

# Invasion pathway switching of plasmodium falciparum reveals post-transcriptional regulation of merozoite proteins

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**Invasion pathway switching of  
*Plasmodium falciparum*  
reveals post-transcriptional regulation of  
merozoite proteins**

**CLAUDIA KUSS**

School of Biological Sciences

A thesis submitted to the Nanyang Technological University  
in fulfillment of the requirement for the degree of  
Doctor of Philosophy

**2009**



## **Abstract**

The clinical symptoms of the parasitic disease malaria are caused by the intra-erythrocytic stage of the *Plasmodium* spp life cycle. The invasive form of the malarial parasite for erythrocytes is the merozoite. Erythrocyte invasion is a multi-step process and proteins required for this event are transcribed during the schizont stage and subsequently expressed at the merozoite stage. The merozoite is able to invade erythrocytes using a wide range of invasion pathways. Invasion pathways are determined by erythrocyte and merozoite properties. Erythrocyte characteristics and availability of erythrocyte receptors are crucial criteria for successful invasion. Each parasite clone possesses unique invasion properties: firstly the parasite clone does not necessarily express all homologues of the invasion protein family; secondly, the amount of a particular protein being expressed may vary; and thirdly some proteins are predominantly expressed over others.

The *Plasmodium falciparum* clone W2mef is able to switch from a sialic acid-dependent to independent (W2mef/NM) invasion pathway when selected in the presence of neuraminidase-treated erythrocytes. Neuraminidase cleaves sialic acid from erythrocyte surface proteins thus limiting the availability of erythrocyte receptors. Some aspects of the molecular mechanism underlying this switch has been determined by Stubbs et al., (2005) and Gaur et al., (2006) by transcriptional assessment of the schizont stage and subsequent expression analysis of identified candidate molecules. Recent studies have though shown that invasion molecules may not only be regulated at the transcriptional stage, but also during protein expression. By using a novel-quantitative proteomic approach (iTRAQ) this present study investigates merozoite protein expression profiles and reveals differences in protein expression between W2mef and W2mef/NM. Of the 600 proteins that were quantitatively analysed 88 proteins show differential expression in W2mef/NM versus W2mef. Furthermore, this study exploits the ability of the W2mef clone to switch between invasion pathways as a tool to uncover the gene regulation mechanism by directly comparing gene transcription during the schizont stage and protein expression at the merozoite stage. Post-transcriptional regulation appears to

determine the amount of the protein expression. Many invasion molecules such as MSP-1 RhopH complex, RAMA, ASP etc. were up-regulated in W2mef/NM at the protein expression level but did not show any apparent regulation at the transcriptional stage. Some of these expression changes can be explained by analyzing the influence of neuraminidase treatment on erythrocytes. Neuraminidase-treated erythrocytes exhibit reduced erythrocyte surface charge, change of shape and deformation compared to untreated erythrocytes. Based on this study it is apparent that neuraminidase treatment of erythrocytes induces extensive changes to which the parasite needs to adjust. Within this context the biological relevance of the observed proteomic changes will be discussed.

These results give insight into the multiple levels of gene regulation and demonstrate the importance of adhesive proteins such as MSP-1 as a foundation for vaccine design and therapeutic intervention.

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Many Antibodies have been used in this study and I would like to thank Dr. Kuhlemeier (Bern, Switzerland) for eI5A antibody; Dr. Anthony Holder (London, UK) for providing MSP1, MSP7, Clag3.1, RhopH2 and MTIP antisera, Dr. Julien Rayner (Cambridge, UK) for GAP45; Dr. Xiahong Gao (NTU, Singapore) for RH-1 antibody and MR4 for plasmepsin 2 and histone 3 antibodies.

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## **Abbreviations**

|                |  |
|----------------|--|
| aa             | amino acid   |
| ABRA           | acidic basic repeat antigen                          |
| ACD            | anti-coagulant detergent                             |
| ACT            | artemisinin combination therapy                      |
| AFM            | atomic force microscopy                              |
| AMA-1          | apical membrane antigen-1                            |
| AP2            | activator protein 2                                  |
| ASP            | Apical sushi protein                                 |
| ATP            | adenosine triphosphate                               |
| CDPK1          | calcium dependent protein kinase 1                   |
| C <sub>t</sub> | threshold cycle                                      |
| DBL-domain     | Duffy binding like-domain                            |
| DBP            | Duffy binding protein                                |
| DRM            | detergent resistant membrane                         |
| DTT            | Dichloro-diphenyl-trichloroethane                    |
| EBA            | Erythrocyte binding antigen                          |
| EBL            | erythrocyte binding ligand                           |
| EGF-domain     | epidermal growth factor-domain                       |
| EI             | elongation index                                     |
| EMP-1          | erythrocyte membrane antigen-1                       |
| GAP45          | Glideosome associated protein 45                     |
| GPI-anchor     | glycosylphosphatidyl-anchor                          |
| HIV            | human immunodeficiency virus                         |
| HLA antigens   | human leukocyte antigens                             |
| HPLC           | high performance liquid chromatography               |
| IMC            | inner membrane complex                               |
| ITN            | insecticide-treated bed nets                         |
| iTRAQ          | isobaric tags for relative and absolute quantitation |
| Kb             | kilo bases   |
| kDa            | kilo Daltons   |

|        |  |
|--------|--|
| KO     | knock out                                  |
| MEME   | Multiple Em for motif elicitation          |
| min    | minutes                                    |
| MS     | Mass spectrometry                          |
| MSP    | Merozoite surface antigen                  |
| MTIP   | myosin A tail domain interacting protein   |
| MyoA   | myosin a                                   |
| m/z    | mass-to-charge ratio                       |
| NAT    | natural antisense transcripts              |
| NM     | neuraminidase                              |
| Nt     | nucleotide                                 |
| PKB    | protein kinase B                           |
| PV     | parasitophorous vacuole                    |
| PVM    | parasitophous vacuole membrane             |
| RAMA   | roptry associated membrane antigen         |
| RAP    | roptry associated protein                  |
| REX    | Ring exported protein                      |
| RBL    | reticulocyte binding ligand                |
| RH     | reticulocyte-binding homologue             |
| RhopH  | high-molecular mass roptry protein complex |
| ROM    | rhomboid protease                          |
| RON    | Rhoptry neck protein                       |
| RT-PCR | real time PCR                              |
| SCX    | strong cation exchange chromatography      |
| SDS    | sodium dodecyl sulfate                     |
| SERA   | serine repeat antigen                      |
| SUB    | subtilisin                                 |
| TM     | transmembrane domain                       |
| TP     | Time point                                 |
| TRAP   | thrombospondin related anonymous protein   |
| UTR    | untranslated region                        |

|          |   |
|----------|---|
| Var      | variant antigens  |
| W2mef/NM | W2mef clone adapted to grow in neuraminidase treated erythrocytes |
| WHO      | world health organization   |
| w/o      | without   |

## **Personal Bibliography**

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## Appendix

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## **Chapter 1**

### **Introduction**

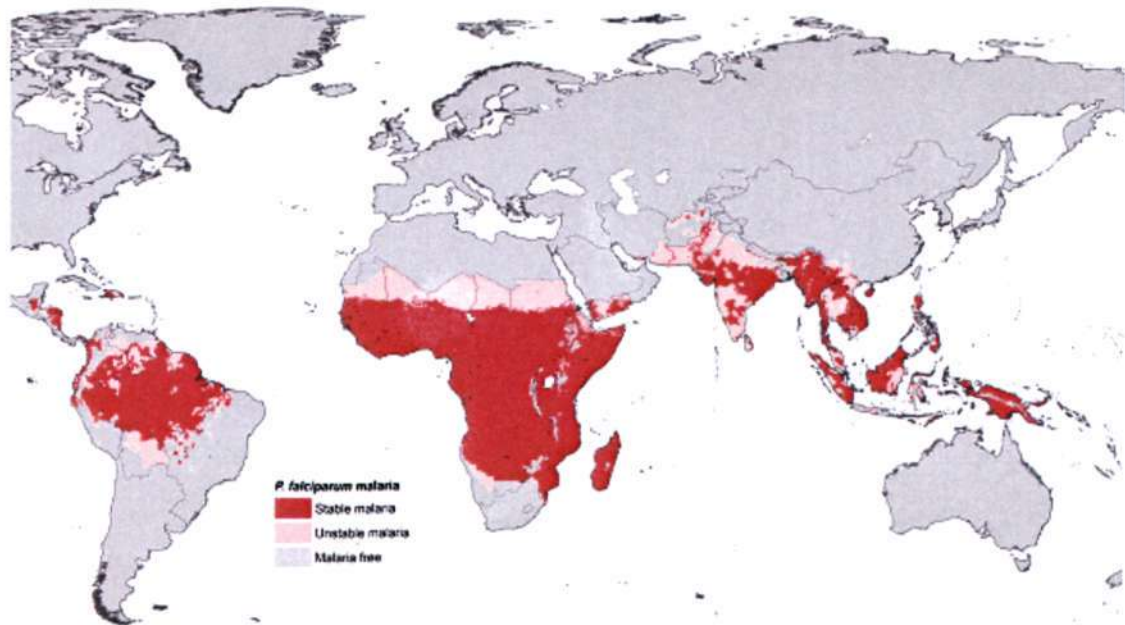
#### ***1. 1 Impact of malaria***

Ever since the existence of humanity, malaria has been a threatening enemy. The earliest evidence that malaria occurred in humans has been found in Egyptian mummies dating back as far as 3200 BC. This disease has influenced history by infecting and killing emperors, popes, nobility and artists. It is believed that Alexander the Great died from malaria in 323 BC as well as the German Kings Otto II and Heinrich, Mongolian ruler Genghis Khan, Italian poet Dante and Pope Leo X as well as many others ([http://www.malariasite.com/malaria/history\\_parasite.htm](http://www.malariasite.com/malaria/history_parasite.htm)). Still today, there are 500 million cases a year (Snow *et al.*, 2005) and over 2 million deaths annually (Breman, 2001). Co-infection with human immunodeficiency virus (HIV) accounts for severity of the pathogenesis and, unfortunately, further deaths (Smith *et al.*, 2004).

Approximately 40% of the world's population is at risk of becoming infected with malaria in tropical and subtropical regions (Figure 1.1.). Primarily pregnant women and children under the age of five suffer most from the disease. Underprivileged residents are especially at great risk with the majority of malaria cases occurring in the poorest 20% of the world's population with catastrophic economic consequences for the patients (Breman *et al.*, 2004).

The aetiological organism causing malaria is the *Plasmodium* parasite, which was identified by Alphonse Laveran in 1880. Malaria parasites belong to the phylum Apicomplexa (Levine, 1971). Apicomplexan parasites are obligate intracellular protozoan organisms, which, depending on the species, are able to invade a wide range of different host cells, for example erythrocytes (*Plasmodium*) and fibroblasts and other nucleated cells (*Toxoplasma*). A defining feature of all members of this





**Figure 1. 1. Global distribution of *P. falciparum*.**

(Adapted from Guerra *et al.*, (2008). Populations at risk defined by parasite incidence, temperature and aridity. Areas were defined as stable malaria (Dark Pink, Annual parasite incidence  $>0.1$  per 1,000 pa), unstable malaria (light pink, Annual parasite incidence  $<0.1$  per 1,000 pa) and no risk (grey).

phylum is the unique presence of organelles found at the anterior end of their invasive forms. This apical complex plays an important role in the invasion of the host cell (Bannister *et al.*, 1986; Sam-Yellowe, 1996). The genus *Plasmodium* belongs to the family *Plasmodiidae*, order *Haemosporidiidae*, phylum *Apicomplexa*. More than 120 species of *Plasmodium* have been identified in mammals, reptiles, and birds and are recognised taxonomically by the presence of two types of asexual division: schizogony in the vertebrate host; and sporogony in the insect vector.

Human malaria is mainly caused by four *Plasmodium* species. *P. falciparum* is most pathogenic, responsible for the most severe form of malaria and cause of malaria-related deaths. *P. vivax* is the most geographically widespread form with less severe symptoms than *P. falciparum* but with chronic long lasting infection due to dormant liver stages residing within the host for many years after infection (Krotoski *et al.*, 1986). The less predominant but not to be underestimated species are *P. malariae* and *P. ovale*. Similar appearance and disease pattern as *P. vivax* have been noted for *P. ovale*. *P. malariae* differs from the other human malaria species by its very slow development in both vertebrate and insect hosts. A fifth species has recently risen to prominence and may become a threat to humans. Field studies in Borneo and Peninsula Malaysia reported human infection with *P. knowlesi*, so far only known to infect primarily rhesus monkeys (Cox-Singh *et al.*, 2008; Singh *et al.*, 2004; Vythilingam *et al.*, 2008).

Only one mosquito genus, the female *Anopheles* mosquito, is able to act as vector for malaria. *Anopheles* contains around 430 species but only 70 species are known to be of major importance as vectors. *Anopheles* have a virtually world-wide distribution, primary in tropical and subtropical regions at elevations below 2500 m.

## ***1. 2 Malaria control and drug treatment***

Malaria can be controlled in the context of blocking malaria transmission by eliminating the mosquito vector or preventing mosquito bites.

Dichloro-diphenyl-trichloroethane (DDT) is a reliable insecticide and was commonly used from the 1940s to the 1970s, until it was banned in many countries mainly due to its toxicity after chronic exposure. The unsystematic use of DDT resulted in serious ecological problems. It has been suggested that nearly all living beings, including both plant and animal, are contaminated by organochlorine insecticides like DDT (Alawi *et al.*, 1992; Beall and Nash, 1971; Bouwman *et al.*, 1990; Bouwman *et al.*, 1994; Hernandez *et al.*, 1993; Somogyi and Beck, 1993). Furthermore, the long persistence of DDT in the environment and reported resistance of insect species led to use of other insecticides in place of DDT in many developed countries. In recent years DDT has been reintroduced as a major malaria control mechanism in Africa by indoor residual spraying and has been recommended by WHO (World Health Organisation) since 2006. (Attaran and Maharaj, 2000; Rogan, 2000; Ross, 2005). The main reasons for re-visiting the use of DDT include that it has a longer action time, and thus fewer household sprayings are needed.

The combination of both insecticide-treated bed nets (ITNs) and indoor residual insecticide spraying (IRS) is most effective against the indoor-resting Anopheline mosquitoes (Lengeler, 2004; WHO, 2006). ITNs reduce the need for DDT spraying but does not abolish it completely (Bremán *et al.*, 2004). These measures already show some success in selected areas (Killeen and Smith, 2007; Killeen *et al.*, 2007; Mufunda *et al.*, 2007; Ridl *et al.*, 2008; Sharp *et al.*, 2007a; Sharp *et al.*, 2007b; Walker and Lynch, 2007).

While ITN and IRS show great promises, the tremendous numbers of infections and mortalities indicate the need for drugs and vaccines. The actions of components from Cinchona bark and *Artemisia annua* against malaria have been known for centuries and effective extracts of these plants were used to cure malaria. Quinolines such as chloroquine are derivatives from the active substance (quinine) from the Cinchona tree. Chloroquine affects the parasite haemoglobin degradation



(Foley and Tilley, 1997; Warhurst and Hockley, 1967). Artemisinin, extracted from *Artemisia annua* has potentially multiple modes of action. Artemisinin, interferes with calcium transporters (Uhlemann *et al.*, 2005), depolarizes the mitochondrial membrane (Li *et al.*, 2005) and inhibits hemoglobin degradation (Pandey *et al.*, 1999).

Establishing a standard intervention strategy against malaria has been very difficult since the parasite developed resistance to almost all currently used anti-malarials (amodiaquine, chloroquine, mefloquine and sulfadoxine-pyrimethamine). A current treatment protocol given to patients depends on the parasite strain and the severity of the disease. Today, artemisinin combination therapy (ACT, combination of artemisinin or one of its derivatives with one or two anti-malarial drugs of a different class) are recommended by the WHO. Combination therapy has the advantage of multiple components with independent modes of action. Furthermore, different targets improve therapeutic efficacy and also reduce the development of resistance against components of the drug.

In addition to therapeutic drugs, a number of prophylactic drugs also exist. The most common are Lariam™, with mefloquine as active component, and more recently Malarone™ which uses the combination of atovaquone and proguanil hydrochloride. Which drug is prescribed depends on the parasite in the area, known resistance against the drug, as well as side effects of the drug.

### ***1. 3 Naturally acquired immunity and disease pathology***

By definition, a parasitic organism benefits from the prolonged relationship with its harmed host. Therefore, the parasite's principle objective is not to kill, but reside within and be partially protected from the host's immune surveillance. Here, antigenic variation (detailed explanation is below) allows the host's immune system to eradicate the main parasite population, but a subpopulation will survive this attack and assure the advancement of a chronic infection of the host (Saul, 1999).

Only in stable malaria endemic regions does an extreme burden of mortality and morbidity lie on infants and children under the age of five. The inhabitants of these endemic areas have acquired a very specific immunity against malaria during their early age. These individuals spend most of their lifetime parasitized but are otherwise in a healthy state reviewed in Druilhe and Perignon (1994) and Struik and Riley (2004). Babies of immune women will be relatively immune against infection for a certain period of time of ~3 months after birth (Fried *et al.*, 1998). In the case of heavy transmission, these babies may become infected but rarely develop any symptoms associated with the severe disease. After these first three months, children become receptive for more rigorous features of malaria. These severe attacks apart from the 48 hour high fever cycles in *P. falciparum*, involve convulsions, shock, hypoglycaemia, respiratory distress, metabolic acidosis, hepatic dysfunction, renal failure and severe anaemia (Miller *et al.*, 1994). Cytoadherence of infected erythrocytes to blood vessels is connected with the most severe symptoms of cerebral malaria and complications during pregnancy leading to microvascular hindrance in the brain and placenta, respectively. In cerebral malaria the sequestered erythrocytes can breach the blood brain barrier, and possibly resulting in coma (Adams *et al.*, 2002). Cerebral malaria is very often deadly and placental malaria entails many unfortunate complications for the foetus sometimes resulting in death. From the 4<sup>th</sup> year onwards the severe clinical manifestations will decrease while a high level of parasites reside within the body (Gupta *et al.*, 1999). An absolute immunity to malaria infection is probably never reached.

It is also suggested that immunity is strain specific: children, who are immune to some *Plasmodium* clones, remain susceptible to the disease. An individual needs to experience a repertoire of strains in order to gain substantial immunity.

The principle by which individuals in holo-endemic areas could gain natural acquired immunity is by humoral immunity: neutralising antibodies against the merozoite surface proteins and surface proteins of the *Plasmodium falciparum*-

infected erythrocyte result in maintenance of a low level parasite population (Marsh *et al.*, 2004).

On the other hand immunity can also be T-cell mediated. This plays an important role mainly in pre-erythrocytic stages where processed parasite antigens are presented in association with HLA molecules on the surface of hepatocytes. T-cells in turn recognise these HLA-peptide complexes and recruit NK-cells which eliminate the infected cells reviewed in Overstreet *et al.*, (2008).

### **1. 4 Malarial vaccine development**

Malaria researchers all over the world have one common goal: to obtain a better understanding about the parasite physiology and eventually create an effective vaccine that will offer protection against the disease.

#### **1. 4. 1 Whole organism vaccines**

The biggest hopes for producing an effective malaria vaccine came from studies performed in 1973; human volunteers were immunised with irradiated sporozoites which activated an immune response and resulted in robust protection against a subsequent *P. falciparum* infection (Clyde *et al.*, 1973a; Clyde *et al.*, 1973b). The principle of attenuating sporozoites by  $\gamma$ -irradiation is to initiate multiple random mutations in the parasites by which liver stage development is blocked. In experimental vaccine studies, immunised volunteers harbour a heterogeneous population of attenuated sporozoites. On the other hand, over-irradiated sporozoites do not guarantee protection and also lose their ability to infect hepatocytes (Silvie *et al.*, 2002).

There are obvious limitations to this whole organism strategy as it is difficult to obtain the irradiated sporozoites in large scale with no *in vitro* culture system for sporozoites available at present. There are also concerns about safety, the genetic



stability and lack of preservation. A study in *P. berghei* parasites gave some promising prospects for the implementation of a whole organism vaccine with a directed manipulation of the parasite. Deletion of the *uis3* gene resulted in the parasite losing the ability to mature into liver stage merozoites and arrested development (Mueller *et al.*, 2005). A booster dose of these genetically modified sporozoites gave persistent sterile protection against malaria (Mueller *et al.*, 2005).

Nevertheless, attenuated sporozoites are to date the only “vaccine” capable of inducing proven long-lasting sterile protection against malaria transmission (Morrot and Zavala, 2004).

#### **1.4. 2 Subunit vaccines**

Next to whole organism vaccine development investigations are under way for subunit vaccine strategies. Subunit vaccines are partial or complete antigens (peptides or proteins) selected from the pathogen and used to generate a protective immune response. Another variation of subunit vaccine are DNA vaccines. Here, genomic DNA (Wang *et al.*, 1998) or recombinant attenuated DNA virus (Schneider *et al.*, 1998) ) can be used to create candidate DNA-based vaccines. But antibody responses have reportedly been quite low after DNA-based challenges (Le *et al.*, 2000).

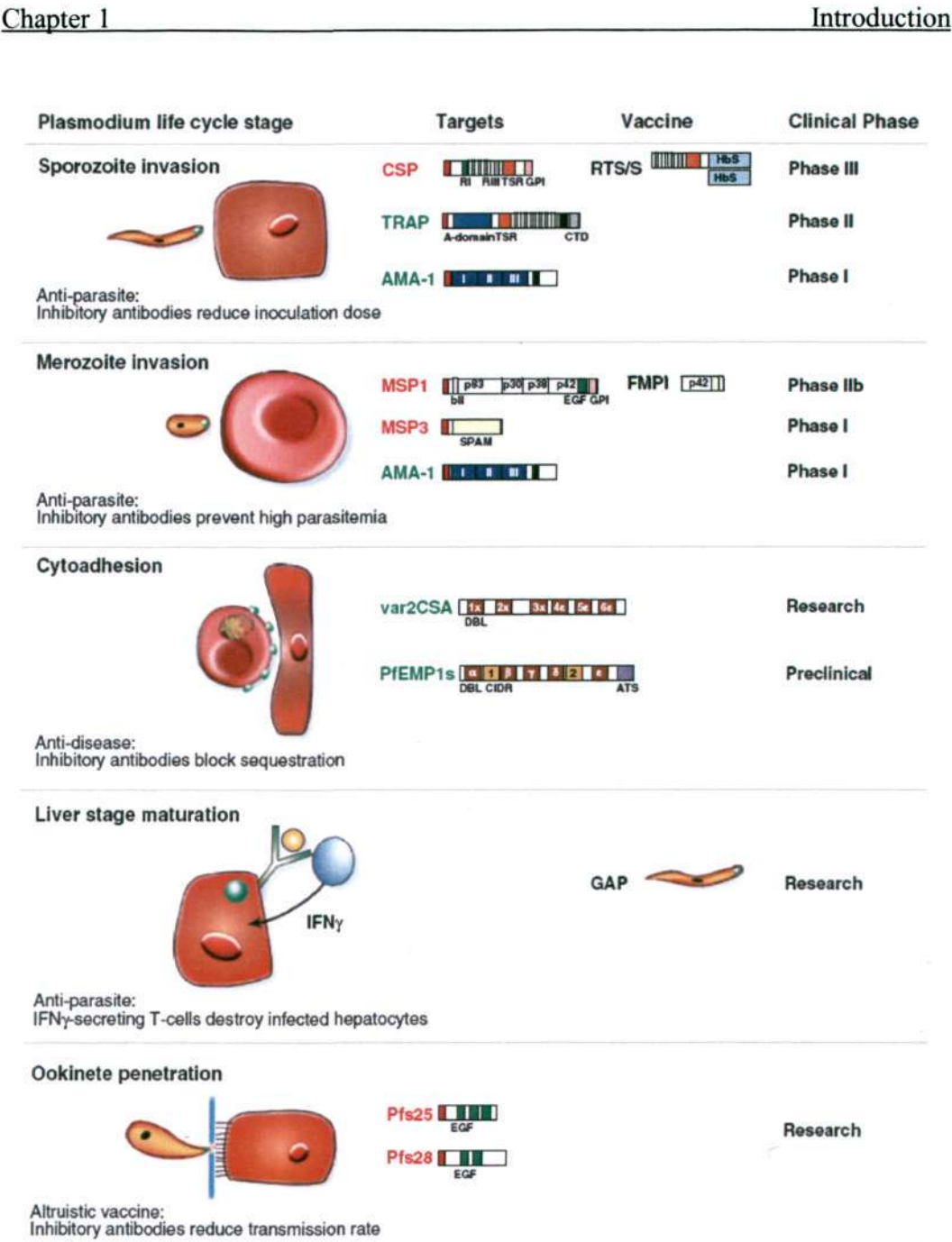
The first malaria antigens discovered were sporozoite and merozoite surface antigens. These are still the main targets for subunit vaccine strategy. These vaccine candidates are summarised in Figure 1.2.

Notably, the most advanced CSP-based (circumsporozoite protein) subunit vaccine is the RTS,S vaccine a pre-erythrocytic vaccine by GlaxoSmithKline. Promising results from clinical trials in various regions in Africa support the conduct of PHASE III clinical trials (Abdulla *et al.*, 2008; Bejon *et al.*, 2008). Additional antigens to build on the CSP fragment are advantageous and improve the

immunogenicity. Initially RTS,S with the adjuvant AS02A efficiency was 30% in children of 1 to 4 years (Alonso *et al.*, 2004). Recently a higher protection was achieved by a different adjuvant AS01E in 894 children aged 5 to 17 months in Tanzania and Kenya. The combination of RTS,S/AS01E was safe and led to a 53% reduction of clinical malaria during the 8 months after vaccination (Bejon *et al.*, 2008). Another study in Tanzania investigated the safety and efficiency of RTS,S/AS02D in combination with other vaccines given at 8, 12 and 16 weeks after birth (Abdulla *et al.*, 2008). The result of the study showed that antibody levels of the other vaccines were not influenced by RTS,S/AS02D and reduced incidence of malaria first infection during 6 month after birth of 65.2%.

The understanding of immunity in malaria infected individuals as well as antigen processing, genetic engineering and protein expression methods and delivery system are continuously improving – and the search for the “holy grail” of malaria vaccine research continues.



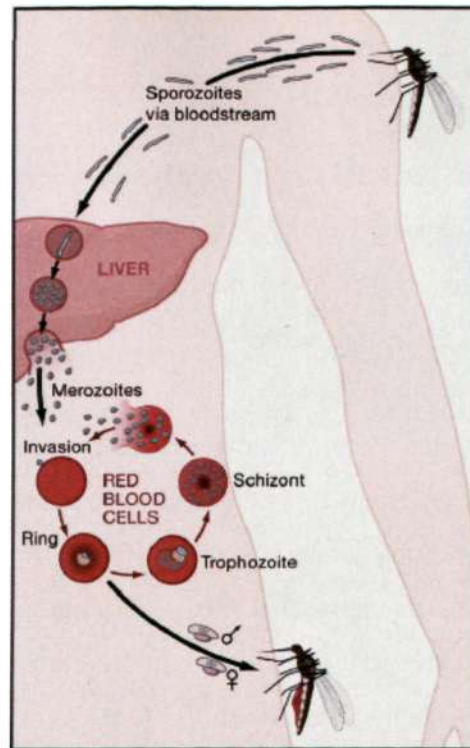


**Figure 1. 2. Major malaria vaccine candidates.**  
Figure adapted from Matuschewski and Mueller (2007). The major vaccine candidates are listed according to the life cycle stage. The primary structure and functional domains of vaccine targets and lead vaccines are illustrated (detailed explanation in Matuschewski and Mueller, 2007). The status of the vaccine development is indicated in the Phase column.

### ***1. 5 Life cycle of malaria parasite***

The life cycle of malaria parasites is an ingenious process involving two host systems (Figure 1.3). The definitive host, the mosquito, is required to guarantee sexual reproduction (sporogony) and the intermediate host is represented by the vertebrate host for asexual development.

The female *Anopheles* mosquito bites a malaria-infected vertebrate host and ingests blood containing infected erythrocytes and gametocytes. Gametocytes are gamete-forming cells and the first stage of sexual development. In the mosquito gut the decreased temperature and the mosquito factor xanthurenic acid (Garcia *et al.*, 1998) triggers the gametocytes to differentiate into female and male gametes. Male and female gametes fuse together and form a zygote. The zygote develops to an ookinete, which is motile and able to cross the gut wall into the haemocoel and encysts. There, another transformation occurs into an oocyst from which sporozoites are released. Sporozoites then migrate to the salivary glands. When the infected *Anopheles* bites a new victim, sporozoites get injected with her saliva. After only two minutes in the host's bloodstream most of the sporozoites have migrated to the liver and invaded hepatocytes. Within these cells a parasitophorous vacuole is formed by the parasite to separate the intruder from the surrounding cytoplasm. In this environment the parasites develop from sporozoite to schizont. Each schizont contains approximately 30,000 to 50,000 merozoites. The enlarged liver cell ruptures and releases the merozoites into the bloodstream. Once merozoites enter the blood vessels it takes as little as 30 seconds to invade an erythrocyte. Within the erythrocyte the merozoites undergo asexual multiplication to create erythrocytic merozoites. In *P. falciparum*, each cell releases 16 to 32 merozoites into the bloodstream where they repeatedly invade other erythrocytes. Some parasites are able to develop into gametocytes, the sexual form of the parasites. The life cycle is completed when the mosquito ingests blood with gametocytes and gametes are formed.



**Figure 1. 3. *Plasmodium* life cycle.**

Figure adapted from Cowman and Crabb (2006). An infected *Anopheles* mosquito injects sporozoite-containing saliva into the host's bloodstream. The sporozoites invade the liver cells immediately and multiply by cell division. After some time, which can vary depending on the *Plasmodium* species, sporozoites develop into merozoites, which give rise to a structure known as a schizont. This maturation term follows a rupture of the liver cells and the merozoite's release. Once in the bloodstream the merozoites enter the red blood cells and undergo asexual reproduction. In *P. falciparum*, every 48 hours infected red blood cells rupture to release merozoite forms of the parasite, and these merozoites invade and multiply within new erythrocytes. Not all of the merozoites develop into schizonts, some form gametocytes. When the female mosquito bites an infected human, it takes up gametocytes along with blood. The gametocytes immediately mature into female and male gametes inside the mosquito gut and fuse to form a zygote. After this sexual reproduction the motile zygote (ookinete) crosses the gut wall into the haemocoel and encysts. Sporozoites are discharged and reach the salivary gland. The infected *Anopheles* has to bite a new victim to complete the life cycle.



## ***1. 6 Invasion of erythrocytes***

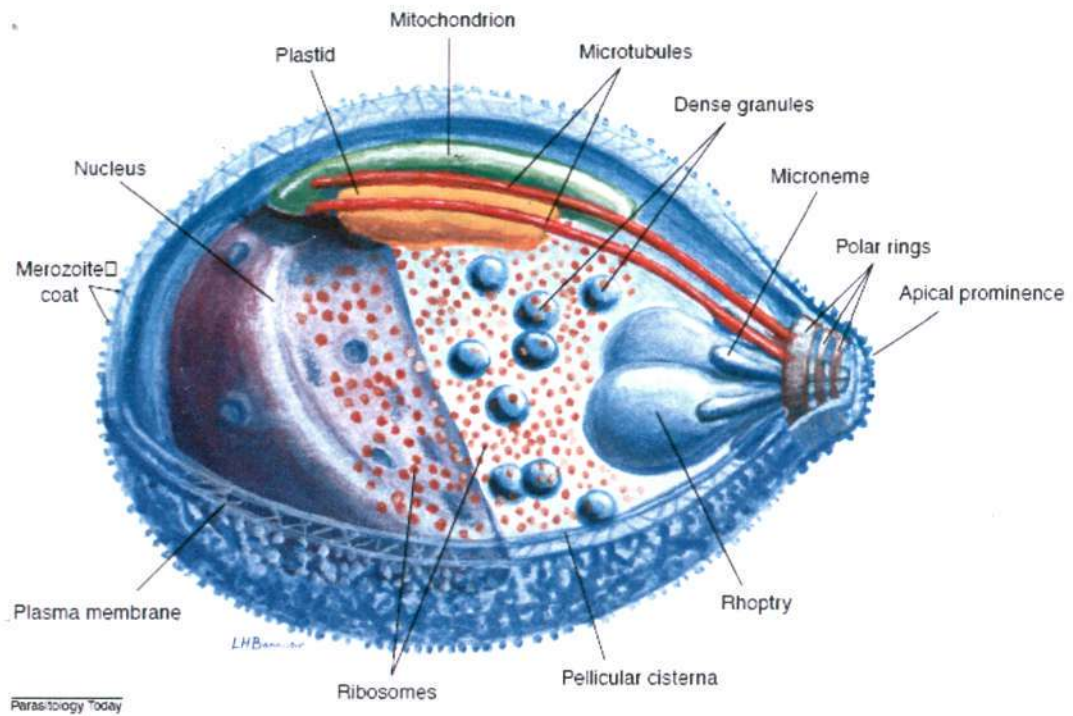
The invasion of erythrocytes by *Plasmodium* merozoites is a specialised and well synchronised sequence of events. The parasite requires the erythrocyte for nutrition and uses it as a habitat for multiplication. The free merozoite (Figure 1.4.) has to act rapidly to gain entry into the erythrocyte to guarantee its survival and to avoid detection by the host immune system.

The first interaction between merozoite and erythrocyte starts with random binding of a merozoite to the surface of erythrocytes (Figure 1.5). It has been shown by microscope image recording that any part of the merozoite surface can mediate this first attachment (Dvorak *et al.*, 1975). This attachment appears reversible, to give the parasite the opportunity to find a “suitable” erythrocyte. Parasite proteins, like merozoite surface proteins, are thought to mediate this initial interaction (Holder and Freeman, 1984; Holder *et al.*, 1985b; Miller *et al.*, 1975a; Narum and Thomas, 1994). After successful binding, reorientation occurs by deforming the host cell in such a way that the erythrocyte surface is wrapped around the merozoite thus placing the apical end in contact with the erythrocytic membrane (Lew and Tiffert, 2007). This is followed by the formation of a tight junction: indicating the irreversible interaction between the merozoite and host cell. During this step the merozoite secretes micronemal as well as rhoptry proteins thereby facilitating invasion (Bannister *et al.*, 1986).

The micronemes and the rhoptries as well as dense granules are part of the apical complex, a unique feature of the invasive form of the parasite also found in invading sporozoites. Molecules from the rhoptry bulb participate in parasitophorous vacuole (PV) formation (Hadley *et al.*, 1983). Penetration occurs when the parasite moves into the PV and enters the erythrocyte driven by an actin-myosin-motor (Keeley and Soldati, 2004).

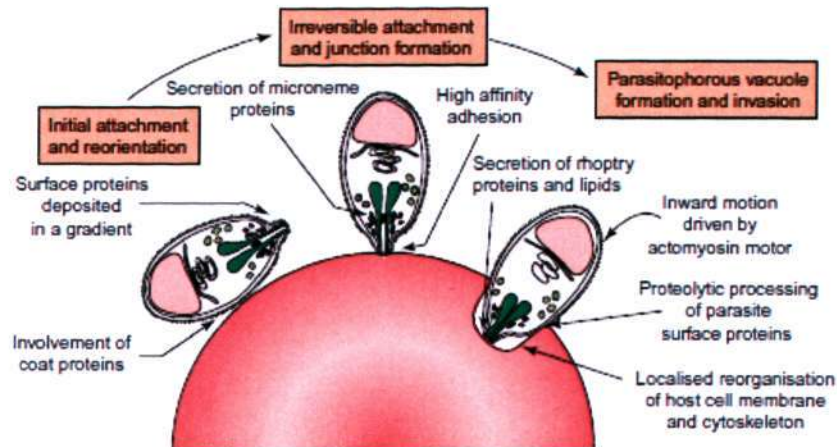
Electronmicroscopic images show that the tight junction moves from the anterior to

the posterior end (Miller *et al.*, 1979; Mitchell and Bannister, 1988). During this movement the interacting molecules are cleaved off by proteases allowing entry of the parasite (Bannister *et al.*, 1975; Harris *et al.*, 2005; O'Donnell *et al.*, 2006). After successful penetration the erythrocyte membrane reseals and the dense granules discharge their contents to expand the PV (Bannister *et al.*, 1975; Torii *et al.*, 1989).



**Figure 1. 4. Cartoon of merozoite structure.**

Figure adapted from Bannister *et al.*, (2000). The 3-dimensional structure of the merozoite and its major internal components are shown. Important features of the merozoite are the apical organelles located at the apical prominence. The apical organelles include of Rhoptries, Micronemes and Dense Granules and their contents are important for the invasion process into erythrocytes.



**Figure 1. 5. Illustration of main steps of erythrocyte invasion by the merozoite.**

Figure adapted from Chitnis and Blackman (2000). The initial attachment starts with random binding of the merozoite to the surface of the erythrocyte. Parasite proteins like merozoite surface protein 1 (MSP-1), apical membrane antigen 1 (AMA-1) are thought to mediate this initial interaction. After successful binding, reorientation occurs placing the apical end in contact with the erythrocyte membrane. This is followed by the formation of a tight junction- indicating the irreversible interaction between the merozoite and host cell. During this step the merozoite secretes micronemal as well as rhoptry proteins. Lipids from the rhoptry organelles generate the parasitophorous vacuole (PV). Penetration occurs when the parasite moves into the PV and enters the erythrocyte driven by an actin-myosin-motor. During this transaction the tight junction moves from the anterior to the posterior end. After successful penetration the erythrocyte membrane reseals and the dense granules discharge their contents to expand the PV.



## **1. 7 Organisation of the erythrocyte surface**

Outside the protective erythrocyte the merozoite is very fragile and has to gain rapid entry into the host cell. The multitude of interactions performed by merozoite and erythrocyte are very well orchestrated and precise. The parasite has adapted to use the erythrocyte as a host cell and the “interplay” between them has evolved over thousands of years. The parasites recognise the erythrocyte as a host cell, some species can distinguish between young and old erythrocytes, and even specific receptors and “sugar” molecules can be recognised and be bound to by the specialised parasite. Therefore it is very important to understand the erythrocyte surface in structure, receptor composition and overall properties.

### **1. 7. 1 Erythrocyte membrane and cytoskeleton**

The erythrocyte membrane gives the cell the ability to undergo extensive, reversible deformations while maintaining its structural composition. This very elastic cell responds quickly to applied stress and is competent in enduring excessive membrane stretching without fragmentation and retaining a constant membrane surface area. The distinctive nature of the erythrocyte is due to an interconnected structure: the plasma membrane composed of amphiphilic lipid molecules forming a bilayer is vertically tied to an elastic network of skeletal proteins via transmembrane proteins (Figure 1.6), (Mohandas and Evans, 1994). It has also been suggested that there is a direct interaction of skeletal proteins with the anionic phospholipids (An *et al.*, 2004; Rybicki *et al.*, 1988). The general belief about the lipid bilayer organization is the lipid raft model. It is suggested that erythrocytes contain detergent-resistant membrane (DRM) rafts. These rafts are thought to form buoyant ‘islands’ of proteins and lipids held together by a cholesterol-rich microenvironment (Brown and London, 1998; Friedrichson and Kurzchalia, 1998; Simons and Ikonen, 1997; Varma and Mayor, 1998).



Proteins belonging to the DRM include major integral membrane proteins such as band 3, but not others such as glycophorin A (Samuel *et al.*, 2001). Even though glycophorin A and band 3 are directly interacting with each other during their biosynthesis and trafficking to the plasma membrane (Groves and Tanner, 1992), an immediate link within the erythrocyte membrane is still under investigation (reviewed in Williamson and Tøye, 2008). The asymmetric distribution of phospholipids is of functional relevance since it interacts with skeletal proteins spectrin and protein 4.1R, modulating the anchor point of the skeletal network to the bilayer.

### ***Erythrocyte transmembrane proteins***

More than 50 transmembrane proteins of varying quantity, ranging from a few hundred to approximately one million copies per erythrocyte, have been identified. Around 25 of these proteins are referred to as blood group antigens (Reid and Mohandas, 2004). Transmembrane proteins display functional diversity in forming transporters, adhesins involved in interactions of erythrocytes with other erythrocytes and endothelial cells, or function in cell signalling events and in some yet to be defined functions (Reid and Mohandas, 2004).

Band 3, glycophorin C and D are the only membrane spanning proteins known to be connected with the membrane skeleton. Band 3 is a multi-spanning membrane protein with 12 to 14 transmembrane (TM) domains. It resides in the erythrocyte membrane as both dimers and tetramers and it is the tetrameric form that binds protein 4.2R and ankyrin. Band 3 has mainly two functions in the erythrocyte membrane, one is to passively exchange bicarbonate-chloride across the membrane and the second is to stabilise the erythrocyte membrane (Alper *et al.*, 2002; Tanner, 1997, 2002).

### ***Vertical interactions at the erythrocyte surface***

Band 3 associates the bilayer to the cytoskeleton through the interaction of its cytoplasmic domain with ankyrin. Ankyrin is an adaptor protein that links integral membrane proteins to spectrin-actin based membrane skeleton. This linkage is required to maintain integrity of the plasma membrane (Bennett, 1983; Nicolas *et al.*, 2003).

Glycophorin C and D interact with protein 4.1R, calmodulin and p55 (Marfatia *et al.*, 1994; Marfatia *et al.*, 1995; Nunomura *et al.*, 2000a; Reid *et al.*, 1990). P55 is a palmitoylated membrane phosphoprotein and acts as linker molecule between spectrin and glycophorins.  $\text{Ca}^{2+}$  saturated calmodulin regulates the binding of protein 4.1R (Nunomura *et al.*, 1997; Nunomura *et al.*, 2000b). Protein 4.1 is a multifunctional protein in the cytoskeleton and stabilises the membrane shape by its vertical and horizontal interactions (An *et al.*, 1996; Arpin *et al.*, 1994; Baines, 2006). About 90% of glycophorin C present in the erythrocyte is bound to the cytoskeleton and the remaining 10% moves freely within the membrane. These vertical interactions form important structures by regulating the coherence between bilayer and cytoskeleton. This linkage is crucial as disruption of it results in lipid loss and decreased membrane surface area, hence impairing the capacity of erythrocytes to deform. The deficit of membrane surface area is the major cause of decreased cell survival in various erythrocyte disorders (Mohandas and Chasis, 1993). On the other hand, increased number of linkages leads to increased membrane coherence and increased membrane stiffness (Knowles *et al.*, 1994; Mohandas *et al.*, 1992).

### ***Horizontal interactions on the erythrocyte surface***

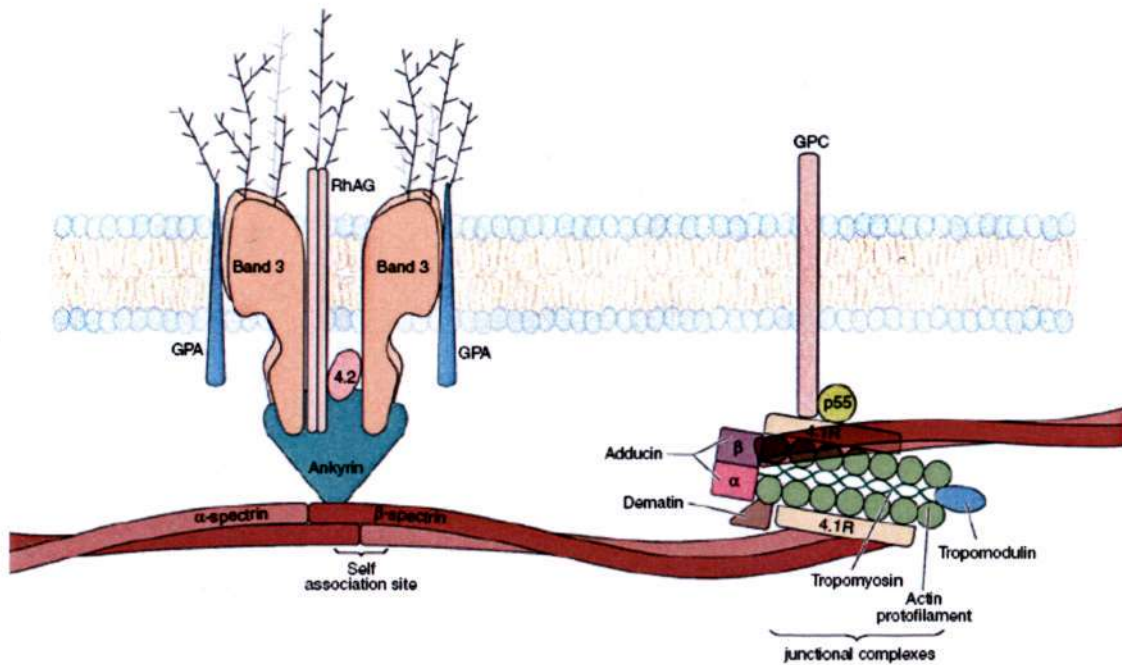
The major component of the skeletal network is composed of  $\alpha$ - and  $\beta$ -spectrin, actin, protein 4.1R, tropomyosin and tropomodulin (Bennett, 1989; Bennett and Baines, 2001).  $\alpha$ - and  $\beta$ -spectrin form an anti-parallel heterodimer by strong lateral interaction between repeats near the C-terminus of  $\alpha$ -spectrin with repeats near the

N-terminus of  $\beta$ -spectrin (Ursitti *et al.*, 1996). Helical repeats of spectrin are structurally heterogeneous. Deformation of the cytoskeleton provokes unfolding of these specific repeats resulting in membrane elasticity (An *et al.*, 2006; Johnson *et al.*, 2007). The physiological structure of spectrin in the skeletal network is a tetramer (DeSilva *et al.*, 1992; Speicher *et al.*, 1993). Disruption of the spectrin heterodimer interaction destabilises the membrane-cytoskeleton network (An *et al.*, 2002; Liu and Palek, 1980).

The other end of the 100 nm long spectrin dimer forms a junctional complex with actin and protein 4.1R (An *et al.*, 2005; Karinch *et al.*, 1990; Ungewickell *et al.*, 1979). Actin filament length within the cytoskeleton is highly controlled by tropomyosin and formed by 14 to 16 actin monomers (Fowler, 1996). The weak interaction of actin with the N-terminus of  $\beta$ -spectrin is promoted by protein 4.1R (Ohanian *et al.*, 1984). The spectrin-actin-protein 4.1R complex is a significant controller of membrane mechanical integrity.

The general concept of membrane properties are: the unfolding and refolding of spectrin repeats accounts for the extraordinary elasticity, obstruction of this interplay influences the overall membrane rigidity. The vertical interactions between lipid bilayer and cytoskeleton play an important role in maintaining membrane coherence. However the lateral connections between spectrin dimer and spectrin-actin-protein 4.1R are the main regulatory units of mechanical stability of the erythrocyte. The combination of both properties is important for the erythrocyte to undergo extensive deformation which is needed while circulating inside the bloodstream.





**Figure 1. 6. Erythrocyte membrane and cytoskeleton.**

Figure adapted from An and Mohandas (2008). Transmembrane molecules Band 3 and Glycophorin (GPA and GPC) are embedded in a layer of amphiphilic lipid molecules. These transmembrane molecules act as tethering sites to connect with the two dimensional elastic network of skeletal proteins. Spectrin exists as a dimer of  $\alpha$  and  $\beta$  spectrin and due to elongations of the spectrin in the direction of extension the erythrocyte is able to deform and band.



### ***1. 8 Erythrocyte receptors involved in merozoite invasion***

There are large numbers of diverse receptors on the erythrocyte, some of these have been shown to be utilised by the merozoite during the invasion process. These include glycophorin A, B, C, D, band 3 and the so far uncharacterised receptors X, Y, Z and E (Dolan *et al.*, 1990; Dolan *et al.*, 1994; Mayer *et al.*, 2001; Narum *et al.*, 2002; Thompson *et al.*, 2001).

Glycophorins have a single transmembrane domain and large amounts of sialic acid residues on their extracellular domain. The predominant receptor is glycophorin A, a sialoglycoprotein with a high abundance of approximately  $10^6$  copies per cell (Gardner *et al.*, 1989; Steck and Dawson, 1974). Glycophorin A is largely responsible for the negative charge of the erythrocyte membrane (Eylar *et al.*, 1962). Thus, a supplementary function is to lower cell-cell interactions and erythrocyte aggregation in circulation (Rogers *et al.*, 1992a). *Plasmodium* takes advantage of this high abundance of glycoproteins and the sialic acid residues and uses them as binding sites prior to entry. There are two ways in which sialic acid is bound to glycoproteins: *O*-linked groups bound to serine (S) or threonine (T) residues; and *N*-linked groups bound to asparagine (N) residues (Pasvol, 1984; Vanderberg *et al.*, 1985). The different glycophorins differ in their *O*- or *N*-glycosylation sites (Kudo and Fukuda, 1989; Pasvol, 1984; Vanderberg *et al.*, 1985).

### ***1. 9 Invasion molecules of the merozoite***

The invasion of the merozoite into the erythrocyte is a sophisticated and well-orchestrated process and it requires a multitude of partner molecules to form interactions with erythrocyte receptors. Table 1.1 summarises the cognate invasion molecules. Some of these molecules are only associated with erythrocyte invasion by evidence of their expression at the merozoite stage and/or their localisation with other invasion molecules or localisation at organelles associated with invasion, but

**Table 1.1. Invasion molecules expressed at merozoite stage.**

Adapted and modified from Cowman and Crabb (2006). Invasion molecules are listed according to their localisation/features.

| Name   | Accession Number | Function  |
|--|------------------|---|
| <b>GPI-Anchored (known or putative) surface proteins</b> |                  |   |
| MSP-1  | PFI1475w         | Binds to Band 3 of the RBC, associated with MSP6 and MSP7, polymorph                    |
| MSP-2  | PFB0300c         | Highly polymorphic; two major alleles functionally identical; species-specific function |
| MSP-4  | PFB0310c         | Unknown function; C-terminal single EGF domain  |
| MSP-5  | PFB0305c         | Unknown function; Not required for invasion   |
| MSP-8  | PFE0120c         | Unknown function  |
| MSP-10   | PFF0995c         | Unknown function; Surface and apical appearance-terminal double EGF domain              |
| Pf38   | PFE0395c         | Member of 6-cys family, also present in the apical organelles                           |
| Pf92   | PF13_0338        | Cysteine-rich surface protein   |
| Pf12   | PFF0615c         | Membrane protein pf12 precursor; Member of 6-cys family                                 |
| Pf113  | PF14_0201        | Putative apical and/or surface protein  |
| <b>Peripheral surface or parasitophorous vacuole</b>     |                  |   |
| MSP-3  | PF10_0345        | Trafficked with MSP-9 to the surface, unknown function                                  |
| MSP-6  | PF10_0346        | Associated with MSP-1, unknown function   |
| MSP-7  | PF13_0197        | MSP-1 binding protein; strongly associated; unknown function                            |
| MSP-7 like   | PF13_0196        | Putative MSP1 binding protein, associated with detergent-resistant membrane protein     |
| MSP-7 like   | MAL13P1.173      | Putative MSP1 binding protein   |
| MSP-7 like   | MAL13P1.174      | Putative MSP1 binding protein   |
| MSP-7 like   | PF13_0193        | Putative MSP1 binding protein   |
| MSP-7 like fragment                                      | MAL13P1.175      | Putative MSP1 binding protein   |
| MSP-9  | PFL1385c         | ABRA, trafficked with MSP-3 to the surface, putative protease                           |
| S-Antigen  | PF10_0343        | Unknown function  |
| GLURP  | PF10_0344        | Unknown function  |
| Pf41   | PFD0240c         | Member of 6-cys family; apical end of merozoites  |



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|                               |            |  |
|-------------------------------|------------|--|
| H101                          | PF10_0347  | Chr10 locus “MSP3-like” protein; no known function   |
| H103                          | PF10_0351  | Chr10 locus “MSP3-like” protein; no known function   |
| SERA3                         | PFB0350c   | Cysteine protease domain with active-site serine, not essential  |
| SERA4                         | PFB0345c   | Cysteine protease domain with active-site serine, appears to be essential  |
| SERA5                         | PFB0340c   | Cysteine protease domain with active-site serine, chymotrypsin like proteolytic activity, appears to be essential, |
| SERA6                         | PFB0335c   | Cysteine protease domain with active-site cysteine, appears to be essential  |
| <b>Microneme proteins</b>     |            |  |
| EBA-140/BAEBL                 | MAL13P1.60 | Binds to Glycophorin C (Gerbich antigen) on the surface of RBC   |
| EBA- (140)                    | MAL7P1.176 | Binds to Glycophorin C (Gerbich antigen) on the surface of RBC   |
| EBA-165/PEBL                  | PFD1155w   | Pseudogene   |
| EBA-175                       | MAL7P1.176 | Binds to Glycophorin A on the surface of RBC   |
| EBA-181/JESEBL                | PFA0125c   | Binds trypsin-resistant receptor W on RBCs   |
| AMA-1                         | PF11_0344  | Mediates reorientation of the RBC  |
| PTRAMP                        | PFL0870w   | Unknown function   |
| MTRAP                         | PF10_0281  | Motor-associated protein, binds to aldolase, thrombospondin related  |
| ASP                           | PFD0295c   | Processed in tow fragments, contains a adhesive “sushi” domain, hence the name apical sushi protein, ASP           |
| SUB2                          | PF11_0381  | Subtilisin-like serine protease; MSP-1/AMA-1-processing “shedase”  |
| <b>Rhoptry bulb proteins:</b> |            |  |
| RAP1                          | PF14_0102  | Escorter of RAP2, associated with RAP2 and RAP3, truncation disrupts trafficking of the complex                    |
| RAP2                          | PFE0080c   | Associated with RAP1, unknown function   |
| RAP3                          | PFE0075c   | Associated with RAP1, unknown function   |
| RhopH1(3.1)                   | PFC0110w   | Associated with RhopH2/H3, unknown function; CLAG3.1   |
| RhopH1(3.2)                   | PFC0120w   | High-molecular-weight rhoptry complex; CLAG3.2   |
| RhopH2                        | PFI1445w   | Associated with RhopH1, unknown function   |
| RhopH3                        | PFI0265c   | Associated with RhopH1, unknown function   |
| Rhop148                       | PF13_0348  | Unknown function   |

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|                              |             |  |
|------------------------------|-------------|--|
| RhopH1                       | MAL7P1.229  | Cytoadherence linked asexual protein; unknown function   |
| RhopH1(2)                    | PFB0935w    | Unknown function; CLAG2  |
| RhopH1(9)                    | PFI1730w    | Association with RhopH complex, involved in cytoadherence; CLAG9   |
| RAMA                         | MAL7P1.208  | GPI anchored, processed form binds to the surface of the RBC, associated with RAP1 and RhopH+C11                     |
| ROPE                         | PFB0145c    | Spectrin-like molecule   |
| Rhoptry protein              | PF14_0637   | rhoptry protein, putative  |
| <b>Rhoptry Neck Proteins</b> |             |  |
| Rh1                          | PFD0110w    | Binds to the surface of RBC ("receptor Y"), associated with Rh2b   |
| Rh2a                         | PF13_0198   | Reticulocyte binding ligand 2 homolog a; Might be involved in signalling microneme release upon invasion             |
| Rh2b                         | MAL13P1.176 | Reticulocyte binding ligand 2 homolog b; Involved in invasion pathway through "receptor Z"                           |
| Rh3                          | PFL2520w    | Reticulocyte-binding protein; Probable transcribed pseudogene  |
| Rh4                          | PFD1150c    | Differential expression allows invasion-pathway switching  |
| Rh5                          | PFD1145c    | Reticulocyte-binding ligand, erythrocyte binding ( binding to erythrocyte)   |
| RON2                         | PF14_0495   | Rhoptry neck protein-2 form a complex with AMA-1 at moving junction in <i>T. gondii</i> and <i>P. falciparum</i>     |
| RON4                         | PF11_0168   | Rhoptry neck protein-4 form a complex with AMA-1 at moving junction in <i>T. gondii</i> and <i>P. falciparum</i>     |
| RON5                         | MAL8P1.73   | Rhoptry neck protein-5 form a complex with AMA-1 at moving junction in <i>T. gondii</i>                              |
| Pf34                         | PFD0955w    | GPI anchor, associated with parasite-derived detergent-resistant microdomains (DRMs)                                 |
| <b>Mononeme</b>              |             |  |
| ROM4                         | PFE0340c    | Rhomboid protease, shedding of EBA-175 and other members of the erythrocyte-binding EBL-family of merozoite proteins |



their function during the invasion process is still unknown. The major players during the invasion process are summarised in the following sections.

### **1.9. 1 Merozoite surface and primary interactions with the erythrocyte**

#### ***Merozoite surface protein***

Interactions between molecules located on the extra-cellular merozoite surface play a central role prior to invasion by initiating the first contact with the host cell. The merozoite surface is covered with numerous ligands forming branches of fibrils ranging from 12 to 15 nm in size (Mitchell and Bannister, 1988). Some of these filaments belong to the family of merozoite surface proteins (MSP). So far, ten MSPs have been identified (Black *et al.*, 2001; Black *et al.*, 2003; Drew *et al.*, 2005; Holder *et al.*, 1985b; Marshall *et al.*, 1997; Marshall *et al.*, 1998; McColl *et al.*, 1994; Mills *et al.*, 2002; Oeuvray *et al.*, 1994; Pachebat *et al.*, 2001; Stahl *et al.*, 1986; Trucco *et al.*, 2001; Weber *et al.*, 1988). The most studied member of this family is the 195 kDa MSP-1. MSP-1 is regarded as one of most important vaccine candidates for targeting erythrocyte invasion by the parasite (Stoute *et al.*, 2007; Valderrama-Aguirre *et al.*, 2005).

The erythrocyte interaction partner of MSP-1 is thought to be anion exchanger band 3 (Goel *et al.*, 2003). This interaction is independent of sialic acid. Synthesised band 3 peptide binds to native MSP-1<sub>42</sub> and recombinant MSP-1<sub>19</sub>. Band 3 peptides inhibit the invasion of erythrocytes by *P. falciparum* 3D7 clone (Goel *et al.*, 2003). The same group revealed that MSP-9 binds to the same region on band 3 as MSP-1 (Li *et al.*, 2004b). The two regions MSP9/D1a and MSP9/D2 interact with the 5abc fragment. Furthermore, the same segment that mediates the binding to band 3 is also implicated in the formation of a co-ligand complex of native MSP-1<sub>42</sub> and MSP-9 (Kariuki *et al.*, 2005). MSP-9 is also referred as ABRA (acidic basic repeat antigen)

and is associated with the merozoite surface during schizont rupture and most likely functions as a protease on the erythrocyte surface (Kushwaha *et al.*, 2002).

To date the function of MSP-1 is still unknown. Attempts to knock out MSP-1 failed, providing evidence to the importance of this unique ligand (O'Donnell *et al.*, 2000).

MSP-1 undergoes two processing steps. The first cleavage occurs during or just before schizont release (Holder *et al.*, 1999). Thereby the protein is cleaved into a complex comprising subunits of ~83, 30, 38 kDa and 42 kDa. These four fragments reside together as a complex on the surface of the merozoite. During red blood cell invasion secondary processing takes place where the 42 kDa fragment is cleaved by a calcium-dependent serine protease into two polypeptides: an N-terminal 33 kDa; and C-terminal 19 kDa fragment (Blackman and Holder, 1992). Only the C-terminal fragment is bound to the parasite membrane via its GPI anchor. The 19 kDa fragment remains on the parasite surface as it invades the new RBC. By analysing the amino acid sequence and later NMR, it was seen that MSP-1<sub>19</sub> is composed of two epidermal growth factor (EGF)-like domains (Blackman *et al.*, 1991; Morgan *et al.*, 1999). Out of a total of roughly 45 amino acids this domain contains six cysteine residues, which are paired in three disulfide bonds. By this means the protein structure gains stability.

The native core MSP-1 complex was isolated by chemical cross-linking indicating a molecular weight of ~500kDa and it is suggested that MSP-1 naturally occurs as a dimer (Sanders *et al.*, 2007). This finding is consistent with previous NMR study on *P. vivax* MSP-1<sub>42</sub> fragment (Babon *et al.*, 2007).

In the context of the immune response, inhibitory antibodies are able to bind the EGF region. Consequently the C-terminus is a target of the protective immune responses by inhibiting erythrocytes invasion (Holder *et al.*, 1999). Vaccination of mice with the 42 kDa region of *P. yoelii* MSP-1 (MSP-1<sub>42</sub>) or its 19 kDa C-terminal

processing product (MSP-1<sub>19</sub>) can raise protective antibody responses in mice (Ahlborg *et al.*, 2002). Furthermore, specific antibodies against both antigens inhibit *P. falciparum* growth *in vitro* (Bergmann-Leitner *et al.*, 2006; Blackman *et al.*, 1990; Chang *et al.*, 1992; Singh *et al.*, 2006a; Woehlbier *et al.*, 2006). MSP-1<sub>19</sub> antibodies from immune inhabitants of malaria endemic areas also appear to play a principal role in invasion inhibition (O'Donnell *et al.*, 2001).

In addition to the co-ligand complex with MSP-9, MSP-1 is in direct contact with proteolytic processed fragments of MSP-6 and MSP-7 on the merozoite surface (Kauth *et al.*, 2006; Trucco *et al.*, 2001). After its own proteolytic shedding, MSP-1 subunit 38 interacts with the C-terminal region of MSP-6<sub>36</sub> and subunits 30, 38 and 83 with MSP-7. MSP-1 and MSP-7 precursors already form a complex during biosynthesis. MSP-7 undergoes extensive proteolytic processing and after the second processing step a 33kDa product remains bound on MSP-1 and is further cleaved into 22kDa (and in some parasite lines an additional 19kDa) fragment. The same portion of MSP-6 C-terminus bound to MSP-1 is required to form a tetramer structure for MSP-6<sub>36</sub> (Kauth *et al.*, 2006).

MSP-7 and five MSP-7-like members belong to the MSP-7 multigene family in *P. falciparum*. MSP-7 gene can be disrupted without affecting MSP-1 in any way (Kadekoppala *et al.*, 2008). No significant change was observed in the MSP-7 null parasite regarding change of invasion pathway or differential expression of other invasion molecules. However, loss of MSP-7 reduced the invasion efficiency dramatically (Kadekoppala *et al.*, 2008). The same approach used in *P. berghei* showed an increased preference of MSP-7 KO parasites for reticulocytes (Tewari *et al.*, 2005).

MSP-1, MSP-8, and MSP-10 have a double EGF-like domain at the C-terminus and a GPI anchor in common and these structures are considered potential targets for protective immunity.



MSP-8 is primarily expressed during the ring stage and is probably involved in PV formation (Drew *et al.*, 2005).

MSP-3 was first detected by screening a cDNA library with a pool of human immune serum from individuals from Papua New Guinea (McColl *et al.*, 1994), and it was also observed that anti-MSP-3 antibodies inhibit *in vitro* growth (Oeuvray *et al.*, 1994). MSP-3 is highly polymorphic and contains three blocks of four heptad repeats, a hydrophilic region and a putative leucine zipper domain (McColl *et al.*, 1994). MSP-3 was the first *P. falciparum* merozoite surface gene disrupted by truncating 37 aa from the C-terminus thus removing the putative leucine zipper sequence (Mills *et al.*, 2002). The truncation interfered with the trafficking of MSP-3 to PV and no MSP-9 was found on the merozoite surface leading to a reduction of erythrocyte invasion.

The last MSP so far identified is MSP-10, which shares common features with MSP-1 such as a GPI anchor, two EGF-like domains and proteolytic processing. In addition, MSP-10 fusion protein are recognised by human immune sera (Black *et al.*, 2003).

MSP-2, 4 and 5 are not as well characterised and there is very little data available defining the roles of these molecules.

### ***AMA-1***

Another major player during the early interaction of the merozoite and erythrocyte is apical membrane antigen-1 (AMA-1) (Deans *et al.*, 1984; Marshall *et al.*, 1998; Narum and Thomas, 1994; Peterson *et al.*, 1989; Peterson *et al.*, 1990).

Both monoclonal and polyclonal antibodies to AMA-1 can block merozoite invasion (Coley *et al.*, 2006; Thomas *et al.*, 1984) but do not interfere with the initial attachment (Mitchell *et al.*, 2004). This fact implies that AMA-1 may play a



role during the reorientation event. More insight about the role of AMA-1 is given by the study in *T. gondii* where it has been found in a complex of proteins in the moving junction (Alexander *et al.*, 2005; Lebrun *et al.*, 2005; Maryse Lebrun, 2005). Also in *P. falciparum* AMA-1 has been shown to interact with RON4 in the moving junction (Alexander *et al.*, 2006). The moving junction forms a “walking ring” around the merozoite and specific merozoite surface complexes are removed while the parasite enters the PV (Dubremetz *et al.*, 1985).

It is also suggested that the association with RON4 implies a function at some point after initial attachment which is leading up to the tight junction formation (Mital *et al.*, 2005; Mitchell *et al.*, 2004). The main function of AMA-1 may be to bring the apex in close proximity to the erythrocyte surface, thus allowing parasite ligands originating from rhoptries and micronemes to form interactions with receptors.

Knock out attempts in *T. gondii* and *P. falciparum* have failed, underlying the importance of this gene during invasion (Mital *et al.*, 2005; Triglia *et al.*, 2000).

AMA-1 consists of three domains. It is a type I integral membrane protein with a C-terminal cysteine rich region (domain I), a central domain (domain II) and a duplicated cysteine rich region at the N-terminus (domain III) (Bai *et al.*, 2005; Chesne-Seck *et al.*, 2005; Hodder *et al.*, 1996; Nair *et al.*, 2002; Pizarro *et al.*, 2005). The structure of domain I and II consists of an apple domain (PAN fold), a feature of many binding ligands (Pizarro *et al.*, 2005). In common with other invasion related proteins, AMA-1 is proteolytically processed during merozoite release (Howell *et al.*, 2001; Narum and Thomas, 1994) from an 83kDa precursor protein to a 66kDa fragment. AMA-1 originates at the micronemes and repositions to the surface (predominantly at the apical pole) upon merozoite egress (Bannister *et al.*, 2003; Healer *et al.*, 2002; Hodder *et al.*, 2001). During invasion a large portion is shed in a quantitative manner by subtilisin 2 and leaves a 48kDa ectodomain on the merozoite surface (Harris *et al.*, 2005; Howell *et al.*, 2003).

MSP-1 and AMA-1 are produced from single copy genes and are essential for the parasites continued existence (Triglia *et al.*, 2000). The two genes have distinctive roles very early during invasion with no alternative system to substitute their function.

### 1.9. 2 Micronemal proteins

#### *Apical sushi protein*

Apical sushi protein (ASP) is a micronemal protein with a single sushi domain. A sushi domain is defined by 60 consensus residues with four cysteines forming intramolecular disulphide bonds. The domains are involved in protein-protein and protein-ligand interactions (Kirkitadze and Barlow, 2001). The peak of transcription of ASP is at the end of schizogony and the protein is proteolytically processed (O'Keeffe *et al.*, 2005). ASP contains a signal peptide and a hydrophobic region which shares similarities to the GPI-anchor (O'Keeffe *et al.*, 2005). Usually there are multiple sushi domains found in this family of proteins in other species and the direction of one sushi domain relative to the other sushi domains appears to be of importance for function (Brodbeck *et al.*, 1996; Klickstein *et al.*, 1988; Krych *et al.*, 1991).

The family of proteins known as erythrocyte binding ligands (EBLs) is also expressed in micronemes. They are explained in detail at a later stage as their expression is associated with distinct invasion pathways.

### 1.9. 3 Rhoptry proteins

#### *RhopH complex*

Within the rhoptry organelle, and more specifically within the rhoptry bulb, proteins from the high molecular weight complex (HMW or high-molecular mass rhoptry protein complex, RhopH) are expressed (Campbell *et al.*, 1984; Coppel *et al.*, 1987;

Doury *et al.*, 1994; Holder *et al.*, 1985b; Ling *et al.*, 2004). The RhopH complex is comprised of RhopH1 (155kDa), RhopH2 (140kDa) and RhopH3 (110kDa) (Hienne *et al.*, 1998; Lustigman *et al.*, 1988).

Upon invasion, these proteins are relocated and found to be present at the erythrocyte membrane and probably contribute to PV formation (Sam-Yellowe 1988 and 1991). Their explicit role during the invasion process is still elusive but erythrocyte binding of the complex occurs in a trypsin-chymotrypsin sensitive manner (Hiller *et al.*, 2003; Ndengele *et al.*, 1995; Sam-Yellowe *et al.*, 1988).

Five genes have been identified to encode for RhopH1. These are members of the rhoph/clag (cytoadherence linked asexual gene) gene family: *clag2*; *clag3.1*; *clag3.2*; *clag8*; and *clag9* (Kaneko *et al.*, 2001). But not all proteins encoded by members of the *clag* gene family are present in the RhopH complex at the same time. Only one of the *clag* members is needed to form one RhopH complex. Thus, five forms potentially exist to create a RhopH complex (Kaneko *et al.*, 2005). It has been shown that all merozoites in a single schizont express at least *clag9*, *clag2* and *clag3.1*, with each of them being able to form a complex with RhopH2 and RhopH3 (Kaneko *et al.*, 2005).

### ***RAP proteins***

Members of the low molecular mass rhoptry protein complex (LMW) are rhoptry associated proteins 1-3 (RAP1, 2 and 3). They form a RAP1-RAP2-RAP3 complex. The members of this complex are expressed in the rhoptry bulb and towards the end of the invasion process found in the PV of the newly invaded erythrocyte (Baldi *et al.*, 2000).

The function of RAP proteins is still unknown, but antibodies against RAP1 can inhibit invasion of erythrocytes *in vitro* (Harnyuttanakorn *et al.*, 1992; Howard *et al.*, 1998; Schofield *et al.*, 1986). Experiments with the truncated form of RAP1



have shown that truncated RAP1 can be transferred to the rhoptries but is not able to form a complex with RAP2 (Baldi *et al.*, 2000). This fact may imply that the inhibition effect may not be specific against RAP function but result from a steric hindrance of invasion (Cowman *et al.*, 2002). A recent publication demonstrates the erythrocyte binding function of RAP1 and RAP2 (Sterkers *et al.*, 2007).

### ***RAMA***

A low abundance rhoptry protein is the rhoptry associated membrane antigen (RAMA) (Topolska *et al.*, 2004). RAMA is initially synthesised as a 170kDa precursor and later proteolytically processed to yield a 60kDa product. During the invasion process this 60kDa form is anchored via GPI-tail in the rhoptry membrane and later on secreted to interact with the erythrocyte membrane (Pinzon *et al.*, 2008; Topolska *et al.*, 2004). The relatively early transcription of RAMA, 15-20 hours post-invasion, led to the assumption that RAMA is involved in rhoptry biogenesis (Topolska *et al.*, 2004). Active immunisation of mice with RAMA did not provide protection against *P. yoellii* parasites (Kats *et al.*, 2008). Direct interaction of with other rhoptry proteins is proposed for RhopH3 and RAP1 (Topolska *et al.*, 2004). These proteins exist in complex themselves (RhopH3 forms a complex with RhopH1 and RhopH2; and RAP1 with RAP2 and RAP3). Thus a huge macromolecular complex is formed around RAMA, as it is the only protein in this complex with a GPI anchor (Topolska *et al.*, 2004).

Another protein expressed in the rhoptry bulb is the repetitive organellar protein (ROPE). This protein shares some characteristics with spectrin and a role in spectrin mimicry is proposed during PV formation (Werner *et al.*, 1998).

### ***RON***

Rhoptry neck protein (RON) was first identified in *Toxoplasma*. As the name implies the protein is expressed at the Rhoptry neck. There are five orthologues



found in *Plasmodium* (RON1-5). RON2 and RON4 have been associated with AMA-1 and were localised in the moving junction between merozoite and erythrocyte (Alexander *et al.*, 2006; Baum *et al.*, 2008b). RON contains a homology region with RhopH1, but it does not appear to be involved in the RhopH complex formation (Cao *et al.*, 2008). In addition, the *Toxoplasma* orthologues of RON2, RON4 and RON5 have been shown to bind AMA-1 in *Toxoplasma*. RON5 has not been further characterised in *P. falciparum*.

The second gene family expressed in the rhoptry neck is the reticulocyte binding ligand (RBL) family. As for EBL, members of RBL characterise erythrocyte invasion pathways and are introduced in detail in a later section.

### ***1. 10 Role of calcium during invasion of erythrocytes***

A range of experiments appear to confirm the requirement of increased  $\text{Ca}^{2+}$  during invasion and point to  $\text{Ca}^{2+}$  induced pre-invasion enfolding action of the erythrocyte surface (Chiu and Lubin, 1989; Tiffert *et al.*, 2000).

By analysing video imaging, Lew and Tiffert (2007) proposes a hypothesis of dynamic events prior to invasion with elevated intracellular  $\text{Ca}^{2+}$  initiating the invasion process (Lew and Tiffert, 2007). There, a random initial contact between the apical end of a merozoite and the surface of the targeted RBC generates a weak attachment. Ligand interactions between GPI-anchored merozoite coat proteins (Sanders *et al.*, 2005; Sanders *et al.*, 2006) and integral membrane proteins of the RBC membrane (e.g. MSP-1- band 3 interaction) form or activate a  $\text{Ca}^{2+}$  permeability pathway in the area of contact.  $\text{Ca}^{2+}$  influx diffuses from the point of entry into the RBC elevating  $\text{Ca}^{2+}$  locally. Elevated  $\text{Ca}^{2+}$  generates a dynamic response of the cortical spectrin-actin mesh. This in turn initiates an enfolding motion centred at the point of attachment and embracing a variable area of the merozoite surface. This motion results in disruption of the weak initial attachment inactivating  $\text{Ca}^{2+}$  and relaxing the local deformation. The same sequence is repeated

until by chance, the apical duct is in close position to the erythrocyte membrane and forms a tight junction.

As mentioned earlier, while there is a lot of information about ligand-receptor interaction, only little is known about the cross-communication of molecules involved during invasion and their ultimate initiation. There exists some evidence that an optimum calcium level is crucial for successful parasite entry (McCallum-Deighton and Holder, 1992; Moreno and Docampo, 2003). Many putative signalling molecules have been discovered by subsequent genome analysis (Anamika, 2005; Aravind *et al.*, 2003; Gardner *et al.*, 2002b; Ward *et al.*, 2004). A central signalling molecule in mammalian cells is protein kinase B (PKB). There is a *P. falciparum* orthologue termed PfPKB but it seems that *Plasmodium* has developed a unique regulation mechanism which differs to that of mammalian cells (activation upon phosphorylation by PDK1) (Vanhaesebroeck and Alessi, 2000). PfPKB lacks the phosphoinositide-interacting pleckstrin homology domain present in mammalian PKB (Kumar *et al.*, 2004). *P. falciparum* PKP is activated by calcium/calmodulin (Vaid and Sharma, 2006).

A second kinase, the calcium dependent protein kinase 1 (PfCDPK1) has been proven to be essential during erythrocytic asexual cycle (Kato *et al.*, 2008). It has been recently shown that two sites of GAP45 are phosphorylated by this kinase (Green *et al.*, 2008). CDPK1 can phosphorylate recombinant MTIP suggesting an important role in parasite motility (Kato *et al.*, 2008). Protein phosphorylation appears to be important activation process of proteins involved in motility and invasion in general. Indeed many groups are focusing on further identification of phosphorylated proteins.

### ***1. 11 Role of proteases during invasion***

Protease activity is mandatory for successful invasion to disconnect interactions formed between parasite ligands and host cell receptors. Two classes of proteases

have been associated with invasion: the Subtilisin-like superfamily; and a family of intra-membrane serine proteases, the Rhomboids.

### 1.11. 1 Subtilisin-like superfamily

The cleavage of at least two essential merozoite surface proteins, MSP-1 and AMA-1, is thought to occur continuously during invasion as the parasite progresses into the emerging PV. This shedding is performed by a single proteolytic cleavage at a juxtamembrane and is mediated by the sheddase SUB2 (Harris *et al.*, 2005). The enzyme is localised in the micronemes just before invasion is initiated. During the movement of the junction from the anterior to the posterior end, SUB2 relocates with the moving junction in an actin-dependent manner (Harris *et al.*, 2005). During the invasion process the AMA-1 and MSP-1-complex are quantitatively cleaved from the parasite surface. The MSP-1 cleavage site is adjacent to the doublet EGF-domain resulting in a membrane bound MSP-1<sub>19</sub> fragment that will ultimately be carried into the newly invaded host cell (Blackman *et al.*, 1990). For AMA-1, shedding occurs exactly 29 residues from of the transmembrane domain with only a very small segment (TMD and cytoplasmic domain) of this protein carried into the erythrocyte (Howell *et al.*, 2001; Howell *et al.*, 2003; Howell *et al.*, 2005).

The second member of this family, SUB1, has its peak of expression during late schizont stage and plays a key role during schizont egress by processing and ultimately activating serine repeat antigen (SERA 4, 5 and 6 and potentially other SERA members) function for digestion of the PV (Yeoh *et al.*, 2007). In the study by Yeoh *et al.*, (2007) the subtilisin specific inhibitor MRT12113 was more forceful in preventing merozoite invasion than in retarding egress. The authors propose that SUB1 may perform further functions other than SERA maturation. The analogy between SUB1 recognition motif and the processing site of the MSPs suggest it potentially mediates processing of various merozoite surface proteins prior to merozoite release (Yeoh *et al.*, 2007). The function of SUB3 remains unknown.



### 1.11. 2 Rhomboid proteases

*Toxoplasma* was the first apicomplexan in which it was discovered that trans-membrane adhesins are processed by a rhomboid protease (Urban and Freeman, 2003). The family of proteases specifically recognise the motif IA/GG. This specificity is conserved for all microneme proteins of other apicomplexans including *Plasmodium* species (Dowse *et al.*, 2005; Dowse and Soldati, 2005). There are eight different members of ROM identified in *Plasmodium* (Wu *et al.*, 2003). Four members are shared with *T. gondii*: ROM1, ROM3, ROM4 and ROM6. The distinct members are expressed throughout the life cycle. Only the location of ROM1 has been determined. It is localised in distinct organelles in close proximity to subpellicular network, termed mononemes (Singh *et al.*, 2007). Two members, ROM1 and ROM4, have been shown to process invasion molecules. These two Rhomboid proteases cleave ectodomains specifically of 14 adhesion molecules involved during invasion by intra-membrane proteolysis (Figure 1.7) (Baker *et al.*, 2006; O'Donnell *et al.*, 2006). ROM1 was identified to cleave AMA-1. Together ROM1 and ROM4 can cleave EBL family members as well as TRAP and RBLs. The importance of this group of proteases is emphasised by the fact that a knockout of ROM4 was not successful, implicating that shedding of adhesins is critical for *P. falciparum* survival (O'Donnell *et al.*, 2006).





Figure 1. 7. Rhomboid protease recognition site.

Adapted and modified from Baker *et al.*, (2006). Sequence alignment of 14 *Plasmodium* adhesin transmembrane domains analysed by Baker 2006 (in capital letters) compared to those of *Drosophila* Spitz and *Toxoplasma* MIC2 (Micronemal invasion ligand in *Toxoplasma*). The intra-membrane rhomboid protease was first discovered in EGF r receptor ligand Spitz (Dm Spitz). The two vertical lines indicate the putative membrane boundaries. The amino acid residues contributing to rhomboid cleavage are shown in green, while those that interfere with cleavage are in red.

## ***1. 12 Molecular motor and moving machinery***

Apicomplexan parasites feature a unique way of entering the host cell using an actin-myosin based gliding motility (Baum *et al.*, 2006a; Baum *et al.*, 2006b; Fowler *et al.*, 2004; Morrissette and Sibley, 2002; Opitz and Soldati, 2002; Soldati *et al.*, 2004). Extensive work on the molecular motor has been done in the related organism *Toxoplasma* and the concept is adapted to *Plasmodium*. The existence of this motor is underlined in the observation of translocation of molecules from the anterior to the posterior end during host cell entry (King, 1988). The movement is an energy dependent process. It is still unclear from where the ATP is derived. There are lines of evidence supporting the important role of host cell ATP as erythrocytes lacking ATP are resistant to malaria infections (Dluzewski *et al.*, 1983).

Actin I has been identified in merozoites by mass spectrometry (Schmitz *et al.*, 2005) and approximately one third is globular actin (monomeric) and the rest exists in the filamentous (F-actin) form (Field *et al.*, 1993; Webb *et al.*, 1996). Experiments using cytochalasin B, an actin polymerisation inhibitor, accentuate the need for filamentous actin during gliding motility (Miller 1979). Hereby, merozoites treated with cytochalasin B are able to recognise and attach to erythrocytes but do not proceed beyond junction formation (Field *et al.*, 1993; Miller *et al.*, 1979). It has been very difficult to visualise polymerised actin in apicomplexa (Bannister and Mitchell, 1995). Schmitz *et al.*, (2005) produced striking evidence supporting the presence of actin filaments. They are unconventionally short with only 100nm of length visualised by electron micrographs (Schmitz *et al.*, 2005). The unique polymerisation properties of *Plasmodium* actin I were studied by using the recombinant protein (Schuler *et al.*, 2005). Actin I only polymerised in the presence of gelsolin and phalloidin (Schuler *et al.*, 2005). *In vivo* actin polymers are most probably stabilised by other supporter proteins such as capping proteins or polymerisation factor formin 1 which is localised at the barbed end during filament elongation (Baum *et al.*, 2008b). The globular actin polymerises into F-actin in a head to tail organisation creating a molecular polarity from plus to minus end

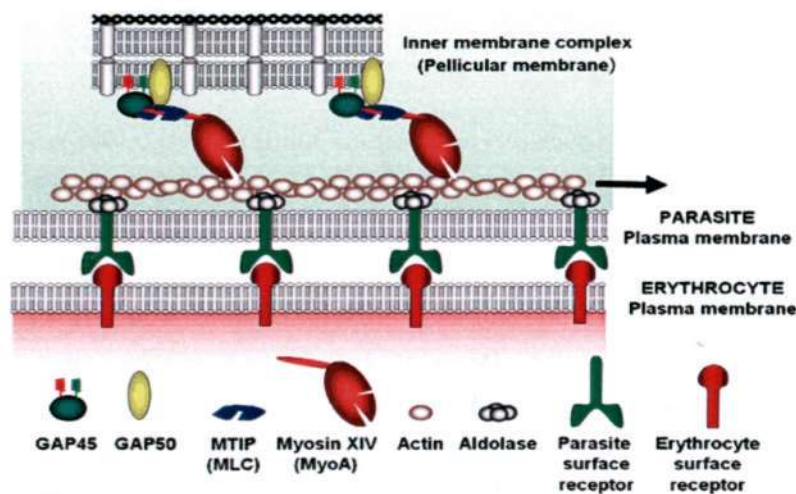
(Pollard and Borisy, 2003). The formed F-actin is arranged in a right-handed helical spiral (Vale, 2003). The way in which actin is bundled up sets the parameter for motility direction along the micro-filaments from the plus end to the minus end.

The counterpart of actin to provide the linear driving force is myosin A, a class XIV myosin only found in apicomplexa (Pinder *et al.*, 1998). *Plasmodium* contains six myosins (Ben Mamoun *et al.*, 2001; Bozdech *et al.*, 2003; Chaparro-Olaya *et al.*, 2005; Le Roch *et al.*, 2003b; Vale, 2003), with only two myosins expressed during the merozoite stage, MyoA and MyoD, (Chaparro-Olaya *et al.*, 2005).

The MyoA function has been elucidated and it is highly expressed in the invasive merozoite in the supra-alveolar space (Pinder *et al.*, 2000; Pinder *et al.*, 1998). MyoA protein is structured in three domains: an ATP hydrolysing and actin binding head, which eventually produces the locomotion along the actin filaments; a neck, where the characteristic IQ motif usually promoting the binding of myosin light chain is degenerated (Herm-Gotz *et al.*, 2002; Mooseker and Cheney, 1995); and a very short 15 aa tail that is linked to motor molecules anchored in the IMC via MyoA domain interacting protein (MTIP) (Bergman *et al.*, 2003; Green *et al.*, 2006). The MyoA head binds to polymerised actin by hydrolysing ATP and was demonstrated by the addition of specific myosin ATPase inhibitor butane-2,3-monoxime (BDM) resulting in invasion inhibition (Pinder *et al.*, 1998). Further evidence for the importance of myosin as a motor molecule was given by conditional knock out of MyoA in *Toxoplasma* where the modified parasite lost its gliding motility (Meissner *et al.*, 2002). An *in vitro* motility assay measured a translocation velocity of  $3.51\mu\text{m s}^{-1}$  for MyoA (Green *et al.*, 2006). This is equivalent to the speed observed during microscopic video imaging (Dvorak *et al.*, 1975).

During the invasion event, it is believed the motor assembles and acts in the gap between the plasma membrane and the underlying inner membrane complex (IMC) and is linked to the surface of the erythrocytes via the actin-binding protein aldolase





**Figure 1. 8. Model of the invasion motor complex.**

Figure adapted from National Institute for Medical Research (NIMR) website ([http://www.nimr.mrc.ac.uk/physbiochem/veigel/motility/motor\\_complex/](http://www.nimr.mrc.ac.uk/physbiochem/veigel/motility/motor_complex/)). After initial attachment to the erythrocyte surface, the parasite penetrates erythrocytes by the powering force of an actin-myosin-motor. Micronemal proteins like TRAP (thrombospondin-related anonymous protein) are anchored in the parasite plasma membrane and build the connection to erythrocyte via its erythrocyte binding partner. The major components lie in the space between the inner membrane complex and the merozoite plasma membrane. The cytoplasmic tail of TRAP is connected to the actin-myosin-motor via aldolase. Globular actin is bundled up in to filaments which can be bound by aldolase. The other major component of this motile complex is myosin A which is connected to the IMC (inner membrane complex) via MTIP (myosin tail-domain interacting protein) and MTIP is connected to GAP45 and GAP50. GAP 50 has transmembrane domain and stabilises the complex in the IMC. The direction of the movement is given by the direction of the actin polymerisation. The motile force applied by myosin A (MyoA) on actin results in moving the parasite relative to its extra-cellular anchor point. An array of actin-myosin-motors may bind to the filament to generate the movement. Finally, to release the adhesins (TRAP) from the host cell and permit forward movement, a protease from the micronemes is necessary to cleave the adhesins within the transmembrane regions in the parasite plasma membrane.

and cell surface ligands (Figure 1.8) (Jewett and Sibley, 2003; Pinder *et al.*, 2000; Pinder *et al.*, 1998). The central role during this event is held by polymerised actin and its complementing partner myosin (Opitz and Soldati, 2002). These two components necessitate an anchoring point with the IMC, mediated by the interaction of the MyoA-tail “stump” and MTIP (Bergman *et al.*, 2003; Bosch *et al.*, 2007b; Green *et al.*, 2006; Jones *et al.*, 2006). In turn, MTIP is associated with Glideosome associated protein 45 (GAP45) forming a complex with Glideosome associated protein 50 (GAP50) (Jones *et al.*, 2006). GAP50 has been characterised in *Toxoplasma* as an integral membrane protein attached to the IMC and its cytoplasmic domain is essential for the anchoring of the myosin complex to sub-cellular domain (Gaskins *et al.*, 2004).

How exactly these diverse parts of the glideosome are linked to one another and what triggers the assembly, as well as what makes the complex stable during the movement, have not been elucidated. Also the role of the GAP45 protein remains to be determined. With myosin steadily coupled to the IMC, it is up to actin to interact indirectly with the cell surface adhesion molecules that mediate the transient physical interactions between the parasite and the erythrocyte.

The bridging molecule between actin and membrane bound cell surface adhesins is aldolase, a multifunctional protein able to bind F-actin (Jewett and Sibley, 2003; Wang *et al.*, 1996). The cytoplasmic tail of thrombospondin related anonymous protein (TRAP), an adhesin originating in the micronemes, and its homologues have been identified to interact with aldolase (Bosch *et al.*, 2007a; Buscaglia *et al.*, 2003; Buscaglia *et al.*, 2006; Buscaglia *et al.*, 2007; Jewett and Sibley, 2003).

TRAP homologues are found in different stages of the *Plasmodium* life cycle (Dessens *et al.*, 1999; Trottein *et al.*, 1995; Yuda *et al.*, 1999) and again, are conserved throughout the phylum of apicomplexa parasites (Spano *et al.*, 1998; Tomley *et al.*, 1991; Wan *et al.*, 1997). TRAP homologues share a conserved motif, the thrombospondin repeat (TSR) (Muller *et al.*, 1993). The specific binding of



TRAP to aldolase is mediated by only the six C-terminal residues of the TRAP cytoplasmic tail (Bosch *et al.*, 2007a; Buscaglia *et al.*, 2007). Furthermore one single tryptophan within the binding motif is crucial for the interaction with the catalytic pocket of aldolase (Buscaglia *et al.*, 2007). TRAP family members are type I integral membrane proteins and relocate during the invasion process from micronemes to zoite surface (Gantt *et al.*, 2000; Rogers *et al.*, 1992b). There are two homologues found in merozoites: Merozoite TRAP (MTRAP) (Baum *et al.*, 2006b) and *Plasmodium* thrombospondin-related apical merozoite protein (PTRAMP) (Thompson *et al.*, 2004). Both are expressed at high levels schizont stage parasites, released onto the merozoite surface prior to invasion, and processed during invasion: MTRAP by intra-membrane cleavage by a rhomboid protease and PTRAMP is proteolytically processed by SUB2 (Baum *et al.*, 2006b; Green *et al.*, 2006; Thompson *et al.*, 2004). In addition MTRAP is putatively essential as demonstrated by repeatedly unsuccessful efforts to knock out the endogenous gene (Baum *et al.*, 2006b). It has only been demonstrated for MTRAP that the cytoplasmic tail binds *in vitro* to both recombinant and native aldolase (Baum *et al.*, 2006b). It is controversial which member is the “true” TRAP in merozoites.

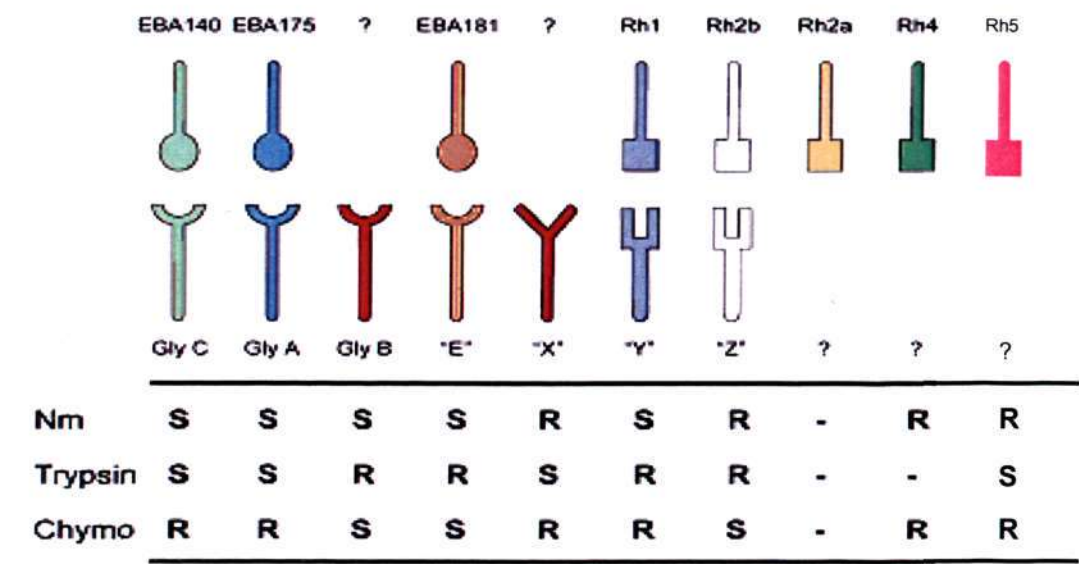
Researchers have so far failed to prove the connection of the EBL-family or RBL-family with the motor machinery. No other adhesion molecule other than TRAP was identified to bind aldolase by pull-down assays (Buscaglia *et al.*, 2003). How EBL or RBL are connected to the glideosome remains unresolved even though there is a degenerated tyrosine-based motif and a high content of acidic amino acids in the cytoplasmic tail of TRAP and EBA-175 (Di Cristina *et al.*, 2000). This fact gave cause to create a chimeric protein of EBA-175 with the 54aa cytoplasmic tail substituted by the last 37aa of TRAP (Gilberger *et al.*, 2003a). The altered EBA175/TRAP retained its function and it is suggested that a similar mechanism of binding to a connecting molecule like aldolase exists. Besides, the receptor recognised by merozoite-expressed TRAP has not been elucidated, but there is mounting evidence published about EBL and RBL members binding to erythrocyte receptors (Iyer *et al.*, 2007a). Like TRAP and MTRAP, EBLs and RBLs are cleaved



during invasion just above their TM-domain by rhomboid protease. The purpose of shedding is to disengage the interaction of the merozoite with the erythrocyte and to allow the driving force of the glideosome to move the merozoite into the erythrocyte.

### ***1. 13 Secondary interactions with the erythrocyte - Selection of host cell***

The first interaction between merozoite and erythrocyte surface is followed by a more specific contact as the tight junction is formed. Proteins from rhoptries, micronemes and dense granules, located at the apical end of the merozoite, initiate this irreversible interaction with the host cell (Preisler *et al.*, 2000). These protein superfamilies have the ability to recognise and bind to certain receptors located on the erythrocyte membrane. A summary of the binding preference of EBL and RBL ligands is shown in Figure 1.9.



**Figure 1. 9. Receptor-ligand interactions involving known invasion molecules and their sensitivity to Neuraminidase (Nm), Trypsin and Chymotrypsin (Chymo).**

Figure adapted from Baum *et al.*, (2005) and modified. S indicates sensitivity of invasion to this enzyme (inhibits invasion/ligand-binding). R indicates resistance (does not inhibit invasion/ligand-binding). The “?” indicates unknown ligands or receptors. Unknown receptors E, X, Y, and Z known only from their enzyme sensitivities. Dashes in table indicate that no data is available.

### 1.13.1 EBL-family

The Duffy receptor was the first receptor identified to be utilised by *Plasmodium* spp to penetrate erythrocytes (Miller and Carter, 1976). The parasite protein was thus named after the receptor it binds to: Duffy binding protein (DBP) and can only bind to Duffy blood group antigen (Wertheimer and Barnwell, 1989). A good model to study DBP is the simian parasite *P. knowlesi*. Human erythrocytes deficient in Duffy receptor were resistant to invasion by *P. knowlesi* merozoites (Mason *et al.*, 1977; Miller *et al.*, 1975b; Wertheimer and Barnwell, 1989), underlining the significance of this receptor-ligand interaction during invasion. *P. vivax* expresses one DBP, *P. knowlesi* expresses three and this family further expanded to six homologues in *P. falciparum*. They all share the same amino acid motif for receptor recognition and are classified into the same superfamily (Adams *et al.*, 1992) (Figure 1.10.). This family is named the erythrocyte binding ligand (EBL) family in *P. falciparum* and encodes the following members: EBL-1, EBA-175, PEBL (EBA-165), BAEBL (EBA-140), JESEBL (EBA-181), and MAEBL. All *eb1* genes are located at the subtelomeric regions on different chromosomes (Gardner *et al.*, 2002b).

The overall exon-intron structure is conserved among the different members. The individual members are thought to bind different receptors on the RBC thus providing functional homologues of each other. All homologues of EBL, except MAEBL are located in the micronemes (Adams *et al.*, 1990; Blair *et al.*, 2002; Gilberger *et al.*, 2003b; Narum *et al.*, 2002; Sim *et al.*, 1992). MAEBL is unique and may have a different role compared to the other members of this family; it is expressed within the rhoptries and occurs on the merozoite surface at the late schizont stage as well as sporozoite stage (Blair *et al.*, 2002; Ghai *et al.*, 2002; Kappe *et al.*, 2001; Noe and Adams, 1998). Furthermore, all EBL proteins, except MAEBL, are characterised by two cysteine rich regions, a signal peptide, a transmembrane domain and a short cytoplasmic tail at the C-terminus. The cysteine rich region at the N-terminus contains the DBL domain (Duffy binding like domain) with the specific attraction to an intrinsic receptor (Adams *et al.*, 1992; Adams *et*

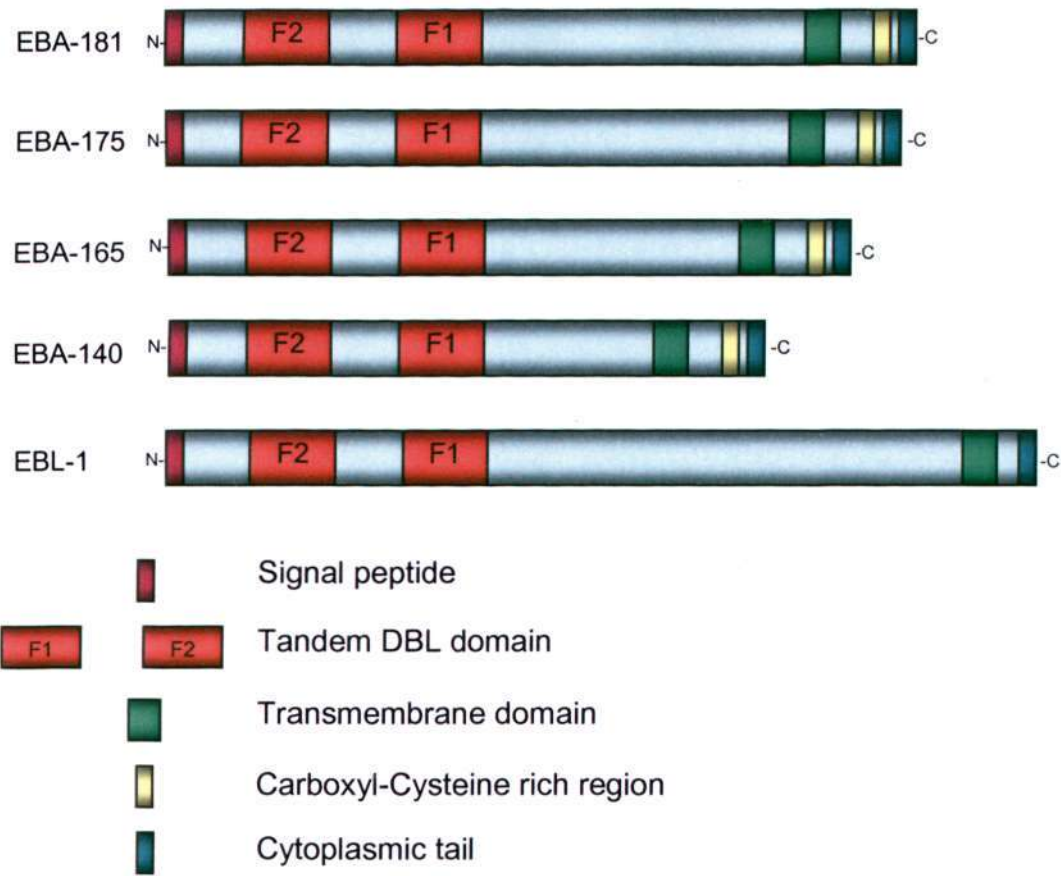


*al.*, 2001; Di Cristina *et al.*, 2000). In *P. falciparum* the DBL domain is duplicated and is subdivided in F1 and F2 and is characterised by 12 conserved cysteines.

Interestingly the same DBL domain is present in another superfamily involved in cytoadherence: the variant erythrocyte membrane protein 1 (EMP-1) family. PfEMP-1 encompasses around 59 homologues in *P. falciparum*, and proteins are localised on the surface of infected erythrocytes with their DBL motif evolutionary diversified into four domains (Michon *et al.*, 2002).

The second cysteine rich region (carboxyl-cysteine rich region) of EBLs shares high amino acid homology among the members and neighbours the transmembrane domain at the C-terminus. The region is characterised by eight cysteines however EBL-1 has only four (Adams *et al.*, 2001). The exact function of this domain remains to be determined but it seems to play an important role in directing the EBLs to the micronemes (Gilberger *et al.*, 2003a; Treeck *et al.*, 2006). Interestingly, the high-resolution X-ray crystal structure of EBA-175 region VI shares structural similarities with the intracellular KIX-binding domain of CREB-binding protein, a transcriptional co-activator protein (Withers-Martinez *et al.*, 2008).

The most studied member of the EBL family of invasion ligands is EBA-175. Camus and Hadley were the first to discover this 175kDa protein in parasite culture supernatants (Camus and Hadley, 1985). They further characterised this protein by defining the specificity to bind to sialic acid residues. Later on, glycophorin A was identified to be the particular host cell receptor of which EBA-175 recognises the sialic acid residues and the peptide backbone (Chitnis and Miller, 1994; Dolan *et al.*, 1994; Klotz *et al.*, 1992; Orlandi *et al.*, 1992; Sim *et al.*, 1994). X-ray crystallographic studies gave insights into the EBA-175 region II and glycophorin A interaction and it is proposed that EBA-175 functions as a dimer that assembles around the dimeric glycophorin A ectodomain during erythrocyte binding (Singh *et al.*, 2006b; Tolia *et al.*, 2005).



**Figure 1. 10. Schematic representation of five EBL family members.**

Generally, EBL family proteins are comprised of Signal peptide, a duplicated DBL-domain (Duffy binding like domain), a Transmembrane domain, a carboxyl cysteine rich region and cytoplasmic domain.

There is evidence that only the F2 domain executes binding activity (Ockenhouse *et al.*, 2001; Sim *et al.*, 1994). Antibodies raised against the recombinant DBL domain and anti-peptide antibodies specific for a region close to the cysteine rich region of EBA-175 inhibit merozoite invasion (Narum *et al.*, 2000; Sim *et al.*, 1990). Antibodies against recombinant EBL proteins are acquired in an age-associated manner. Some individuals predominantly produce inhibitory antibodies against EBA-175, which does not necessarily protect from malaria infection (Persson *et al.*, 2008). It is suggested that variation in invasion phenotype might have evolved as a mechanism for immune evasion by *P. falciparum*. A wide inhibitory response against multiple parasite ligands may be mandatory to gain a sustained immunity. Polymorphisms in both tandem DBL domains of EBA-175 have no impact on receptor selection.

Another well-studied member of the EBL family is EBA-140 (BAEBL) (Narum *et al.*, 2002). EBA-140 from different *Plasmodium* clones exhibits five nucleotide polymorphisms in its F2 domain with the result being four different receptors as interacting partners (Mayer *et al.*, 2002) including glycophorin C and D (Lobo *et al.*, 2003; Mayer *et al.*, 2001; Narum *et al.*, 2002; Thompson *et al.*, 2001).

EBA-181 (Jesebl) binding characteristics have been described as sialic-acid-dependent, trypsin resistant and chymotrypsin sensitive (Gilberger *et al.*, 2003b). In addition it has been reported that EBA-181 is binding to S-s-U cells (glycophorin B null erythrocytes), excluding that the preferred receptor is glycophorin B. The unique erythrocyte binding nature of EBA-181 suggests that it binds to an uncharacterised receptor named glycophorin E. Furthermore it has been shown by Blot overlay and histidine pull-down experiments that recombinant EBA-181 protein binding to an erythrocyte protein 4.1R which in fact resides within the erythrocyte cytoskeleton and is not exofacial (Lanzillotti and Coetzer, 2006; Lauterbach *et al.*, 2003). This finding may point out the complexity and multifunctional role of EBA-181 during invasion. Similar to Baebl, EBA-181 is



highly polymorphic with eight polymorphisms detected in the erythrocyte binding domains (Mayer *et al.*, 2004).

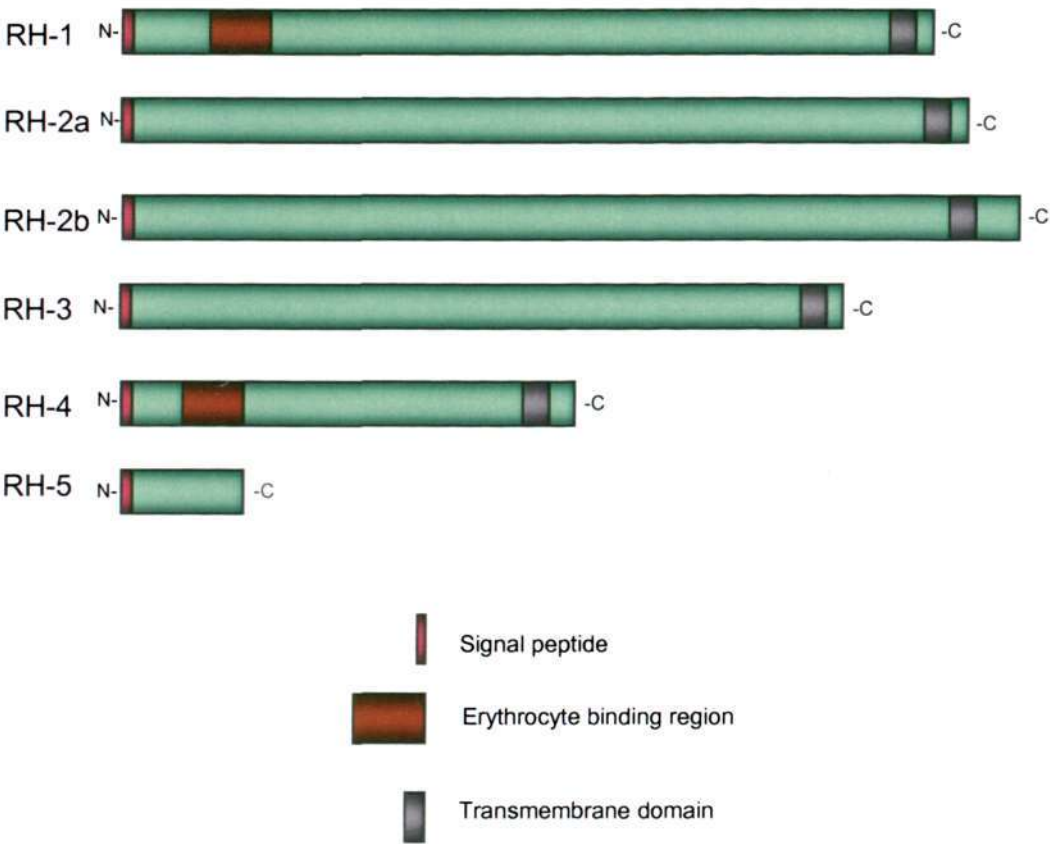
As for the other two EBL proteins, no specific erythrocyte receptor has been associated with EBL-1 and EBA-165 (Pebl). Both of the genes are referred as pseudogenes as there are no functional proteins detected but transcripts are made. For EBL-1 there is one and EBA-165 there are two 5' frame shift mutations (Drummond and Peterson, 2005; Rayner *et al.*, 2004; Triglia *et al.*, 2001b). Nevertheless, EBA-165 transcript has been detected in high abundance in parasites using the sialic acid independent invasion pathway (Gaur *et al.*, 2006; Stubbs *et al.*, 2005).

### 1.13. 2 RBL family

A second gene family known to be involved in erythrocyte recognition and receptor binding is the reticulocyte binding ligand (RBL) family (Galinski *et al.*, 2000). An RBL protein was first characterised in *P. yoelii* in studies where monoclonal antibodies protected mice from virulent infection (Freeman *et al.*, 1980) and was named after this species (Py) and its molecular weight (235kDa): Py235 (Borre *et al.*, 1995; Keen *et al.*, 1990).

The copy number of RBL varies significantly between different *Plasmodium* species, in *P. falciparum* there are six members: RH-1 (Rayner *et al.*, 2001), RH-2a, RH-2b (Rayner *et al.*, 2000; Triglia *et al.*, 2001a), RH-3 (Taylor *et al.*, 2001), RH-4 (Kaneko *et al.*, 2002) and RH-5 (Hayton *et al.*, 2008) while in *P. yoelii* there are >15 (Carlton *et al.*, 2002; Iyer *et al.*, 2007b).

All members of this family have a conserved two exon and one intron structure and belong to type I membrane proteins (Green and Holder, 2000; Sinha *et al.*, 1996) (Figure 1.11), except for RH-5 which lacks the transmembrane domain



**Figure 1. 11. Schematic representation of RBL family members.**  
Comparison of the 6 RBL members revealed presents of signal peptide, and a transmembrane domain is shared by 5 members with the exception of RH5. The specific erythrocyte binding region is known for only RH-1 and RH-4.

(Desimone *et al.*, 2008). There is a putative signal peptide located at the 5' end; a 500 aa homology region towards the carboxyl end has the highest level of conservation between the different homologues (Keen *et al.*, 1994; Ogun and Holder, 1994; Rayner *et al.*, 2001). The cytoplasmic tail domain next to the TM at the C-terminus is conserved with 6 aa in all members except RH-5 (Desimone *et al.*, 2008). The functional relevance of this cytoplasmic tail has not been determined and seems to be different from the cytoplasmic domain of EBA-175 which is important for functional ligand–receptor interaction (Desimone *et al.*, 2008; Gilberger *et al.*, 2003a; Treeck *et al.*, 2006). RBLs are large proteins with a molecular mass >200kDa (except RH-5). Different RBL proteins are thought to enable the parasite to invade erythrocytes using structurally diverse receptors.

Most RBLs are localised in the club-shaped rhoptries (Rayner *et al.*, 2000; Rayner *et al.*, 2001) except for one homologue, RH-4 which seems to be expressed in another compartment at the apical end (Kaneko *et al.*, 2002). Interestingly, the expression profile of RBLs in different *P. falciparum* lines varies, and this may in part be responsible for the differences in invasion phenotype seen in the different parasites. Furthermore not all RBL members are present in the genome in the different *P. falciparum* clones and it is assumed that not all members of the RBL family are required for erythrocyte invasion (Duraisingh *et al.*, 2003b).

The possible function of the RBL is suggested by the observation in *P. knowlesi* of long filamentous engagements of the merozoite apical end with the erythrocyte in the absence of junction formation (Aikawa and Miller, 1983; Miller *et al.*, 1979). Based on this fact it is suggested that RBLs bring the apex into close proximity with the erythrocyte which may lead to signal micronemes releasing their contents and ultimately enabling the DBL to form a junction (Galinski and Barnwell, 1996; Iyer *et al.*, 2007a).

The best characterised member of the RBL family in *P. falciparum* is RH-1. It binds in a sialic acid-dependent manner to a trypsin resistant receptor referred to as



receptor Y (Rayner *et al.*, 2001; Taylor *et al.*, 2002; Triglia *et al.*, 2005). Antibodies raised against this predominant protein inhibit invasion (Rayner *et al.*, 2001). The binding region of RH-1 is a small 334 aa region featuring an N-terminal binding domain and a C-terminal coiled coil region. Antibodies raised against this region are protective against invasion *in vitro* (Gao *et al.*, 2008).

No particular receptor could be assigned to RH-2a, although antibodies against RH-2a and RH-2b can block invasion in various strains (Triglia *et al.*, 2001a).

Furthermore, RH-2b has been shown to interact with chymotrypsin sensitive but neuraminidase and trypsin resistant erythrocytes by gene knock out experiments (Duraisingh *et al.*, 2003b). A receptor with these characteristics has been allocated as receptor Z (Duraisingh *et al.*, 2003b). RH2a shares approximately 80% sequence identity with RH-2b with large differences found in the C-terminal region. At present, there is no evidence that RH-2a acts as a functional protein (Duraisingh *et al.*, 2003b).

Through sequence similarities RH-4 was discovered as a member of the RBL family, despite its much shorter sequence: RH-4 is only 2/3 the size of other RBL members. In 2007 Gaur reported a small 261 aa sequence as a binding motif for erythrocytes. There is some overlapping sequence between RH-1 and RH-4 binding sites, but a generalised binding motif for RBLs needs to be determined. RH-4 has been elucidated in microarray and Real Time PCR studies as the primary transcript up-regulated in the sialic-acid-independent invasion pathway and western blot analysis confirmed the higher levels of the protein product (Gaur *et al.*, 2006; Stubbs *et al.*, 2005). The molecular basis for this finding is still unknown.

RH-3 is considered to be a pseudogene with only a transcribed product detected in intra-erythrocytic stages due to two 5' frame shift mutations in the gene (Taylor *et al.*, 2001).

The last identified member of the RBL family is RH-5 (Cowman and Crabb, 2006). It is much smaller than its homologues, only 65kDa in size, and also lacks the transmembrane domain emphasising the idea that RH-5 co-functions with another merozoite protein (Rodriguez *et al.*, 2008). Nevertheless, RH-5 binds a sialic-acid-independent receptor on the erythrocyte surface. Antibodies against RH-5 do not interfere with invasion efficiency of the parasite (Rodriguez *et al.*, 2008).

Localisation studies revealed in addition to rhoptry location that RH-5 is found at the tight/moving junction of the invading parasite and erythrocyte (Baum *et al.*, 2008a). This observation supports the hypothesis of RBL co-function with members of the EBL family.

A role of RH-5 in host receptor selection and binding of sialic acid residues was demonstrated by studying *Aotus* monkeys infection of a genetic cross of *P. falciparum* clones 7G8 and GB4 (Hayton *et al.*, 2008). The two clones differ in their virulence to the primate *Aotus nancymae* (7G8 non-infective, GB4 highly virulent). DNA transfection and linkage analysis revealed polymorphism in RH5 to be responsible for the virulence.

Further evidence provided experiments from human erythrocytes treated with neuraminidase for removal of sialic acid residues can bind RH-5 fragment when it contains I204 but not when it contains K204 and interestingly untreated human erythrocytes were able to bind both forms of the fragment. It is proposed by the same group that the ionic bond between the positively charged lysine and a negatively charged sialic acid potentially support an important ligand-receptor interaction. Disruption of this gene has not been successful (Cowman and Crabb, 2006; Hayton *et al.*, 2008).

Only speculation exists about the molecular and biochemical interaction of RBL proteins with erythrocyte counterparts. The general sequence preservation between RBL homologues is very low. A recent domain mapping study identified a putative

nucleotide binding region within all members of the RBL family. This domain is called the Nucleotide Binding Domain 94 (NBD94). This novel domain has been expressed as a recombinant protein and demonstrated to bind specifically to ATP (Ramalingam *et al.*, 2008). NBD94 has an increased binding to ATP and a lower affinity to bind ADP. From these results the authors suggest that RBL undergo structural changes during ATP/ADP binding which may be essential for invasion.

### ***1. 14 The role of antigenic variation in immune evasion by Plasmodium spp***

The subtle system of antigenic variation allows the parasite to alter the presentation of an antigen in order to avoid detection by the immune system. The most studied models of gene regulation and antigenic variation are the *variant (var)* genes which are expressed as erythrocyte membrane antigen-1 (PfEMP-1) on the infected erythrocyte surface. PfEMP-1 facilitates the infected erythrocyte to cytoadhere and sequester into deep tissues and organs, thus avoiding clearance by immune effectors produced by and residing in the spleen. *Var* genes can switch from a silent to an active mode without detectable DNA alterations (Scherf *et al.*, 1998). Out of the 59 *var* genes, only one member is expressed at a time and presented on the surface of the infected erythrocyte (Gardner *et al.*, 2002a; Scherf *et al.*, 1998; Smith *et al.*, 1995). The switch-rate of *var* genes has been calculated to occur at 2.4% per generation, with the majority of *var* genes expressing the same PFEMP-1 in the clonal population (Biggs *et al.*, 1991; Chen *et al.*, 1998; Roberts *et al.*, 1992).

Clonal phenotypic variation is also found in the invasive form of the parasite and was first discovered in the rodent malaria *P. yoelii* (Preiser *et al.*, 1999). The proposed model specifies that each merozoite descended from a single schizont expresses a distinctive member of the *Py235* multigene family. Members of the *Py235* family are also found in sporozoites and hepatic merozoites and there is a switch of *Py235* homologue expression from one stage to the next (Preiser *et al.*, 2002). This variability also contributes to antigenic variation in that a different



subset of Py235 is present in sporozoite and hepatic stages compared to the numerous erythrocytic merozoites. In a more recent study it was shown that there is a direct correlation between the amount of Py235 proteins expressed and efficiency of erythrocyte invasion (virulence), thus the parasites possess a mechanism to control Py235 expression to evade the immune system (Iyer *et al.*, 2007b).

The second gene family also associated with phenotypic variation is the EBL family. The variation of expression of EBL occurs at the population level. A subpopulation of parasites always expresses a different EBL variant than the majority. This gives the parasite the advantage to rapidly adapt to changes in host receptor availability.

Members of both families (RBL and EBL family) are expressed in a variant manner which reflects the erythrocyte receptor heterogeneity and thus allows a broader spectrum of host cell infection (Cortes *et al.*, 2007; Duraisingh *et al.*, 2003a; Stubbs *et al.*, 2005).

An alternative explanation for the ability of the parasite to regulate different members defining one specific invasion pathway is to contribute to evasion of protective host immune response. Parasite entry using one specific invasion pathway can be achieved by using a number of different parasite ligands. When the host immune system recognises and blocks one member, the parasite has the capacity to switch to another member and gain entry to erythrocyte.

Recently, another gene family, the clag gene family, and the mutually exclusive expression of clag 3.1 and clag 3.2 have been associated with phenotypic variation (Cortes *et al.*, 2007).

### ***1. 15 Different invasion properties of P. falciparum***

One could describe the underlying receptor-ligand interaction during *P. falciparum* merozoite invasion as a “key-lock” mechanism where the erythrocyte comprises many locks, in the form of receptors, and the parasites a large pool of “universal” keys, in the form of erythrocyte binding ligands.

Many polymorphisms on the erythrocyte surface have arisen due to evolutionary selection pressure (Wang *et al.*, 2003; Williamson and Toye, 2008). These polymorphisms reduce the erythrocyte infectivity or even make the host completely resistant to infection, such as individuals which are unable to express the Duffy blood group antigen. The Duffy receptor is a major receptor utilised by *P. vivax* malaria to penetrate erythrocytes (Wertheimer and Barnwell, 1989). The parasite is unable to enter the host cell when this receptor is absent. Another limiting factor for this parasite is the age of the erythrocyte. Young erythrocytes, termed reticulocytes, have the highest abundance of receptors on their surface. Erythrocytes are more limited in their metabolic activity than other body cells and over time, the erythrocyte loses receptors, or parts of them, without having the capacity to renew them (Ripoche and Sim, 1986). Unlike *P. vivax*, *P. falciparum* is able to invade erythrocytes using a range of different ligand-receptor interactions, defining specific invasion pathways. These interactions are made by distinct protein families located at the apical end of the merozoite, i.e. the erythrocyte binding ligand (EBL) and the reticulocyte binding homologue (RBL) families. These interactions are very specific and the preference of ligand-receptor utilisation defines the invasion pathway.

Interestingly there are not only differences in invasion properties between various *Plasmodium* species but also among different clones within one species. These differences can be studied in culture by enzymatic disruption of receptors, or parts of receptors, presented on the erythrocyte surface. There are three main enzymes used, effecting different types of receptors: neuraminidase disrupts the sialic acid residues from O-linked tetrasaccharides of glycoproteins (Miller *et al.*, 1977); trypsin principally cleaves extracellular exposed peptide chains at the carboxyl side

of lysine and arginine, except when either is followed by proline (Anstee, 1981); while chymotrypsin effects the aromatic amino acids tyrosine, tryptophan and phenylalanine of externalised erythrocyte membrane proteins. As an example, the enzyme cleavage sites of glycophorin A are summarised in Figure 1.12.



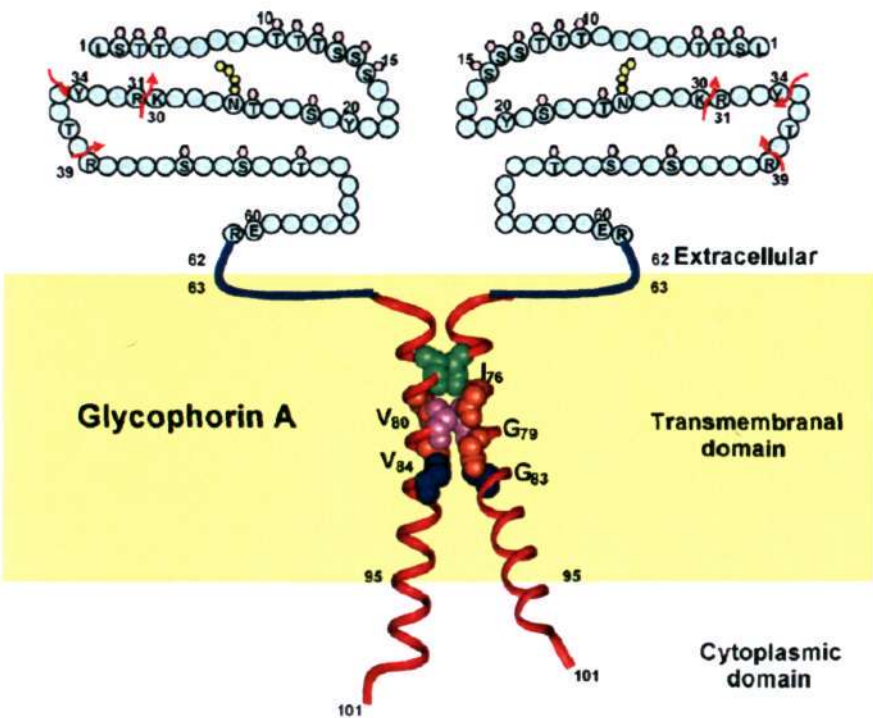


Figure 1. 12. Glycophorin A with enzyme cleavage sites.

Figure adapted from Pinzon *et al.*, (2008). Dimeric glycophorin A with extracellular domain (schematic), transmembrane domain and cytoplasmic domain depicted from the X-ray crystallographic structure. The enzyme cleavage sites (arrow) exist at arginine 39 (R39) and arginine 31 (R31) for trypsin, tyrosine 34 (Y34) for chymotrypsin, 16 O-linked monosaccharides linked to threonine (T) and serine (S) residues for neuraminidase, and N-linked to tetrasaccharides attached to asparagine residues.

## ***1. 16 Hierarchy of molecular interactions***

An indeterminate number of receptors exist on the erythrocyte surface and even the number of merozoite ligands detected to be involved in invasion is still growing. Hence, the indeterminacy of possibilities of invasion pathways makes it difficult to study invasion by *P. falciparum*. Variation in expression of merozoite proteins can change the invasion pathway. One tool of modern molecular biology used to overcome these hurdles is gene knock out techniques. Gene knockout experiments have been of great help to establish the invasion pathway characteristics by controlled silencing of certain genes. The loss of a parasite ligand will cause a switch to another ligand and subsequently a change to another invasion pathway. For instance, silencing of predominant ligands associated with certain invasion pathways like EBA-175, RH-1 and RH-2b ultimately leads to a change in the invasion pathway of most parasite lines (Duraisingh *et al.*, 2003a; Duraisingh *et al.*, 2003b; Reed *et al.*, 2000; Triglia *et al.*, 2005). In contrast the knockout of invasion ligands RH-2a and EBA-140 does not change the invasion pathway (Duraisingh *et al.*, 2003b; Maier *et al.*, 2003). In the same manner silencing of EBA-140, clag 2, clag 3.1 and clag 3.2 also has not resulted in detectable changes in invasion pathway (Cortes *et al.*, 2007).

The model of hierarchal expression of ligands arises where dominant members of a gene family are expressed and thus determine the preferred invasion pathway of this parasite line (Baum *et al.*, 2005). It is proposed that due to the spatial restriction at the apical pole this hierarchy of molecular interaction occurs (Baum *et al.*, 2005; Duraisingh *et al.*, 2003a; Triglia *et al.*, 2005). Non-dominant members will only be used when the expression of the prevailing ligand is abrogated.

Determination of the nature of the invasion pathway is dependent on the enzymatic characteristics. It is important to note, that different invasion pathways could still have similar enzymatic profiles. Other useful tools are the powerful specific inhibitory antibodies directed towards parasite ligands. The combination of knock

out, antibodies and enzymatic treatment have resulted in gaining great knowledge about invasion pathways and their underlying molecular biology.

### ***1. 17 Invasion pathways***

Field studies conducted in different countries all over the world demonstrate that the parasites do not restrict themselves to one invasion pathway. Studies performed in India, The Gambia, Brazil, Tanzania, Kenya and Senegal (Baum *et al.*, 2003; Bei *et al.*, 2007; Deans *et al.*, 2007; Jennings *et al.*, 2007; Lobo *et al.*, 2004; Nery *et al.*, 2006; Okoyeh *et al.*, 1999) showed that the preferred pathway differs depending on the area. For instance: Indian parasites predominantly use sialic-acid-independent and/or trypsin-resistant invasion pathways (Okoyeh *et al.*, 1999), while Gambian, Brazilian and Tanzanian isolates preferably invade via sialic-acid-dependent, trypsin-sensitive pathways (Baum *et al.*, 2003; Bei *et al.*, 2007; Lobo *et al.*, 2004). On the other hand, sialic-acid-independent, trypsin-sensitive invasion was predominant among Kenyan isolates (Deans *et al.*, 2007). The study on Senegalese isolates discovered invasion is mainly conducted via trypsin-sensitive pathway, with different sensitivity to other enzymes (Jennings *et al.*, 2007).

Altogether, work from field studies and modified *in vitro* culture established that in principal high levels of EBA-175/EBA-140, or RH-1 are linked with corresponding low levels of RH-2a/RH-2b/RH-4 and have characteristics of sialic-acid-dependent pathway. On the other hand, when high levels of RH-2a /RH-2b, or RH-4 and low levels of EBA-175, EBA-140 and RH-1 are expressed, the parasite utilises a sialic-acid-independent pathway. Sialic-acid-dependent invasion pathway can be trypsin-sensitive or trypsin-resistant depending on whether EBL proteins or RH-1 are dominant in the parasite line.



### ***1. 18 Sialic acid and invasion pathway switching***

Assorted *P. falciparum* clones have shown diverse invasion phenotypes when exposed to enzyme treated erythrocytes. From previous enzyme studies (Dolan *et al.*, 1994; Gaur *et al.*, 2003; Hadley *et al.*, 1987; Mitchell *et al.*, 1986) it is reported that single *P. falciparum* lines differ in terms of their requirement of sialic acid for their erythrocyte invasion. The most studied member of the EBL superfamily is EBA-175, the ligand for glycophorin A (Dolan *et al.*, 1994). Glycophorin A is a very abundant glycoprotein on the surface of erythrocytes and the interaction with EBA-175 is defined by sialic acid residues found on the receptor. EBA-175 therefore defines a sialic-acid-dependent pathway (Camus and Hadley, 1985).

In order to learn more about the nature of the sialic-acid-dependent pathway, EBA-175 knockouts in sialic-acid-independent 3D7 and W2mef clones have been created. For 3D7 $\Delta$ 175 no change in invasion pathway was observed and W2mef $\Delta$ 175 switched to utilisation of sialic-acid-independent pathway. 3D7 $\Delta$ 175 and W2mef $\Delta$ 175 are not able to invade chymotrypsin-treated erythrocytes as efficient as their parent clones (Duraisingh *et al.*, 2003a). The receptor properties were described as trypsin-resistant and chymotrypsin-sensitive and the receptor named receptor Z (Duraisingh *et al.*, 2003b; Rayner *et al.*, 2001).

The *P. falciparum* Dd2 strain can switch from the sialic-acid-dependent to sialic-acid-independent invasion pathway (Dolan *et al.*, 1990). This selection mechanism is initiated when Dd2 is forced to invade neuraminidase treated erythrocytes, which lack sialic acid side chains on glycophorins and the strain is then called Dd2/NM. The same phenomenon was shown in W2mef (Stubbs *et al.*, 2005). W2mef is the parent clone of Dd2 and ultimately derived from Indochina III isolate. This strain is able to shift with a frequency of  $10^{-4}$  to a sialic-acid-independent pathway. The selected subclone is genetically stable even when growing for several cycles in normal erythrocytes (Dolan *et al.*, 1990). As mentioned earlier, invasion pathway switching is a selection process, meaning a minor subpopulation of Dd2 has the

capacity to invade NM treated erythrocytes. This was calculated by Soubes *et al.*, (1997) with the result of 1 in 217 (0.46%) of naturally occurring Dd2/NM parasites in the Dd2 population (Soubes *et al.*, 1997). This fantastic asset of Dd2 and W2mef gives these parasites the advantage to immediately respond to host receptor changes and ensures a continued survival.

The underlying molecular mechanism behind invasion pathway switching was exploited by Stubbs *et al.*, (2005) and Gaur *et al.*, (2006). Both groups independently showed (in the W2mef and Dd2 clones respectively) that RH-4 is the major interacting ligand enabling the pathway switch. These two studies have exploited the involvement of ligands during invasion pathway switching by studying microarray transcripts.

Another study using 3D7 subclones (subclone 3D7 A invades erythrocytes sequentially treated with neuraminidase and trypsin; subclone 3D7 B does not have this ability) revealed the involvement of other proteins such as erythrocyte binding antigen 140 (EBA-140) and RhopH complex proteins. The study also showed for the first time evidence that there is epigenetic silencing involved in invasion pathway switching (Cortes *et al.*, 2007).

## **Aims**

Some *P. falciparum* strains such as W2mef are able to adapt to erythrocytes which lack their preferred interacting partner. This is a selection process where the parasite population switches from sialic-acid-dependent to the sialic-acid-independent invasion pathway (Dolan *et al.*, 1990; Gaur *et al.*, 2006; Stubbs *et al.*, 2005). Invasion of erythrocytes is a cascade of events involving numerous molecules, interacting partners and signalling. It is only rational to believe that switching an invasion pathway would implicate many parts of this ingenious multi-step process. Studies of transcript levels revealed only RH-4 and the pseudogene EBA-165 as highly up-regulated in the sialic-acid-independent pathway (Gaur *et al.*, 2006; Stubbs *et al.*, 2005). The up-regulation of the transcript is also reflected in the protein expression of RH-4. The sialic-acid-dependent ligand RH-1 is unaffected on transcript levels but is reduced on expression levels (Gao *et al.*, 2008). This fact already indicates that not many changes take place during transcription but occur at the protein level. Post-transcriptional regulation is thought to play a key role of controlling invasion molecules. Therefore, transcriptional analysis on its own is not sufficient to study gene regulation.

The objective of this thesis is to obtain reliable quantification of protein expression and their comparison with transcript levels from genetic identical cultures during invasion. Another focus is to identify the regulatory mechanism in terms of post-transcriptional motifs responsible for protein regulation in W2mef/NM.

Furthermore, enzymatic treatment may have many effects on erythrocytes beyond removing a receptor and this could also impact on how parasites respond. Biomechanical and biophysical techniques will be used to study the changes induced by neuraminidase treatment such as micropipette aspiration, zeta potential measurement and Atomic force microscopy.



## **Chapter 2**

### **Materials and Methods**

#### ***2. 1 Cultivation of W2mef***

*Plasmodium falciparum* W2mef clone was obtained from MR4 Malaria Resource Centre. Cultivation of *Plasmodium* clones follows Trager and Jensen (1976) with slight modifications: purified human erythrocytes were kept at 1 % hematocrit (packed erythrocyte proportion in suspension) in 37°C pre-warmed RPMI-1640 (Gibco) media supplemented with 0.25% Albumax II (Gibco), 2.3 g/l sodium bicarbonate (Sigma), 0.5 mM hypoxanthine (Sigma) and 50 mg/l gentamycin (Gibco) (Trager and Jensen, 1976). Medium with the above stated supplements but without Albumax was used as washing medium for fresh erythrocytes or parasitized erythrocytes. Medium was replaced daily and erythrocytes added when necessary. The parasites were grown in 175 cm<sup>2</sup> culture flasks (Nunc) unless otherwise stated. The parasitemia should not exceed 10% in one flask and should be diluted to 1% parasites in the flask. The parasite culture was maintained under low oxygen conditions of 88% nitrogen, 5% oxygen and 7% carbon dioxide in a 37°C incubator (Thermo Election Corporation). The growth of the culture was monitored microscopically by Giemsa (Sigma) stained smear.

#### ***2. 2 Parasitemia***

One drop (10 µl) of the culture was applied to a slide to make a thin film then fixed in methanol for about 30 sec and air-dried. The slide was placed in a staining jar and stained with Giemsa solution (1 in 5 dilution in H<sub>2</sub>O). After 5 min, the slide was rinsed thoroughly under running tap water and dried in an upright position. The smear was observed with an immersion oil objective at 100 × (Olympus). At least 1000 erythrocytes were counted.

$$\text{Parasitemia} = \frac{\text{"infected RBC"}}{\text{"total \# of RBC"}} \cdot 10$$

### ***2. 3 Erythrocytes***

Erythrocytes were obtained from a malaria negative donor. 350 ml of blood was drawn into a blood bag (GMS) containing anti-coagulant detergent (ACD). The blood was aliquoted into 50 ml tubes (Greiner bio one, cellstar) and stored at 4°C until use. Erythrocytes were used in culture up to a maximum of 30 days after blood was drawn. Before the erythrocytes were added to the culture, serum and ACD was removed and the erythrocytes were washed twice with RPMI 1640 w/o Albumax at 2400 rpm for 5 min (Centrifuge 5810 R, Eppendorf, swing bucket rotor A-4-62). The cells ready for use were stored at 4°C at 50% hematocrit in RPMI 1640 w/o Albumax.

### ***2. 4 Enzymatic treatment of erythrocytes***

Human erythrocytes were washed three times with RPMI 1640 w/o Albumax. 250 µl Erythrocytes were resuspended in RPMI 1640 w/o Albumax at 50% hematocrit in 1.5 ml eppendorf tubes. Neuraminidase (Calbiochem, 1 U/ml) was added to each eppendorf tube at concentrations of 0.01, 0.1, 1.0, 5, 10, 25, 50 and 67 mU/ml respectively, and incubated while rotating at 37°C for 1 hour. The treated erythrocytes were pelleted and washed 3 times with RPMI 1640 w/o Albumax. The cells were then ready for use and stored at 4°C at 50% hematocrit in RPMI 1640 w/o Albumax.

### ***2. 5 Separation of schizonts***

Parasites were washed in RPMI w/o Albumax and resuspended at 20% hematocrit. 5 ml of parasites were placed gently on top of 10 ml 70% isotonic Percoll (Sigma) in RPMI 1640 w/o Albumax in 50 ml tubes (Greiner bio one, cellstar). Isotonic

Percoll consists of 90% Percoll and 10% 10× PBS. The gradient was centrifuged at 2100 rpm for 10 min w/o brake (Centrifuge 5810 R, Eppendorf, swing bucket rotor A-4-62). After the centrifugation the schizont layer was removed and washed 2 times with RPMI 1640 w/o Albumax by centrifugation at 2400 rpm for 3 min, brake 3. Giemsa-stained smear was performed to ensure the pellet contained only schizonts. Schizonts were used immediately for invasion assays or synchronisation.

## **2. 6 Parasite synchronisation**

Schizonts purified by Percoll treatment were cultured with fresh erythrocytes. Two hours later, the parasites were monitored for ring stage parasites. Once ring stage parasites were visible 5% sorbitol (Sigma) was added 1:1 to parasitized erythrocytes at room temperature for 10 min in order to remove all parasites older than 24 hours (Hoppe *et al.*, 1991). The parasites were washed 3 times in RPMI 1640 w/o Albumax at 2400 rpm for 3 min, brake 3, supernatant was removed and placed back in culture and fresh erythrocytes added.

## **2. 7 Harvesting of parasites for RNA preparation**

*P. falciparum* clone W2mef and W2mef/NM were cultured as described above. Cells were synchronised with 5% sorbitol (Sigma) after 4h and 20h post-invasion on two consecutive cell cycles. The cells were harvested from 32 h post-invasion until reinvasion every 2h, resulting in 10 time points (TP) for W2mef and 10 TP for W2mef/NM.

The cells were centrifuged (2400 rpm, 3 min) and washed with phosphate buffered saline (PBS) to yield 1 ml of packed, parasitized erythrocytes. Cell pellets were rapidly frozen in liquid nitrogen and stored at -80°C until used for RNA extraction.



## ***2. 8 Obtaining free merozoites***

Synchronised schizonts without erythrocytes were resuspended in RPMI 1640 with Albumax, placed back in culture flasks, gassed and incubated at 37°C. The culture was observed by Giemsa-stained smear. Once free merozoites were visible, the culture was transferred to a 50 ml centrifuge tube and centrifuged twice at 2400 rpm for 3 min. Supernatant was transferred to another 50 ml tube and centrifuged at 5000 rpm for 4 min. Subsequently, to remove all schizonts the merozoite pellet was immediately resuspended in RPMI-1640 w/o Albumax and passed through an amicon pressure unit (10 ml with 2.0 µm TTTP isopore membrane filter; Milipore).

The pellet was observed by Giemsa-stained smear to ensure that only merozoites were present in the pellet. The pellet was washed twice in 500 µl 1× PBS then transferred into 1.5 ml Eppendorf tubes and centrifuged at 5000 rpm for 4 min (Eppendorf centrifuge 5415 D). Finally the pellet was resuspended in 10 mM Tris-Cl and centrifuged at 5000 rpm for 4 min. The supernatant was removed and the pellet was snap frozen in liquid nitrogen prior storage at - 80°C.

## ***2. 9 Neuraminidase Invasion assay***

Schizont stage parasites were isolated from synchronous parasite culture and added in duplicate to a 96 well flat-bottomed microtiter plate (Iwaki). Different concentrations of NM-treated erythrocytes or normal erythrocytes were added to give final parasitemia of 2% and hematocrit of 4% in a total volume of 150 µl/well. After 24 h the invasion efficiency was determined by counting the number of successfully invaded erythrocytes per 1,000 erythrocytes on Giemsa-stained thin smears.

## ***2. 10 Adaptation and growth of parasites in enzyme-treated erythrocytes***

Tightly synchronised late schizonts, approximately 45 h post-invasion were separated and mixed with 10 mU/ml neuraminidase-treated erythrocytes to yield a 1% starting parasitemia. The culture was maintained at 2% hematocrit in RPMI 1640 with Albumax in a 25 cm<sup>2</sup> flask (Nunc). The culture was incubated at 37°C. 50 µl of fresh, treated erythrocytes were added to the culture twice a week. Media was changed on a daily basis. Once the parasitemia reached 8-10%, the culture was expanded to a larger flask (75 cm<sup>2</sup> or 175 cm<sup>2</sup>).

## ***2. 11 MSP-1 invasion inhibition assay***

Synchronised late stage schizonts of W2mef or W2mef/NM were purified as described above and 160 µl of parasite suspension was added in duplicate in a 96 well flat-bottomed microtiter plate (Iwaki) containing 40 µl of serial dilution from 1:10 to 1:200 of MSP-1<sub>19</sub> antiserum. The final hematocrit of NM-treated or untreated erythrocytes was 5%. 1000 erythrocytes were counted for the presence of rings on Giemsa-stained thin smears 24 h post-invasion. Invasion in the presence of antiserum was compared with positive control of invasion of the same parasite clones into normal erythrocytes in RPMI 1640 with Albumax. Invasion inhibition is presented relative to control in percentage of inhibition. Data shown are from two separate experiments.

## ***2. 12 Western blot analysis***

W2mef and W2mef/NM merozoites were obtained as described above. Merozoite pellets were thawed on ice, resuspended in 100µl 2× SDS loading buffer. The lysate was 3 times frozen and thawed, boiled at 100 °C for 10 min and separated on a 6 to 12% gradient acrylamide gel. Following electrophoresis the acrylamide gels were transferred on nitrocellulose membrane (Biorad) by Biorad semidry transfer

chamber with a buffer containing 1× tris-glycine and 20% methanol, for 30 min at 18 mV and then for another 30 min at 25 mV. The membranes were blocked for 1 h at RT in 5% milk powder in TBST 0.05% (Tris-based saline with 0.05% Tween 20). All primary antibodies were diluted in the blocking buffer and incubated o/n at 4°C, followed by 3 times 10 min wash in TBST 0.05%. The dilutions for the primary and secondary antibodies are listed in Table 2.1. Secondary antibodies were diluted in blocking buffer and incubated for 1 h at RT and washed 3 times in TBST 0.05% for 10 min. For detection Pierce Chemiluminescences Pico Kit (Pierce) is used and exposed to Kodak medical X ray film general green purpose or for higher sensitivity Amersham hyperfilm TM ECL (GE healthcare).

Table 2. 1. Dilutions of primary and secondary antibodies.

| Primary Antibody    | Dilution | Secondary antibody Dilution | Source/ Reference               |
|---------------------|----------|-----------------------------|---------------------------------|
| Actin               | 1:500    | Anti rabbit 1:6000          | Sigma-Aldrich                   |
| MTIP                | 1:10000  | Anti rabbit 1:6000          | (Jones <i>et al.</i> , 2006)    |
| Clag 3.1            | 1:3000   | Anti rabbit 1:6000          | (Kaneko <i>et al.</i> , 2005)   |
| RhopH2              | 1:5000   | Anti rabbit 1:6000          | (Holder <i>et al.</i> , 1985a)  |
| MSP-7               | 1:2000   | Anti rabbit 1:6000          | (Pachebat <i>et al.</i> , 2007) |
| MSP-1 <sub>19</sub> | 1:5000   | Anti rabbit 1:6000          | (Blackman <i>et al.</i> , 1990) |
| eIF5a               | 1:5000   | Anti rat 1:2000             | (Chamot and Kuhlemeier, 1992b)  |
| Histone 3           | 1:5000   | Anti rabbit 1:6000          | MR4                             |
| Plasmepsin          | 1:2000   | Anti rabbit 1:6000          | MR4                             |

2. 13 DNA extractions from cultured parasites

The ring-stage culture was centrifuged at 2400 rpm for 5 min. Supernatant was removed and the infected erythrocyte pellet was utilised for DNA extraction by using the Easy-DNA Kit (Invitrogen, Version F, Protocol #2-30 minutes DNA Extraction from Blood samples). Ethanol was added for DNA precipitation. Finally



the DNA pellet was resuspended in 100  $\mu$ l 1 $\times$  TE buffer containing Tris-Cl and ethylenediaminetetraacetic acid (EDTA) and stored at 4 °C.

## ***2. 14 PCR genotyping at MSP-1 and MSP-2 polymorphic regions***

DNA (0.5  $\mu$ g) was amplified in an Eppendorf mastercycler in a final volume of 50  $\mu$ l containing 200  $\mu$ M of each deoxynucleotide (dNTP), 1  $\mu$ M of forward and reverse primer, and 2.5 units of *Taq* polymerase (Promega) in 2.25 mM MgCl<sub>2</sub> and 5 $\mu$ l 10 $\times$  buffer (Promega). The primary PCR was performed for 32 cycles (2 min at 95°C, 1.5 min at the appropriate annealing temperature, and 2 min at 72°C).

The nested PCRs were carried out with 1 in 100 dilution of the primary PCR product. Amplification was done for 17 cycles (2 min at 95°C, 1.5 min at the appropriate annealing temperature, and 2 min at 72°C). The sequence of the various primers and the annealing temperatures used are listed in Table 2.2.

The products of the genotyping PCR were analysed for size polymorphism on a 1.5% metaphor agarose gel with ethidium bromide.

**Table 2. 2. Primer sequences and annealing temperature for PCR genotyping.**

Adapted and modified from Zwetyanga *et al.*, (1998).

| Target         | Primer  | Annealing temperature | Oligonucleotide Sequence in 5' to 3' direction |
|----------------|---------|-----------------------|--|
| MSP-1 external | Forward | 55°C                  | CACATGAAAGTTATCAAGAACTTGTC                     |
|                | Reverse |                       | GTACCTCTAATTCATTTGCACG                         |
| MSP-1 K1       | Forward | 65°C                  | GAAATTACTACAAAAGGTGCAAGTG                      |
|                | Reverse |                       | AGATGAAGTATTTGAACGAGGTAAAGTG                   |
| MSP-1 MAD-20   | Forward | 67°C                  | GAACAAGTCGAAGAGCTGTTA                          |
|                | Reverse |                       | TGAATTATCTGAAGATTTGTACGTCTTGA                  |
| MSP-1 RO-33    | Forward | 68°C                  | GCAAATACTCAAGTTGTTGCAAAGC                      |
|                | Reverse |                       | AGGATTTGCAGCACCTGGAGATCT                       |
| MSP-2 external | Forward | 55°C                  | ATGAGGGTAATTAACATTGTCTATTATA                   |
|                | Reverse |                       | ATATGGCAAAAGATAAAACAAGTGTTGCTG                 |
| MSP-2 A        | Forward | 58°C                  | GCAGAAAGTAAGCCTTCTACTGGTGCT                    |
|                | Reverse |                       | GATTTGTTTAGGCATTATTATGA                        |
| MSP-2 B        | Forward | 58°C                  | GCAAATGAAGGTTCTAATACTAATAG                     |
|                | Reverse |                       | GCTTTGGGTCCTTCTTCAGTTGATTC                     |

Primers which are not indicated as external were used for the nested PCR

2. 15 Microarray

Microarray slides were prepared essentially as described by Eisen and Brown (1999).

2.15.1 Preparation of polylysine-coated slides

Standard glass microscope slides (Gold Seal MicroSlides) were placed vertically in metal racks in a glass beaker. 400 g NaOH was dissolved in 1600 ml ddH<sub>2</sub>O and 2400 ml of 95% ethanol to give 2l alkaline wash solution. The slides were completely submerged in this solution for at least 2 h while rotating at 60 rpm on a

rotation platform. The slides were then cleaned extensively 5 times in ddH<sub>2</sub>O while rotating for 5 min. Coating solution was prepared in a plastic beaker: 300 ml of poly-L-lysine (0.1% (w/v) in H<sub>2</sub>O; Sigma), 350 ml sterile filtered PBS and 2850 ml ddH<sub>2</sub>O. The slide rack was transferred into the coating solution and rotated gently for 45 min. The slides were washed 5 times in ddH<sub>2</sub>O and plunged up and down for 30 sec. The slide rack was transferred to a centrifuge and centrifuged for 5 min, room temperature, at 600 rpm in an eppendorf centrifuge 5810. The slides were stored at room temperature in a tightly sealed slide box. It is necessary to store the slides for at least 2 weeks to allow the surface to become adequately hydrophobic in order to maintain the small size of the printed DNA spots on the slide.

### 2.15.2 Printing microarray slides

A new microarray for the *P. falciparum* genome was designed by Hu *et al.*, (2007) consisting of 10166 long oligonucleotide elements for 5363 genes with one unique oligonucleotide every 2 kb per gene (Hu *et al.*, 2007). 3× SSC (buffer containing sodium chloride and sodium citrate) was added to the customised oligonucleotides (invitrogen) to yield 20 µM. The oligonucleotides were lyophilised and stored at -20°C in 384 well microtitre plates. Prior to printing, all oligonucleotides were hydrolysed at room temperature in H<sub>2</sub>O up to a concentration of 20 µM using a Gilson 940 workstation.

The oligonucleotides were printed on the prepared glass slides using a VersArray Chipwriter pro system (Biorad) according to manufacturer's protocols.

### 2.15.3 Post-processing of printed slides

The oligonucleotide spots are not necessarily evenly formed and for a more uniform distribution, the spots are rehydrated and snap-dried. The slides were hydrated one by one in slide warmer (Cole Parmer) containing a hydration tray with 0.5× SSC at 42°C for 90 sec with the printed side facing down. The slides were immediately



snap-dried on a flat metal surface in a heat block at 100°C. Oligonucleotides are cross-linked to the slide to enhance binding to the hydrophobic surface by UV irradiation. The slides are placed array side up in a Stratagene UV 1800 Stratalinker and exposed to 800 mJ energy-start-modus.

To reduce background noise the remaining free lysine groups are modified to minimise their ability to bind labelled probe DNA. The slides were placed into a metal rack and soaked in 65°C pre-warmed 3× SSC with 0.2% SDS for 5 min. The slides are instantly plunged for 30 sec in ddH<sub>2</sub>O and then in 95% ethanol. For the blocking solution, 5.5 g succinic anhydride (Sigma) were dissolved in 500ml ddH<sub>2</sub>O and 335ml 1,2-methyl pyrrolidinone (Sigma) was added. Immediately after the succinic anhydride dissolved, 1 M sodium borate, pH 8.0 was added, and mixed until the solution was clear. The slides were immediately immersed in the blocking solution and plunged up and down for 30 sec and then rotated gently for 5 min on a rotating platform. The slides were washed in ddH<sub>2</sub>O by plunging up and down for 1 min and placed back into the blocking solution, plunged for 30 sec and then rotating for 5 min. The slides were washed again in ddH<sub>2</sub>O for 1 min then transferred to another ddH<sub>2</sub>O-containing dish and moved up and down for 30 sec. Lastly, the slides were transferred to a chamber containing 95% ethanol and moved up and down for 30 sec and then centrifuged to dry at 600 rpm for 5 min. The slides were then ready for hybridization.

#### 2.15.4 RNA preparation and microarray hybridization

RNA extraction and hybridizations were performed according to Bozdech *et al.*, (2003b). Total RNA was prepared directly from frozen pellets of parasitized erythrocytes (10 different TP of W2mef and W2mef/NM, respectively), 1 ml of cell pellet was lysed in 10 ml Trizol reagent (Invitrogen) and 2 ml of chloroform added and incubated on ice for 10 min, followed by a centrifugation at 3500 rpm for 10 min w/o brake at 4°C. The supernatant was transferred to 14 ml polypropylene round bottom centrifuge tube (Beckman) and equal volume of isopropanol was

added. After storage at  $-20^{\circ}\text{C}$  o/n, the precipitated RNA was centrifuged at 9000 rpm for 1 h at  $4^{\circ}\text{C}$  in JA20 rotor with adaptors in Beckmann Coulter Avanti centrifuge. The resulting pellet is incubated on ice for 10 min with the addition of 70% ethanol. After centrifugation at 9000 rpm for 10 min at  $4^{\circ}\text{C}$ , the pellet was dried for 1 h under vacuum at RT. The RNA pellet is resuspended in 100  $\mu\text{l}$  ddH<sub>2</sub>O and stored at  $-80^{\circ}\text{C}$ . The quality of the RNA was assessed by gel-electrophoresis and spectrometric measurement of the absorbance at 260 nm and 280 nm. The ratios of  $A_{260}/A_{280} > 1.8$  and no observation of RNA degradation on the agarose gel were considered good quality of RNA and were used for hybridizations.

For the hybridization experiments, 12  $\mu\text{g}$  of total pooled reference RNA (W2mef parasites of all developmental intra-erythrocytic stages) or sample RNA (single TP W2mef or W2mef/NM) was used for first-strand cDNA synthesis as follows: RNA was mixed with a mixture of random hexamer (pdN9) oligonucleotides and oligo-(dT20) at final concentration 125  $\mu\text{g}/\mu\text{l}$  for each oligonucleotide and heated at  $70^{\circ}\text{C}$  for 10 min and incubated on ice for 10 min. Reverse transcription was initiated by adding dNTPs to a final concentration of 200 mM dATP and 100  $\mu\text{M}$  each: dCTP, dGTP, dTTP and 5-(3-aminoallyl)-2'-deoxyuridine-5'-triphosphate, (aa-dUTP, Sigma), with 150 units of reverse transcriptase (Fermentas). The reaction was carried out at  $42^{\circ}\text{C}$  for 2 h and the remaining RNA was hydrolysed with 0.1 mM EDTA and 0.2 M NaOH at  $65^{\circ}\text{C}$  for 15 min. The resulting aa-dUTP-containing cDNA was coupled to Cy3 or Cy5 (Amersham) in the presence of 0.1 M NaHCO<sub>3</sub> pH 9.0. Coupling reactions were incubated for a minimum of 1 h at room temperature. The labelled product was purified using QIAquick PCR purification system (Qiagen). Samples from individual TP of either W2mef or W2mef/NM (coupled to Cy5) (Amersham) were hybridized against the W2mef reference pool (coupled to Cy3) (Amersham). Microarray hybridizations were incubated for 14–16 h using Maui hybridization system (Bio Micro Systems). cDNA microarray hybridizations were performed in duplicate. Arrays were washed in 2x SSC with 0.2% SDS and then 0.1x SSC at room temperature for 3 min per wash. The

microarrays were scanned with a GenePix 4000B scanner and the images analysed using GenePix Pro 3.0 software (Axon Instruments, Union City, CA).

Data were acquired and analysed by GenePix Pro 3 (Axon Instruments). The raw array data was stored and normalised using the NOMAD microarray database (<http://ucsf-nomad.sourceforge.net/>). The array features were unflagged and those with median intensities greater than the local background plus 2 times the standard deviation of the background were extracted from the database.

## ***2. 16 Real-time PCR and reverse transcription***

The same RNA was used for Real-time PCR as used for microarray analysis as described in 2.15.4. 5 µg of RNA was treated with 1 unit DNase (Fermentas), 1 µl 10× DNase buffer and 9 µl H<sub>2</sub>O and incubated at 37°C for 30 min. The enzyme reaction was inactivated with 1 µl EDTA at 65 °C for 10 min. The DNase treated RNA was converted to cDNA using random Primers (Invitrogen), 10mM dNTP (iDNA) and 13 µl H<sub>2</sub>O. The reaction was heated at 65 °C for 5 min. After 10 min at room temperature 5x first strand buffer, 0.1M DTT and 200 units of Superscript reverse transcriptase III (Invitrogen) were added, kept for 5 min at room temperature and incubated at 42°C for 2 hours. The reaction was inactivated at 70°C for 15 min. In addition a negative control without reverse transcriptase was performed. Primers were designed using Primer 3 software (<http://frodo.wi.mit.edu/>) to have a T<sub>m</sub> of 55-65°C, a GC-content of 30-60%, a length of 20-26bp and produce a product of 150-250 bp. Primers used for real time PCR are listed in Table 2.3.



Table 2. 3. Primers used for Real Time PCR

| Target                                      | Forward Primer                 | Reverse Primer              |
|---|--------------------------------|-----------------------------|
| Actin 1<br>(PFL2215w)                       | TGCACCACCAGAGAGAAAAT           | ACGATGAATCAGGACCAAGT        |
| High mobility group<br>protein MAL8P1.72    | CCCATTAGCTCCAAAGAGAGC          | CCCCAAGCTTCACCTATCAA        |
| purine nucleotide<br>phosphorylase PFE0660c | CGGTGTAGGTTACAGCAGGAT          | CACCAACAGCTGGGAAATCT        |
| REX<br>(PFI1735c)                           | AATCGGGTGCTCCATACAAG           | CGTCTTTTGTCCCTGTTCTG        |
| Hypothetical protein<br>(PFD0090c)          | CCCGAAACGATATGGAAAAG           | TGTGGCTGGCTTATTAACACC       |
| ETRAPM 14.1<br>PF14_0016                    | TTCAGCGAAAGCACAAATTC           | TGAGCAATATTTGCCATTCC        |
| MESA<br>(PFE0040c)                          | TGAAACTGAACCACCGATGA           | TTTCGGATTCTTGCATTTC         |
| MTIP<br>(PFL2225w)                          | TGCATCTTACAATGCTCGAAA          | TGCATCACCCCAAGTTGTAA        |
| GAP-45<br>(PFL1090w)                        | CACTGGTGCCGTACAACAAA           | TTTCATCATGACGTTACACAA       |
| Plasmepsin 2<br>(PF14_0077)                 | GCAATTCAACATTTGATGGATTAA<br>AC | CCACACATTACACTACAAAAGAGAAG  |
| AMA-1<br>(PFI1_0344)                        | ATCATACATTGCTACTACTGC          | TATTCCTTCATCATCATTATC       |
| Clag 3.1<br>(PFC0120w)                      | ACTCACGGACTTGCTGCTGA           | AAAGCATTTTCCATATCATATCTTCT  |
| eIF5A<br>(PFL0210c)                         | TGG AGC ATC ACA AAC GTA CCC    | ATGCTC TTTCAACAT TACATGACCA |
| MSP-1<br>(PFI1475w)                         | TTCGTGCAAATGAATTAGACGTAC       | GGATCAGTAAATAAACTATCAATGT   |
| Histone 3<br>(PF13_0185)                    | CATTTAATAGGGACAAGTC            | GTGTATAACGAAATTGCTC         |

Individual Real-time PCR reactions were carried out in 15 µl volumes using MiroAmp optical 8-tube strip (0.2ml) with optical caps (Applied Biosystems) with 7.5 µl Sybr Green master mix (Applied Biosystems), 6.4 µl H<sub>2</sub>O, 10 µM forward and reverse primer 0.3 µl each, gDNA or cDNA 0.5 µl. The incorporation of fluorescent SYBR green dye (Applied Biosystems) was measured with Applied Biosystems 7500 Real-Time PCR System using the following protocol:

Table 2. 4. Real Time PCR protocol

| Cycles | Temperature | Time in min |
|--------|-------------|-------------|
| 1      | 50.0 °C     | 2:00        |
| 1      | 95.0 °C     | 10:00       |
| 40     | 95.0 °C     | 0:15        |
| 40     | 58.0 °C     | 1:00        |
| 40     | 60.0 °C     | 1:00        |
| 1      | 95.0 °C     | 0:15        |
| 1      | 60.0 °C     | 1:00        |
| 1      | 95.0 °C     | 0:15        |
| 1      | 60.0 °C     | 0:15        |

Standards of 10-fold dilutions specific to the gene of interest were included in each run and the  $C_t$  value at each dilution was measured. Since only single copy genes are analysed, standards were obtained by PCR amplification from W2mef genomic DNA using the same primers as in the Real-Time PCR. Standards were quantified by spectrophotometry (Nanodrop). The product was specific and resulted in a single peak dissociation curve. In each Real-Time PCR,  $H_2O$  control and control without the SYBR green master mix were included. Each individual gene was analysed in triplicate and the  $C_t$  of each sample was recorded at the end of the reaction. The average and standard deviation (SD) of three  $C_t$ s per gene were calculated when the  $SD < 0.38$  (Pfaffl, 2001). In order to measure relative efficiency amplifications on the dilution series a reference primer was used (housekeeping gene ornithine aminotransferase) and target genes. The average of the  $C_t$  for the reference gene and target gene was calculated and  $\Delta C_t$  determined ( $\Delta C_t = C_t$  target gene -  $C_t$  reference gene). The log DNA dilution versus  $\Delta C_t$  was plotted. When the absolute value of the slope was  $< 0.1$ , the relative quantification  $\Delta \Delta C_t$  was calculated for each gene as described by Livak and Schmittgen (Livak and Schmittgen, 2001):  $\Delta \Delta C_t = (C_t \text{ target gene} - C_t \text{ reference gene})_{W2mef/NM} - (C_t \text{ target gene} - C_t \text{ reference gene})_{W2mef}$ . Results were plotted in fold change W2mef/NM vs. W2mef.

## **2. 17 Protein preparation for ITRAQ**

### **2.17.1 Carbonate extraction method**

This method was modified from Fujiki *et al.*, (1982). Pelleted merozoites from W2mef and W2mef/NM cultures were resuspended in a buffer consisting of 50 mM Tris/HCl, pH 7.3 and a mixture of protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). The sample was lysed by freezing in LN<sub>2</sub> followed by graining in a mortar at least 3 times. Unbroken cells were removed by centrifugation at 2500× g for 10 min. The supernatant was diluted with ice cold 1M sodium carbonate (pH 11) to a final concentration of 0.1 M and stirred slowly on ice for 1 h. The carbonate treated membranes were collected by ultracentrifugation in a Beckman Coulter Optima ultracentrifuge TLA 120.1 rotor using polycarbonate tubes at 125,000×g for 1 h at 4 °C. The supernatant was discarded and the membrane pellet was resuspended and washed in 50 mM Tris/HCl, pH 7.3. The pellet was collected by centrifugation at 100,000 x g for 30 min (Beckman Coulter Optima ultracentrifuge TLA 120.1 rotor). The wash was repeated with deionised H<sub>2</sub>O and centrifuged at 100,000 x g for 30 min. Finally the pellet was dissolved in 1% SDS with 50 mM HEPES buffer (pH 7.3). Solubilised proteins of W2mef and W2mef/NM were quantitated and 100µg were labelled with iTRAQ reagent as described below.

### **2.17.2 Urea-based protein extraction and isobaric labelling**

All chemicals were purchased from Sigma-Aldrich (St Louis, MA) unless otherwise stated. Pelleted merozoites were resuspended in a buffer consisting of 500mM TEAB (Triethylammonium bicarbonate) (pH 8.5), 8M urea and a mixture of protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). The sample was lysed by freezing in LN<sub>2</sub> followed by graining with a mortar that was repeated at least 3 times, then centrifuged at 13,000 x g for 30min and protein was recovered in the supernatant. Proteins were reduced using 5 mM Tris-(2-carboxyethyl) phosphine for 1 h at 37°C and cysteine disulphide bond formation blocked (or



alkylated) with methylmethanethiosulfate (MMTS) at a final concentration of 10mM for 10 min at RT according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). The sample was then diluted to 1 M urea, tolerable for trypsin digestion. Trypsin was added according to the ratio of 50:1 (protein:trypsin), and incubated overnight at 37°C. The resulting peptides were dried and resuspended in 30 µl of 500 mM TEAB (pH8.5) and 100 µl of ethanol, isotopically labelled for 1 h at room temperature by adding the content of one vial of iTRAQ reagent to each 100 µg protein. The labelling reaction was stopped by the addition of 100 µl H<sub>2</sub>O and incubated for 20 min. The labelled samples were combined, dried by speed vacuum centrifugation and stored at -80°C until further MS analysis. The labelling scheme was 114: W2mef/NM; 115: W2mef; 116: W2mef/NM; 117: W2mef.

### 2.17.3 SCX (strong cation exchange) chromatography

SCX chromatography was performed on a Shimadzu Prominence<sup>TM</sup> UFLC unit (Kyoto, Japan) using a 200×4.6 mm (5-µm particle size, 200-Å pore size) PolySULFOETHYL A<sup>TM</sup> column (PolyLC, Columbia, MD). A 50 min gradient was designed using a combination of 10 mM KH<sub>2</sub>PO<sub>4</sub> in 25% acetonitrile, pH 2.85 (buffer A) and with 500 mM KCl, pH 2.85 (buffer B). The gradient consisted of 5 min of isobaric buffer A followed by four linear gradients of buffer B: 0% to 10% over 2 min, 10% to 15% over 20 min, 15% to 40% over 8 min, and 40% to 100% over 5 min. 100% buffer B was sustained for 5 min. The gradient ended with 100% buffer A for 5 min. A total of 30 fractions were collected, lyophilised and desalted using SEK-PAK C18 cartridges (H<sub>2</sub>Os Corporation, Milford, MA). Eluents were lyophilised and stored in -80 °C before mass spectrometer analysis.

### 2.17.4 Mass Spectrometry

The LTQ-FT ultra (hybrid ion trap Fourier transform high resolution mass spectrometer) or LTQ-Orbitrap XL (linear ion trap and Orbitrap™ hybrid Fourier Transform Mass Spectrometer) (Thermo Electron) was coupled with an online Shimadzu UFLC system utilising nanospray ionization (Michrom Bioresources Inc., Auburn, CA). Peptides were first enriched using a Zorbax 300SB C18 column (5mm×0.3mm, Agilent Technologies, Santa Clara, CA) followed by elution into an integrated nano-bored column (75µm×100mm, New Objective, Woburn, MA) packed with C18 material (5µm particle size, 300Å pore size, Michrom BioResources Inc.). Mobile phase A (0.1% formic acid, 100 ddH<sub>2</sub>O) and mobile phase B (0.1% formic acid, 100% acetonitrile) was used to establish the 60-min gradient: 0-3 min: 5-8% B, 3-40 min: 8-30% B, 40-44 min: 30-60% B, 44-45 min: 60-80% B, 45-49 min: maintain 80% B, 49-50 min: 80-5% B, 50-60 min: maintain 5% B. The MS was operated in the data dependent mode. A full survey scan (350-2000m/z range) was acquired in the 7-Tesla FT-ICR cell at a resolution of 100,000 for LTQ-FT; or in the Orbitrap cell at a resolution of 60,000 for LTQ-Orbitrap, and maximum ion accumulation time of 1000 msec. The linear ion trap was used to collect peptides where 10 most intense ions were selected (ion selection threshold of 500 counts) for collision-induced dissociation (CID) and pulsed-Q dissociation (PQD), which were performed concurrently with a maximum ion accumulation time of 200 msec. Dynamic exclusion was activated for this process, with a repeat count of 1 and exclusion duration of 30s. For CID, the activation Q was set at 0.25; isolation width (m/z) 2.0; activation time 30ms; and normalised collision energy of 35%. In the case of PQD, activation Q was set to 35%, default change state 5; isolation width (m/z) 2.0; normalised collision energy 35%; and activation time 0.100ms.

For Q-TOF analysis, mass spectrometric analysis was performed using a QStar Elite Hybrid Quadrupole time-of-flight tandem mass spectrometer, Q-TOF-MS/MS (Applied Biosystems, Framingham, MA, USA; MDS-Sciex, Concord, Ontario, Canada), coupled with a Tempo nanoLC-system comprising a combination of an

autosampler, a 10-port column switching valve, and a dual channel split-less LC system with two binary gradient pump powered by Eksigent (Dublin, CA, USA). Samples were first trapped and desalted with Zorbax 300SB-C18 enrichment column (5mm x 0.3mm, Agilent Technologies, Germany), before further separated onto an integrated nano-bored C18 column (75µm i.d. x 7cm, New Objective, Woburn, MA, USA), packed with Magic C18 particles (5µm, 300-Å pore size, Michrom BioResources, Auburn, CA, USA). The elution gradient comprised of a linear gradient from 5-35% of buffer B (100% acetonitrile in 0.1% formic acid) for 90 min, followed by 35-80% of buffer B for 10 min, before maintaining at 80% of buffer B for 6 min, and finally equilibrating at 95% of buffer A (0.1% formic acid in H<sub>2</sub>O) for 14 min. The nano-LC flow rate was set at 300 nl/min. The mass spectrometer was set to perform data acquisition in the positive ion mode, with a selected mass range of 300-1600 m/z. The three most abundant peptides (with +2 to +4 charge states) above a 10 count threshold were selected for tandem mass spectrometry analysis, and dynamically excluded for 30 sec with ±50mmu tolerance. The time of summation for single MS/MS event was set to maximum of 2 sec and the feature of dynamic background subtraction (DBS) was activated in the Analyst 2.0 software. The external mass calibration using Renin Substrate Tetradeca-peptide (Porcine, Mw = 1757.9) (Sigma-Aldrich, St Louis, MO, USA), also known as angiotensinogenase, was carried out everyday at the same time interval.

### 2.17.5 Data analysis

The extract\_msn programme found in Biowork 3.3 (Thermo Electron) was used to extract the spectra (in dta format) from the raw data of LTQ-FT ultra or LTQ-Orbitrap XL. These dta files were then compiled into a mascot generic file (mgf) using an in-house written programme (by Prof. Siu Kwan Sze), in order to convert MH<sup>+</sup> values to m/z ratios. Intensity values and fragment ion m/z ratios were not manipulated. This data was used to obtain protein identities by searching against the PlasmoDB *P. falciparum* protein database (version 5.4) by means of an in-house



MASCOT server (version 2.2.03) (Matrix Science, Boston, MA). In order to estimate the rate of false positives (FP), the search was performed with 'target' (forward PlasmoDB *P. falciparum* sequences) and 'decoy' (reverse PlasmoDB *P. falciparum* sequences) database as described elsewhere Elias and Gygi (2007). The search was limited to: maximum 2 missed trypsin cleavages; mass tolerances of 10ppm for peptide precursors; and 0.8 Da mass tolerance for fragment ions. Fixed modification was MMTS at cysteine residue; whereas variable modification was oxidation at methionine residue; iTRAQ labelling at N-terminal, lysine or tyrosine residues; and phosphorylation at serine, threonine or tyrosine residues. Only proteins with a MOWSE score of 42, corresponding to  $p < 0.05$  were considered significant. The peptide/protein list(s) obtained was exported to Microsoft Excel for further analysis

Raw data from QStar was searched using ProteinPilot software v2.0.1 (Applied Biosystems; MDS-Sciex). The search was carried out against a concatenated target-decoy PlasmoDB *P. falciparum* database (version 5.4), and Paragon algorithm in ProteinPilot software was used as the default search engine. Sample type was set to 4-plex iTRAQ peptide labelled. Other selected parameters were: trypsin as digestion agent; MMTS for cysteine modification; and up to 2 amino acids substitutions per peptide using BLOSUM 62 matrix. The mass tolerance was predefined in ProteinPilot software, with 0.1 Da for MS and 0.15 Da for MS/MS. Miss-cleavage was left undefined to include the possibility of more than 80 biological modifications. Proteins confidence was set at 95% confidence interval (equivalent to a ProtScore of 1.3).

## ***2. 18 Atomic force microscopy (AFM)***

Treated (10 mU/ml NM) and untreated erythrocytes were diluted in PBS and a thin smear on a glass slide (Sigma) was prepared. The smear was dried o/n under dehumidifying conditions. The AFM system utilised was a Dimension 3100 model with Nanoscope IIIa controller (Veeco, Santa Barbara, CA). A triangular cantilever

(200 mm long and 18 mm wide arms) (Model ORC8, Veeco) with tip radius of curvature less than 20 nm and a spring constant of 0.05 N/m. Both height and deflection images were captured at a resolution of 512×512 and scan rate of 0.5 to 2 Hz depending on the scan scale which can range from tens of microns to hundreds of nanometres. All the AFM scanning images were recorded, processed and analysed using Nanoscope 5.1 software (Veeco, Santa Barbara, CA).

## ***2. 19 Zeta potential***

The erythrocytes were treated for 1 h in different concentrations of Neuraminidase (1, 2, 5 and 10 mU/ml) as described above. The electro-kinetic potential (Zeta potential) was measured with Zetizer Nano ZS (Malvern Instruments). Around  $5 \times 10^6$  cells/ml were resuspended in 10 mM sucrose-PBS buffer and transferred to DTS1060 disposable zeta cell (Malvern instruments). The zeta potential was measured in triplicate and the mean and standard deviation was calculated.

## ***2. 20 Erythrocyte deformability measurements***

All measurements were performed by Heng Li Tze (Division of Bioengineering, Nanyang Technological University, Singapore). Erythrocyte deformability was analysed using RheoSCan-D200 (SEWTON Meditech, Inc.). Various shear stresses of diffraction of erythrocytes in a microchannel were measured based on the microfluid and laser-diffraction method. The detection was performed with a CCD camera and in all measurements a RSD-K01 disposable kit was used.  $1 \times 10^6$  erythrocytes were suspended in 600 µl of Rheoscan-D RSD-P01 medium. Measurements were performed in duplicate. The deformability of untreated erythrocytes was used as a reference. The cell suspension was transferred into the ektacytometer chamber, and cell deformability was determined at 37°C. Cell deformability was assessed by calculating the elongation index (EI) at shear stresses ranging from 0 to 20 Pa. EI determined at various shear stresses represents the degree of elongation of erythrocytes at the corresponding shear force.

## **2. 21 Chromosomal distribution of genes**

The accession numbers for 61 up-regulated proteins were pasted into Acuity software using distribution blot and the location of all 61 genes on 14 chromosomes of *P. falciparum* extracted.

## **2. 22 Motif search in untranslated region**

Sequences upstream and downstream of the coding region of 88 differentially expressed proteins were downloaded from PlasmoDB ([www.plasmodb.org](http://www.plasmodb.org)). For 5'UTR 1000 nt upstream ATG codon and for 3'UTR 500 nt downstream the stop codon were retrieved. Untranslated regions were considered only on the encoded DNA strand. All sequences used are in the 5' to 3' orientation. The sequences were uploaded into MEME (multiple Em for motif elicitation) <http://meme.sdsc.edu/meme/meme.html> (Bailey and Elkan, 1994). MEME is a motif search tool designed to identify over-represented motifs in any given sequence using an expectation maximisation algorithm. For the search of 61 up-regulated proteins in 5'UTR the search was split into two sets with 1 to 500 nt and 501 to 1000 nt upstream of the encoded gene, as MEME is restricted to 60,000 characters only. For the other search sets, 1000 nt upstream of the transcriptional start site were used in a MEME search. The search options were set at motif length 4 to 8 nt and maximum number of motifs to be found as 6 and search on the given strand. The distribution of the motif was set at both "any number of the repetition" and "zero or one occurrence per sequence". From the job output only motifs were regarded as significant with an E-value of  $\geq 0.1$ . The E-value gives an estimate of the likelihood one would find this motif in a similar set of random sequences. Individual motifs identified by MEME were searched in FIMO (Find individual motif occurrences, [http://meme.sdsc.edu/meme4\\_1/cgi-bin/fimo.cgi](http://meme.sdsc.edu/meme4_1/cgi-bin/fimo.cgi)) for occurrences in untranslated regions in other sets of genes (down-regulated and no expression change genes were searched for motifs in up-regulated genes; up-



regulated and no expression change for motifs identified in down-regulated genes). The  $p$ -value output threshold was set as 0.0001.

The input matrix from MEME for each individual motif was search against a database of target motifs (Jaspar core) in TOMTOM ([http://meme.nbcr.net/meme4\\_1/cgi-bin/tomtom.cgi](http://meme.nbcr.net/meme4_1/cgi-bin/tomtom.cgi)). The comparison function was set at Pearson correlation coefficient.

## **Chapter 3**

### **Differences in merozoite protein expression in sialic acid-dependent and -independent W2mef clones**

Different parasite clones use different invasion pathways. The W2mef clone uses sialic acid residues on the erythrocyte as receptors to bind and enter the host cell. When sialic acid is not available on the erythrocyte surface, W2mef is able to adapt to the changed conditions. Part of the molecular basis for this selection was revealed by Stubbs *et al.*, (2005). Comparison of sialic acid-dependent and -independent invasion pathways concentrated mainly on variation of transcription levels and highlighted the role of two protein families: RBL and EBL (Stubbs *et al.*, 2005, Gaur *et al.*, 2006). Both protein families are regarded as the major players during invasion pathway switching. Transcription levels of RBL and EBL have been surveyed in a number of clones. While the sialic acid-independent ligand RH-4 is transcriptionally up-regulated, the sialic acid-dependent ligand RH-1 remains at the same level in both clones (W2mef and W2mef/NM) as demonstrated by Microarray and RT-PCR data (Stubbs *et al.*, 2005, Gaur *et al.*, 2006). Protein levels were assayed by Gao *et al.*, (2008), using anti-RH-1 antiserum raised against the erythrocyte binding domain of RH-1. In this study, reduced levels of RH-1 protein expression in the W2mef/NM clone were observed via western blot analysis. Furthermore, it was demonstrated that W2mef merozoite invasion was inhibited while there was very little impact on invasion of the W2mef/NM clone. After invasion pathway switching, there was a discrepancy between the W2mef/NM RH-1 transcription and expression levels and post-transcriptional regulation may account for this observation.

Further complexity in regulation of invasion molecules was investigated by variant expression in several genes encoding members of the RhopH, EBL and RBL gene families using isogenic subclones of *P. falciparum* 3D7. It was identified that members of the EBL, Rhoph1 and RBL families are present in either an active or a

silenced state. This silencing of some members of the RhopH family was transmitted epigenetically (Cortes *et al.*, 2007). Two members of the RhopH1 complex were mutually exclusively expressed, depending on the invasion pathway used by the parasite. It is speculated that a clonal silencing of multigene invasion molecules is epigenetically controlled.

In addition, expression of invasion molecules underlies a functional hierarchy, in which some ligands are dominant over others (Baum *et al.*, 2005). The conclusion is that epigenetic and post-transcriptional mechanisms are important during control of expression in *Plasmodium* (Cortes *et al.*, 2007). Post-transcriptional mechanisms regulate protein levels and orchestrate the invasion characteristics of merozoites. Accordingly, a better understanding of regulation at the posttranscriptional level of invasion molecules is essential and has not yet been exploited in a broad spectrum.

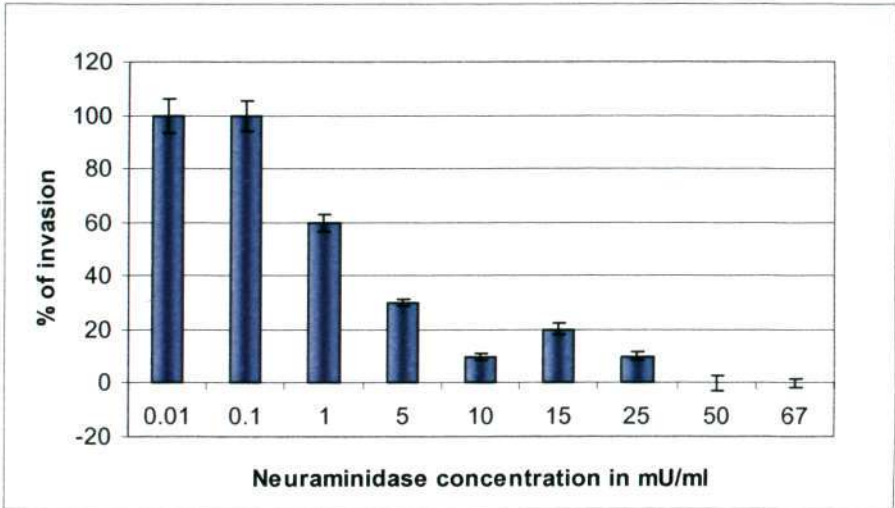
This chapter will focus on the variation in expression levels of W2mef and W2mef/NM merozoite proteins and concentrates mainly on proteins up-regulated in W2mef/NM. The “switching system” from sialic acid-dependent to sialic acid-independent of the W2mef clone is used as a tool to investigate proteomic changes during erythrocyte invasion.

## Results

### ***3.1 Adaptation of W2mef to NM-treated erythrocytes***

The sialic acid-dependent W2mef clone was selected to invade erythrocytes treated with different concentrations of NM ranging from 0.01 to 67 mU/ml (Figure 3.1). At concentrations of 0.01 and 0.1 mU/ml no effect on invasion was observed. The invasion rate gradually drops at concentrations of 1 to 10 mU/ml. The invasion efficiency stays below 20% from 10 mU/ml treated erythrocytes up to the highest concentration of 67 mU/ml NM with almost no invasion at 50 and 67 mU/ml. Thus the concentration of 10 mU/ml was used for all subsequent treatments of erythrocytes as it was the concentration with the greatest impact on W2mef invasion efficiency.

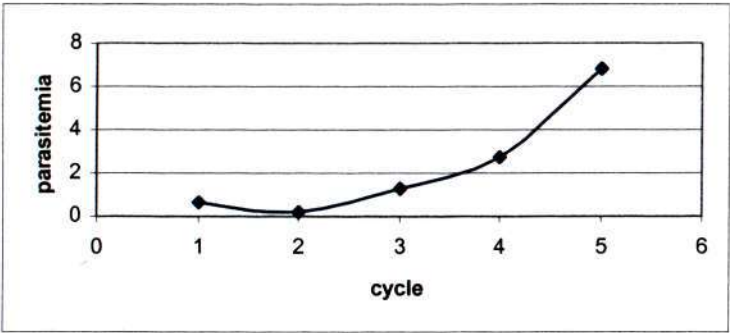




**Figure 3. 1. W2mef invasion of erythrocytes treated with different concentrations of neuraminidase.**

The percentage of invasion is relative to invasion of untreated control erythrocytes. The concentration of 10 mU/ml was used for subsequent adaptation of W2mef to grow in neuraminidase-treated erythrocytes.

After 3 cycles of growth in neuraminidase-treated erythrocytes, W2mef had adapted to the new invasion conditions and invasion rates of 3- to 6-fold higher were achieved in the subsequent invasion cycles (Figure 3.2). The adapted clone was re-named 'W2mef/NM'.



**Figure 3. 2. Adaptation of W2mef to neuraminidase-treated erythrocytes.**  
Starting material was 1% W2mef schizonts. Parasitemia after 5 invasion cycles of W2mef parasites into neuraminidase-treated erythrocytes is shown. After 3 cycles the parasite adapted to the new conditions and regained its exponential growth rate.

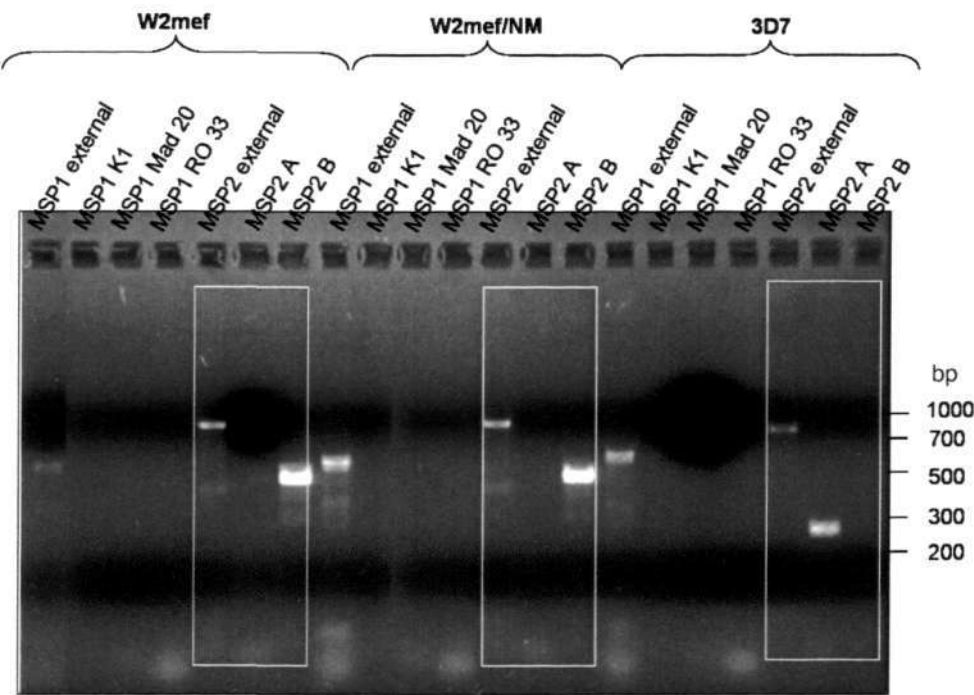


### ***3. 2 W2mef and W2mef/NM are isogenic parasites with different invasion properties***

W2mef and W2mef/NM were continuously grown over several cycles. To guarantee that over time no cross-contamination with other *P. falciparum* clones occurred, DNA was extracted on a regular basis and the genotype was analysed (Figure 3.3). The genotyping method is based on the sequence polymorphism in *mep-1* and *mep-2* genes. These polymorphisms are strain specific and are used as genetic markers to distinguish between *P. falciparum* clones (Felger *et al.*, 1993; Mercereau-Puijalon *et al.*, 1991; Snewin *et al.*, 1991; Snounou and Beck, 1998; Viriyakosol *et al.*, 1995; Wooden *et al.*, 1992). *Mep-1* contains a variable nucleotide sequence and a variable number of repeats in block 2. Three allelic families with specific differences in block 2 have been identified: K1, MAD20 and RO33. The names derive from clones where these polymorphisms were first identified. Different repeat sequence units characterise the K1 and Mad 20 whereas RO33 contains a unique sequence. Similarly, there are two MSP-2 allelic families, FC27 and 3D7, which differ in the nucleotide sequence and copy number of repeats of the central domain of the gene.

The primary PCR for MSP-1 gave a product of ~650 bp in all clones. No product was observed for the nested PCR of K1, MAD20 and RO33 for any of the three clones. MSP-2 gave a product of ~ 850 bp. No band was observed in W2mef and W2mef/NM for the MSP-2 3D7 polymorphism (MSP2A). In 3D7 clones as expected a band was observed of ~250 bp in MSP2A. W2mef and W2mef/NM showed a product migrating at ~450 bp corresponding to the FC27 allele (MSP2B) and no band was observed in 3D7.

Genotype profiles of W2mef and W2mef/NM were identical for the analysed MSP-1 and MSP-2 polymorphic regions and differ from the profile observed in 3D7 in MSP2 allelic polymorphism. No cross-contamination with 3D7 was observed.



**Figure 3. 3. PCR Genotyping of MSP-1 and MSP-2 polymorphic regions of *Plasmodium falciparum* W2mef, W2mef/NM and 3D7 clone.**

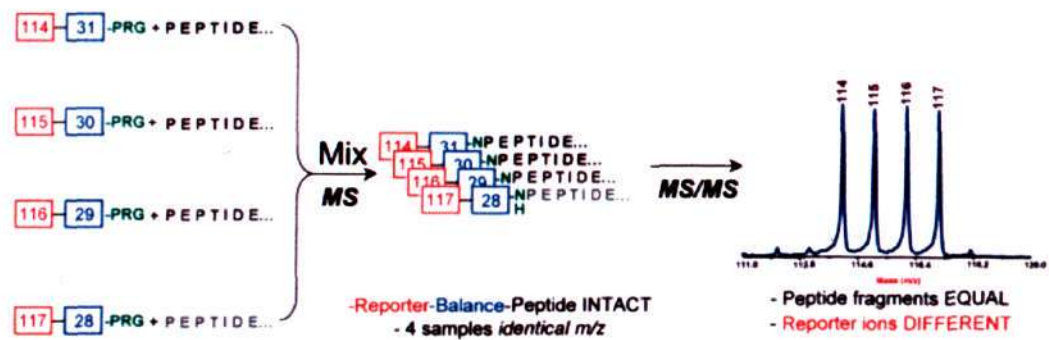
Genotyping was performed on three *P. falciparum* parasite lines W2mef, W2mef/NM and 3D7. MSP1 K1, MSP1 MAD20 and MSP1 RO33 are internal PCR for MSP-1 and MSP2A and MSP2B are internal PCR for MSP-2. The white box indicates MSP-2 external and internal PCR products. W2mef and W2mef/NM are from the same genetic background that differs from 3D7.

### ***3. 3 Proteomic iTRAQ results of differentially expressed proteins***

For proteomic studies, free merozoites were obtained from the cultures of W2mef and W2mef/NM. Merozoite proteins were extracted in Triethylammonium bicarbonate with 8M urea and quantitated to assure equal amounts of proteins in W2mef and W2mef/NM were analysed. (This extraction method is termed “Urea extraction”.)

A novel mass spectrometric-based technique to determine the relative quantitative abundance of proteins, iTRAQ (isobaric tags for relative and absolute quantitation), was used (Ross *et al.*, 2004). In brief, the basic principle of iTRAQ is the covalent linkage of each peptide with a distinctive tag which will be separated during tandem MS. The amount of such tags corresponds to the abundance of the peptide thereby allowing relative quantification and identification of the peptide from each sample. iTRAQ reagents consist of a reporter group, a balance group and a peptide reactive group (PRG) (Figure 3.4). The PRG covalently links the iTRAQ reagent isobaric tag to each lysine side chain and the NH<sub>2</sub>-terminal group of any peptide. The balance group ensures that the iTRAQ reagent-labelled peptide displays the same mass. In an experiment with four distinct reporter reagents, the mass ranges from  $m/z$  114.1 to 117.1, while the balance group ranges from 28 to 31 Da. The combined mass of the whole molecule is maintained at 145.1 Da by varying the balance group to ensure identical behaviour of tagged peptides during chromatography and in MS mode. During peptide fragmentation in the MS/MS mode, the balance moiety is lost and the four reporter tags produce a signal at  $m/z$  114.1, 115.1, 116.1 and 117.1 in the “quiet range” of the spectrum. The relative area of each peak corresponds with the proportions of the labelled peptide in the sample allowing relative quantitation and identification of each peptide. The rest of the fragment ions in the spectrum are used for peptide identification.

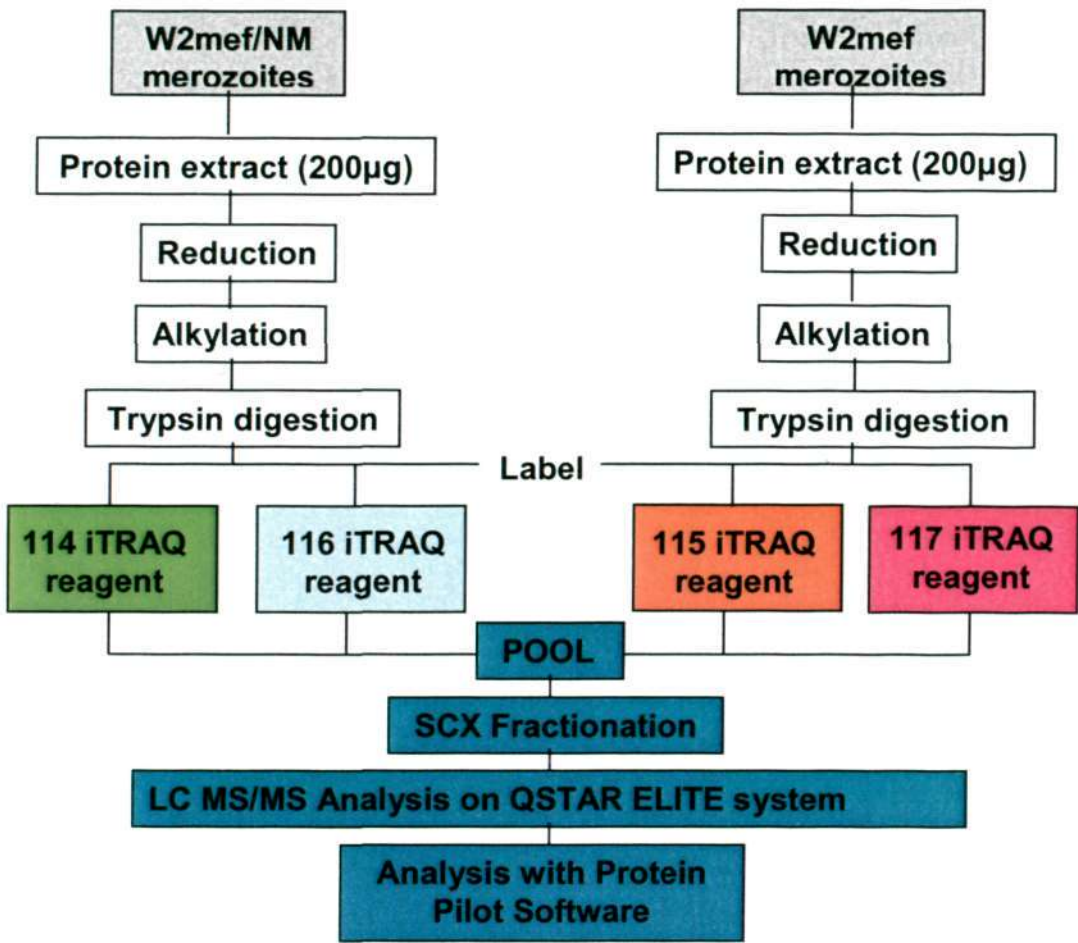




**Figure 3. 4. iTRAQ principal.**

Figure adapted from Applied Biosystems application note. Four iTRAQ reagents are used in this study. iTRAQ reagents consist of a reporter group, a balance group and a peptide reactive group. The tag is covalently bound to each lysine side chain and N-terminal group of each peptide. The balance group ensures that an iTRAQ reagent-labelled peptide displays the same mass. In the MS/MS mode the tag is cleaved and the number of separated tags reflects the amount of peptide in the sample.

Here, four iTRAQ reagents, two tags (115 and 117) of iTRAQ reagent to label W2mef and two other tags (114 and 116) to label W2mef/NM peptides were used (Figure 3.5). This gave the benefit to complete the analysis with a duplicated sample in one mass spectrometry experiment. After incubation, the four labelled peptides were combined together. This combined pool of labelled peptides was then subjected to SCX (strong cation exchange chromatography) analysis by HPLC. After SCX separation, each fraction should contain peptide from all four tags, and the amount should be equal, unless intrinsically one of the conditions contains less peptide due to a biological reason. Fractionated samples from SCX were dried and after resuspension split into three parts. For the mass spectrometric analysis triplicate measurements were performed to increase the number of distinctive peptides identified and quantified.



**Figure 3. 5. Workflow of quantitative proteomic survey of merozoite proteins using iTRAQ technology.**

All samples were treated in exactly the same manner to minimise quantitation errors introduced during the workflow. The protein samples from W2mef and W2mef/NM merozoites were solubilised, reduced and alkylated then digested with trypsin. The tryptic peptides of W2mef/NM were labelled with iTRAQ reagents 114 and 116 and peptides from W2mef labelled with 115 and 117. After the covalent iTRAQ reagent binding, the samples were pooled and separated into 20 fractions by strong cation exchange (SCX) chromatography. Each fraction was separated by 1h reversed-phase gradient and MS/MS data acquired on Q-Star elite mass spectrometer. The data was analysed for protein identification and relative quantitation by ProteinPilot software.

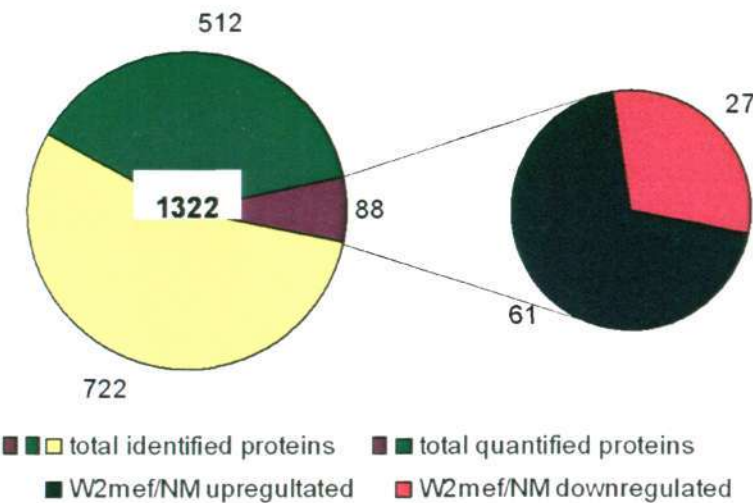


### 3.3. 1 Protein identification

Multiple peptides per protein can be labelled, allowing high confidence in quantitation of the sample during MS/MS. Protein identities were obtained by searching the PlasmoDB *P. falciparum* database (version 5.4) by means of an in-house MASCOT server (version 2.2.03). Proteins with a 95% confidence interval were used for statistical analysis. Only proteins with several peptides (or the same peptide detected multiple times) allowed statistical measurements of error factor (EF) and p-value. Only peptides with a MOWSE (molecular weight search) score of 42 (MOWSE calculates peptide masses for each entry in the database and compares them with the set of experimental data. MOWSE uses empirically determined factors to assign a statistical weight to each individual peptide match.), corresponding to a p-value<0.05 and EF of <2 were regarded as statistically reliable and were included in the analysis. An average of ~6 peptides was identified per protein.

In this proteomic study of W2mef and W2mef/NM, a total of 1322 merozoite proteins were detected using the “urea” protein extraction. Of this, fewer than 50% (600) were able to be quantitatively analysed in a reliable fashion (Figure 3.6.). 88 proteins showed differences in expression levels in W2mef and W2mef/NM.

In order to expand the analysis to membrane-bound proteins an additional protein extraction procedure was performed. The extraction method differs from the previous “urea” protein extraction method as the proteins are first extracted in carbonate-based buffer, pelleted and then solubilised in SDS-based buffer. This method has been proven to give satisfying results in other fields (Fujiki *et al.*, 1982; Molloy *et al.*, 2000; Speers *et al.*, 2007). Unfortunately, this method was not successful using *Plasmodium* merozoites and only 30 proteins with very low confidence were identified, which were also found in the previous set of “urea” extracted proteins (data not shown). Therefore, the subsequent analysis presented here focuses on the protein set identified using the “urea” extraction method.



**Figure 3. 6. Merozoite proteins identified and quantified by iTRAQ technology.**

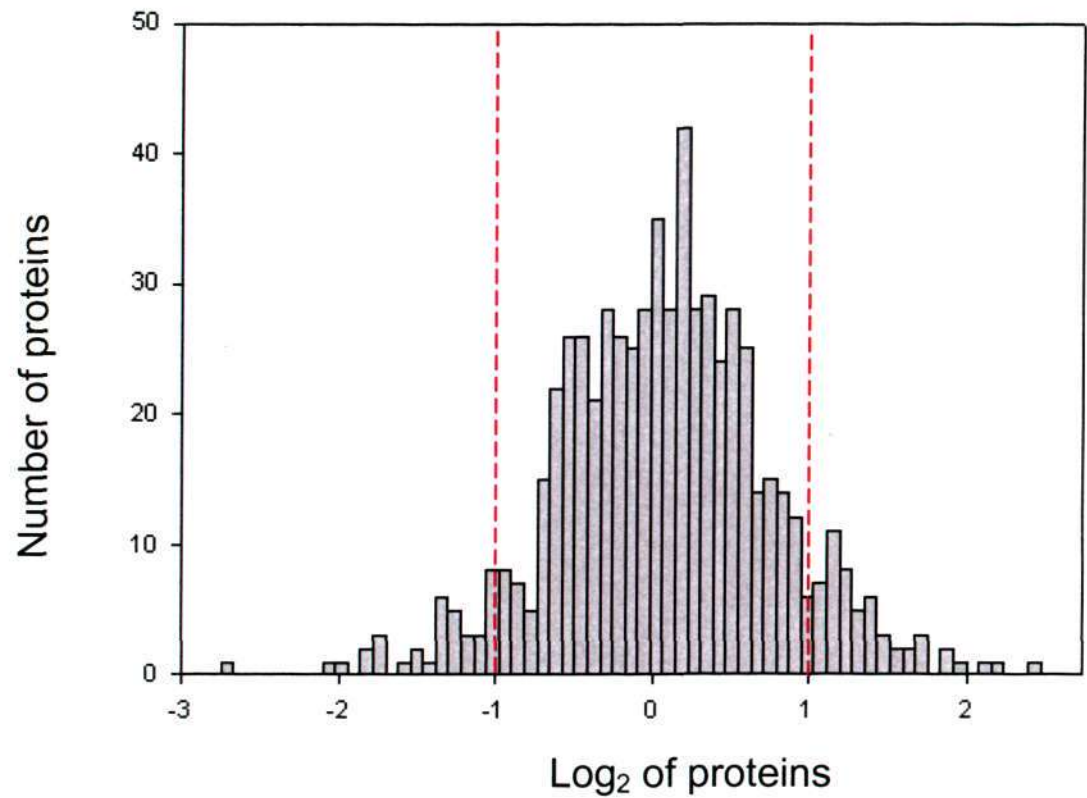
The pie diagram on the left represents all 1322 identified proteins. Of this 600 were quantitatively analysed. 88 proteins were differentially regulated, as illustrated by the pie diagram on the right with 27 up- and 61 down-regulated proteins in W2mef/NM compared to W2mef.

### 3.3. 2 Comparisons of W2mef/NM and W2mef merozoite protein expression

The histogram illustrating overall distribution of merozoite proteins is presented in Figure 3.7. The average of 114/115 and 116/115 ratios were calculated and the  $\text{Log}_2$  ratio plotted. The  $\text{Log}_2$  ratio of W2mef/NM versus W2mef protein expression levels follows a normal distribution. The biological significance for up- and down-regulation is set at a ratio of 2. This is set as a statistical cut-off for proteomic analysis in the present study. Thus only those proteins are considered that have differential expression of at least 2-fold in at least one 114/115 or 116/115 were considered as differentially expressed. The 2-fold change is a more stringent test than the confidence interval (mean plus or minus two times standard deviation) which would only result here in a cut-off of 1.6 (Chong *et al.*, 2006; Gan *et al.*, 2007). Thus the differentially expressed proteins in this set of data were identified with a high level of confidence.

The vertical red dashed lines demonstrate the 2-fold change in regulation at  $\text{Log}_2(-1)$  and  $\text{Log}_2(1)$ . Most of the 600 proteins show no change in regulation and are equally expressed in both clones. Less than 5% of all quantified merozoite proteins are down-regulated in W2mef/NM versus W2mef in their expression profile and more than 10% show significant changes and are up-regulated in W2mef/NM. The highest fold change is at  $\text{Log}_2(2.48)$  and the lowest is at  $\text{Log}_2(-2.75)$ . An overview of the differentially expressed proteins in W2mef/NM is shown in Table 3.1 for up-regulated proteins and Table 3.2 with a list of down-regulated proteins.





**Figure 3. 7. Histogram of overall protein distribution.**

The Log<sub>2</sub> ratio of W2mef/NM versus W2mef protein expression levels of 600 proteins identified by iTRAQ is plotted. The vertical red dashed lines demonstrate the 2-fold change in regulation at Log<sub>2</sub>(-1) and Log<sub>2</sub>(1). This stringent cut-off resulted in a confident data set where the proteins with > Log<sub>2</sub>(-1) demonstrate the down-regulated proteins and the proteins < Log<sub>2</sub>(1) are up-regulated proteins in W2mef/NM.

### 3.3. 3 Overview of function of differentially regulated proteins

The 88 differentially regulated proteins were found to be clustered into different biological functions (Figure 3.8). A majority of the differentially expressed proteins in W2mef/NM and W2mef are hypothetical proteins (23%). These proteins are assigned as 'hypothetical', indicating that they show no significant homology to characterized genes from any other species. The *Plasmodium* genome comprises about 65% hypothetical genes (Carlton *et al.*, 2002; Gardner *et al.*, 2002a; Hall *et al.*, 2005). Therefore it is not surprising that the majority of proteins of interest (differentially regulated) are hypothetical.

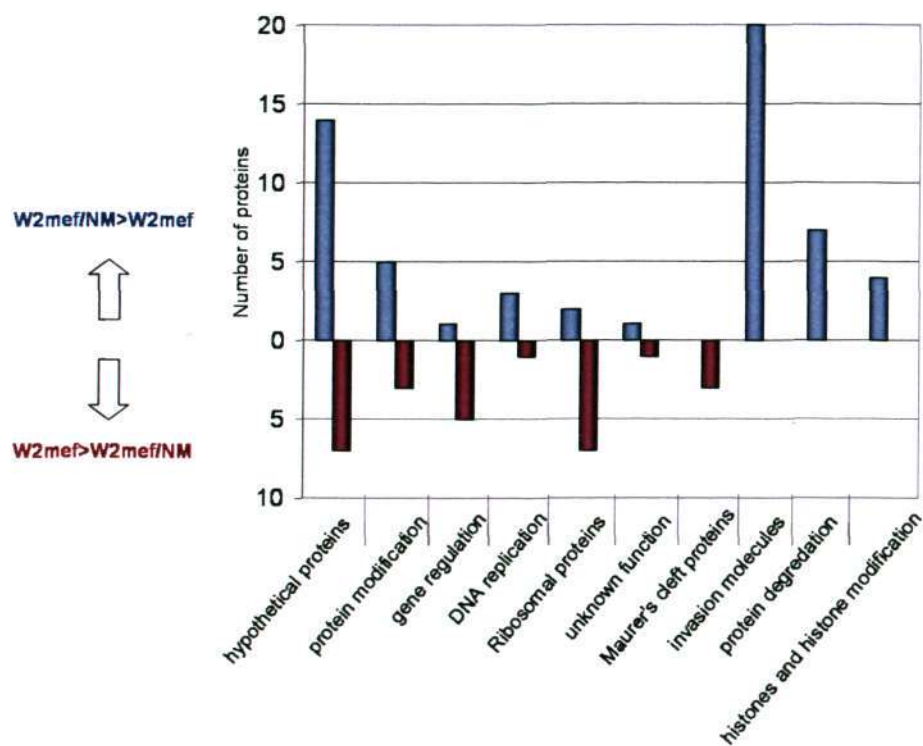


Figure 3. 8. Overview of function of differentially regulated proteins in W2mef/NM.

The 88 differentially regulated proteins were grouped according to their function. The blue bars represent the up-regulated proteins and the purple bars correspond to the down-regulated proteins in W2mef/NM.



### ***Up-regulated proteins***

#### ***Invasion Molecules***

Known invasion molecules, i.e. proteins expressed on the merozoite surface coat and apical organelles, are found to be up-regulated in W2mef/NM. These include proteins such as MSP-1 and MSP-7 from the merozoite surface coat. Both proteins form a complex on the merozoite surface. MSP-7 levels are 5.6 times higher in W2mef/NM compared to W2mef and the level of up-regulation is the greatest of all proteins detected (Table 3.1).

Proteins expressed in the rhoptry bulb are also up-regulated in W2mef/NM. These include members from HMW-complex: RhopH1 (Clag 2, Clag 3.1, Clag 3.2, and Clag 9), RhopH2, RhopH3 and LMW-complex (RAP1, RAP2 and RAP3) as well as RAMA. RhopH3 and RAP1 are known to be associated with RAMA and form a macromolecular RhopH-RAP-RAMA complex (Topolska *et al.*, 2004). This fact gives a high confidence to the data set. The exact function of this complex still needs to be elucidated, though a role during PV formation is speculated. In addition some of these proteins have an erythrocyte binding activity (RhopH3, RAP1, RAP2 and RAMA during invasion, Clag 9 during cytoadhesion) (Ling *et al.*, 2004; Ndengele *et al.*, 1995; Pinzon *et al.*, 2008; Sam-Yellowe *et al.*, 1988; Sam-Yellowe and Ndengele, 1993; Sterkers *et al.*, 2007).

RON2 (PF14\_0495) and RON5 (Mal8P1.73) are up-regulated Rhoptry neck proteins (Accession number still refers to hypothetical protein in PlasmoDB database) and RON2 is associated with AMA-1 at the moving junction (Cao *et al.*, 2008). RON2 contains a homology region with RhopH1, but it does not appear to be involved in the RhopH complex formation (Cao *et al.*, 2008). RON2 and RON5 (and also RON4) form a complex with AMA-1 in *Toxoplasma* and are involved in creation of the moving junction during invasion process (Alexander *et al.*, 2005; Alexander *et al.*, 2006; Boothroyd and Dubremetz, 2008; Lebrun *et al.*, 2005). RON5 has not yet been further characterised in *P. falciparum*.

Three members of the merozoite moving machinery are also up-regulated in W2mef/NM. These proteins are myosin A, MTIP and GAP45. These proteins associate together during the invasion process. Coronin and calmodulin are also associated with the motor complex. Coronin is an actin-interacting protein and it is associated with filamentous actin (Tardieux *et al.*, 1998). The exact role of coronin remains unclear. More is known about calmodulin. It activates protein kinase B which in turn phosphorylates GAP45 (Vaid *et al.*, 2008).

The “core molecules” of the merozoite actin-myosin motor are up-regulated and lead to the assumption that the W2mef/NM parasite requires a more powerful “driving force” than W2mef. The desialiated erythrocyte surface is altered and creates a stronger barrier for the parasite compared to normal cells. The W2mef/NM parasite has to overcome this hindrance by up-regulation of motor proteins.

### ***Protein degradation***

The proteases falcipain 3 and plasmepsin 2, and Leucine aminopeptidase have been found to be up-regulated in W2mef/NM. Leucine aminopeptidase is a haemoglobin degrading enzyme (Gardiner *et al.*, 2006). Falcipain 1, a falcipain 3 homologue, has a potential role in erythrocyte invasion and falcipain-specific inhibitors blocked erythrocyte invasion (Greenbaum *et al.*, 2002). It has been proposed that translational and post-translational mechanisms account for the activity at the merozoite stage as mRNA transcripts are only detected at the ring stage.

Furthermore, there seems to be an association between falcipain and plasmepsin. It has been shown that falcipain 2 and falcipain 3 are able to mature plasmepsin 2 *in vitro* (Drew *et al.*, 2008). Plasmepsin is a type 2 integral membrane protein and acts in the food vacuole. Its primary function is haemoglobin degradation and degradation of endosomal vesicles delivered to the digestive vacuole (Bonilla *et al.*, 2007a; Bonilla *et al.*, 2007b). Plasmepsin 1 and 2 perform the initial cleavage of hemoglobin in the digestive vacuole of the intra-erythrocytic parasite and falcipain appears to digest the fragmented form of hemoglobin. Most published literature

concentrates on hemoglobin digestion, but one study pointed out an alternative substrate for plasmepsin 2, other than hemoglobin.

Le Bonniec *et al.*, (1999) expressed the recombinant plasmepsin 2 and found that it digested native spectrin at pH 6.8. Additional experiments revealed that the cleavage occurs within the SH3 motif of the spectrin  $\alpha$ -subunit. Furthermore degradation of actin and protein 4.1R from erythrocyte ghosts was observed (Le Bonniec *et al.*, 1999). Confocal microscopy of schizont-infected erythrocytes localised plasmepsin 2 at the periphery of the mature schizonts (Le Bonniec *et al.*, 1999). This observation may suggest a role during egress.

In the present study plasmepsin and falcipain were detected at the merozoite stage. One could speculate that in addition to their function as essential genes for hemoglobin degradation, they may also degrade other host proteins such as those of the erythrocyte membrane or cytoskeleton and are therefore needed for invasion. The reason for this might be the altered erythrocyte surface (see chapter 4) and therefore the counterpart (to be degraded protein) is also changed and the parasite compensates for this change by up-regulation of degrading enzymes. Another role might be activation of other processes essential for invasion.

Interestingly, a number of proteins from the 20S core protease from the proteasome are also up-regulated in W2mef/NM. In total four proteins that are part of the 20S proteasome were detected, one from the alpha type subunit (proteasome subunit alpha type 5) and three from the beta subunit (proteasome precursor, proteasome beta subunit, 20S proteasome beta 4 subunit). Furthermore there are three additional proteasome subunits of the 20S proteasome found in the dataset but with low statistical significance (data not shown).



***Protein modification***

An increased number of protein regulatory- and histone modification molecules were observed in W2mef/NM.

A number of proteins implicated in protein modifications are found in both, up- and down-regulated proteins. Protein modification could probably account for differential regulation of the proteins found to be up- and down-regulated. Of interest is the serine/ threonine protein kinase which is responsible for protein phosphorylation. This kinase is also activated by calcium/calmodulin which may be another reason for the up-regulation of calmodulin. This kinase has not been further characterised and what substrate is used is only speculation.

A second kinase up-regulated in W2mef/NM is cAMP-dependent protein kinase (PKA). This kinase belongs to cyclic-nucleotide- and calcium/phospholipid-dependent kinase. This kinase has been further characterised (Li and Cox, 2000) and is found in other organism as a key element of the signal transduction pathway (Taylor *et al.*, 1990).

Two other protein modifying components are cyclophilin and peptidyl prolyl *cis-trans* isomerase which carry out a similar function (Bell *et al.*, 2006). Cyclophilin has a peptidyl prolyl *cis-trans* isomerase activity (Fischer *et al.*, 1989; Takahashi *et al.*, 1989) and catalyses the slow *cis-trans* isomerisation of prolyl imide bonds in peptide chains during protein folding (Fischer *et al.*, 1984). Cyclophilin may also have a function in protein-protein interactions and specifically binds proline-containing peptides. Cyclophilin is also a target for the anti-malaria drug cyclosporine (Berriman and Fairlamb, 1998).

***Histone and histone modification***

Proteins belonging to the category of histone and histone modification were found to be up-regulated in W2mef/NM. Four proteins (histone 3 putative, histone 2b and

histone 2a, high mobility group protein) are up-regulated in W2mef/NM. Alterations in histone arrangements of a gene may account for transcriptional changes. These changes are probably relevant after the invasion of the erythrocyte. High mobility group protein is a nuclear factor and is able to modify histones.

### **Metabolism**

Enolase and triose-phosphate isomerase are involved in glycolysis. Enolase is a highly abundant protein and appears in many proteomic investigations in *Plasmodium* spp. Enolase is a multifunctional protein. Its primary function is glycolysis but it has been localised on the merozoite surface and associated with cytoskeletal and membrane fractions in *P. yoelii* (Pal-Bhowmick *et al.*, 2007a). An increasing number of metabolic enzymes have been reported to have multiple functions (Moore, 2004; Sriram *et al.*, 2005).

Anti-enolase antibodies are common in sera among adults in endemic regions in eastern India. There is a correlation between the increase in anti-enolase antibodies and age, where 96% of adults were positive for anti-enolase antibodies but none of the children. Furthermore, rabbit anti-enolase antibodies inhibited *in vitro* growth of a culture of *P. falciparum* and mice immunised with rabbit anti-enolase antibodies showed protection against a challenge with the 17XL *P. yoelii* (Pal-Bhowmick *et al.*, 2007b).

It is postulated that post-translational modification (especially phosphorylation) account for the recruitment of enolase for functions other than glycolysis (Foth *et al.*, 2008; Pal-Bhowmick *et al.*, 2007b). Enolase appears to bind to actin (Schmitz *et al.*, 2005). This fact leads to the speculation that enolase may be a linker molecule between the actin-myosin motor and microneme/rhoptry adhesins, similar to the function of aldolase. Aldolase has also a glycolytic function but acts as a linker molecule between TRAP and the actin-myosin motor during erythrocyte invasion.

Thioredoxin is a peroxidase and active in the redox metabolism. This enzyme might be activated by oxidative stress. *Plasmodium* lacks the antioxidant enzymes catalase and glutathione peroxidase and thioredoxin compensates the loss.

Purine nucleotide phosphorylase is a major component of the purine metabolism.

Two proteins are involved in metabolism: triose-phosphate isomerase is active during glycolysis and in purine metabolism along with purine nucleotide phosphorylase.

### ***DNA replication***

Three proteins involved in DNA replication were found to be up-regulated in W2mef/NM: replication factor C; proliferating cell nuclear antigen; and DNA topoisomerase II.

### ***Gene regulation***

Eukaryotic initiation factor 5a (eIF5a) is conserved and an essential translation initiation factor in many organisms and possesses a unique amino acid: hypusine. Spermidine is needed to activate hypusine in eIF5a. Interestingly there are observations that suggesting eIF5a acts in post-transcriptional processing of a specific subset of mRNAs required for cell viability and efficient proliferation (Caraglia *et al.*, 2003; Park *et al.*, 1994; Rosorius *et al.*, 1999; Shi *et al.*, 1996). Furthermore, eIF5a functions as a cofactor of the human immunodeficiency virus type 1 (HIV-1) Rev mRNA transport factor (Ruhl *et al.*, 1993). In mammalian cells eIF5a can induce p53 mediated apoptosis (Li *et al.*, 2004a). Altogether these results from different species propose diverse roles for eIF5a not just translation initiation (Zanelli *et al.*, 2006).



**Table 3. 1. List of up-regulated proteins in W2mef/NM from iTRAQ**

Proteins are arranged in descending order according to their average ratio of up-regulation in both signature ion ratios 114/115 and 116/115. The yellow highlight indicates proteins used in western blot analysis.

| <b>Accessions</b> | <b>Merozoite proteins</b>                     | <b>Fold change<br/>W2mefNM/W2mef</b> |
|-------------------|---|--------------------------------------|
| PF13_0197         | Merozoite Surface Protein 7 precursor, MSP7   | 5.6                                  |
| PF11_0280         | small nuclear ribonucleoprotein F, putative   | 4.6                                  |
| PFF0860c          | histone h2a                                   | 4.3                                  |
| PFL2225w          | myosin A tail domain interacting protein MTIP | 3.9                                  |
| PFI1545c          | proteasome precursor, putative                | 3.6                                  |
| PF11_0162         | falcipain-3                                   | 3.3                                  |
| PF07_0112         | proteasome subunit alpha type 5, putative     | 3.2                                  |
| MAL7P1.208        | rho-try-associated membrane antigen, RAMA     | 3.2                                  |
| PF10_0119         | hypothetical protein                          | 3.1                                  |
| PF14_0323         | calmodulin                                    | 3                                    |
| PF14_0545         | thioredoxin                                   | 2.9                                  |
| PF10_0170         | hypothetical protein                          | 2.9                                  |
| PF13_0185         | histone h3, putative                          | 2.8                                  |
| PFI1475w          | merozoite surface protein 1, precursor        | 2.8                                  |
| PF13_0233         | myosin a                                      | 2.7                                  |
| PFE1285w          | hypothetical protein, conserved               | 2.7                                  |
| PFB0680w          | hypothetical protein                          | 2.6                                  |
| PF10_0039         | hypothetical protein                          | 2.6                                  |
| PFL2460w          | coronin                                       | 2.6                                  |
| MAL8P1.72         | high mobility group protein, putative         | 2.6                                  |
| PFD0295c          | apical sushi protein, ASP                     | 2.5                                  |
| PFE0080c          | rho-try-associated protein 2, RAP2            | 2.5                                  |
| PFI0265c          | RhopH3  | 2.5                                  |
| PF10_0155         | enolase                                       | 2.4                                  |
| PFL1090w          | gliding-associated protein 45, GAP45 putative | 2.4                                  |
| PF14_0607         | hypothetical protein                          | 2.4                                  |
| PFE0660c          | purine nucleotide phosphorylase, putative     | 2.3                                  |
| PFC0110w          | Cytoadherence linked asexual protein 3.1      | 2.3                                  |
| PF11_0164         | peptidyl-prolyl cis-trans isomerase           | 2.3                                  |
| PFC0120w          | Cytoadherence linked asexual protein, 3.2     | 2.3                                  |
| PFI1445w          | High molecular weight rho-try protein-2       | 2.3                                  |
| PF14_0146         | ribonucleoprotein, putative                   | 2.3                                  |
| PF14_0102         | rho-try-associated protein 1, RAP1            | 2.3                                  |
| MAL8P1.142        | proteasome beta-subunit                       | 2.3                                  |
| PFL0210c          | eukaryotic initiation factor 5a, putative     | 2.2                                  |
| PF14_0378         | triose-phosphate isomerase                    | 2.2                                  |

## Chapter 3

## Results

|            |  |      |
|------------|--|------|
| PF14_0077  | plasmepsin 2                                     | 2.2  |
| PF14_0660  | hypothetical protein                             | 2.2  |
| PF14_0439  | leucine aminopeptidase, putative                 | 2.2  |
| PFL1945c   | early transcribed membrane protein 12, ETRAMP12  | 2.2  |
| PF11_0287  | hypothetical protein                             | 2.2  |
| PFB0935w   | cytoadherence linked asexual protein 2           | 2.2  |
| PF13_0058  | RNA-binding protein, putative                    | 2.2  |
| PF14_0578  | hypothetical protein                             | 2.1  |
| PF14_0495  | hypothetical protein, RON2                       | 2.1  |
| PF11_0062  | histone H2B                                      | 2.1  |
| PFA0210c   | hypothetical protein, conserved                  | 2.1  |
| PF14_0527  | hypothetical protein                             | 2.1  |
| PFD0720w   | hypothetical protein, conserved                  | 2    |
| PF13_0328  | proliferating cell nuclear antigen               | 2    |
| PFE0075c   | rhoptry-associated protein 3, RAP3               | 2    |
| PF14_0316  | DNA topoisomerase II, putative                   | 2    |
| MAL8P1.73  | hypothetical protein, conserved, RON5            | 2    |
| PFC0975c   | cyclophilin, peptidyl-prolyl cis-trans isomerase | 2    |
| PFI1685w   | cAMP-dependent protein kinase catalytic subunit  | 1.9* |
| PF14_0676  | 20S proteasome beta 4 subunit, putative          | 1.9* |
| PFI1730w   | cytoadherence linked asexual protein 9(CLAG9)    | 1.9* |
| PF14_0476  | serine/threonine protein kinase, putative        | 1.9* |
| PF14_0257  | hypothetical protein, conserved                  | 1.8* |
| MAL7P1.119 | hypothetical protein, conserved                  | 1.8* |
| PFB0840w   | replication factor C, subunit 2                  | 1.8* |

\*The 7 proteins with lower than 2-fold ratios were taken into account because they had significant 2-fold up-regulation in one of the signature ion ratios.



### ***Down-regulated proteins***

#### ***Maurer's cleft proteins***

Surprisingly, proteins which are usually associated with Maurer's clefts were found to be down-regulated. The merozoite proteomic data set from Le Roch *et al.*, (2003b) detected these proteins in merozoites as well. This finding indicates that they have potentially multiple roles during the parasite asexual life cycle. These proteins are SBP1 (skeleton binding protein 1), MAHRP-1 (membrane associated histidine rich protein 1) and REX (ring exported protein). A potential role of these proteins might be to establish the transport mechanism for the young parasite and therefore they are needed early on during the intra-erythrocytic cycle.

#### ***Gene regulation***

Spermidine synthetase is also down-regulated in W2mef/NM. This is a regulatory enzyme that activates eIF5A.

Further regulatory proteins that were found to be down-regulated are: cleavage and polyadenylation specificity factor protein; eIF3 subunit 10; and eIF3 37.28 kDa subunit.

#### ***Merozoite apical prominence***

PF70 protein is a merozoite protein expressed at the apical pole (Ma *et al.*, 1996) and the exact function is not known.

#### ***Ribosomes***

Ribosomal RNA (rRNA) has an active role in the assembly, structure, and interaction of the ribosomal subunits and a direct role in performing and directing protein synthesis (Brimacombe, 1992; Holmberg *et al.*, 1994; Mitchell *et al.*, 1992; Noller, 1991; Noller *et al.*, 1992).

Ribosomal proteins from 40S and 60S subunit are predominantly down-regulated in W2mef/NM. At this point the reason for this observation is unclear. It has been reported in yeast that degradation of mature ribosomes can be regulated by a



mechanism termed ribophagy due to starvation (Kraft *et al.*, 2008). This selective mechanism involves the ubiquitin-proteasome pathway for degradation of the ribosomes. The post-translational modification targets the ribosome with ubiquitin; this modified protein will be recognised and ultimately targeted by the proteasome. Any form of nutritional starvation in W2mef/NM could be ruled out since W2mef and W2mef/NM were cultured and treated the same way.

In addition the merozoite pellets used for iTRAQ were derived from multiple flasks which were continuously cultured. Down regulation of ribosomes may be a result of stress response of the merozoite during schizont extraction. Extraction takes some time and could create stress in the merozoite which could potentially result in targeting ribosomes to ubiquitination and degradation. However, other stress factors such as heat shock proteins were not up-regulated indicating that stress related reaction of the parasite can be excluded.

The observation by Kraft *et al.*, (2008) may implicate that a selective regulatory mechanism exists which can adjust the requirements for the cell. Elimination of ribosomal subunits limits the ribosomal biogenesis and protein translation. Overall, W2mef/NM has more protein being generated for the invasion process and these ribosomes were needed for biosynthesis of all the up-regulated proteins. Thus after the required proteins have been made, the system may eradicate the ribosomes because they are no longer required. As parts of the proteasome are up-regulated in W2mef/NM it can be speculated that one of the reasons the proteasome machinery is up-regulated, is to degrade excess ribosomes and regulate in this way the number of ribosomes per cell.

Stage-specific transcription of rRNA has been observed for the rodent malaria parasite *P. berghei*. This mechanism allows the parasite to reduce the total number of gene units that are simultaneously active (Gunderson *et al.*, 1987; McCutchan *et al.*, 1988; McCutchan *et al.*, 1995; Rogers *et al.*, 1996; Thompson *et al.*, 1999). Based on the differences in expression pattern and nucleotide sequence of the rRNA

gene units the existence of three types of structurally different ribosomes in *Plasmodium* has been postulated. The A-type ribosomes are present in the liver and blood stages of the parasite, and the O- and S-type ribosomes are the predominant types produced during development in the mosquito (Li *et al.*, 1997). Due to the structural differences among the distinct rRNA types, it is hypothesised that they are also functionally different (Gunderson *et al.*, 1987; Rogers *et al.*, 1996; Thompson *et al.*, 1999). The reason for this phenomenon of differential rRNA transcription is not clear. It is postulated that these different rRNA offer the parasite the another mechanism of post-transcriptional control of protein expression (Dame and McCutchan, 1984; Gunderson *et al.*, 1987; Thompson *et al.*, 1999). The expression of different ribosomes gives the parasite the chance to change a life cycle stage by controlling the population of messenger rRNAs that are translated.

Otherwise, through upholding of functionally diverse ribosome types, the parasite can express proteins at an optimum level during growth in the different host at different life cycle stages. It is assumed that structurally distinct ribosomes may simply result from the different accessibility of the genome to the transcriptional apparatus during different stages of the life cycle. Unfortunately, direct evidence for the existence of functional differences among the three ribosome types of *Plasmodium* is still missing.

In summary this proteomic investigation of W2mef and W2mef/NM merozoite proteins produced a robust data set and identified many functionally diverse proteins. Many of these identified proteins exist in complexes and the similar expression patterns of these proteins supports the accuracy of this data set. Other abundant proteins like AMA-1 and actin are present in equal levels in both clones providing further evidence for a robust dataset.

**Table 3. 2. List of down-regulated proteins in W2mef/NM from iTRAQ**

Proteins are arranged in descending order according to their average ratio of down-regulation in both signature ion ratios 114/115 and 116/115.

| Accessions | Merozoite proteins   | Fold change<br>W2mefNM/W<br>2mef |
|------------|--|----------------------------------|
| PFC0290w   | 40S ribosomal protein S23, putative                                    | -10.6                            |
| PFE0185c   | 60S ribosomal subunit protein L31, putative                            | -4.1                             |
| PF11_0043  | 60S acidic ribosomal protein p1, putative                              | -4.0                             |
| MAL8P1.95  | hypothetical protein, conserved  | -3.5                             |
| PFF0700c   | 60S ribosomal protein L19, putative                                    | -3.3                             |
| PF14_0434  | hypothetical protein   | -2.9                             |
| PF13_0170  | glutaminyl-tRNA synthetase, putative                                   | -2.7                             |
| PFE0065w   | skeleton binding protein1, PfSBP1                                      | -2.7                             |
| MAL13P1.41 | membrane associated histidine-rich protein, MAHRP-1                    | -2.6                             |
| PFD0770c   | ribosomal protein l15, putative  | -2.5                             |
| PFI1735c   | ring exported protein, REX   | -2.5                             |
| PF13_0095  | DNA replication licensing factor mcm4-related                          | -2.5                             |
| PF14_0579  | ribosomal protein L27, putative  | -2.4                             |
| PFD0090c   | hypothetical protein, conserved in <i>P.falciparum</i>                 | -2.4                             |
| MAL7P1.81  | Eukaryotic translation initiation factor 3 37.28 kDa subunit, putative | -2.4                             |
| PF13_0224  | 60S ribosomal subunit protein L18, putative                            | -2.4                             |
| PF10_0025  | PF70 protein   | -2.3                             |
| PFI1780w   | hypothetical protein   | -2.1                             |
| PF14_0364  | cleavage and polyadenylation specifity factor protein, putative        | -2.0                             |
| PF08_0074  | DNA/RNA-binding protein Alba, putative                                 | -2.0                             |
| PF10_0242  | hypothetical protein   | -2.0                             |
| PFI1740c   | hypothetical protein   | -2.0                             |
| PF13_0232  | Casein kinase II regulatory subunit, putative                          | -1.9*                            |
| PF07_0007  | hypothetical protein, conserved  | -1.9*                            |
| PFA0555c   | UMP-CMP kinase, putative   | -1.9*                            |
| PF11_0301  | spermidine synthase  | -1.9*                            |
| PFL0625c   | Eukaryotic translation initiation factor 3 subunit 10, putative        | -1.8*                            |

\*Five proteins with lower than 2-fold ratios where taken into account because they had significant 2-fold up-regulation in one of the signature ion ratios.



### ***3. 4 Validation of iTRAQ results by an alternative method***

The quantitative results derived from the iTRAQ approach were verified by semi-quantitative western blot analysis. Several proteins were selected where antibodies against these antigens were available. RhopH2, Clag3.1, MSP7, MSP-1<sub>19</sub>, MTIP, GAP45, Plasmepsin 2, Histone 3 and eIF5A (Figure 3.9) were tested and confirmed the up-regulation of these proteins in W2mef/NM. W2mef and W2mef/NM merozoites were lysed in 2 times SDS buffer and applied to a gel appropriate for each protein. The same lysate was used for all western blot analyses. According to the iTRAQ method actin is equally expressed in W2mef and W2mef/NM and was subsequently used as a control protein to assure equal loading of W2mef and W2mef/NM merozoite lysates in western blot analysis. All selected proteins were more up-regulated than 2-fold in both iTRAQ reagents 114 and 116 (both tags used for W2mef/NM peptides) and showed a significant stronger signal in W2mef/NM in the semi-quantitative immunoblot experiment.

#### **3.4. 1 Proteins belonging to the moving machinery GAP45 and MTIP**

GAP45 showed a significantly stronger signal in W2mef/NM compared to W2mef. The fold change observed using the iTRAQ technology was 2.4 but visual observation of the western blot suggests a much more significant difference in W2mef/NM versus W2mef.

MTIP revealed a faint signal in W2mef and an approximately 2-4 times stronger signal in W2mef/NM. ITRAQ technology detected 3.9 fold more protein in W2mef/NM versus W2mef.

### 3.4. 2 RhopH2 and clag3.1 and merozoite surface proteins

A very strong signal is detected for RhopH2 in W2mef/NM. This signal is much more intense than the 2.3-fold change detected with iTRAQ. W2mef/NM signal in western blot is significantly stronger than the signal detected for W2mef.

Clag 3.1 belongs to the complex of RhopH1 and the expected size of this complex is around 140kDa. At that size only a weak band for W2mef and a stronger band for W2mef/NM were detected.

MSP-1<sub>19</sub> has 2.8 fold more protein in W2mef/NM than in W2mef measured by iTRAQ technology. Immunoblot analysis revealed that there is much more protein in W2mef/NM than in W2mef with a very faint band in W2mef and a strong band in W2mef/NM.

The greatest level of up-regulation in W2mef/NM as detected by iTRAQ technology was found in protein MSP-7 with a 5.6-fold increase. Western blot analysis confirmed this. There is almost no signal detected in W2mef but a strong signal is observed in W2mef/NM lysate.

### 3.4. 3 Plasmepsin 2 and histone 3 and eIF5A

Plasmepsin 2 is 2.2 fold more abundant in W2mef/NM analysed with iTRAQ technology. The signal in western blot analysis is very strong in W2mef/NM and almost no visual signal is detectable in W2mef with anti-plasmepsin 2 antibody.

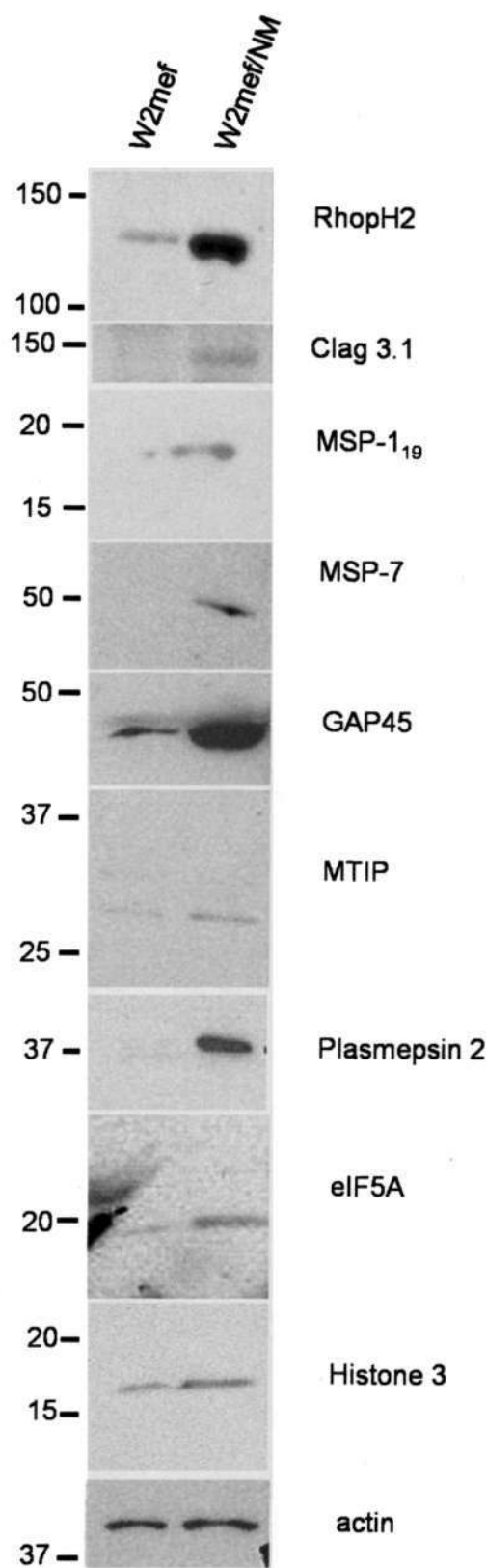
Histone 3 has a 2.8 fold higher expression measured with iTRAQ and the western blot analyses revealed a similar expression pattern for W2mef and W2mef/NM.

For eIF5A is a 2.2 fold change higher expression in W2mef/NM observed. This is also confirmed by western blot analysis with roughly 2 to 3 fold stronger signal in W2mef/NM parasite lysate with the protein detected at ~19kDa. Originally this

antibody has been raised against eIF5A from tobacco plant (Chamot and Kuhlemeier, 1992a). This antibody has been recently used in *P. falciparum* to detect isoforms of this protein in two dimensional gel electrophoresis 2D gels (Foth *et al.*, 2008). The anti-eIF5A antibody showed some unspecific signals in the corner of the western blot.

A new batch of anti-actin antibody from Sigma was purchased but did not give satisfying results. Without a reliable control the western blots for the down-regulated proteins could not be pursued.





**Figure 3. 9. Validation of differential expression of selected proteins by western blot.**

The increased expression of eight proteins (determined to be up-regulated in W2mef/NM using the iTRAQ system) was validated by semi-quantitative immunoblot with specific antibodies against these merozoite proteins. Actin is used as a loading control.

## Discussion

ITRAQ is a powerful quantitative proteomic technique and is commonly employed to study large-scale proteomic changes in diverse biological systems (Ross *et al.*, 2004). This novel technique has been used here for the first time on the malaria asexual developmental stage. The present study demonstrates that iTRAQ technology is well suited to generate comparative quantitative proteomic data from *P. falciparum* merozoites displaying changes in their invasion properties. This dataset identified 1322 merozoite proteins with 600 quantitatively assessed. Proteins exhibiting more than a 2-fold change in at least one of the signature ion ratios 114/115 or 116/115 were considered to be differentially expressed and resulted in identification of 88 proteins. The majority of the 88 proteins: 61 proteins were found to be up-regulated in W2mef/NM and 27 proteins were down-regulated in W2mef/NM compared to W2mef.

The proteomic data obtained here was analysed in two ways: firstly by quantitative comparison of W2mef and W2mef/NM proteins, and secondly through comparison of proteomic differences in mRNA data obtained by microarray. The latter will be discussed in detail in Chapter 5. Therefore, it was important to choose the best suited proteomic approach which will allow both types of analyses to be performed. Presently, there are three quantitative shotgun proteomic approaches available: stable isotope labelling or ICAT (isotope-coded affinity tags); SILAC (stable isotope labelling with amino acids in cell culture); and the proteomic approach used in this study - iTRAQ.

In brief, SILAC cell cultures are subjected to media containing isotopically labelled amino acids and heavy and light label are incorporated into the synthesised proteins by the cell. The advantage of this method is that there is no risk of contaminating the sample during protein extraction as the proteins are already labelled (Ong *et al.*, 2002).

ICAT uses light and heavy ICAT reagents that label all cysteines in a protein (Gygi *et al.*, 1999). The ICAT principle is similar to that of iTRAQ. ICAT consists of 3 elements: a reactive group, which labels specifically cysteine; an isotopically coded linker; and a biotin tag that is utilised during affinity isolation of labelled proteins/peptides. Two samples can be simultaneously analysed by labelling with light and heavy ICAT reagents. After labelling, the proteins are combined and subjected to trypsin digestion and subsequent avidin affinity chromatography to isolate peptides labelled with isotope-coded tagging reagents.

MudPIT technology provides another alternative to SILAC, ICAT and iTRAQ, although it is considered to be semi-quantitative. This method uses the combination of two liquid chromatography steps: strong cationic exchange and reverse-phase high performance liquid chromatography. After the second chromatography, the sample is directly sprayed into the mass spectrometer for analysis. Comparative protein profiling can be achieved by metabolic labelling with  $^{14}\text{N}$  and  $^{15}\text{N}$ . The drawback of this technique is that MudPIT data alone is only semi-quantitative. The number of peptides detected and identified is relative to the actual protein abundance within the cell (Washburn *et al.*, 2001). The data thus needs to be adjusted by the software to get quantitative results. Therefore MudPIT alone is not optimal for a comparison utilising mRNA data.

A gel-based technique to quantitate expression differences is two-dimensional difference gel electrophoresis (2D-DIGE) (Unlu *et al.*, 1997). This technique utilises fluorescent synthetic N-hydroxysuccinimidyl (NHS) ester derivatives of the cyanine dyes (Cy 2, Cy3, Cy5) to label protein samples. These dyes react with primary amine groups (N-terminal  $\alpha$ -amino and lysine  $\epsilon$ -amino groups) in the target protein. The first dimension separates proteins by isoelectric point and the second dimension by molecular weight. Gels are then scanned and analysed for differences with specific software.



The disadvantages of the 2D approach are that it is not very suitable for hydrophobic proteins, especially high molecular weight proteins, as the SDS-containing sample is incompatible with the first dimension of the 2DE procedure. Furthermore it appears that there is a bias in identifying more abundant proteins. To overcome this, it has become the trend to enrich for diverse fractions or pre-treat the sample to increase the number of spots on the gel. In addition, resolution is a key issue. The resolution of spots is weaker towards the edges of the gel than in the centre. Using narrow-range pH gradients for the first dimension expands the resolution in the given range but increases the number of gels required to cover the entire pH range (Di Luccia *et al.*, 1991; Esteve-Romero *et al.*, 1996).

The gel format is also crucial to the resolution, in general the larger the gel the better the proteins are resolved. Reproducibility is another key issue in 2D gel proteomics. 2D gel analysis is very complex and involves numerous technically difficult steps. Variability between experiments introduces artefacts to the final result. Fluorescent labelling with CyDyes is very sensitive and can cause high background signal by detection of contaminants such as dust or residues on the casting glass plates. Some proteins appear to be preferentially labelled with one CyDye over another regardless of structural similarity, identical reactive groups and labelling under denaturing conditions (Krogh *et al.*, 2007). Lastly, the identification of the protein spot of interest must be performed by mass spectrometry. This step in itself requires strict, almost sterile conditions to limit sample contamination while also ensuring that the spot should contain a sufficient amount of protein to enable identification. The DIGE is a minimal label technique where the labelled minority and unlabelled majority of a protein do not exactly co-migrate. Therefore the gels need to be post stained with a general protein staining technique (e.g. SybroRuby). Despite these disadvantages, it is a very visual method and only those proteins with quantitative differences would be necessary to identify.

A major advantage of the iTRAQ strategy over the other proteomic strategies is that the identification and quantification occur at the same point during MS-mode, resulting in higher confidence in peptide identification and quantitation.

An advantage of iTRAQ is that multiple samples can be analysed simultaneously. Here, two samples were compared in the same mass spectrometric experiment and thus can be compared for quantitative differences. As well as reducing the time needed for mass spectrometry analysis it also negates day-to-day experimental variation.

One study compared the substrate discovery rate of ICAT versus iTRAQ (Dean and Overall, 2007). ITRAQ technology identified 9 times more proteins than ICAT. This could be explained by the fact that the b and y ions derived from peptides labelled with the four iTRAQ reagents are identical. The MS/MS intensity is higher and results in higher confidence in peptide identification. In comparison, ICAT and SILAC MS/MS spectra for differentially labelled peptides are acquired independently.

On the other hand, during treatment and analysis of a highly complex sample containing many abundant proteins, the majority of the iTRAQ or ICAT label will bind the proteins in higher abundance, leaving considerable fewer labels for tagging less abundant proteins.

The major advantage of SILAC is that 100% incorporation of the labelled amino acid is observed whereas chemical labelling during ICAT and iTRAQ can only result in a maximum labelling of 95 % of proteins.

Overall, due to the high confidence in peptide quantification and easy handling, iTRAQ appeared to be the best suited technique for the present study and did indeed result in a representative and quantitative dataset.

Proteomic studies in the context of invasion and invasion pathway switching have typically relied on a “protein by protein approach” to investigate proteomic changes among candidates identified by transcriptional methods (Stubbs *et al.*, 2005, Gaur *et al.*, 2006, Cortes *et al.*, 2007). This strategy has resulted in a very limited set of identified proteins. The objective of the present work was to identify and quantify as many merozoite proteins as possible and reveal differences in protein expression between sialic acid-dependent and -independent W2mef clones. As a result none of the proteins we have identified as differentially regulated have previously been associated with invasion pathway switching. Only the *clag* gene family was found to be mutually expressed in different 3D7 subclones but this occurs at the transcriptional level (Cortes *et al.*, 2007).

It is somewhat surprising that more members of the RBL family were not identified in this study as they are directly implicated in invasion pathway switching. Only one peptide of RH-1 protein was identified, however, the change protein in expression was not significant, in contrast to a western blot analysis reported by Gao *et al.*, (2008).

Given that hydrophobic proteins are especially difficult to solubilise and extract, the first step in proteomics is to apply the best suited protein extraction method to the sample to solubilise as many proteins as possible. Merozoite pellets were subjected to the urea extraction method. 8M urea buffer is usually used to solubilise proteins prior 2DE. The 8M urea does interfere with trypsin digestion and the sample therefore needed to be diluted to a trypsin-tolerable concentration of 1M urea. An alternative protein extraction method was also tested, based on a carbonate-SDS procedure. The aim was to solubilise difficult proteins where the 8M urea method was not effective. The carbonate-extraction method has been applied to solubilise membrane-bound proteins in many other organisms (Fujiki *et al.*, 1982; Molloy *et al.*, 2000; Speers *et al.*, 2007). Unfortunately, upon repeated attempts this method only resulted in extraction and solubilisation of a small number of proteins already present in the set of 8M urea solubilised proteins.



There are several reports outlining the difficulties encountered in extracting merozoite proteins and various extraction methods have been applied. Florens *et al.*, (2002) used a semi-quantitative approach to study all developmental stages in *P. falciparum* and found 839 proteins in the merozoite stage. Gelhaus *et al.*, (2005) visualised merozoite stage proteins by 2DE and analysed 132 proteins. After identification by mass spectrometry 94 proteins were identified as parasite-specific (Gelhaus *et al.*, 2005).

Previous studies also failed to detect RBL members in various extraction methods. Florens *et al.*, (2002) were unable to detect any of the RBLs in merozoites, but identified RH-3 in sporozoites. One study analysed proteins from rhoptry-enriched fractions of three rodent malaria parasites (Sam-Yellowe *et al.*, 2004). Even here, Rhoptry bulb proteins such as RhopH 1- and 2-related proteins were detected but this study failed to detect RAMA, clag 9 and Rhoptry neck proteins such as Py235 which is an RBL homologue in rodent malaria parasite. The reason why it is so difficult to detect RBL proteins is still unclear. Only one member of the EBL family was quantitatively evaluated, EBA 181, with virtually identical quantities detected in W2mef and W2mef/NM extracts.

Not only is the protein extraction method critical, also the process of merozoite isolation is a crucial step for reaching maximum protein extraction. During the preparation of the merozoite pellet, it is possible that some contamination from erythrocyte cell debris or parasites or some premature egression of parasites occurred. Furthermore the process of merozoite collection takes around 25 min. By the time the merozoite is extracted from the culture it may have lost viability and thus the ability to invade erythrocytes. It is possible that protein loss due to degradation takes already place in these unviable merozoites. Once free merozoites were seen on the Giemsa-stained smear, the centrifugation and membrane filtration were performed. The “freshness” of these merozoites is not known. It could be that these merozoites had just ruptured, or the fragile mature schizont ruptured during

the centrifugation process and released even “fresher” merozoites. The invasion of the erythrocyte by the *P. falciparum* merozoite *in vitro* takes on average ~1 min (Gilson and Crabb, 2009). The viability of merozoites likely lasts only a few minutes. It is feasible that the merozoites did not survive the extraction method and degradation and lysis had already begun. This scenario may have occurred more often during W2mef/NM extraction as it contains more proteasome components. But this is not the case as the majority (~85%) of quantified proteins in W2mef and W2mef/NM do not change their expression levels and no overall reduction of proteins was observed. In addition differential expression was observed in specific sets of proteins which many of them can be associated with invasion.

Previous studies on merozoite proteomics identified novel proteins of a rhoptry-enriched fraction from three rodent parasites by MudPIT (Multidimensional Protein Identification Technology) (Sam-Yellowe *et al.*, 2004).

Two other investigations on merozoite proteins used distinctive parts of a *P. falciparum* late schizont preparation to identify GPI-anchored membrane proteins also using MudPIT technology (Sanders *et al.*, 2005) along with two-dimensional gel electrophoresis (2DE) in combination with mass spectrometry (Gilson *et al.*, 2006). Another broad proteomic analysis of merozoite proteins used a total merozoite preparation to identify merozoite proteins by 2DE in combination with MALDI-TOF MS (Gelhaus *et al.*, 2005).

All these investigations identified novel proteins at the merozoite stage. Until today, only one study has used semi-quantitative MudPIT in a comparative way. In that study the merozoite proteome was compared to other developmental stages of *P. falciparum* (Florens *et al.*, 2002).

Analysis of the technical replicate capacity of iTRAQ revealed an average of ~11% in variation of protein expression (Gan *et al.*, 2007). MS variance in each experiment was reported at an average of 0.1% (Gan *et al.*, 2007). In the present study, the relative protein regulation was calculated from the average value of the

duplicate analysis (114/115 and 116/117 for W2mef against W2mef/NM), and in addition, the sample was measured by triplicate injections in the MS mode. Therefore by taking technical replication and MS variance into account, we only considered those proteins which had a regulation of equal to or more than 80% variation (i.e. 2-fold and above) to be significantly regulated by intrinsic biological activities (Table 3.1 and 3.2). No normalisation was required since the ProteinPilot software had already taken into account the internal bias caused by the labelling efficiency and other physical variants.

The proteomic results manifest that there are more changes in protein level than there are, as previously reported, at the transcriptional stage. When NM is used in invasion pathway studies this enzyme is supposed to remove sialic acid from erythrocyte receptors. A reasonable proposition is that the changes induced by NM alter the erythrocyte in a more radical way than simply influencing the receptor availability.

The effect of NM on erythrocytes will be discussed in detail in the following chapter.

The parasite adjusts to these alterations on the erythrocyte surface by regulating the proteins required for invasion. These altered conditions seem to make it more difficult for the parasite to invade the desialiated erythrocyte. As the parasite invades the NM-treated erythrocyte, it interacts with the erythrocyte membrane and the interacting proteins that are now altered. As a matter of fact, amongst the proteins up-regulated in W2mef/NM are merozoite surface proteins. MSP-1 forms a complex on the merozoite surface along with other MSPs and mediates the initial interaction before entry. Rhoptry neck proteins (RONs) are associated with AMA-1 and are located at the moving junction.

The “core proteins” of the actin-myosin motor are expressed in higher quantities in W2mef/NM than in W2mef. It seems that the W2mef/NM parasite requires a more



powerful “driving force” to overcome the altered and probably more rigid erythrocyte surface. When the parasite has more actin-myosin motor components, would it have more unknown surface molecules where the motor is linked (e.g. TRAP homologue)?

Furthermore, proteases and proteins from the rhoptry bulb are also up-regulated in W2mef/NM. Proteins expressed in the rhoptry bulb are involved in establishing PV in the erythrocytes and proteases may degrade some of the altered erythrocyte membrane molecules.

In summary it appears that every single step of merozoite invasion has been influenced by the loss of sialic acid on the erythrocyte surface. The next question to be addressed is: how does the removal of sialic acid impact the erythrocyte cytoskeleton? The cytoskeleton is linked to transmembrane molecules such as band 3 and Glycophorin C which are sialic acid-containing proteins. The sialic acid removal may alter the conformation of these proteins or may influence the binding of band 3/ Glycophorin C to the cytoskeleton. To what extent alteration occurs in erythrocytes treated with only 10 mU/ml NM will be discussed in the following chapter.

## **Chapter 4**

### **Biophysical and biomechanical properties of erythrocytes and their impact of proteomic changes during invasion of desialiated erythrocytes**

Invasion of the parasite into the erythrocyte involves many interactions within a very limited time. Proteomic analysis detected at least 20 invasion proteins with many of them known to be to interact with the erythrocyte surface/cytoskeleton molecules. To understand why all these changes have occurred in W2me/NM it is important to be aware of the influence of neuraminidase treatment to erythrocytes.

Sialic acid removal from erythrocytes by neuraminidase has been widely used as a tool to investigate invasion pathway properties of various *P. falciparum* strains. The action of neuraminidase was mainly seen as interfering with those receptor-ligand interactions that utilise sialic acid on peptide backbone. Little credence was given to downstream effects on erythrocyte properties that may have been induced by neuraminidase.

Changes on the surface of erythrocytes may have additional effects on membrane and cytoskeleton and may influence their function. Another major function of the components of the cytoskeleton and membrane is maintenance of shape and stability as well as retaining the flexibility and deformability.

It is a reasonable proposition that sialic acid loss decreases the surface charge of the erythrocyte thus inducing structural changes of some erythrocyte membrane spanning proteins or rearrangements of the erythrocyte surface and membrane (Nordt *et al.*, 1978). Sialic acid is linked to glycophorin C and band 3. For instance; Glycophorin C contains sialic acids and its cytoplasmic tail interacts with protein 4.1R which in turn is linked to the spectrin network. When the sialic acids are lost,

the structure of Glycophorin C could be altered because of the overall changed protein charge. This could lead to some downstream effect on the cytoskeleton network, hence the binding of modified Glycophorin C to protein 4.1R might be weakened and the cytoskeleton network is extenuated.

The same scenario could be possible for band 3 which is linked to spectrin via ankyrin and protein 4.2R. When band 3 is structurally altered and the connection to ankyrin and/or protein 4.2R is destabilised, the spectrin network might be more fragile. These changes may have an impact on shape, rigidity and deformability of the neuraminidase-treated erythrocyte.

Therefore neuraminidase treatment of erythrocytes might not be the ideal approach to study differences in invasion pathway switching as many components of the erythrocyte surface are modified. The parasite needs to react to these modifications invoked by neuraminidase and adjusts the implicated proteins by its regulatory system. Some of these adjustments probably account for the changes in regulation seen in the proteomic survey. Therefore it is important to determine the nature of the changes induced by sialic acid removal.

This chapter directs attention to the changed properties of desialiated erythrocytes due to neuraminidase treatment and the resultant biological consequences for W2mef/NM invasion.

## Results

### ***4. 1 Electrokinetic potential***

Erythrocytes have a negative surface charge which results primarily from the presence of carboxyl groups of sialic acids on the cell surface (Cook *et al.*, 1961; Eylar *et al.*, 1962). Neuraminidase cleaves sialic acids from sialoproteins (Gottschalk, 1960) and clearly decreases the electrophoretic mobility of erythrocytes (Jan and Chien, 1973) which can be measured as the zeta potential.



The zeta potential is the potential across the ion layer around a charged colloidal particle.

The zeta potential has been measured in previous studies (Luner *et al.*, 1975; Seaman *et al.*, 1977; Williams and Domen, 1990). This present study uses a relatively low concentration of neuraminidase (1 to 10 mU/ml) compared to other studies. The activity of enzymes varies between manufacturers and different batch preparations. It is important to establish that a lower concentration of neuraminidase has already an effect on erythrocytes surface charge.

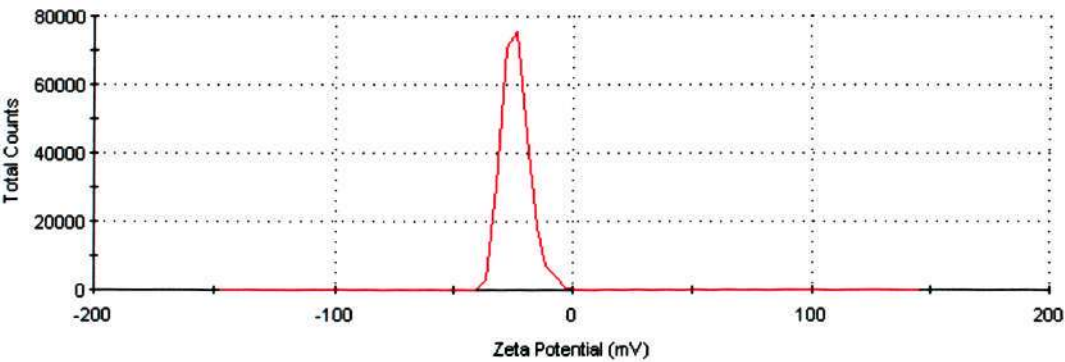
Therefore different concentrations of neuraminidase ranging from 1 mU/ml to 10 mU/ml were used for the treatment. For each measurement,  $5 \times 10^6$  erythrocytes are resuspended in an electrolyte with an ionic strength of 10 mM. The particle velocity was measured using the technique of laser Doppler anemometry. The frequency shift of an incident laser beam caused by the moving erythrocytes was measured as particle mobility. The particle mobility was converted to the Zeta potential by the input of the dispersant viscosity and the application of the Helmholtz-Smoluchowski approximation (Rice and Whitehead, 1965).

The measurement at the NM concentration of 10 mU/ml demonstrates the uniformity of the NM treatment reflected by a single peak (Figure 4.1.). The majority of the erythrocytes have a similar zeta potential. The zeta potential follows a Gaussian distribution.

Figure 4.2 shows the decrease of the zeta potential of untreated and treated erythrocytes in a neuraminidase-concentration dependent manner. For each concentration measurements were performed in triplicate and the average values with the standard deviation as error bar was plotted.

The high reduction of ~20% of the negative charge was observed when treated with 1 mU/ml compared to normal erythrocytes. The erythrocytes become more “positive” with increasing concentrations of neuraminidase. Almost 50% of the

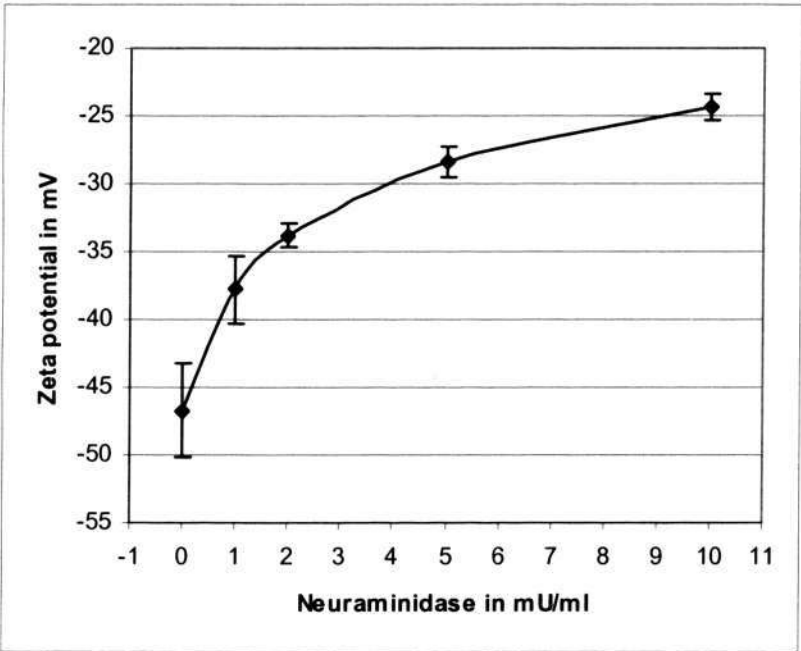
negative charge was removed when a concentration of 10 mU/ml was used. The concentration of 10 mU/ml was used in all enzyme treatments for the growth of W2mef/NM.



**Figure 4. 1. Zeta potential distribution of one single measurement.**

The electrokinetic potential of a single measurement of 10 mU/ml NM treated erythrocytes is shown in total counts of erythrocyte versus the zeta potential. The measurement displays a normal distribution.





**Figure 4. 2. Zeta potential of erythrocytes treated with different concentrations of neuraminidase.**

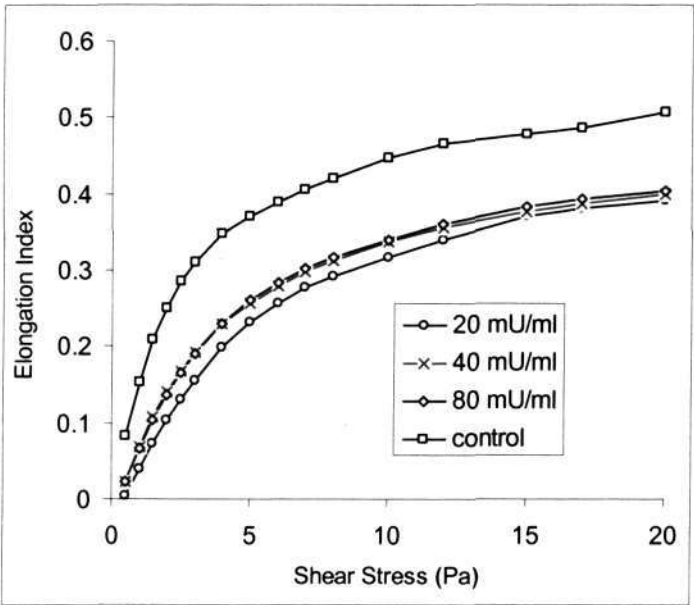
The zeta potential is plotted versus the neuraminidase concentration. Each concentration was measured in triplicate and the average values were plotted. The standard deviation was calculated for each concentration and indicated as an error bar.

## 4. 2 Deformability of desialiated erythrocytes

These measurements were performed by Heng Li Tze in Dr. Bjoern Neu's laboratory at School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore.

The deformability of erythrocytes primarily depends on the composition of the membrane and cytoplasm. Quantitative assessment of the deformation property can be done by measurement with an ektacytometer. With this method, erythrocytes are exposed to increasing shear stress while the laser diffraction pattern through the suspension is documented. The resulting elongation index (EI) is directly related to erythrocyte deformability at various shear stresses (Groner *et al.*, 1980).

Figure 4.3 depict the extent of deformability of desialiated erythrocytes of different concentrations (20, 40 and 80 mU/ml) as compared to normal erythrocytes. Altogether, desialiated erythrocytes yield almost identical EI relative to concentration due to saturation effect. To see a progressing effect a lower concentration of NM should be used. Unfortunately problems with the instrument occurred and the measurements with lower concentration of NM could not be performed. The EI after neuraminidase treatment was lower compared to untreated erythrocytes, indicating a decreased deformability of neuraminidase treated erythrocyte. The EI increases with increasing shear stress, although, the slope of untreated and treated erythrocyte are similar.



**Figure 4. 3. Deformability of erythrocytes treated with different neuraminidase concentrations.**

The deformability of erythrocytes treated with different concentrations of neuraminidase is determined from the elongation index at increasing shear stress.



### ***4. 3 Morphology analysis of erythrocyte surface by atomic force microscopy (AFM)***

Rearrangements and alteration of the erythrocyte membrane and cytoskeleton due to sialic acid loss was investigated by AFM. A number of groups have used AFM to study cytoskeleton and in more detail the spectrin network (Almqvist *et al.*, 1994; Liu *et al.*, 2003; Swihart *et al.*, 2001; Takeuchi *et al.*, 1998; Yamashina and Katsumata, 2000). AFM is a high resolution microscope where the surface of the erythrocyte is scanned with a cantilever (probe). The force between the tip and the sample lead to a deflection of the cantilever and is further transformed to produce an image.

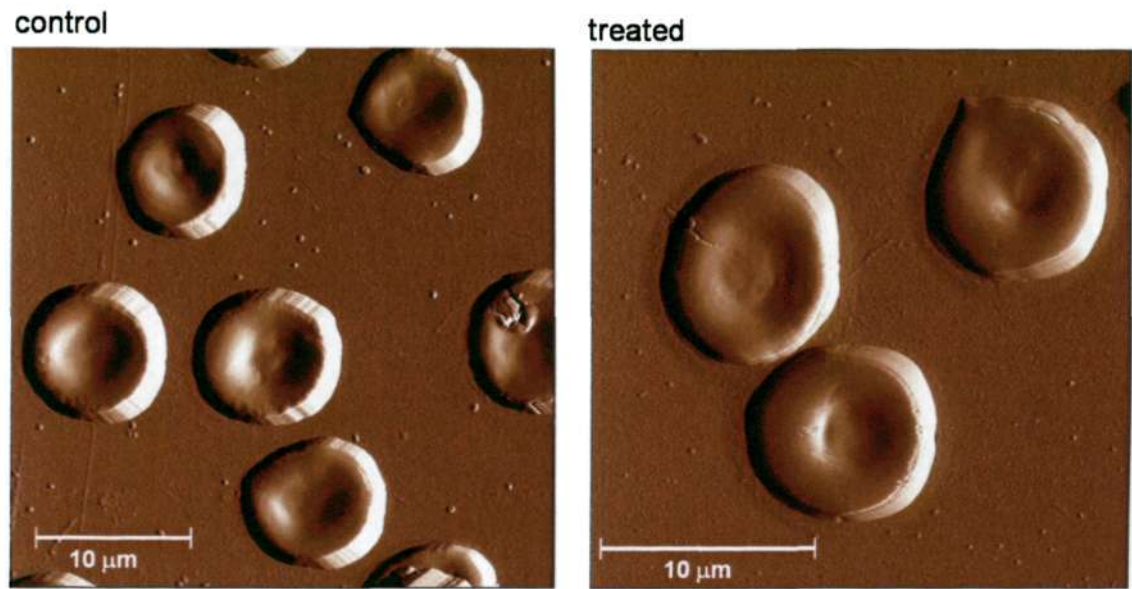
Freshly drawn erythrocytes were treated with 10 mU/ml neuraminidase for 1 hour at 37°C and washed extensively. Control erythrocytes were also incubated for 1 hour at 37°C with no enzyme added. Until smeared, the erythrocytes were stored in RPMI-1640 w/o Albumax at 4°C. Thin blood smears were taken after 1 hour, 1 day and 1 week after completion of the enzymatic treatment.

The erythrocytes have a similar appearance in control and treated samples from smears taken after 1 hour and 1 day of treatment (Figure 4.4 and 4.5).

Differences can be seen when pictures from treated and control erythrocytes taken after 1 week (Figure 4.6). The control erythrocytes look like in the erythrocytes from 1 h and 1 day but the desialiated erythrocytes become echinocyte-like shaped. The boundaries have an irregular and lobate shape.

Figure 4.7 visualises single erythrocyte after 1 week of treatment (4.7 A untreated and 4.7 B NM treated). The magnified picture of control erythrocyte shows the spectrin network as a white elevated mound.

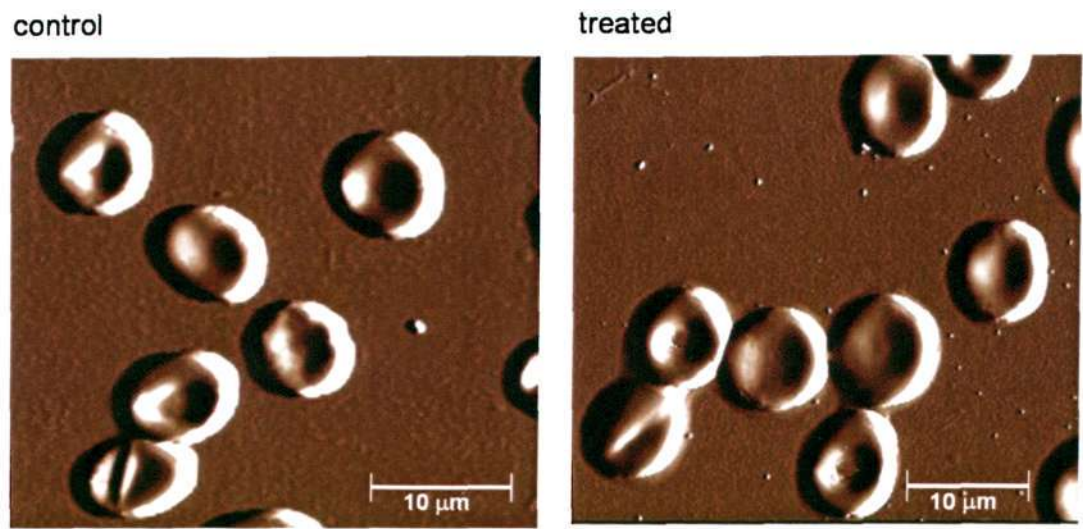
The zoomed in picture of the NM treated echinocyte-like erythrocyte shows a similar white elevated mound of network with randomly distributed white protuberance. At this point it is not clear what these spots are. They could be aggregates of erythrocyte surface proteins. In addition, it appears that the overall spectrin network is more condense. The dark spot in the bottom corner of the magnified picture is probably a mark from smearing. There are some dust particles present on the slide. During the preparation of the smear, the erythrocytes are pressed down and the dust particles leave marks on the surface. Only a few high resolution pictures are taken. To make more solid statements about the erythrocyte constitution further images need to be evaluated.



**Figure 4. 4. AFM images of erythrocytes 1h after NM treatment.**

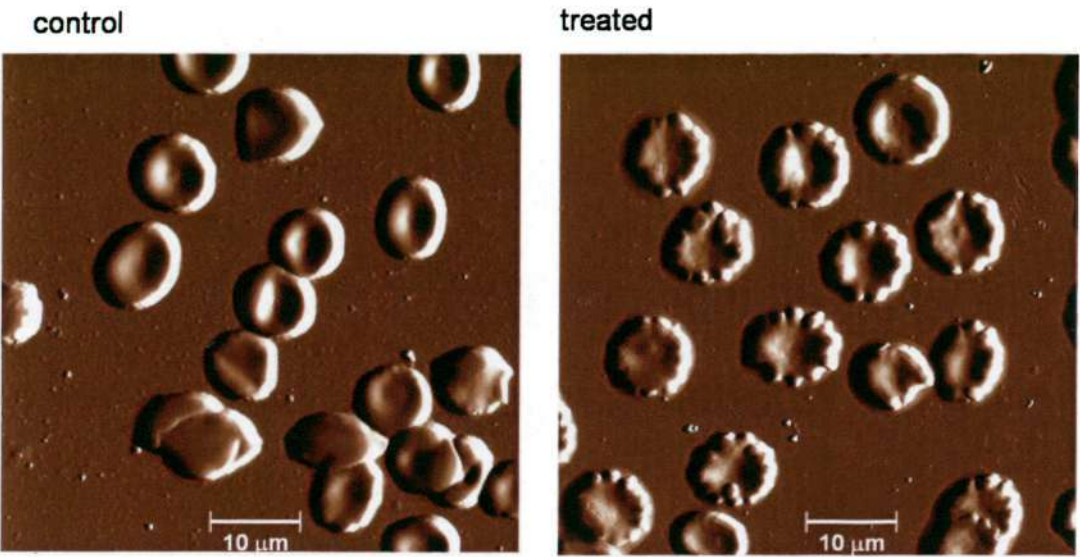
Fresh erythrocytes were treated with 10 mU/ml NM. One hour after the enzyme treatment was completed the smear was taken for both treated and control erythrocytes (no treatment) and dried for at least 24 hours prior AFM image acquisition.





**Figure 4. 5. AFM images of erythrocytes 1day after NM treatment.**

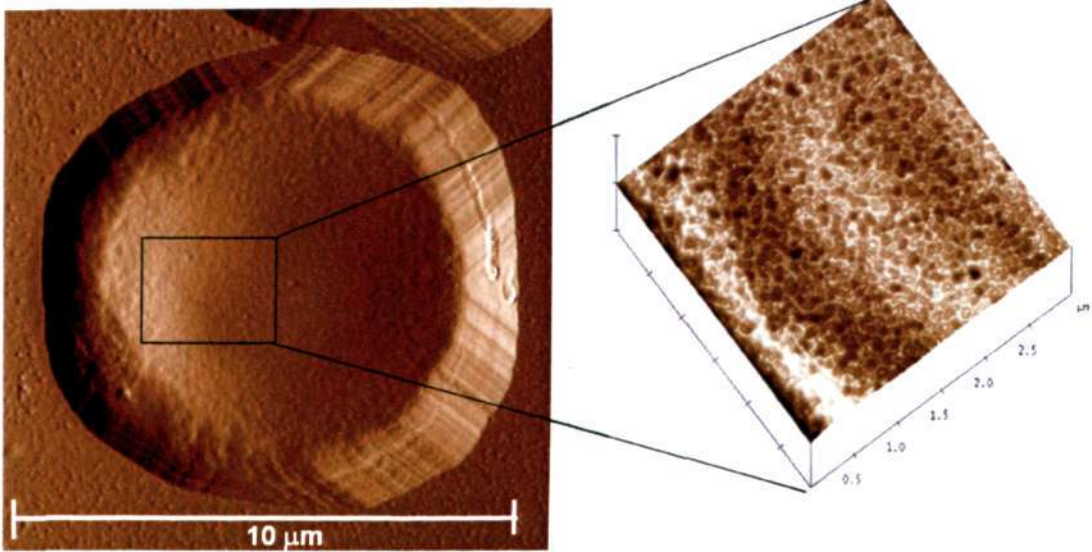
Fresh erythrocytes were treated with 10 mU/ml NM. One day after the enzyme treatment was completed the smear was taken for both treated and control erythrocytes (no treatment) and dried for at least 24 hours prior AFM image acquisition.



**Figure 4. 6. AFM images of erythrocytes 1 week after NM treatment.**

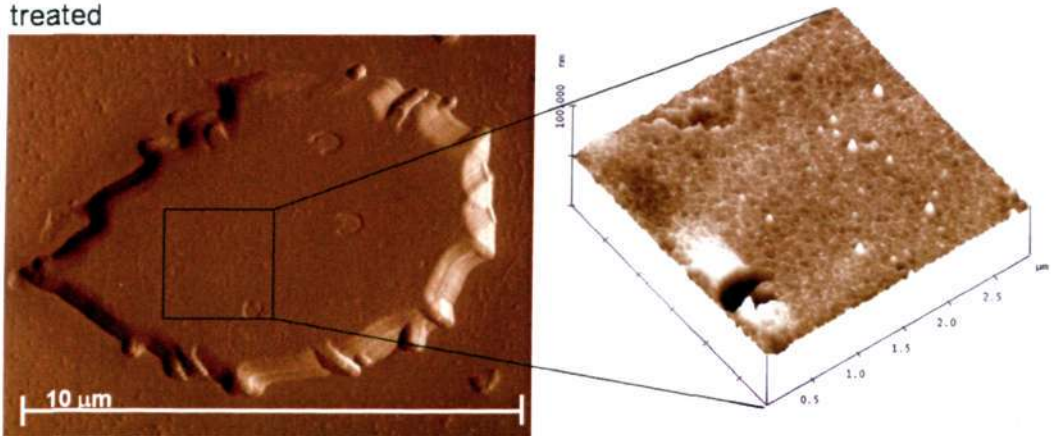
Fresh erythrocytes were treated with 10 mU/ml NM. One week after the enzyme treatment was completed the smear was taken for both treated and control erythrocytes (no treatment) and dried for at least 24 hours prior AFM image acquisition.

control



B

treated



**Figure 4. 7. Erythrocyte AFM images and magnified surface of erythrocytes 1 week after NM treatment.**

Fresh erythrocytes were treated with 10 mU/ml NM. One week after the enzyme treatment was completed the smear was taken for both (A) control (no treatment) and (B) treated erythrocytes and dried for at least 24 hours prior AFM image acquisition.



#### ***4. 4 Physiological relevance of MSP-1 during invasion of desialiated erythrocytes***

The biological relevance of any of these 61 proteins expressed in higher levels in W2mef/NM during invasion of desialiated erythrocytes is unknown. On the other hand 1/3 of the 61 up-regulated proteins are thought to be directly involved in the invasion process. One of these proteins is MSP-1, one of the most studied molecules in *Plasmodium* research. MSP-1 is a key invasion molecule and involved in the initial attachment of the parasite with the erythrocyte. This is also the only molecule from this list where the erythrocyte binding molecule has been identified (Goel *et al.*, 2003).

This present study observes that W2mef/NM expresses more MSP-1 molecules than W2mef. Invasion of merozoite into erythrocyte brings the merozoite into very close proximity to the erythrocyte. A number of physical forces act in this small space between parasite and merozoite. These non-specific forces can be electrostatic repulsion, steric effects and possibly depletion interaction. For example, when sialic acid is removed, the reduction of the surface charge of the erythrocyte increases the agglutinability of erythrocytes. Thus with increased erythrocyte aggregation the steric effects and electrostatic repulsion are reduced. These altered biophysical forces during the first contact of the erythrocyte with the merozoite may account for the increased number of MSP-1 ligands expressed in W2mef/NM.

What is the biological significance to have higher amounts of MSP-1 in W2mef/NM? When the MSP-1 binding partner on the erythrocyte is altered, the W2mef/NM parasite needs to balance the induced differences by expressing a larger amount of MSP-1. W2mef/NM requires more MSP-1 to enter NM treated erythrocytes than W2mef needs for normal erythrocytes. Hence, W2mef/NM would have an excess of MSP-1 protein during invasion of normal erythrocytes. W2mef/NM parasites should not be impaired to perform invasion as much as W2mef in the presence of an inhibitor molecule.

This hypothesis was tested by adding MSP-1<sub>19</sub> specific antibody to the parasite culture. The inhibitory effect of the antibody towards W2mef and W2mef/NM invasion into NM treated and normal erythrocytes were accessed.

#### ***4. 5 MSP-1 antiserum invasion inhibition assay***

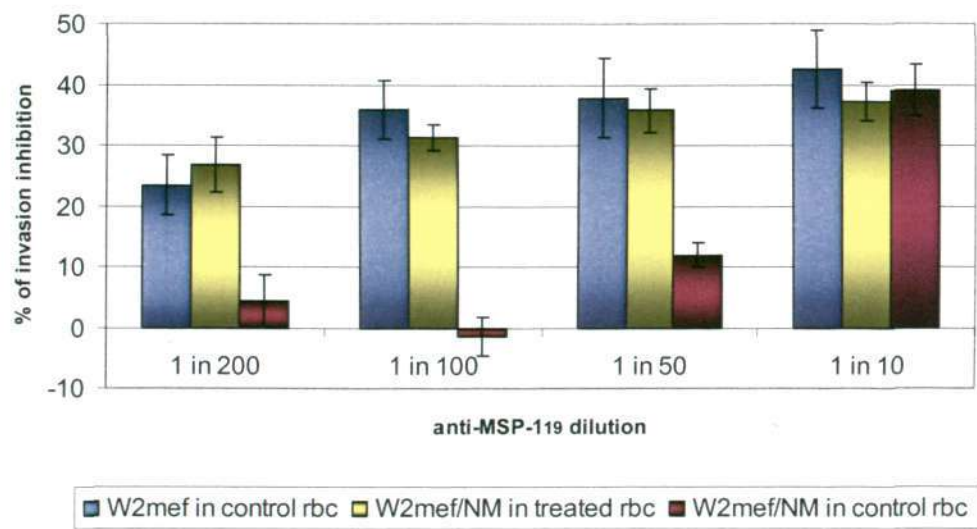
Purified schizont stage parasites and increasing concentrations of antibody against MSP-1 were incubated with erythrocytes for 16 hours. The result was evaluated from blood smears and summarised in Figure 4.8.

The MSP-1 antibody used was raised against the 19 kDa portion known to be implicated during the invasion process. The antibody was used in dilutions ranging from 1:200 to 1:10. Invasion efficiency in the presence of antibody was tested for: W2mef invasion in untreated (control) erythrocytes, W2mefNM invasion in control and 10 mU/ml NM treated erythrocytes. The results were normalised to W2mef or W2me/NM, respectively without addition of MSP-1 antibody.

As expected, the invasion of W2mef in control erythrocytes was significantly inhibited in all antibody concentrations used. The inhibitory effect of MSP-1 antibody in W2mef (control) increases with increasing concentration of the antiserum. The highest inhibition was achieved at 1:10 dilution with ~40% inhibition.

The profile of W2mef/NM invasion in treated erythrocytes was similar to W2mef in control erythrocytes.

W2mef/NM invasion in untreated erythrocytes showed no noteworthy inhibitory effect with increasing antibody concentrations until the 1:10 dilution. Here, the invasion was inhibited similar to the other tested clones at around 40%. It appears that the highest inhibition with this particular antibody was achieved at ~40%.



**Figure 4. 8. MSP-1 invasion inhibition assay.**

The reinvasion of parasites into erythrocyte at the presence of different concentration of MSP-1 antibody was assessed. The invasion inhibition was plotted in relation to control parasite without antibody added. “Treated rbc” are erythrocytes treated with 10mU/ml NM. Error bars indicate the standard deviation of the triplicate experiment.



## Discussion

The previous chapter exploits the proteomic differences of W2mef clone during invasion into desialiated erythrocytes compared to normal erythrocytes. The present chapter focuses on the NM treated erythrocytes and elaborates differences concerning biophysical properties of NM treated erythrocytes compared to normal erythrocytes. The biological changes of the merozoite observed in Chapter 3 are brought in connection with overall changes of the desialiated erythrocyte structure.

One of the differentially expressed proteins is MSP-1 with higher expression levels in W2mef/NM. This protein is implicated in the invasion process very early on and is one of the few parasite ligands with a known interacting partner on the erythrocyte surface (band 3). This protein is 2.8-fold up-regulated in W2mef/NM compared to W2mef. The W2mef/NM parasite has altered surface properties in comparison to W2mef with reduced electrostatic repulsion and steric effects. If these forces are the cause for higher expression levels of MSP-1 is not known and the exact reason why MSP-1 is up-regulated in W2mef/NM remains to be determined.

Another altered parameter caused by sialic acid removal is the increased aggregation of erythrocytes and the reduced steric repulsion (steric effect) as a result from the changed surface structures. These are not favourable conditions for MSP-1 erythrocyte binding and therefore more MSP-1 is required to adjust to the new environment. The amount of MSP-1 needed to invade normal erythrocyte is the same for W2mef and W2mef/NM. An antibody against MSP-1 blocks a certain amount of MSP-1 on the merozoite surface from binding to erythrocyte receptor. Once the critical level of blocking is reached, invasion into erythrocytes by the merozoite is blocked. To ensure proper parasite-merozoite interaction, more MSP-1 is needed for the invasion of NM treated erythrocyte and therefore higher concentrations antibody is required to have an inhibitory effect of W2mef/NM invasion into NM treated erythrocyte.

MSP-1<sub>19</sub> antibody was used in a concentration dependent manner in an invasion competition assay with W2mef and W2mef/NM in control and NM-treated erythrocytes. W2mef in control erythrocyte and W2mef/NM in treated erythrocytes invasion efficiency was inhibited with increasing concentration of antibody. W2mef/NM invasion into control erythrocyte tolerated MSP-1 antibody concentration up to 1 in 50 without any apparent inhibitory effect. It appears from these results that MSP-1 antibody inhibition regardless of parasite clone is saturated at around 40% at an antibody concentration of 1 in 10. W2mef invasion into normal erythrocytes and W2mef/NM invasion into treated erythrocytes are equally inhibited by MSP-1 antibody. The amount of MSP-1 is just enough to interact with the erythrocyte. This interaction can be blocked by the MSP-1 antibody.

This result would indicate that W2mef invasion into normal erythrocytes is similarly reduced by the antibody as W2mef/NM invasion into NM treated erythrocytes. W2mef/NM has more MSP-1 available for invasion. When W2mef/NM invades normal erythrocytes the amount of antibody is not as sufficient in 1 in 50 dilution to block the excess of available MSP-1.

In conclusion, the merozoite is up-regulating MSP1 expression in W2mef/NM to adjust to changes on the erythrocyte surface that reduce the efficiency of MSP1 function (possibly initial attachment). In such a situation anti-MSP1 antibodies would be expected to have a similar effect on W2mef invading untreated erythrocyte and W2mef/NM invading NM treated erythrocyte. In contrast W2mef/NM parasites would have an excess of MSP1 available when invading untreated erythrocytes making it more difficult of the available MSP1 –sera to inhibit invasion efficiently.

The proposed binding partner of MSP-1 is band 3. This protein is highly abundant on the erythrocyte surface and contains sialic acid residues. The proposed interacting portion of band 3 with MSP-1 is independent of sialic acid (Goel *et al.*, 2003). When MSP-1 interaction is really independent of sialic acid residues why is

MSP-1 up-regulated? One could speculate that the sialic acid removal of band 3 alters the overall band 3 conformation and more MSP-1 is required to gain a similar interaction as with normal erythrocytes.

Under regular conditions biological surfaces, including the erythrocytes surface, are negatively charged. The erythrocyte has a negative membrane curvature which is sensitive to chemical alterations and intrinsic disturbances of any nature. On the periphery of the erythrocyte reside sialic acids which mainly account for the negative electrical surface charge of the erythrocyte (Eylar *et al.*, 1962; Seaman and Uhlenbruck, 1963).

At first, the reduction of the surface charge of erythrocytes treated with different concentration of NM is observed. Enzymes like neuraminidase can be utilised to remove sialic acid residues from erythrocytes. The reduction of the surface charge occurs in a concentration-dependent manner. This finding is in agreement with earlier published data (Luner *et al.*, 1975; Seaman *et al.*, 1977; Williams and Domen, 1990).

The reduced surface charge affects other biophysical properties of the erythrocyte e.g. rigidity or deformability characteristics. When the erythrocyte deforms, the erythrocyte is able to undergo distortion and is still able to restore biconcave shape without fragmentation or loss of integrity (Mohandas and Chasis, 1993).

This study tested the deformability of erythrocytes treated with different concentrations of NM by analysing the elongation index at different shear stress. The result is in tune with previous publication observing a reduced deformability of NM treated erythrocytes (Wen *et al.*, 2000). The elongation index is saturated at a low concentration of 20 mU/ml. This shows that a low enzyme concentration is sufficient to have a drastic effect on erythrocyte deformation.



In general, there are two types of erythrocyte shape changes, echinocyte and stomatocyte, which ultimately result in spherocytes. These abnormalities can be caused by integral membrane proteins like band 3 and glycophorin C, skeletal proteins as spectrin or protein 4.1R, or anchor proteins ankyrin or protein 4.2R. If any of these components are altered, the normal deformability or rigidity of the erythrocyte could be impaired.

In this study no change in shape was observed after 1 hour and 1 day storage of NM-treated erythrocytes. The visual changes observed by AFM demonstrated an echinocytic shape of the NM treated erythrocytes only observed 1 week after treatment. Grebe *et al.*, (1988) and Alhanaty and Sheetz (1984) also noted curvature changes of erythrocyte resulting in a stomatocytic shape change. In contrast to this work and earlier studies, Glaser (1982) induced echinocyte formation and observed no influence of NM, but has evidence that the transmembrane potential changes influence the cell shape (Glaser, 1982). But when desialiated erythrocytes have an altered surface potential why is there no effect seen?

Previous publications measured the shape alterations by flow cytometry and were only able to make a statement about spherical index (Piagnerelli *et al.*, 2003). They reported that the NM-treated erythrocytes are more spheric than normal erythrocytes but no further statement is made. All together, it is clear that neuraminidase has an effect on erythrocyte shape. However, previous publications did not state how much time elapsed after treatment and analysis. Here only a visual method is used where it is difficult to assess tendency towards a spheric shape after 1 hour or 1 day post-treatment, but there is a clear effect observed after 1 week of storage compared to untreated control erythrocytes.

The erythrocytes used to grow W2mef/NM parasites are stored at 4°C up to 1 week after treatment. Therefore it was important to test if there are any changes observable. Interestingly, the echinocytic effect occurred only after 1 week and not immediately or after 1 day of storage after repeated smears. One possible

explanation could be that the storage in at 4°C has an influence on the altered cell shape. Neuraminidase is not active at 4°C and even after extensive washing any residual enzyme would not have any action. Nevertheless, low temperature storage for more than 6 days can reduce surface charge of erythrocyte (Godin and Caprani, 1997). The erythrocyte is probably already altered on the molecular level just after the treatment. These changes become only after 1 week of storage obvious. The effect of sialic acid removal and the additional storage for seven additional days probably enhanced the effect on shape changes towards echinocyte.

Furthermore, it is known that smearing normal erythrocyte on glass can result in so called echinocytogenic glass artefact where normal washed erythrocyte can have an echinocytic transformation in close vicinity to glass surfaces. This may influence the appearance of NM treated cells as well.

The obvious echinocytic effect was only seen by AFM. When smears are taken from the growing W2me/NM culture the echinocytic effect was not observed. This has something to do with the preparation of the smear. Only erythrocyte suspended in PBS are smeared for AFM visualisation. Erythrocytes taken from culture are suspended in culture medium containing glucose and albumax which are favourable to maintain the natural shape of the erythrocyte.

The resting erythrocyte maintains a biconcave shape with only minimal energetic effort (Canham, 1970). The shape of the cell and the membrane flexibility depend on the erythrocyte cytoskeleton, with its main components spectrin, actin, and protein 4.1. (Bennett, 1990; Elgsaeter *et al.*, 1986; Mohandas and Evans, 1994). These proteins interact with each other and form contacts with transmembrane proteins like Band 3 and Glycophorin C. If any of these interactions is imbalanced or disturbed, this will have consequences for the overall stability of the erythrocyte. Though, changes in shape and elasticity and deformability are in one way or another linked to the major element in the erythroid cytoskeleton, spectrin. In the following paragraphs different possible scenarios are discussed which may account for the

increased rigidity and incapability to deform of desialiated erythrocytes: the inability of spectrin to stretch, phosphorylation of cytoskeletal components and blockage of band 3/glycophorin A mobility.

The basic spectrin molecule is a heterodimer, which can form interactions between dimers which lead to the formation of tetrameric and oligomeric structures of higher complexity. Under physiological conditions, the spectrin tetramers are in rapid dynamic equilibrium with the spectrin dimers. Shear-induced cell wall deformation produces more dimers (An *et al.*, 2002). Upon unloading, the dynamic stability is shifted toward tetramers and the stiffness of the cell wall increases. Spectrin is highly expandable and allows the cell flexibility and elasticity (Shotton *et al.*, 1979). An important feature of the long filamentous spectrin dimer is the sequence of 36 repeats, 20 in  $\alpha$ -spectrin and 16 in  $\beta$ -spectrin. Unfolding of the least stable spectrin repeats might affect membrane elasticity (Johnson *et al.*, 2007). These findings support the concept that the unfolding and refolding of distinct spectrin repeats make a major contribution to the elasticity of the normal erythrocyte membrane.

Furthermore, spectrin is also involved in the maintenance of dynamic (phase-state) asymmetry in erythrocyte membranes (Williamson *et al.*, 1982). This is probably the result of multiple weak spectrin–lipid interactions along the length of the spectrin molecule that are continuously broken and reformed (Mc Kiernan *et al.*, 1997; O'Toole *et al.*, 1999). The connections between spectrin and inner monolayer lipids probably contribute to the elasticity of the circulating erythrocyte (Markin and Kozlov, 1988). Disruption of these interactions, lead to instability of the membrane and as a consequence, a reduced deformability.

The stability of the membrane skeleton also depends on the interactions between spectrin and protein 4.1. These interactions occur through a binding site on the beta chain of spectrin apparently close to actin and calmodulin binding sites. Other



interactions occur between the hydrophobic segment of spectrin and membrane lipids.

The rigidity can be enormously increased by ATP-depletion (Devaux, 1988). These observations suggest that the membrane flexibility is maintained by constant phosphorylation of the cytoskeletal components. Thus, phosphorylation reduces the bending of ankyrin to band 3 and of protein 4.1 to glycophorin. The partial dissociation of the network from the bilayer mediated by phosphorylation appears to be essential for the maintenance of the high degree of elasticity. These findings propose a regulatory role for the phosphorylation of skeletal proteins in the dynamic regulation of erythrocyte membrane properties.

The hypothesis is that an external change of the erythrocyte periphery has an impact on the underlying cytoskeleton. The theory is that a binding event on one side of the bilayer may cause a protein conformational change of the transmembrane protein on the opposite side of the bilayer, which in turn may commence or regulate subsequent metabolic, binding, or chemical events. The role of the transmembrane protein conformational change is “information” transfer. The question arises: does a similar conformational change happen to transmembrane proteins, when sialic acids are removed?

Anderson *et al.*, (2002) provided evidence for transmembrane conformational change by exploiting the influence of lectin bound to glycophorin in regard to shape changes. The discocyte↔echinocyte shape conversion is reversible. Both directions of this morphology change are efficiently blocked by lectins specific for glycophorin’s external saccharides (Lovrien and Anderson, 1980). Specific lectins bound to distinct parts of the Glycophorin blocked the erythrocyte shape at the echinocyte or discocyte status (Anderson *et al.*, 2002).

The cytoplasmic tail of Glycophorin A is very short with a domain of only 39 residues. The Glycophorin A cytoplasmatic tail does not have the capacity to bind to

any part of the cytoskeleton, it has been suggested that Glycophorin A and spectrin interact by dynamic tension and induces rigidification (Butterfield *et al.*, 1983).

Band 3 and glycophorin A are in close proximity to one another and cooperative interaction takes place (Nigg *et al.*, 1980; Telen and Chasis, 1990). Furthermore, band 3 is able move to a certain degree with the erythrocyte membrane (lateral mobility). The regulation of this lateral mobility appears to be regulated by the cytoplasmatic domain of band 3 and the erythrocyte skeleton (Golan and Veatch, 1980; Sheetz *et al.*, 1980). It is further speculated that a similar interaction between band 3 and the skeleton is perhaps induced by glycophorin A receptor ligands (Knowles *et al.*, 1994). However, glycophorin A and band 3 are “mobile” molecules and only become immobilised as a result of glycophorin A receptor ligand binding.

An alternative hypothesis that can account for the immobilisation of glycophorin A and band 3 and the assistant increase in rigidity is that ligand binding to glycophorin A induces a direct interaction between this protein and the membrane skeleton. This interaction in turn draws the skeletal network closer to the membrane bilayer. This change in network configuration disables the spectrin network to undergo conformational change required for deformation (Knowles *et al.*, 1994).

Decreased membrane deformability is a characteristic feature of erythrocytes in hereditary ovalocytosis (Mohandas *et al.*, 1984). Ovalocytosis is very common endemic area especially in Southeast Asia (Amato and Booth, 1977). Membranes of ovalocytes are 4 to 8 times less elastic than membranes normal erythrocytes (Mohandas *et al.*, 1984; Mohandas *et al.*, 1992). A genomic deletion of 27 bp encoding amino acids 400 to 408 of band 3 at the boundary between the cytoplasmic and first transmembrane domains has been associated with ovalocytosis (Jarolim *et al.*, 1991; Mohandas, 1992; Mohandas *et al.*, 1992; Schofield *et al.*, 1992; Tanner *et al.*, 1991).

This deletion causes immobilisation of band 3 and increased rigidity of the altered erythrocytes (Mohandas *et al.*, 1984; Mohandas *et al.*, 1992; Saul *et al.*, 1984).

Secondary structure analysis of this altered band 3 suggests that a flexible portion of cytoplasmic domain is removed which may alter the conformation of the cytoplasmic domain thus provide a different interaction with the skeletal network (Mohandas *et al.*, 1992). These altered interactions between band 3 and the spectrin network may cause the membrane rigidification.

In the human body, discoid shaped erythrocytes circulate in the vein system and are forced by the fluid flow to travel through narrow capillaries to transport oxygen to tissues. The ability of these cells to undergo extensive passive deformation under fluid shear stress is an important physiological property. The erythrocyte surface basically consists of membrane and cytoskeleton. During the 120 days of their lifespan, the enucleated erythrocyte irreparably loses surface molecules, like sialic acid, over time (Bocci, 1976). Normocytes (mature erythrocytes) compared to reticulocytes (young erythrocytes) contain about 10% less sialic acid (Cohen *et al.*, 1976; Seaman *et al.*, 1977). NM treatment is an attractive means to study sialic acid loss of the erythrocyte surface. Hence, the removal of sialic acid with neuraminidase from the erythrocyte glycocalyx produces “artificially aged” erythrocytes. It is important to note that neuraminidase has a more radical effect on sialic acid removal than the natural loss would have (Nordt *et al.*, 1978). It appears that neuraminidase treatment invokes changes within the erythrocyte beyond “artificial cell aging” (Nordt *et al.*, 1978).



## **Chapter 5**

### **Regulation of differential merozoite protein expression**

The complete genome sequence of *P. falciparum* was resolved in 2002 (Gardener *et al.*, 2002). The availability of the genome sequence has been of high value for malaria researchers and opened up the way for transcriptional analysis using DNA microarray. This approach has shown that genes coding for members of multi-gene families or which are functionally related are frequently transcriptionally co-regulated. The *P. falciparum* genome appears to encode fewer conserved transcription factors but instead appears to contain an increased number of genes for RNA-binding proteins and proteins involved in the chromatin remodelling machinery (Coulson *et al.*, 2004). Accordingly, it appears that many genes in *Plasmodium* spp. are regulated at the level of translation (Hall *et al.*, 2005; Le Roch *et al.*, 2004), or at the level of RNA stability (Shock *et al.*, 2007). The modifications at the RNA level result in a discrepancy between gene transcript and proteins expression product. Quantitative proteomics is needed to uncover protein expression levels. Comparative analysis of gene transcription and quantitated protein expression can uncover transcriptional, post-transcriptional or post-translational regulation.

Broad proteomic analyses of *P. falciparum* and *P. berghei* of different discernable life-cycle stages have been published (Florens *et al.*, 2002; Hall *et al.*, 2005; Khan *et al.*, 2005; Le Roch *et al.*, 2004). Three studies have taken on the challenge to compare transcript and protein levels in *P. falciparum*, *P. berghei* and *P. yoelii* (Hall *et al.*, 2005; Le Roch *et al.*, 2004; Tarun *et al.*, 2008). It has been speculated that gene expression in *Plasmodium* spp. is preferentially regulated post-transcriptionally through mRNA degradation, translational repression, and epigenetic mechanisms (Hakimi and Deitsch, 2007; Mair *et al.*, 2006; Shock *et al.*, 2007).

Gene regulation in the context of invasion comes from a study using isogenic subclones of 3D7 (Cortes *et al.*, 2007). In this study it was demonstrated that members of the EBL, Rhoph1 and RBL families are present in either an active or a silenced state. The two members of the RhopH1 complex were mutually exclusive expressed, depending on the invasion pathway used by the parasite. It was proposed that a clonal silencing of multigene invasion molecules is epigenetic controlled and probably evoked to avoid the host immune response.

Earlier studies using Dd2 and W2mef parasites demonstrated a switch from sialic-acid-dependent to -independent invasion is linked with the up-regulation of RH-4 expression. While the sialic-acid-independent ligand RH-4 gets up-regulated, the sialic-acid-dependent ligand RH-1 remains at the same level in both clones (W2mef and W2mef/NM) demonstrated by microarray and RT-PCR data (Gao *et al.*, 2008). In these studies, changes in other genes were just around- or within the 2-fold range. Both investigations used a single schizont stage sample for the microarray experiment. The schizont stage is defined as the multi-nucleation of the parasite and is subjectively determined from Giemsa stained blood smears. The timeframe of the schizont stage ranges from around 32 hours to 48 hour post-invasion. Single TP microarray has limitations and demands a precise synchronisation and extraction of schizonts at the same developmental stage. The wide schizont stage window probably account for the masking of transcriptional changes by the shift in progression of each gene itself. As transcription is process over time and for example a shift of gene regulation could not be observed in a study with compiled schizont stages.

A recent study compared gene transcription profiles of *P. falciparum* with *P. vivax* over 48 hour cycle and uncovered a shift in the peak of transcription of a number of genes (Bozdech *et al.*, 2008). This could also be the case for genes in W2mef/NM compared to W2mef, but this can only be investigated by a comprehensive time course experiment.

Altogether these three studies Cortes *et al.*, (2007), Stubbs *et al.*, (2005) and Gaur *et al.*, (2006) limited their work to transcriptional studies and only followed the protein expression profiles for candidates identified by transcriptional analysis. In this chapter transcriptional profiles of the entire schizont stage are analysed and correlated to the protein expression data. Therefore the use of isogenic parasite strain with altered invasion characteristics provides a valuable tool to study gene regulation at the merozoite stage. The transcription of a gene is correlated with the expression data of the protein product and differences are identified. Furthermore this chapter presents putative regulatory motifs of differentially regulated proteins in untranslated regions by *de novo* identification.

## Results

### ***5. 1 Microarray of schizont stage parasite***

During the schizont stage, the parasite is actively transcribing genes important for merozoite development including invasion molecules. In order to follow gene transcription during schizogony, individual time points (TP) from synchronous schizont stages starting from 32 hours post-invasion (every 2 hours) until reinvasion from W2mef (sialic-acid-dependent) and W2mef/NM (sialic-acid-independent) were collected. Each TP from W2mef and W2me/NM was hybridized against the reference pool (W2mef all stages) and analysed. This was performed in duplicate. A newly designed oligo-microarray was used consisting of 10166 long oligonucleotide elements for 5363 genes with one unique oligonucleotide every 2 kb per gene (Hu *et al.*, 2007). The dataset was filtered with median intensities greater than the local background plus 2 times the standard deviation of the background for each channel. This gave a set of a total of 5059 genes for the analysis. Furthermore, the data set was smoothed by using the average values of the duplicated microarray experiment.



### **5.1.1 Correlation of W2mef and W2mef/NM transcriptome datasets**

At first it was important to establish that the individual datasets obtained by microarray technology were in at the same developmental stage in order to allow transcription comparison of W2mef and W2mef/NM.

Two approaches are used to establish the correspondence between the TP of W2mef and W2mef/NM. First the maturation of trophozoites to segmented schizonts was visually inspected by Giemsa-stained thin blood smears taken from each TP. The uniform development of the stages from trophozoite to schizont was observed in both clones.

The second approach was the statistical evaluation by Pearson correlation (Table 5.1). All genes of one TP in W2mef are set in correlation with all genes of a TP in W2mef/NM.

A Pearson correlation closer to 1 is well correlated and a correlation close to -1 is anti-correlated. In general, the same TP in W2mef correlates with the same TP in W2mef/NM (fields with blue colour) and used for the further analysis of genes. The largest disparity was found in 38h and 40h (TP4) post-invasion with an intermediate correlation coefficient.

Table 5. 1. Pearson correlation of individual time points taken from W2mef and W2mef/NM for all genes

|                  | W2mef<br>TP1 | W2mef<br>TP2 | W2mef<br>TP3 | W2mef<br>TP4 | W2mef<br>TP5 | W2mef<br>TP6 | W2mef<br>TP7 | W2mef<br>TP8 | W2mef<br>TP9 | W2mef<br>TP10 |
|------------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|---------------|
| W2mef/NM<br>TP1  | 0.935231     | 0.8664414    | 0.67060729   | 0.1432152    | -0.333556    | -0.5231484   | -0.491295    | -0.4462209   | -0.3934734   | 0.44503627    |
| W2mef/NM<br>TP2  | 0.8159067    | 0.822406     | 0.70459024   | 0.217214     | -0.1889786   | -0.4526062   | -0.458977    | -0.4522031   | -0.4217591   | 0.19439707    |
| W2mef/NM<br>TP3  | 0.5580991    | 0.6319001    | 0.68285855   | 0.3995081    | 0.1348409    | -0.1894214   | -0.248852    | -0.3064211   | -0.3245502   | -0.0558686    |
| W2mef/NM<br>TP4  | 0.2763663    | 0.3959463    | 0.54516515   | 0.4888009    | 0.4062922    | 0.0987102    | 0.0059072    | -0.0773675   | -0.1269985   | -0.1860033    |
| W2mef/NM<br>TP5  | -0.477941    | -0.360538    | -0.109043    | 0.4074999    | 0.7816322    | 0.7513912    | 0.6938998    | 0.616892     | 0.5090521    | -0.1430237    |
| W2mef/NM<br>TP6  | -0.583425    | -0.526353    | -0.3156859   | 0.3101008    | 0.752827     | 0.8716619    | 0.8618365    | 0.8268644    | 0.6894429    | 0.07356689    |
| W2mef/NM<br>TP7  | -0.470269    | -0.474318    | -0.3634677   | 0.1982684    | 0.5908039    | 0.7889638    | 0.8056029    | 0.8520961    | 0.76471      | 0.34276861    |
| W2mef/NM<br>TP8  | -0.287582    | -0.335651    | -0.313074    | 0.1232702    | 0.4098065    | 0.623413     | 0.7009896    | 0.801099     | 0.7248338    | 0.55475017    |
| W2mef/NM<br>TP9  | -0.22331     | -0.2981      | -0.3295613   | 0.083342     | 0.296455     | 0.5435586    | 0.6345951    | 0.7465232    | 0.6857824    | 0.64645854    |
| W2mef/NM<br>TP10 | -0.076664    | -0.175771    | -0.2497194   | 0.0322472    | 0.155057     | 0.3826677    | 0.5076727    | 0.629249     | 0.5878861    | 0.71530485    |

### ***Correlation of individual gene transcripts***

Two approaches were used to analyse schizont gene transcription. First, the best fit Pearson correlations were used to correlate gene transcripts at the individual gene level between W2mef/NM to W2mef. Here, the similarity between W2mef and W2mef/NM transcriptional development per gene was calculated. Genes with low Pearson correlation (close to -1) have a greater transcriptional disparity (total opposites) than genes with good correlation (close to 1, series is almost identical). Two curves with identical shape, but different magnitude, will still have a correlation of 1.

The Pearson correlation coefficient ( $r$ ) of all 5059 genes from the microarray was calculated and categorised in three arbitrary intervals: low correlation, intermediate correlation and high correlation (Figure 5.1). These intervals were defined previously based on visual inspections and the same classification can be applied on this data set (Bozdech *et al.*, 2008). Genes were plotted according to their Pearson correlation coefficient.

The majority of genes (80%) are well correlated as they are in the range between  $r=0.5$  to 1. Less than 18% (905 genes) are intermediately correlated and only 79 genes have a very low correlation (-1 to -0.8).



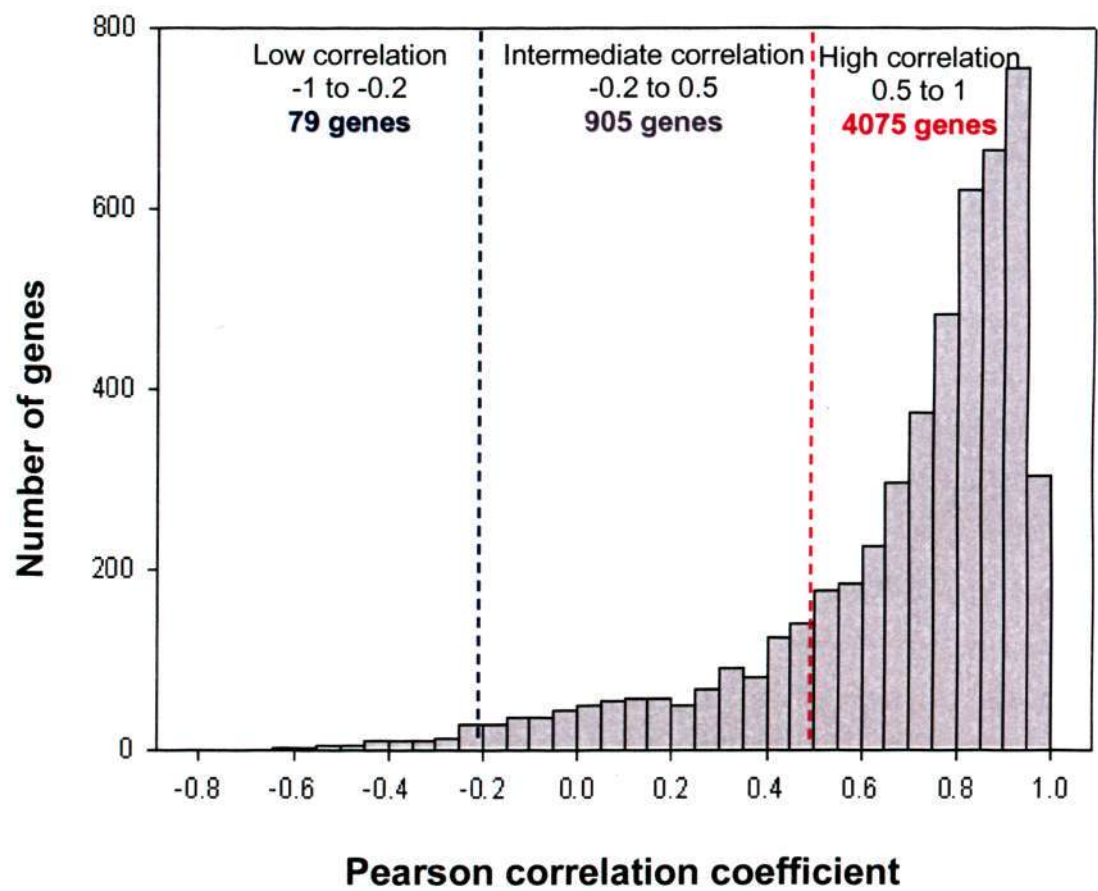


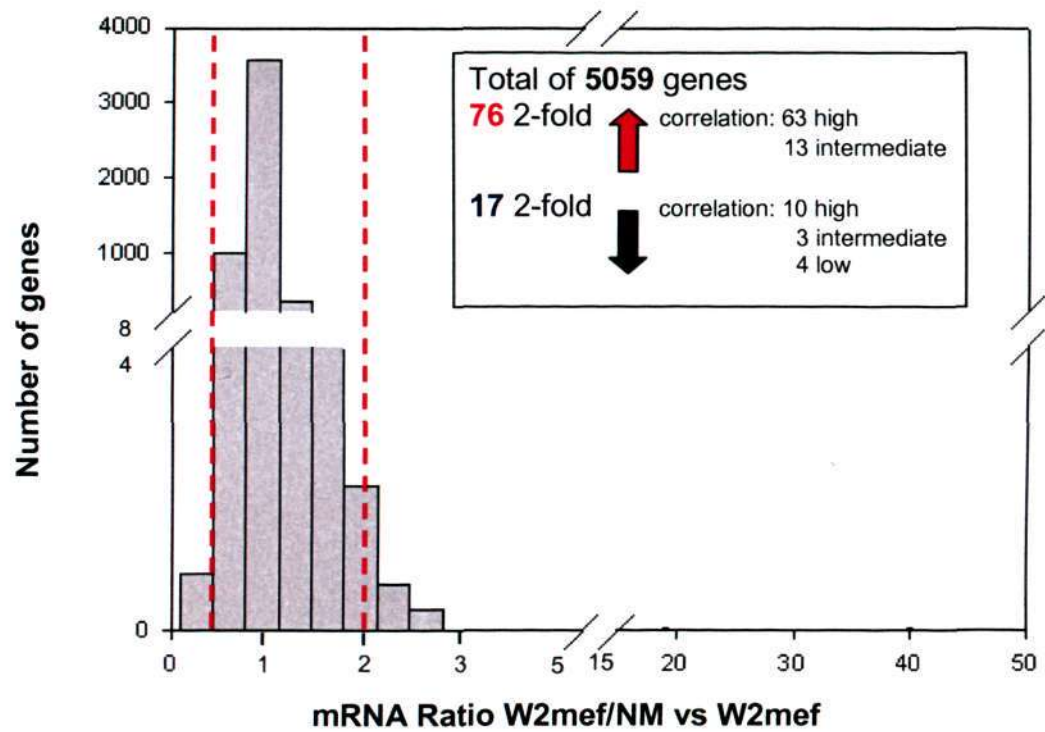
Figure 5. 1. Histogram of Pearson correlation of genes.

Histogram showing the distribution of Pearson correlation coefficients of 5059 gene transcripts calculated gene transcription profiles determined by microarray technology.

### ***Comparative analysis of transcriptional changes between W2mef and W2mef/NM***

Pearson correlation only provides information about the timing of transcription but not about the amount of mRNA transcript. The second approach was used to obtain changes in total mRNA transcription at the individual gene level by calculating ratios for up- and down-regulated genes. Thereby, the sum of all ten TP of W2mef/NM was divided by the sum of all ten TP of W2mef (Figure 5.2). Most of the genes (4964) show no change in transcript levels. The significance for up or down-regulation was set at 2-fold difference between the 2 samples (W2mef/NM and W2mef) over the period of ten data points. This is a stringent setting and only approximately 2% of genes disclose changes in transcription with 76 up-regulated and 17 down-regulated genes in W2mef/NM.

Out of the 76 up-regulated genes in W2mef/NM 63 have a high Pearson correlation, 13 an intermediate correlation. Similar to the 17 down-regulated genes: the majority has a high Pearson correlation (10), 3 genes are intermediate correlated, and 4 transcripts have a low Pearson correlation. The highest change in transcription showed RH-4 with 40 and EBA-165 with 19. This is in correspondence with previously reported transcriptional changes by Stubbs *et al.*, (2005) and Gaur *et al.*, (2006).



**Figure 5. 2. mRNA ratio W2mef/NM vs. W2mef of 5059 genes.**  
The box indicates 2-fold up-regulation (red arrow) and down-regulation (blue arrow) of genes. The red dashed line at 0.5 and 2.0 indicate the 2-fold change.



## ***5. 2 Transcriptional analysis of quantified merozoite proteins***

Chapter 3 describes quantitative proteomic data from W2mef and W2mef/NM. The transcriptional profile of these quantified proteins was used in a comparative analysis.

All 600 quantified proteins are also identified in the microarray survey and all further transcript analysis is based on this set of 600 proteins.

In order to get an idea about transcriptional differences in both clones W2mef and W2mef/NM, Pearson correlation was performed at the individual gene level only for the 600 identified molecules from the proteomic dataset (Figure 5.3 A).

A high Pearson correlation between 0.5 and 1 was observed for most of the transcripts with similar expression (540 genes), an intermediate correlation was observed in 55 genes and only 5 genes show a low Pearson correlation. Examples of transcription profiles for each of the Pearson correlation sections was represented in Figure 5.3 B,C and D. RAP18 is a GTP binding protein and involved in intracellular vesicular trafficking (Figure 5.3 B) (Quevillon *et al.*, 2003). The negative correlation is due to anti-progression of the transcriptional trend. Besides, the differences between the two clones are not very large, with only 0.6-fold alteration at the peak amplitude at TP 4.

An example for intermediate Pearson correlation is shown in Figure 5.3 C. Histone 2a is one of the four core proteins in the chromatin and modifications of Histones can regulate transcription of genes. Transcription profile of Histone 2a in W2mef peaks around TP7 whereas the peak of transcription in W2mef/NM is around two hours earlier at TP5.

A very similar transcriptional development in W2mef/NM and W2mef is observed for serine/threonine protein kinase, with a Pearson correlation of 0.93 Figure 5.3 D.

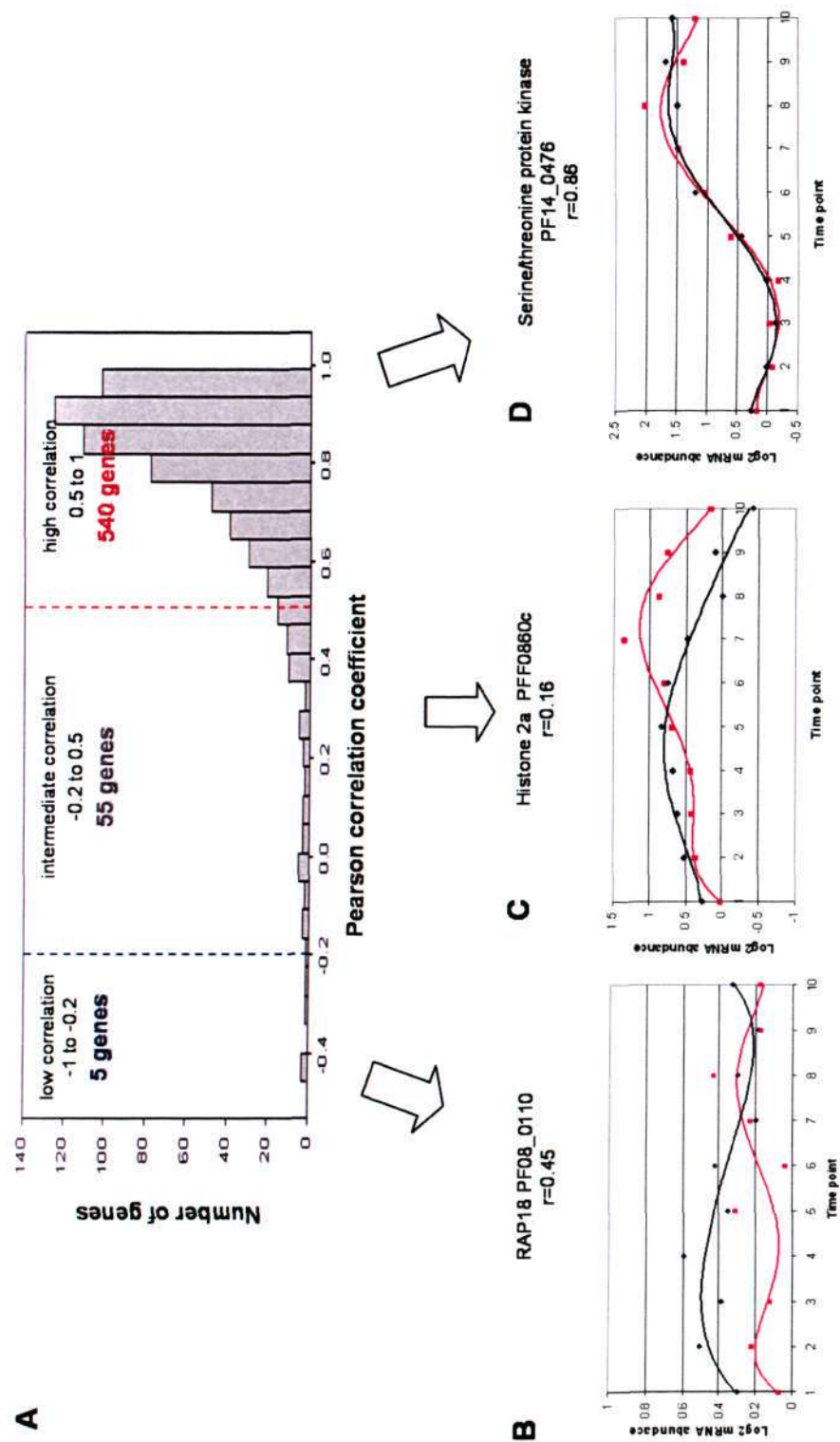


Figure 5.3. Overview of correlation of 600 genes with proteomic data.

- A. Pearson correlation of transcripts with proteomic data.
- B. Example of transcriptional profile one low correlated gene: RAP18 PF08\_0110.
- C. Example of transcriptional profile one intermediate correlated gene: Histone 2a PFF0860c.
- D. Example of transcriptional profile one high correlated gene: Serine/threonine protein kinase PF14\_0476.

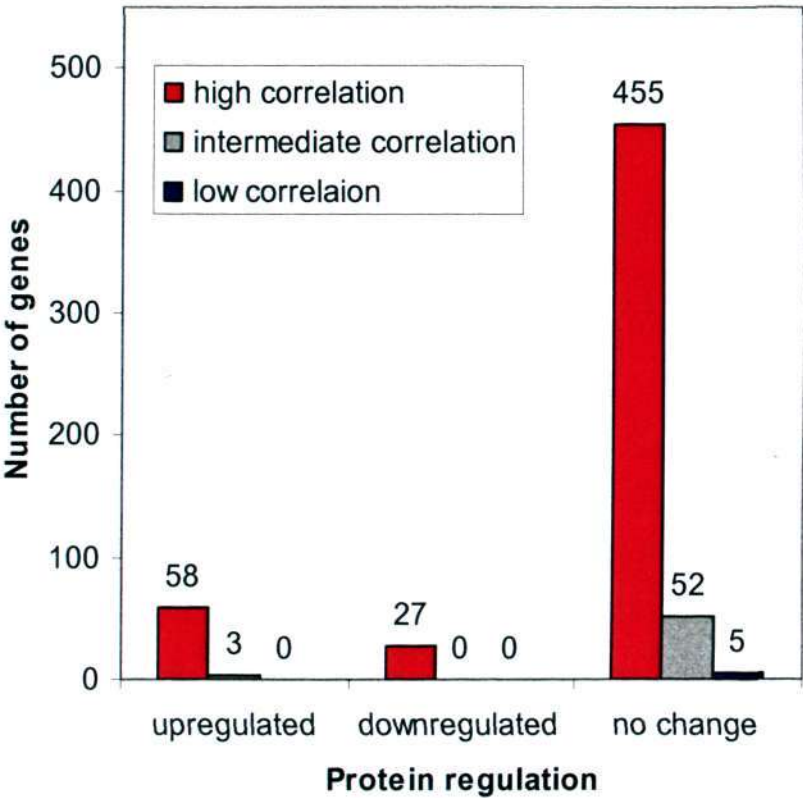
### **5.2. 1 Comparison of transcriptional regulation with proteomic expression data**

The Pearson correlation of the 600 genes with proteomic data was categorised in up-regulation, down-regulation and proteins with no change (Figure 5.4) as described before in section “Correlation of individual gene transcripts”. The vast majority of genes regardless of the category have high Pearson correlation. These high correlated genes show no change in there transcriptional profile.

In the category of up-regulated proteins the majority (58) of genes show high person correlation and only 3 genes display an intermediate person correlation.

All 27 down-regulated genes have a high Person correlation. In the section with no expression changes, 455 genes have a high person correlation and 52 intermediate Pearson correlation and only 5 genes express a low correlation coefficient.





**Figure 5. 4. Pearson correlation of 600 genes with proteomic data categorised by their protein expression regulation.**

High Pearson correlation: 0.5 to 1

Intermediate Pearson correlation: -0.2 to 0.5

Low Pearson correlation: -0.2 to -1

***Transcriptional distribution of genes with protein expression data***

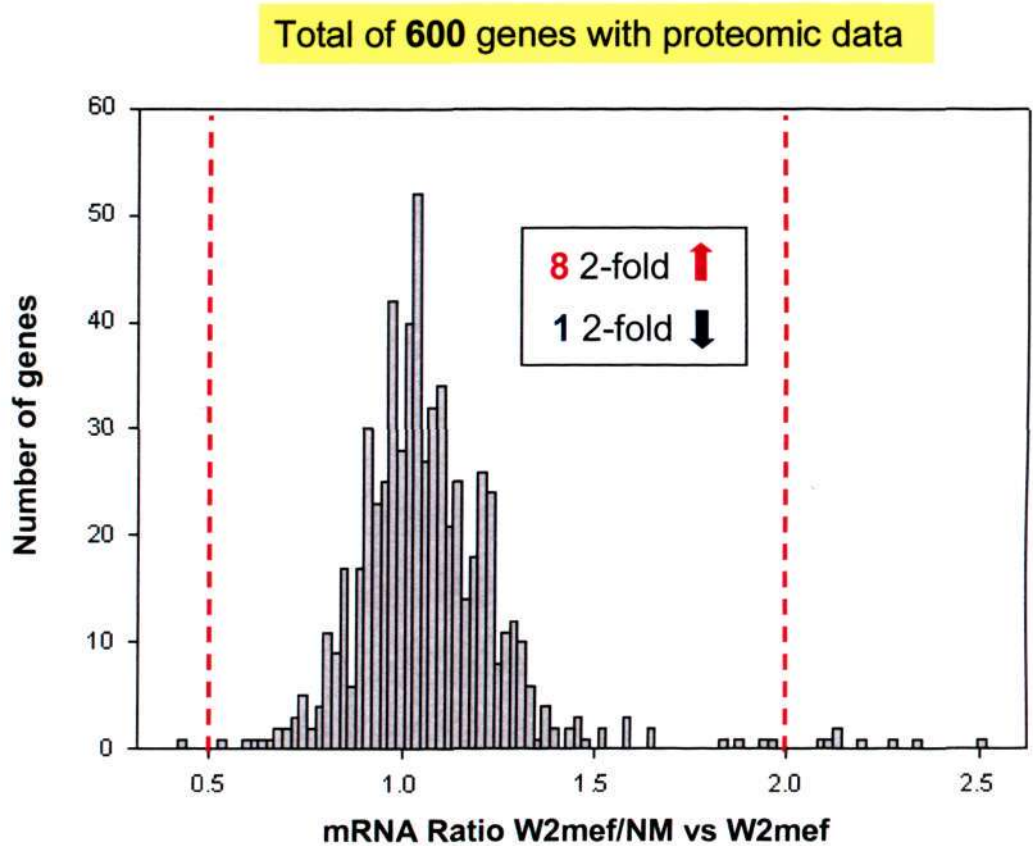
Changes of the amount of mRNA transcript for the 600 genes was summarised in Figure 5.5. The majority of these genes show no change in transcription between W2mf/NM and W2mef. Only one transcript was more than 2-fold down-regulated and 8 transcripts were more than 2-fold up-regulated. The respective genes are listed in Table 5.2 and 5.3.

The 600 genes are categorised in three groups according to their corresponding protein expression: up-regulated proteins, down-regulated proteins and proteins with no expression differences between W2mef/NM and W2mef (Figure 5.6).

Here again, most of the genes exhibit no change in transcriptional profile. None of the up-regulated proteins demonstrate any transcriptional differences. They fall within the 2-fold up- and 2-fold down-regulation.

Four genes are up-regulated in transcript levels in down-regulated protein category and none of the genes with the protein down-regulation showed a significant down-regulation in their transcripts.

In the category of proteins with no change of expression, five genes are significantly up-regulated and one was more than 2-fold down-regulated. 506 of 512 genes follow the trend of having no change in expression and transcription.



**Figure 5. 5. Distribution of mRNA ratios of genes with proteomic data.**  
The total of 600 genes follows the normal distribution. The red dashed line at 0.5 and 2.0 indicate the 2-fold change.



Table 5. 2. Up-regulated gene transcripts.

|           |   |
|-----------|---|
| PF10_0025 | PF70 protein  |
| PFE0065w  | PFE0065w  skeleton binding protein1, PfSBP1             |
| PFI1740c  | PFI1740c  hypothetical protein                          |
| PFI1735c  | PFI1735c  ring exported protein, REX                    |
| PFB0120w  | PFB0120w  early transcribed membrane protein 2, ETRAMP2 |
| PF11_0509 | ring-infected erythrocyte surface antigen, putative     |
| PFE0120c  | PFE0120c  Merozoite Surface Protein 8, MSP8             |
| PF14_0016 | early transcribed membrane protein 14.1, etramp14.1     |

Table 5. 3. Down-regulated gene transcript

|          |   |
|----------|---|
| PFE0040c | PFE0040c  Mature parasite-infected erythrocyte surface antigen (MESA) or PfEMP2 |
|----------|---|

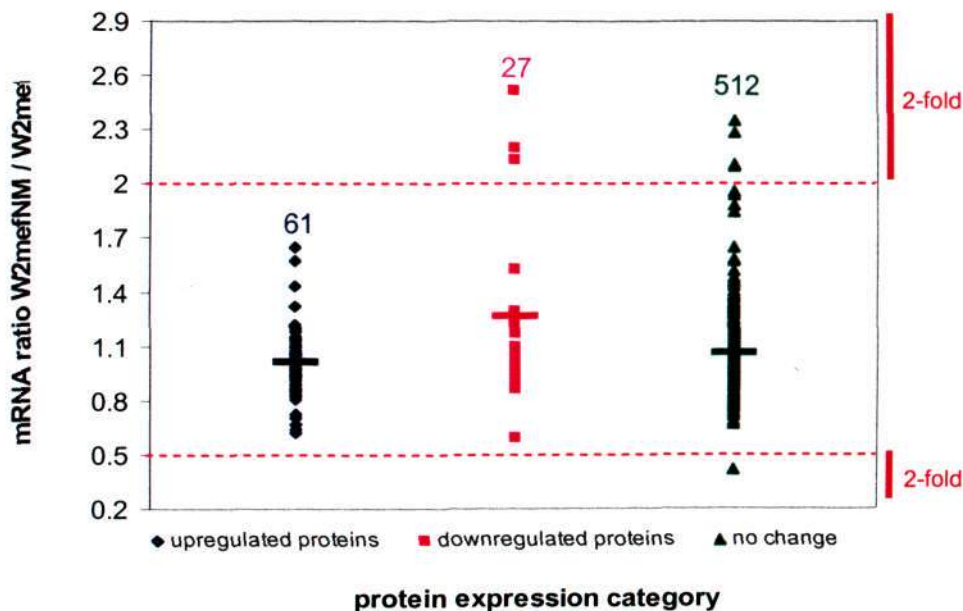


Figure 5. 6. Transcriptional distributions of genes with protein expression data.

None of the gene transcripts is differentially transcribed in the category of up-regulated proteins. Differential transcription is observed in down-regulated proteins with four genes up-regulated. Five gene transcripts are up-regulated and one is down-regulated in the category with no protein expression.

### ***5. 3 Validation of transcripts of selected proteins analysed in Chapter 3 by Real-time PCR***

In addition to microarray data, Real-time PCR was used to validate the transcriptional data for some selected genes. The cDNA of the individual TP1-10 for W2mef/NM and W2mef were pooled respectively. The gene ornithine aminotransferase (Figure 5.7) with a Pearson correlation coefficient of 0.99 is equally transcribed in both clones and was used as standard gene. The threshold cycle ( $C_t$ ) was calculated for all three samples per gene of interest and subtracted by the standard gene.

In Chapter 3 some proteins were tested for protein expression levels and independently evaluated by western blot analysis. The immunoblot result confirmed the higher expression of these proteins. The overall transcript levels of these higher expressed proteins are not altered as listed from microarray analysis as ratio of all 10 TP of W2mef/NM vs. W2mef in Table 5.4. Real time PCR was performed to validate the transcriptional data from the microarray (Figure 5.8). The majority of genes show no significant change in transcription profile (AMA-1, plasmepsin 2, eIF5a, Histone 3, clag 3.1 and Gap-45). A change around 2-fold was observed for MSP-1, actin and MTIP. This is considered to be insignificant as a 2-fold change is actually only 1 cycle difference in the real time PCR experiment. Overall, real time PCR confirms that there are no differences in gene transcription between W2mef/NM and W2mef in the selected genes.



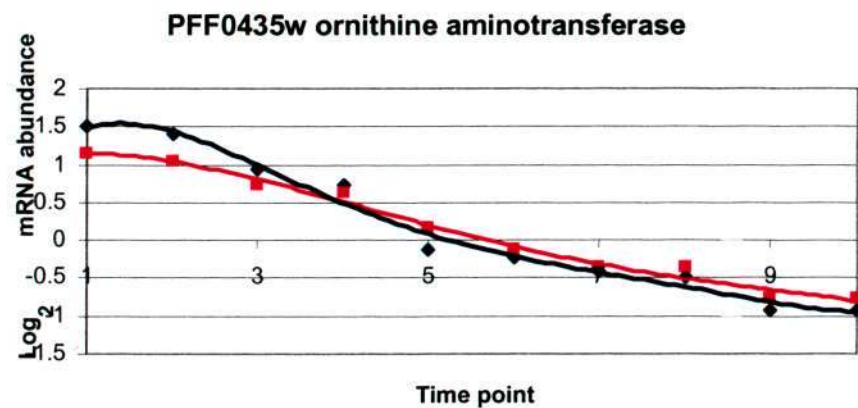
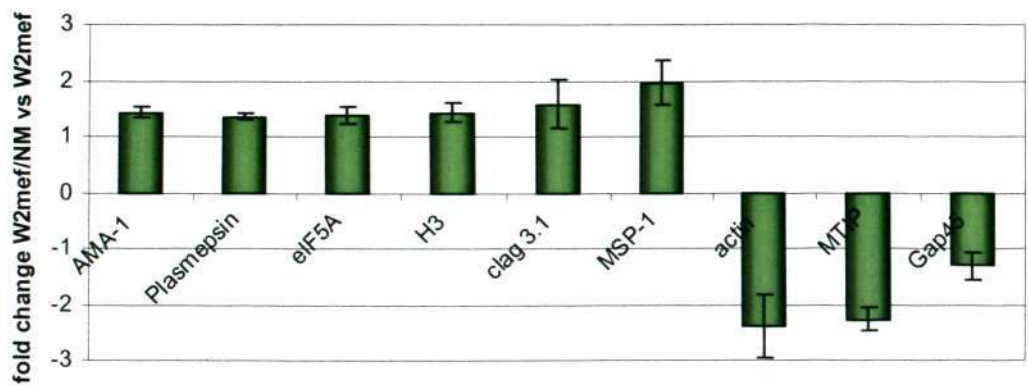


Figure 5. 7. Ornithine aminotransferase transcriptional trends from microarray experiment.

W2mef/NM and W2mef exhibit an almost identical transcription of this gene. Therefore, this gene is used as the standard gene for real time PCR.

Table 5. 4. Selected invasion molecules with expression and transcription data

| Accession | Name         | Protein expression<br>(iTRAQ)<br>Fold change<br>W2mef/NM vs. W2mef | Microarray ratio<br>W2mef/NM vs. W2mef |
|-----------|--------------|--|--|
| PF13_0197 | MSP-7        | 5.6  | 1.2                                    |
| PFL2225w  | MTIP,        | 3.9  | 1.0                                    |
| PF13_0185 | histone h3   | 2.8  | 1.0                                    |
| PF14_0077 | plasmepsin 2 | 2.2  | 1.0                                    |
| PFL1090w  | GAP-45       | 2.4  | 0.8                                    |
| PFC0110w  | Clag 3.1     | 2.3  | 1.2                                    |
| PFL0210c  | eIF5a        | 2.2  | 1.0                                    |
| PFI1445w  | RhopH2       | 2.3  | 0.9                                    |
| PFI1475w  | MSP-1        | 2.8  | 1.0                                    |



**Figure 5. 8. Validation of selected proteins by Real Time PCR.**

Gene transcription ratio of W2mef/NM vs. W2mef of seven genes is given relative to house keeping gene ornithine aminotransferase. Standard deviation of triplicate is indicated as error bar. cDNA of combined 10 TP was used to generate the data.



### ***5. 4 Correlation of expression data to transcription profile***

The top and the bottom transcript from each expression category were selected to analyse the correspondence of transcript and expressed protein.

In the category of up-regulated proteins the highest expression ratio W2mef/NM vs. W2mef was found in MAL8P1.72 (Figure 5.9. A). The transcriptional profile does not vary too much during the schizogony. W2mef/NM is clearly higher transcribed throughout the observed time then W2mef. The sum of W2mef/NM vs. W2mef from microarray is 1.65. Real time PCR data confirmed this by analysis of the transcription fold change of W2mef/NM vs. W2mef of 2 compared to housekeeping gene (Figure 5.9. C). Thus, the same trend of gene transcription and expression is observed.

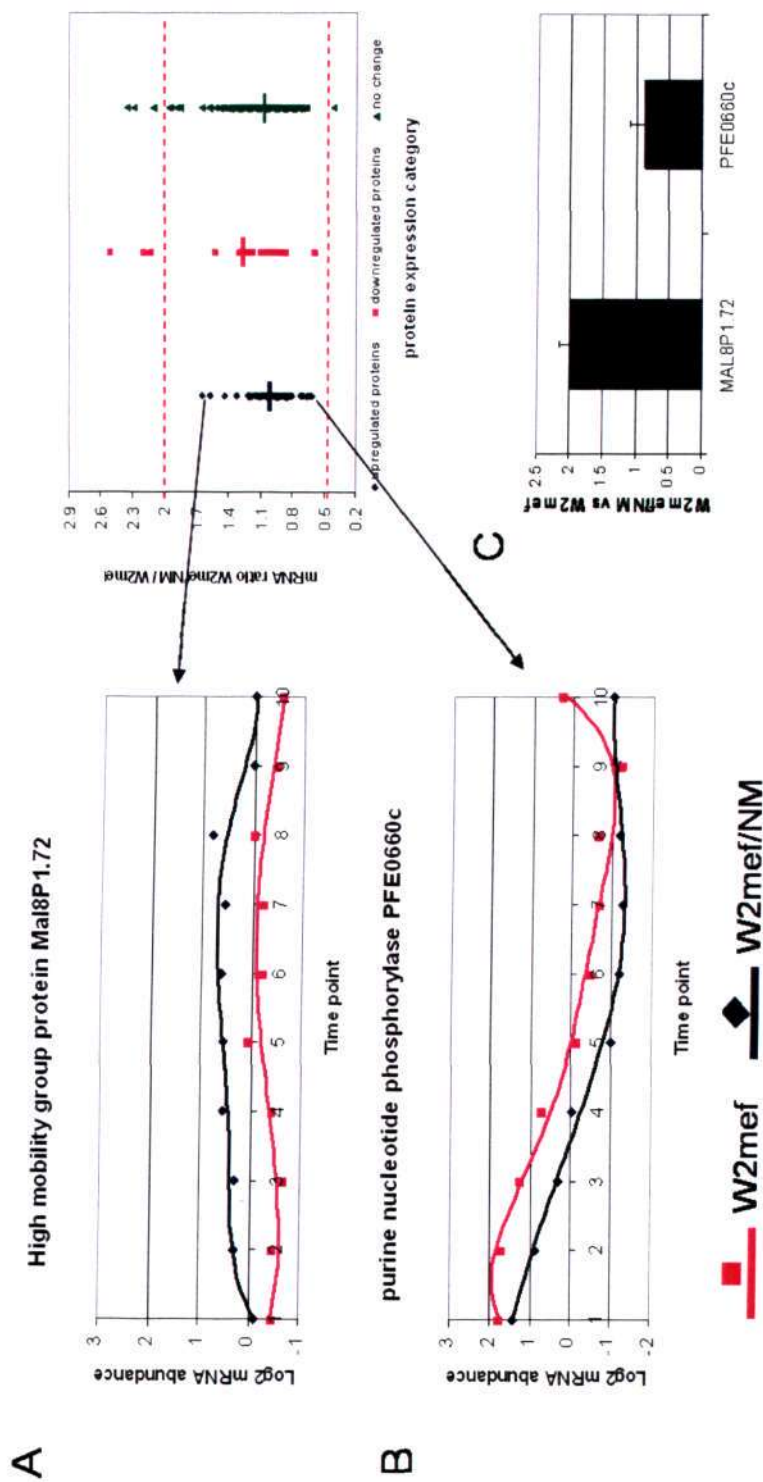
The down-regulated gene is PFD0660c. W2mef PFD0660c transcript is slightly more abundant than W2mef/NM during the schizogony (Figure 5.9. B). The sum of W2mef/NM vs. W2mef is 0.65. The RT-PCR exhibits also a trend towards higher W2mef in transcript levels (Figure 5.9. C).

The top up-regulated transcript in the category of down-regulated proteins is PFI1735c (Figure 5.10. A). The transcriptional profile is the same for both clones the first six hours during schizont stage and then W2mef/NM transcription elevates. RT-PCR revealed transcript levels of 1.5 fold change.

PFD0090c transcription is lower in W2mef/NM in microarray and RT-PCR this is in consistent with the proteomic expression data (Figure 5.10. B and C).

The protein with no expressional change showed the largest transcriptional change in PFE0120c (Figure 5.10. A). From the fifth TP onwards, more transcript of W2mef/NM is detected in microarray, but RT-PCR detected more W2mef transcript (Figure 5.11. C). Anyhow, the expressional data displays no change on the protein level.

PFE0040c transcript is down-regulated throughout the timeframe observed. This is also confirmed by RT-PCR (Figure 5.11. B and C).



**Figure 5.9. Transcriptional profile of highest and lowest transcript of up-regulated proteins.**  
**A.** Transcriptional profile of W2mef and W2mef/NM of High Mobility group protein Mal8P1.72.  
**B.** Transcriptional profile of W2mef and W2mef/NM of purine nucleotide phosphorylase PFE0660c.  
**C.** Real time PCR of gene transcription for (Mal8P1.72) and (PFE0660c). Gene transcription ratio of W2mef/NM vs. W2mef is given relative to housekeeping gene ornithine aminotransferase.



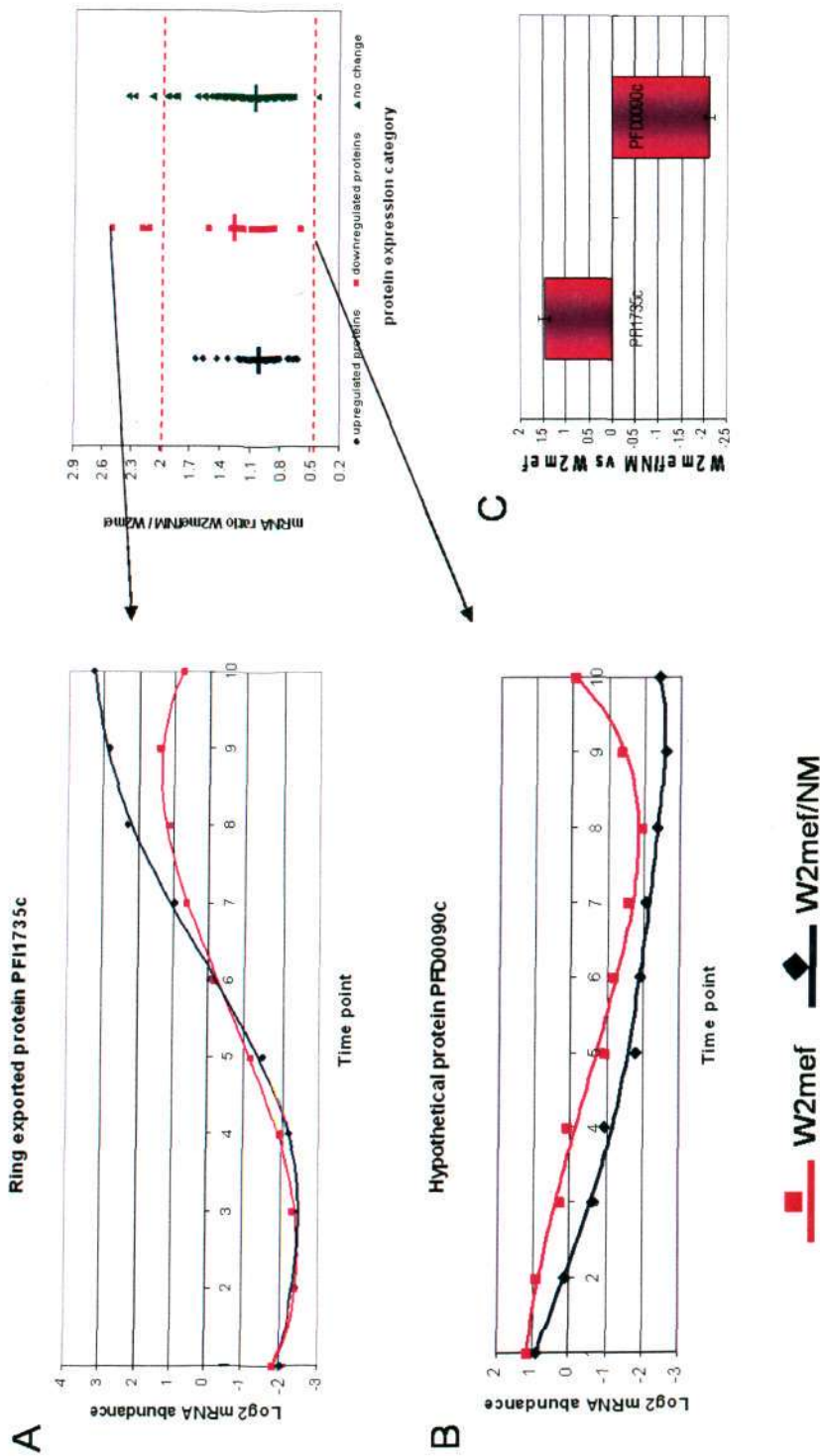


Figure 5. 10. Transcriptional profile of highest and lowest transcript of down-regulated proteins.

A. Transcriptional profile of W2mef and W2mef/NM of PF11735c.

B. Transcriptional profile of W2mef and W2mef/NM of PFD0090c.

C. Real time PCR of gene transcription for PF11735c and PFD0090c. Gene transcription ratio of W2mef/NM vs. W2mef is given relative to housekeeping gene ornithine aminotransferase.

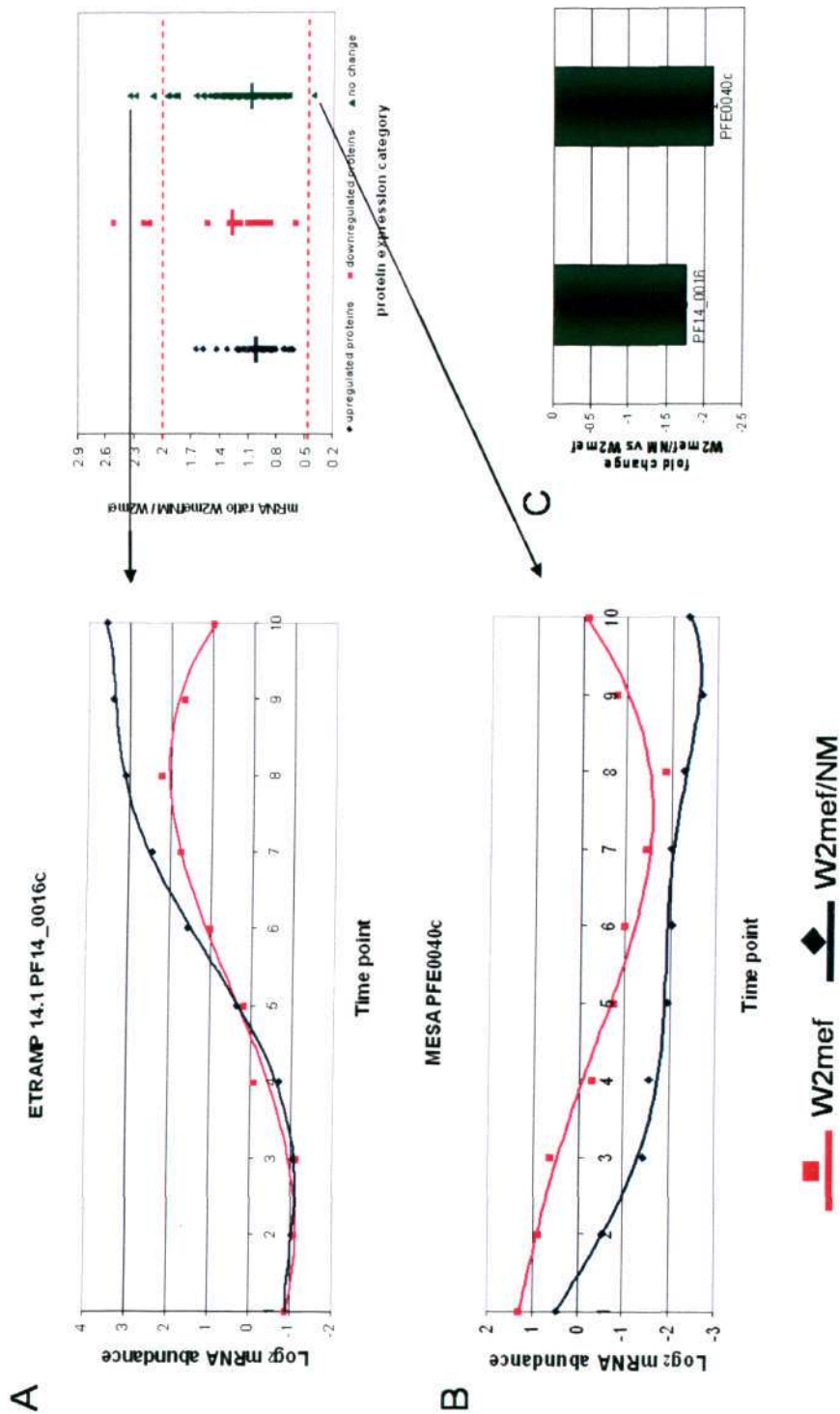


Figure 5.11. Transcriptional profile of highest and lowest transcript with no change in protein expression.

A. Transcriptional profile of W2mef and W2mef/NM of PF14\_0016c.

B. Transcriptional profile of W2mef and W2mef/NM Mesa PFE0040c.

C. Real time PCR of gene transcription for ETRAMP 14.1 PFE1120c and MESA PFE0040c. Gene transcription ratio of W2mef/NM vs. W2mef is given relative to housekeeping gene ornithine aminotransferase.

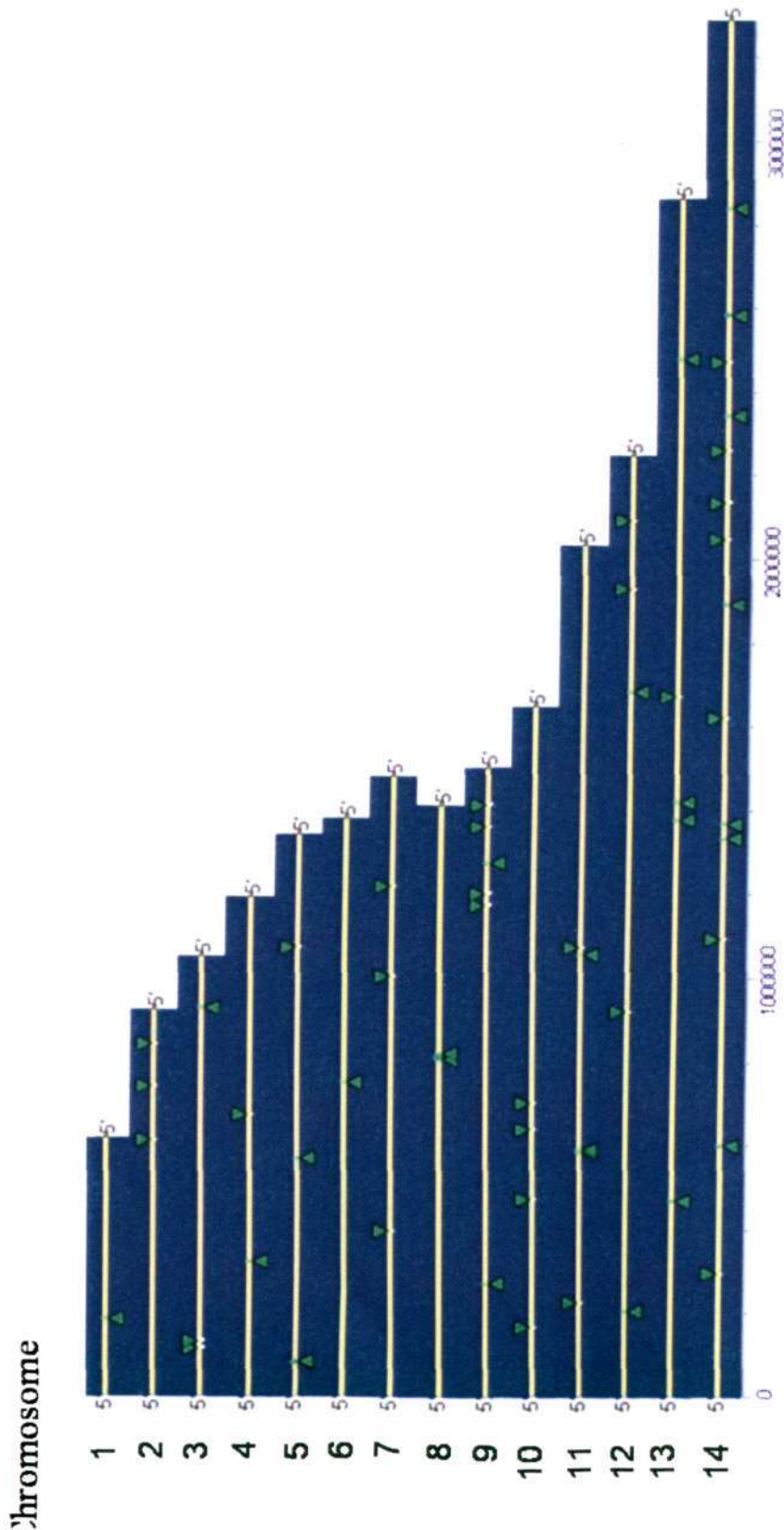
### ***5. 5 Chromosomal distribution of up-regulated proteins***

The close proximity of gene on a chromosomal region can activate mechanisms for co-regulation of these genes. One example is the up-regulation of EBA-165 in W2mef-NM in association with the up-regulation of RH-4 (Stubbs *et al.*, 2005). While no functional protein has been detected for EBA-165, this gene is considered a pseudogene (Triglia *et al.*, 2001b). Both genes are located at the subtelomeric region on chromosome 4 in a head to head orientation. It is proposed the EBA-165 is transcriptionally regulated by the up-regulation of RH-4 due to their close gene location proximity.

The 61 up-regulated proteins exhibit very diverse functions. To elucidate why the 61 proteins are up-regulated on the protein level, it is of importance to analyse their chromosomal gene location.

Figure 5.12 is a schematic picture of *P. falciparum* 14 chromosomes with sense and anti-sense strand. Locations of genes are indicated by green arrow. The 61 up-regulated genes are evenly scattered along the chromosomes and show no evident association with any particular chromosomal region.





**Figure 5. 12. Chromosomal location of 61 genes corresponding to up-regulated proteins.**  
All 14 *P. falciparum* chromosomes are represented by yellow bars corresponding in size to the size to the individual chromosome. The position of each green arrow reflects a relative location of each gene within the *P. falciparum* chromosomes. The tip of the arrow indicates sense or anti-sense location.

## ***5. 6 Characterisation of regulatory elements in untranslated regions***

There is no association found in gene location and increased expression of the 61 up-regulated proteins. Thus the increased protein abundance might be driven from regulatory elements common among these genes. Several levels of regulation of gene expression exist. The changes of protein abundance can occur not only during transcription but also at the level of protein expression. The proteomic data revealed 88 differentially expressed proteins and there is no evidence that transcriptional events account for these changes. Therefore one needs to include that protein expression can be regulated by post-transcriptional regulation.

After DNA is transcribed into mRNA a number of mechanisms could constitute to regulate how much RNA is going to be translated into protein product. Regulatory elements by means of RNA binding proteins can regulate some of these processes by recognising a specific RNA motif or secondary structure, typically at the 5'- and 3' UTR of the transcript. These RNA binding proteins recognise specific region in the mRNA. These regulatory processes include: capping (protection of mRNA from 5'exonuclease), alternative splicing (removing introns to make RNA stable for translation) and polyadenylation (stabilises RNA by "buffering" 3'exonuclease activity). Transcription-related regulatory motifs occur frequent in the upstream regions in eukaryotes and multiple copies of the motif are associated with transcriptional activity (van Helden *et al.*, 1998; Zhang *et al.*, 2003). *In silico* identification of putative regulatory motifs that may have biological function needs to be determined by follow up experiments. This experimental design has been applied at a study identifying transcriptional regulatory elements in *P. falciparum* (Iengar and Joshi, 2009; Young *et al.*, 2008).

This section identifies putative post-transcriptional regulatory elements of differentially expressed proteins with similar functions.

### 5.6. 1 Regulatory element search with MEME

The motif discovery tool MEME is used to identify statistically over-represented motifs in a given set of sequences (Bailey and Elkan, 1994). Over-represented motifs are sequences which occur more frequent than expected in a given set of sequences based on its frequency in the genome.

This motif discovery search tool was used before to identify regulatory sequences in *Plasmodium* (Fonager *et al.*, 2007; Gunasekera *et al.*, 2007; Iengar and Joshi, 2009; Le Roch *et al.*, 2004; Young *et al.*, 2008). The average length of intergenic region are determined as 1694 and 1000 nt upstream the translational start site were considered for motif search (Young *et al.*, 2008), but the data input of MEME is limited to 60,000 characters. For the search of 61 up-regulated proteins in 5'UTR the search was split in two sets with 1 to 500 nt and 501 to 1000 nt upstream the encoded gene. For the other search sets, 1000 nt upstream the translational start site were used in MEME search. All motifs displayed were analysed by FIMO for presence in other sequences. The Tomtom programme searches the query motif against a database of known motifs.

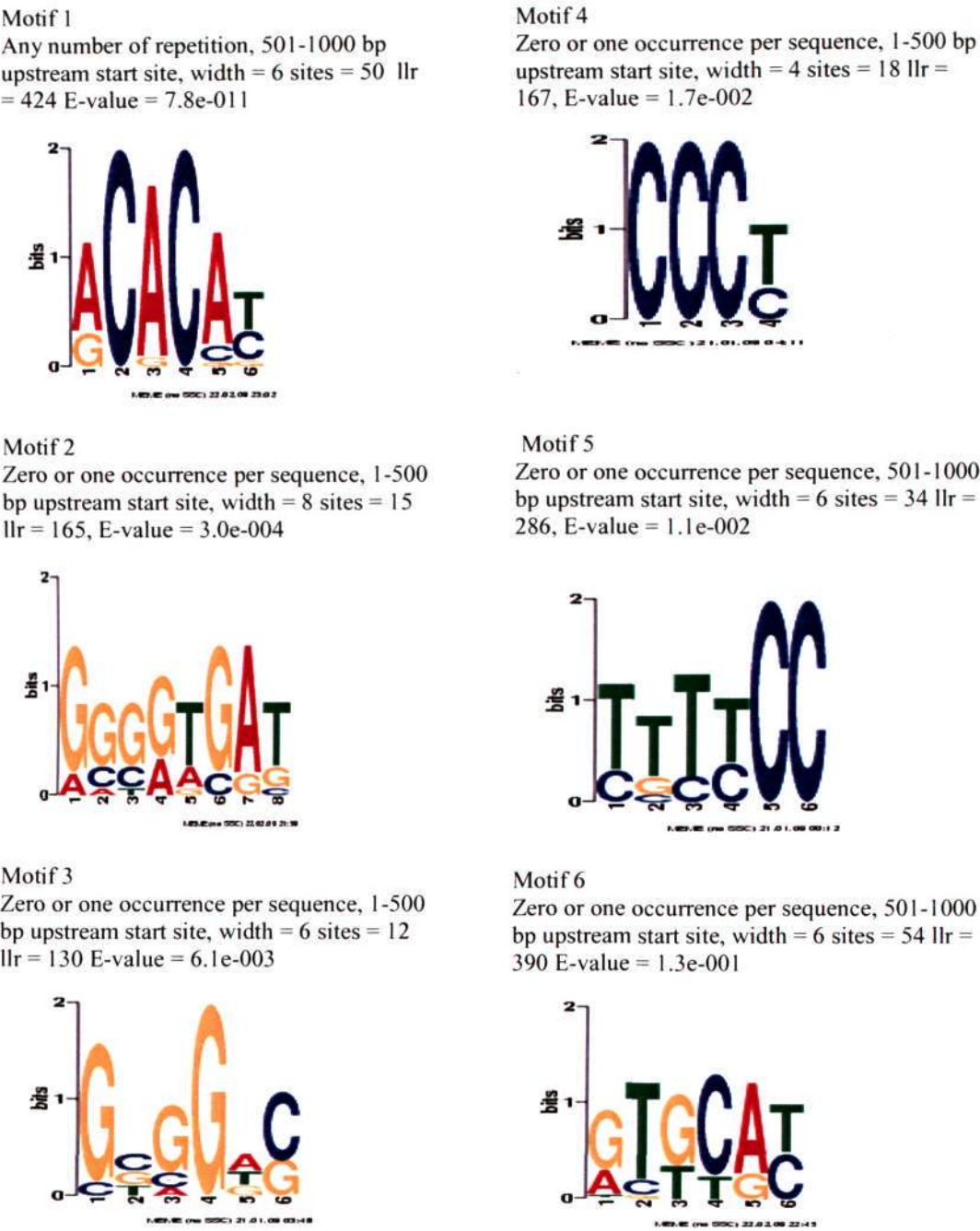
#### *Motifs in 5'UTR in up-regulated proteins*

Six conserved sequences found in MEME are shown in Figure 5.13. The motifs are arranged in order of their conservation in descending order with the most conserved motif at first.

Previous studies analysed genes which are functionally different and are transcribed at the same time. The 61 genes analysed have in common that they are all expressed at the same time in the merozoite stage. Otherwise they are functionally diverse and the peak of transcription within the schizont stage can be different too.



Therefore the 61 genes were dissected in different functional groups (as in Chapter 3 Figure 3.7.) and analysed for conserved motif. The search was only performed when the group of proteins contained at least four different proteins.



**Figure 5. 13. Six motifs identified for up-regulated proteins in 5'UTR.**  
5'UTR sequences of 61 up-regulated proteins were analysed by MEME. Caption identifies the mode of the run: “any number of the repetition” or “zero or one occurrence per sequence”. The range 1-500 bp or 501-1000 bp indicates the region in the UTR upstream from the start site, where the motif was found. Width: Motif length, Sites how many times this motif was identified, llr: log likelihood ratio, E-value: expectation value

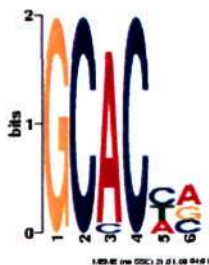
***Motif in invasion proteins***

20 up-regulated proteins were used to identify motifs from their DNA sequence. Three motifs are found with high confidence and are listed with descending order of their E-value Figure 5.14. The first two motifs (as well as the sixth motif from Figure 5.13) share similarity with the motif identified by (Gunasekera *et al.*, 2007). The authors identified a motif in the 5'UTR "TGCAC" in functional related proteases. High yields in transfection assays with a construct containing this motif indicated a potential role of this motif in gene expression (Gunasekera *et al.*, 2007). Furthermore the same motifs share a similarity to a motif identified for cell invasion PFM18.1 "GTGCA" (Young *et al.*, 2008). The authors claim that this is a motif specific for rhoptry proteins. An exact match of this motif was identified when 1-850 bp upstream of the translational start site of the 5'UTR is analysed. Here the "GTGCA" motif has 19 matches including proteins from different expression locations other than rhoptries (Table 5.5). Furthermore, as mentioned earlier, a similar motif was identified in 54 up-regulated proteins (Figure 5.13, motif 6).



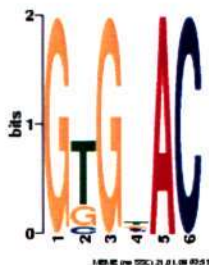
Motif 1

Any number of the repetition width = 6 sites = 18 llr = 185 E-value = 1.1e-008



Motif 2

Zero or one occurrence per sequence width = 6 sites = 13 llr = 129 E-value = 6.7e-003



Motif 3

Any number of the repetition width = 6 sites = 28 llr = 243 E-value = 8.2e-003

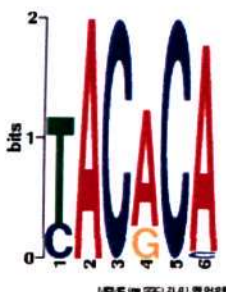


Figure 5. 14. Three putative regulatory motifs in up-regulated invasion proteins.

Caption identifies the mode of the run: “any number of the repetition” or “zero or one occurrence per sequence”

Width: motif length

Sites how many times this motif was identified

llr: log likelihood ratio E-value: expectation value

Table 5. 5. GTGCA motif identified in 1-850 bp from the translational start site in 19 up-regulated proteins in W2mef/NM.

| Accession number | bp relative to start site | P-VALUE  | SITES       |              |             |
|------------------|---------------------------|----------|-------------|--------------|-------------|
| PFB0840w         | 138                       | 4.47E-05 | AATTTTTTAGT | <b>GTGCA</b> | TAAATTGAAT  |
| MAL7P1.119       | 383                       | 4.47E-05 | CACAAATCCA  | <b>GTGCA</b> | CAACAGTGCT  |
| PFI1685w         | 412                       | 4.47E-05 | TGGTTTAGTA  | <b>GTGCA</b> | ATCAAAGTGT  |
| MAL8P1.73        | 151                       | 4.47E-05 | CTGCCCTAAA  | <b>GTGCA</b> | TATATAATAT  |
| PFE0075c         | 257                       | 4.47E-05 | TGCATGTGAA  | <b>GTGCA</b> | AAATAAAAAA  |
| PFD0720w         | 271                       | 4.47E-05 | ATATAGAAGT  | <b>GTGCA</b> | AAGTTCTAAT  |
| PF11_0062        | 215                       | 4.47E-05 | ATATTTGCAT  | <b>GTGCA</b> | TATTAAAAAGT |
| PF13_0058        | 19                        | 4.47E-05 | TTTATTAAGT  | <b>GTGCA</b> | TAATTTTTTAT |
| PFB0935w         | 734                       | 4.47E-05 | TTCTCTTTCG  | <b>GTGCA</b> | TTGTTTTCTA  |
| PF11_0287        | 349                       | 4.47E-05 | ATATATGTAT  | <b>GTGCA</b> | ATTTTTTTTTT |
| PFL1945c         | 173                       | 4.47E-05 | TTATAAATTC  | <b>GTGCA</b> | TAATATACAT  |
| PF14_0439        | 417                       | 4.47E-05 | AAAAGCCTTT  | <b>GTGCA</b> | TGGTTAATAA  |
| PFC0120w         | 788                       | 4.47E-05 | TTATATATTT  | <b>GTGCA</b> | ATATATCAAA  |
| PF11_0164        | 508                       | 4.47E-05 | TTTTTATTGT  | <b>GTGCA</b> | TTAAGTATTG  |
| PFE0080c         | 129                       | 4.47E-05 | TGCATATGAA  | <b>GTGCA</b> | AATGATAATA  |
| PFI1475w         | 8                         | 4.47E-05 | ATATATTA    | <b>GTGCA</b> | CTAAAGGAAA  |
| PF14_0323        | 589                       | 4.47E-05 | TTATTCCAAT  | <b>GTGCA</b> | TGATAAAAGA  |
| PF13_0197        | 803                       | 4.47E-05 | TAAAATTTTT  | <b>GTGCA</b> | AATCAAATTT  |
| PF14_0660        | 303                       | 5.13E-05 | TTAACAATTC  | <b>GTGCG</b> | TATGGTTTTT  |

***Motif in proteins involved in degradation***

Seven sequences from the proteasome were analysed for conserved motif and two motifs were found in all given sequences (Figure 5.15). The second motif shares similarities with HMG-IY motif identified in plant *Pisum sativum*, human ETS1 and *Drosophila melanogaster* dl\_1 as identified by Tomtom.

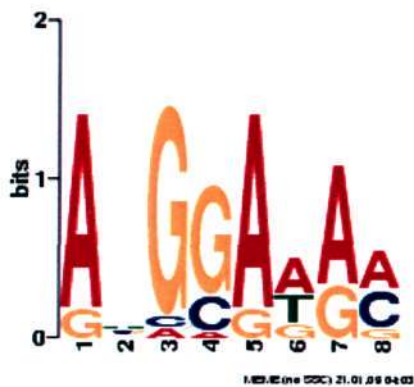


Chapter 5

Results

Motif 1

Any number of the repetition width = 8 sites = 22 llr = 187 E-value = 3.2e-003



Motif 2

Zero or one occurrence per sequence width = 6 sites = 7 llr = 71 E-value = 2.9e-002

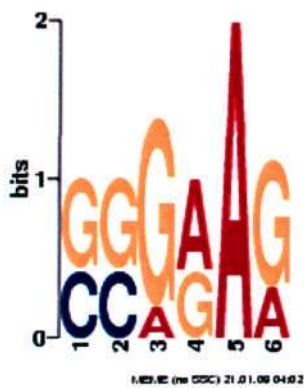


Figure 5. 15. Two putative regulatory motifs in up-regulated proteasome proteins in 5'UTR.

The first motif has similarities to other motifs analysed by TOMTOM.

Caption identifies the mode of the run: “any number of the repetition” or “zero or one occurrence per sequence”

Width: motif length

Sites how many times this motif was identified

llr: log likelihood ratio, E-value: expectation value

***Histone and histone modification and protein modification***

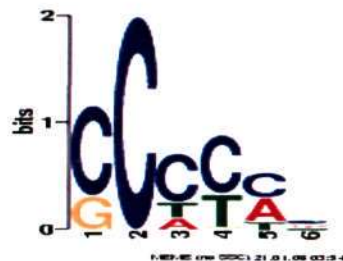
The motif search for proteins in the category histone and histone modification and protein modification did not result in any significant motif.

***Motifs in down-regulated proteins***

Common motifs were also found in the 5'UTR region of 27 down-regulated genes. Figure 5.16 displays 4 motifs. The motifs are arranged in order of their conservation in descending order with the most conserved motif at first. The first two motifs were found in all 27 down-regulated genes.

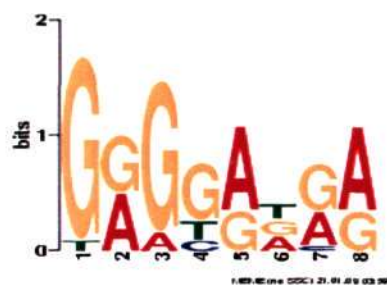
Motif 1

Any number of the repetition width = 6  
sites = 34 llr = 326 E-value = 2.0e-017



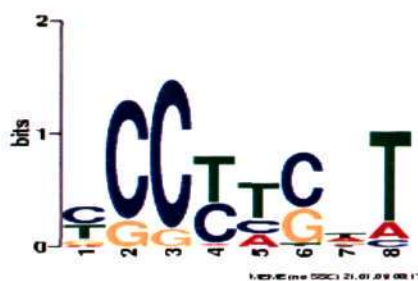
Motif 3

Any number of the repetition width = 8 sites = 18  
llr = 199 E-value = 5.9e-009



Motif 2

Zero or one occurrence per sequence  
width = 8 sites = 27 llr = 259 E-value = 9.4e-017



Motif 4

Zero or one occurrence per sequence width = 8  
sites = 20 llr = 198 E-value = 5.9e-006

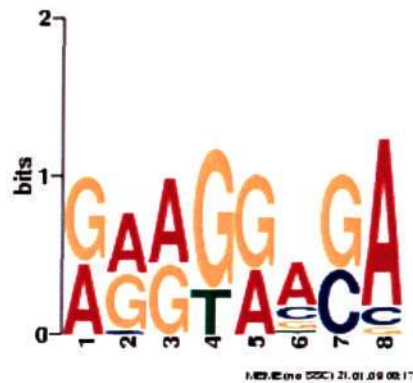


Figure 5. 16. Four putative regulatory motifs in 5'UTR in down-regulated proteins.

Caption identifies the mode of the run: “any number of the repetition” or “zero or one occurrence per sequence”

Width: motif length

Sites how many times this motif was identified

llr: log likelihood ratio

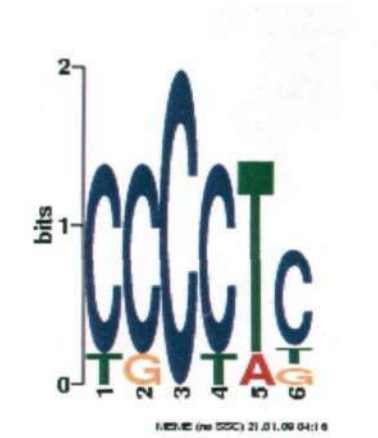
E-value: expectation value



***Motif in ribosome down-regulated proteins***

Only one motif was found to be significant and it is conserved in all seven sequences (Figure 5.17). Analysis by TOMTOM identified similarities with human NFKB1 and MZF1\_5-13 motif as well as *Drosophila melanogaster* motif usp.

Motif 1  
Zero or one occurrence per sequence width = 6 sites = 7 llr = 84 E-value = 7.5e-009



**Figure 5. 17. Putative regulatory motif in ribosome down-regulated proteins.**

Caption identifies the mode of the run “zero or one occurrence per sequence”

Width: motif length

Sites how many times this motif was identified

llr: log likelihood ratio

E-value: expectation value

***Down-regulated regulatory proteins***

No significant motif was found in down-regulated regulatory proteins.

**5.6. 2 Motifs in 3'UTR**

Motifs accounting for gene repression can be found in the 3'UTR region. Thus, down-regulated proteins were surveyed for signature motifs. Downstream of the coding sequence are also conserved sequences found. It was attempted to find motifs up to 500 bp downstream the translational stop site in the 3'UTR using MEME motif finder.

***Up-regulated proteins***

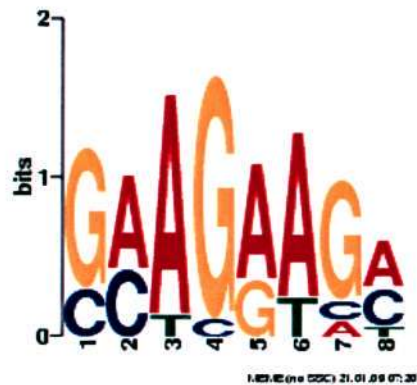
Only one motif in the 3'UTR was identified (Figure 5.18). In the different functional categories only one motif was identified in the invasion proteins (Figure 5.19).

***Down-regulated proteins***

No significant motif was found.



Motif 1  
Zero or one occurrence per sequence width = 8 sites = 61 llr = 433  
E-value = 1.1e-004



**Figure 5. 18. Putative regulatory motif in 3'UTR of 61 up-regulated proteins.**

Caption identifies the mode of the run “zero or one occurrence per sequence”

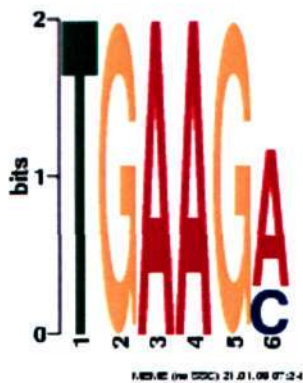
Width: motif length

Sites how many times this motif was identified

llr: log likelihood ratio

E-value: expectation value

Motif 1  
Any number of the repetition width = 6 sites = 43 llr = 313 E-value = 5.2e-004



**Figure 5. 19. Putative regulatory motif in 3'UTR of up-regulated invasion proteins.**

Caption identifies the mode of the run “any number of the repetition”

Width: motif length

Sites how many times this motif was identified

llr: log likelihood ratio

E-value: expectation value

## Discussion

So far, most studies on invasion pathway switching focused on differences in transcriptional regulation of gene expression and only analysed selected protein candidates. This present study uses a different approach: first identification of changes during protein expression and then correlation of these differences to transcriptional changes. The quantification of changes in merozoite protein expression and their comparison with schizont stage transcript levels from isogenic cultures were used to identify evidence for post-transcriptional regulation. The clustering of protein expression and gene transcription data by functional categories (Chapter 3, Figure 3.8.) revealed a discrepancy in correlation and suggested that the regulatory mechanisms function at the post-transcriptional level.

This observation is in agreement with previous studies correlating transcript levels with protein expression from discerned life stage in *P. falciparum*. The poor correlation of mRNA abundance with the protein product leads the authors to the conclusion that post-transcriptional regulation is an important mechanism to regulate gene expression in *Plasmodium*.

Post-transcriptional mechanism can be exhibited in various complex ways between transcription and translation of a gene. Post-transcriptional regulation can influence protein synthesis and degradation, respectively (Greenbaum *et al.*, 2003; Pratt *et al.*, 2002). In the following, different regulatory mechanisms will be discussed which are potentially relevant for this present work. A number of regulatory mechanisms have been analysed in eukaryotic systems which can be applied to *Plasmodium* spp.: Protein degradation by ubiquitin- proteasome pathway, mRNA decay, alternative splicing, mRNA motifs in UTR, translational repression, natural anti-sense transcripts, post-transcriptional modifications (e.g. phosphorylation).

The discrepancy between protein expression and gene transcription could be explained by the half life of the mRNA as the result of varied protein synthesis and

mRNA degradation. Furthermore, protein turnover can vary significantly too. The cell can control the rates of synthesis or degradation for structurally diverse proteins that have similar functions (Pratt *et al.*, 2002). Shock *et al.*, (2007) provided evidence that the half life of mRNA during the intra-erythrocytic stage varied significantly, and increased from ring stage to schizont stage. It is suggested by these authors that the limited presence of components of the RNA degradation machinery towards the end of schizogony may be responsible for the prolonged time of transcripts (Shock *et al.*, 2007). This mechanism of regulating mRNA half life may be used by the parasite to adjust protein expression levels. However, this is not the case here, as the absolute mRNA transcripts were measured by microarray and no contradiction between W2mef and W2mef/NM mRNA transcript levels was observed.

Alternative splicing is also a mechanism to regulate mRNA levels in the parasite (Chakrabarti *et al.*, 2007; Lu *et al.*, 2007; Upadhyay *et al.*, 2005). Alternative splicing could lead to a non-functional protein product, but if this is the case this study would still detect the peptide of this protein in the proteomic analysis. More likely to be the case here is that the spliced product is out of frame for translation and will be degraded.

Work on mammalian genes revealed that the use of different distinct promoter sites upstream of a gene can control the splicing pattern of the transcript (Cramer *et al.*, 1999; Roberts *et al.*, 1998; Tasic *et al.*, 2002).

The exact transcriptional start site is not known for many plasmodial genes. There is a possibility that a similar mechanism exists as it has been revealed for calmodulin transcriptional control of invasion molecules.

Calmodulin is one of the up-regulated proteins in W2mef/NM. Transcriptional initiation and alternative splicing of *calmodulin* has been of research interest in a number of studies. A motif similar to the G-box motif GAGGGGA of



approximately 30 bp upstream of the most distal transcriptional start site was identified (Militello *et al.*, 2004; Polson and Blackman, 2005). In addition, two independently regulated promoter activities within the *pfcam* were also identified (Crabb *et al.*, 1997; Polson and Blackman, 2005). The sequence contained promoter activity in both orientations and can enhance the activity of an adjacent promoter located in a head-to-head orientation. Transcription initiation of the *pfcam* gene can occur at multiple sites in the 5'UTR. It is proposed by Polson and Blackman (2005) that there is a relationship between the site used for initiation of transcription and correct splicing. Transcripts initiated from the ORF-proximal sites were less likely to be spliced than those initiated from the upstream sites (Polson and Blackman, 2005). A shift towards the use of the ORF proximal transcriptional start sites results in lower splicing efficiency. This might provide a mechanism at the transcriptional level for the down-regulation of steady-state calmodulin mRNA levels which occurs towards the latter stages of intra-erythrocytic growth (Orfa Rojas and Wasserman, 1995). Furthermore, the *P. falciparum* transcription-initiation complex has a low degree of sequence-specificity for the sites of initiation but preferentially acts downstream of long poly(dA)poly(dT) tracts (Polson and Blackman, 2005).

Translational repression is another way of post-transcriptional modification. Translational repression is the accumulation of “silenced” mRNA together with RNA binding proteins to form mRNP as mRNA processing (P) bodies (Parker and Sheth, 2007). The authors reported that post-transcriptional gene silencing may play a role in regulating translation during sexual stages. This mechanism of regulation was exploited by compression of gametocyte transcriptome with the proteomes of gametocytes and ookinetes. Nine genes were identified with accumulation of transcripts in gametocytes but not translated occurred until the ookinete stage. Furthermore a 47-base motif in *P. berghei* was found within 500bp downstream of the open reading frames of six of the nine post-transcriptional silenced genes (Hall *et al.*, 2005). This motif is a known *cis*-activating motif for Puf RNA binding proteins involved in translational repression through the formation of messenger ribonucleoprotein (mRNP) (Braks *et al.*, 2008; Fan *et al.*, 2004; Hall *et al.*, 2005).

Two homologues of Puf have been identified in *P. falciparum* being expressed at gametocytes and ookinetes stage (Cui *et al.*, 2002; Fan *et al.*, 2004). In general translational repression is mediated by *cis*-activating proteins in the 3'UTR. *Cis*-activating sequences for repression can also be found in 5'UTR of mRNA as demonstrated for the *P. berghei pb25* gene (Braks *et al.*, 2008). In addition to *cis*-acting elements, *trans*-activating factors may also be involved in translational repression. The DEAD-box RNA helicase (DOZI) in the *P. berghei* female gametocyte is similarly implicated in transcriptional repression via its RNA binding activity and mRNP formation (Coller and Parker, 2005; Khan *et al.*, 2005). Comparison of the wild-type and DOZI gametocytes transcriptome revealed down-regulation of 370 genes. In addition, a cohort of 92 genes was up-regulated in the DOZI gametocytes which suggest a further role for DOZI in regulating mRNA degradation processes.

Generally, the fate of repressed mRNA in the P bodies is degradation. However, in some events, mRNA in P body status can also be temporarily stored and re-enter translation. This has been observed for yeast cells under stress conditions (Bhattacharyya *et al.*, 2006; Brengues *et al.*, 2005). Translational repression may play a role during intra-erythrocytic development and is probably induced by oxidative stress (Shaw *et al.*, 2007).

Taken these facts together, the mechanism of translational repression may be potentially be used by the parasite to react instantly to environmental changes (e.g. invasion into desialiated erythrocytes).

A range of mechanism of temporal gene regulation is mediated by natural anti-sense transcripts (NAT) (Gunasekera *et al.*, 2004; Lu *et al.*, 2007; Militello *et al.*, 2008; Patankar *et al.*, 2001). The combination of serial analysis of gene expression (SAGE) tag libraries and strand-specific northern blots and RT-PCR as well as a variety of microarray formats signified the existence of NAT in *P. falciparum* with 17% anti-sense tags (Militello *et al.*, 2008; Patankar *et al.*, 2001). The transcription



of *msp2* anti-sense NAT appears to be temporally inversely related to that of the sense *msp2* transcript (Kyes *et al.*, 2002). Interestingly anti-sense tags are over-represented in two functional classes, translation and proteolysis, and on the other hand under-represented in mitochondrial genes (Gunasekera *et al.*, 2004; Patankar *et al.*, 2001). Some of the so far identified anti-sense tags overlap with identified up-regulated proteins in this present study: Histone 2b, MSP-1 (Gunasekera *et al.*, 2004), RAP1 and calmodulin (Patankar *et al.*, 2001). It still remains to be determined how anti-sense tags mediate control of temporal RNA expression. The second open question is in what way the regulation takes place: does it promote degradation of target mRNA, inhibition of translation initiation, or in contrast, they play a more progressive role by binding to either chromatin, its modifiers or RNA polymerase II (Lapidot and Pilpel, 2006; Werner, 2005).

Post-translational modification of target translational products can also occur by the addition of functional groups such as phosphorylation, acetylation, ubiquitination, and lypoylation. Identification of these modifications by proteomics is difficult and hence, very little is known about their role in *Plasmodium* spp. In the recent years protein kinases and their substrates became of research interest. Phosphorylation as a mechanism of activation of invasion motor molecules has been investigated (Green *et al.*, 2008; Kato *et al.*, 2008).

Another way of post-translational modification is ubiquitination. This modification is reversible and involves covalent conjugation of mono-, multi- or poly-ubiquitin. The fate of the targeted protein is predominantly proteasomal destruction, however, a wide range of implementation in other cellular processes has been demonstrated as well (Ciechanover, 2005). The degradation of proteins can act as a mechanism of actively down-regulate protein expression. Ubiquitination pathways have been recently identified in *P. falciparum* and appear to be developmentally regulated and essential for intra-erythrocytic development (Horrocks and Newbold, 2000; Ponts *et al.*, 2008). The up-regulation of the proteasome, especially components of the 20S

proteasome in W2mef/NM, gives room to speculate that some proteins which are down-regulated might be targeted by ubiquitin and are degraded by the proteasome. SUMO (small ubiquitin-like modifier) is an ubiquitin like protein (structurally similar to ubiquitin) and conjugation of SUMO is involved in a variety of cellular functions such as transcriptional regulation, nuclear location and signal transduction (Gill, 2003; Goodson *et al.*, 2001; Hong *et al.*, 2001). A number of SUMO targets have been identified by Mass spectrometric approach in blood stage malaria *P. falciparum* strain (Issar *et al.*, 2008).

Two RNA binding molecules identified here are putative RNA-binding protein (PF13\_0058) and High mobility group protein (PFI1445w). The function and mode of binding for this protein has not been characterised. This protein could potentially control translation of merozoite proteins.

High mobility group protein (HMGP) is expressed at the end of schizogony and at the sexual stage of the parasite. It also belongs to the up-regulated proteins in W2mef/NM. High mobility group protein is involved in transcriptional controlling of gene expression in sexual stage of *P. yoelii* and it is important in oocyst development in the mosquito (Gissot *et al.*, 2008). Based on these facts one could speculate that HMGP could potentially control transcription of specific mRNA at the schizont stage.

Many eukaryotes control protein expression by regulatory elements in untranslated regions of the gene. The parasite contains uncommonly AT-rich intergenic sequences which make it difficult to adapt regulatory mechanism from other eukaryotic systems. Analysis of the *P. falciparum* genome revealed very limited pool of proteins for transcriptional initiation (Aravind *et al.*, 2003; Coulson *et al.*, 2004). *Plasmodium* harbours some genes, which function in parasite-specific processes only (e.g. invasion into erythrocytes). It appears that the parasite has its own unique regulatory machinery. *In silico* regulatory element discovery is based on the postulation that co-transcribed genes involved in related biological functions



are more likely to be regulated by a similar mechanisms. Extensive studies have been made in *S. cerevisiae* and all evidence is pointing to the supposition that functionally related genes are also regulated in a similar way (Cora *et al.*, 2004; Ettwiller *et al.*, 2003; van Helden *et al.*, 1998). The same observations have been made in *Plasmodium* and the authors also come to the conclusion that functionally related genes (such as invasion molecules) are usually regulated in a similar way (Le Roch *et al.*, 2003a; Young *et al.*, 2008). Young *et al.*, (2008) identified motifs which were only found in the sets of genes which are expressed in specific invasion organelles, rhoptries and micronemes. These proteins are thought to be involved in erythrocyte invasion. Two motifs were of particular interest PfM20.1 (ACAACCT) and PfM18.1 (NGGTGCA). PfM20.1 was found upstream in five of the eight gene products expressed in micronemes. Motif PfM18.1 was found within 1200 nt upstream of the start codon of nine of the ten rhoptry genes. This motif commonly occurs as a duplicate separated by six or seven nucleotides, GTGCA(N5–6)GTGCA and is well conserved in other *Plasmodium* species *P. yoelii*, *P. berghei* and *P. vivax*.

The motif PfM18.1 was also identified in this study. Meme was only able to identify a single “GTGCA” motif. Glam2 (Gaped local alignment of motifs) available from MEME suite did not identify this motif as it was masked by over-represented AT-rich motifs. Furthermore, results from this present study postulate that more proteins than only rhoptry expressed protein share sequence similarity with this motif. In addition, a variant of PFM18.1 has been characterised by Voss *et al.*, (2003) and termed SPE2. SPE2 is involved in *var* gene expression regulation by silencing *var* genes during the intra-erythrocytic cycle. Similarity of GTGCA is also shared with the motif “TGCAC” identified by Gunasekara *et al.*, (2007) and Sunil *et al.*, (2008). This motif is commonly identified by functionally related proteases.

Altogether it appears that different variants of this motif exist, which act during the intra-erythrocytic development. The slight modification of each of the motifs could account to be specific for a set of functional related molecules.

Non-coding regions especially intergenic regions can harbour important information relevant for gene regulation. Therefore, proteins were clustered according to function and non-coding regions up-stream and down-stream of the gene were analysed for conserved regulatory motif. Furthermore, *Plasmodium* 5'UTR appear to be longer than in other eukaryotes (Wesseling *et al.*, 1989). Long non-coding sequences are also encoded in mature mRNA (Horrocks and Lanzer, 1999; Lanzer *et al.*, 1992; Watanabe *et al.*, 2002). The long 5'UTR contains further information by having enhancer and repressor elements that can alter the mRNA stability. The existence of these elements has been demonstrated by deletion of some sequence upstream the promoter sequence resulting in increased or repressed gene activity (Voss *et al.*, 2003).

*In silico* research for over-represented sequences in untranslated regions has been developed. It has been suggested to use multiple motif discovery programmes to minimise the false positive rate (Iengar and Joshi, 2009). In this present study only one programme was used. The MEME programme gave satisfactory results in other *Plasmodium* studies (Iengar and Joshi, 2009; Le Roch *et al.*, 2004; Young *et al.*, 2008).

The *P. falciparum* genome is extremely AT-rich. The overall GC content is around 20%. It is assumed that regions with enriched GC nt would harbour important information and therefore the GC content in exons is slightly higher with ~24% and intergenic regions have a lower GC content of only 14% (Gardner *et al.*, 2002a). When upstream and downstream untranslated regions are analysed for over-represented motifs, presented GC containing motifs were regarded as significant motifs as GC is “wisely” used by the parasite. On the other hand, AT rich motifs like “AAAAA”, “ATATAT” or TTTTTTT were disregarded as they are widely found in the *P. falciparum* genome and not unique for the analysed sequences. The upstream regions of co-expressed or functionally-related genes were analysed for over-represented motifs common to a particular set of genes but not abundant in the remainder of the analysed genes.

This study identified over-representation of motifs in co-expressed genes. The results provide an explanation for the contradiction between the large variation in *P. falciparum* gene expression and the reported rarity of specific transcription factors. It can be explained by a combinatorial mode of gene regulation, in which every gene is regulated by multiple factors.



## **Chapter 6**

### **Conclusion and future perspectives**

Invasion of erythrocytes by the *Plasmodium* parasite involves a cascade of events for successful penetration. The parasite is able to adapt to changes in its environment i.e. the parasites can switch from one invasion ligand to another when the preferred erythrocyte receptor is absent. The regulation and controlling of gene expression during this process is of great interest. Previous work indicates regulation of intra-erythrocytic cycle at the post-transcriptional level (Le Roch *et al.*, 2004; Nirmalan *et al.*, 2004).

This present study is the first comparative approach using transcriptional analysis and quantitative proteomics of invasion molecules during invasion pathway switching. 88 merozoite proteins were identified as differentially regulated in sialic-acid-independent clone W2mef/NM. Apparently these identified proteins show differential regulation only at protein expression levels and not during transcription. This finding suggests a mechanism of post-transcriptional control during invasion. The sequence of these proteins were analysed for over-represented motifs in their 5'- and 3'UTR.

A number of different motifs were identified. The role of these putative regulatory elements can be tested by analysing expression capacity in W2mef and W2mef/NM transfections of 5'UTR and 3'UTR sequence of selected differentially regulated molecules. In the case there are differences seen, the identified motif in the 5'UTR region could be deleted and the impact of this motif on regulation can be analysed by transfection.

Another mode of how these genes are regulated is NAT. The presents of NAT in *P. falciparum* has been introduced in the discussion of Chapter 5. The existence of antisense transcripts has been shown for four genes also identified in this study. It



would be very interesting what mode of regulation these antisense transcripts have in these genes. In the context of invasion pathway switching, NAT may or may not be the reason for differential regulation, as these genes have the same genetic background. However, it would be interesting to investigate differences of the level of antisense transcription by Real-time PCR. There might be regulatory elements on the antisense strand which regulate the amount of antisense transcript being made.

Another approach to study gene regulation is to investigate the amount of accumulated mRNA in P-bodies which might be a way of translational repression. Specific probes against some candidate genes with differential expression in W2mef and W2mef/NM can be generated for analysis by fluorescent in situ hybridisation (FISH).

MSP-1 is expressed in higher levels in W2mef/NM. The effect seen by the MSP-1 antibody in W2mef invasion of normal erythrocytes, W2mef/NM invading NM-treated erythrocytes versus W2mef/NM invasion of normal erythrocytes could be tested by invasion inhibitory antibody AMA-1. There exists AMA-1 antibodies which have an invasion inhibitory effect (Pizarro *et al.*, 2005). This antibody could be used as a negative control to proof the hypothesis about the role of MSP-1 in invasion explained in the discussion in Chapter 4. The two parasites should have a similar invasion inhibition effect by the antibody regardless of the erythrocyte they invade.

Though many invasion molecules and therewith erythrocyte interacting molecules are expressed in much higher levels in W2mef/NM underlining the fact that additional proteins are involved (apart from EBL and RBL family members) in the highly complicated process of invasion pathway switching. A high number of hypothetical proteins and proteins which have not yet been associated with invasion (e.g. MAHRP, REX etc.) were differentially expressed. It will be important to study these proteins as they have a significant role during invasion.

The results of this study suggest that NM has much broader effect than simply sialic acid removal. This study established that NM has a much higher impact on the erythrocyte surface and underlying cytoskeleton. Further analysis needs to be performed by AFM with higher magnification and exposure of the erythrocyte cytoskeleton similar to the study by Liu *et al.*, (2005). This would aid to identify more precise differences in the spectrin network and higher magnification could potentially give some information about the aggregates seen in this present study.

Furthermore it could be tested if spectrin is predominantly a dimeric or tetrameric form in desialiated erythrocytes by immunoblot analysis of erythrocyte samples. Under physiological conditions, spectrin is primarily in the tetramer form (Liu and Palek, 1980). Furthermore ankyrin binds the tetramer form ten times stronger than the spectrin-dimer which probably also influences the overall stability (Kennedy *et al.*, 1991). When the desialiated erythrocyte contains more dimers and less spectrin-ankyrin interactions, the cytoskeleton is more weakened than in the normal erythrocyte. As the tetramers are double the size of the spectrin dimers it could be easily tested if the dimer-tetramer equilibrium is shifted towards spectrin dimers.

To distinguish between differences invoked by invasion pathway switching and differences induced by altered properties of desialiated erythrocytes, further *P. falciparum* clones should be analysed. For example invasion pathway induced alteration could be analysed by parasite invasion of normal erythrocytes. The sialic-acid-dependent clone FCR3 should have a similar expression pattern as W2mef and in turn 3D7 should be similar to sialic-acid-independent W2mef/NM. Thereby proteins involved during invasion pathway switching could be identified by immunoblot analysis.

The loss of sialic acid is also associated with aging of erythrocytes (Cook *et al.*, 1961; Eylar *et al.*, 1962). It is believed that reduced surface charge and reduced deformability of older erythrocytes act as senescent signs and the cell will be ultimately removed from circulation (Beck WS 1973, Weed 1970, Danon 1971).



However, the loss of sialic acid of “old” erythrocytes is only 10%. It would be interesting to test the invasion efficiency of W2mef and W2mef/NM in “old” and “young” erythrocytes. Erythrocytes of different “age” can be extracted by density gradient. W2mef/NM should have similar efficiency to invade “old” erythrocytes as desialiated erythrocytes. An impairment should be seen for W2mef invasion into old and desialiated erythrocytes and a higher efficiency should be observed for W2mef invasion into young erythrocytes.

Nevertheless, the treatment of erythrocytes with neuraminidase does not reflect a natural phenomenon usually encountered by the parasite; receptor heterogeneity as well as variation of erythrocyte rigidity are regular phenomenon the parasite needs to overcome. Differences in the common blood group antigens as well as the presence or absence of the Duffy blood group antigens are just some examples of the natural diversity of the erythrocyte surface (Anstee, 1990). In addition red blood cell abnormalities like ovalocytosis, thalassaemia or glucose 6-phosphate dehydrogenase-deficiency (reviewed in An and Mohandas, 2008) that have been shown to have an impact on the biomechanical properties of the erythrocyte are commonly encountered by the parasite. Ovalocytosis frequency is high in malaria endemic areas particularly in Southeast Asia (Mohandas *et al.*, 1984). Decreased membrane deformability seen in ovalocytosis is linked to a genomic deletion in band 3 (Mohandas *et al.*, 1984; Mohandas *et al.*, 1992; Saul *et al.*, 1984) that results in immobilization of band 3 and thereby increased rigidity of the erythrocytes (Mohandas *et al.*, 1992). In the case of ovalocytes, malaria parasite appear to still invade these cells as efficiently as normal erythrocytes, reflected in high levels of parasitemia in vivo (Allen *et al.*, 1999). In contrast culture adapted clones show distinct differences in their ability to invade ovalocytes with one study showing that only the neuraminidase and trypsin resistant parasite line 3D7-A being able to invade these cells efficiently while the genetically identical clone 3D7-B can not (Cortes *et al.*, 2004). This is analogous to the invasion pathway switching in the clone W2mef. The comparative transcriptional analysis of these two 3D7 clones has

been performed (Cortes *et al.*, 2007). In addition a quantitative proteomic approach could be performed on these clones to test whether post-transcriptional mechanism of gene expression regulation also exists.

The combination of quantitative proteomics as well as microarray analysis has provided new insights into the regulation of gene expression in *P. falciparum* that would not have been observed by utilising either technique on their own. Furthermore, the findings have also led to the observation that biophysical and biomechanical properties of erythrocyte provide additional challenges for the merozoite during the invasion process. In response to these changes in the erythrocyte the parasite up-regulates specific proteins important in the movement of the merozoite through the junction as well as the establishment of the parasitophorous vacuole. Finally, the observation that increased expression of MSP1 can have a negative impact on the efficiency of invasion inhibitory antibodies has important implications for vaccine development. It strongly suggests that changes in expression levels of potential vaccine targets can be used by the parasite to overcome immune pressure and thereby serve as another mechanism of immune evasion.



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