Functional significance of the STEVOR multigene family of plasmodium falciparum

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NANYANG
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**FUNCTIONAL SIGNIFICANCE OF THE STEVOR MULTIGENE
FAMILY OF *PLASMODIUM FALCIPARUM***

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SCHOOL OF BIOLOGICAL SCIENCES

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MULTIGENE FAMILY OF *PLASMODIUM FALCIPARUM***

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University in Partial Fulfilment of the Requirements for
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TABLE OF CONTENTS

Acknowledgements.....	1
Table of Contents.....	5
List of Figures.....	11
List of Tables.....	15
List of Abbreviations.....	17
Abstract.....	19
1 INTRODUCTION	23
1.1 Malaria: The Big Picture.....	25
1.2 The Parasite Life Cycle.....	27
1.2.1 The Asexual Life Cycle.....	27
1.2.2 The Sexual Life Cycle.....	27
1.3 Immunity to Malaria.....	30
1.4 Protein Trafficking in <i>Plasmodium falciparum</i>	32
1.4.1 Overview of Structural Components of Protein Trafficking.....	32
1.4.2 Overview of Sequence Motifs involved in parasite protein export.....	35
1.5 Antigenic Variation.....	36
1.5.1 The <i>pir</i> superfamily of Variant Antigens.....	37
1.5.2 The Variant Antigens of <i>P.falciparum</i>	38
1.5.2.1 The <i>var</i> genes.....	38
1.5.2.2 The <i>rifins</i>	40
1.5.2.3 The STEVOR multigene family of <i>Plasmodium falciparum</i>	41
1.5.2.3.1 STEVORs, Antigenic Variation and Immune evasion.....	42
1.5.2.3.2 STEVOR: Transcription and Protein Expression.....	43
1.5.2.3.3 STEVOR: Functional Properties.....	44
1.5.2.4 The PfMC2TM family.....	47

1.5.2.5 The SURFINs.....	47
1.5.2.6 The PHIST proteins.....	47
1.6 Rosetting and Sequestration in <i>P.falciparum</i>	48
1.6.1 Rosetting, Sequestration and Parasite Pathology.....	48
1.6.2 Rosetting and Sequestration: Molecular basis of <i>P.falciparum</i> cytoadherence..	50
1.7 Rigidity, parasite maturation and the RBC cytoskeleton.....	52
1.7.1 Asexual Development and Rigidity Modulation.....	52
1.7.2 Gametocyte Development and Rigidity Modulation.....	55
1.8 Aims and Objectives.....	59
2 MATERIALS AND METHODS.....	61
2.1 Materials.....	63
2.1.1 List of Chemicals/Reagents/Kits.....	63
2.1.2 List of Instruments/Equipment/Software.....	64
2.1.3 Lab Generated Antibodies/Strains.....	65
2.1.3.1 Parasite Clones.....	65
2.1.3.2 Anti- STEVOR polyclonal antibodies against the semi-conserved region.....	66
2.2 Methods.....	67
2.2.1 Synchronous Production of <i>Plasmodium falciparum</i> gametocytes <i>in vitro</i>	67
2.2.2 Parasite Transfections.....	67
2.2.3 RT-PCR for Validation of Transfectants.....	68
2.2.4 Western Blotting.....	68
2.2.5 Immuno-fluorescence Assays.....	69
2.2.6 Atomic Force Microscopy.....	69
2.2.6.1 Procedure for AFM tip functionalization.....	70
2.2.6.2 Procedure for Cell Sample Preparation.....	70
2.2.7 Rigidity Assays using micropipette aspiration.....	70
2.2.8 Immuno-precipitation Experiments.....	71
2.2.9 Rosetting Selection of Parasites and set up of Rosetting Assays.....	72
2.2.10 Cos-7 Transfections and Cell Binding Assays.....	73

3 RESULTS

3.1 Topology & Distribution of STEVOR on the iRBC membrane using AFM...	77
3.1.1 Validation of antibody specificity and tip functionalization protocol.....	78
3.1.2 Determination of Threshold Parameters in Control Experiments.....	79
3.1.3 Scanning of iRBCs at Different Stages and Resolutions and Mapping of the Distribution of the STEVOR proteins.....	80
3.1.4 Comparison of STEVOR Expression on the iRBC membrane of parasites at the Different stages of asexual maturation.....	84
3.1.5 Spatial Distribution of STEVOR at the iRBC membrane.....	87
3.1.6 Topology of the STEVOR protein.....	91
3.1.7 Conclusion and Discussion.....	94
3.2 Role of STEVOR in parasite Rosetting.....	96
3.2.1 STEVOR and Glycophorin C in rosetting at the late asexual stages of parasite maturation.....	96
3.2.1.1 Validation of the ability of anti-STEVOR antibodies to interfere with rosetting.....	97
3.2.1.2 Effect of Glycophorin C knock-down on STEVOR mediated rosetting.....	99
3.2.1.3 Interaction of Glycophorin C with the semi-conserved region of STEVOR.....	101
3.2.2 Rosetting at the gametocyte stages of parasite development.....	103
3.2.3 Conclusion and Discussion.....	107
3.3 STEVOR, Parasite Rigidity and Cytoskeletal Interactions.....	109
3.3.1 Transfection and Validation Experiments.....	111
3.3.2 Rigidity Measurements.....	113
3.3.3 Investigations of Cytoskeletal Interactions of STEVOR at the iRBC membrane.....	119
3.3.4 Mass-Spectrometry Results and Validation.....	122

3.3.5 Effect of the Cytoplasmic Domain of STEVOR on molecular clustering.....	124
3.3.6 Conclusion and Discussion.....	128
4 FINAL DISCUSSION AND CONCLUSIONS.....	131
5 FUTURE DIRECTIONS.....	143
6.BIBLIOGRAPHY.....	149
APPENDIX.....	165
Appendix I.....	167
Appendix II.....	168
LIST OF CONFERENCES/WORKSHOPS ATTENDED.....	169
LIST OF PUBLICATIONS.....	171

LIST OF FIGURES

1.1 The <i>Plasmodium</i> Life Cycle.....	28
1.2 Schematic Representation of a Parasite Infected RBC.....	32
1.3 Expression Profile of the three major multigene families of <i>Plasmodiumfalciparum</i>	38
1.4 Predicted Domain structure of the STEVOR proteins of <i>P.falciparum</i>	41
1.5 Schematic Representation of the membrane-cytoskeletal contacts in a mature, uninfected RBC.....	52
1.6 Schematic Representation of the proteins involved in <i>P.falciparum</i> knob formation.....	54
1.7 <i>P.falciparum</i> Gametocyte Development.....	56
1.8 Deformability of iRBCs and Splenic filtration.....	57
3.1 Western Blot of 3D7 lysates using anti-STEVOR and anti-PfEMP1 antibodies...	78
3.2 Validation of the Tip-Functionalization Protocol used for AFM scanning of Samples.....	79
3.3 Determination of Threshold values using Bare versus Antibody functionalized tips.....	79
3.4 Force Frequency Histograms of 5A versus A4 Infected Cells.....	80
3.5 Height and Adhesion Maps of a late Stage iRBC (32*32 pixels).....	83
3.6 The Different Stages of Infection of the RBC and the Increase in Adhesion Frequency of STEVOR seen on the Membrane as the Parasite Matures Within the iRBC.....	86
3.7 Occurrence of Sequential Multiple Bond Rupture Events During force Mapping Experiments.....	88
3.8 A Representative Force Map of a Total of 16384 Adhesion Events on the iRBC surface.....	89
3.9 Alternate Topologies of the STEVOR protein on the iRBC Surface.....	92
3.10 Fixed and Live Staining Using antibodies Against the Semi-Conserved (SC) and Predicted Cytosolic (CYT) domains of the STEVOR proteins.....	93
3.11 Live IFA using anti-S1 (STEVOR) antibody to Stain 5A schizonts.....	97
3.12 Rosetting Selection and the Subsequent Increase in Rosetting Frequency of 5A Parasites.....	97
3.13 Effect of anti-STEVOR antibodies on Parasite Rosetting (5A clone).....	98

3.14	FACS Validation of Glycophorin C knockdown in RBCs used for the Rosetting Assays.....	99
3.15	Effect of Glycophorin C knockdown on the Rosetting Ability of 5A parasites in a Rosetting Assay.....	100
3.16	Effect of GlyC knockdown on the interaction of STEVOR-transfected COS-7 cells with RBCs.....	102
3.17	Validation of STEVOR Expression at the Gametocyte Stages of the 5A Clone.....	104
3.18	Gametocyte Rosetting Assays (quantification and images) at Stages I-V of the 5A clone.....	105
3.19	IFA Showing the Absence of STEVOR Expression in the A4 Clone.....	109
3.20	Sequence of the PFF0850c protein Showing the Cytoplasmic Domain of STEVOR.....	110
3.21	IFA using the anti-S2 Antibody and the Anti-Cytoplasmic Domain Antibody to Stain A4FL and A4TD Schizont Stage Parasites.....	112
3.22	Proposed Role of the Cytoplasmic Domain of STEVOR in Rigidity Increase.....	113
3.23	Effect of the Truncation of the Cytoplasmic Domain of STEVOR on Parasite Membrane Rigidity at the Late Asexual Stages.....	114
3.24	The Presence or Absence of STEVOR on the iRBC membrane at the Different Stages of Gametocyte Development of the A4FL and A4TD Strains.....	116
3.25	Comparison Between Rigidity of A4, A4FL and A4TD strains at Different Stages of Gametocyte Development Using Micropipette Aspiration.....	117
3.26	Giemsa Smears Showing the Effect of the Saponin Lysis Protocol Used on the iRBC.....	119
3.27	The Association Between p55 and Glycophorin C Detected in the pull-down Protocol used.....	120
3.28	Western Blot Detecting STEVOR in the pull-down and Input fractions using α - S2 Primary Antibodies.....	120
3.29	Silver Stain of Co-immunoprecipitation samples Using anti-STEVOR Antibody on Membrane Fractions of A4FL Schizonts.....	121
3.30	Western Blot for Detection of STEVOR and Actin in A4FL and A4TD fractions.....	123

3.31	Adhesion Maps Generated During the AFM scan of A4FL and A4TD schizonts.....	125
3.32	Cluster Size of STEVOR for the A4FL and A4TD Strains.....	126

LIST OF TABLES

3.1 Tip Specifications and Parameters Used to Scan Samples

After Calibration of the AFM Tip.....	81
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3.2 Summary of Parameters Used in Order to Perform a High Resolution

Scan of the iRBC.....	84
-----------------------	----

3.3 RT-PCR of A4FL and A4TD Clones using Primers Against the Cytoplasmic Domain of STEVOR.....

3.4 MS Analysis of Pull-down Fractions Using anti-STEVOR antibodies.....	111
	122

LIST OF ABBREVIATIONS

Abbreviation	Full-Form
A4 FL	A4-Full Length
A4 TD	A4-Truncated Domain
AFM	Atomic Force Microscope
CD36	Cluster of Differentiation 36
CD4	Cluster of Differentiation 4
CK-II	Casein Kinase II
Clag 9	Cytoadhesion Linked Asexual Gene 9
CR1	Complement Receptor 1
CYT	Cytosolic/Cytoplasmic Domain
DDT	Dichloro-Diphenyl-Trichloroethane
GlyC	Glycophorin C
ICAM-1	Immuno Cell Adhesion Molecule 1
IDC	Intra-erythrocytic Developmental Cycle
IMC	Inner Membrane Complex
iRBC	infected Red Blood Cell
KAHRP	Knob Associated Histidine-Rich Protein
LC-MS/MS	Liquid Chromatogram-Tandem Mass Spectrometry
MACS	Magnet-Assisted Cell-Sorting
MC	Maurers Clefts
MEC	MESA Erythrocytic Cytoskeletal motif
MESA	Mature Erythrocyte Surface Antigen
PEXEL/VTS	Plasmodium Export Element/Vesicular Trafficking Signal
PfEMP1	<i>Plasmodium falciparum</i> Erythrocyte Membrane Protein 1
PfEMP3	<i>Plasmodium falciparum</i> Erythrocyte Membrane Protein 3
PFMC2TM	<i>P. falciparum</i> Maurers Cleft 2 Trans Membrane
PK A	Protein Kinase A
RBC	Red Blood Cell
RESA	Ring-Infected Erythrocyte Surface Antigen
RIFIN	Repetitive Interspersed Sequences
RT-PCR	Reverse-Transcriptase/ Real-Time Polymerase Chain Reaction
SICAv	Schizont-infected Cell Agglutinating variant antigens
STEVAR	Sub-Telomeric Variant Open Reading frame
SURFINs	Surface associated Interspersed Gene
TM	Trans-membrane
vsg	Variant Surface Glycoproteins
WHO	World Health Organization

ABSTRACT

Plasmodium falciparum is a protozoan parasite that is responsible for the most severe form of human malaria. Many protozoan pathogens are capable of exhibiting antigenic variation and the STEVOR proteins constitute one such variant antigen family in *P. falciparum*. Recent work has shown that these proteins are expressed on the surface of parasite-infected RBCs during the late asexual stages (trophozoites and schizonts) and immature gametocyte (III-IV) stages of the parasite life cycle. While the asexual cycle is responsible for the increase in parasite numbers and the symptoms of malaria in the human host, the gametocyte stages are important for parasite transmission from human to mosquito. The focus of this project was to study some of the functions and characteristics of the STEVOR proteins at these two stages of parasite development.

The initial aim of the project was to confirm the topology of the protein and its distribution on the surface of the late asexual stages. Here, we used the technique of Atomic Force Microscopy to show that the amount of STEVOR on the parasite surface increases from late trophozoite to bursting schizont. We also demonstrated its ability to cluster on the surface of these cells and confirmed its topology as a single transmembrane domain containing molecule. This information then led us to study the roles of the cytosolic and surface exposed domains of the protein. Using cell binding assays, we confirmed the interaction of the surface exposed semi-conserved region of STEVOR with Glycophorin C on the uninfected RBC. Thus, we confirmed that STEVOR is an important ligand in mediating parasite rosetting. It has already been demonstrated that rosetting contributes to the severity of the disease mediated by *P. falciparum*. Here, we confirmed the involvement of yet another receptor-ligand pair in this phenomenon. This lends further support to already existing evidence that the parasite uses multiple ligands and multiple receptors on the uninfected RBCs to mediate this process.

Our next aim was to study the function of the internal, cytosolic domain of STEVOR. Previous work has shown that the presence of STEVOR on the iRBC surface increases its rigidity at the late asexual as well as immature gametocyte stages of the parasite life cycle. However, the basis of this interaction was unknown. Using micropipette aspiration, we showed that the truncation of the cytoplasmic domain of STEVOR led

to a partial but significant decrease in the rigidity of the iRBC membrane at the late asexual stages. These results were consistent even at the gametocyte stages of the parasite life cycle. Subsequently, we sought to elucidate the mechanism behind this decrease at the late asexual stages. To this end, a pull-down assay was developed using antibodies against the STEVOR proteins. In these assays, membrane fractions of late schizonts were loaded onto a column and the elutions were analysed by LC-MS/MS in order to identify any interaction partners of STEVOR. We showed that while the full length molecule interacted with actin at the iRBC surface, this interaction was lost when the cytosolic domain was truncated. Thus, we showed that the cytosolic domain mediated the increase in rigidity through its interaction with actin. Interestingly, we also pointed out that although the cytosolic domain of the protein was involved in an interaction with cytosolic actin, its truncation did not abolish the clustering of the molecule at the iRBC surface.

In summary, this work shows that the variant STEVOR proteins are multifunctional. We have characterized some interactions of the molecule with host cell components and elucidated the molecular mechanism, by which two different domains of the protein can mediate distinct functions. Both these functions of rosetting and rigidity-increase are important to parasite pathology. Confirmation of the single TM topology of the protein also provides evidence that the STEVOR proteins are representatives of the PIRs, a superfamily of variant antigens that is conserved in all human and rodent *Plasmodium* species.

1.INTRODUCTION

1.1 Malaria: The Big Picture

Malaria literally means ‘bad air’ in Italian. It is a disease of the human blood system transmitted from person to person by the female *Anopheles* mosquito, its biological vector. It is one of the most widespread tropical diseases known to date, caused by the apicomplexan protozoan parasite *Plasmodium* (CDC, *Laveran and the Discovery of the Malaria Parasite*, September 2015). There are five known species of Plasmodia that can infect humans; *P. vivax*, *P. knowlesi*, *P. ovale*, *P. malariae* and *P. falciparum* (Ahmed & Cox-Singh, 2015). The two major species that cause clinical symptoms in humans are *Plasmodium vivax* and *Plasmodium falciparum*, with *P. falciparum* causing the more severe form of the disease. (Jones, Malcolm K, Good, 2006). On average, 3.3 billion people are at risk worldwide (*World Malaria report*, 2015) and occurrences are especially severe in sub-Saharan Africa and South East Asia. The absence of a good vaccine and an increase in drug resistant parasites add to the threat of an increasing burden of disease. *P. berghei*, *P. chabaudi* and *P. yeolii* cause rodent malaria and are used as important models to study the mechanisms and symptoms of the disease *in vivo*. *P. reichenowi* and *P. gaboni* are the two known chimpanzee parasite species, with *P. reichenowi* being the one most closely related to *P. falciparum*. Since it is very difficult to culture *P. vivax*, most *in vitro* studies of human malaria have focussed on *P. falciparum* (Bass & Johns, 1912).

To date, attempts to eliminate malaria have been unsuccessful. In 1955, the WHO attempted to carry out an elimination campaign using DDT along with chloroquine as an insecticide for the mosquito vector and drug against the parasite, respectively. Their attempts met with initial success, but ultimately culminated in the emergence of resistant vectors and parasites. In 1976, they acknowledged the futility of the attempt. Today, even more people suffer from the disease than at the commencement of the program (Eugene Nester et al., 2003).

The difficulty in eliminating the disease is due to the complexity of the life cycle of the parasite, which has the ability to change into different invasive forms. Throughout its growth within the red blood cell (RBC), the parasite exports a variety of different proteins to the RBC surface, thus changing its antigenic properties (Jones, Malcolm K, Good, 2006). This presents a great challenge to the immune system of the host.

Current treatments for the disease include quinine derivatives and artemisinin combination therapy. Parasites that are resistant to both approaches have arisen, and there is a need for the development of better therapeutics to tackle the infection (Amaratunga et al., 2012; Le Roch et al., 2003). Only one vaccine for the prevention of the disease in children has recently been developed and is in the process of being licensed for use. The formulation is known as RTS,S/AS01 and is composed of a fusion protein between the Hepatitis B surface antigen and the circumsporozoite (CSP) protein of the malarial parasite (Segal et al., 2015; Wilby, Lau, Gilchrist, & Ensom, 2012). However, this vaccine is largely meant for the subset of the population that lives in malaria endemic areas. This highlights the need for the continuous development of better preventives and therapeutics to combat the disease.

1.2 The Parasite Life Cycle

1.2.1 The asexual life cycle

As shown in Figure 1.1, when a *Plasmodium* infected female *Anopheles* mosquito takes a blood meal, sporozoites are injected into the dermis of the victim. These invasive forms are capable of migrating through the dermis and into the bloodstream of the host. Once the parasite reaches the liver, it gains entry into the organ, first by migrating through the Kupffer cells, and ultimately invades the hepatocytes, where it develops into invasive forms known as merozoites. These merozoites have the capacity to replicate through an iterative Intra-erythrocytic developmental cycle (IDC), where each released merozoite can subsequently invade another uninfected RBC. This leads to a substantial increase in parasite numbers within the bloodstream of the host. The cycle begins with the entry of the extracellular merozoite into the RBC in a process known as invasion. This process is mediated by a number of different protein interactions between the merozoite and the target RBC. It ends in the complete entry of the merozoite into the RBC and the resealing of the RBC membrane. At this point, the free form of the parasite is no longer exposed to the immune system of the host. The infected RBC membrane, however, is continuously remodelled by parasite proteins as it develops through the ring, trophozoite and schizont stages, at which point the RBC ruptures, releasing merozoites. The released merozoites are capable of invading new RBCs. This entire cycle of development takes about 48 hours for *P. falciparum* parasites, and the clinical symptoms of waves of fever and shivering, which are typical of malarial infections, coincide with this event of RBC rupture (Pulliam et al., 2003).

1.2.2 The sexual life cycle

The process described above constitutes the asexual cycle of *P. falciparum* development. The merozoites that arise from this process, however, are not infectious to the *Anopheles* mosquito, and in order for the parasite to be successfully transmitted, it must undergo development into the gametocyte stage. Thus, in the body of the host, the parasite develops into male and female gametocytes, the mature stages of which are infectious to the *Anopheles* vector (Bousema & Drakeley, 2011). Certain factors, such as anemia and high parasitemia, among others, are thought to have an important

role in determining the commitment of the parasite to the gametocyte stage. The parasite commits to gametocytogenesis at the ring stage of the lifecycle (Baker, 2010). Thereafter, it progresses through a developmental program that is very different from the asexual one. Only the ring and mature stages of gametocyte development are detectable in peripheral circulation, with the immature stages sequestered away. Upon uptake of the mature stage by the vector, the gametocytes rupture into gametes within the mosquito midgut. The female gametocyte gives rise to a single macrogamete and the male gametocyte gives rise to 8 microgametes. Fusion of the male and female gametes results in the formation of an ookinete, which can burrow through the midgut wall of the mosquito. The growth and division of the oocyst within the mosquito midgut then results in the formation of haploid sporozoites, which migrate towards and reside within the salivary gland of the vector. The bite of this mosquito then transmits the sporozoites to the next victim. The above cycle constitutes the sexual or sporogonic cycle of parasite development.

Figure 1.1: The *Plasmodium* life cycle (showing parasite development and replication within the human host). [Image Source : Jones, Malcolm K, Good, 2006]

During the period of growth within the human host, the parasite is exposed to constant surveillance and attack by the vertebrate immune system. In order to survive and replicate successfully, it must find a way to overcome this challenge. *P. falciparum* achieves this by two main mechanisms of (1) rosetting and deep tissue sequestration and (2) antigenic variation of the proteins exposed to the immune system. These two

phenomena and their contribution to disease will be discussed in more detail in the following sections of this chapter.

1.3 Immunity to Malaria

Studies have shown that it is extremely difficult to achieve sterilizing immunity to malarial infections. However, in endemic areas, antibodies that are protective against severe disease are naturally acquired by the population during childhood, so that disease episodes during adult life are mild. This shows that the infected RBC presents antigenic targets to the host immune system, against which it is possible to mount an effective adaptive immune response (Wyler & Brown, 1977). It is proposed that the difficulty in attaining sterilizing immunity is due to the capacity of the parasite to continuously present polymorphic targets to the host.

In areas where malaria is endemic, the local population has certain mutations that are protective against the disease. These mutations affect certain RBC characteristics like shape and oxygen carrying capacity, for example sickle cell anaemia (Ayi, Turrini, Piga, & Arese, 2004) and the thalassaemias (Wambua et al., 2006). Also, it has been shown that mutations in RBC surface receptors, for example, Duffy blood group negativity (protective against *P. vivax* infections) (R. Wang et al., 2005) and Gerbich negativity (Maier et al., 2003) in addition to mutations in the CR1 receptor (Cockburn et al., 2004), are protective against severe episodes. Yet other mutations have been identified in molecules that are involved in immunological signalling, for example, the Interferons α and β and IL-4 (Koch et al., 2002).

Rodent species are used as important models in order to study immune responses to malaria, and much information about how the host responds to the infection in terms of innate and adaptive responses has been gleaned from studies of these infections. In *P. chabaudi chabaudi* AS infections, NK cells have been shown to be necessary in order to lower parasitemia levels during the acute phase of the infection (Mohan, Moulin, & Stevenson, 1997). The authors showed that cytokine production by these cells was an important mechanism to control parasitemia during the acute phase of infection (Langhorne, Simon-Haarhaus, & Meding, 1990). It has also been shown that mice depleted of the CD4⁺ T cell population were unable to control the rising parasitemia levels or clear the parasite, whereas depletion of other T cell populations only had a minor effect on parasite numbers (Seixas, Fonseca, & Langhorne, 2002). Thus, the antibody response is known to be important for immunity against the development of severe infection. It has been shown in certain studies that antibodies

against merozoite surface proteins that are known to be involved in invasion (for example, MSP-1 and AMA-1) are capable of parasite neutralization. However, there is limited evidence to show that the protective response induced by AMA-1 immunization is antibody independent, despite the fact that it is CD4⁺ T cell dependent (Xu et al., 2000). Recently, the roles of anti-PfRh5 antibodies in invasion-blocking and as possible effective vaccine candidates have also been demonstrated (Douglas et al., 2011). However, the exact mechanisms that stimulate this protective response and the processes and effector cells that these antibodies activate remain vague.

In general, it has proved difficult to translate the results of studies in mice to human systems. Many antigens, which have been shown to generate effective protection against severe disease in mice, do not show consistent results in human trials. In order to address this problem, humanized mouse models are being developed in order to study immune responses to malaria in detail, in the context of a human immune system. Recent work in RICH (RBC supplemented, immune cell-optimized humanized) mice has helped to conclusively show that NK cells play a role in the reduction of parasitemia in the host body (Chen et al., 2014). Additional breakthroughs have also been made and it is now possible to support a *P. falciparum* infection in these humanized mice *in vivo* (Amaladoss et al., 2015).

1.4 Protein Trafficking in *Plasmodium falciparum*

Once the *P. falciparum* merozoite invades the erythrocyte, it must generate its own intracellular organelles and machinery in order to survive. The mature RBC itself being optimized for the function of oxygen delivery, lacks this internal cellular architecture (Gratzler et al, 1984). Upon invasion, the merozoite enters with part of the RBC membrane that later goes on to form the parasitophorous vacuolar membrane (PVM), within which it develops (Atkinson & Aikawa, 1990). The pre-existing merozoite membrane becomes the parasite membrane (PM). Thus, the parasite now has three membranes that separate it from the extracellular milieu of the host, viz, the PM, the PVM and the RBC membrane, as shown in Figure 1.2.

Figure 1.2: Schematic representation of a parasite infected RBC. The figure shows the different sub cellular compartments and membranes of the *P. falciparum* infected erythrocyte. [Copyright: Andrew Osborne, UCL, UK].

Depending on their function, different parasite proteins are present in these three distinct compartments and their respective membranes. The parasite uses different signals and probably different mechanisms in order to target them to the right sub cellular location. However, the process of protein trafficking in *P. falciparum* is incompletely understood, due to its complexity. The following sections provide a brief update about what is known about this process, with a focus on proteins that are exported to the iRBC membrane.

1.4.1 Overview of Structural Components of Parasite Protein Trafficking

Certain parts of the parasite protein trafficking process are known to be analogous to the classical vesicle-mediated pathway of higher eukaryotes. For example, the

Brefeldin A (BFA) toxin can be used in order to inhibit protein transport that occurs within the confines of the parasite, indicating that it uses a COP I/COP II-dependent process of trafficking within this sub compartment (Baumgartner, Wiek, Paprotka, Zauner, & Lingelbach, 2008). Recent work has identified the members involved in this process, including parasite v and t SNAREs, synaptin and dynamin. In mammalian cells, these components are known to be important for vesicular trafficking and fusion. EM has also revealed the presence of an endoplasmic reticulum that spreads throughout the parasite cytoplasm and that is continuous with the nuclear envelope. A rudimentary structure that resembles the Golgi network has also been observed (Bannister, Hopkins, Margos, Dluzewski, & Mitchell, 2004).

It is thought that all proteins that need to cross the parasite membrane are first released into the PV. Whether those that remain in the PV localize to a separate sub compartment from those that are trafficked further into the PVM or RBC cytosol remains unclear.

It has been shown that the PTEX (Plasmodium Translocon of Exported Protein) machinery is capable of translocating proteins beyond the PVM. The PTEX is a parasite translocon that is found on the vacuolar side of the PVM, having five known protein components and a size of about 1.2 MDa (de Koning-Ward et al., 2009). It has been found to be essential in all *Plasmodium* species, and deletion mutants of the three core components of PTEX are lethal (Elsworth et al., 2014). Thus, using this machinery, parasite proteins are exported past the PVM and into the RBC cytosol.

Within the RBC cytosol, several membrane-bound structures have been identified. One such structure is the Tubular Vesicular Network (TVN), and it has been found to spread through the cytoplasm of the iRBC (Behari & Haldar, 1994). However, inhibition of the function of this organelle does not abrogate protein export to the iRBC membrane and beyond. Thus, its function is not essential for protein trafficking. Other, better studied vesicle-like structures known as the Maurer's Clefts (MC) have also been localized to the iRBC cytoplasm. These structures have been shown to remain associated with the RBC ghost after parasite rupture. Reports have shown that they form stable associations with the iRBC membrane, via links to the RBC cytoskeleton. Many proteins that are destined for the iRBC membrane are thought to become part of these membrane-bound structures first (Spycher et al., 2006). In

addition to the MC, smaller, single membrane-bound vesicular structures have also been identified within the RBC cytosol (Haeggström et al., 2004).

There are two models that have currently been proposed in order to describe the transport of proteins from the PVM to the MC and then beyond. The first one is the vesicular trafficking model, in which vesicles that bud from the PVM go on to fuse with the MCs. This model predicts that the MCs later fuse with the plasma membrane of the RBC, in order to allow the transport of proteins to the iRBC membrane or extracellular space. The final step of how the parasite achieves the transport of resident MC proteins to the iRBC membrane is not clear since no direct evidence of membrane fusion between these two compartments has been found as yet. However, *P. falciparum* homologues of COP II and NSF proteins have been found in the RBC cytosol and could be involved in MC mediated vesicular transport (Hayashi et al., 2001). An MC resident protein, PfSBP1, has been identified and is thought to interact with the host cytoskeletal proteins (Blisnick et al., 2000), in order to perform the function of vesicle docking. The second model proposes that the MCs are structures that are composed of singular tubules and stacks of membranes, which are continuous with the PVM. In this case, the proteins are predicted to be trafficked from the PVM to the MCs by lateral diffusion. However, this model also suggests that the final step of transport to the iRBC membrane and beyond involves membrane fusion. Recently, however, preliminary evidence has shown that the MCs are not continuous with the PVM (Grüning et al., 2011).

Variant antigens like RIFINs and STEVOR (Kaviratne, Khan, Jarra, & Preiser, 2002; Przyborski et al., 2005) that are found on the surface of the iRBC have also been shown to be present in the MC. Other proteins like KAHRP, which is found on the cytoplasmic side of the iRBC membrane, and PfEMP1, which is on the extracellular side, have been found in aggregates in the iRBC cytosol, only transiently associated with the MC (Knuepfer, Rug, Klonis, Tilley, & Cowman, 2005). The current hypothesis is that such proteins have the ability to refold spontaneously after synthesis, upon interaction with other proteins at their final destination. Several chaperone complexes like Hsp40 of parasite origin and Hsp70 of host origin are likely to be exploited while these proteins are in transit within the iRBC cytosol (Botha et al., 2011).

No pathways for the retrograde transport of proteins from the iRBC membrane back into the cytosol have been identified. However, reports have shown that certain proteins localized to RBC rafts can undergo backward transport, although the mechanisms that enable this remain unknown.

1.4.2 Overview of protein sequence motifs involved in parasite protein export

In eukaryotes, the major way to export proteins is to recruit them into the secretory pathway. This is done by way of an ER signal sequence, generally present in proximity to the N-terminal ends of proteins. A similar signal sequence has been found in the parasite protein PfEXP1, and it has been shown that this sequence is enough to direct the export of proteins beyond the parasitophorous vacuole (PV).

Many proteins that are directed to the iRBC cytosol and membrane have been shown to contain a pentameric sequence known as the PEXEL (Plasmodium Export Element)/VTS (Vesicular Targeting Sequence) motif. This motif has the consensus sequence RxLxE/Q/D and is generally found proximal to the N-terminus of the protein. Both, soluble as well as membrane-bound proteins have been shown to contain this motif (Marti, Good, Rug, Knuepfer, & Cowman, 2004). An ER resident parasite protease, known as Plasmepsin V, has been shown to be essential for the cleavage of proteins containing this motif and their subsequent transport via the PTEX machinery. Plasmepsin V has been shown to cleave the protein after the first leucine of the PEXEL motif (Russo et al., 2010). However, PEXEL-negative exported proteins (PNEPs) have also been identified, although the mechanisms that allow their transport are unclear. Among these are PfSBP-1 and PfEMP1, and it has been shown that certain amino acids at their N-terminal ends block the action of Plasmepsin V, thus precluding their cleavage and downstream processing by this enzyme (Boddey et al., 2013). The exact processing pathway that these proteins undergo prior to export is as yet unclear. Additionally, a PLASMED sequence has been described in *P. yeolii*. The elucidation of this signal has helped to identify many additional proteins that are targeted for host cell export in these parasites (Siau et al., 2014). Thus, it is likely that the *P. falciparum* parasite also uses many other sequences for host cell targeting that are as yet uncharacterized.

1.5 Antigenic Variation

Antigenic variation is defined as the capacity of an infectious agent to change the molecules exposed to the host's immune system over the course of an infection (Deitsch, Lukehart, & Stringer, 2009). It is a strategy that allows the parasite to establish a chronic infection within the body of the host. This becomes especially important in case of parasites that rely on a vector for transmission. It is also extremely important to the malarial parasite's survival strategy as, in this case, transmission is seasonal as well.

Different *Plasmodium* species are known to establish chronic infections of varying durations within the body of the host. For example, *P. malariae* can establish a chronic infection that can last for upto 30 years, while *P. vivax* infections are known to last for upto 5 years. *P. falciparum* can establish infections that can last for upto 3 years (Snounu, Jarra and Preiser, 2000).

Multiple mechanisms can lead to antigenic variation, depending on the type of organism. In general, this can be achieved by two main mechanisms of phase variation and true antigenic variation that involves multigene families. Phase variation constitutes the simpler mechanism of antigenic variation, wherein a particular antigen is expressed on the surface of the cell during one phase and is turned off during the alternate phase (Deitsch et al., 2009). Classical examples are the expression of flagella in *Salmonella* and the surface proteins of *Neisseria* and *T. palladium*.

Within protozoans, *Trypanosoma brucei* and *Giardia lamblia* along with *P. falciparum* are examples of well-studied organisms that exhibit the capacity for true antigenic variation. The *T. brucei* repertoire of approximately 1000 variant surface glycoprotein (vsg) genes represents the best studied example. In this system, the parasite expresses a single vsg variant on its surface, at any given point. Expression of these genes is thus regulated in a mutually exclusive manner, with most parasites of a given population expressing a single member of the gene family (Stockdale, Swiderski, Barry, & McCulloch, 2008).

In *Plasmodium*, the first report describing that parasite derived proteins can be targets of the acquired immune response was for the SICA-vars of *P. knowlesi* (Howard, Barnwell, & Kao, 1983). It was also observed that different surface proteins were

expressed during waves of the infection. These antigens were later shown to be the cause of chronic *P. knowlesi* infections as well. Studies by Hommel *et al.* described that the shizont and trophozoite stages of *P. falciparum* were also capable of displaying different antigenic properties. This led to further studies and ultimately the discovery and characterization of the *var* gene family of *P. falciparum*. The expression of the *var* genes (encoding the PfEMP1 proteins) of *P. falciparum* is also regulated in a mutually exclusive manner (Dzikowski, Templeton, & Deitsch, 2006). The striking feature of the PfEMP1 proteins, however, is that along with contributing to antigenic variation of the *P. falciparum* parasite, different members have been shown to have different host receptor binding capabilities (Pasloske & Howard, 1994). This aspect is an important contributor to the severity of the disease and is discussed in more detail in the following sections of the report.

Although no homologues of *var* have been found in other species of *Plasmodium*, a superfamily of variant antigens exists in human and rodent species (Janssen, Barrett, Turner, & Phillips, 2002). This superfamily is known as the *pir* (Plasmodium interspersed repeats) and is discussed in further detail below (Janssen, Phillips, Turner, & Barrett, 2004). Interestingly, the *stevor* and the *rifin* genes of *P. falciparum* are thought to belong to this group of variant antigens, given their size and the predicted presence of at least one TM domain (Jemmely, Niang, & Preiser, 2010).

1.5.1 The *pir* superfamily of variant antigens

The *pirs* were first described as a large superfamily of variant antigens with representatives in all human and rodent species of *Plasmodium* (Janssen *et al.*, 2002). The *vivax interspersed repeats* (*vir*) genes of *P. vivax* were the first group of variant antigens to be identified that belong to this superfamily. Subsequently, other members were identified in rodent species (*P. chabaudi*, *P. berghei* and *P. yoelii*) as well as in the simian parasite, *P. knowlesi*. All members of this family show a sub-telomeric location within the genome of the parasite (Janssen *et al.*, 2004). The largest subfamily of *pirs* is represented by the *yirs* (*yoelii* interspersed repeats), which comprise 838 members, and the smallest by the *kirs*, which comprise only 68 members. All members of this superfamily have a conserved three exon structure with at least one TM domain and a short cytoplasmic tail.

The *pirs* are thought to have diverse functions, with different members being expressed during different stages of the parasite life cycle. In addition, certain variants have been shown to localize to the surface of the iRBC during parasite development, whereas others have a more internal location. In general, efforts to localize these proteins to the surface of the RBC have proved troublesome, due to their sequence variability and high copy number. It has, however, been shown in the case of the *yirs* that certain members are localized to the schizont surface and that the host immune response alters their transcription pattern (Cunningham et al., 2005).

1.5.2 The variant antigens of *P. falciparum*

In addition to the *var* genes, the *P. falciparum* parasite has other multigene families that are postulated to contribute to antigenic variation. This includes the *rif*, *stevor* and *Pfmc-2tm* gene families. Figure 1.3 shows the expression profile of the different multigene families at the different stages of the parasite's asexual life cycle.

Figure 1.3: Expression profile of the three major multigene families of *Plasmodium falciparum*. The timing of gene transcription and protein expression of the PfEMP1s, RIFINs and STEVORs is represented over the asexual cycle of parasite development.

1.5.2.1 The *var* genes

The *var* genes constitute a multigene family of approximately 60 members in the reference clone 3D7 that encode the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) proteins (Gardner et al., 2002). They were first identified as high molecular weight proteins of the iRBC that could be surface-labelled using radioactive

iodine by Howard *et al.* in 1988 (Howard *et al.*, 1988). Each protein is composed of varying combinations of Duffy Binding Ligand (DBL) and Cysteine rich Inter-Domain Regions (CIDR) domains (Baruch *et al.*, 1997; Peterson, Miller, & Wellems, 1995). These domains display a high degree of variability among each other, and it is this feature that is thought to contribute to the ability of PfEMP1 to confer different cytoadhesive properties to the iRBCs (Deitsch & Hviid, 2004; Pasloske & Howard, 1994) and also contribute to its antigenic variability (Biggs *et al.*, 1991).

Most studies have shown that the *var* genes are expressed in a mutually exclusive manner (Dzikowski *et al.*, 2006) at the mRNA as well as protein level; although, there are some recent reports that contradict this and have found that multiple *vars* may be transcribed at a single parasite level. It is thought that one of the ways in which the parasite is able to alter the antigenicity as well as cytoadhesive properties of the iRBC is by switching the expression of the *var* genes (Biggs *et al.*, 1991). Different PfEMP1 proteins have been found to interact with a variety of cell surface receptors on the iRBC as well as on the endothelial cells, like CD36, CR1, ICAM-1 CSA and thrombospondin (Baruch, Gormely, Ma, Howard, & Pasloske, 1996).

An age-matched study by Pete Bull *et al.* in Kenyan children shows that antibodies to the variant of PfEMP1, expressed during an episode of clinical malaria, are less likely to be present in the pre-existing serum of the same child than in children of similar age and within the same community (Peter C. Bull *et al.*, 1998). It has also been shown that antibodies against PfEMP1 can agglutinate RBCs in a variant specific manner (P C Bull, Lowe, Kortok, & Marsh, 1999). In addition, Cham *et al.* show that antibodies against variants of PfEMP1 are acquired sequentially over the course of an infection, with antibodies against those variants that are known to be responsible for severe disease acquired first (Cham *et al.*, 2009).

The PfEMP1 proteins also contain a cytoplasmic acidic terminal segment (ATS). This domain has been shown to be highly conserved between members of the multigene family (Baruch *et al.*, 1996). It is this domain that is known to be important for the anchoring of the PfEMP1 proteins to membrane protrusions known as knobs (Waller, Cooke, Nunomura, Mohandas, & Coppel, 1999). PfEMP1 is extremely prominent on the surface of the iRBC due to its presentation in the context of the knobs. However, not all the PfEMP1 found on the membrane is anchored within the knobs; there are

reports to show that the expression of this protein is possible on the iRBC surface in knobless strains. However, in this case the amount of PfEMP1 on the surface decreases (Horrocks et al., 2005). The interactions between proteins that constitute the knobs and the iRBC cytoskeleton and their contribution to iRBC membrane rigidity are discussed in detail in the following sections of this chapter.

1.5.2.2 The *rifins*

Rifins constitute the largest known family of variant antigens in *P. falciparum*, being present in about 150 copies in the 3D7 genome (Gardner et al., 2002). They are present at the sub-telomeric ends of all 14 chromosomes. The encoded proteins are of low molecular weight, about 27-45 kDa in size. They may be separated into a cysteine-rich N-terminal segment and a C-terminal region that contains a hypervariable loop region, predicted to be flanked by two transmembrane regions. However, recent evidence supports the presence of a single TM domain, with the semi-conserved and hypervariable regions of the protein exposed to the extracellular milieu (Goel et al., 2015a). Overall, *rifins* can be categorized into two broad sub-families, the *rif A* and *rif B*. The presence of an insert downstream of the PEXEL motif and within the semi-conserved region in the A type RIFINs distinguishes them from type B RIFINs. The A type RIFINs are trafficked to the iRBC membrane (Newbold, 1999; Petter et al., 2007) via the parasite MCs, while the B type RIFINs have been shown to be more internally localized. A recent study shows that a B type RIFIN is expressed at the merozoite tip and on the surface of the gametes (Mwakalinga et al., 2012).

The earliest reports of rosetting at the asexual stages implicated low molecular weight proteins (Fernandez, Hommel, Chen, Hagblom, & Wahlgren, 1999); and the RIFINs were earlier known as the ‘Rosettins’ of the iRBC. However, their role in rosetting has only been recently confirmed in a study by Goel *et al.* (2015), which shows their ability to bind to the iRBC in a blood group A antigen-dependent manner (Goel et al., 2015). A substantial humoral immune response to the RIFINs has also been detected through the course of a *P. falciparum* infection and that this response may lead to milder, asymptomatic infections (Abdel-Latif, Cabrera, Köhler, Kremsner, & Luty, 2004; Abdel-Latif, Dietz, Issifou, Kremsner, & Klinkert, 2003; Abdel-Latif, Khattab, Lindenthal, Kremsner, & Klinkert, 2002). There is also evidence to show that the *rifins*

are transcribed at the gametocyte stage, however, whether they are exposed on the iRBC surface at these stages is as yet unclear (C. W. Wang et al., 2010).

1.5.2.3 The *Stevor* multigene family of *P. falciparum*

Stevors constitute the third largest multigene family of *Plasmodium falciparum* (Gardner et al., 2002). They were first characterized by Cheng *et al.* (1998) in terms of copy number, protein domain prediction and chromosomal location (Cheng et al., 1998). This gene family is comprised of 33 members in the reference clone 3D7, and they are present at the sub-telomeric ends of all 14 chromosomes, in proximity to the *vars* and the *rifs* (Cheng et al., 1998). About 2-3 members of *stevor* have been shown to be transcribed at a single parasite level, and multiple variants of the protein are also detectable (Blythe et al., 2008; Khattab & Meri, 2011; Khattab et al., 2008; Niang, Yan Yam, & Preiser, 2009). Studies have shown that certain *stevor* variants are conserved across strains, indicating that these members may be under specific selective pressure. The *stevor* genes encode a protein that is approximately 35-40 kDa in size (Cheng et al., 1998). Previous bioinformatic predictions indicated the presence of a signal peptide, PEXEL motif, hypervariable (HV) region and two TM domains that flank a semi-conserved (SC) region as shown in Figure 1.4.

Figure 1.4: Predicted domain structure of the STEVOR proteins of *P. falciparum*. The percentage values indicate the degree of conservation between different members of the family [Adapted from Niang et al., 2009]

Given its property of sequence variation, it was thought that the HV region is exposed to the extracellular side of the iRBC surface. Taken along with the prediction of a 2 TM structure, this would indicate that the SC region is internally located. *Stevors* share this 2 TM prediction with the *rifs* and the *pf-mc2tms*. However, recent biological evidence challenges this prediction and instead suggests a model that comprises a single TM domain, with both, the HV and SC regions exposed on the extracellular side of the iRBC surface, and a short cytoplasmic tail anchoring the protein (Niang et al., 2009). This model is confirmed by part of the work presented in this thesis. Interestingly, the single TM domain structure was also confirmed for the RIFINs (Goel et al., 2015), and most members the PIR superfamily of variant antigens also share this single TM structure (Jemmely, Niang, & Preiser, 2010).

1.5.2.3.1 STEVORs, antigenic variation and immune evasion

The STEVOR proteins were shown to be expressed during the sporozoite, trophozoite, schizont, merozoite and gametocyte stages of parasite development (McRobert et al., 2004), indicating that they may perform a distinct function at each stage. At the late trophozoite and schizont stages, they were previously shown to be localized to the MC of the iRBC (Kaviratne et al., 2002). Further studies that involved the use of field isolates and the selection of parasites for *stevor* expression then showed that the trafficking of the protein did not stop at the MC, but rather that it reached the surface of the iRBC (Blythe et al., 2008; Niang, Yan Yam, & Preiser, 2009). Within a population of the reference clone 3D7, different STEVORs were shown to be expressed by individual parasites, thus demonstrating that STEVORs mediate antigenic variation of the iRBC (Niang et al., 2009). It has also been shown that the variant of STEVOR expressed on the surface of the infected schizont is distinct from the one expressed on the merozoite surface (Blythe et al., 2008; Khattab & Meri, 2011; Khattab et al., 2008). Further supporting the role of STEVOR in antigenic variation was another study that showed using RT-PCR that sub-populations of the NF-54 parasite isolate expressed different variants of STEVOR and that expression switching also occurred between different isogenic clones at a rate of approximately 2 percent (Lavazec, Sanyal, & Templeton, 2007).

During the asexual replication cycle, the peak of *stevor* transcription occurs at around 28 hours post invasion (mid-trophozoite stage), as seen in parasites maintained under conditions of continuous culture (Kaviratne et al., 2002). Recently, it has been shown in patient isolates that another peak of transcription occurs at the early ring stage (Bachmann et al., 2012). Interestingly, the same variants of the gene family were expressed during both peaks. This indicates that the protein may have an additional role during the early stages of parasite development within clinical isolates. Two independent studies have demonstrated that expression of STEVOR proteins is up regulated in patient isolates as compared to culture strains (Bachmann et al., 2012; Blythe et al., 2008). This further lends weight to the idea that STEVOR is involved in immune evasion.

However, very little is known about STEVOR and its ability to mediate protective immunity. A single study, using four recombinant STEVOR variants when tested against the sera of infants, showed a correlation between presence of anti-STEVOR antibodies and increased episodes of parasitemia (Schreiber et al., 2008). This study, however, used only four variants that are not representative of the entire repertoire diversity in a bacterial recombinant protein production assay. In addition, sera of different age groups were not tested. More extensive studies need to be carried out before this data can be properly interpreted.

1.5.2.3.2 STEVOR: transcription and protein expression

Not many studies have focussed on the regulation of *stevor* gene expression; although, recent work shows that they are regulated independently of *var* gene transcription. This is unlike the regulation of the *rif* genes, where it has been shown that their expression is linked to the subset of *var* genes transcribed. Also, similar sub-nuclear localization of transcriptionally active *var* and *rif* promoters was also shown, while no such evidence is available for *stevor*. However, the co-regulation of antigenic variants may restrict the overall repertoire of the parasite; and there is no available explanation for why the parasite might do this. It has been shown that a common activation factor may be involved in regulation of all four VSA families of *P. falciparum*, including PfEMP1. Episomal promoters of each of these families were shown to be capable of decreasing expression of chromosomal members of the other three families. The exception to this is the dynamics between the regulation of the *pfmc2tm* gene family and *stevor*, where it was shown that high levels of episomal *stevor* promoters lead to upregulation of the *pfmc2tm* genes. It is as yet unclear which epigenetic marks mediate the activation and silencing of the *stevor* loci at the chromosome level.

The presence of the PEXEL motif at the N-terminus of the protein was an early indication that it is trafficked beyond the confines of the parasite and into the iRBC cytosol. At the late trophozoite stage, the protein has been located to the Maurer's clefts (MC) of the parasite (Kaviratne et al., 2002; Przyborski et al., 2005) and later to the iRBC membrane (Blythe et al., 2008; Niang et al., 2009). Through its transit from the parasite to the iRBC membrane, STEVOR has been found to be associated with the membrane fraction of the parasite lysate, suggesting that it is always present as an

integral membrane protein. Additionally, in support of this model of trafficking, the STEVOR TM domain has been shown to be essential for the transport of the protein through the MC to the iRBC surface (Przyborski et al., 2005). Here, it was also shown that STEVOR co-localizes with PfSBP1 and PfEMP3. A recent study demonstrated that the PfPTP1 protein, which is known to be important for trafficking of PfEMP1 to the iRBC membrane via the MCs, is also essential for STEVOR transport. The authors show that in a parasite line lacking PfPTP1, STEVOR is not detectable on the iRBC surface (Rug et al., 2014).

1.5.2.3.3 STEVOR: functional properties

STEVOR has long been suspected to have host receptor binding capacity on the iRBC surface. Initial studies on rosetting and RBC binding suggested that low molecular weight proteins on the surface of the iRBC were responsible for this process (Fernandez et al., 1999). However, the prominence of the knobs on the iRBC and their presentation of PfEMP1, taken along with the fact that different PfEMP1 variants can easily be selected for based on different cytoadhesive properties, led the focus away from the small variant proteins that were previously implicated. While it is well established that rosetting is a phenomenon that is not restricted to *P. falciparum*, no homologues of the *vars* have been found in Plasmodium species apart from *P. knowlesi*. This suggests that there are other players that are involved in this process that have been neglected so far. *P. falciparum* exports a host of other variant proteins to the iRBC surface apart from the PfEMP1s. Recent work carried out on the RIFINs that shows their involvement in rosetting also emphasizes the need to study the other proteins exported by the parasite (Goel et al., 2015). Part of the work in this thesis deals with the interaction of STEVOR with Glycophorin C and its role in parasite rosetting. Interestingly, STEVOR is also present at the apical tip of the merozoite, and its role in parasite invasion was recently confirmed (Niang et al., 2014). RIFINs have also been localized to the merozoite tip, although their role in invasion has not been characterized as yet.

In addition to its function of RBC binding, STEVOR has also been shown to contribute to increased iRBC membrane rigidity at the late trophozoite and schizont stages, during which it is expressed on the iRBC membrane (Sanyal, Egée, Bouyer,

Perrot, Safeukui, Bischoff, et al., 2012). However, the mechanism underlying STEVOR's effect on membrane rigidity remains unknown. *P. falciparum* parasites increase the rigidity of the iRBC they infect as they mature through the asexual cycle of replication, with the membrane of the schizont-infected RBC being the most rigid. Why the *P. falciparum* parasite modifies the rigidity of the iRBC membrane is not clear yet, and there are likely to be several proteins that contribute to the process. It is possible that the increased rigidity of the iRBC membrane at the late stages of parasite infection may aid the sequestration process within host tissues, and thus they can resist being carried into the peripheral circulation by blood flow within the host body. The alternate hypothesis is that sequestration is a phenomenon that is necessary for *P. falciparum* iRBCs because of their increased rigidity and their subsequent inability to pass through the splenic circulation. Thus, if they remain sequestered, they are then able to avoid splenic clearance. In support of the claim that multigene families may be involved in avoidance of splenic clearance, it has been shown that in splenectomised patients, there is an absence of multigene family expression (Bachmann et al., 2009). The question of whether STEVOR is also involved in host endothelial cell binding and sequestration or not at these stages remains unanswered.

STEVOR proteins are also expressed during the gametocyte stage of parasite development. Interestingly, it has been shown that the *rifs* and *vars* are also transcribed at these stages (Petter et al., 2007; Sharp et al., 2006) and that a subset of the varC genes are specifically transcribed during gametocytogenesis (Sharp et al., 2006). Sharp et al. provide some evidence that the *stevor* variants expressed at the gametocyte stage are similar to those expressed at the asexual stages, apart from the downregulation of a single variant in the gametocyte culture. In this work, the authors used quantitative RT-PCR in order to study whether certain variants of *stevor* were upregulated in gametocytes as compared to their asexual progenitors in a population of 3D7 parasites (Sharp et al., 2006). However, recent studies provide evidence that limiting dilution of a mixed population gives rise to clones that are able to express different variants of *stevor* (Niang et al., 2009). Thus, whether the expression of *stevor* variants changes in gametocytes when compared with asexual stages would be better evaluated using these clones. Additionally, these studies were carried out only in culture strains and not in field isolates. It has already been shown in the case of asexual parasites that the

expression profile of the *stevors* differs in field isolates (Bachmann et al., 2012; Blythe et al., 2008).

Gametocytes represent the transmission stage of *Plasmodium*. *P. falciparum* undergoes a unique and lengthy process of gametocytogenesis from Stage I to Stage V, with only stage V gametocytes being found in the peripheral circulation. STEVOR has been shown to be expressed on the iRBC surface from Stages III-IV, while it disappears from the membrane of Stage V parasites (Tibúrcio, Niang, et al., 2012). During this maturation process, the parasite modifies the rigidity of the iRBC membrane, with the stage V parasite being the least rigid. It is thought that the stage V gametocyte loses its rigidity in order to allow its unobstructed circulation through the spleen and peripheral blood, while post-mortem studies have shown that stages I-IV sequester within the bone marrow (Joice et al., 2014).

STEVOR proteins have been localized to the iRBC membrane of gametocytes. However, no information is available on the protein trafficking pathway employed, as stage III and IV gametocytes have been shown to lack MCs, which constitute the classical protein trafficking apparatus during the asexual development of the parasite. Recent studies have shown that STEVOR contributes to this increase in iRBC membrane rigidity seen from Stage III-IV of gametocyte maturation. Techniques like microspherulization and ektacytometry were used in order to measure the rigidity of the maturing parasites (Tibúrcio, Niang, et al., 2012). The loss of rigidity seen in the Stage V gametocyte has been linked to the removal of STEVOR from the iRBC membrane. However, no information is currently available to address which domains of the protein are involved in these changes in rigidity; and part of the work presented in this thesis attempts to fill this gap. Interestingly, the RIFINs have also been shown to be expressed in all stages of gametocyte development, except at stage V (Mwakalinga et al., 2012).

Thus, the *stevors* and *rifs* have been shown to be important to several areas of *P. falciparum* growth and function in the human host. Further studies that focus on the mechanisms and functions of these small variant antigens are likely to fill some gaps that exist in the overall understanding of *P. falciparum* mediated disease and pathology.

1.5.2.4 The Pf-MC2TM family

Although the three major variant antigen families of *P. falciparum* described above have been localized to the iRBC surface, there is another, smaller family comprising of 13 members that has been localized to the MC of the parasite. These are the PfMC-2 TM proteins, named for their localization in the iRBC and their conserved 2 TM topology (Sam-Yellowe et al., 2004). No identifiable function has been ascribed to these proteins as yet, although they have also been found to be exported to the iRBC membrane. Surprisingly, microarray data showed that all 13 variants are transcribed at the trophozoite stage of parasite growth. However, RT-PCR experiments demonstrated that only certain variants were upregulated in comparison with the rest and that this gene family was also capable of variant switching (Lavazec et al., 2007). Recent genetic clustering and alignment studies have suggested the presence of a conserved and most likely functional MC-TYR domain between members of this family (Frech & Chen, 2013).

1.5.2.5 The SURFINS

Another family of 10 proteins has been identified, which also localize to the surface of iRBCs (Winter et al., 2005). Recent reports show that these proteins are also present on the merozoite tip (Mphande et al., 2008). Genetic alignment studies propose that they are evolutionarily related to the *SICAvars* of *P. knowlesi*, and may share a common origin with the PfEMP1s of *P. falciparum* as well (Frech & Chen, 2013).

1.5.2.6 The PHIST proteins

These proteins constitute a family of 72 members that are thought to be involved in remodelling of the RBC. The genes that encode these proteins are sub-telomerically located, and homologues of this family have also been found in other *Plasmodium* species. All members have a two exon structure, with the conserved PHIST domain being encoded by part of the second exon. Certain members in *P. falciparum* are proposed to have RBC cytoskeleton binding properties. Interestingly, they have also been shown to have the ability to modify the deformability and rigidity of the iRBC.

1.6 Rosetting and Sequestration in *P. falciparum*

1.6.1 Rosetting, sequestration and parasite pathology

Rosetting is an important aspect of *P. falciparum* induced pathology; however, the ability to form rosettes is not limited to this parasite. It has been shown that all species of *Plasmodium* that are capable of infecting humans can rosette (Lowe, Mosobo, & Bull). In addition, the rodent parasite *P. chabaudi* is used as an important *in vivo* model to study this aspect of parasite pathology. Rosetting is defined as the phenomenon during which the infected RBC binds to uninfected RBCs, engaging surface receptors on both types of cells. It is thought that in this way, the parasite manages to achieve two goals. The first among these is to evade the host immune system and to limit its access to the infected RBC and released merozoites. The second one is to ensure parasite survival and a drastic increase in parasitemia, as the merozoites from the bursting schizont can rapidly invade the uninfected RBCs that are part of the rosette (Wahlgren et al., 1989). However, the exact function of rosette formation is unknown, since there are contradicting reports on whether rosette formation actually leads to an increase in parasite numbers. Although a single study has shown a correlation between the ability to form rosettes and increased parasitemia in African children (J Alexandra Rowe, Obiero, Marsh, & Raza, 2002), there are others that do not support this conclusion. In addition, it was also shown that the capacity to rosette does not positively co-relate with the ability to evade invasion inhibitory antibodies (Deans & Rowe, 2006). The fact that field isolates show differences in their capacity to rosette further complicates the issue. However, rosetting has been directly linked to the ability of *P. falciparum* to cause complicated disease and severe infection in several studies (Wahlgren, Carlson, Helmby, Hedlund, & Treutiger, 1992). Investigations have shown that isolates from patients that have suffered from complicated malaria have a higher rate of rosetting than those that haven't.

A model that could explain the fact that rosette formation leads to complicated and severe disease without causing a direct increase in parasitemia was proposed by Kaul *et al* (Kaul, Roth, Nagel, Howard, & Handunnetti, 1991). This model suggests that

rosette formation occurs on top of iRBCs that are already bound to the endothelium (sequestered), in order to obstruct blood flow in the thin capillaries of vital organs. This could then lead to the symptoms of severe disease such as coma, respiratory distress, renal and hepatic failure. In addition, the study also showed that rosetting occurs mainly in the venules of isolated mesoecum vasculature and not in the arterioles, suggesting that the lower venous blood pressure enables the formation of these rosettes. In contrast with rosetting, the capacity for endothelial sequestration is unique to *P. falciparum* from amongst those species that infect humans. Of all the stages of *P. falciparum* development in the body of the human host, only rings and mature stage V gametocytes have been detected in the peripheral circulation, suggesting that all other stages sequester within internal sites. The immature gametocytes of *P. falciparum* are also known to sequester during their development in sites such as the bone marrow and spleen (Baker, 2010; Bousema & Drakeley, 2011). Thus, it can be seen that the parasite employs the mechanism of sequestration at both stages of development. It is thought that these mechanisms are employed in order to avoid the splenic circulation at both stages. In support of this claim, it has been shown that the mature asexual as well as immature gametocyte stages can be detected in the peripheral circulation in splenectomized patients. Thus, a loss of the sequestration phenotype is seen in such cases (Bachmann et al., 2009).

The asexual stages of *P. falciparum* are known to sequester in different sites (David, Hommel, Miller, Udeinya, & Oligino, 1983), the most prominent being the brain endothelium. By sequestering in the thin capillaries of the brain, these parasites block blood flow and this contributes to one of the most severe symptoms of *P. falciparum* infection, i.e., cerebral malaria. This often results in coma and death in patients, who develop the severe form of the infection (Pasloske & Howard, 1994). The puzzle of why certain infections develop into severe ones and others don't is further complicated by differences in host responses and inflammatory factors; and although cytoadherence is linked to severe infection, it is not the only contributing factor. *P. berghei* ANKA infection of C57BL/6J mice replicates many of the symptoms of severe malaria, including cerebral malaria, seen in humans and is used as an important investigative model (De Oca, Engwerda, & Haque, 2013).

The molecular basis of rosetting and endothelial sequestration is discussed in detail in the following section.

1.6.2 Rosetting and sequestration: molecular basis of *P. falciparum* cytoadherence

Studies have shown that several components of the uninfected and infected RBCs are involved in rosette formation. Among these, the receptors expressed on both types of cells play an important role. It has been shown that CR1 is an important ligand on the uninfected RBC. Rowe *et al.* (year) showed that a deficiency of this receptor lead to a reduction in parasite rosetting. Subsequently, Cockburn *et al.* showed that this mutation could protect the endemic population from severe malaria (Cockburn et al., 2004).

Many studies have been carried out on the molecular mechanisms that mediate rosetting. Till date, PfEMP1 has been identified as one of the major parasite ligands involved in this process. Several host receptors such as CR1 are known to interact with PfEMP1 and mediate this process (J A Rowe, Moulds, Newbold, & Miller, 1997).

However, not many studies have been carried out in order to characterize the roles of other variant antigen families in this process. Previous studies have identified the RIFINS as rosettins of the iRBC (Fernandez et al., 1999). Recent work by Goel *et al.* suggests that these are indeed rosetting ligands for the blood group antigen A on the uninfected RBC (Goel et al., 2015). Part of the work presented in this thesis deals with the role of the STEVOR proteins in host cell receptor binding. It is likely that the phenomenon of rosetting seen *in vivo* is due to the synergistic contribution by different parasite ligands rather than being the effect of a single type of molecular interaction. It is also likely that the parasite uses several ligands on its surface to mediate this process in order to overcome the problem of host cell receptor diversity. Each of the parasite ligands identified during the process of rosetting so far are able to engage different surface receptors on the uninfected RBC. Also, many of the receptors on the uninfected RBC like CR1, Glycophorin C and the blood group A antigen are under selection pressure in areas where *P. falciparum* infection is endemic (Cockburn et al., 2004; Maier et al., 2008).

While rosetting is not unique to *P. falciparum* from amongst those *Plasmodium* species that cause infection in humans, the process of endothelial sequestration is. Since late stage parasites are sequestered, an increase in parasite numbers is not reflected in Giemsa smears of peripheral blood. PfHRP2 is thus used as a reliable indicator of true parasite load within the body of a patient. Recent studies that have

used this indicator have shown that when samples of patients who suffered from severe *P. falciparum* malaria were analysed, an increased load of sequestered parasite biomass was observed in comparison with the controls (Cunnington, Bretscher, Nogaro, Riley, & Walther, 2013). Currently, a link has been established between endothelial sequestration and severity of disease (Avril, Brazier, Melcher, Sampath, & Smith, 2013).

Several parasite ligands have been shown to be important for endothelial sequestration. These include both, variant proteins on the IE surface as well as invariant adhesins and modified host cell receptors. Currently, the PfEMP1 and ICAM-1 pair represents the most well-studied host-ligand interaction that has been implicated in endothelial sequestration (Rogerson et al., 1999). However, current studies show that this interaction and binding between the two molecules is not unequivocal; and in certain studies, it has been shown that changes in the variant of PfEMP1 expressed do not lead to a reduction of ICAM-1 mediated cytoadherence of the parasites. EPCR is another receptor on the host cell that has been found to complicate this interaction between PfEMP1 and ICAM-1 (Turner et al., 2013).

Several invariant parasite proteins such as sequestrin (Ockenhouse, Klotz, Tandon, & Jamieson, 1991) and clag 9 have also been implicated in the process of endothelial sequestration (Trenholme et al., 2000). In addition, the parasite also modifies the host cell band 3 protein into a protein called Pfadhesin. Known endothelial receptors for these proteins are CD36 and TSP (Eda, Lawler, & Sherman, 1999).

Taken together, this information shows that the parasite uses a variety of different receptors in order to mediate the process of host cell binding. These studies demonstrate the importance of studying this phenomenon of cytoadhesion mediated by *P. falciparum* parasites. It can be speculated that interventions that are able to disrupt these interactions will serve to reduce the high levels of morbidity and mortality that are characteristic of *P. falciparum* infections.

1.7 Rigidity, Parasite Maturation and the RBC Cytoskeleton

1.7.1 Asexual development and rigidity modulation

The RBC is specialized for the function of oxygen delivery to all parts of the body. In order to carry out its role optimally, it has a biconcave shape which gives it a larger

surface area to volume ratio; approximately 40 % higher than a sphere of the same volume. It also has an extremely flexible membrane, as it must be able to squeeze through very narrow capillaries. This is made possible by the special properties of the RBC cytoskeletal proteins. The RBC cytoskeleton is composed of tetrameric spectrin filaments, which interact with short actin fibres that are anchored to the RBC membrane at certain points (DeSilva, Peng, Speicher, & Speicher, 1992; Speicher & Marchesi). The main points of anchorage are provided by the Glycophorin C (TM), band 4.1, p55 complex (Nunomura, Takakuwa, Parra, Conboy, & Mohandas, 2000) and the band 3 (TM), ankyrin, band 4.1 complex (Bennett, 1983). Reversible protein phosphorylation ensures the stability of this network. The overall flexibility of the structure is conferred by the ability of the spectrin filaments to slide against each other between these points of anchorage. The strength that the underlying cytoskeletal structures confer to the RBC membrane and the cohesion between the cytoskeleton and membrane also prevent vesiculation and rupture (An, Lecomte, Chasis, Mohandas, & Gratzer, 2002). Thus, under normal conditions, the RBC is highly deformable (Fowler, 2013).

Figure 1.5: Schematic representation of the membrane- cytoskeletal contacts in a mature, uninfected RBC. The organization of specific TM and adaptor proteins and their resulting interactions with spectrin and actin gives the RBC membrane its flexibility. [Image source: Nunomura et al.,2000)

The *P. falciparum* parasite is known to modify the structural properties of the RBC drastically and increase the rigidity of the iRBC membrane. As the parasite matures from ring to schizont, the rigidity of the iRBC increases progressively (Nash, O'Brien, Gordon-Smith, & Dormandy, 1989). However, not all species of *Plasmodium* cause an

increase in the rigidity of the iRBC, and in contrast, *P. vivax* infection is known to lower it. This difference is thought to contribute to the variation in pathology of the two types of infections.

Once infected with *P. falciparum*, the RBC undergoes a change in shape from discoid to spherical. The intracellular development of the parasite also increases the viscosity of the plasma. Previously, this increase in rigidity of *P. falciparum* iRBCs was thought to be a result of the parasite's growth within the iRBC, and its reduction of the surface area to volume ratio of the erythrocyte. In addition, further studies have shown that many parasite proteins are capable of interaction with the existing RBC membrane and cytoskeletal structures. Several proteins are known to contribute to this increase in rigidity seen at the asexual stages.

As described earlier, *P. falciparum* exports various proteins to the RBC surface that are essential for its survival and development. Using these proteins, the parasite also changes the permeability of the RBC membrane to different substances. Many of these exported proteins are capable of interaction with the existing RBC transmembrane and cytoskeletal structures. Proteomic studies have shown that around 200 proteins are exported to the host cell cytoplasm by the parasite (van Ooij et al., 2008), and five of these have been shown to interact directly with the spectrin cytoskeleton. Primary among these are RESA, MESA, KAHRP, PfEMP1 and PfEMP3 (Cooke, Mohandas, & Coppel, 2004; Xinhong Pei, Xinhua Guo, Ross Coppel, Souvik Bhattacharjee, Kasturi Haldar & Narla Mohandas, 2007). In this way, the parasite is known to modify the properties of the RBC membrane and rigidify it (Nash et al., 1989).

At the ring stage, a parasite protein called RESA is present at the iRBC membrane. This protein interacts with the spectrin dimer and prevents the tetrameric form of spectrin from dissociating. Ektacytometry was used in order to show that this modification confers an increased tolerance for mechanical stress on the iRBC (Xinhong Pei, Xinhua Guo, Ross Coppel, Souvik Bhattacharjee, Kasturi Haldar & Narla Mohandas, 2007). The most well-characterized proteins that contribute to increased rigidity at the late asexual stages (once RESA disappears) are represented by the components of the 'knob' complex. Knobs are the structures that are responsible for anchoring PfEMP1 proteins to the surface of the iRBC (S S Oh et al., 2000; Voigt et al., 2000), and they form prominent protrusions of the iRBC membrane that are

easily visible under the microscope. Extensive studies have shown that structures at the base of the knob are capable of interaction with the spectrin filaments and adaptor proteins of the iRBC cytoskeleton and membrane. For example, it is known that parasite derived MESA (Mature Erythrocyte Surface Antigen) is capable of interaction with the RBC adaptor protein band 4.1. This interaction is known to modify the affinity of p55 for the band 4.1-Glycophorin C complex at the iRBC membrane, and this probably affects downstream signalling events in the iRBC. Based on this finding, a MEC (MESA erythrocyte cytoskeleton) binding motif was identified. This motif was found in at least 13 other parasite proteins that are thought to be exported (Kilili & LaCount, 2011).

Figure 1.6: Schematic representation of the proteins involved in *P. falciparum* knob formation Knobs found on the iRBC membrane and their ability to alter the organization of the underlying RBC cytoskeleton. [Image source: Maier et al., 2009]

Extensive work has been carried out on these knob structures and their ability to anchor the PfEMP1 protein at the iRBC membrane. The major component of this structure has been shown to be the KAHRP protein. This protein is capable of interaction with the PfEMP1 conserved C-terminal ATS domain (Voigt et al., 2000). Although, earlier studies showed that the ATS of PfEMP1 could interact directly with KAHRP, recent work shows that a PHIST protein is involved in the interaction (Oberli et al., 2014). KAHRP has also been shown to interact with the spectrin-actin-band 4.1

junction of the RBC cytoskeleton. It is thought that it is this interaction that is responsible for the increase in rigidity of the iRBC membrane caused by the knobs (S.Steven Oh et al., 2000). It has been shown that PfEMP1 can also be presented on the iRBC surface in the absence of knobs. In this case, the C-terminal ATS domain of the protein interacts directly with the cytoskeletal structures of the RBC in order to anchor itself to the membrane.

1.7.2 Gametocyte development and rigidity modulation

The gametocytes of *P. falciparum* represent the transmission stage of its life cycle. The mosquito vector takes up the mature stage V gametocytes from an infected human. Once inside the mosquito midgut, these parasites must respond to a range of different environmental conditions in order to continue their life cycle (Alano, 2007). The gametocyte must therefore be well-equipped with mechanisms that allow it to quickly switch between two very different environments. The change in transcription and protein expression profiles seen when the parasite commits to gametocytogenesis and leaves the iterative IDC is mainly due to this requirement (Baker, 2010).

The gametocyte also prepares for the impending change in environment by altering its RNA and protein expression profiles. Several proteins known to be important for gametocyte development and gamete function such as Pfs16, Pfg377, Pfs25 and Pfs28 have been identified at these stages. Some of the newly transcribed RNA molecules have also been shown to be gender specific.

Currently, the asexual stage of *P. falciparum*, which is responsible for the symptoms of a malarial infection, is relatively well studied and continues to receive much attention. In comparison, however, the gametocyte stage has received much less attention, partly due to the fact that it is not responsible for any of the clinical symptoms seen and is not known to contribute to the severity of the disease in an infected individual. However, the development of interventions that can target this stage are of great importance in reducing the disease burden at the population level.

P. falciparum gametocytes take much longer to develop within the human host as compared to other *Plasmodium* species. The period of development lasts for about 12-14 days before the mature stage V gametocyte can be detected in the peripheral circulation and is ready for uptake by the *Anopheles* vector during its blood meal.

These gametocytes are known to develop through five distinct stages. The first stage is morphologically very similar to the trophozoite stage of asexual development; however, the remaining four stages deform the RBC extensively and can be distinguished microscopically from the asexual stages with relative ease, as shown in Figure 1.7 (Hawking et al, 1971).

Figure 1.7: *P. falciparum* gametocyte development, showing the distinctive morphology of the different stages. [Image source: Hawking et al, 1971]

In fact, the *falciparum* parasites have been named for the falciform or crescent shape of the stage V gametocyte. The ability of the parasite to significantly alter the shape of the RBC at the gametocyte stages is thought to be due to the presence of the inner membrane complex (IMC), which lies just under the parasite plasma membrane. It has been shown that this structure arises from the redistribution of the outer parasite ER, consistent with the localization of GAP50 (an ER marker) to the IMC. In association with this IMC is an assembly of microtubules, which is seen until Stage IV of development. It has been shown that this microtubular complex disassembles at stage V of the gametocyte, and this phenomenon is thought to contribute to the formation of the crescent or banana shape of the gametocyte at this stage (Dixon, Dearnley, Hanssen, Gilberger, & Tilley, 2012).

It has been hypothesized that the shape-shifting that is characteristic of *P. falciparum* gametocytes is in order to allow the circulating Stage V to subvert splenic detection. This is consistent with the fact that the parasite also becomes more deformable at this stage, probably in order to allow the parasite to pass easily through the splenic sinuses, as shown in Figure 1.8.

Figure 1.8: Deformability of parasite infected RBCs and Splenic filtration.

The postulated ability of the deformable uninfected RBCs and stage V gametocytes to pass through the splenic sinuses, while the less deformable trophozoites get trapped [Image Source: Dixon et al., 2012].

Recent work has suggested that a reduction in intracellular cAMP kinase-dependent signalling in the Stage V gametocyte is responsible for the increased membrane deformability of this stage. In this study, drugs were used in order to increase the cAMP kinase-dependent signalling in stage V gametocytes and it was observed that the increase in deformability of these parasites was lost in comparison with the control (Ramdani et al., 2015).

Since the success of the parasite depends on a large extent to the effectiveness of its transmission between host and vector, the parasite must ensure that the gametocyte stage is well protected from the host's immune response during its lengthy period of development. It is probably in order to achieve this objective that the immature gametocytes (I-IV) sequester in the deep circulation of the host, and the parasite is thought to maintain its rigidity through these stages in order to contribute to this phenomenon of sequestration. Post mortem studies conducted on patients, who have succumbed to *P. falciparum* infection, provide *in vivo* proof that the immature

gametocyte stages sequester in sites such as the bone marrow (Joice et al., 2014) and spleen.

The absence of knobs at all stages of gametocyte development has been reported recently (Tibúrcio, Silvestrini, et al., 2012). It is known, however, that stage I and II gametocytes express PfEMP1 on their surface, albeit to a lower degree than seen in asexual stages. Stage III and IV gametocytes, however, show the absence of PfEMP1 on their surface. At these stages, STEVOR proteins are expressed on the iRBC surface and are also known to contribute to the lower deformability and increased rigidity of the iRBC membrane (Tibúrcio, Niang, et al., 2012). At stage V, STEVOR surface expression is not seen and an increase in iRBC deformability has been reported. It will be interesting to study the importance of the different domains of STEVOR at these stages of parasite development, in order to further dissect its role in rigidity and sequestration.

1.8 Aims and Objectives

The work in this thesis focuses on the functions, topology and distribution of the STEVOR proteins on the iRBC surface, at both, the asexual as well as gametocyte stages of parasite development. Earlier work in the lab and outside showed that STEVOR was likely to be involved in rosetting and rigidity modulation of the iRBC. In this work, we sought to delineate one possible mechanism for rigidity modulation carried out by STEVOR. The central hypothesis at the start of the work was that STEVOR is a multi-functional protein that is able to mediate distinct functions using its different domains. In this work, we focus on the cytoplasmic and semi-conserved domains of the protein.

The aims of this project were to:

- Map the distribution of STEVOR on the iRBC surface and confirm its topology.
- Confirm the interaction of STEVOR with Glycophorin C.
- Study the effect of the cytoplasmic domain of STEVOR on parasite rigidity.
- Study the interaction of the cytoplasmic domain of STEVOR with the RBC cytoskeleton.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 List of Chemicals/Reagents/kits

Media and supplements:

1. RPMI
2. DMEM
3. FBS
4. Albumax
5. Hypoxanthine
6. Glutamine
7. Penicillin/Streptomycin
8. Gentamycin
9. Luria Bertani (LB) Broth and Agar
10. Ampicillin
11. Kanamycin
12. WR99210 drug for DHFR resistant *P. falciparum* transfectants

Chemicals and Reagents:

1. Sorbitol
2. Percoll
3. Phosphate Buffered Saline (PBS)
4. Sodium Dodecyl Sulphate (SDS)
5. Ammonium Persulphate (APS)
6. Tris-Cl buffered salts
7. Sodium Hydroxide pellets
8. Acetic Acid
9. Absolute Methanol
10. Absolute Ethanol
11. Saponin
12. Para formaldehyde (PFA)
13. Bovine Serum Albumin (BSA)
14. Gluteraldehyde
15. Protease inhibitor

Kits and Commercial Antibodies:

1. DyNa beads conjugated to protein G (Life Technologies)
2. Silver Staining Kit (Biorad)
3. Western ECL detection substrate (Thermoscientific)
4. Mouse monoclonal anti-p55 antibody (Abcam)
5. Mouse anti-actin antibody (Abcam)
6. HRP conjugated anti-mouse secondary antibody
7. HRP conjugated anti-rabbit secondary antibody
8. FITC conjugated anti-rabbit secondary antibody (Sigma)
9. PE conjugated anti-rabbit secondary antibody (Sigma)
10. FITC conjugated anti-mouse secondary antibody (Sigma)
11. PE conjugated anti-mouse secondary antibody (Sigma)

2.1.2 List of Instruments/Equipment/Software

1. JPK Atomic Force Microscope
2. Bruker AFM MLCT tips for force spectroscopy and scanning
3. Zeiss Confocal Microscope
4. Olympus DIC microscope connected to micropipette aspiration apparatus
5. BIORAD SDS PAGE casting and running apparatus
6. Western blot development machine
7. JPK data analysis software
8. Image J processing software
9. MACS magnetic apparatus and columns (Miltenyl Biotech)
10. Magnetic Eppendorf rack used with DyNa beads (Lifetech)

2.1.3 Lab generated antibodies/strains

2.1.3.1 Parasite clones

The 3D7 isogenic clone 5A was isolated by performing a limiting dilution of a 3D7 mixed culture population (Niang et al, 2009). In such a way, parasites that stably express known variants of *stevor* were isolated and characterized. Of these, clone 5A was used for some of the work carried out in this thesis. Its transcriptional profile with respect to *stevor* expression is shown in Figure 2.1.

Figure 2.1: The transcriptional profile of *stevor* in the 3D7 isogenic clone 5A (Niang et al., 2009).

2.1.3.2 Anti-Stevor polyclonal antibodies (rabbit) against the semi-conserved region.

Anti-STEVOR polyclonal antibodies S1 and S2 were raised against the conserved regions of the two genes PF10_0395 and PFF0850c as shown in Figure 2.2 (Niang et al, 2009). These antibodies were designed in order to recognize the maximum number of *stevor* variants, and hence the semi- conserved region was used as the target epitope.

Figure 2.2: Semi-conserved regions against which anti-STEVOR antibodies were raised. The regions against which the anti-STEVOR polyclonal antibodies S1 (STEVOR 1) and S2 (STEVOR 2) were raised (Niang et al., 2009).

2.2 METHODS

2.2.1 Synchronous production of *Plasmodium falciparum* gametocytes *in vitro*

The different strains of *P. falciparum* used were first grown to high parasitemia for 3-4 asexual replication cycles after thawing. Medium used was made up of 1X 1640 RPMI supplemented with 10 % albumax. Synchronization of late stage parasites was carried out using MACS enrichment (Miltenyl biotech, as per manufacturer's instructions). The following flowchart was then followed until the desired stage of gametocytes was detected by microscopy of thin giemsa smears. Gametocytes were then purified by either MACS or percoll gradient purification and then used for different experiments.

Figure 2.3: Protocol for the synchronous production of gametocytes. Flowchart showing the protocol followed for the synchronous generation of gametocytes (Fivelman et al., 2007).

2.2.2 Parasite Transfections

Two separate plasmids containing the *stevor* (PFF0850c) gene were generated: one having the full length gene (FL) and the other lacking the last 17 amino acids that represent the cytoplasmic domain of the protein (TD). Both versions of the gene were under the control of the *hsp86* promoter of *P. falciparum*. 100 µg of each plasmid was

used to transfect the A4 cell line previously shown to lack STEVOR expression. Highly synchronized A4 culture at approximately 10 % early rings was used for the transfection. Blood was changed to fresh blood (< 7 days old) one cycle prior to carrying out the transfection. 200 µl of blood was used per transfection and each transfection was carried out in duplicate. Electroporation of the ring stage parasites was carried out using the following settings: 0.31 kV, 960 mF in 0.2 cm cuvettes. Parasites were then put back in culture in T25 small flasks and selected for positive transfectants using 5 nM of WR99210.

2.2.3 RT-PCR for validation of transfectants

cDNA synthesis from the RNA of the parasites was carried out using the Biorad iscript RT supermix. The following PCR conditions were used- priming was carried out for 5 minutes at 25 °C, reverse transcription was carried out for 30 minutes at 42 °C and reverse-transcription (RT) inactivation was carried out at 85 °C for 5 minutes. The resulting cDNA was then used for amplification of the STEVOR constructs from both parasites using qRT-PCR. The STEVOR specific primers used are listed in Appendix II.

2.2.4. Western blotting

Samples were prepared in 4 M urea buffer containing bromophenol blue and loaded onto a 10 % - 12 % SDS denaturing gel. The gel was run at 100 V for 3 hours. Wet transfer onto a nitrocellulose membrane was performed at 150V for 2 hours at 4 °C. Blocking of the membrane was then carried out using 5 % skimmed milk in 1X PBST for 1 hour.

Primary antibody incubation using rabbit (1:5000)/ mouse (1:2000) anti-S1/ mouse anti-actin (1:10,000) was then carried out under mild rocking conditions overnight at 4 °C.

The membrane was then washed 3X with 1X PBST for 10 minutes. Secondary antibody incubation with HRP conjugated anti-mouse antibody (1:10,000 dilution)/ HRP conjugated anti-rabbit antibody (1:10,000 dilution) was carried out at room temperature under mild rocking conditions for 1 hour. The membrane was then washed 3X with 1X PBST for 10 minutes.

Chemiluminiscent substrate was added and incubation was carried out in the dark for 5 minutes under mild rocking conditions. Development was then carried out using the Amersham system according to manufacturer's instructions. Exposure time was varied from between 2 minutes to 10 minutes, depending on the strength of the signal seen.

2.2.5. Immuno-fluorescence Assays

The different gametocyte stages / schizont stages as generated above by MACS/percoll purification were prepared. For live IFA, the cells were used directly. In the case of fixed IFAs, the cells were first fixed with 2 % PFA under rotating conditions for 20 minutes at room temperature. Three washes using 1X PBS were then carried out. Blocking was done at room temperature in 1 % BSA for 45 minutes under rotating conditions. Cells were then incubated with primary antibody (1:50 for fixed IFA and 1:20 for live IFA; diluted in PBS-1 %BSA) for 1 hour at 37 °C. Cells were then washed in 1X PBS 3 times, 10 minutes each wash. Secondary antibody was added and then incubation was carried out at 37 °C for 1 hour. The secondary antibody dilutions used were Alexa 488 conjugated anti-mouse antibody (1:400 dilution)/Alexa 594 conjugated anti-rabbit antibody (1:400 dilution). DAPI was added to the secondary antibody solutions at a dilution of 1:500. Imaging was carried out at 100X magnification using the Zeiss confocal microscope at the appropriate wavelengths.

2.2.6 Atomic Force Microscopy

Different parasite stages were enriched using MACS. Imaging and force mapping experiments with the AFM set up were carried out at different stages of infection. AFM tips were functionalized with anti-STEVEOR antibody molecules to probe the test samples from 5A, A4 and A4-FL and A4-TD clones. MACS enriched and suitably diluted samples of iRBCs at different stages were prepared on thin coverslips and experiments were carried out in a fluid environment on the JPK AFM set up (JPK Instruments AG). Depending upon the kind of experiments (i.e., imaging or force mapping), scanning parameters were optimized in real time in order to obtain a stable and specific signal. A number of control experiments were also performed in order to obtain levels of background fluctuations and non-specific interactions. These parameters were used as the threshold values during data analysis. Collected force curve scans were processed using JPK SPM data processing software (JPK

Instruments AG) in order to obtain visual-quantitative information about cell surfaces and spatial adhesion maps.

2.2.6.1 Procedure for AFM tip functionalization

The AFM tip was first plasma treated for 10 minutes, after which an incubation with 20 μ l BSA-biotin was carried out overnight. The tip was then washed for 2 minutes on a belly dancer using PBS. It was then incubated with NHS-PEG4-Biotin cross-linker for 45 minutes. A gentle wash with PBS was then carried out. Rabbit anti-S1 (1:200) was added directly after removing PBS and incubated for 1 hour. The tip was washed in PBS again for 2 minutes and the extra activated amine groups were blocked by incubating the tip with Glycine for 30 minutes. A final wash using PBS was carried out for 2 minutes.

2.2.6.2 Procedure for Cell Sample Preparation

The cover slip on which the sample was coated was first washed with ethanol using the belly dancer for 5 min, after which it was dipped in DI water. Any additional moisture was absorbed using kimwipes, after which it was left to dry. Plasma treatment of the coverslip was then carried out for 5 minutes, followed by a 30 μ l PHA-E coating and an incubation of 1 hour. The coverslip was then washed thrice with PBS. Enriched schizont stage iRBCs were added on top of the PHA-E coating and incubated for 1 hour at room temperature. Unbound RBCs were washed off using complete culture medium to prevent any floating cells.

2.2.7 Rigidity assays using micropipette aspiration

Micropipette aspiration of live cells was first described as a technique to estimate elastic shear modulus of human RBCs by Robert Hocmuth in 1993 (Technique reviewed in Hocmuth et. al, 2000). In this technique, a glass micropipette with an inner wall having a diameter of a few micrometres is used in order to partially aspirate the membrane of the iRBC. Negative pressure is applied to the cells using a fluid pump in order to generate the suction force necessary for the aspiration to occur. The leading edge of the cell being aspirated into the mouth of the pipette can be tracked using a microscope and camera attached to the apparatus. A total of 80-100 frames were acquired per cell. Care was taken to only include those frames in the analysis for which the length aspirated into the micropipette was less than its diameter. The data

analysis module used was fitted using the hemispherical cap model. The analysis uses the length of the cell aspirated, time taken for aspiration, diameter of the pipette and suction pressure as variables in order to estimate the Elastic Shear Modulus of the cell membrane, in accordance with Laplace's law (Hochmuth et al, 2000). The higher the estimated shear modulus, the more rigid the cell.

Cultured iRBCs (asexual and gametocyte stages) were centrifuged at 2200 rpm for 3 minutes (acceleration=9; deceleration=2) at room temperature and then diluted to a 1:100 ratio with 1 %BSA in PBS. The dilution step is carried out in order to ensure that the cell sample contains a thin suspension that is not crowded, so that single cells can easily be aspirated through the mouth of the micropipette. The BSA is added to preserve the morphology of the RBCs.

Diluted cells were then placed in a chamber constructed with two coverslips and parafilm stacks. This chamber was then kept on the stage of the microscope and the mouth of the micropipette was inserted between the coverslips of the chamber. The appropriate suction pressure was applied in order to aspirate the cells. Recording of images was started once the leading edge of the cell membrane began to be aspirated into the micropipette. 80-100 frames were captured per cell at constant intervals.

2.2.8 Co-immunoprecipitation experiments

Parasites were cultured at high parasitemia (8 %-10 %) and the infected mid-late stage schizonts were purified using percoll density gradient centrifugation. Small volumes (1-2 microlitre) of percoll purified samples were then used for giemsa smears in order to validate the enrichment protocol. The rest of the sample was first fixed with 1 % PFA, lysed with 0.02 % saponin for 10 minutes on ice, and then centrifuged at high speed (17000 g) in the cold room. Supernatant was collected and then sheared with 0.5% Triton X and used as the membrane fraction of the iRBCs for the co-immunoprecipitation (protocol as in Chandramohanadas et al., 2009). In order to confirm removal of parasite membrane a giemsa smear was prepared from the residual pellet, also showing that the rest of the parasite was intact (Figure 3.26).

Antibodies used for the co-immunoprecipitation were conjugated to Protein G Dyna beads for 45 minutes at 37 °C as recommended by the manufacturer's protocol. Beads were then washed 3X for 5 minutes each in order to remove excess antibody. The

resultant mixture of antibody conjugated to beads was split into aliquots of 50 µl each. The membrane fraction of the parasites obtained using the lysis procedure described above was incubated with these antibody conjugated beads for 1 hour at 37 °C and then overnight at 4 °C. Beads were then thoroughly washed 3X for 15 minutes each under rotating conditions using 1X PBS in order to remove any nonspecific binding partners. Samples were transferred to fresh Eppendorf tubes prior to elution using urea buffer (4 M final concentration) containing bromophenol blue. Elution of bound sample from beads was carried out for 10 minutes at 72 °C before loading onto a 10 % SDS gel. The gel was run at 120 V for 2 hours, followed by silver staining in order to detect the separated proteins. Specific bands from the silver stained gel were then cut out and sent for MS analysis by LC-MS/MS.

2.2.9 Rosetting selection of parasites and set up of rosetting assays

Parasites were selected for rosetting using a 0.5 % gelatin solution in iRPMI. A culture of mid-late schizonts at 8-10 % parasitemia and 1ml packed cell volume (PCV) of RBCs was resuspended in 10 ml of the gelatin-iRPMI solution and then incubated for 60 minutes in a water bath at 37 °C. Parasites that are rosetting positive (R+) tend to settle at the bottom of the tube, while those that do not rosette(R-) remain suspended in a diffused solution above the R+ pellet. The two phases (R+ and R-) were separated into separate falcon tubes and washed with iRPMI at 2200 rpm for 5 minutes. The respective parasite samples were cultured further, and after gaps of 7 days rosetting selection was carried out using the R+ samples up to a total of 3-4 times until high rosetting frequency of parasites was achieved. These parasites were then used in rosetting assays as described below.

Rosetting positive cultures were enriched at the mid-late schizont stage using MACS. A 1:10 ratio of infected RBCs: uninfected RBCs was maintained at a 10 % haematocrit in order to provide the excess RBCs required for rosetting to occur. A solution of iRPMI containing 20 % FBS was used as the binding medium, and the resulting cell suspension was incubated at 37 °C for 60 minutes under rotating conditions. Cells were then diluted appropriately to avoid crowding and imaged using the DIC microscope. Resultant rosettes were then counted and rosetting frequency was quantified under various conditions. A minimum of 100 iRBCs were counted per experiment.

2.2.10 Cos-7 Transfections and cell binding assays

All transfections were carried out on 12 well plates once a confluency of 40 %-50 % COS-7 cells were obtained. For the transfections, serum free and antibiotic free medium was used, which was changed 5 hours after transfection to fully supplemented medium. Lipofectamine LTX was used as the transfection reagent and a total of 0.8 µg of plasmid DNA (ST3C, empty vector and PvDBPII) was used to transfect the cells. Each transfection was carried out in triplicate for each assay. Cells were allowed to grow for one day before the binding assays were carried out. Before performing the assays, the cells were washed thoroughly 3X with iRPMI. For the cell binding assays, binding medium containing 20 % FBS was used. A dilution of 1:500 of the respective RBC fraction (Normal, GlyC k/d or empty vector) was used to make the RBC stock solutions. 1ml of binding medium was added to each well. 50 µl of the RBC stock solution was then added to each well. Cells were incubated for 2 hours at 37 °C and then washed thoroughly with PBS until no floating cells were visible. Imaging was carried out at 40X using a DIC microscope. A single COS-7 cell having a minimum of two RBCs bound to it was counted as a single rosette.

3. RESULTS

3.1 Topology and Distribution of STEVOR on the iRBC Membrane Using AFM

Earlier work carried out on STEVOR has shown that these proteins are associated with the MC of the parasite and that they localize to the RBC membrane at the late trophozoite and schizont stages (Kaviratne et al 2002, Przyborski et al, 2005, Blythe et al 2008, Niang et al, 2009). The peak of RNA transcription has been shown to be at 28 hours post invasion (Kaviratne et al., 2002). However, there are no studies to quantify protein expression on the iRBC membrane as the parasite matures from trophozoite to schizont. Also, the question of how the protein is distributed here remains unanswered. The work presented in this chapter attempts to address these two questions using AFM and also clarifies the topology of the protein.

Previously, it has been shown that PfEMP1, the most well-studied variant antigen on the surface of the iRBC can cluster (Voigt et al., 2000). It is thought that this clustering and the resulting distribution of the protein on the iRBC membrane are linked to parasite pathology.

Atomic force microscopy, which is a subset of scanning probe microscopy techniques, can detect intermolecular interactions at very high resolutions (Whited & Park, 2014). As described earlier, the setup comprises of a tip that is in contact with the sample, which is the iRBC in these experiments. Upon sample scanning, a deflection of the tip results in a proportionate and measurable deflection of a laser beam that is directed at the tip. This deflection information is then converted into force spectroscopy curves and high resolution images of the sample. For the experiments in this chapter, the AFM tip was functionalized using anti-S1 antibodies. Control experiments were carried out using a bare/non-functionalized tip (coated with BSA). Further controls comprise a set of measurements taken using the functionalized tip to scan the surface of uninfected RBCs. Values obtained from these control experiments were used in order to set the threshold parameters, above which specific interactions were obtained and measured. Presented in this chapter are the detailed results obtained from all control, validation and experimental sets of data.

Work carried out to show the increased expression of STEVOR on the asexual parasite surface as the parasite matures was done in collaboration with Himanshu Singh, SMART. During the analysis, it was noticed that STEVOR may cluster on the iRBC

surface. Independent experiments were carried out in order to demonstrate this phenomenon of clustering.

3.1.1 Validation of antibody specificity and tip functionalization protocol

In order to scan the surface of the infected RBC for STEVOR expression and distribution, an AFM tip functionalized with rabbit anti-S1 antibody was used. The western blot of parasite lysate shown in Figure 3.1 confirms that S1 detects a band at 35 kDa that is specific to STEVOR. Importantly, this image shows that the antibody is not capable of cross reacting with the other major variant surface antigen on the parasite membrane, i.e, PfEMP1 (which is also known to cluster).

Figure 3.1: Western blot of 3D7 lysates using anti-STEVOR and anti-PfEMP1 antibodies. The blot shows the inability of the STEVOR antibody to cross react with PfEMP1 (No detection of bands in the first two panels around 250 kDa; antibodies S1 and S2). The right panel shows the typical band pattern for PfEMP1 as seen in a Western blot (Antibodies: Ani-ATS) (*Niang et al, 2014*).

Additionally, confocal microscopy was carried out on the tip used to scan the sample, in order to confirm that the antibody bound reliably to the tip using the functionalization protocol described in Section 2.2.6.1. Phycoerythrin (PE)-conjugated rabbit secondary antibody was used against S1 and the results are shown Figure 3.2. Fluorescence confocal imaging was carried out at a wavelength of 578 nm.

Figure3.2: Validation of the tip functionalization protocol used for AFM scanning of samples. Showing the coating of the AFM tip with anti-STEVR antibodies. The triangular surface shows the cantilever. The glowing red dot at the far end shows the tip used to scan the sample. Scale bar: 0.15 mm.

3.1.2 Determination of threshold parameters in control experiments

Scanning of cells in order to detect background interactions was carried out using various sets of controls. Bare tips were used to scan the surface of parasite infected RBCs in order to calibrate levels of non-specific interactions between tip and sample. Similarly, scans were also carried out of uninfected RBCs using S1 functionalized tips. Background interactions in comparison with the interactions observed during actual experimental conditions are shown in Figure 3.3. During analysis, these control values were used as threshold parameters, above which specific interactions were obtained and analysed.

Figure 3.3: Interaction frequencies in an AFM scan using bare versus antibody functionalized tips. The values obtained for the scanning of the iRBC with a bare tip were used in order to determine the threshold parameters above which specific interactions were quantified (data pooled from 5 cells each).

Figure 3.3 shows the percentage of interactions (pooled from scans of 5 cells each) that were found to be above the 20 pN threshold. The threshold was set at 20 pN, as the maximum number of interactions for the bare tip were found to be below this value (between 0-20 pN). Only very few interactions using the bare tip were found to be above 20 pN, as shown in Figure 3.3. Only those interactions that were above the 20 pN threshold were included in the following analyses.

In an additional set of control experiments, the A4 clone (STEVR negative; derived from IT04) (Roberts et al., 1992) was used in order to confirm that the interactions detected by the functionalized tip were also below the determined threshold in this case. Presented below is a comparison between a scan performed on an A4-infected RBC and a 3D7-derived 5A infected RBC.

Figure 3.4 Force frequency histograms of 5A versus A4 infected cells.

A: Showing the scan of a 3D7-derived 5A infected RBC using the S1 functionalized tip. Maximum number of interactions occur between 70-90 pN for this cell. B: Showing the scan of an IT04 derived A4 infected (STEVR negative) RBC using the S1 functionalized tip. Maximum number of interactions occur below 30 pN for this cell.

3.1.3 Scanning of iRBCs at different stages and resolutions and mapping of the distribution of the STEVR proteins

The 3D7-derived 5A clone was used for the following study of STEVR expression and distribution. Infected RBCs at different stages were used and the expression and distribution of the protein was mapped using the anti-S1 functionalized tip. The

contact mode of the instrument was used to scan the surface of the RBCs, and experiments were carried out in the Force Mapping channel of the instrument.

Summarized in Table 3.1 are the specifications of the tip used for sample scanning and the calibration parameters. Calibration of the tip was carried out under the Force spectroscopy channel of the instrument.

Tip / Parameters	Type / value
Type of tip, Cantilever type	MLCT-Bruker, Cantilever C (triangular)
Resonant frequency range	4-10 kHz
Spring constant range	0.005-0.02 N/m

Table 3.1: Tip specifications and parameters used to scan samples after calibration of the AFM tip.

Calibration using the above parameters was carried out every time the tip was re-mounted or a new tip was used. Care was taken to ensure that the final accepted parameters were within the above recommended ranges.

The calibration parameters and their relevance to the experiment are explained briefly below:

Resonant frequency range: This parameter is used in order to determine the actual spring constant of the tip under experimental settings. It represents the frequency at which the vibrations of the tip fit a normal distribution. Briefly, the instrument vibrates the tip at a range of different frequencies. Those that fit within the above recommended range are used in order to determine the spring constant of the tip.

Spring Constant: In order to determine the spring constant, the tip is raised slightly above the sample surface and then vibrated at a frequency range as described above. It represents the vibration of the cantilever only due to environmental conditions and not sample contact. Since tip deflection causes a proportionate deflection of a laser beam, the instrument measures deflection in terms of voltage. Once the spring constant has been determined, it uses this value in order to convert the voltage to a force value.

Sensitivity: This is another parameter that is calibrated before experimental data is collected. As mentioned above, tip deflection is originally measured by the instrument as a change in voltage due to a proportional deflection of the laser beam. In order to calibrate sensitivity, the tip is first kept in contact with a blank area of the coverslip (no cell present). The instrument is then allowed to run once. This allows the conversion of voltage to distance.

After calibration, large area ($10\text{ }\mu\text{m} \times 10\text{ }\mu\text{m}$) scans were carried out at low resolution (32 pixels*32 pixels) and higher pulling speeds initially, in order to locate the complete infected RBC and to identify regions of high adhesion. A representative image of one such scan acquired in the height and adhesion channels is shown in Figure 3.5.

From the adhesion and height maps shown in Figure 3.5, those areas on the iRBC having high adhesion values were selected. Care was taken to ensure that the edges of the cell were avoided and that the scan was being performed on the surface of the cell. Once this was achieved, the scans of the selected area were repeated at lower pulling speeds and at high resolution (128 pixels*128 pixels). $1\text{ }\mu\text{m} \times 1\text{ }\mu\text{m}$ areas were scanned at this resolution.

Figure 3.5: Height and adhesion maps of a late stage iRBC (32 pixels*32 pixels).
A: A height map of an iRBC (*P. falciparum* infected) during a low resolution scan. Lighter areas represent regions that have increased height when compared to darker areas (shown in the scale bar). B: An adhesion map of the same iRBC. Areas of high adhesion are represented by lighter areas whereas low adhesion areas are darker (shown in the scale bar). Yellow circles demarcate the cell boundary in both A and B. The yellow boxes in both A and B represent areas at the centre of the iRBC where a high resolution scan was performed. α -S1 antibody was used to functionalize the tip used for the above scans.

Table 3.2 summarizes the parameters used for the high resolution scan.

Parameters	Value
Relative Setpoint	0.15 nN
Z length	1.5 μm
Z movement	Constant speed
Extend Speed	5.0 $\mu\text{m/s}$
Extend Time	0.2 s
Extend Delay	0.05 s
Delay mode	Constant force

Table 3.2: Summary of parameters used in order to perform a high resolution scan of the iRBC. Instrument settings used during the high resolution scans. Scan size: 1 μm *1 μm . Resolution: 128 pixels*128 pixels.

These scans yielded a spatial resolution of about 8 nm. This allowed the finely tuned mapping of adhesion sites on the cell surface. A detailed analysis of each force curve and the bond break pattern allowed a very close look at the distribution of STEVOR on the surface of the cell.

Two different pieces of information could be obtained from the scans performed above. The data obtained enabled the mapping of the total amount of protein on the iRBC surface in a stage specific manner. In addition, the high resolution scans enabled a closer look at the molecular distribution. Presented in the following sections are the analyses and results from the above experiments.

3.1.4 Comparison of STEVOR expression on the iRBC membrane of parasites at the different stages of asexual maturation

In collaboration with Himanshu Singh, SMART

Single cells from the 3D7-derived 5A clone were scanned at different stages of asexual maturation. The cells were sorbitol synchronized one cycle prior to scanning. These included 10 cells at the ring stage and 20 cells at the trophozoite and schizont stages.

Late stages were first enriched using MACS and then diluted appropriately before sample preparation as described in section 2.2. Represented in Figure 3.6A are images of the iRBC at the different stages of asexual maturation, acquired in the height and adhesion channels.

The interactions obtained from the above scans were then quantified. First, they were separated into force bins of 0-20 pN, 20-100 pN, 100-500 pN and >500 pN. The number of interactions obtained for each bin was plotted in a stage specific manner, as shown in Figure 3.6B. The increase in STEVOR at the iRBC membrane as the parasite matures is clearly visible. In addition, a graph of cumulative specific adhesion frequency is shown, in order to show the increase of STEVOR on the parasite surface as it matures. Also compared and used as a threshold were the number of interactions obtained using the functionalized tip to scan uninfected RBCs. It can be seen from this control that not many non-specific interactions were obtained.

From this work it can be seen that as the parasite matures, the amount of STEVOR localized to the iRBC membrane increases. The experiments presented in the next section attempt to study its spatial distribution on the membrane.

A

B

Figure 3.6: The different stages of infection of the RBC and the increase in adhesion frequency of STEVOR seen on the membrane as the parasite matures within the iRBC. A: The lower panel represents the adhesion obtained, and the increase in high adhesion spots can be seen clearly. The yellow circles represent those areas of the iRBC (2 μ m) that were scanned at high resolution (128 pixels*128 pixels); shown in the lowermost panel. B: Left: Showing the increase in the number of high-force interactions as the parasite matures. Right: Showing the overall increase in STEVOR at the membrane as the parasite matures from ring to schizont. Data from the different trophozoite stages were pooled and analysed. Error bars represent Standard Error. A total of 100 cells were scanned. Scale bar = 5 μ m. Done with Himanshu Singh, SMART.

3.1.5 Spatial distribution of STEVOR at the iRBC membrane

Scans of the schizont infected RBC at 128 pixels*128 pixels enabled the visualization of its surface at a spatial resolution of approximately 8 nm. Additionally, while carrying out the force mapping experiments, an interesting pattern of bond rupture events was observed upon tip retraction at certain areas of the surface. This pattern indicated that there were multiple simultaneous and sequential bond rupture events occurring at certain areas of the membrane. Shown on the adjacent page are a few examples of the force curves obtained.

From the cumulative force frequency histogram, a single bond break event was estimated to occur at 36 pN. Further manual filtration and analysis of specific bond rupture events and the total force required for the tip to detach from the cell surface during retraction strengthened the argument for the occurrence of clustering. More than 10^5 specific adhesion events were taken into consideration during this analysis.

Figure 3.7: Occurrence of sequential multiple bond rupture events during force mapping experiments. The blue arrows point to the bond rupture events in the force curves. Light red curves represent the extension of the tip, while those in dark red show the retraction of the tip from the cell surface.

In addition to the analysis of single force curves, it was also observed that certain areas of the membrane showed many bond rupture events in series, i.e, there were several bond rupture events occurring in succession at certain locations. Shown in Figure 3.8 is a representative force map obtained at a resolution of 128*128 pixels. Certain areas that have multiple high adhesion spots have been circled. Each scan yields a total number of 16,384 interactions.

Figure 3.8: Representative force maps of a total of 16384 adhesion events on the iRBC surface.

A whole cell scan at 32 pixels*32 pixels was carried out (shown in the upper panel). The yellow circle demarcates the area occupied by the whole iRBC. The black box represents that area on the iRBC that was scanned at 128 pixels*128 pixels (lower panel). Each pixel in the lower panel represents a single interaction. Circled in black are areas of the membrane where clustering is visible. Lighter adhesion spots represent higher force values.

Figure 3.8.1: The collection and interpretation of data from AFM experiments. A and B: Low resolution scans carried out at 32-64 pixels in the height (A) and adhesion (B) channels. Areas of high adhesion were chosen (black box in B) in order to perform high resolution scans at 128*128 pixels, as shown in C. D represents a single interaction collected at each pixel during the high resolution scans. Bond break patterns were analysed and filtered and data was summarized to define the clustering phenomenon. Increase in STEVOR expression as suggested by specific bond break and adhesion events during surface scanning as the parasite matures is shown in E.

Taken together, this information suggested that the STEVOR molecules are capable of clustering on the surface of the iRBC. The clustering phenotype is likely to be relevant to the function of the protein under physiological conditions, as it is possible that this phenotype is able to strengthen any interactions with receptors on the host cell surface under flow conditions.

The data from the AFM experiments combined with a series of IFA experiments also enabled the clarification of the topology and the domain structure of the protein. These results are presented in section 3.16.

3.1.6 Topology of the STEVOR protein

Earlier bioinformatic predictions showed that the STEVOR protein probably contains two-transmembrane domains, similar to the *rifs* of *P. falciparum* (Cheng et al., 1998). However, as mentioned in chapter 2, the S1 and S2 antibodies were generated against the semi conserved region of the protein. Interestingly, while using these antibodies in live IFA experiments as well as in the AFM force mapping scans described above, the semi-conserved region was found to be exposed to the extracellular milieu. In the AFM experiments, surface scanning of iRBCs was carried out, using an AFM tip functionalized with antibodies (S1/S2) against the semi-conserved region of STEVOR. In these scans, only those motifs exposed on the surface of the infected cell can be detected. Generation of adhesive forces and specific bond break patterns during these scans, as shown in Figure 3.7 and 3.8, indicated that these antibodies were capable of detecting STEVOR on the extracellular surface of iRBCs. Taken together, this information suggests that it is the semi-conserved region of the protein that is exposed to the extracellular milieu. The STEVOR proteins being variant in nature, also contain a hypervariable region. It is thought that this domain is involved in immune evasion, and thus it was assumed that it is exposed to the extracellular milieu as well (Niang et al., 2009). For both, the hypervariable and semi-conserved regions of the protein to be exposed extracellularly, it must contain only a single TM domain. The other possibility is that the protein contains two TM domains, with both, the semi-conserved region and the predicted cytosolic domain exposed to the extracellular milieu. In this case, the hypervariable region would be internal.

The previously predicted two TM domain architecture of the protein is shown in Figure 3.9. Shown alongside is the architecture of the protein as confirmed by the experiments in this chapter, containing only a single TM domain.

Figure 3.9: Alternate topologies of the STEVOR protein on the iRBC surface. Left: Showing the previously predicted two TM architecture of the protein. Right: Showing the single TM domain architecture, with both, the HV and SC domains exposed to the extracellular milieu.

In order to negate the possibility of both, the semi-conserved and predicted cytosolic domains being exposed to the extracellular surface, with the hyper variable domain being cytosolic, a series of fixed and live IFAs using antibodies against the different domains was carried out, as shown in Figure 3.10.

In these experiments, specific antibodies against the SC region (same as those that were used for the AFM scans) were used along with specific antibodies against the cytosolic domain of the protein (described in Section 2.2; for validation (ELISA titre and IFA) see Appendix1). Only those domains that are exposed to the extracellular surface of the iRBC show positive staining in live IFA experiments; whereas those domains that are located facing the cytosolic side of the iRBC only show positive staining in fixed IFA experiments.

Figure 3.10: Fixed and live staining using antibodies against the semi-conserved (SC) and predicted cytosolic (CYT) domains of the STEVOR protein. The images clearly show that the antibodies against the predicted cytosolic domain only show positive staining in a fixed (permeabilized) IFA. Staining using these antibodies is absent in live IFA experiments, as shown in the bottom panel. Scale bar: 5 μ m.

Staining patterns shown in Figure 3.10 clearly indicate that the predicted cytosolic domain of the protein is indeed internal. Also, the live IFA experiments shown using antibodies against the semi-conserved region further support the exposure of this domain to the extracellular milieu and confirm the single TM architecture of the protein.

3.1.7 Conclusion and Discussion

The work described in this section used the Atomic force microscope as a tool to investigate the quantitative properties of STEVOR on the iRBC membrane. While it has been established that the peak of *stevor* transcription occurs at around 28 hpi (Kaviratne et al., 2002), the quantitative profile of protein expression on the iRBC membrane remained unclear. Previously studied variant antigens like PfEMP1 have been shown to perform important functions at the iRBC membrane (Pasloske & Howard, 1994). In addition, due to their variant nature it has been hypothesized that they are involved in immune evasion (P C Bull et al., 1998). Similarly, the STEVORs also represent one of the major variant antigen families of *P. falciparum* and the localization, distribution and topology of the protein on the iRBC membrane is relevant to its functional properties.

The experiments described above confirm that STEVOR appears at the iRBC membrane at the late trophozoite stage of asexual maturation and also show that there is an increase of the protein on the iRBC membrane as the parasite develops into a bursting schizont. This information lends weight to the hypothesis that STEVOR may be a parasite sequestration and cytoadhesion ligand, since it is known that late stage asexual parasites sequester (David et al., 1983). The work also indicates that it is able to form clusters at the iRBC surface and this clustering phenotype is likely to be relevant to its possible function of sequestration under physiological conditions. It is possible that the clustering of the protein enables it to better perform other functions like cytoadhesion and rigidity increase of the iRBC membrane.

Taken along with a series of immunofluorescence experiments performed, this work provides an interesting insight into the topology of the protein at the iRBC surface and confirms that it contains only a single TM domain. This topology allows the semi conserved and hypervariable regions of the protein to be available to the extracellular milieu. The hypervariable region has been hypothesized to be involved in host immune evasion, although there are a limited number of studies that have investigated this aspect of protein function (Schreiber et al., 2008). This work shows that it is indeed available for host immune factors and antibodies to bind to.

In this work, the use of the AFM has greatly enhanced our understanding of the distribution of STEVOR on the infected red cell surface. This allowed us to take a

better look at the context in which STEVOR might be presented. Although it is possible to achieve a confirmation of surface expression through immuno fluorescence experiments, a much higher resolution is achieved through the use of AFM. However, the limiting factor is the number of scans that can be achieved through this approach. The use of electron microscopy could also be useful for high resolution scanning of the iRBC membrane in order to gain more insight on the protein presentation context.

The next two sections of this chapter focus on the functions of each, the semi-conserved (extracellular) and the cytoplasmic (intracellular) domains of the STEVOR protein, and seek to elucidate their roles in host cell receptor binding and parasite rigidity, respectively.

3.2 Role of STEVOR in Parasite Rosetting

3.2.1 STEVOR and Glycophorin C in rosetting at the late asexual stages of parasite maturation

Earlier work has shown that variant antigens, specifically PfEMP1 are capable of binding to uninfected RBCs in order to form rosettes. Work in the lab has shown that STEVORs are also expressed on the surface of the iRBC at the late trophozoite and schizont stages of parasite development (Niang et al., 2009). In addition, it was shown that antibodies raised against these proteins are capable of inhibiting rosette formation, pointing to a possible role for STEVOR in binding to uninfected RBCs. When these RBCs were treated with proteases in order to cleave surface receptors, the binding decreased and it was also shown that purified Glycophorin C and recombinant STEVOR are capable of interaction in a co-immunoprecipitation experiment. Work also showed that recombinant Glycophorin C is capable of competing with uninfected RBCs for rosette formation, and also that a clone lacking STEVOR (A4) has decreased ability to rosette (Niang et al., 2014). Taken together, this information suggests that Glycophorin C is indeed a receptor for STEVOR mediated parasite rosetting.

The work presented in this section confirms the interaction of STEVOR with Glycophorin C using rosetting assays and cell binding assays. In these assays, Glycophorin C knock-down RBCs were used and rosetting frequencies between knock down and normal RBCs were compared. RBC knock-downs of Glycophorin C were generated by Selasi Dankwa, Harvard School of Public Health. Also, the semi-conserved region of STEVOR was used to transfect COS-7 cells and RBC binding ability to these cells was also compared.

In addition, the ability of STEVOR expressing gametocytes at different stages of maturation were compared for their ability to rosette. A single study carried out by Matts Wahlgren has previously shown that gametocytes do not rosette (Wahlgren et al, 1992). However, the parasites used in this study were not selected for the expression of specific variant antigens and the basis for the absence of rosetting at the gametocyte stages of development has not been established. Gametocytes are also known to sequester in the bone marrow of infected patients (Joice et al., 2014), however their interaction with specific receptors at these stages and the molecular mechanisms that mediate this are unclear. Studies have also shown that the variant of STEVOR

expressed at the gametocyte and the asexual stages remains the same(Sharp et al., 2006). In this work, the same clone that was used for the asexual stage rosetting assay was triggered for gametocytogenesis and their ability to rosette was compared. A detailed description of these experiments and the results obtained are presented below.

3.2.1.1 Validation of the ability of anti STEVOR antibodies to interfere with rosetting

The 5A clone described in chapter 2 was used in order to carry out these experiments. This clone was previously selected for STEVOR expression. Figure 3.11 shows the IFA of STEVOR protein expression in the 5A iRBC.

Figure 3.11: Live IFA using anti-S1 (STEVOR) antibody to stain 5A schizonts. Showing STEVOR expression on the surface of schizont infected 5A parasites. Scale bar=5μm.

This clone was then used for further rosetting selections as described previously in section 2.2. Shown below is the increase in rosetting frequency after each selection. These selections were carried out at intervals of one week each. The increase in rosetting frequency from approximately 30% to approximately 75% can clearly be seen in Figure 3.12.

Figure 3.12: Rosetting selection and the subsequent increase in rosetting frequency of 5A parasites. Gelatin floatation was used in order to separate rosetting positive parasites from those that do not rosette. Rosetting frequency of the parasites increased significantly after 3 selections.

Rosetting assays were then carried out with the above selected parasites, and anti-STEVR antibodies were used to block the interaction between infected and uninfected RBCs. During these assays, the presence of excess un-infected RBCs was ensured. Frequencies obtained were compared with assays in which an isotype control antibody was used. The results are presented in the graph below and show that the anti-STEVR antibody is specifically capable of inhibiting rosette formation in these assays (Figure 3.13). These assays also provide proof that the parasites used form rosettes in a STEVR-dependent manner.

Figure 3.13: Effect of anti-STEVR antibodies on parasite rosetting (5A clone). Ability of rabbit anti-STEVR antibody to inhibit the formation of rosettes in the selected 5A strain. Isotype IgG antibody (rabbit) was used as a control. A: Representative images. B: Graphs of quantification. Experiments were carried out in triplicate; error bars indicate standard error. 100 infected cells were counted per experiment. Scale bar=5µm.

3.2.1.2 Effect of Glycophorin C knockdown on STEVOR mediated rosetting

Next, the same pre-selected population of 5A parasites was used in order to quantify their ability to rosette with a population of uninfected RBCs that lack Glycophorin C. The extent of Glycophorin C k/d in these cells is shown in Figure 3.14 (done by Selasi Dankwa, Harvard School of Public Health).

Figure 3.14: FACS validation of Glycophorin C knockdown in RBCs used for the rosetting assays. (Done by Selasi Dankwa, Harvard School of Public Health) The left panel shows the FACS data of cell staining using anti-Glycophorin C antibodies. An 87% knockdown of this receptor was achieved. On the right is the data from a FACS experiment using anti-Glycophorin A as a positive control to stain the same population of RBCs. As can be seen, there was no knockdown of this receptor.

Normal uninfected RBCs were used as a positive control in these rosetting assays and comparison of results was carried out in a blinded study. Gly C- cells represent the population of cells that were transfected with the empty vector lacking the construct for the deletion of GlyC. All experiments were carried out in triplicate. Representative images of the assays and graphs of the quantification are shown in Figure 3.15.

Figure 3.15: Effect of GlyC knockdown on rosetting ability of 5A parasites in a rosetting assay.

A: Representative images of GlyC knockdown (GlyC-) RBCs versus normal RBCs (positive control) and those containing an empty plasmid (GlyC +). Scale bar=5 μ m. Three independent blinded trials were carried out. 100 infected cells were counted in each trial. B: Quantification of the results obtained. Significant difference between rosetting ability of GlyC negative and positive Control RBCs ($p < 0.05$). Error bars represent standard error.

The results show that the rosetting ability of the GlyC - population was significantly reduced in comparison with the cell population that displayed GlyC on their surface. This indicates that GlyC is an important ligand on the surface of un-infected RBCs during the process of parasite rosetting.

3.2.1.3 Interaction of Glycophorin C with the semi conserved region of STEVOR

The antibodies used in the rosetting assays described above to block STEVOR mediated rosetting are targeted against the semi conserved region of the protein. Earlier work in the lab has also shown that when the different regions of STEVOR are expressed on the surface of COS-7 cells, the semi-conserved region of the protein is the most efficient at mediating the binding of uninfected RBCs.

In the work described below, the semi-conserved region of *stevor* (Pf10_0395c) gene was used in order to transfect the COS-7 cells. The ability of uninfected RBCs having a GlyC k/d (Gly C-) phenotype to bind to these cells was then compared with that of uninfected RBCs transfected with the empty vector (GlyC+). Each time, transfections of the *stevor* (Pf10_0395c) gene were carried out simultaneously using the same conditions for all wells (Section 2.2). The *pvdbpII* gene was transfected in parallel and the ability of the different types of RBCs to bind to these cells was used as a positive control to demonstrate that the assay was functional. Thus, PvDBPII was used as a positive control for the demonstration of rosetting (Figure 3.16).

These assays were done in order to confirm the results of the rosetting assay and to demonstrate the direct interaction of GlyC with the semi-conserved region of STEVOR.

The results obtained are presented in Figure 3.16. A panel of representative images is shown, followed by the graphs of the quantification. During the quantification process, a single COS-7 cell binding to more than 2 uninfected RBCs was counted as a single rosette and the results were compared. All assays were performed in triplicate.

These results support the findings from the previous section that implicate GlyC as an important ligand for *P.falciparum* rosetting. Additionally, they also show that the SC region of STEVOR is capable of interaction with GlyC. This may also explain why this region shows some degree of conservation between variants of the STEVOR family.

Thus, the results from this section show that the STEVOR variant protein of *P.falciparum* is able to mediate rosetting at the late asexual stages of parasite development via its interaction with the GlyC receptor on the surface of the uninfected RBCs. Interestingly, it is the semi-conserved region of the protein that is involved in

this interaction. The next section of this chapter aims to study the rosetting phenotype at the gametocyte stages of parasite maturation, especially at stages III and IV.

Figure 3.16: Effect of GlyC knockdown on the interaction of STEVOR transfected COS-7 cells with RBCs. A: Representative images of different samples in the COS-7 cell binding assay. Results clearly show the decreased ability of GlyC negative cells to bind to COS-7 cells transfected with the semi-conserved region of STEVOR (ST3C). Scale bar=25µm. The black arrow in the magnified image points to the uninfected RBCs bound to the transfected COS-7 cells. B: Quantification of the results obtained. Blinded experiments were carried out in triplicate. $P < 0.01$. Error bars represent Standard Error.

3.2.2 Rosetting at the gametocyte stages of parasite development

Earlier studies have shown that the STEVOR proteins are expressed on the surface of Stage III and IV gametocytes. Since their surface expression has been co-related with rosetting ability of the asexual stage parasites, the work presented in this section of the chapter aims to answer the question of whether they mediate the same function at the gametocyte stages of development as well. In the context of gametocyte rosetting, it has been shown in a single study carried out by Mats Wahlgren (Wahlgren et. al, 1992) that they do not rosette. However, taken along with the information that STEVOR expression is down regulated in cultured parasites (Blythe et al., 2008), the lack of rosetting seen previously could be due to the absence of variant antigen expression in the parasites that were used previously for the study.

In the work presented here, the pre-selected 5A strain was used in order to study gametocyte rosetting. The ability of the different stages of gametocytes of this strain to rosette was compared in an assay similar to the one used for asexual parasite rosetting comparison. A detailed description of the experiments carried out and the results obtained is presented below.

Asexual stage parasites were selected three times to obtain a rosetting frequency of about 70-80%, as described above. These selected parasites were then used in order to carry out gametocyte differentiation as described in section 2.2. The different stages of gametocytes were then validated for STEVOR expression as shown in Figure 3.17. Gametocytes of different stages were enriched using the MACS system and incubated with excess un-infected RBCs in order to improve the likelihood of rosette formation. Asexual parasite rosetting was used as a positive control in each assay. The assays were carried out in triplicate. Any cell bound to more than two uninfected RBCs was quantified as a single rosette.

Graphs of the quantification and a few representative images of low frequency rosettes that were seen are presented in Figure 3.18. One iRBC bound to more than two un-infected RBCs was counted as a single rosette. The assay was carried out in triplicate.

Figure 3.17: Validation of STEVOR expression at the gametocyte stages of the 5A clone.

A: Showing STEVOR staining at the five different stages of gametocyte development in the 5A clone.

B: Clearly shows STEVOR on the surface of the stage III-IV gametocyte, as previously published.

Scale bar=5 μ m.

Figure 3.18: Gametocyte rosetting assays (quantification and images) at stages I to V of the 5A clone. Top panel: Graphs of the comparison between rosette formation at the gametocyte (blue bars) versus asexual stages (Orange bars) for the 5A clone. Error bars represent standard error. A total of 100 cells were counted. Bottom panel: Figure showing low frequency rosettes at the different gametocyte stages (I-V). Scale bar=5μm.

These results demonstrate that the gametocytes do not show any significant capacity to rosette when compared with the asexual stages. However, the gametocytes were able to form some rosettes at a low frequency. Quantification of rosettes was carried out after cells were appropriately diluted. This was done in order to ensure that cells that are sticking together were not mistaken for rosettes. Levels of gametocyte rosetting were found to be independent of stage, although there was a slight but insignificant increase in the ability of stage V to form rosettes. These results indicate that rosetting ability of the cell is not solely dependent on the ligands it expresses, but is more likely to be a combination of factors including distribution of the different proteins on the membrane.

3.2.3 Conclusion and Discussion

The work presented in this section confirms the involvement of STEVOR in parasite rosetting. It also confirms the interaction of STEVOR on the infected RBC with Glycophorin C on the uninfected RBC during this process. The results also show that the STEVOR proteins cannot perform the same function with equal efficiency at Stages III and IV of gametocyte development, as the number of rosettes seen in the assays performed with these cells was greatly reduced. This was despite the fact that parasites that were previously selected for the asexual rosetting phenotype were used in order to trigger gametocytogenesis. Previous studies have shown that the same variants of STEVOR are transcribed at both, the asexual and gametocyte stages of parasite development (Sharp et al, 2006). However, four STEVOR variants are known to be transcribed in the 5A clone used in this work (Niang et al, 2009). It could be that the variant that is trafficked to the parasite surface at the gametocyte stage is different from the one that is present on the membrane of the asexual stages. This is possible, as it has been shown that the variant of STEVOR present on the merozoite surface is different from the one at the schizont surface during asexual maturation (Khattab & Meri, 2011; Khattab et al., 2008). It is also possible that the rosetting phenotype is not solely dependent on direct protein binding interactions but also requires the ligands involved to be distributed and presented on the cell surface in a certain context; and also maybe in certain amounts. The work described above by Sharp et al did show that the transcription of STEVOR during gametocytogenesis was decreased as compared to what was shown for asexual parasites(Sharp et al., 2006). It is well known from earlier work that the shape and membrane characteristics of gametocytes are very different from those of the asexual stage parasites and this may also interfere with the ability of these cells to rosette.

Previous studies have shown that PfEMP1 is also capable of mediating rosetting at the asexual stages of parasite development, via the CR1 receptor on the uninfected RBC. Recent studies have also shown the involvement of A type RIFINS in binding to the blood group A receptor on the uninfected RBC (Goel et al, 2015). The work presented in this section confirms the interaction of STEVOR with Glycophorin C. This part of the thesis was published in the paper by Niang et al.,2014(Niang et al., 2014).This shows that the three major variant antigen families of *P.falciparum* are capable of mediating a redundant function using distinct receptors on the uninfected RBC.

In this work, the technique of receptor knockdown was used for the first time in order to demonstrate a direct decrease in rosetting efficiency due to the absence of a single receptor. Although these studies were carried out in static binding assays, similar techniques used in flow assays can provide a better understanding of the interplay between host and pathogen in vivo.

Rosetting can contribute to *P.falciparum* sequestration within the deep circulation of the host. Studies also show that the *P.falciparum* parasite modulates the rigidity of the RBC as it matures through the cycle of asexual development, with the late stages being the most rigid. This increased rigidity is linked to parasite sequestration under conditions of physiological flow. The next section of this chapter addresses the role of the cytoplasmic domain of STEVOR and its contribution to the increase in membrane rigidity known to be mediated by STEVOR (Sanyal et al, 2012, Tiburcio et al, 2012).

3.3: STEVOR, Parasite Rigidity and Cytoskeletal Interactions

Previous studies have shown that STEVOR proteins at the iRBC membrane are responsible for the increase in parasite rigidity seen at the late asexual stages of the parasite life cycle as well as at stages III-IV of gametocyte development (Sanyal et al, 2012, Tiburcio et al, 2012). Currently, the domain involved in this rigidity increase and the mechanisms that underlie it remain unknown. The aim of the work presented in this chapter is to address the role of the cytoplasmic domain of STEVOR and its contribution to parasite rigidity. Although the *rifs* and *stevors* are known to align with each other during BLAST analysis, they show distinct differences in their cytoplasmic domains, making this an interesting avenue for study. Also, the cytoplasmic domain of *stevor* has been shown to have 80% sequence conservation among members of the multigene family (Cheng et al., 1998), suggesting an important function across all variants.

In order to specifically answer the question of how the cytoplasmic domain contributes to STEVOR function, two transgenic parasite lines were generated. Transfections were carried out into the A4 parasite clone that does not express STEVOR. An IFA of the A4 clone confirming the absence of STEVOR expression is shown below.

Figure 3.19: IFA showing the absence of STEVOR expression in the A4 clone. α -MSP 1 was used as a positive control. Anti-STEVOR (S1+S2) antibody was used at a dilution of 1:50 each. Scale bar=5 μ m.

Two constructs encoding different forms of PFF0850c were used (FL and TD), one of which lacks the 17 amino acids (TD) that encode the cytoplasmic domain, as shown in Figure 3.20. The PFF0850c construct was chosen as it was identified as one of the *stevor* variants that was highly expressed in sub-cloned populations of 3D7 (Niang et al., 2009).

<p>MNIYYVKILIFTFLVNTLILSHNENCQNNQYNISLIKNNQTQRTTIKPRLLAQTKNHNPHY</p> <p>HNDPELKEMIDKLNKEAIKKYQKIHEPYEQLQELVEKKGTPVGEHGTEPMSTIEKELLE</p> <p>TYEKMFGDKKDIMLKSGMPPNDDDRSDKSITCECTDINNPDLTKAKSKDKYLRKLEGGCT</p> <p>RGICTCSVGSAFLTLIGLAAAKKAAVAFAFSAPYDACVSSTSLFYIFDSGTLASALKVGST</p> <p>CASVATDMAGTVSSAATTAIIPFSISIIYVLIVITVLLIILYIWLYRRRKNSWKHECKKHLC</p> <p>K</p>
<p>MNIYYVKILIFTFLVNTLILSHNENCQNNQYNISLIKNNQTQRTTIKPRLLAQTKNHNPHY</p> <p>HNDPELKEMIDKLNKEAIKKYQKIHEPYEQLQELVEKKGTPVGEHGTEPMSTIEKELLE</p> <p>TYEKMFGDKKDIMLKSGMPPNDDDRSDKSITCECTDINNPDLTKAKSKDKYLRKLEGGCT</p> <p>RGICTCSVGSAFLTLIGLAAAKKAAVAFAFSAPYDACVSSTSLFYIFDSGTLASALKVGST</p> <p>CASVATDMAGTVSSAATTAIIPFSISIIYVLIVITVLLIILYIWLY</p>

Figure 3.20: Sequence of the PFF0850c gene showing the cytoplasmic domain of STEVOR. Showing the sequence of the gene transfected into A4. Top: Showing the full length gene that was transfected in order to generate the A4FL strain. Bottom: Showing the truncated construct transfected in order to generate the A4TD strain. Black: Signal peptide. Blue: protein sequence. Red: Cytoplasmic domain.

3.3.1 Transfections and Validation Experiments

Transfections were carried out into the A4 clone, as described in section 2.2 of this thesis. Plasmid maps containing the STEVOR gene that were used for transfection are also shown.

Parasite transfections were episomal, and in order to validate the truncation of the cytoplasmic domain in the A4 TD as versus the A4 FL strain, RT-PCR reactions using primers against the cytoplasmic domain were carried out, as shown in the table below. Results indicate the successful truncation of the cytoplasmic domain in the A4 TD strain.

STRAIN	FOLD CHANGE-RT PCR	PRIMER PAIR
A4-FL	5.95871	Against CTD
A4-TD	1.209994	Against CTD

Table 3.3: RT-PCR of A4FL and A4TD clones using primers against the cytoplasmic domain of STEVOR. The table shows the successful truncation of the cytoplasmic domain (CTD) in the A4 TD strain as compared to the A4FL strain. [Primer sequences in Appendix 2]. Housekeeping gene used for normalization: Ubiquitin conjugating enzyme (UCE).

In order to validate the export and trafficking of proteins in both strains, live and fixed IFA experiments were carried out using antibodies against the semi-conserved domain of the protein, which should be present in both strains and exposed extracellularly if the protein is properly trafficked to the membrane. Results are shown in Figure 3.21 A. This figure shows that both, the A4-FL and A4-TD strains show positive staining for STEVOR when antibodies against the surface exposed semi-conserved region are used. This confirms that the STEVOR proteins are properly exported and trafficked to the extracellular surface of the iRBC in both transfected strains. In addition, fixed IFA experiments using antibodies against the cytoplasmic domain of the protein were also performed on both strains, in order to confirm its absence in the A4 TD strain as shown in Figure 3.21 B. This figure shows that while STEVOR positive staining can be seen in the A4-FL strain, the A4-TD strain shows negative staining when antibodies against the cytosolic domain of the protein are used. This further confirms the successful truncation of the cytosolic domain in the A4-TD strain.

A

B

Figure 3.21: IFA using the anti-S2 antibody and the anti-cytoplasmic domain antibody to stain A4FL and A4TD schizont stage parasites. A: Showing live staining using antibodies against the SC domain in both transfectants. B: Showing staining using the anti-cytoplasmic domain antibody in the A4FL strain. No staining is seen in the case of the A4TD strain, confirming the truncation of the cytoplasmic domain. Scale bar=5 μ m.

3.3.2 Rigidity Measurements

The central hypothesis of this section is that the cytoplasmic domain of STEVOR interacts with the cytoskeleton. This interaction could then have an impact on the overall rigidity of the iRBC membrane. In the case of the A4 FL strain (containing the cytoplasmic domain), this effect is expected to be different from that of the A4TD strain (lacking the cytoplasmic domain).

Figure 3.22: Proposed role of the role of the cytoplasmic domain of STEVOR in rigidity increase. Describing the rationale behind the truncation of the cytoplasmic domain of STEVOR and the experiments carried out in this section.

In order to validate this model, single cell micropipette aspiration was used to study late stage asexual parasites as well as gametocytes of each strain for rigidity differences. Parasites of all strains were synchronized using percoll-sorbitol to ensure that all cultures were at approximately the same stage of development. Cells were diluted appropriately in order to easily allow the aspiration of single cells. A flow rate of 9ml/hour was used in order to aspirate the cell membrane of the asexual stage parasites. For all cells, the side of the parasite membrane that was chosen for aspiration corresponded to the part of the iRBC that was completely occupied by the

parasitophorous vacuole. A total of 12 cells per strain was included in the analyses. Rigidity was estimated in terms of elastic shear modulus and compared. As shown in Figure 3.23, at the late trophozoite to schizont stage, the A4-FL strain is significantly more rigid than the A4-TD strain. However, there is some increase in rigidity seen in the A4-TD strain; indicating that the cytoplasmic domain is only partially responsible for the increase in rigidity due to the presence of STEVOR that is seen at these stages. As expected and previously reported, the A4 strain (no STEVOR) was the least rigid.

Figure 3.23: Effect of the truncation of the cytoplasmic domain of STEVOR on parasite membrane rigidity at the late asexual stages. Top panel shows a representative image of the single cell micropipette aspiration technique. Scale bar=5 μ m. 100 frames per cell were collected. A pooled average of 48 cells across strains is shown in the graph. Significant differences in rigidity as measured across strains is represented. Error bars represent Standard Error; $P<0.001$.

This result indicates that the cytoplasmic tail of the STEVOR protein is partially responsible for the increase in membrane rigidity seen at the late stages of parasite development, although its truncation does not completely abolish the increase in rigidity mediated by STEVOR. This is presumably because the rigidity increase is

mediated by both, the cytoplasmic as well as the transmembrane domain of the protein.

STEVOR has also been shown to be involved in the change in rigidity between Stages III, IV and V of *P.falciparum* gametocytogenesis. We there for triggered the A4FL and A4TD clones developed above for gametocytogenesis. First, cells were validated for the presence of STEVOR at the iRBC surface as shown in Figure 3.24. These experiments employ the technique of live IFA staining using antibodies against the extracellularly-exposed semi-conserved region of the protein in order to detect the stages at which STEVOR is present on the iRBC surface. This figure shows that, as expected, STEVOR was trafficked to the surface of the Stage III and IV gametocytes (Figure 3.24B), while it remained internally localized in stages I/II and V (Figure 3.24 A and C). While in stages I and II STEVOR is in the process of being trafficked to the surface of the iRBC, some staining is visible in the iRBC cytosol. However, as can be seen in Figure 3.24 C, the staining pattern of STEVOR in stage V gametocytes is mainly localized to the internal parasitophorous vacuole. Also, STEVOR disappears from the membrane at these stages, showing that it may be cleaved off the surface in order to facilitate the increase in deformability seen in stage V gametocytes.

Single cell micropipette aspiration as described above was carried out on the gametocytes of the A4, A4TD and A4FL strains and compared. The results are shown in Figure 3.25. In these experiments, stages were grouped into 3 bins, i.e., I/II, III/IV and V. This was done in order to highlight the contribution of STEVOR to the changes in membrane rigidity. Differences between stages III/IV and V of all strains are important to the analysis since the protein appears on the membrane at stages III/IV and cannot be seen on the surface of stage V, as shown in Figure 3.24.

Figure 3.24: The presence or absence of STEVR on the iRBC membrane at the different stages of gametocyte development of the A4FL and A4TD strains. A: Live and fixed staining of stage I and II gametocytes. STEVR is not detected at the iRBC membrane at these stages, although it is present internally. B: Fixed and live staining of Stage III and IV gametocytes. STEVR is present at the iRBC membrane as well as internally at these stages. C : Live and fixed staining of Stage V gametocytes. STEVR disappears from the iRBC membrane but can still be detected internally. The rounded edges of the parasite seen in panel C was used as an indication of the transition between Stages IV(pointed) and V(rounded). Scale bar=5 μ m.

Figure 3.25: Comparison between rigidity of A4, A4FL and A4TD strains at different stages of gametocyte development using micropipette aspiration. A: Representative images showing the different gametocyte stages (I-V) at which rigidity was measured. Scale bar=5μm. B, C, D: Showing the differences in rigidity between stages of the same strain. E: Showing differences in rigidity between stages III/IV of the A4FL, A4TD and A4 strains. Data from 90 cells (10 cells per stage per strain) was included in the analysis.

Figure 3.25A shows the morphological differences that can be seen between the five different stages of gametocytogenesis. These differences allowed us to easily identify one stage from the other. Importantly, the pointed edges that are characteristic of Stage IV are absent in Stage V where the ends of the cell are rounded instead. This characteristic was used to effectively distinguish the switch between stages IV and V in our experiments. Figure 3.25B, C and D show a comparison of elastic shear modulus at the different gametocyte stages of the A4FL, A4TD and A4 strains respectively. While it can be seen that in all cases the rigidity does not differ significantly at Stages I/II, significant differences between the rigidity of stages III/IV, (when STEVOR has been shown to be on the surface) can be seen.

From the final analysis presented in Figure 3.25, it can be seen that the differences in rigidity between stages III/IV and V of the A4TD strain are less significant than for the A4FL strain. Overall, the A4TD strain shows an increased deformability compared to the A4FL strain at those stages at which the STEVOR proteins are trafficked to the membrane. Importantly, this change in deformability was not seen at stages I and II, where STEVOR is not trafficked to the iRBC surface. As expected and previously published, rigidity differences between stages III/IV and V of a STEVOR negative strain were not significant (Tibúrcio, Niang, et al., 2012).

The results suggest that the cytoplasmic domain of STEVOR is capable of mediating changes in rigidity even at the surface of the gametocyte stages of parasite development. However, its contribution is partial and as for the asexual stages, it is likely that the increase in rigidity seen due to the presence of STEVOR at the membrane is probably due to the interactions of both, the cytoplasmic tail and the transmembrane domain with different cytoskeleton related proteins.

The following sections focus on the elucidation of the mechanism by which the cytoplasmic domain is likely to mediate an increase in membrane rigidity. The main aim of the following experiments was to study any interactions of STEVOR with cytoskeletal proteins.

3.3.3 Investigation of Cytoskeletal Interactions of STEVOR at the iRBC membrane

In order to further investigate the interactions between STEVOR and other proteins at the iRBC membrane, an immuno-precipitation experiment was carried out using anti-STEVEOR antibodies. Here, parasite proteins were cross linked and then lysis was carried out so as to separate the membrane fraction (described in section 2.2.8). Giemsa smears showing the lysed RBC after removal of the membrane are shown below.

Figure 3.26: Giemsa smears showing the effect of the saponin lysis protocol used on the iRBC. A: Showing the purification and enrichment of late stage iRBCs. Red arrows point to the RBC membrane. B: Smear of the pellet fraction after RBC lysis. Showing that the saponin treatment used (0.02%) did not lyse the internal parasite. Scale bar=8 μ m. The supernatant containing the membrane fraction was used to load the column. [Protocol for saponin lysis: Section 2.2.8]

The membrane fraction was used in a magnetic bead system conjugated to anti-S1 antibodies in order to detect interacting partners of STEVOR on the iRBC membrane. For this experiment; and in order to prove specific detection of interacting partners, the system was first optimized using anti p55 antibodies. P55 is an integral membrane protein and has known interacting partners on the surface of the iRBC. It is known to interact with Glycophorin C (35 kDa) on the RBC membrane, and this interaction is reliably detected using anti-p55 in the co-immunoprecipitation experiment, as shown in Figure 3.27. The red arrows in this figure point to the bands at 55kDa and 35kDa; that represent p55 and Glycophorin C respectively. The two additional bands seen at 50kDa and 25kDa represent the heavy and light chains of the antibody respectively.

Figure 3.27: The association between p55 and Glycophorin C detected in the co-immunoprecipitation protocol used. The co-immunoprecipitation protocol was validated using this well established interaction between p55(55kDa) and Glycophorin C (35kDa) .

Next, standardization was carried out for the STEVOR co-immunoprecipitation using four different anti- STEVOR antibodies. Of these, rabbit α -S1 worked reliably. The Western Blot in Figure 3.28 shows that the anti-S1 antibody is capable of recognizing a 35 kDa band in the membrane fraction that represents STEVOR in the A4FL strain. The Western Blot showing that this band is recognized by the anti-STEVR antibody (α -S2) is shown below.

Figure 3.28: Western blot detecting STEVOR in the co-immunoprecipitation and input fractions using α -S2 primary antibody. A specific band at 35 kDa in both, the input and co-immunoprecipitated fractions was seen. 1:3 and 1:10 indicate the extent to which the sample was diluted before loading.

Along with the STEVOR band, three highly specific bands were seen between 50kDa-30kDa on the gel. The corresponding image of the silver stained gels is shown in Figure 3.29. Arrows indicate the location of the bands that were cut and sent for mass spectrometry (MS) analysis. The red box indicates the region from which these bands were cut. Once the specific bands of interest were cut out from the gel, the pieces were pooled as a single fraction and sent for M/S analysis. Also seen in the gel are the bands at 50kDa and 25kDa, which represent the heavy and light chains of the antibody respectively. The IgG isotype control also shows additional background bands which are visible in the S1 co-immunoprecipitated fraction as well. Since these bands were present in both, the control and S1 fractions they were removed from the analysis and weren't sent for LC-MS/MS.

Figure 3.29: Silver stain of co-immunoprecipitated samples using anti-STEVOR antibody on membrane fractions of A4FL schizonts. Showing specific pull-down bands detected reliably in three biological replicates. Isotype controls (IgG) are shown alongside the samples of interest (A4FL). The box in red outlines the bands that were sent for M/S analysis.

Attempts to carry out the staining of the gel using Coomassie blue dye were unsuccessful. Thus, the more sensitive technique of silver staining was carried out. This shows that although our co-immunoprecipitation assay is highly specific for STEVOR, very low amounts of interacting partners could be co-immunoprecipitated. Fractions loaded onto the magnetic bead column as described previously were from approximately 3ml PCV blood in culture at 8-10% parasitemia. It is likely that some of

the interactions of STEVOR were lost in our assays due to the low abundance of the protein on the membrane.

3.3.4 Mass-Spectrometry results and validation

Mass spectrometry (LC-MS/MS) was carried out for the bands obtained in the A4FL strain co-immunoprecipitated fraction shown in Figure 3.29. The MASCOT algorithm was used in order to perform the analysis. Proteins from the sample were compared to the human and *P.falciparum* proteomes. Presented in the table below are the results obtained. As mentioned before, gel pieces from the three specific bands were pooled as a single fraction and sent for analysis.

Protein Description	Accession Number	Score
Actin, cytoplasmic 1 OS=Homo sapiens GN=ACTB PE=1 SV=1 - [ACTB_HUMAN]	P60709	62.58
Apolipoprotein A-I OS=Homo sapiens GN=APOA1 PE=1 SV=1 - [APOA1_HUMAN]	P02647	86.91
Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2 - [ALBU_HUMAN]	P02768	545.4

Table 3.4: MS analysis of co-immunoprecipitated fractions using anti-STEVOR antibodies. The results of the LC-MS/MS analysis from the A4FL pull-down fraction. The Swiss-Prot database was used as the reference. Only those hits that have a p value ≤ 0.05 (Score ≥ 52) are shown.

The table shows only those proteins identified that had a significant score during analysis. Of these, serum albumin and apo-lipoprotein are known to be present in human blood and serum. Since the focus of these experiments was to identify interacting cytoskeletal partners, the presence of actin in the co-immunoprecipitated fraction was relevant to this study, and was also within the expected molecular weight range, since the bands between 40-50kDa were sent for analysis. In order to confirm and validate the LC-MS/MS results, a western blot was performed using anti-STEVOR and anti-actin antibody. Co-immunoprecipitated fractions of the A4FL and A4TD strains were compared and the result is shown in Figure 3.30.

Figure 3.30: Western blot for detection of STEVOR and actin in A4FL and A4TD fractions. Showing the presence of actin in the A4FL co-immunoprecipitated fraction. The band is absent in the A4TD fraction. Both fractions were loaded at a 1:3 dilution. A panel including the co-immunoprecipitated result after elution of A4FL on IgG coated beads has been included to eliminate the possibility of non-specific binding.

Figure 3.30 shows that while STEVOR is detectable in both fractions at similar levels, Actin is co-immunoprecipitated only in the A4FL fraction; as no band is detectable in the A4TD fraction using the anti-actin antibody. Although the anti-STEVOR and anti-ACTIN exposures shown in Figure 3.30 are from separate membranes, both were taken from the same co-immunoprecipitated fractions. They were loaded onto separate gels and transferred onto separate membranes since both primary antibodies used were anti-mouse. Use of the anti-rabbit primary antibody was avoided as the beads used for the co-immunoprecipitation experiments were bound to rabbit anti-STEVOR antibodies. Also, the predicted molecular weights of STEVOR and actin are 35 kDa and 42 kDa respectively. Therefore, in order to ensure that the results could be interpreted clearly, the samples were loaded separately.

The results from this section indicate that the cytoplasmic domain of the STEVOR protein is capable of interaction with actin, and the differences in the results obtained from the two different parasite lines (A4FL and A4TD) in the co-immunoprecipitation experiments performed above as well as from the rigidity assays can be accounted for by the absence of this domain. This work also suggests that the increase in rigidity of the iRBC membrane mediated by STEVOR, however, is probably due to the combined effect of both, the cytoplasmic as well as TM domains. Truncation of the cytoplasmic domain does not completely abolish the increase in rigidity caused by the full length protein.

3.3.5 Effect of the cytoplasmic domain of STEVOR on molecular clustering

As described in section 3.1 of this thesis, STEVOR was shown to cluster on the surface of the iRBC at the late stages of asexual development. Earlier work has shown that PfEMP1 clusters on the iRBC surface due to its interactions with components of the knob complex and other proteins. In the case of STEVOR, no such secondary interaction has been shown to be responsible for the clustering phenomenon. The work in this sub-section focuses on addressing whether the cytoplasmic domain of the protein has an effect on this phenotype.

In order to study this, the A4 FL and A4 TD strains described above were used. In both cases, cells were tightly synchronized using double sorbitol at 4 hour intervals. Next, cells between 38-40 hours post invasion (late schizont stages) were collected using percoll purification and washed thoroughly. Tight synchronicity of both cell populations was ensured so that the effects of stage-dependent variation between samples could be minimized. Cells were then fixed for 15 minutes in 1%PFA and washed again. PFA was used at low concentrations for a short period of time to ensure partial fixation. This was done so that the cells do not burst while the scan is being carried out. Further sample processing was carried out as described in section 2.2 of this thesis. AFM scanning experiments using an anti-S1 functionalized tip were then performed. The same parameters and scan optimizations were used as described in section 3.1. These scans allowed a spatial resolution of approximately 8nm on the iRBC surface, thus also enabling an estimation of cluster size on the surface.

In the above experiments, a total of 50,000 interactions per sample were analysed. 1 μ m*1 μ m scans of 5 cells per strain were carried out. Each scan yielded a total of 16,384 interactions. Only the specific adhesion events were accounted for in the analysis. A representative area of one such scan of each type of cell is presented Figure 3.31. No difference in cluster size could be observed between the two cell types from the pattern of adhesion events seen on the surface of the cell. In order to quantify bond rupture events, each interaction was manually filtered for specific adhesion events and the number of bond breaks per interaction was counted. This represented the number of simultaneous bond rupture events seen per sample (Cluster size 2-3). In addition, the percentage of sequential bond ruptures seen was also quantified (Cluster size >3). A comparison of cluster size between strains is presented in Figure 3.32.

Figure 3.31: Adhesion maps generated during the AFM scan of A4FL and A4TD schizonts.
A: Showing an area of the map on the surface of the A4FL iRBC. B: Showing an area of the map on the surface of the A4TD iRBC.

Figure 3.32: Cluster size of STEVOR for the A4FL and A4TD strains. A comparison of cluster sizes between the A4FL and A4TD strains. No significant difference can be seen in the peak cluster size between strains.

Figure 3.31 shows an AFM adhesion map using anti-STEVOR antibodies of a representative cell from both, the A4FL (A) and A4TD (B) strains. Lighter spots represent areas of higher adhesion. The black circles show clusters that can be seen on the surface of both cells. From this figure, it is evident that there is not much difference in the ability of STEVOR to cluster on both strains. In order to confirm that there are no differences that have been missed by visual analysis of the adhesion map, each bond break event was manually filtered and quantified. The results are shown in Figure 3.32. As can be seen in Figure 3.32A, the peak size of STEVOR clusters is at two, similar to what is seen for the A4TD strain (Figure 3.32B). Additionally, a comparison was carried out between the levels of clustering seen in both strains and the results are presented in Figure 3.32C. In this comparison, no significant differences in cluster size were found between the two strains.

From the above experimental results it can be seen that there is no significant difference in the size of STEVOR clusters between strains. The ‘size’ indicated, however, is relative and is not an indication of the number of molecules involved in the cluster, as this information cannot be accurately calculated from these experiments. A higher resolution technique with a defined stoichiometry of interaction between

antibody and sample can accurately provide this information. However, the aim of these experiments was to provide a comparative analysis of cluster size between the A4FL and A4TD strains. Our analysis showed that the cytoplasmic domain of the STEVOR protein is probably not involved in determining cluster size. This indicates that the oligomerization of STEVOR on the membrane surface might be due to inter-molecular hydrophobic interactions between segments of the TM domain of the protein.

3.3.6 Conclusion and Discussion

The deformability of the RBC is known to be dependent on several properties of the membrane and the underlying cytoskeleton. Previous studies have shown that the *P. falciparum* parasite modifies and alters these properties during its cycle of intra-erythrocytic development. Studies have also shown that the STEVOR protein in particular is able to contribute to the increased rigidity and decreased deformability of the iRBC membrane (Sanyal, Egée, Bouyer, Perrot, Safeukui, Buffet, et al., 2012; Tibúrcio, Niang, et al., 2012). The work in this section shows that the truncation of the cytoplasmic domain of this protein can lead to a decrease in the rigidity of the membrane, at both, the asexual as well as gametocyte stages of parasite development. Interestingly, it also shows that although this domain is able to contribute to a change in membrane deformability, its truncation only partially abolishes the increased rigidity mediated by the full length protein. This result was consistent even at the gametocyte stages (III and IV) of parasite development.

Although the decrease in rigidity seen upon truncation of the cytoplasmic domain is not complete, in the pull-down experiments described, the interaction with actin was completely lost in the A4TD strain. This shows that the cytoplasmic domain is probably necessary for the interaction of STEVOR with actin and provides a possible mechanism for the reduction in rigidity seen due to its truncation. It could also be that the interaction of actin with the truncated form of STEVOR is weakened, and thus lost in our experiments. Actin is one of the major cytoskeletal proteins and its interaction with STEVOR may, in some way, affect the extent of its polymerization or the manner in which it is able to regulate and interact with other downstream proteins. Several native RBC proteins are known to interact with actin and modify its polymerization properties. These interactions are capable of modulating the deformability of the cell and the membrane (Gokhin et al., 2015).

The decrease in rigidity seen upon truncation of the cytoplasmic domain seems to be consistent even at the gametocyte stages. It will be interesting to study the underlying interactions at these stages of development, since the overall rigidity of immature gametocytes is known to be higher than that of the late asexual stages.

Previous studies have shown that PfEMP1 (also a *P. falciparum* variant antigen) is anchored to the RBC membrane on protrusions called knobs. These protrusions are

known to increase the rigidity of the iRBC membrane. The knobs are a multi-protein complex and certain associated proteins such as MESA are known to have interactions with native cytoskeletal components of the RBC. The work described in the section above provides the first report of another variant antigen, i.e, STEVOR, and its ability to interact with the RBC cytoskeleton.

In this work, engineered parasites were generated using episomal transfections, and the contribution of the cytoplasmic domain of STEVOR to iRBC membrane rigidity was studied using the technique of micropipette aspiration. In addition, preliminary AFM scans were conducted in order to check whether the cytoplasmic domain of the protein could have any impact on the clustering phenotype. A good confirmation of these results can be provided by conducting ektacytometry and electron microscopy on these parasites. While ektacytometry can provide population level parameters for membrane rigidity experiments, EM can provide confirmatory high resolution images of the clustering phenotype.

Another aspect that could contribute to the increase in membrane rigidity mediated by STEVOR is the clustering ability of the molecule. In the experiments described as part of this section, the contribution of the cytoplasmic domain to this phenotype was also investigated. No significant difference in cluster size was seen between the strain containing the cytoplasmic domain and the one that lacked it. Thus, we show here that STEVOR is able to cluster by a mechanism that is independent of its cytoplasmic domain. This supports the argument that the TM domain of the protein is also involved in the rigidity increase of the membrane and also suggests that it is this domain that is involved in mediating the interactions that lead to the clustering of the molecule.

4. FINAL DISCUSSION AND **CONCLUSIONS**

To date, malaria has proved to be a difficult disease to tackle. It represents one of the primary causes of morbidity and mortality in the tropical and sub-tropical regions of the world. Of all the *Plasmodium* species that cause malaria infection in humans, *P. falciparum* is known to be the most virulent (*World Malaria Report*, 2015).

The *P. falciparum* parasite radically modifies the RBC within which it matures by establishing various sub cellular compartments and protein trafficking organelles (Atkinson & Aikawa, 1990). The membrane of the iRBC is also modified by the presence of several parasite proteins, many of which are exposed to the immune system of the host. Among these are the variant antigens that are hypothesized to participate in host immune evasion. *P. falciparum* has three major variant antigen families, i.e., STEVOR (encoded by the *stevor* genes) (Cheng et al., 1998), PfEMP1 (encoded by the *var* genes) (Howard et al., 1983) and RIFIN (encoded by the *rifs*) (Cheng et al., 1998). Although the *vars* are transcribed first with the *rifs* following them, both proteins seem to appear on the iRBC surface at the same time (Kyes, Rowe, Kriek, & Newbold, 1999). PfEMP1 and RIFINs have been found on the iRBC surface at the early trophozoite stages of parasite growth, while the STEVORs follow them at the late trophozoite stage (Blythe et al., 2008; Niang et al., 2009). Thus, at the late schizont stages of development, all three of these variant antigens are present on the parasite surface.

Studies have demonstrated that the variant antigens of *Plasmodium* are trafficked to the iRBC surface in an MC-dependent manner (Przyborski et al., 2005). There is recent evidence to show that PfSBP1, the MC resident protein of *P. falciparum* has homologues in other rodent *Plasmodium* species as well, indicating that trafficking mechanisms may be well conserved between species (Conference communication, BIOMALPAR XI abstract book). It has recently been shown that the PfPtp1 protein is responsible for transporting all three variant antigen families to the surface of the iRBC and that disruption of its function leads to the absence of these proteins on the iRBC surface (Rug et al., 2014). Despite having a common protein that trafficks them to the iRBC surface, the timing of their arrival on the iRBC surface is different. This is partially due to the fact that the timing of transcription of STEVOR is different from that of the *vars* and the *rifs* (Kaviratne et al., 2002). However, in this work we show that the amount of STEVOR on the iRBC membrane increases as the parasite matures from trophozoite to bursting schizont. This suggests that the parasite employs a very

finely tuned mechanism to ensure that only a certain amount of protein reaches the surface in a stage-dependent manner. It is not known whether the PfEMP1s and RIFINs are also subjected to such regulation, although it has been shown that the knob structures increase in size and number on the parasite surface as it matures (Li, Mansoor, Tan, & Lim, 2006). In addition, it has also been shown that the trafficking of PfEMP1 stops at around 30-36 hpi (Kriek et al., 2003). However, all three variant antigens are still present within the MC, while they can also be localized to the iRBC membrane. This is a clear indication that all of the protein present within the MC does not reach the membrane. Despite the fact that the amount of STEVOR increases on the membrane of the iRBC as the parasite matures, a very large amount of the protein remains internal, as can be seen from the fixed IFA experiments performed as part of this thesis. It could be that the mechanism of transport to the membrane is concentration-dependent, and that an increase in the amount of protein produced internally leads to a subsequent increase in the total amount that finally reaches the iRBC surface. The question of how the parasite achieves the final step of protein transport from the MC to the iRBC surface is unknown, as there is no evidence of membrane fusion between the two compartments.

From the work carried out in this thesis that used Glycophorin C knockdown RBCs to study their effect on STEVOR mediated rosette formation, it was confirmed that the two molecules are capable of interaction with each other (Niang et al, 2014). This indicates that the STEVOR proteins are capable of mediating other functions apart from their postulated role as evaders of host immunity. Recently, it has also been shown that the RIFINs (A type) have the ability to bind uninfected RBCs using the blood group A antigen (Goel et al, 2015). Work done much earlier has also shown that PfEMP1 is able to mediate rosetting of the iRBC by binding to CR1 on the red cell surface (J A Rowe et al., 1997). Thus, all three variant antigens are capable of mediating the same function on the iRBC, while using different receptors on the surface of the host cell. It is of note that the rosetting ligands on the host cell surface are largely glycosylated (Barragan, Kremsner, Wahlgren, & Carlson, 2000; A. Rowe, Berendt, Marsh, & Newbold, 1994), suggesting that the parasite ligands may contain lectin-like domains. Interestingly, lectins are also known to be important mediators of the host immune responses (Toscano et al., 2012). Another interesting avenue for study would be the distribution of the three antigen families on the iRBC surface with

respect to each other. There are currently no studies to address the question of whether these antigens are capable of modulating the cytoadhesive properties of the iRBC based on the context in which they are presented on the surface relative to one another. For example, it will be interesting to study whether the STEVORs and RIFINs are located in proximity to the knobs on the parasite membrane.

Many studies have sought to establish links between the rosetting phenotype and severe disease. There is some evidence to show that in cases of complicated *P. falciparum* malaria, parasites have an increased ability to rosette (Wahlgren et al., 1992). Anaemia, coma, renal failure and hepatic dysfunction are common indicators of complicated disease. It is thought that the ability of the parasite to sequester in the fine capillaries of these organs and block blood flow contributes to these symptoms. Thus far, there have been many studies on the role of PfEMP1 and its ability to mediate rosetting and cytoadhesion (Sherman, Eda, & Winograd, 2003). It has been shown that distinct subsets of the *var* genes are able to mediate this phenotype, and receptors for the PfEMP1 proteins on the surface of RBCs and endothelial cells have been identified and extensively studied (Abdi et al., 2014). The occurrence of symptoms that complicate the disease, however, are not restricted to *P. falciparum* malaria, and neither is the rosetting phenotype. Taken along with the information that apart from *P. knowlesi*, no other species has homologues of the *var* genes (Frech & Chen, 2013), this suggests the involvement of other proteins and ligands that can mediate these processes in different *Plasmodium* parasites. For example, members of the *pir* superfamily, have been shown to be present in all human and rodent species of *Plasmodium* (Janssen et al., 2002, 2004), and the *stevors* and *rifs* are thought to be representative members in *P. falciparum* (Jemmely et al., 2010). The works by Niang *et al.* (year) and Goel *et al.* (year), confirming that the STEVORs and RIFINs are both single TM containing proteins, further strengthen the argument that these proteins represent the PIRs in *P. falciparum*. There is recent evidence to show that disease caused by *P. vivax* can also lead to complications like anemia and rosetting has been implicated as a contributing mechanism (Marín-Menéndez et al., 2013). Interestingly, Glycophorin C has been identified as a ligand for *P. vivax* mediated rosetting as well (Lee et al., 2014); although the receptor on the parasite surface remains unidentified. A recent study shows that a panel of VIRs, when expressed on the surface of mammalian cells, can bind to different endothelial cells in a static adhesion assay (Bernabeu et al.,

2012). Here, the authors studied three different VIR proteins and showed that each one has a different sub cellular localization, similar to what has been shown for the STEVORs and the RIFINs. As mentioned above, the variant of the STEVOR protein found on the merozoite tip was shown to be different from the one on the schizont surface (Blythe et al., 2008; Khattab & Meri, 2011; Khattab et al., 2008). Similarly, it was shown for the RIFINs that members of the RIFIN A family are present on the iRBC surface, while proteins belonging to the RIFIN B family remain internally localized (Petter et al., 2007).

Recent work on the STEVOR proteins has shown that the rosettes they form are capable of conferring a selective advantage on the growth of the parasite, since in this case a link has been established between rosetting and invasion (Niang et al., 2014). However, this link between rosetting and increase in parasitemia could not be established for PfEMP1 mediated rosettes. This may be associated with the fact that while STEVOR is expressed on the merozoite tip (Khattab et al., 2008; Niang et al., 2014), there is no evidence for the expression of PfEMP1 on the merozoite surface. Whether rosette formation mediated by the RIFINs is linked to a direct increase in parasitemia is as yet unknown.

Interestingly, two of the three receptors involved in rosetting, i.e., Glycophorin C (Gerbich antigen) (Maier et al., 2003) and the A antigen are under selection pressure (Cserti-Gazdewich, Mayr, & Dzik, 2011). This may be because of the selective advantage that it confers on the host. A certain variant of PfEMP1 is known to mediate endothelial sequestration by engaging host ICAM-1 (Baruch et al., 1996), but no evidence of this endothelial binding ability has been reported for the STEVORs or RIFINs. This is probably due to a lack of studies that have focussed on this aspect of protein function. It is possible that different members of these multigene families are also capable of binding different host cell ligands and that the parasite modulates the expression of variants of all three families based on host receptor availability.

Additionally, both multigene families use redundant receptors in order to mediate rosetting, i.e. Glycophorin C is also a known ligand for EBA 140 (Rydzak, Kaczmarek, Czerwinski, & Lukaszewicz, 2015) and the blood group A antigen is also a known receptor for EBA 175. Thus, the same receptor is engaged by parasite proteins during the process of rosetting and invasion. It is important to note that while

all three proteins (PfEMP1s, RIFINs and STEVORs) have been implicated in resetting (Goel et al., 2015; Niang et al., 2014; J A Rowe, Moulds, Newbold, & Miller, 1997), only the STEVORs have been conclusively shown to participate in both, rosetting as well as invasion (Niang et al, 2014). It will be interesting to further study the binding regions and kinetics of these different sets of receptor-ligand interactions, in order to obtain some perspective on why the parasite uses multiple receptors for the same function.

Taken together, this information suggests that the physiological relevance of these distinct types of rosettes may be different. It could be that the first goal of the parasite is endothelial sequestration, following which rosettes are formed on these already sequestered parasites in order to facilitate an increase in parasitemia and immune evasion; however links between rosetting, parasitemia and immune evasion are controversial. The avenue of molecular presentation and its effect on host receptor binding efficiency also remains unexplored, except in the case of the PfEMP1 antigen. It has been shown that PfEMP1 is clustered on the parasite surface through its interaction with KAHRP and the associated structures of the knob complex (S S Oh et al., 2000; Voigt et al., 2000) and that an absence of KAHRP leads to a compromised ability of the iRBCs to cytoadhere (Crabb et al., 1997). This interaction can lead to a higher avidity interaction with host cell receptors. Here, we show that STEVOR is also capable of clustering on the surface of the iRBC. The physiological relevance of this interaction remains unexplored, although it is likely that the clustering of the molecule is also linked to an increase in host receptor binding avidity.

Apart from host receptor binding, rigidity modulation of the iRBC membrane is another factor that is purported to be important for parasite sequestration under physiological conditions. It has been suggested that the *P. falciparum* parasite sequesters in order to avoid splenic clearance. The two mechanisms of host cell receptor binding and rigidity increase of the iRBC are thought to be linked in order to allow the parasite to successfully evade the host immune system and avoid splenic filtration. It has been shown that the expression of multigene families is downregulated in the absence of the spleen (Bachmann et al., 2009), suggesting that these families play a role in avoiding the splenic clearance of the parasite.

Previous work has shown that the overexpression of STEVOR on the surface of the iRBC can lead to an increase in membrane rigidity at the late asexual stages as well as at stage III and IV of gametocyte development (Sanyal, Egée, Bouyer, Perrot, Safeukui, Buffet, et al., 2012; Tibúrcio, Niang, et al., 2012). In this work, we show that the absence of the cytoplasmic domain of STEVOR caused a significant but incomplete reduction in parasite membrane rigidity of the iRBC membrane. The results were consistent at both, the late asexual stages and stages III and IV of gametocyte development.

At the late asexual stages of parasite development, studies have shown that the PfEMP1 variant protein is anchored by the knob structure to the membrane and the underlying cytoskeleton of the erythrocyte. These structures are partially responsible for the increase in rigidity of the RBC upon parasite growth and maturation. It has been shown that the KAHRP protein, which is a component of the knobs, is able to interact with the conserved cytoplasmic ATS domain of PfEMP1 and that it is through this association that the anchorage of PfEMP1 to the knobs is possible (Voigt et al., 2000). Experiments have demonstrated that the recombinant KAHRP protein is capable of binding to the RBC spectrin-actin-band 4.1 cytoskeletal junction (S. Steven Oh et al., 2000). It has also been shown through simulations that it is possible for this binding and the resulting increase in vertical contacts between the RBC membrane and cytoskeleton to in turn increase the rigidity of the iRBC membrane (Zhang et al., 2015). However, in the absence of the knobs, the ATS domain (cytoskeletal) is also capable of binding independently to the spectrin-actin network. Although the PfEMP1 proteins show a high degree of sequence variability between their extracellular domains, the cytosolic ATS domain is highly conserved (Su et al., 1995). This degree of conservation amongst cytoplasmic domains seems to be a common feature between the PfEMP1s, RIFINs and the STEVORs (Cheng et al., 1998; Kyes et al., 1999; Su et al., 1995). This suggests that these domains of the protein may play an important role in presenting the antigen on the iRBC surface. Here, we demonstrate that the cytoplasmic domain of STEVOR is involved in interactions with cytoskeletal actin. Taken along with the work done earlier on PfEMP1, it can be seen that the cytosolic domains of the variant antigens are capable of interactions with cytoskeletal proteins of the RBC. This may represent a conserved mechanism by which they are able to

mediate the increase in rigidity of the iRBC membrane. Here, we also show in a series of co-immunoprecipitation experiments that STEVOR is capable of interacting with actin on the membrane of late schizonts. This interaction was abolished upon truncation of the cytoplasmic domain of the protein, suggesting a possible mechanism by which STEVOR mediates the increase in membrane rigidity of the parasite. Previous work has also shown that the dynamism of the actin filaments is an important factor that controls the behaviour of the red cell membrane (Gokhin et al., 2015). Many studies have focussed on the role of the spectrin cytoskeleton and its effect on RBC membrane dynamics; however, there are many recent studies that show the importance of actin and its associated proteins in regulating cytoskeletal structure.

The parasite also heavily modulates the shape and rigidity of the iRBC during gametocyte development. *P. falciparum* undergoes a lengthy process of gametocytogenesis involving five stages (Baker, 2010). Of these, only stage V is found in the peripheral circulation and studies of infected patients have found that stages I-IV sequester within internal organs such as the bone marrow (Joice et al., 2014). Recent work has also shown the expression of the *vars*, *rifs* and *stevors* during the gametocyte stages of *falciparum* development (Mwakalinga et al., 2012; Tibúrcio, Niang, et al., 2012; Tibúrcio, Silvestrini, et al., 2012). Similar to the late asexual stages of development, it is thought that stage I-IV gametocytes become more rigid and sequester in order to avoid splenic clearance. Earlier work has demonstrated that the presence of STEVOR on the surface of stage III and IV gametocytes leads to an increase in membrane rigidity (Tibúrcio, Niang, et al., 2012). Consistent with the results obtained at the late asexual stages, we also show that the truncation of the cytoplasmic domain of STEVOR leads to a partial reduction in membrane rigidity at Stages III and IV of gametocyte development.

Recent work has also shown that the increased deformability of the stage V gametocyte is caused by a decrease intracellular cAMP mediated signalling at these stages. The authors showed that when the cAMP levels of stage V gametocytes was artificially increased, the deformability of these cells was compromised, establishing a direct link between the signalling event and cell deformability. They also showed that cAMP dependent kinase activity mediated by a PfPKA (*P. falciparum* Protein Kinase A) was responsible for the increased rigidity of the immature gametocytes (Ramdani et

al., 2015). Several proteins on the iRBC surface may be targets of this kinase dependent phosphorylation, including STEVOR. Taken along with the experiments carried out in this work that show that the cytoplasmic domain of STEVOR is capable of interaction with actin at the schizont stage, the post translational reversible phosphorylation of the protein may play a role in its interaction with the cytoskeleton. Interestingly, the CBS Netphos server predicts that a conserved Serine at amino acid position 291 (part of the cytoplasmic domain of STEVOR) has a phosphorylation potential above the determined threshold. It has also been shown for PfEMP1 that Casein Kinase-II dependent phosphorylation modulates its interaction with the spectrin cytoskeleton. Therefore, it is likely that the parasite uses the mechanism of reversible phosphorylation in order to modulate cytoskeletal interactions.

Interestingly, although *P. falciparum* increases the rigidity of the iRBC during the first IV stages of gametocyte development, it does this without the use of knobs (Tibúrcio, Silvestrini, et al., 2012), which are known to increase the rigidity of asexual stage parasites. Trypsin digestion of membrane fractions shown that although PfEMP1 is present on the surface of stage I and II gametocytes, it is not presented in the context of the knobs at these stages. This may be because during gametocytogenesis, the parasite needs to modify the rigidity of the stage V gametocyte drastically, and because of this it chooses to do this by using proteins that can easily be cleaved off the membrane at stage V. Since STEVOR has been directly linked to changes in rigidity of the iRBC membrane, it is important to study whether it is also a possible target for such cleavage.

Unlike at the trophozoite stage, *P. falciparum* displays PfEMP1 and STEVOR on its surface at different stages of gametocyte maturation, but not simultaneously (Tibúrcio, Niang, et al., 2012; Tibúrcio, Silvestrini, et al., 2012). This may be because both proteins act as ligands for different host cell receptors, and help the parasite to home or sequester within specific body sites during maturation. As such, mechanisms of multigene family transcription at the gametocyte stages remain unexplored. While PFEMP1 has been shown to bind to certain endothelial receptors at the asexual stages, its role in gametocyte adhesion is as yet unclear. A single study showed that a specific subset of the *var* gene repertoire was up regulated in gametocytes; however, the authors reported that the same *stevors* were transcribed at the asexual as well as

gametocyte stages (Sharp et al., 2006). However, more studies on sub-cloned parasites and field isolates need to be carried out in order to confirm this result. STEVOR's role in endothelial cell binding, especially at the gametocyte stage is also unclear. In this work, we report that it is unable to perform the role of uninfected RBC binding with equal efficiency at these stages despite the fact that it is present on the surface of stage III and IV gametocytes. This could be due to the difference in distribution of the protein at these stages or due to the extensive shape change of the iRBC that occurs at stage III and IV of gametocyte development. At these stages, the iRBC is elongated and spindle shaped, in contrast to the spherical shape of the asexual iRBC. The mechanisms underlying the differences in host receptor binding at both stages will be an interesting future avenue for study and can shed some light on the relatively poorly understood biology of the *P. falciparum* gametocytes.

In conclusion, the work from this thesis reveals certain common features that STEVOR shares with the other variant antigens of *P. falciparum*, i.e., the RIFINs and the PfEMP1s. It confirms the interaction of the protein with Glycophorin C during the process of resetting (Niang et al., 2014) and also confirms its topology as a single TM containing protein on the surface of the late stage asexual parasites. The work also shows that the cytoplasmic domain of the protein interacts with actin and that it is able to cluster on the iRBC surface in a manner that is independent of this interaction. Confirming previous studies that show the importance of STEVOR on the iRBC surface in the increase of membrane rigidity at both, the asexual and gametocyte stages, it demonstrates that the cytosolic domain of the protein plays a role in this increase. In summary, it sheds some light on the multiple functions and interactions of this less well studied molecule, in the hope that it will be used to achieve a better understanding of the enigma that is *P. falciparum*.

5. FUTURE DIRECTIONS

The work presented in this thesis studied various aspects of STEVOR biology at both, the asexual and gametocyte stages. During these studies, several interesting aspects of the molecule were revealed.

We showed that STEVOR has the ability to cluster on the surface of the iRBC at the late asexual stages, in a manner that doesn't depend on the presence of the cytosolic domain. This indicates that the clustering of the molecule may be dependent on the TM domain of the protein. Mutations of this TM domain could define the intermolecular interactions that are important for the clustering to occur; and also how the disruption of these interactions could affect the rigidity of the iRBC membrane. Another interesting avenue would be to study whether the clustering of the molecule also affects the strength of the interactions between STEVOR and Glycophorin C, during the process of rosetting. Such studies could help to elucidate the biological relevance of the clustering phenotype.

In addition, this work revealed the interaction of the cytoplasmic domain of STEVOR with cytoskeletal actin of the iRBC, providing one mechanism for how the molecule affects the rigidity of the membrane. Recent work published by Catherine Lavazec's group indicates that the rigidity of gametocytes is modulated by kinase activity that leads to changes in cAMP levels within these cells. The cytoplasmic domain of STEVOR has been proposed as an interesting candidate for phosphorylation, and one of the highly conserved Serine residues that is predicted to be phosphorylated is present at amino acid residue 291, within the cytoplasmic domain of the protein. Previous studies have shown that the cytoplasmic domain of PfEMP1 is a target for phosphorylation by Casein kinase II; and that reversible phosphorylation could affect its interaction with cytoskeletal structures. In fact, reversible protein phosphorylation has been shown to be a commonly used mechanism to regulate the interactions of several cytoskeletal structures with each other. It will be interesting to see whether this is the case for STEVOR as well, and whether the mutation of this Serine residue leads to a change in the ability of the molecule to interact with actin.

Although this work provides the first report of the interaction of STEVOR with actin, the effect of this interaction on the overall organization of the actin cytoskeleton as well as its effect on the dynamic behaviour of actin is not known. The use of high resolution microscopy and recombinant protein production could help to clarify these

downstream effects of this interaction and thus lead to a better understanding of the biological consequences of this interaction.

In this work we also showed that the truncation of the cytoplasmic domain of STEVOR had an effect on the rigidity of the iRBC membrane even at the gametocyte stages. However, the distribution of the molecule on the gametocyte surface and the effect of the truncation of the cytoplasmic domain on this distribution, if any, remains unknown. The biology of *P. falciparum* gametocytes is not well understood, and studies that contribute to this field may reveal some interesting insights about the parasite.

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APPENDIX

Appendix I: Validation of anti-CYT STEVOR antibody

Antibody	Peptide Sequence	ELISA Titre
α-STV CYT	RRRKNSWKHECKKHLCK	400K

Figure A1: Validation of the α -STEVOR CYT domain antibody by IFA and ELISA. The figure above shows the IFA of the STEVOR negative strain (A4) in which there is no staining obtained using the polyclonal rabbit antibody (company manufactured) against the cytoplasmic domain of STEVOR. However, in the 5A clone which is positive for STEVOR expression, the antibody is able to detect the protein. The table shows the ELISA titre obtained using the specific peptide for detection.

Appendix II: Primer Sequences for RT-PCR

Primer Used	Sequence
UCE-F	TTGGTGTTAGTGGCTCACC
UCE-R	TACTTTGGGTGGTCTGTTGG
PFF0850c FL-F	ACAAAACCTGTAGGTGAACATGG
PFF0850c FL-R	TGTCATCATCATTTGGGGGCA
PFF0850c TD-F	GAACTGTTTCGAGTGCTGCT
PFF0850c TD-F	TAAATGTTTCTTGCATTCATGCTTC

Figure A2: Primer sequences used for validation of A4-TD and A4-FL parasite transfectants using RT-PCR. The table shows the sequences of the forward and reverse primers used to validate STEVOR transcription in the A4FL and A4TD clones. UCE (Ubiquitin Conjugating Enzyme) was used as the housekeeping gene.

List of Conferences/Workshops Attended

EMBL BioMalPar XI conference, May 2015. Student Oral presentation

(EMBL Travel Fellowship Awarded)

Title of Talk: *P.falciparum* STEVOR and its functions on the iRBC membrane.

APOCB Congress, Singapore, February 2014. Poster presentation.

(APOCB Student Fellowship Awarded)

Title of Poster: Quantitative study of *STEVOR* surface expression during the asexual stages of *P.falciparum* malaria using Atomic Force Microscopy

Singapore Malaria Network Meeting, Singapore, February 2012.

List of Publications

STEVOR is a *Plasmodium falciparum* erythrocyte binding protein that mediates merozoite invasion and rosetting.

Cell Host Microbe 2014 Jul;16(1):81-93 Makhtar Niang, Amy Kristine Bei, **Kripa Gopal Madnani**, Shaaretha Pelly, Selasi Dankwa, Usheer Kanjee, Karthigayan Gunalan, Anburaj Amaladoss, Kim Pin Yeo, Ndeye Sakha Bob, Benoit Malleret, Manoj Theodore Duraisingh, Peter Rainer Preiser.

Expression and physiologically relevant functional characterization of STEVOR during late asexual stages of *Plasmodium falciparum* (*Manuscript Submitted*).

Himanshu Singh, **Kripa Gopal Madnani**, Ying Bena Lim, Jianshu Cao, Peter R. Preiser, Chwee Teck Lim