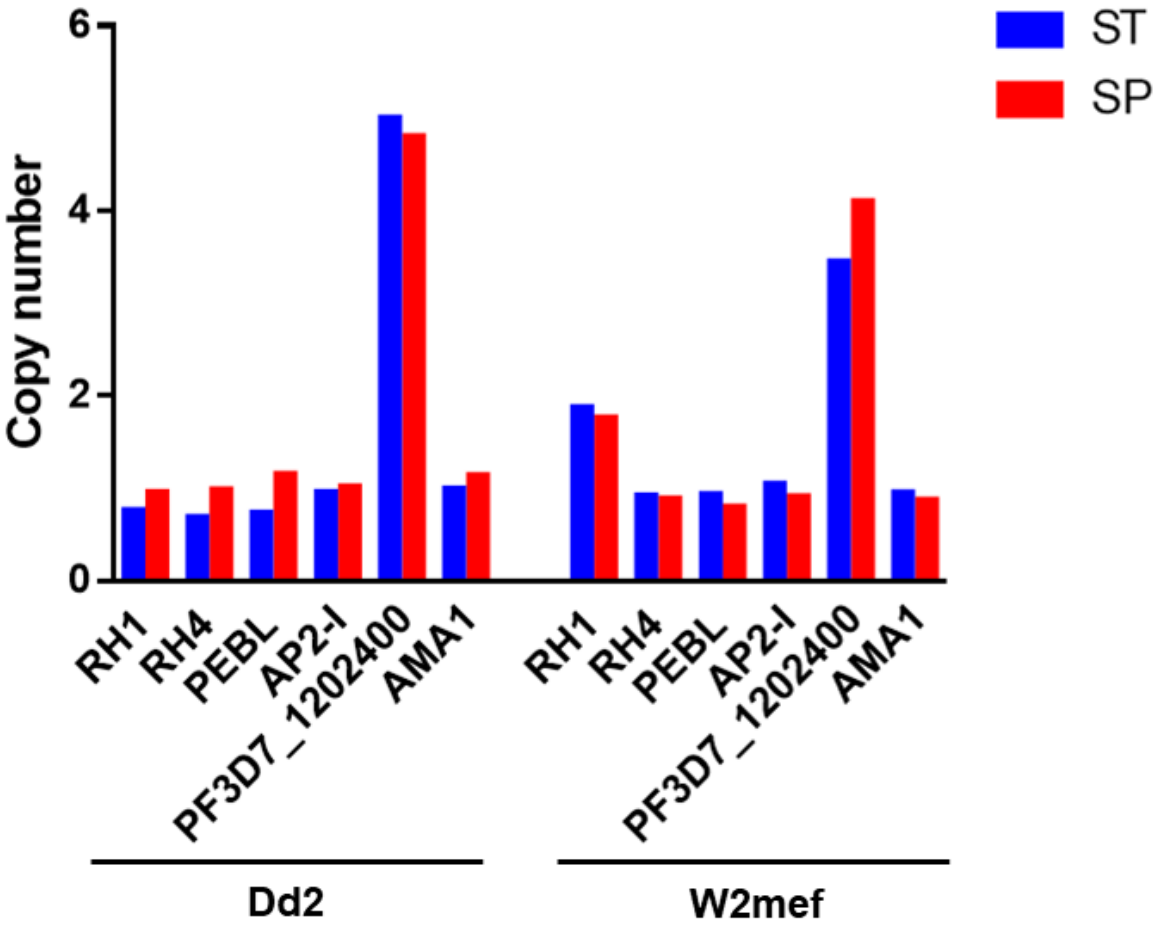

Figure 4. 9: Copy numbers of up-regulated genes remain unchanged.
The copy numbers of genes whose expression levels were significantly higher in *Suspended* compared to *Static* Dd2 and W2mef were estimated by comparison to the single copy number gene seryl-tRNA synthetase of 3D7. AMA1 which is single copy in both strains was used as an internal control. All experiments were run in triplicate.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND FUTURE PERSPECTIVES.

5.1 DISCUSSION

The presence of multiple proteins that are functionally redundant is a major hurdle to the development of an effective blood stage vaccine against *P. falciparum* malaria. This functional redundancy has long been attributed to host-immune factors targeting invasion related proteins (Persson *et al.*, 2008; Wright and Rayner, 2014). However, the potential contribution of non-immune factors to the variation in the expression and use of invasion related genes is poorly characterized. *P. falciparum* Dd2 and W2mef have the unique ability to switch to SA-independent invasion phenotypes when cultured continuously in Nm-treated erythrocytes (Dolan *et al.*, 1990; Stubbs *et al.*, 2005). In addition, deletion of the SA-dependent invasion ligand EBA-175 has also been shown to induce a similar phenotypic switch (Reed *et al.*, 2000; Duraisingh *et al.*, 2003a; Stubbs *et al.*, 2005), indicating that this change was driven by limiting access to the SA-dependent invasion pathway.

This study describes another remarkable feature of these parasites, which is their ability to spontaneously switch to the SA-independent phenotype, simply by shaking the cultures, without removing SA or its ligands. It was confirmed by flow cytometry that levels of SA molecules on the erythrocyte surfaces were not significantly affected by culturing in suspended conditions. However, the shaking may be increasing the stringency of binding to SA, and the decreased affinity may be mimicking SA removal or EBA-175 deletion, which then triggers the switch in phenotype in the parasite. The potential relevance of this phenomenon *in vivo* is significant, given that parasites in the blood of an infected person are essentially in suspension for a majority of their

asexual stage. Thus, the dynamics of the parasite phenotypes in peripheral circulation compared to tissue-sequestered ones would be interesting for further investigation.

The phenotypic switch in *Suspended* parasites was accompanied by a substantial increase in sensitivity to chymotrypsin and a modest but significant increase in resistance to trypsin relative to *Static* parasites, which gave a clue about the possible receptors involved. EBA175-deleted W2mef parasites also expressed a similar chymotrypsin sensitivity (Duraisingh *et al.*, 2003a; Persson *et al.*, 2008), which suggests a more significant role for glycophorin B (Dolan *et al.*, 1994; Dankwa *et al.*, 2017) in mediating invasion by *Suspended* compared to *Static* parasites. Expression of EBL1, the putative ligand for glycophorin B, however remains unchanged. This suggests that either the basal levels of EBL1 are sufficient for glycophorin B-mediated invasion or another glycophorin B-binding ligand is involved, as mooted elsewhere (Dankwa *et al.*, 2017). The enzyme sensitivity data also implicates CR1, which is resistant to neuraminidase but sensitive to chymotrypsin (Tham *et al.*, 2012). Furthermore, a putative RH2b-binding receptor has been characterized as being resistant to trypsin and sensitive to chymotrypsin (Duraisingh *et al.*, 2003b; Aniweh *et al.*, 2016), hence its involvement in mediating erythrocyte invasion by *Suspended* parasites is also possible.

The role of CR1 in mediating SA-independent invasion by *Suspended* parasites was confirmed by receptor inhibition experiments which clearly demonstrated that anti-CR1 antibodies potently abrogated invasion into Nm-treated erythrocytes. These results are consistent with previous observations that have shown CR1 to be the major SA-independent receptor for both laboratory-adapted parasites and field isolates (Stubbs *et al.*, 2005; Spadafora *et al.*, 2010; Tham *et al.*, 2010; Awandare *et al.*, 2011b; Mensah-Brown *et al.*, 2015). In addition, data presented here also confirmed the critical role played by basigin in mediating erythrocyte invasion by all *P. falciparum*

parasites tested to date (Crosnier *et al.*, 2011; Mensah-Brown *et al.*, 2015; Zenonos *et al.*, 2015). However, it was clear that the role of basigin was equally important for invasion by *Suspended* and *Static* parasites, suggesting that a role for the RH5-basigin interaction in mediating the phenotypic switch is unlikely.

The ability of Dd2 and W2mef to switch invasion phenotype under selection on Nm-treated erythrocytes or EBA175 knockout has been correlated to the up-regulation of RH4 and PEBL (Stubbs *et al.*, 2005; Gaur *et al.*, 2006). In this study, *Suspended* Dd2 and W2mef parasites also showed a similar upregulation in RH4 and PEBL gene expression relative to the corresponding *Static* cultures, further confirming that gentle agitation of the parasites over a period of time elicited identical molecular changes to those observed using the previously described methods (Stubbs *et al.*, 2005; Gaur *et al.*, 2006).

Of potential interest, our studies observed significant upregulation of RH1, and AP2-I, which have not been previously associated with switching of invasion phenotypes. Nonetheless, RH1 expression is increased alongside RH4 when EBA175 is knocked-out in 3D7 (Lopaticki *et al.*, 2011). Given the role of RH1 in signaling EBA175 release (Gao *et al.*, 2013a), the upregulation of RH1 in the absence of EBA175 is possibly to amplify signaling events aimed at inducing the downstream release of EBA175 for invasion through the EBA175-GPA route to occur. In the context of the present work, increased expression of RH1 similar to the absence of EBA175 suggests that the suspended parasites may not be using either EBA175 or the other members of the EBAs during erythrocyte invasion.

Additionally, RH1 binding is accompanied by increased Ca^{2+} concentration (Gao *et al.*, 2013a); a condition required for successful erythrocyte invasion (Singh *et al.*, 2014; Weiss *et al.*, 2015; Aniweh *et al.*, 2016; Volz *et al.*, 2016; Aniweh *et al.*, 2017; Cowman *et al.*, 2017). Furthermore,

strong erythrocyte deformation mediated by the EBAs and RH4 is a requirement for merozoite invasion of erythrocytes (Weiss *et al.*, 2015). Erythrocyte deformation is believed to aid tighter interactions which fastens the parasite to the erythrocyte surface prior to invasion (Cowman *et al.*, 2017). The calcium-dependent parasite phosphatase, calcineurin, has been proposed to mediate strong attachment during erythrocyte invasion and increased concentrations of the enzyme is required for successful invasion in *Suspended* compared to *Static* cultures (Paul *et al.*, 2015b). It is a possibility that RH1 upregulation is a mechanism to provide the optimal Ca^{2+} concentration required for the action of calcineurin or other calcium-dependent events during erythrocyte invasion in suspended cultures. However, further investigations are required to determine the potential relevance of this observation to erythrocyte invasion.

The apicomplexan apetala2 (ApiAP2) DNA-binding domain containing proteins are a family of transcription factors modulating diverse events during the life cycle of *Plasmodium* parasites, including gametocyte formation, ookinate development and sporogony (Sinha *et al.*, 2014; Modrzynska *et al.*, 2017; Kent *et al.*, 2018). The AP2-I member of the ApiAP2s has previously been shown to mediate the expression of *P. falciparum* invasion related genes such as MSP4, MSP5, RAP2, etc. (Santos *et al.*, 2017). The present study observed a significant increase in the expression of AP2-I in *Suspended* compared to *Static* parasites. This coincides with a similar expression pattern for RH4 in the two parasite lines. However, AP2-I was shown not to bind to the promoters of both RH4 and AMA1 (Santos *et al.*, 2017). A plausible theory is that AP2-I may be mediating the expression of other molecules such as transcription factors and histone modifiers, leading to the activation and expression of RH4. Another working theory is that AP2-I interacts with bromodomain protein 1 to mediate the expression of genes, of which the invasion related

genes are inclusive (Josling *et al.*, 2015; Santos *et al.*, 2017). However, the current data is not adequate to conclude on the significance of AP2-I upregulation in *Suspended* parasites.

It has been demonstrated that the regulation of RH4 expression is through epigenetic mechanisms involving the trimethylation of histone H3K9 (Jiang *et al.*, 2010; Coleman *et al.*, 2012). The MSP2 genotyping data suggest that there were no changes at the DNA level in *Suspended* parasites compared to the corresponding *Static* parasites. Furthermore, the copies of all the up-regulated genes remained similar for both static and suspended parasites, suggesting that the increased expressions observed were due to gene regulation mechanisms independent of the DNA sequence. Together, these observations indicate that similar epigenetic mechanisms may be mediating the upregulation of genes in the *Suspended* cultures. Therefore, the relationships between culture agitation and histone methylation will be of significant interest in ongoing investigations of this spontaneous phenotypic switching phenomenon.

5.2 CONCLUSION

This study has demonstrated another unique feature of *P. falciparum*, which is its ability to alter the type of ligand-receptor interactions used for erythrocyte invasion under different culturing conditions. The data shows that Dd2 and W2mef parasites cultured under flow conditions switch from their dependency on EBA175, and possibly the other EBAs, to the RH4-CR1 route for erythrocyte invasion, thus demonstrating the plasticity of malaria parasites to differing external conditions. These results have implications on the strategies used in assessing *Plasmodium* phenotypes and highlights the importance of considering *in vivo* physiological conditions during *in vitro* assays, and the prioritization of invasion related ligands in a multi-component invasion blocking malaria vaccine.

5.3 FUTURE PERSPECTIVES AND RECOMMENDATIONS

The results presented thus far demonstrate that the variation in invasion phenotypes is due to the differential expression of invasion related genes by the two parasite lines. However, this study was limited to erythrocyte invasion and the major ligands that are known to mediate invasion. Moving forward, it would be necessary to ascertain the extent to which culture agitation affects the global biology of the parasite. To this end, the entire transcriptome of parasites from the two culturing conditions have been sequenced and the data is currently being analyzed to identify all possible genes which are differentially expressed. Functional annotation of these genes will provide comprehensive information for the identification of biological processes affected by the different culturing conditions.

Given that the MSP2 genotyping and gene copy number variation assays showed no genomic aberrations, it is reckoned that the up-regulation of genes leading to the invasion phenotype variation is due to epigenetic gene regulation mechanisms. To this effect, we hope to identify epigenetic markers which could account for the differential gene expression observed and their positions within the genome. Identification of these markers and other molecular players involved could provide therapeutic targets.

The current data is limited in respect of the physiology of the erythrocyte under the different culturing conditions. Though it has been demonstrated that the levels of sialic acids and complement receptor 1 were not affected by either culturing condition, it is possible that the biophysical properties of erythrocytes under the two conditions are different, and thus could account for the phenotypic differences observed. Thus, in future studies, the effect of the different culturing methods on the physiology of the erythrocytes will be investigated.

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APPENDIX

APPENDIX A

Table A. 1: Primers for gene expression analyses by RT-qPCR.

Ligand	Forward (sense) primer (5'→3')	Reverse (antisense) primer (5'→3')
Actin-1*	AGCAGCAGGAATCCACACA	TGATGGTGCAAGGGTTGTAA
AMA1	CAATCAACGAACATAGGGAAC	TGGTCTATTGGATATGCGTG
AP2-I	ATTAACACCAGTTGAAGGGG	CTGCCAAACTATGAGCCTTA
Calcineurin B	AATCCCCTAGTTAAAAGGGTT	AGGCAAATTTCTTCTTCTGG
EBA-140	GACAAGTTCCTTCAGAGGAT	CACCCCTACCATTTTCACTA
PEBL	AGGGCATTATGTAAGCTGGA	AAGCATTTCCAAACTCCCA
EBA-175*	TTCGTGATGAGTGGTGGAAA	GGCAATAATCATCACCCCATT
EBL-1	TCCTTCATCTTCGAAAGCTC	TTCCCCCACCAGAACTATTA
L18	ATTCTGAAATGGCTGGTAGG	TGGTAAATGCAATGCTGGA
MSP1	TCCCAGAAGCAAAAGTTACA	GAGCCTGAAGTTGGTATTTG
PF3D7_1202400	AAGCATTCGAAAAGGGGTAT	CCAAATGTCCCAAAACAACA
PF3D7_1215200	GTTGGTAGGGTCTTGATCG	CCTACTAAACCAATACCAGGG
RH1*	ATGTATTTTGCCAGTGGAAT	TTGTGTGCTTTTATCATCCA
RH2b	GTAACAGAAGAAGATTACACGG	AAACAACTCCTCCAGCATTA
RH4*	AGGAAAATCTTCAGGGAAAG	TCTGAATGTGCATTATCTGC
RH5*	ACGAAGAATCAAGAAAATAATCTGACGTTACT	TGTTGAATGATCTTTAGCATTATTTGTTTTTATATTCTCTTT

*Primer sequence adopted from Lopaticki *et al*., (2011).

Table A. 2: RT-qPCR reaction setup

Component	Volume for 10 µl reaction	Final concentration
Luna Universal One-Step Reaction Mix (2X)	5	1X
Luna WarmStart RT Enzyme Mix (20X)	0.5	1X
Forward primer (10X)	0.4	0.4 µM
Reverse primer (10X)	0.4	0.4 µM
Template RNA	2.5	5 µg
Nuclease-free Water	1.2	

Table A. 3: Reaction conditions for RT-qPCR

Cycle step	Temperature (^{0} C)	Time	No of Cycles
Reverse transcription	55	10 minutes	1
Initial denaturation	95	1 minute	1
Denaturation	95	10 seconds	45
Extension	60	1 minute	
Melt curve	60-95	Various	1

Table A. 4: PCR reaction setup for MSP2 genotyping

Component	Volume for 25 µl reaction	Final concentration
5X One Taq Standard Reaction Buffer	5	1X
10 mM dNTPs	0.5	200 µM
10 µM Forward Primer	0.5	0.2 µM
10 µM Reverse Primer	0.5	0.2 µM
One Taq DNA polymerase	0.125	0.625 units/ 25µl PCR
Template DNA	5	200 µg
Nuclease-free water	13.375	

Table A. 5: Reaction conditions for MSP2 genotyping

Reaction step	Temperature (^{0} C)	Time	No of Cycles
Initial denaturation	94	30 seconds	1
Denaturation	94	15 seconds	30
Annealing	50	30 seconds	
Extension	68	1 minute	
Final extension	68	5 minutes	1

Table A. 6: MSP2 genotyping

Strain	**Forward primer (5'→3')**	**Reverse primer (5'→3')**	**Amplicon size (bp)**
Dd2	ATGAAGGTAATTAAAACA TTGTCTATTATA	ATATGGCAAAAGATAAAAC AAGTGTTGCTG	864
W2mef			864
3D7			811

APENDIX B

PUBLICATION

Awandare, G. A., Nyarko, P. B., Aniweh, Y., Ayivor-Djanie, R., and Stoute, J. A. (2018). *Plasmodium falciparum* strains spontaneously switch invasion phenotype in suspension culture. Scientific reports, 8(1), 5782.