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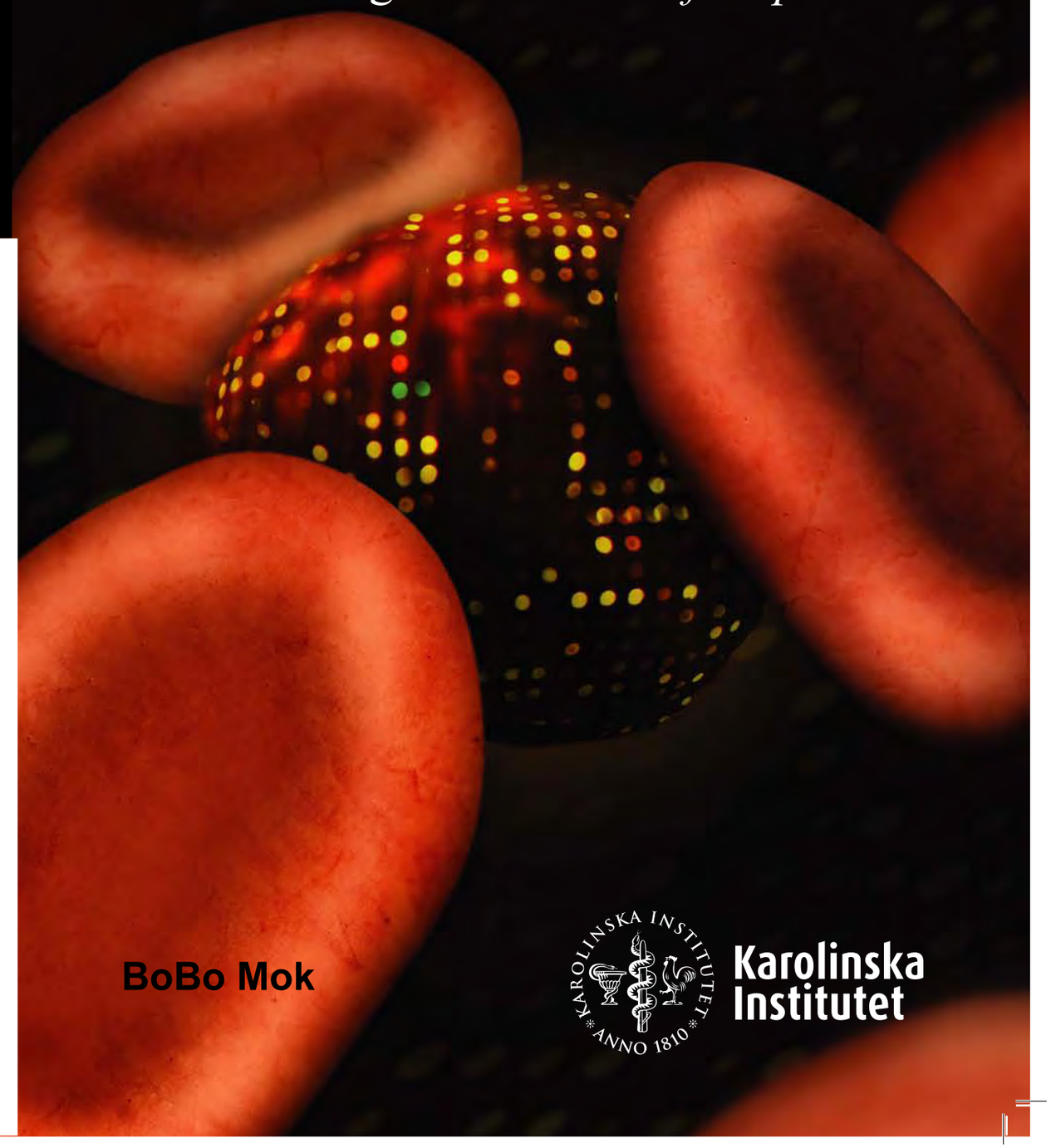
2007

Genomic and Transcriptomic Variation in Blood Stage *Plasmodium falciparum*



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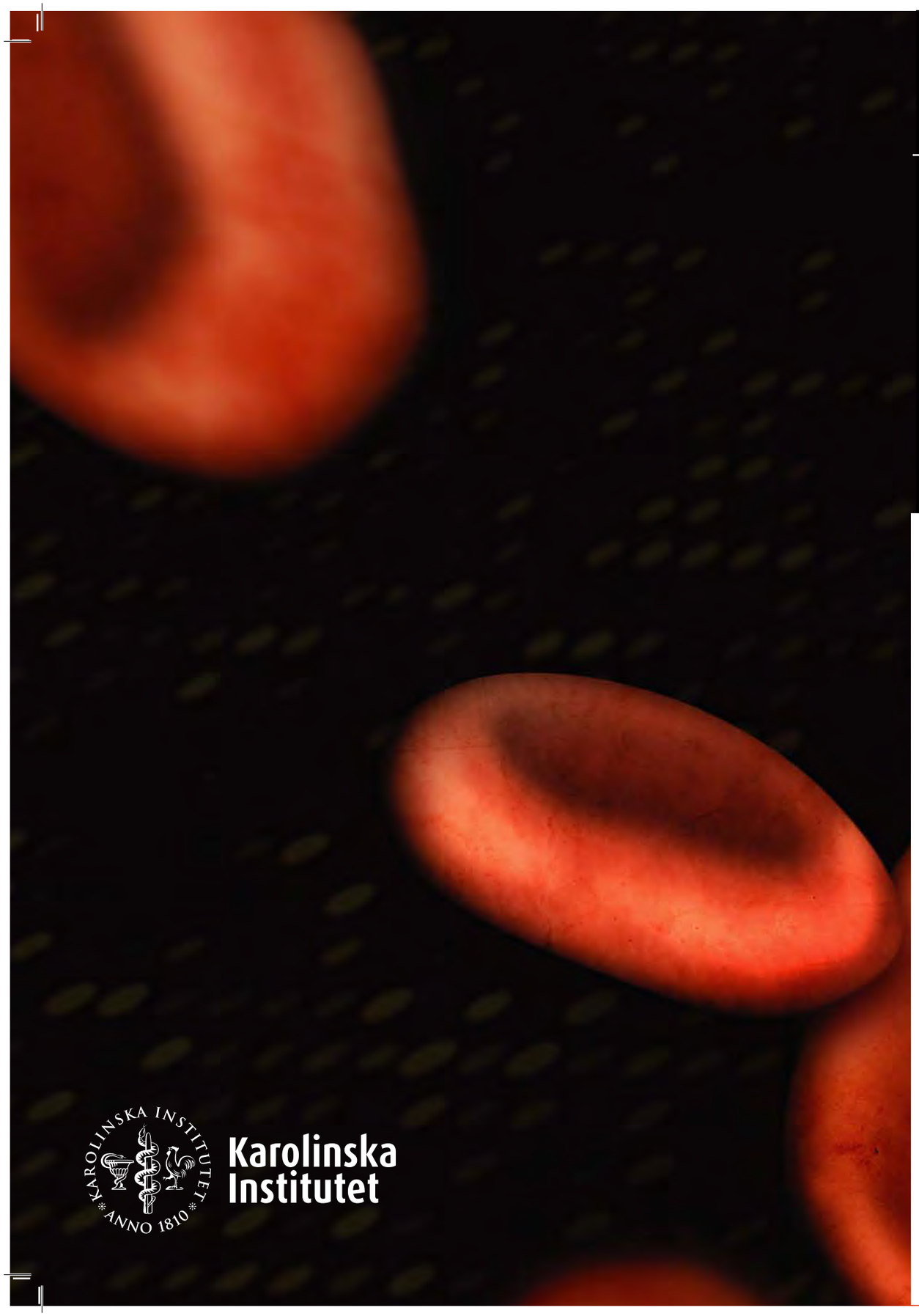

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This thesis is dedicated to my beloved grandparents

ABSTRACT

Malaria research has entered a postgenomic era since October 2002, when the complete genomic sequence of *Plasmodium falciparum* strain 3D7 was published. A massive amount of information generated by the *P. falciparum* genome project has facilitated the development of many novel platforms for profiling different levels of biological aspects. In this thesis, by employing various high throughput approaches, I aimed at understanding the genetic basis of phenotype variation in *P. falciparum*.

Genomic diversity of *P. falciparum* strains originating from different geographical areas was studied using microarray-based comparative genomic hybridization (CGH). Here I show presence of multiple genome wide-copy number polymorphisms (CNPs) covering 82 genes. The genes, amplified in up to six copies, encode molecules involved in cell-cycle regulation, cell division, drug resistance, erythrocyte invasion, sexual differentiation and unknown functions. Our results suggest that *P. falciparum* employs gene duplications and deletions as general strategies to enhance its survival and spread.

Even though the *P. falciparum* subtelomeres are considerably variable, segmental duplication were observed within these regions. High-resolution mapping and CGH data of the subtelomeric regions revealed a block of genes that had been amplified nine times on multiple subtelomeres of *P. falciparum*. These duplicated segments (SD) are of more than 10 kilobases in size and span six genes, including three known variant gene families: *var, rif* and *pfmc-2tm* and three hypothetical genes (*n-, o-, q-gene*). Sequencing data revealed that both inter- and intragenic regions are highly conserved across the species, despite their variation in copy numbers. One of the hypothetical genes within the SD, the *n-gene*, encoding a PEXEL/VTS-containing two transmembrane proteins was found to be transcribed at an early stage of the asexual erythrocytic cycle and its transcriptional levels were correlated to the gene dosage. The ubiquity and uniqueness of these SDs in the *P. falciparum* subtelomeres, and the conserved nature of the gene content within, suggests an important role in plasmodia speciation.

A major part of the parasite virulence attributes to the extensive host cell modification of blood stages *P. falciparum*. Two 3D7 isogenic clones with distinct adhesive and antigenic phenotypes (rosettes formation for 3D7S8.4 and CD36 binding for 3D7AH1S2) were isolated by micropanipulation. To have a global view of gene expression governing their phenotypes, we have employed a *P. falciparum* genome array, supplemented with a panel of in-house oligonucleotides, for comparative transcriptomal analysis. Fifteen genes were found highly differentially expressed (greater than a 5-fold change) encoding proteins for apical organellar (Gbph2, GBP-related antigen), cell-rescue, defense/virulence (RESA-2, RIFIN, PfEMP1), DNA/RNA processing (RNA methylase), erythrocyte invasion (SERA-5) and a number of hypothetical proteins. A number of short and full-length *var* transcripts were differentially expressed between the clones; yet, only one full-length transcript was dominant in both rings and trophozoites. In fact, *var* genes were found at the top of the list of the highly differentially expressed gene in between the two clones and its protein product, PfEMP1, is believed to be the most important determinant of antigenic phenotypes in *P. falciparum*.

To understand better the mechanisms and dynamics of *var* gene switching, we propagated 3D7S8.4 and 3D7AH1S2 under the same conditions for more than one year without enrichment or panning. We found that, upon long-term culturing, both parasites switch to express a common *var* gene (*var2csa*) matched by the loss of PfEMP1 surface expression and host cell-binding. The *var2csa* gene repositioned in the perinuclear area upon activation away from the telomeric clusters and heterochromatin to express spliced, full-length RNA. We suggest switching to *var2csa* to be an inherited trait that allows for small populations of *P. falciparum* to express new *var* genes. The process may coordinate the variant-antigen repertoire and thus protect against its rapid exhaustion.

Keywords: antigenic variation; comparative genomic hybridization (CGH); copy number polymorphism (CNP); microarray; *P. falciparum*; PfEMP1; subtelomeric regions; segmental duplication; switching; *var*

LIST OF PUBLICATIONS

This thesis is based upon the following papers:

I. Ulf Ribacke, **Bobo W. Mok**, Valtteri Wirta, Johan Normark, Joakim Lundeberg, Fred Kironde, Thomas G. Egwang, Peter Nilsson, Mats Wahlgren:
"Genome wide gene amplifications and deletions in *Plasmodium falciparum*,"
Molecular & Biochemical Parasitology 155 (2007): 33–44.

II. **Bobo W. Mok**, Ulf Ribacke, Ellen Sherwood, Mats Wahlgren:
"Highly conserved segmental duplication of variable copy numbers in the subtelomeres of distinct *Plasmodium falciparum,*"
Submitted.

III. **Bobo W. Mok**, Ulf Ribacke, Gerhard Winter, Ben H. Yip, Chuen-Seng Tan, Victor Fernandez, Qijun Chen, Peter Nilsson, Mats Wahlgren:
"Comparative transcriptomal analysis of isogenic *Plasmodium falciparum* clones of distinct antigenic and adhesive phenotypes,"
Molecular & Biochemical Parasitology 151 (2007): 184–192.

IV. **Bobo W. Mok**, Ulf Ribacke, Niloofar Rasti, Fred Kironde, Qijun Chen, Peter Nilsson, Mats Wahlgren:
"*var* gene switching in *Plasmodium falciparum* as an Inherited trait,"
Submitted.

ABBREVIATIONS

ATS	acidic terminal segment
BLAST	basic local alignment search tool
CHO	Chinese hamster ovary cell
CNV	copy number variation
CNP	copy number polymorphism
CSA	chondroitin sulphate A
Ct	cycle threshold
FIKK	kinases with phenylalanine, isoleucine, lysine and lysine amino acid motif
GBP	glycophorin binding protein
ICAM-1	intercellular adhesion molecule 1
IE	infected erythrocyte
KAHRP	knob associated histidine rich protein
kb	kilobases
LOESS/LOWESS	locally weighted regression
MESA/PfEMP2	mature parasite infected erythrocyte surface antigen / *Plasmodium falciparum* erythrocyte membrane protein 2
PEXEL/VTS	*Plasmodium falciparum* export element / vacuolar translocation signal
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PfEMP3	*Plasmodium falciparum* erythrocyte membrane protein 3
PfMC-2TM	*Plasmodium falciparum* Maurer's clefts 2-transmembrane protein
qPCR	real-time quantitative polymerase chain reaction
RESA	ring-infected erythrocyte surface antigen
RIFIN	repetitive interspersed protein
RT-PCR	reverse-transcriptase polymerase chain reaction
SEP/ETRAMP	small exported protein / early transcribed membrane protein
SERA	serine repeat antigen
STEVOR	subtelomeric variant open reading frame protein
SURFIN	surface-associated interspersed protein
TSP	thromobospondin

CONTENTS

A hundred years after the first parasite was identified in the red blood cell of a malaria patient, the genetic codes for the plasmodia have eventually been disclosed.

Yet, do we know much about these parasites?

1 BACKGROUND

1.1 Malaria

1.1.1 Facts

Malaria is considered to be one of the most serious health problems in tropical and sub-tropical regions; approximately forty percent of the world's population is affected. There are between 350 - 500 million clinical episodes of malaria each year. More than one million deaths, most of them in children under five, are attributed to this disease (Snow *et al.*, 2005; WHO, 2005). The global incidence of the disease is now increasing due to emerging insecticide-resistant vectors, widespread drug resistances, population movements, environmental disturbances, wars and disintegrating health services. Malaria is not just a disease commonly associated with poverty, but is also a major burden to economic and social development in Africa, costing the continent US$ 12 billion each year (Sachs and Malaney, 2002).

1.1.2 Infection and Transmission

Malaria is caused by protozoan parasites belonging to the genus *Plasmodium* of approximately one hundred species (Levine, 1988). *Plasmodium* exhibits a heteroxenous life cycle involving a vertebrate host and an arthropod vector. The vertebrate hosts include reptiles, amphibians, rodents, birds, monkeys and humans. In mammalian *Plasmodium* species, the vectors are anopheline mosquitoes. Four *Plasmodium* species account for almost all human infections: *Plasmodium falciparum (P. falciparum); Plasmodium vivax; Plasmodium ovale;* and *Plasmodium malariae*. *Plasmodium falciparum* is responsible for the most severe disease and mortality.

1.1.3 Beating malaria

To ensure a better prevention and control of malaria, a comprehensive approach is necessary. Such an approach involves providing long-lasting insecticide-treated bed nets

and Indoor Residual Spraying, providing early access to diagnosis and effective antimalarial drugs such as Artemisinin-based combination therapy (ACT) and offering a packet of interventions through strengthened antenatal care services for pregnant women. In addition, education to empower communities with the knowledge and resources, development of effective vaccines as well as launching of multilateral efforts for initiatives, networks and funding are ultimately crucial to combat this disease.

1.2 *Plasmodium falciparum*

1.2.1 Life Cycle

The life cycle of *P. falciparum* is complex and involves several life-stages, which take place partly in the vertebrate host and partly in the female mosquito vector. It initiates when a sporozoite is injected into the blood of a human host during mosquito feeding. The sporozoite enters the circulatory system and within thirty-to-forty-five minutes invades a liver cell. After invasion, the sporozoite multiplies in the liver cell, and thousands of progeny, called merozoites, are released into the circulatory system following the rupture of the host hepatocyte. The merozoites then recognize specific proteins on the surface of the erythrocytes and invade the cell facilitated by the apical organelles. Within the erythrocyte, the merozoite develops through a ring- and mature-trophozoite stage, and then undergoes a series of asexual divisions to produce a large segmented schizont. The schizont then ruptures and releases sixteen-to-thirty-two daughter merozoites, which immediately attach and invade more erythrocytes; this process may in theory be repeated indefinitely but either the host dies or the immune-system counteracts the growth of the parasite. A small proportion of merozoites do not divide but develop into sexual stages, the male and female gametocytes, which circulate into the blood stream until they are taken up by the mosquitoe in the process of feeding. Female macrogametocytes and male microgametocytes transform into gametes, meet and fuse in the gut of the mosquito-forming zygotes. On the epithelial lining the zygote develops into an oocyst and undergoes several rounds of multiplication, and within one to two weeks release more than one thousand sporozoites. The motile sporozoites then migrate into the salivary glands of the mosquitoe, from where they can be injected into a

new host during the subsequent blood meal (Figure 1). The mosquitoe may bite two- to three times during the 30 days of average lifespan in nature.

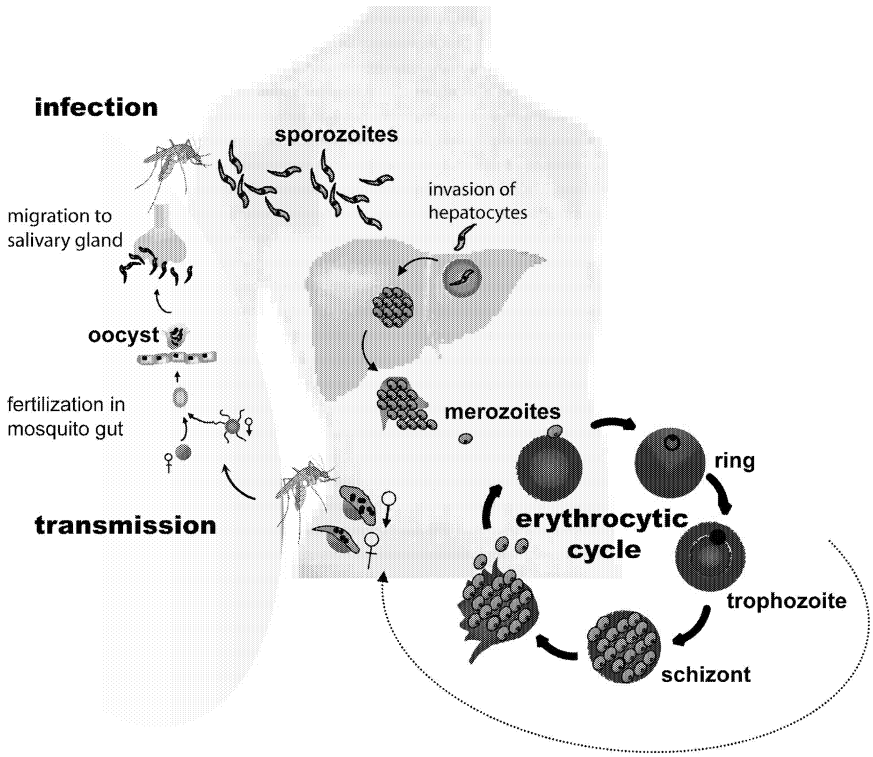

Figure 1. Life cycle of *Plasmodium falciparum*

1.2.2 Clinical features of *P. falciparum* infection

The consequences of *P. falciparum* infection can be broadly classified into three groups: asymptomatic infection; uncomplicated malaria; and severe malaria. Asymptomatic infection occurs when an individual is able to tolerate the presence of parasitaemia without having any clinical symptoms; as a result of repeated exposure to malaria, immune responses gradually develop that protect against serious disease. Most of the episodes (90% of the infection) in endemic areas lead to uncomplicated malaria with mild clinical symptoms such as fever, malaise, headache, myalgia, and minor gastrointestinal symptoms. The non-specific fever is mediated by the host cytokine responses upon the rupture of infected erythrocytes (IEs) induced by the release of parasite material and metabolites, haemozoin and cellular debris. Severe malaria is a complex multisystem disorder; its clinical manifestations are listed in Table 1 (Warrell *et al.*, 1990).

Table 1. Clinical manifestations of severe malaria

cerebral malaria (unrousable coma often with convulsions)
severe normocytic anaemia
pulmonary edema
renal dysfunction or failure
hypoglycaemia
circulatory collpase and shock
severe haemoglobinuria (black water fever)
acidaemia /acidosis
spontaneous bleeding / disseminated intravascular coagulation
placental disfunction, maternal anaemia, fetal death and reduced birthweight
jaundice
hyperpyrexia
extreme weakness

It has been shown that excessive proinflammatory cytokine production such as tumour necrosis factor (TNF) and Interferon gamma (IFN-α) plays an important role in immunopathogenic processes (Clark *et al.*, 1997). The downstream mediators, such as nitric oxide (NO) that the host normally uses to control parasites, when in vast amounts, would interfere with neurotransmission and lead to vasodilation of cerebral vessels implicated in cerebral malaria (Clark *et al.*, 1991). The rupture of schizonts to release

new merozoites and the glycosylphosphatidylinositol (GPI), a potential malaria toxin (Tachado *et al.*, 1996), could trigger excessive proinflammatory cytokine cascades. TNF could also enhance the expression of endothelial molecules such as endothelial leukocyte adhesion molecule 1 (ELAM-1), vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) (Ockenhouse *et al.*, 1992) which, along with other receptors, promote the binding of IEs and lead to microvasculature occlusion – a process, perhaps considered to be the most important virulence factor of *P. falciparum*, contributing to severe malaria.

1.2.3. Sequestration

Sequestration of *P. falciparum* IE in the mircovasculature is due to the binding of IE to cells, proteins or glycoproteins. It is mostly confined to the IE during the last half of the asexual life cycle (trophozoite and schizont stages) but may also occur with ring-stage infected erythrocytes. Sequestration depends on:

- **Cytoadhesion**– the adhesion of IE to microvascular endothelial cells in various organs, such as brain, placenta, intestine, lung, liver and skin;
- **Rosetting** – the adhesion of IE to other non-infected erythrocytes; also giant-formation (giant-rosetting) with the adhesion of IE to each other and to uninfected erythrocytes (Carlson *et al.*, 1990; David *et al.*, 1988; Udomsangpetch *et al.*, 1989b);
- **Autoagglutination** - the adhesion of IE to other infected erythrocytes (Fernandez *et al.*, 1998; Roberts *et al.*, 1992);
- **Platelet-mediated clumping** – the binding of IE to each other mediated by the platelet (Wahlgren *et al.*, 1995; Pain *et al.*, 2001).

Sequestration is believed to confer a fitness benefit to the parasites such as evasion of splenic clearance. However, while this survival strategy may improve its ability to reinvade and proliferate, it may also causes severe adverse effects to the host. For example, excessive sequestration in the microvasculature of the brain is part of the pathophysiology of cerebral malaria, which is one of the major causes of death from malaria infections; also, sequestration of IE in the placenta causes complications

associated with pregnancy malaria. The downstream effects of sequestration might include microvascular obstructions, hypoxia, and focal release of parasite toxins and inflammatory mediators (Chen *et al.*, 2000b; Rasti *et al.*, 2004). A number of host molecules have been identified that can act as receptors for the adhesion of IE (Table 2).

Table 2. PfEMP1 domains and the host receptors

Receptor location	Host Receptor	PfEMP1 domains	References
Endothelium	CD36	CIDR1α	Barnwell *et al*., 1989; Baruch *et al.*, 1996; Baruch *et al.*, 1997
	ICAM-1	DBL2β – C2	Berendt *et al.*, 1989; Baruch *et al.*, 1996; Smith *et al.*, 2000
	PECAM/CD31	DBL2δ, CIDR1α	Treutiger *et al.*, 1997; Chen *et al.*, 2000
	VCAM-1	?	Ockenhouse *et al.*, 1992
	P-selectin	?	Udomsangpetch *et al.*, 1997
	E-selectin	?	Ockenhouse *et al.*, 1992
	TSP	?	Roberts *et al.*, 1985; Baruch *et al.*, 1996
	Heparan Sulphate	DBL1α	Vogt *et al.*, 2003
	CSA	DBL3x	Robert *et al.*, 1995; Rogerson *et al.*, 1995; Buffet *et al.*, 1999
	Non-immune IgG, IgM	?	Scholander *et al*., 1996
RBC	Heparan Sulphate	DBL1α	Vogt *et al*., 2004
	Blood group antigen A	DBL1α	Carlson and Wahlgren, 1992; Barragen *et al.*, 2000; Chen *et al.*, 2000
	Blood group antigen B	?	Carlson and Wahlgren, 1992; Barragen *et al.*, 2000
	CR1	DBL1α	Rowe *et al.*, 1997
	CD36	?	Handunnetti *et al.*, 1992; van Schravendijk *et al.*, 1992
	Non-immune IgG, IgM	?	Scholander *et al*., 1996
Syncytiotrophoblast	CSA	CIDR, DBL3, DBL2x, DBL3x, DBL5ε, DBL6ε	Fried and Duffy, 1996; Buffet *et al.*, 1999; Reeder *et al.*, 2000; Gamain *et al.*, 2005; Rasti *et al.*, 2006
	Hyaluronic acid	?	Beeson *et al.*, 2000; Duffy et al., 2005
	Non-immune IgG	DBL2β, DBL2x, DBL6ε	Flick *et al.*, 2001; Rasti *et al.*, 2006
	Non-immune IgM	DBL2β, DBL2x, DBL5e, DBL6ε	Creasey *et al.*, 2003; Rasti *et al.*, 2006; Semblat *et al.*, 2006
Platelet	CD36	?	Pain *et al.*, 2001
Serum	Non-immune IgM	CIDR1α	Chen *et al.*, 2000
	TSP	?	Robert *et al.*, 1995; Baruch *et al.*, 1996
B cell	Non-immune Ig	CIDR1α	Donati *et al.*, 2004

1.2.4 Modification of infected erythrocyte surface

The surface of the erythrocytes displays several dramatic morphological changes upon infection. In the first hours of intra-erythrocytic development, there is no noticeable change outside the parasitophorous vacuole or in the erythrocyte membrane. At around twelve-to-fourteen hours of post-invasion, alteration of the IE surface begins to be seen. First, the membrane of the IE increases in rigidity and is reduced in deformability, which makes the IE difficult to pass through the microvasculature. As the parasites mature, the permeability of the IE increases which allows nutrient uptake, metabolite removal and regulation of the cytosolic ion concentration through special ion channels or pores in the host erythrocyte plasma membrane (Deitsch and Wellems, 1996; Staines *et al.*, 2007). Furthermore, a number of parasite-derived polypeptides, such as PfEMP1, RIFIN,

SURFIN and STEVOR, are secreted from the parasites and inserted into the erythrocyte membrane during intracellular development. All these parasite proteins significantly change the structure of the IE membrane.

The most significant structural modification of the IE surface is the formation of knobs (Trager *et al.*, 1966), an electron-dense protrusion on the IE membrane with approximately 100 nm in diameter (Luse and Miller, 1971). While the rest of the IE surface membrane is negatively charged, the knobs are positively charged (+20 mV) (Aikawa *et al.*, 1996). Knobs develop as the parasite matures from ring-trophozoite to late-trophozoite (Winograd and Sherman, 1989). Various components were found in the knobs including knob-associated histidine-rich protein (KAHRP) (Kilejian, 1979; Pologe *et al.*, 1987), *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) (Baruch *et al.*, 1995; Leech *et al.*, 1984), MESA/PfEMP2 (Coppel *et al.*, 1986; Howard *et al.*, 1987) and PfEMP3 (Pasloske *et al.*, 1993). Studies have shown that knobs are the binding sites of IE to other cell surfaces (Aley *et al.*, 1984; MacPherson *et al.*, 1985) where fibrillar material is linking the cells together (Scholander *et al.*, 1996). Reduction of PfEMP1 expression on the IE surface has been observed in knobless parasites (presumably due to the inefficient traffic of PfEMP1 in the absence of knobs) (Horrocks *et al.*, 2005), its presence is however not essential for IE cytoadherence or rosetting (Biggs *et al.*, 1989; Ruangjirachuporn *et al.*, 1991; Ruangjirachuporn *et al.*, 1992; Udomsangpetch *et al.*, 1989a; Udomsangpetch *et al.*, 1989b).

After invading the red blood cells, *P. falciparum* becomes surrounded by a parasitophorous vacuolar membrane (PVM). Thus the parasite proteins have to traverse the parasite membrane and PVM in order to reach the host cell's cytosol. A conserved host-targeting signal bearing a five-amino-acid core motif (RXLXE/D/Q) was identified in *P. falciparum* recently. This motif (PEXEL/VTS) together with a signal sequence in front is predicted to be targeting the parasites' protein to export to the erythrocyte (Hiller *et al.*, 2004; Lopez-Estrano *et al.*, 2003; Marti *et al.*, 2004). Over four hundred putative parasite proteins (the secretome) having the PEXEL/VTS motifs are present in the *P. falciparum* genome, evidencing the complexity of erythrocyte modification in this parasite (Table 3).

Table 3. *P. falciparum* proteins containing a PEXEL/VTS motif in 3D7 parasites

Protein or protein family	no. of proteins
*Pf*EMP1 (no leader signal sequence)	~60
Rifin	>130
STEVOR	>30
*Pf*MC-2TM	13
PHIST	>60
FIKK kinases	20
DNAJ/heat shock (RESA-related)	17
Glycophorin binding proteins	3
Knob associated histidine-rich protein (KAHRP)	1
Other proteins known to be exported to the erythrocyte (MESA/*Pf*EMP2, PfHRPII, *Pf*EMP3)	3
Other annotated proteins (ABC transporter, phosphatases)	4
Hypothetical proteins	~80-190

Information based on Gardner *et al.*, 2002, Hill *et al.*, 2004, Marti *et al.*, 2004, Sam-Yellowe *et al.*, 2004, Schneider and Mercereau-Puijalon, 2005, Sargeant *et al.*, 2006 and PlasmoDB

1.3 *Plasmodium falciparum* genome

1.3.1 *P. falciparum* genome sequencing project

In 1996, an international consortium formed by teams of malaria researchers and genome sequencing centers was formed to launch the whole genome sequencing project of *P. falciparum*. Responsibility for sequencing the genome was shared between three sequencing centers: Wellcome Trust Sanger Institute (UK); Institute for Genomic Research (TIGR)/Naval Medical Research Institute (USA); and Stanford University (USA). The process for the first few years was tardy, with certain regions of the genome containing stretches of Adenosines and Thymidines causing particular difficulties. Yet with technical improvements, the sequence of chromosomes 2 and 3 were completed by the TIGR in 1998 (Gardner *et al.*, 1999) and the Sanger in 1999 (Bowman *et al.*, 1999), respectively; and the full genome was unveiled in 2002 (Gardner *et al.*, 2002). Additional *P. falciparum* genome sequence projects on other strains are now under way, including the Hb3, Dd2 strains (Broad Institute of Harvard and MIT), It strain and Ghanaian isolate (The Plasmodium genome project, Wellcome Trust Sanger Institute).

The genome sequencing was done on *P. falciparum* clone 3D7 and the haploid genome was found to have a size of approximately 22.8-megabases. The nuclear genome consists of fourteen chromosomes, encodes at least 5,400 genes. The (A + T) genome composition of *P. falciparum* is 80.6 percent (90% in the non-coding regions), the most AT-rich genome sequenced to date. The average length of genes in *P. falciparum* was 2.3 kb, which is almost twice the size of that in *P. yoelii* (1.3 kb) and in other organisms (1.3 to 1.6 kb). In addition, a greater proportion of genes longer than 4 kb are found in *P. falciparum* (15.5%) compared to yeasts (3.0% in *S. pombe* and 3.6% in *S. cerevisiae*). The reason for increased gene length in this parasite is still unknown.

The subtelomeres

Plasmodium falciparum chromosomes vary considerably in length, ranging from 610 kb (chromosome 1) to 3.3 Mb (chromosome 14). The gene content of the chromosomes was found to be similar across the species, despite extensive variation occurring in the subtelomeric regions of different plasmodia. Because many genes involved in antigenic variation are located in the subtelomeric regions, an understanding of subtelomere structure and functional properties of genes within is essential for elucidation of the mechanisms underlying the generation of antigenic diversity. The subtelomeric regions of the chromosomes are however structurally highly conserved. Each chromosomal end can be divided into five different subtelomeric blocks (SB1-SB5): SB-1 is comprised of 7-mer (GGGTTT/CA) repeats; SB-2 is composed of sub-blocks of repeats of various sizes, including the telomeric-associated repetitive elements (TAREs 2-5) and other sequences; SB-3 consists of Rep20 element (or TARE 6) which is a twenty-one base-pair degenerate tandem repeat occurring in random order; SB-4 also has short tandem repeats, and it is the main subtelomeric compartment where the variable genes are located. A number of variant genes have been identified in this region, including *var* (Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995), *rif*, *stevor* (Cheng *et al.*, 1998; Fernandez *et al.*, 1999; Kyes *et al.*, 1999), *surf* (Winter *et al.*, 2005), *pfmc-2tm* (Sam-Yellowe *et al.*, 2004), *phist* (Daily *et al.*, 2005; Sargeant *et al.*, 2006), *fikk* (protein kinase) (Nunes *et al.*, 2007; Schneider and Mercereau-Puijalon, 2005), *hsp* (heat shock protein) (Acharya *et al.*, 2007; Botha *et al.*, 2007) and *sep/etramp* (Birago *et al.*,

2003); SB-5 does not include tandem repeats and it is a long-shared region only on a few telomeres, still it extends up to 120kb in some chromosomes and contains members of the variant gene families. The conserved arrangements of the subtelomeric regions can mediate chromosomal-end clustering and alignments, and thus facilitate non-allelic recombination (ectopic recombination) between telomeric-located genes (Freitas-Junior *et al.*, 2000; Freitas-Junior *et al.*, 2005; O'Donnell *et al.*, 2002).

The centromeres

Using the etoposide-mediated topoisomerase-II cleavage as a biochemical marker, the centromeric region of each fourteen chromosomes was mapped (Chen *et al.*, 1984; Kelly *et al.*, 2006). The predicted centromeres encompass the extremely AT-rich element (≈ 97%) with a sharply defined size range of 2.3 to 2.5 kb. Only one such region was found in each of the fourteen chromosomes.

1.3.2 The post-genomic era

Comparative genomics

Comparative genomics is the analysis and comparison of genomes from different species. Through this, we can have a better understanding of genome organization, gene functions and how species have evolved.

The first attempt to look at the conservation of gene synteny between the *P. falciparum* and rodent malaria species was done by Carlton and colleagues (Carlton *et al.*, 1998). Allied with the completed and virtually-completed genome sequencing projects of different *Plasmodium* species were: two human parasites, *P. falciparum* and *P. vivax*; three rodent parasites, *P. yoelii, P. chabaudi* and *P. berghei*; one avian parasite, *P gallinaceum*; and two non-human primate parasites, *P. reichenowi* and *P. knowlesi*, more comprehensive comparative genomic analyses have been described (Carlton *et al.*, 2002; Hall *et al.*, 2005; Kooij *et al.*, 2005). Approximately 85 percent of the predicted *P. falciparum* genes have orthologues in at least one of the rodent *Plasmodium* parasites, predominately found in the syntenic central regions of the chromosomes. Conservation among species breaks down at the ends of chromosomes where species-specific genes are clustered, many of them involved in host-parasite interaction (Kooij *et al.*, 2005). For

example, the subtelomeric regions of *P. falciparum* contain a repertoire of unique gene families, e.g. the *var* gene family, is absent in other *Plasmodium* species. Using probabilistic models on the basis of protein predictions and gene-structure analysis, a major multigene superfamily termed *pir* was identified; members include the *vir* (*P. vivax*) (del Portillo *et al.*, 2001), *yir* (*P. yoelii)*, *cir* (*P. chabaudi)*, *bir* (*P. berghei)* (Janssen *et al.*, 2002), *kir* (*P. knowlesi)* (Janssen *et al.*, 2004) *and rif* (*P. falciparum*) (Cheng *et al.*, 1998). Yet, phylognetic anaylses revealed that members of the PIR superfamily fall into different clades: YIR/CIR/BIR sequences are clustered together; KIR and VIR sequences formed their own groups whereas RIFIN, which is more divergent from other PIR members, is categorized to a distinct branch (Janssen *et al.*, 2004). More than six hundred copies of *vir* present in the *P. vivax* genome and different subfamilies (A to F) have been categorized based on their sequence similarity (del Portillo *et al.*, 2001), no homologue has yet been found in *P. falciparum*. Nevertheless, a recent study showed that *surf* and *pfmc-2tm* gene families belong to a particular clade of *vir* gene subfamilies based on their structural similarities (Merino *et al.*, 2007); in addition, *surf* was found to have similar structure characteristics with another *P. vivax* gene, *pvstpl* (del Portillo *et al.*, 2001; Winter *et al.*, 2005). Taken together, these data implicate that species-specific evolution of the antigenic genes, most likely under the pressure from a different host-immune environment, has led to the diverse repertoire of antigens found in different species.

Functional genomics

Conventional methods for functional studies have been hampered due to the limitation of genetic tools for the haploid genome and the complexity of the parasite life cycle. Rather than using a traditional gene-by-gene approach, we now prefer to consider many genes at a time, and try to get an integrated picture of the cell or the parasite as a whole. Recent advances in high-throughput techniques such as genome-wide transcription profiling, proteome analysis and computational tools allow exploitation of the genome sequence data in a systematic way, leading to a more comprehensive survey of the *Plasmodium* life cycle.

(a) Transcriptome

Since the disclosure of the genome sequence of *P. falciparum*, transcriptome analysis has become a rapid and cost-effective tool for investigating the biology of the malaria parasite. Indeed, just before the completion of the *P. falciparum* genome sequence project, several genome-wide gene expression studies of the *P. falciparum* had been accomplished. The first high-throughput transcriptome analysis study was performed using shotgun DNA microarray constructed by 3648 PCR inserts to compare the gene expression profile between asexual- (trophozoites) and sexual-stages (gametocytes) of the parasites (Hayward *et al.*, 2000). Later, serial analysis of gene expression (SAGE) was applied to characterize the global transcriptional profile of erythrocytic stage of parasites; the SAGE library comprises numerous short sequence tags corresponding to at least four thousand different genes of 3D7 strain parasites (Munasinghe *et al.*, 2001; Patankar *et al.*, 2001).

Two whole genome coverage transcriptional analyses were published one year after the release of the *P. falciparum* genome. Despite alternatives, microarray formats were used in these studies: (1) High density 25-mer oligonucleotide array synthesized *in situ* by photochemistry and mask-based photolithography (also called GeneChip®, Affymetrix); and (2) 70-mer oligonucleotide array with oligonucleotides spotted on a glass slide (Bozdech *et al.*, 2003a; Le Roch *et al.*, 2003), both studies showed comparable gene expression patterns for the eythrocytic stages (www.plasmoDB.org). These two whole-genome microarrays have become common tools for investigating the biology of the malaria parasites. For example, a number of studies used microarray to identify genes responsible for the gametocyte transformation (Eksi *et al.*, 2005; Silvestrini *et al.*, 2005; Young *et al.*, 2005) with the aim to block parasite transmission; investigation of transcriptional changes in *P. falciparum* during drug treatments or under environmental perturbation, such as temperature elevation (Oakley *et al.*, 2007), have also been performed. Furthermore, genome sequence information had also been integrated in the transcriptome analysis to identify regulatory elements governing gene expression in *P. falciparum* (Coulson *et al.*, 2004; Young *et al.*, 2005).

(b) Proteome

Global profiling of protein expression has become feasible since the invention of mass-spectometry and the availability of peptide predictions from the genome database. Two general strategies can be adopted in proteomic studies: (1) protein samples can be first separated through one- or two-dimensional electrophoresis, followed by excision of the protein bands/spots, proteolytic digestion, and submitted for mass analysis (gel-based); or (2) protein mixtures can be directly digested with proteases and the peptides generated successively separated using liquid chromatography (non-gel based).

Along with the release of the *P. falciparum* genome sequence, two stage-specific proteomic studies of the *P. falciparum* life cycle, with slightly different approaches, were published on the same issue (Florens *et al.*, 2002; Gardner *et al.*, 2002; Lasonder *et al.*, 2002). It is noteworthy that both analyses unexpectedly identified several antigenic variants (PfEMP1 and RIFIN) in the sporozoites that had previously been described present in the blood-stage parasites only; although it is controversial, this observation has been further confirmed by transcription analysis (Le Roch *et al.*, 2003). One of the explanations is that sporozoites do not replicate until they reach the host liver and develop into merozoites, and thus they have to express antigenic variants to escape immune detection; similarly, another variant protein family SURFIN was found differentially expressed both in *P. falciparum* merozoites and infected red cells (Winter *et al.*, 2005). These observations suggest that antigenic variation is not restricted to the IE surface, but also a phenomenon in other stages of *P. falciparum* life cycle.

Furthermore, components of the subcellular parasite compartments have been identified through proteomic studies, such as rhoptry-riched fractions (Sam-Yellowe 2004), Maurer's cleft (Vincensini *et al.*, 2005), infected erythrocyte-surface (Winter *et al.*, 2004) and infected erythrocyte-membrane (Florens *et al.*, 2004).

Bioinformatics

Today, massive amounts of data generated by high-throughput and genome-wide studies are being launched daily. Specially designed computational tools are therefore inevitably crucial for the efficient interpretation of these biological data. In the case of *P. falciparum*, this is made possible by the availability of an integrated database

(www.plasmoDB.org) providing tools for accessing and analyzing from genomic to functional genomics datasets (Bahl *et al.*, 2002; Kissinger *et al.*, 2002). To an even broader approach, the transcriptomes of various apicomplexan parasites have been integrated in a database allowing interspecies comparisons regarding protein motifs, predicted subcellular localization signals, transmembrane regions or upstream promoter elements (Watanabe *et al.*, 2007).

From genome annotation to functional and structural prediction of proteins, bioinformatics have been widely applied in many directions to solve biological questions. Various approaches have been focused on identification of conserved motifs and regulatory elements (Coulson *et al.*, 2004; Gunasekera *et al.*, 2007; Polson and Blackman, 2005) with biological relevance in *P. falciparum*. Recently, a unique motif (PEXEL/VTS), signaling protein translocation across the PV membrane, has been identified through a search in *P. falciparum* databases to identify the consensus sequences of potential *P. falciparum*-exported proteins (Hiller *et al.*, 2004; Lopez-Estrano *et al.*, 2003; Marti *et al.*, 2004). Later, a finer algorithm was constructed to predict the 'exportome' across the *Plasmodium* species (Sargeant *et al.*, 2006).

1.3.3 Genomic variation

Genomic variability can appear in many forms, including single nucleotide polymorphisms (SNPs), variable numbers of tandem repeats, and structural rearrangements (e.g. duplications, deletion, insertion, inversion and translocation). It has been known for sometime that all these types of mutations have different degrees of impact on genome evolution. In particular SNPs and copy number polymorphism/variation (CNP or CNV), have been extensively shown to have phenotypic significance on human genome (Freeman *et al.*, 2006; Redon *et al.*, 2006; Stranger *et al.*, 2007). But how do they contribute to *P. falciparum* genomes and influence the parasite phenotype?

Single nucleotide polymorphisms (SNPs)

SNPs are the commonest source of genetic variation for many living organisms. A frequency of approximately one SNP every 500 - 1000 bp was found among the genome

P. falciparum clones and field isolates (Mu *et al.*, 2002; Mu *et al.*, 2007; Volkman *et al.*, 2007). They are mostly encountered in genes that encode antigenic determinants and drug resistance which are subject to strong selection pressures. On the contrary, reduction in SNPs was found around known drug resistance loci (e.g. *pfcrt* and *dhfr*) (Nair *et al.*, 2003; Wootton *et al.*, 2002) as a result of recent selective sweeps (one strain carrying favorable mutations has recently replaced all others).

SNP analysis have been applied to evolutionary and population size studies on *P. falciparum* (Hughes and Verra, 2001; Jeffares *et al.*, 2007; Joy *et al.*, 2003; Mu *et al.*, 2002; Volkman *et al.*, 2001) as well as being used as markers in linkage analysis of genome-wide population association (Mu *et al.*, 2002; Mu *et al.*, 2007; Volkman *et al.*, 2007). Due to the high frequency of polymorphic genes involved in immune evasion and drug resistance, genome-wide detection of SNPs, allied with suitable bioinformatics tools such as protein structural and functional prediction, might be of great value for identification of potential vaccine candidates and drug targets (Kidgell *et al.*, 2006; Mu *et al.*, 2007; Volkman *et al.*, 2002)

Copy number variation/copy number polymorphism

Copy number variation (CNV) is defined as DNA segments that are of 1 kb or larger in size present at variable copy numbers in comparison with the reference genome (Feuk *et al.*, 2006). CNV can influence gene expression and phenotypic variation as a consequence of altering the gene dosage, disrupting coding sequence or perturbing gene regulation (Freeman *et al.*, 2006). Compared to SNP analysis, fewer studies have been reported on genome-wide CNV in *P. falciparum* genome. A number of reports have shown examples of gene amplifications and deletions using microarray-based comparative genome hybridization approaches, including the study presented in Paper I (Bozdech *et al.*, 2003a; Carret *et al.*, 2005; Kidgell *et al.*, 2006); other examples of amplifications include genes encoding host cell binding and invasion related proteins (e.g. reticulocyte-binding protein encoding gene) as well as multi-drug resistance (e.g. *pfmrd1*). Furthermore, gene deletions occur commonly in long-term propagated parasites, including genes associated with knob formation (including KHARP and PfEMP3 on chromosome 2 left arm) and cytoadherence (including Clag9 on chromosome 9, right

arm). Taken together, gene amplifications and deletions are common strategies for parasite adaptation and survival, a balance between benefit and cost.

Antigenic diversity

Antigenic diversity has dual origins. One is the classical genetic mechanisms of nucleotide replacement and recombination (as described above) that create allelic polymorphisms; as a result, different alleles of antigen-coding genes exist within a mixed population of organisms (Kemp *et al.*, 1990). The second mechanism is antigenic variation, whereby a clonal lineage of parasites switches to express alternate forms of an antigen successively without changing its genotype.

Allelic polymorphism is generated via a number of different mechanisms: point mutations result in SNPs, meiotic recombination involving the exchange of gene fragments and insertion/deletion of tandem repeats. Examples of genes having high levels of genetic polymorphisms are *msp1* (Miller *et al.*, 1993; Tanabe *et al.*, 1987), *msp2* (Fenton *et al.*, 1991; Smythe *et al.*, 1991), *s-antigen* (Saint *et al.*, 1987), *glurp* (Borre *et al.*, 1991) and *csp* (Rich *et al.*, 1997). As a matter of fact, due to the prevalence of repeat-length polymorphisms in these genes, size polymorphism between these alleles is widely used as a marker for parasite genotyping (Arnot *et al.*, 1993; Farnert *et al.*, 2001).

1.4 Antigenic variation in *Plasmodium falciparum*

1.4.1. Introduction of antigenic variation

Antigenic variation is a survival mechanism in which an infectious organism changes its surface proteins for the purpose of host-immune evasion. This strategy is particularly important for organisms to establish a chronic infection in the host in order to allow for efficient transmission. In contrast to the definition of antigenic diversity described earlier, antigenic variation is the process by which a clonal parasite population can switch its antigenic phenotype. Such phenotypic alteration is introduced upon switching of variant genes and expressing one of the family members at a time. Variant antigens are commonly encoded by large families of non-allelic genes, the antigens are highly immunogenic and display poor immunological cross-reactivity.

Classical antigenic variation employing gene-families is a feature of fungi (e.g. *Pneumocystis carinii*, *Candida albicans*) and protozoa (*Giardia lambia*, African trypanosomes and *Plasmodium* species) but many other microbial pathogens including viruses (e.g. Human Immunodeficiency Virus (HIV), rotavirus and enterovirus) and bacteria (e.g. *Borrelia* species, *Neisseria* species and *Chlamydia*) vary their antigens through a number of related mechanisms.

1.4.2. Antigenic variation in *P. falciparum*

Malaria transmission is very much dependent on climates and epidemiological settings. In hyper-endemic areas, where transmission is intense but stops during the dry seasons, the parasites have to establish long and chronic infections to survive in the host for months. In holo-endemic areas on the other hand, where malaria transmission occurs all year long, the parasites have to develop other ways to overcome the high levels of acquired immunity prevalent in the host populations. Successful measures by which the parasites evade host immune systems are the establishment of chronic asymptomatic infection, the paucity of sterile immunity, the continuation of superinfections, or the rescrudescence of parasites (Kaviratne *et al.*, 2003). To achieve all these goals, antigenic variations seem to play an important role.

Early studies of antigenic variation in malaria were performed using schizont-infected cell agglutination (SICA) tests with *Plasmodium knowlesi*. It was demonstrated that chronic malaria was maintained by repeated antigenic changes and that the immunity to infection was contributed by the development of the variant specific antibodies (Brown and Brown, 1965). Many years later, clonal antigenic variation was first shown in *P. falciparum* (Biggs *et al.*, 1991; Roberts *et al.*, 1992) and PfEMP1 was believed to be the major surface variant contributing to the antigenic differences (Howard *et al.*, 1988; Leech *et al.*, 1984; Magowan *et al.*, 1988).

Switching of antigenic variants occurs in natural infections but spontaneous switching may also take place *in vitro,* when no immunological selective pressure acts on the IE (Fernandez *et al.*, 1998; Roberts *et al.*, 1992) (Figure 2). While it is not yet understood how parasites are induced to switch their antigenic profile, there is evidence suggesting that spleen and antibodies might play a role in modulation of antigenic

variation. It was demonstrated that to transfer a *P. falciparum* clone from a splenectomized into a spleen-intact monkey resulted in switching to a different variant type, as well as the changing of its cytoadherence characteristics, from non-binding to binding (David *et al.*, 1983; Hommel *et al.*, 1983).

Antibodies against variable antigens expressed on the IE surface are believed to be important for protection against malaria. In endemic areas, the episodes of malaria in children are more frequent than in adults. Field studies had shown that sera from immune adults were able to recognize most isolates, whereas sera derived from infected children have a more restricted specificity range (Carlson *et al.*, 1990; Barragan *et al.*, 1998; Forsyth *et al.*, 1989; Marsh and Howard, 1986; Marsh *et al.*, 1989). Further, it was observed that immunity to severe malaria develops more rapidly than that to mild malaria (Gupta *et al.*, 1999). Taken together, there are correlations between age, number of exposures and virulence of the variants, and immune protection.

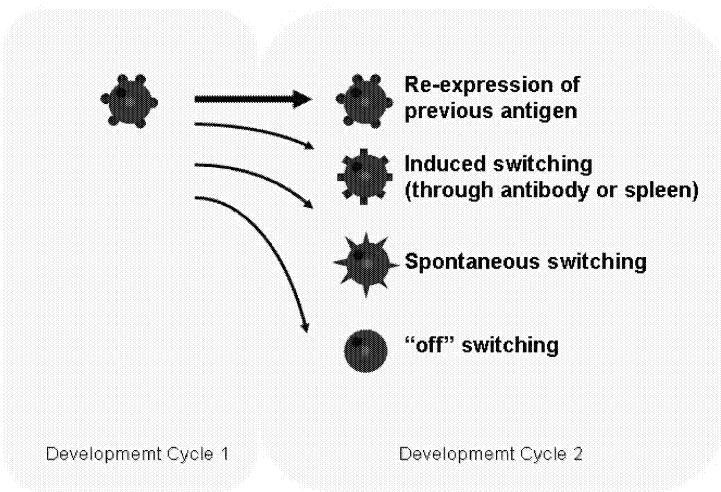

Figure 2. Suggested switching pathways of PfEMP1 in *P. falciparum*

1.4.3 Molecular determinants of antigenic variation in *P. falciparum*

PfEMP1 and the var gene family

Plasmodium falciparum Erythrocyte Membrane Protein 1 (PfEMP1) was first identified on the surface of the *P. falciparum*-infected erythrocytes by immunoprecipitation with variant specific antisera and radioiodinated/metabolically labeled surface protein from intact IE. These surface antigens were of similar biochemical characteristics but exhibited a strain-specific expression (Leech *et al.*, 1984).

Genes encoding PfEMP1 were first cloned in 1995 and were found belonging to a large multigene family collectively called *var* (Baruch *et al.*, 1995; Su *et al.*, 1995); different *var* genes were found expressed in antigenically distinct *P. falciparum* clones (Smith *et al.*, 1995). Genome sequencing information revealed, based on the reference strain 3D7, that the *var* gene family consists of approximately sixty intact gene copies as well as numerous *var* fragments and pseudogenes per haploid genome scattered over all fourteen chromosomes (Gardner *et al.*, 2002). The majority of *var* genes are located subtelomerically with a few copies distributed in the centromeric region of the chromosomes. Although there is extensive sequence diversity among members of the *var* family, they all share the same basic structure. Each *var* gene consists of two exons: exon I encodes the extracellular portion of the protein and a single transmembrane domain; exon II is highly conserved and encodes for a 450-500 amino acid long intracellular acidic terminal segment (ATS). Exon I is hypervariable in sequence and is comprised of an N-terminal segment (NTS); 2-7 domains named Duffy binding like (DBL), because of structural similarity to the Duffy-binding ligands of *P. vivax* and *P. knowlesi* (Adams *et al.*, 1992; Peterson *et al.*, 1995); 1-3 Cystein rich interdomain region (CIDR); and in some variants one to two C2 domains (Smith *et al.*, 2000b). The DBL and the CIDR domains of PfEMP1 have been implicated in binding to various receptors, resulting in the sequestration of IE to different host cells and in different organs (Table 2).

Diversity of the var genes

P. falciparum populations carry vast diversity of *var* forms, whose continual renovation is particularly important for the parasite to evade host-immune attack. Diversity of *var* genes can be generated by non-allelic (ectopic) recombination between

var gene members during sexual reproduction (Freitas-Junior *et al.*, 2000; Taylor *et al.*, 2000b). Telomeres of *P. falciparum* form clusters between four to seven chromosomes which facilitate shuffling between different *var* paralogues, as clustering of the chromosomal ends brings them physically close to each other (Freitas-Junior *et al.*, 2000). The *var* genes can be categorized on the basis of 5' upstream sequence (Ups) and chromosomal locations (Kraemer and Smith, 2003; Lavstsen *et al.*, 2003) (Figure 3). Three major types (A, B, C) and two intermediate types (A/B and B/C) were characterized: Ups A types have Ups A flanking sequences, are exclusively found in subtelomeric regions and are transcribed towards the telomere; Ups B types are flanked by Ups B sequence, consist of subtelomerically *var* genes and transcribed towards the centromere; Ups C types are flanked by type C sequences, located in the centromeric region of the chromosome and transcribed toward the telomere. Further, two *var* genes namely var_{common} (*var1csa;* PFE1640w) (Kyes *et al.*, 2003; Salanti *et al.*, 2002; Winter *et al.*, 2003) and *var2csa* (PFL0030c) (Salanti *et al.*, 2003) are found having distinct 5' upstream sequences and genetic organization and thus be classified as Ups D and Ups E, respectively (Kraemer and Smith, 2003; Lavstsen *et al.*, 2003).

Three distinctive types of *var* genes, *var1csa*, *var2csa* and Type 3 *var* are exceptionally conserved across parasite isolates, suggesting that these atypical *var* genes might undergo self-self recombination. The var_{common} (*var1csa)* gene which was found universally transcribed in most parasites encodes a truncated PfEMP1 product (Winter *et al.,* 2003; Salanti *et al.*, 2003; Salanti *et al.*, 2004; Trimnell *et al.*, 2006; Viebig *et al.*, 2005). The protein encoded by *var2csa* plays a role in CSA binding and pregnancy associated malaria and a gene orthologue is present in *P. reichenowi* that diverged from *P. falciparum* approximately five-to-seven million years ago (Salanti *et al.*, 2003; Salanti *et al.*, 2004; Trimnell *et al.*, 2006; Viebig *et al.*, 2005). In addition, *var2csa* lacks the otherwise common DBL1α domain and, unlike any other *var* gene, the *var2csa* gene contains an upstream open reading frame (uORF) 5' of the promotor region (Kraemer and Smith, 2003; Lavstsen *et al.*, 2003; Trimnell *et al.*, 2006) which has been suggested to be involved in translational regulation (Zhang and Dietrich, 2005).

Figure 3. Schematic representation of 3D7 *var* genes

Gene	Ups	Chr Location	Orientation	Domain Structure	*var* group
PFD0020c	A1	T	T	DBL1α CIDR1α DBL2β C2 DBL3γ DBL4γ DBL5δ CIDR2β ATS	A
PFD1235w	A1	T	T	DBL1α CIDR1α DBL2β C2 DBL3β C2 DBL4γ DBL5δ CIDR2β ATS	A
PF08_0141	A1	T	T	DBL1α CIDR1γ DBL2β C2 DBL3γ DBL4β DBL5ε ATS	A
MAL8P1.207	A1	T	T	DBL1α CIDR1α DBL2β C2 DBL3β C2 DBL4γ DBL5δ CIDR2β ATS	A
PF11_0521	A1	T	T	DBL1α CIDR1α DBL2β C2 DBL3β C2 DBL4δ CIDR2γ ATS	A
PF11_0008	A1	T	T	DBL1α CIDR1γ DBL2γ DBL3δ CIDR2β DBL4β C2 ATS	A
PF13_0003	A1	T	T	DBL1α CIDR1γ DBL2β C2 DBL3γ DBL4δ CIDR2β DBL5β C2 ATS	A
PFA0015c Type 3	A1	T	T	DBL1α DBL2ε ATS	A
PFF0020c Type 3	A1	T	T	DBL1α DBL2ε ATS	A
PFI1820w Type 3	A1	T	T	DBL1α DBL2ε ATS	A
PF08_0140	B1	T	C	DBL1α CIDR1α DBL2β C2 DBL3γ DBL4δ CIDR2β ATS	A/B
PFF1580c	B1	T	C	DBL1α CIDR1α DBL2β C2 DBL3γ DBL4δ CIDR2β DBL5ε DBL6ε DBL7ε ATS	A/B
PFL0020w	B1	T	C	DBL1α CIDR1α DBL2β C2 DBL3γ DBL4β DBL5ε ATS	A/B
PFF0010w	B2	T	C	DBL1α CIDR1γ DBL2β C2 DBL3γ DBL4β DBL5ε ATS	A/B
PFB0010w	B1	T	C	DBL1α CIDR1α DBL2γ ATS	B
PFA0005w	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2β ATS	B
PFA0765c	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2β ATS	B
PFB1055c	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2β ATS	B
PFC0005w	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2β ATS	B
PFC1120c	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2β ATS	B
PFD1245c	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2β ATS	B
PFE0005w	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2β ATS	B
PFF1595c	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2β ATS	B
MAL7P1.212	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2β ATS	B
PF08_0142	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2β ATS	B
MAL8P1.220	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2β ATS	B
PF10_0406	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2β ATS	B
PF11_0007	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2β ATS	B
PFL0005w	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2β ATS	B
PFL2665c	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2β ATS	B
MAL13P1.1	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2β ATS	B
MAL13P1.356	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2β ATS	B
PFI0005w	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2γ ATS	B
PFI1830c	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2γ ATS	B
PF10_0001	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2γ ATS	B
PF07_0139	B1	T	C	DBL1α CIDR1α DBL2δ CIDR2β DBL3ε ATS	B
PFD0005w	B1	T	C	DBL1α CIDR1α DBL2γ DBL3δ CIDR2β ATS	B
PFD0635c	B3	C	T	DBL1α CIDR1α DBL2δ CIDR2γ ATS	B/C
PFD1005c	B1	C	T	DBL1α CIDR1α DBL2δ CIDR2γ ATS	B/C
MAL7P1.50	B1	C	T	DBL1α CIDR1α DBL2δ CIDR2γ ATS	B/C
MAL7P1.55	B1	C	T	DBL1α CIDR1α DBL2δ CIDR2γ ATS	B/C
PF08_0106	B1	C	T	DBL1α CIDR1α DBL2δ CIDR2β ATS	B/C
PFL1955w	B1	C	T	DBL1α CIDR1α DBL2δ CIDR2β ATS	B/C
PFL0935c	B1	C	T	DBL1α CIDR1α DBL2δ CIDR2γ ATS	B/C
PF08_0103	B3	C	T	DBL1α CIDR1α DBL2δ CIDR2β ATS	B/C
PF07_0050	B3	C	T	DBL1α CIDR1α DBL2β C2 DBL3γ ATS	B/C
PFL1950w	B4	C	T	DBL1α CIDR1α DBL2β C2 DBL3δ CIDR2β ATS	B/C
PFD0615c	C1	C	T	DBL1α CIDR1α DBL2δ CIDR2β ATS	C
PFD0625c	C1	C	T	DBL1α CIDR1α DBL2δ CIDR2β ATS	C
PFD1015c	C1	C	T	DBL1α CIDR1α DBL2δ CIDR2β ATS	C
PF07_0048	C1	C	T	DBL1α CIDR1α DBL2δ CIDR2β ATS	C
PFL1960w	C1	C	T	DBL1α CIDR1α DBL2δ CIDR2β ATS	C
PF07_0051	C1	C	T	DBL1α CIDR1α DBL2δ CIDR2β ATS	C
PFD0630c	C1	C	T	DBL1α CIDR1α DBL2δ CIDR2γ ATS	C
PFD1000c	C1	C	T	DBL1α CIDR1α DBL2δ CIDR2γ ATS	C
PF07_0049	C1	C	T	DBL1α CIDR1α DBL2δ CIDR2γ ATS	C
PF08_0107	C1	C	T	DBL1α CIDR1α DBL2δ CIDR2γ ATS	C
PFD0995c	C1	C	T	DBL1α CIDR1α DBL2δ CIDR2γ ATS	C
MAL7P1.56	C2	C	T	DBL1α CIDR1α DBL2δ CIDR2β ATS	C
PFF0845c	C1	C	T	DBL1α CIDR1α DBL2β C2 DBL3δ CIDR2β ATS	C
PFE1640w *var*$_{common}$/*var1csa*	D	T	T	DBL1α CIDR1α DBL2β C2 DBL3γ DBL4ε DBL5γ DBL6β DBL7ε	D
PFL0030c *var2csa*	E	T	T	DBL1x DBL2x DBL3x DBL4ε DBL5ε DBL6ε ATS	E

Switching mechanisms and regulation of var gene expression

Transcriptional switches between different members of the *var* gene family allowing the parasites to alter both the adherent properties of the IEs and their antigenic phenotypes, and therefore avoiding the antibody response of the infected individual and maintaining a persistent infection. At any given time, only a single PfEMP1 protein is expressed in each parasite (Chen *et al.*, 1998b; Kyes *et al.*, 2003; Scherf *et al.*, 1998). It has been demonstrated that mutually exclusive expression of PfEMP1 is regulated at the level of *var* gene transcription, rather than the level of protein synthesis (Chen, 1998; Scherf, 1998; Kyes, 2003; and Voss 2006); unlike in mammalian systems, it has until recently neither been thought regulated upon the negative feedback of the level of the proteins produced (Dzikowski *et al.*, 2006 and Paper IV). In natural infections, switching of *var* genes needs to occur often enough to generate parasite subpopulations that can escape the human immune response, yet be tightly controlled in order to avoid exhaustion of the sixty different proteins encoded within the genome of each individual parasite. The expression dynamics of *var* genes and their switching rates have previously been studied using different approaches including *in vitro* and *in vivo* assays and mathematical modeling (Frank *et al.*, 2007; Gatton *et al.*, 2003; Horrocks *et al.*, 2004; Kaestli *et al.*, 2004; Roberts *et al.*, 1992); the conclusions of these studies, however, were not corroborated with each other. The discrepancies might be in part due to variation in the experimental setups and the approaches used for monitoring variation.

While the details of the mechanism controlling *var* gene expression have not yet been defined, recent evidence supports a role of epigenetic regulation. Epigenetic controls of gene expression refer to alternation in transcriptional states of a gene that is not accompanied by changes in the DNA sequence or position in the genome. In *P. falciparum*, epigenetic regulation of *var* gene expression involves chromatin modification (Chookajorn *et al.*, 2007; Duraisingh *et al.*, 2005), repositioning of *var* loci in subnuclear compartments (Duraisingh *et al.*, 2005; Freitas-Junior *et al.*, 2005; Voss *et al.*, 2006) and activation or silencing by non-coding genetic elements (Calderwood *et al.*, 2003; Deitsch *et al.*, 1999; Frank *et al.*, 2006; Voss *et al.*, 2000; Voss *et al.*, 2003; Voss *et al.*, 2006).

Chromatin modification

Chromatin modifications have been shown to be involved in gene regulation in many organisms (Felsenfeld *et al.*, 1996). In budding yeast, for example, transcriptional silencing is mediated by histone deacetylase, called Silent Information Regulators Proteins (Sir), forming repressive chromatin structures that are confined to specific chromosomal domains (Gartenberg, 2000). Using a chromatin immuno-precipitation (CHIP) assay, a Sir2 homologue was identified in *P. falciparum* (Freitas-Junior *et al.*, 2005); binding of PfSir2 in the promoter region of the subtelomeric *var* gene was found responsible for the silencing of the gene. The authors furthermore demonstrated that H4 accetylation promotes activation of *var* loci, a trait also observed in other organisms (Braunstein *et al.*, 1996; Shankaranarayana *et al.*, 2003). A complementary study by Duraisingh and colleagues demonstrated activation of a subset of *var* genes in PfSir2 knockout parasites, strengthen the regulatory role of PfSir2 in *var* gene silencing (Duraisingh *et al.*, 2005).

Perinuclear location and var gene regulation

Using electron-microscopy and fluorescence *in situ* hybridization (FISH), it has been shown that *var* gene activation is linked with dissociation from the telomeric clusters and relocation to distinct nuclear site, a subnuclear compartment presumably of transcriptional competence (Duraisingh *et al.*, 2005; Ralph *et al.*, 2005; Voss *et al.*, 2006). The dissociation of subtelomeric *var* genes from the area of perinuclear heterochromatin is believed to avoid the telomeric silencing effect (TSE), an important gene regulation phenomenon which occurs in yeast (Andrulis *et al.*, 1998). The dissociated *var* gene then relocates to a transcriptional active site. Once such a site is occupied and active, *var* gene transcription at other loci cannot occur (Dzikowski *et al.*, 2006).

Role of non-coding DNA elements in var silencing

While exon I regions of the *var* genes are variable in sequence, the non-coding regions are highly conserved among different parasite strains, suggesting a conserved functional role of these genetic elements. To examine the association between the *var* promoter and mutually exclusive expression, various plasmid constructs have been

designed in which the upstream regulatory regions and introns of various *var* genes were used to influence the expression of the reporter genes (Calderwood *et al.*, 2003; Deitsch *et al.*, 1999; Dzikowski *et al.*, 2006; Frank *et al.*, 2006; Gannoun-Zaki *et al.*, 2005; Vazquez-Macias *et al.*, 2002; Voss *et al.*, 2000; Voss *et al.*, 2006). It was demonstrated that both the 5' flanking promoter region and the intron within a *var* gene are required to act in cooperation in order to achieve silencing. Further, a number of potential regulatory elements (SPE1, SPE2 and CPE) were identified differentially interacting with different upstream sequence elements (Voss *et al.*, 2003). Silencing of the transcription mediated by the *var* promoters was seen only after the DNA synthesis phase (S-phase) in the cell cycle (Deitsch *et al.*, 2001) where SPE2 was exclusively found (Voss *et al.*, 2003). These observations indicate the cooperative role of DNA-binding complexes and chromatin structure alteration in regulation of *var*-gene transcription.

It is noteworthy that both PfSir2 and perinuclear relocation do not seem to have any effect on internal *var* gene regulation (Freitas-Junior *et al.*, 2005; Ralph *et al.*, 2005). Possibly, the internal and subtelomeric *var* genes might be under different repression kinetics, and the promoters of these *var* types might be regulated by different nuclear factors (Voss *et al.*, 2003). Indeed, *var* genes with a different upstream promoter and chromosomal position have variable intrinsic switching rates, indicating that all *var* genes are not affected by a uniform regulatory machinery (Frank *et al.*, 2007).

Other variant antigens

RIFIN

Another clonal variant protein found on the IE surface is RIFIN (Fernandez *et al.*, 1999; Kyes *et al.*, 1999). RIFINs are immunogenic and widely recognized by sera from semi-immune individuals living in malaria endemic areas (Fernandez et al., 1999; Abdel-Latif *et al.*, 2002). RIFINs are encoded by a highly polymorphic multigene family known as *rif* (repetitive interspersed family). The *rif* family was first identified from a *P. falciparum*-genomic library, which shows a complex and polymorphic pattern when hybridized to restrict enzyme fragments of genomic DNA from different strains (Weber, 1988). All *rif* genes are composed of two exons: an exon I encodes a short signal peptide

of around twenty-to-twenty-five amino acids and an exon II of variable length (~1 kb). The extent of variability in the structures of the *rif* genes were revealed upon the early release of the chromosome 2 and 3 sequencing projects (Bowman *et al.*, 1999; Gardner *et al.*, 1999). Most of the *rif* genes are subtelomerically located with approximately 150 members per genome. Transcription of the *rif* genes peaks at eighteen-to-twenty-three hours post-invasion, nonetheless, some *rif* variants transcripts have also been detected in later stages of the erythrocytic cycle (Bozdech *et al.*, 2003a; Kyes *et al.*, 2000; Kyes *et al.*, 1999; Le Roch *et al.*, 2003) and recently, the presence of RIFINs in the merozoite was observed implying that the merozoite may also partake in antigenic variation (Petter *et al.*, 2007).

STEVOR

Similar to the *rif* gene family, the subtelomeric variant open reading frame (*stevor*) gene family are found also in subtelomeric regions and have the two-exon genetic structure (Bowman *et al.*, 1999; Cheng *et al.*, 1998; Gardner *et al.*, 1999). *Stevor* genes encode for STEVOR proteins of 30-40 kDa, and there are around 30-40 *stevor* copies per haploid genome. Protein products and transcripts of *stevor-genes* have been detected during several stages of the lifecycle: asexual stage parasites; gametocytes; and sporozoites (Florens *et al.*, 2002; McRobert *et al.*, 2004; Sharp *et al.*, 2006; Sutherland, 2001). STEVOR has not been shown to be localized at the surface of the infected RBC but instead migrated to Maurer's cleft just beneath the RBC membrane (Kaviratne *et al.*, 2002). The prediction that STEVOR is a variant membrane-bound protein could imply a role in antigenic variation, but the subcellular location and multi-stages expression raises some doubts as to this possibility (Blythe *et al.*, 2004).

PfMC-2TM

Pfmc-2tm, a recently identified subtelomeric gene family in *P. falciparum* that encodes two-transmembrane proteins located in the Maurer's cleft with approximately 27 kDa. Due to the similarity in the basic protein topology, this protein family, together with RIFIN and STEVOR, belong to the same large superfamily (Lavazec *et al.*, 2006; Sam-Yellowe *et al.*, 2004). Thirteen *pfmc-2tm* gene copies were identified in the 3D7 genome

(Gardner *et al.*, 2002) and it was predicted to have at least ten copies in other strains (Lavazec *et al.*, 2006). Striking sequence conservation was seen among the *pfmc-2tmc* paralogues and their extended flanking regions, except for the hypervariable loop within the *pfmc-2tm* genes predicted to be exposed on the IE surface; this antigenic diversity perhaps is driven by the host immune pressure (Lavazec *et al.*, 2006). Moreover, clonal variation and switching among *pfmc-2tm* have been seen between isogenic clones, signifying its role in antigenic variation (Lavazec *et al.*, 2007).

CLAG

The identification of *clag* originated from the observation of subtelomeric deletions at one end of chromosome 9, which was associated with irreversible loss of cytoadherence and gametocytogenesis (Day *et al.*, 1993). Gene mapping and sequencing of the deleted region revealed that a gene, namely *clag9* to be associated with the adhesion. Subsequently, using targeted gene disruption and antisense inhibition, *clag9* was further proven to be required for cytoadherence to CD36 (Gardiner *et al.*, 2000; Trenholme *et al.*, 2000). Apart from *clag9*, four *clag* paralogues (*clag2, 3.1, 3.2* and *7*) have been identified in *P. falciparum* (Holt *et al.*, 1999; Holt *et al.*, 2001). *clag9* is the most divergent in sequence amongst the *clag* family of genes, still it is conserved between isolates (Manski-Nankervis *et al.*, 2000) and there has been no definite demonstration of the *clag9* protein product on the surface of the IEs; rather, Clag9 is probably a merozoite protein (Mattei *et al.,* 2004).

2 AIMS OF THE THESIS

The overall objectives of this thesis were to increase the understanding of different forms of variation in *Plasmodium falciparum,* at both genomic and transcriptional levels, using genome-wide approaches.

Specific aims of these studies included in this thesis were:

Genomic variation:

1. To investigate the copy number variation in *P. falciparum* strains originating from different geographical areas using microarray-based comparative genomic hybridization (Paper I).

2. To perform *in silico* analysis of the highly polymorphic subtelomeric regions of the 3D7 chromosomes, and to examine them for the presence of segmental duplications (Paper II).

Transcriptomic variation:

3. To generate phenotypically distinct isogenic 3D7 clones, to compare their transcriptomes during the asexual cycle of the development correlating to their adhesive phenotypes (Paper III).

4. To elucidate the switching pattern of *var* genes in the absence of external stimuli and to study the adhesion profiles and transcriptional patterns of the isogenic clones generated in Paper III (Paper IV).

3 EXPERIMENTAL PROCEDURES

The materials and methods used in this thesis are described, with original references, in Papers I- IV. A few of the materials and methods used will be briefly described below:

Parasites

Parasite strains used in these studies include:

- *In vitro* adapted laboratory strains:
  - 3D7AH1, 7GH, Dd2, Hb3, FCR3, F32, TM180, TM284 and R29.
- Isogenic clones generated by micromanipulation, all having 3D7 genetic background:
  - 3D7AH1S1, -S2, -S3 and -S4 (CD36 binder), 3D7S8.4 (rosettes formation) and 3D7S8.4 subclones (3D7S8.4.1-17; no particular binding property).
- Fresh clinical isolates:
  - UAS31, UAS39 and UAM25.

Cloning of parasites

Micromanipulation cloning was carried out using a micromanipulator MN-188 (Narshige), sterile micropipettes of 3-5 mm internal diameter (Sutter Instrument), 12-well tissue culture treated plates and an inverted microscope (Nikon). Each single-picked IE was transferred into an individual well, containing red blood cells at two percent haematocrit in malaria culture medium supplemented with fifteen percent human AB+Rh+ serum. The clones were grown for fifteen days before microscopic examination.

Phenotypic characterization

Phenotypic characterization of the parasites was performed using trophozoite IE at five-to-eight percent parasitaemia or pigmented IEs enriched using a MACS magnetic cell sorter (Miltenyi BioTec). Cell lines and transfectants (CHO-CD36, CHO-ICAM and CHO), soluble receptors (heparin, CD31, CSA, and TSP), as well as placental sections, were used for assessing adhesive properties of IEs.

The P. falciparum genome array

The *P. falciparum* genome array is composed of 6850 70-mer oligonucleotides printed in quadruplicate on CMT-UltraGaps slides (Corning, NY, USA). A complete list of the oligonucleotides used is displayed in Table 4. From which, 294 in-house designed oligonucleotides, including 242 *var*-specific probes, were selected using Array Designer ® Version 1 (Palo Alto, CA, USA). Genomic DNA was labeled with a direct-labeling method using Klenow and Cy3/Cy5-dUTP; whereas an indirect-labeling method (amino-allyl dye coupling) was applied for RNA samples. All hybridization procedures were performed automatically with a Lucidea Automated Slide Processor (Amersham Biosciences); slides were subsequently scanned with an Axon Gene Pix 4000B, hybridization signals were then inspected and quantified using GenePix Pro softwares.

Table 4. List of the array elements used on the *P.falciparum* genome array

Source	Category	Number of probes
Operon	genome set (*P. falciparum* 3D7)	6467
	var genes	75
In-house	*var* genes (*P. falciparum* 3D7)	184
	var ATS (*P. falciparum* 3D7)	13
	15 *var* genes from other strains[a]	45
	stage-specific genes (*P. falciparum* 3D7)	24
	Giardia lamblia	28
Stratagene	spike control	14
		Total = 6850

[a] Three oligos were designed per *var* gene for different *P. falciparum* strains. Clones include: A4tres, Dd2 (*var*1 and *var*7), FCR3S1.2 (*var*1), FCR3 (*var*2, *var*3 and *var*CSA), It4, ItCS2, ItG/FCR3*var*T11, MC (*var*1 and *var*2), R29R (*var*1), tm284S2 (*var*1), *var*ph17

Data analysis

Different approaches were adopted in separate studies:

CGH data were processed in R environment (Gentleman *et al.*, 2004) with a KTH package comprised of aroma (Bengtsson *et al.*, 2004) and LIMMA (Smyth, 2005). The data without background subtraction were normalized using a print-tip dependent LOWESS approach, with *f*=0.5. Two criteria were applied for the detection of copy

number variation: (1) at least two oligonucleotides, mapped consecutively after each other in the genome had to have similar magnitudes of ratio-based differences; (2) oligonucleotides mapped for the antigenic variants or at the highly sequence polymorphic subtelomeric regions of 3D7 genome were disregarded.

For comparative transcriptome analysis for the isogenic clones, the data were processed in R-environment with LIMMA. LOESS was used for the data normalization without background correction. Genes were considered differentially expressed when the signal was greater than two-fold in either direction with a moderated t-statistic p-value (< 0.05), adjusted for multiple comparisons. Genes that showed greater than five-fold differential expression were particularly scrutinized.

For the longitudinal transcriptomal study, GeneSpring 6.1 software (Silicon Genetics) was used for the data analysis. LOWESS was adopted to normalize the data without local background subtraction.

Northern blot

Amplicons of PFD0630c, PFF0845c and PFL0030c were cloned into TOPO PCR II vector (Invitrogen) with a T7 promotor sequence in the anti-sense direction. Digitonin-labeled anti-sense RNA was produced using a Dig RNA labelling kit (Roche). Total RNA (2 μg) from IE was transferred to nylon membranes (Roche) employing a PosiBlot pressure blotter (Stratagene) or by capillary transfer. Blots were pre-hybridized in Dig Easy Hyb buffer and then probed with 100ng/ml Digitonin-labeled anti-sense RNA in Dig Easy Hyb buffer (Roche) at 65°C overnight. After hybridization, membranes were washed twice with 2x SSC, 0.1% SDS at RT and twice with 0.5 x SSC, 0.1% SDS at 65 °C. Detection of RNA was carried out using the Dig Luminescent detection kit (Roche).

Immunoblot

Pigmented IEs were enriched using a MACS magnetic cell sorter and solubilized in reducing SDS sample buffer. Samples were separated on either five percent plus twelve percent SDS-polyacrylamide gels or on three-to-eight percent tri-acetate gradient gels (Invitrogen), and immediately transferred onto nitrocellulose membrane. For immuno-staining, membranes were blocked with five percent non-fat milk powder in PBS and

probed with rabbit polyclonal or mouse monoclonal anti-PfEMP1 sera directed against the conserved C-terminal acidic-terminal-sequence (ATS). For detection, membranes were incubated with a secondary anti-rabbit Ig Horseraddish peroxidase conjugate (1:1500, Amersham) or a secondary sheep anti-mouse Ig-HRP conjugate (1:5000, Pierce) and then developed in 1 M diethanlolamine/1 mM $MgCl_2$ containing 0.5 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.5 mM nitroblue tetrazolium or visualized using ECL system (Amersham), respectively.

Real-time quantitative PCR (qPCR)

CNP or transcriptional difference of a particular gene was evaluated by qPCR using genomic DNA and cDNA, respectively. Primer sets were designed either using Primer Express® software (Applied Biosystem) or acquired from previous studies (Salanti *et al.*, 2003). Primers for endogenous controls such as seryl-tRNA synthetase, β-tubulin and fructose-bisphosphate aldolase were included in different experiments. Various comparative Ct methods were applied according to the experimental setup and analytical purposes.

Fluorescence in situ hybridization

Gel purified PCR products or gene-containing plasmids were labeled using Fluorescein-High Prime Kit (Roche Applied Science). In some cases, Rep20 containing plasmids (pUC9) labeled with Biotin-High Prime kit (Roche Applied Science) were used for indication of telomeric ends. Parasites were isolated from their host erythrocytes using saponin (0.05 % w/v) and smeared as monolayers on microscope slides (Menzel-Gläser). Air-dried monolayers were fixed with four percent paraformaldehyde (PFA) for fifteen minutes at room temperature and treated with RNase (20 mg/ml in 2 x SSC) for thirty minutes at 37°C. Hybridization solution containing 100 ng of labelled and heat denatured probe (in 50 % deionized formamide, 10 % dextran sulphate, 1 x SSC and 250 mg/ml herring sperm DNA) was added to parasite preparations before hybridization at 95°C for three minutes and at 37°C for twelve-to-fifteen hours. After hybridization, parasites were washed several times in deionized formamide / SSC solution with different stringency. Additional steps were included for detection of the biotinylated Rep20 probes. Parasites

were then washed and mounted in Vectashield (Vector Laboratories). Preparations were visualized using a Leica DMRE microscope and imaged with a Hamamatsu C4880 cooled CCD camera.

Bioinformatic analysis

Nucleotide- and amino acid sequence retrievals and BLAST searches were accessed via

- PlasmoDB (www.plasmoDB.org);
- *P. falciparum* Database, the Broad Institute of MIT and Harvard (http://www.broad.mit.edu/annotation/genome/plasmodium_falciparum_spp/MultiHome.html);
- *P. falciparum* Blast Server, the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/P_falciparum);
- NCBI Entrez database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide&cmd=search&term=); and
- geneDB, the Wellcome Trust Sanger Institute (http://www.genedb.org/)

P. falciparum transcription data were acquired from PlasmoDB (www.plasmoDB.org), Derisi Lab Malaria Transcriptome Database (http://malaria.ucsf.edu/) and Expression Profiling of the Malaria Parasite Life Cycle from Winzeler's Lab (http://carrier.gnf.org/publications/CellCycle/).

Orthologous group predictions and gene ontology were obtained from OrthoMCL DB (http://orthomcl.cbil.upenn.edu/cgi-bin/OrthoMclWeb.cgi) (Li *et al.*, 2003) and the Gene Ontology Home (http://www.geneontology.org/), respectively, or via PlasmoDB.

CLUSTAL W sequence alignments were carried out using MacVector™ 7.0 software or BioEdit freeware (Tom Hall, Ibis Biosciences, Carlsbad, CA).

Data deposition

- Information of the *P. falciparum* genome array was deposited in www.ebi.ac.uk/arrayexpress: **A-MEXP-289** (for CGH analysis) and **A-MEXP-75** (for trancriptome analysis).

- Microarry data from different studies were deposited in ArrayExpress with the ascession number:
  E-MEXP-494 (CGH study), **E-MEXP 128** (isogenic clones transcriptomic comparison); and **E-MEXP-1199** (longitudinal transcriptional profiling).
- The application protocol for the Lucidea Automated Slide Processor was launched at: www.mtc.ki.se/groups/wahlgren/microarray.html.

4 RESULTS AND DISCUSSION

Genomic variation in *P. falciparum* (Papers I & II)

The genomic complexity of *P. falciparum*, in particular its ability to generate genetic variants, makes it a successful pathogen. Thus, identification of the genomic differences between distinct *P. falciparum* could increase our understanding of the parasite biology and the pathogenesis of the disease.

4.1 Genome wide gene copy number polymorphism in *P. falciparum* (Paper I)

Using microarray complementary with other technologies (real-time PCR, FISH and DNA sequencing), we have identified twenty-four copy number polymorphisms (CNPs) amongst seven different laboratory strains and two fresh clinical isolates of widely geographical origins, relative to the reference strain 3D7. We demonstrated that CNPs occur all over the genome, with the exception of chromosome 6 and 8. In total, there are fifty genes with increased copy numbers and thirty-two genes decreased in copy number; of these, twenty-one gene duplications and thirteen gene deletions have not previously been identified (Bozdech *et al.*, 2003a; Carret *et al.*, 2005; Kidgell *et al.*, 2006). Many of the CNPs were found to correlate with the phenotype- or origin of the parasite line. As the subtelomeres of *P. falciparum* are known to be highly polymorphic, subtelomeric regions have been excluded in this study. Nevertheless, we did observe an interesting duplication event in the subtelomeric regions of several chromosomes, which is described in Paper II.

Biological relevance of the CNPs

Genes within these twenty-four CNPs encode molecules involved in cell-cycle regulation, drug resistance, erythrocyte invasion, sexual differentiation, metabolic pathway and unknown functions. Most of the identified CNPs (14 out of 24) are shared by more than one parasite, suggesting the presence of genomic hotspots for genomic variation (Table 5).

Table 5. Genomic-wide copy number polymorphism in *P.f.* involved in known biological functions

Biological relevance	CNP	Anotation		Parasites
Knob formation	2.1	PfEMP3 Knob associated histidine-rich protein (KAHRP) Hypothetical proteins	Deletion	FCR3, F32, Dd2
Erythrocyte invasion	4.1	SURFIN 4.1 Reticulocyte binding protein (*Pf*RH1)	Duplication	FCR3, F32
multidrug resistance	5.2[a]	Multidrug resistance protein House keeping proteins[b] Hypothetical proteins	Duplication	FCR3, F32, R29, Dd2, TM180, TM284
Cell cycle regulation	7.1	PelOta protein cwf15 homologue GTP-binding protein Hypothetical proteins	Duplication	TM180, TM284
	10.1	Asparagine-rich antigen eukaryotic translation initiation factor 3 subunit 7 histone deacetylase endonuclease	Duplication	F32
hemoglobin degradation	14.1	Plasmepsin 2 Histo-aspartic protease	Duplication	TM180

[a] CNP5.2 duplications were found to vary in size in different parasites

[b] Six putative house keeping genes found duplicated encode ribosomal large subunit pseudouridylate synthase, DEAD-bod subfamily ATP-dependent helicase, nucleotife binding protein, S-adenosylmethioine-depemdent methyltransferase, 50S ribosomal subunit protein L17 and G10 protein

To examine whether the copy number gains or losses influence the phenotypes of the parasites, several complementary analyses have been conducted. For example, genes (*surf* and *pfrh1*) responsible for erythrocyte invasion were found amplified six times in FCR3 and F32 strains (CNP 4.1); these parasites were later proven to exhibit the highest proliferation rate compared to all tested strains, which indicates their success in invasion and growth. Another example is the amplification of a drug resistance locus (CNP 5.2). The strains lacking the duplications were found sensitive to quinine (3D7AH1, 7G8, UAM25 and UAS31); whereas the parasites that carried multiple *pfmrd1* copies (FCR3, F29, Dd2, TM180 and TM284) were resistant to mefloquine and quinine, with the exception of F32. Taken together, the ubiquity of copy number variation in *P. falciparum* suggests that the parasite employs gene amplifications and deletions as a general strategy to enhance its chances of survival and spread.

4.2 Segmental duplication in *P. falciparum* subtelomeres (Paper I)

Apart from copy number polymorphisms, the ends of each chromosome, in particular the subtelomeric region, create another dimension of genomic diversity. Syntenic comparison of the *P. falciparum* genome with the rodent genomes as well as the full-genome genetic variation map in *P. falciparum* revealed dramatic variability of the subtelomeric regions (Carlton *et al.*, 2002; Hall *et al.*, 2005; Kidgell *et al.*, 2006). Yet,

highly conserved segmental duplication do exist in *P. falciparum* subtelomeres as described herein.

Even though the variable subtelomeric regions were excluded from the analysis in Paper I, we had observed a subtelomeric locus, situated on the right end of chromosome 1 spanning PFA0685c, PFA0690w and PFA0695c in the 3D7 genome, duplicated in a fresh clinical isolate (UAM25). This duplicated gene segment, albeit sequence diverged, carrying the same genetic content and orientation to a block of paralogous genes, which had been amplified eight times at multiple subtelomeres of the 3D7 genome. We named the former duplicon as sequence diverged-SD (sd-SD; depicted in pale-blue, shaded ellipse) and the latter ones as 'SD' (marked in pink, shaded ellipse) (Figure 4). We visualized from the subtelomeric map, that these eight SD span at least ten kilobases and comprise six genes, including three variant gene families (*rif, pfmc-2tm* and *var*) and three hypothetical genes (*n-, o-, q-gene*). ClustalW multiple alignments revealed a profound sequence identity (≈ 99%) shared among the eight SDs in the 3D7 genome, with a hypervariable region (≈ 23 amino acids) only located within the PfMC-2TM, a region presumably encoded for a surface-exposed hypervariable loop whose diversity is driven by the immune pressure.

Sequence variation and copy number polymorphism of the SD

To further elucidate whether such sequence conservation remains across *falciparum* species, we sequenced the *n-, o-, pfmc-2tm* and *q-gene* in five parasites originating from different geographical areas; four additional parasite strains retrieved from public *Plasmodium falciparum* sequence servers were added-in for the analysis. ClustalW multiple alignments of the SD sequences from these ten *P. falciparum* strains confirmed high sequence conservation (≈ 99%) across the *falciparum* parasite. Despite the high level of sequence identity, copy number of SD varies among different strains as observed by quantitative PCR, BLASTN and FISH experiment.

In contrast to SD, the sd-SD, although found duplicated in UAM25, presents as single copy in all the tested strains; the duplication of sd-SD in UAM25 however did not cover PFA0675w (paralogous to the *q-gene*). Remarkably, while the *q-gene* is well

conserved amongst the classical SDs, its paralogue PFA0675w in the sd-SD is genetically variable and contains RESA-like repeats and a DNJ domain.

Role of the SD in speciation and antigenic diversification

Thirteen *pfmc-2tm* genes have been identified in the 3D7 genome; nine of them are located within the SD and the rest are located adjacent in the paralogous gene neighbourhood. The most parsimonious explanation for this observation is that the *pfmc-2tm* gene family was expanded through segmental duplication.

Most of the genes within the SD, except the *q-gene* and a *var* pseudogene, encode PEXEL-containing export proteins. Interestingly, the *q-gene* is the only member having orthologues in other organisms (*P. vivax, P. yoelii, P. chabaudi* and *P. berghei*) and might be one explanation for why the SDs have been selected upon speciation. In other words, the gene might have unique and beneficial purposes on existence of *P. falciparum*.

To determine whether the SD carries any potential biological relevance, we examined the transcription of the SD genes using the transcriptome data generated in Paper III supplement with publicly available databases, and we found only the *n-gene* to be significantly transcribed. Further analysis demonstrated that transcription of the *n-gene*, besides being developmentally regulated, was correlated to the gene dosage in the genome. This suggests that the *n-gene* not only serves as a mediator for non-homologous chromosomal interaction, but could be of functional significance to the parasites.

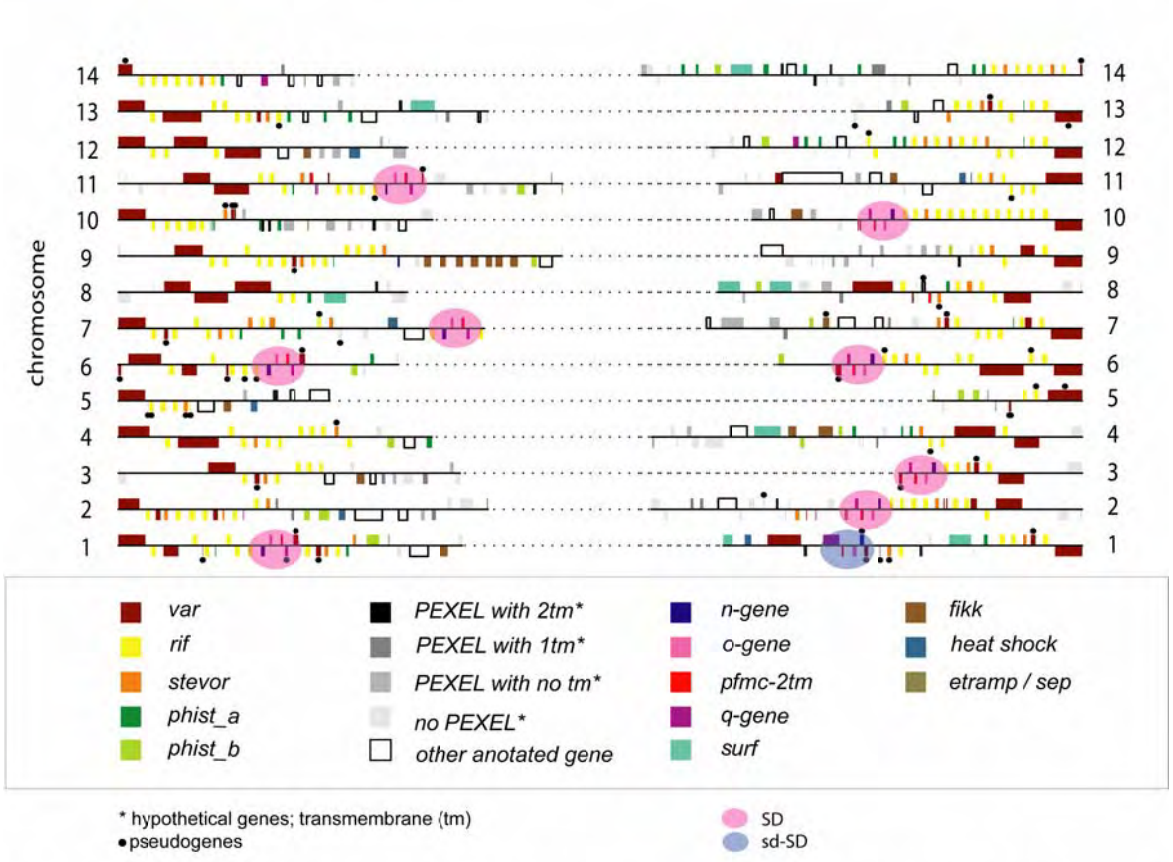

Figure 4. High-resolution display of gene variants compartments in *P. falciparum* subtelomeres

Transcriptomic variation in relation to adhesive characteristics (Papers III & IV)

Antigenic variation is an important mechanism by which a clonal parasite population gains a selective advantage by changing its antigenic profile. To get a better understanding of the molecular basis of antigenic variation, we conducted phenotypic and transcriptomic analysis of two isogenic *P. falciparum* clones generated by single-cell micromanipulation (Figure 5).

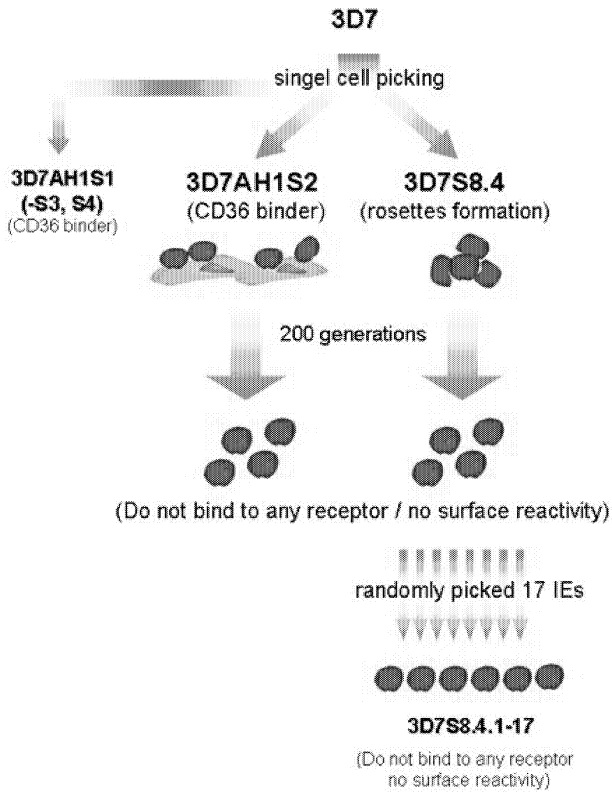

Figure 5. Description of the generation of 3D7 isogenic clones

4.3 Global transcriptional profiles between phenotypically distinct isogenic clones

Two isogenic *P. falciparum* clones, 3D7S8.4 and 3D7AH1S2, were selected for rosette formation and adhesion to CD36-transfected CHO cells, respectively (Figure 5). Comparative transcriptomal analysis revealed a total of 262 genes being differentially transcribed (≥ 2-fold change) in between 3D7S8.4 and 3D7AH1S2; of these, fifteen genes with the largest transcriptional differences (≥ 5-fold change) were identified to encode proteins of a diverse functional profile (Table 6). Two-thirds of the differentially transcribed genes (≥ 5-fold change) were proteins harbouring PEXEL-motifs, signifying their importance in host cell modification. It is not surprising though that a considerable number of differentially expressed genes are subtelomerically located, as these regions are considered to be the most versatile zones of the genome where antigenic variants arise.

Table 6. Differentially transcribed genes in between 3D7AH1S2 and 3D7S8.4 (more than 5-fold change)

parasite stage	3D7AH1S2		3D7S8.4	
	Annotation	Function	Annotation	Function
Ring	**PHISTa**	sexual stage differentiation (Eksi et al, 2005)	SERA-5	erythrocyte invasion (Rosenthal et al, 2004)
	PfEMP1	cell-rescue, denfense/virulence	**RESA-2**	cell-rescue, denfense/virulence
	GBPH2	apical organellar	RNA methylase	DNA/RNA processing
	GBP-related antigen	apical organellar	**PfEMP1**	cell-rescue, denfense/virulence
			Hypothetical protein	
Trophozoite	**PHISTa**	sexual stage differentiation	**PfEMP1**	cell-rescue, denfense/virulence
	PfEMP1	cell-rescue, denfense/virulence	***rif* pseudogene**	cell-rescue, denfense/virulence
	RIFIN-like	cell-rescue, denfense/virulence	Hypothetical protein	
Schizont				

Genes encoded for PEXEL/VTS exported proteins are bolded.

During early- and mid-developmental stages of the parasite's asexual life cycle, *var* genes were the most differentially transcribed genes. Three 3D7AH1S2 sibling clones (3D7AH1S1, -S3 and -S4) with identical binding properties expressed the same *var* gene, implicating a possible intrinsic relationship for PfEMP1 to the adhesive phenotypes. It has been controversial whether a single full-length *var* gene is being exclusively transcribed or multiple *var* genes are present simultaneously (Duffy *et al.*, 2002; Noviyanti *et al.*, 2001). Using the present *P. falciparum* genome microarrays, custom-made with multiple and specific probes covering the entire length of every *var* gene in the genome, we found

that several full-length *var* genes are being transcribed simultaneously but also that short spurious transcripts are present in both parasites. The untranslated full-length *var* genes in fact share the same group of upstream promoter sequences as the coding one, implying that 'loose' transcription (Chen *et al.*, 1998b; Kyes *et al.*, 2003; Scherf *et al.*, 1998) might be attributable to the cross *var* gene transcriptional activation within the same group. Still one *var* transcript is dominant (i.e. PFF0845c in 3D7AH1S2 and PFD0630c in 3D7S8.4) in both at ring- and trophozoite stages that is later translated into the corresponding PfEMP1 (Duffy *et al.*, 2002; Noviyanti *et al.*, 2001).

Members of the *rif* gene family – another multigene family and also a newly described *rif*-like gene (PFC0045w) were found to be differentially transcribed in between the two parasites. Furthermore, we demonstrated that the *rif* genes transcribed were not physically associated to the transcribed *var* genes indicating that *rif* expression may be independent of expressed *var*.

4.4 Inherited *var* switching upon long-term culturing

Antigenic variation of the determinants exposed on the IE surface could be induced by environmental pressure, or may undergo spontaneous switching. Given that parasites of splenectomized human- and animal-hosts do not sequester and do not express PfEMP1 on the infected erythrocyte surface (Barnwell *et al.*, 1983; David *et al.*, 1983; Hommel *et al.*, 1983), the maintenance of antigen expression could also be coordinated by the spleen. However, how *P. falciparum* manages to coordinate the expression of its variable antigens to subsist in the host is intriguing and in part unexplained.

We propagated the two clonal parasites (3D7S8.4 and 3D7AH1S2) under standard *in vitro* condition for ≈ 200 generations without panning or enrichment; during which their changes in transcription and adhesive phenotypes were followed. We found the initially transcribed *var* genes in both clones to be gradually down-regulated, which was intimately linked to an increase of a single *var* transcript, *var2csa* (Figure 6). To further examine the expression of *var* genes in the ≈ 200 generation *P. falciparum*, we randomly sub-cloned 3D7S8.4 by micromanipulation and thereby generated a set of seventeen new parasites (3D7S8.4.1 to 3D7S8.4.17, see Figure 5). A transcription pattern of sixteen

out of seventeen clones showed an identical transcription pattern with a high level of *var2csa* transcription.

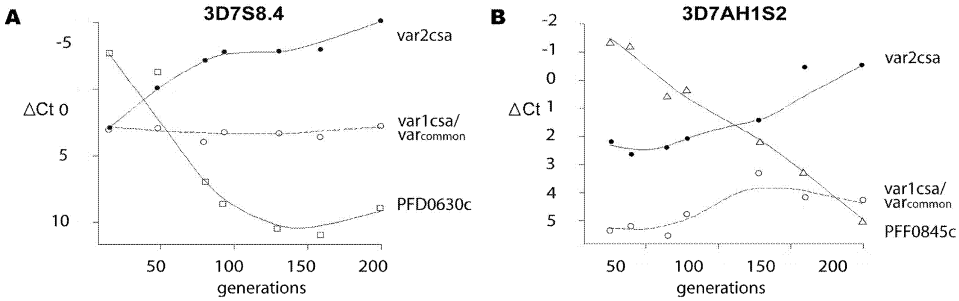

Figure 6. Changes in *var* gene expression in clonal *P. falciparum* (3D7S8.4 and 3D7AH1S2) over 200 generations of *in-vitro* growth

Albeit the high level of spliced and full-length *var2csa* transcripts detected in the long-term cultured parasites (≈ 200 generations), no specific binding to different host receptors and surface reactivity to the pool patient sera / VAR2CSA antisera was seen. To evaluate the data, we subsequently performed immunoblot analysis with parasite crude lysate using an anti-PfEMP1 monoclonal antibody preparation (anti-ATS). We observed a high molecular weight band (~320-340 kDa) in the sub-clones 3D7S8.4.2, 3D7S8.4.17 as well as the long-term cultured 3D7AH1S2 parasites similar to that in CSA-selected parasites (FCR3CSA and GB337CSA). However, despite the high level of *var2csa* transcripts in these clones, the abundance of the translational products was sparse compared to the dominant PfEMP1s present in the other parasites, suggesting that the protein products of the *var2csa* gene in the long-term cultured parasites are significantly controlled through post-transcriptional mechanisms. As a matter of fact, pevious data suggest that post-transcriptional regulation may be of beneficial impact to the parasite (Coulson *et al.*, 2004).

Repositioning of *var* loci within the peri-nuclear area has been found to be associated with the transcription of subtelomerically located *var*-genes (Duraisingh *et al.*, 2005; Freitas-Junior *et al.*, 2005; Voss *et al.*, 2006). Similarly, the *var2csa* gene was here

discovered to reposition from the telomeric clusters and the condensed heterochromatin to occupy a site suggested favoring transcription. In contrast, the two *var* genes which were transcribed in the parasites at the onset of the experiment (PFD0630c and PFF0845c) did not reposition as their transcriptional status changed. The location of these *var* genes immediately after cloning (active) and in parasites obtained after 200 generations (silenced) was shown to be the same with approximately fifty percent of the genes outside and fifty percent within the peri-nuclear heterochromatin. Taken together, the data suggests that the translocation of the *var2csa* gene into a suggested transcription site is of importance for activation, a similar mechanism as proposed in the regulation of VSG in African trypanosomes (Navarro and Gull, 2001). On the other hand, activation of other types of *var* genes, such as the UpsC types PFD0630c and PFF0845c studied here may not require the same machinery.

The *var2csa* gene is suggested to encode an important PfEMP1 ligand associated with the sequestration of IE in pregnancy-associated malaria (Avril *et al.*, 2006; Gamain *et al.*, 2005; Rasti *et al.*, 2006; Salanti *et al.*, 2003; Salanti *et al.*, 2004; Viebig *et al.*, 2005). The *var2csa* gene is however atypical to other *var* genes. Apart from having a different domain-architecture and an uncommon upstream flanking region, it is remarkably conserved across different *plasmodium* species. It is therefore likely that *var2csa* carries multiple important functions including a role in *var*-gene regulation and in the translation into a PfEMP1-species presented at the surface of the IE mediating binding to the syncytiotrophoblasts of the placenta. The transcription of *var2csa* in isolates of children and adults during malaria infections, and the obvious lack of antibody responses to the encoded protein (PfEMP1 VAR2CSA) in these groups of patients suggest the presence of *var* off-switching to occur in nature. Yet, is it compatible with parasite growth that an IE does not express a surface PfEMP1 during an infection? Would the phenomenon as described herein be the explanation to why the parasites of splenectomized human- and animal-hosts do not sequester and do not express PfEMP1 on the infected erythrocyte surface? (Barnwell *et al.*, 1983; David *et al.*, 1983; Hommel *et al.*, 1983)

5 CONCLUSIONS

Using a *P. falciparum* genome array as the groundwork for comparative genomic hybridization and for monitoring transcriptional profiling of the parasites, we describe how:

(1) *P. falciparum* employs gene amplifications and deletions as general strategies to enhance its chances of survival and spread. The copy number variation of the genes may impact the phenotypic variation of the parasite.

(2) The ubiquity and uniqueness of the subtelomeric segmental duplication might have roles in speciation and antigenic diversification of *P. falciparum.*

(3) *P. falciparum* has developed a sophisticated system to modify antigenic and adhesive phenotypes as shown by the complexity of transcriptional differences between the closely related isogenic *P. falciparum* clones.

(4) Off-switching of *var* genes occurs as an inherited trait. This may allow for the coordination of the *var* repertoire and protect against its rapid exhaustion.

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7 REFERENCES

Abdel-Latif, M.S., Khattab, A., Lindenthal, C., Kremsner, P.G., and Klinkert, M.Q. (2002) Recognition of variant Rifin antigens by human antibodies induced during natural Plasmodium falciparum infections. *Infect Immun* **70**: 7013-7021.

Acharya, P., Kumar, R., and Tatu, U. (2007) Chaperoning a cellular upheaval in malaria: heat shock proteins in Plasmodium falciparum. *Mol Biochem Parasitol* **153**: 85-94.

Adams, J.H., Sim, B.K., Dolan, S.A., Fang, X., Kaslow, D.C., and Miller, L.H. (1992) A family of erythrocyte binding proteins of malaria parasites. *Proc Natl Acad Sci U S A* **89**: 7085-7089.

Aikawa, M., Kamanura, K., Shiraishi, S., Matsumoto, Y., Arwati, H., Torii, M., Ito, Y., Takeuchi, T., and Tandler, B. (1996) Membrane knobs of unfixed Plasmodium falciparum infected erythrocytes: new findings as revealed by atomic force microscopy and surface potential spectroscopy. *Exp Parasitol* **84**: 339-343.

Aley, S.B., Sherwood, J.A., and Howard, R.J. (1984) Knob-positive and knob-negative Plasmodium falciparum differ in expression of a strain-specific malarial antigen on the surface of infected erythrocytes. *J Exp Med* **160**: 1585-1590.

Andrulis, E.D., Neiman, A.M., Zappulla, D.C., and Sternglanz, R. (1998) Perinuclear localization of chromatin facilitates transcriptional silencing. *Nature* **394**: 592-595.

Arnot, D.E., Roper, C., and Bayoumi, R.A. (1993) Digital codes from hypervariable tandemly repeated DNA sequences in the Plasmodium falciparum circumsporozoite gene can genetically barcode isolates. *Mol Biochem Parasitol* **61**: 15-24.

Avril, M., Gamain, B., Lepolard, C., Viaud, N., Scherf, A., and Gysin, J. (2006) Characterization of anti-var2CSA-PfEMP1 cytoadhesion inhibitory mouse monoclonal antibodies. *Microbes Infect* **8**: 2863-2871.

Bahl, A., Brunk, B., Coppel, R.L., Crabtree, J., Diskin, S.J., Fraunholz, M.J., Grant, G.R., Gupta, D., Huestis, R.L., Kissinger, J.C., Labo, P., Li, L., McWeeney, S.K., Milgram, A.J., Roos, D.S., Schug, J., and Stoeckert, C.J., Jr. (2002) PlasmoDB: the Plasmodium genome resource. An integrated database providing tools for accessing, analyzing and mapping expression and sequence data (both finished and unfinished). *Nucleic Acids Res* **30**: 87-90.

Bailey, J.A., Yavor, A.M., Massa, H.F., Trask, B.J., and Eichler, E.E. (2001) Segmental duplications: organization and impact within the current human genome project assembly. *Genome Res* **11**: 1005-1017.

Bailey, J.A., Gu, Z., Clark, R.A., Reinert, K., Samonte, R.V., Schwartz, S., Adams, M.D., Myers, E.W., Li, P.W., and Eichler, E.E. (2002) Recent segmental duplications in the human genome. *Science* **297**: 1003-1007.

Baldi, D.L., Good, R., Duraisingh, M.T., Crabb, B.S., and Cowman, A.F. (2002) Identification and disruption of the gene encoding the third member of the low-molecular-mass rhoptry complex in Plasmodium falciparum. *Infect Immun* **70**: 5236-5245.

Barnwell, J.W., Howard, R.J., and Miller, L.H. (1983) Influence of the spleen on the expression of surface antigens on parasitized erythrocytes. *Ciba Found Symp* **94**: 117-136.

Barnwell, J.W., Asch, A.S., Nachman, R.L., Yamaya, M., Aikawa, M., and Ingravallo, P. (1989) A human 88-kD membrane glycoprotein (CD36) functions in vitro as a receptor for a cytoadherence ligand on Plasmodium falciparum-infected erythrocytes. *J Clin Invest* **84**: 765-772.

Barragan, A., Kremsner, P.G., Weiss, W., Wahlgren, M., and Carlson, J. (1998) Age-related buildup of humoral immunity against epitopes for rosette formation and agglutination in African areas of malaria endemicity. *Infect Immun* **66**: 4783-4787.

Barragan, A., Kremsner, P.G., Wahlgren, M., and Carlson, J. (2000) Blood group A antigen is a coreceptor in Plasmodium falciparum rosetting. *Infect Immun* **68**: 2971-2975.

Barry, A.E., Leliwa-Sytek, A., Tavul, L., Imrie, H., Migot-Nabias, F., Brown, S.M., McVean, G.A., and Day, K.P. (2007) Population Genomics of the Immune Evasion (var) Genes of Plasmodium falciparum. *PLoS Pathog* **3**: e34.

Baruch, D.I., Pasloske, B.L., Singh, H.B., Bi, X., Ma, X.C., Feldman, M., Taraschi, T.F., and Howard, R.J. (1995) Cloning the P. falciparum gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes. *Cell* **82**: 77-87.

Baruch, D.I., Gormely, J.A., Ma, C., Howard, R.J., and Pasloske, B.L. (1996) Plasmodium falciparum erythrocyte membrane protein 1 is a parasitized erythrocyte receptor for adherence to CD36, thrombospondin, and intercellular adhesion molecule 1. *Proc Natl Acad Sci U S A* **93**: 3497-3502.

Baruch, D.I., Ma, X.C., Singh, H.B., Bi, X., Pasloske, B.L., and Howard, R.J. (1997) Identification of a region of PfEMP1 that mediates adherence of Plasmodium falciparum infected erythrocytes to CD36: conserved function with variant sequence. *Blood* **90**: 3766-3775.

Baruch, D.I., Gamain, B., Barnwell, J.W., Sullivan, J.S., Stowers, A., Galland, G.G., Miller, L.H., and Collins, W.E. (2002) Immunization of Aotus monkeys with a functional domain of the Plasmodium falciparum variant antigen induces protection against a lethal parasite line. *Proc Natl Acad Sci U S A* **99**: 3860-3865.

Baumgarten, A., Cannon, S., Spangler, R., and May, G. (2003) Genome-level evolution of resistance genes in Arabidopsis thaliana. *Genetics* **165**: 309-319.

Beeson, J.G., Rogerson, S.J., Cooke, B.M., Reeder, J.C., Chai, W., Lawson, A.M., Molyneux, M.E., and Brown, G.V. (2000) Adhesion of Plasmodium falciparum-infected erythrocytes to hyaluronic acid in placental malaria. *Nat Med* **6**: 86-90.

Bengtsson, H. (2004) aroma - An R Object-oriented Microarray Analysis environment.

Bengtsson, H., Jonsson, G., and Vallon-Christersson, J. (2004) Calibration and assessment of channel-specific biases in microarray data with extended dynamical range. *BMC Bioinformatics* **5**: 177.

Berendt, A.R., Simmons, D.L., Tansey, J., Newbold, C.I., and Marsh, K. (1989) Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for Plasmodium falciparum. *Nature* **341**: 57-59.

Biggs, B.A., Culvenor, J.G., Ng, J.S., Kemp, D.J., and Brown, G.V. (1989) Plasmodium falciparum: cytoadherence of a knobless clone. *Exp Parasitol* **69**: 189-197.

Biggs, B.A., Gooze, L., Wycherley, K., Wollish, W., Southwell, B., Leech, J.H., and Brown, G.V. (1991) Antigenic variation in Plasmodium falciparum. *Proc Natl Acad Sci U S A* **88**: 9171-9174.

Birago, C., Albanesi, V., Silvestrini, F., Picci, L., Pizzi, E., Alano, P., Pace, T., and Ponzi, M. (2003) A gene-family encoding small exported proteins is conserved across Plasmodium genus. *Mol Biochem Parasitol* **126**: 209-218.

Blythe, J.E., Surentheran, T., and Preiser, P.R. (2004) STEVOR--a multifunctional protein? *Mol Biochem Parasitol* **134**: 11-15.

Broad Institute of Harvard and MIT (http://www.broad.mit.edu).

Borre, M.B., Dziegiel, M., Hogh, B., Petersen, E., Rieneck, K., Riley, E., Meis, J.F., Aikawa, M., Nakamura, K., Harada, M., and et al. (1991) Primary structure and localization of a conserved immunogenic Plasmodium falciparum glutamate rich protein (GLURP) expressed in both the preerythrocytic and erythrocytic stages of the vertebrate life cycle. *Mol Biochem Parasitol* **49**: 119-131.

Borst, P. (2002) Antigenic variation and allelic exclusion. *Cell* **109**: 5-8.

Botha, M., Pesce, E.R., and Blatch, G.L. (2007) The Hsp40 proteins of Plasmodium falciparum and other apicomplexa: Regulating chaperone power in the parasite and the host. *Int J Biochem Cell Biol*.

Bowman, S., Lawson, D., Basham, D., Brown, D., Chillingworth, T., Churcher, C.M., Craig, A., Davies, R.M., Devlin, K., Feltwell, T., Gentles, S., Gwilliam, R., Hamlin, N., Harris, D., Holroyd, S., Hornsby, T., Horrocks, P., Jagels, K., Jassal, B., Kyes, S., McLean, J., Moule, S., Mungall, K., Murphy, L., Oliver, K., Quail, M.A., Rajandream, M.A., Rutter, S., Skelton, J., Squares, R., Squares, S., Sulston, J.E., Whitehead, S., Woodward, J.R., Newbold, C., and Barrell, B.G. (1999) The complete nucleotide sequence of chromosome 3 of Plasmodium falciparum. *Nature* **400**: 532-538.

Bozdech, Z., Llinas, M., Pulliam, B.L., Wong, E.D., Zhu, J., and DeRisi, J.L. (2003a) The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*. *PLoS Biol* **1**: E5.

Bozdech, Z., Zhu, J., Joachimiak, M.P., Cohen, F.E., Pulliam, B., and DeRisi, J.L. (2003b) Expression profiling of the schizont and trophozoite stages of Plasmodium falciparum with a long-oligonucleotide microarray. *Genome Biol* **4**: R9.

Braunstein, M., Sobel, R.E., Allis, C.D., Turner, B.M., and Broach, J.R. (1996) Efficient transcriptional silencing in Saccharomyces cerevisiae requires a heterochromatin histone acetylation pattern. *Mol Cell Biol* **16**: 4349-4356.

Brown, K.N., and Brown, I.N. (1965) Immunity to malaria: antigenic variation in chronic infections of Plasmodium knowlesi. *Nature* **208**: 1286-1288.

Buffet, P.A., Gamain, B., Scheidig, C., Baruch, D., Smith, J.D., Hernandez-Rivas, R., Pouvelle, B., Oishi, S., Fujii, N., Fusai, T., Parzy, D., Miller, L.H., Gysin, J., and Scherf, A. (1999) Plasmodium falciparum domain mediating adhesion to chondroitin sulfate A: a receptor for human placental infection. *Proc Natl Acad Sci U S A* **96**: 12743-12748.

Calderwood, M.S., Gannoun-Zaki, L., Wellems, T.E., and Deitsch, K.W. (2003) Plasmodium falciparum var genes are regulated by two regions with separate promoters, one upstream of the coding region and a second within the intron. *J Biol Chem* **278**: 34125-34132.

Carlson, J., Helmby, H., Hill, A.V., Brewster, D., Greenwood, B.M., and Wahlgren, M. (1990) Human cerebral malaria: association with erythrocyte rosetting and lack of anti-rosetting antibodies. *Lancet* **336**: 1457-1460.

Carlson, J., and Wahlgren, M. (1992) Plasmodium falciparum erythrocyte rosetting is mediated by promiscuous lectin-like interactions. *J Exp Med* **176**: 1311-1317.

Carlton, J., Silva, J., and Hall, N. (2005) The genome of model malaria parasites, and comparative genomics. *Curr Issues Mol Biol* **7**: 23-37.

Carlton, J.M., Vinkenoog, R., Waters, A.P., and Walliker, D. (1998) Gene synteny in species of Plasmodium. *Mol Biochem Parasitol* **93**: 285-294.

Carlton, J.M., Angiuoli, S.V., Suh, B.B., Kooij, T.W., Pertea, M., Silva, J.C., Ermolaeva, M.D., Allen, J.E., Selengut, J.D., Koo, H.L., Peterson, J.D., Pop, M., Kosack, D.S., Shumway, M.F., Bidwell, S.L., Shallom, S.J., van Aken, S.E., Riedmuller, S.B., Feldblyum, T.V., Cho, J.K., Quackenbush, J., Sedegah, M., Shoaibi, A., Cummings, L.M., Florens, L., Yates, J.R., Raine, J.D., Sinden, R.E., Harris, M.A., Cunningham, D.A., Preiser, P.R., Bergman, L.W., Vaidya, A.B., van Lin, L.H., Janse, C.J., Waters, A.P., Smith, H.O., White, O.R., Salzberg, S.L., Venter, J.C., Fraser, C.M., Hoffman, S.L., Gardner, M.J., and Carucci, D.J. (2002) Genome sequence and comparative analysis of the model rodent malaria parasite Plasmodium yoelii yoelii. *Nature* **419**: 512-519.

Carret, C.K., Horrocks, P., Konfortov, B., Winzeler, E., Qureshi, M., Newbold, C., and Ivens, A. (2005) Microarray-based comparative genomic analyses of the human malaria parasite *Plasmodium falciparum* using Affymetrix arrays. *Mol Biochem Parasitol* **144**: 177-186.

Chen, G.L., Yang, L., Rowe, T.C., Halligan, B.D., Tewey, K.M., and Liu, L.F. (1984) Nonintercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *J Biol Chem* **259**: 13560-13566.

Chen, Q., Barragan, A., Fernandez, V., Sundstrom, A., Schlichtherle, M., Sahlen, A., Carlson, J., Datta, S., and Wahlgren, M. (1998a) Identification of Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) as the rosetting ligand of the malaria parasite P. falciparum. *J Exp Med* **187**: 15-23.

Chen, Q., Fernandez, V., Sundstrom, A., Schlichtherle, M., Datta, S., Hagblom, P., and Wahlgren, M. (1998b) Developmental selection of var gene expression in Plasmodium falciparum. *Nature* **394**: 392-395.

Chen, Q., Heddini, A., Barragan, A., Fernandez, V., Pearce, S.F., and Wahlgren, M. (2000a) The semiconserved head structure of Plasmodium falciparum erythrocyte membrane protein 1 mediates binding to multiple independent host receptors. *J Exp Med* **192**: 1-10.

Chen, Q., Schlichtherle, M., and Wahlgren, M. (2000b) Molecular aspects of severe malaria. *Clin Microbiol Rev* **13**: 439-450.

Cheng, Q., Cloonan, N., Fischer, K., Thompson, J., Waine, G., Lanzer, M., and Saul, A. (1998) stevor and rif are Plasmodium falciparum multicopy gene families which potentially encode variant antigens. *Mol Biochem Parasitol* **97**: 161-176.

Cheung, V.G., and Nelson, S.F. (1996) Whole genome amplification using a degenerate oligonucleotide primer allows hundreds of genotypes to be performed on less than one nanogram of genomic DNA. *Proc Natl Acad Sci U S A* **93**: 14676-14679.

Chookajorn, T., Dzikowski, R., Frank, M., Li, F., Jiwani, A.Z., Hartl, D.L., and Deitsch, K.W. (2007) Epigenetic memory at malaria virulence genes. *Proc Natl Acad Sci U S A* **104**: 899-902.

Clark, I.A., Rockett, K.A., and Cowden, W.B. (1991) Proposed link between cytokines, nitric oxide and human cerebral malaria. *Parasitol Today* **7**: 205-207.

Clark, I.A., al Yaman, F.M., and Jacobson, L.S. (1997) The biological basis of malarial disease. *Int J Parasitol* **27**: 1237-1249.

Coombs, G.H., Goldberg, D.E., Klemba, M., Berry, C., Kay, J., and Mottram, J.C. (2001) Aspartic proteases of Plasmodium falciparum and other parasitic protozoa as drug targets. *Trends Parasitol* **17**: 532-537.

Coppel, R.L., Culvenor, J.G., Bianco, A.E., Crewther, P.E., Stahl, H.D., Brown, G.V., Anders, R.F., and Kemp, D.J. (1986) Variable antigen associated with the surface of erythrocytes infected with mature stages of Plasmodium falciparum. *Mol Biochem Parasitol* **20**: 265-277.

Coppel, R.L., Bianco, A.E., Culvenor, J.G., Crewther, P.E., Brown, G.V., Anders, R.F., and Kemp, D.J. (1987) A cDNA clone expressing a rhoptry protein of Plasmodium falciparum. *Mol Biochem Parasitol* **25**: 73-81.

Coppel, R.L., Roos, D.S., and Bozdech, Z. (2004) The genomics of malaria infection. *Trends Parasitol* **20**: 553-557.

Coulson, R.M., Hall, N., and Ouzounis, C.A. (2004) Comparative genomics of transcriptional control in the human malaria parasite Plasmodium falciparum. *Genome Res* **14**: 1548-1554.

Cowman, A.F., Galatis, D., and Thompson, J.K. (1994) Selection for mefloquine resistance in Plasmodium falciparum is linked to amplification of the pfmdr1 gene and cross-resistance to halofantrine and quinine. *Proc Natl Acad Sci U S A* **91**: 1143-1147.

Creasey, A.M., Staalsoe, T., Raza, A., Arnot, D.E., and Rowe, J.A. (2003) Nonspecific immunoglobulin M binding and chondroitin sulfate A binding are linked phenotypes of Plasmodium falciparum isolates implicated in malaria during pregnancy. *Infect Immun* **71**: 4767-4771.

Daily, J.P., Le Roch, K.G., Sarr, O., Ndiaye, D., Lukens, A., Zhou, Y., Ndir, O., Mboup, S., Sultan, A., Winzeler, E.A., and Wirth, D.F. (2005) In vivo transcriptome of Plasmodium falciparum reveals overexpression of transcripts that encode surface proteins. *J Infect Dis* **191**: 1196-1203.

David, P.H., Hommel, M., Miller, L.H., Udeinya, I.J., and Oligino, L.D. (1983) Parasite sequestration in Plasmodium falciparum malaria: spleen and antibody modulation of cytoadherence of infected erythrocytes. *Proc Natl Acad Sci U S A* **80**: 5075-5079.

David, P.H., Handunnetti, S.M., Leech, J.H., Gamage, P., and Mendis, K.N. (1988) Rosetting: a new cytoadherence property of malaria-infected erythrocytes. *Am J Trop Med Hyg* **38**: 289-297.

Day, K.P., Karamalis, F., Thompson, J., Barnes, D.A., Peterson, C., Brown, H., Brown, G.V., and Kemp, D.J. (1993) Genes necessary for expression of a virulence determinant and for transmission of Plasmodium falciparum are located on a 0.3-megabase region of chromosome 9. *Proc Natl Acad Sci U S A* **90**: 8292-8296.

Dean, F.B., Hosono, S., Fang, L., Wu, X., Faruqi, A.F., Bray-Ward, P., Sun, Z., Zong, Q., Du, Y., Du, J., Driscoll, M., Song, W., Kingsmore, S.F., Egholm, M., and Lasken, R.S. (2002) Comprehensive human genome amplification using multiple displacement amplification. *Proc Natl Acad Sci U S A* **99**: 5261-5266.

Deitsch, K.W., and Wellems, T.E. (1996) Membrane modifications in erythrocytes parasitized by Plasmodium falciparum. *Mol Biochem Parasitol* **76**: 1-10.

Deitsch, K.W., del Pinal, A., and Wellems, T.E. (1999) Intra-cluster recombination and var transcription switches in the antigenic variation of Plasmodium falciparum. *Mol Biochem Parasitol* **101**: 107-116.

Deitsch, K.W., Calderwood, M.S., and Wellems, T.E. (2001) Malaria. Cooperative silencing elements in var genes. *Nature* **412**: 875-876.

del Portillo, H.A., Fernandez-Becerra, C., Bowman, S., Oliver, K., Preuss, M., Sanchez, C.P., Schneider, N.K., Villalobos, J.M., Rajandream, M.A., Harris, D., Pereira da Silva, L.H., Barrell, B., and Lanzer, M. (2001) A superfamily of variant genes encoded in the subtelomeric region of Plasmodium vivax. *Nature* **410**: 839-842.

Delemarre, B.J., and van der Kaay, H.J. (1979) [Tropical malaria contracted the natural way in the Netherlands]. *Ned Tijdschr Geneeskd* **123**: 1981-1982.

Desjardins, R.E., Canfield, C.J., Haynes, J.D., and Chulay, J.D. (1979) Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. *Antimicrob Agents Chemother* **16**: 710-718.

Donati, D., Zhang, L.P., Chene, A., Chen, Q., Flick, K., Nystrom, M., Wahlgren, M., and Bejarano, M.T. (2004) Identification of a polyclonal B-cell activator in Plasmodium falciparum. *Infect Immun* **72**: 5412-5418.

Dudoit, S., Yang, Y.H., Speed, T.P., and Callow, M.J. (2002) Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments. *Statistica Sinica* **12**: 111-139.

Duffy, M.F., Brown, G.V., Basuki, W., Krejany, E.O., Noviyanti, R., Cowman, A.F., and Reeder, J.C. (2002) Transcription of multiple var genes by individual, trophozoite-stage Plasmodium falciparum cells expressing a chondroitin sulphate A binding phenotype. *Mol Microbiol* **43**: 1285-1293.

Duffy, M.F., Reeder, J.C., and Brown, G.V. (2003) Regulation of antigenic variation in Plasmodium falciparum: censoring freedom of expression? *Trends Parasitol* **19**: 121-124.

Duffy, M.F., Byrne, T.J., Elliott, S.R., Wilson, D.W., Rogerson, S.J., Beeson, J.G., Noviyanti, R., Brown, G.V. (2005) Broad analysis reveals a consistent pattern of var gene transcription in *Plasmodium falciparum* repeatedly selected for a defined adhesion phenotype. *Mol Microbiol.* **56**: 774-788.

Duffy, M.F., Caragounis, A., Noviyanti, R., Kyriacou, H.M., Choong, E.K., Boysen, K., Healer, J., Rowe, J.A., Molyneux, M.E., Brown, G.V., and Rogerson, S.J. (2006) Transcribed var genes associated with placental malaria in Malawian women. *Infect Immun* **74**: 4875-4883.

Duraisingh, M.T., Voss, T.S., Marty, A.J., Duffy, M.F., Good, R.T., Thompson, J.K., Freitas-Junior, L.H., Scherf, A., Crabb, B.S., and Cowman, A.F. (2005) Heterochromatin silencing and locus repositioning linked to regulation of virulence genes in Plasmodium falciparum. *Cell* **121**: 13-24.

Dzikowski, R., Frank, M., and Deitsch, K. (2006) Mutually exclusive expression of virulence genes by malaria parasites is regulated independently of antigen production. *PLoS Pathog* **2**: e22.

Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. (1998) Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* **95**: 14863-14868.

Eksi, S., Haile, Y., Furuya, T., Ma, L., Su, X., and Williamson, K.C. (2005) Identification of a subtelomeric gene family expressed during the asexual-sexual stage transition in Plasmodium falciparum. *Mol Biochem Parasitol* **143**: 90-99.

Farnert, A., Arez, A.P., Babiker, H.A., Beck, H.P., Benito, A., Bjorkman, A., Bruce, M.C., Conway, D.J., Day, K.P., Henning, L., Mercereau-Puijalon, O., Ranford-Cartwright, L.C., Rubio, J.M., Snounou, G., Walliker, D., Zwetyenga, J., and do Rosario, V.E. (2001) Genotyping of Plasmodium falciparum infections by PCR: a comparative multicentre study. *Trans R Soc Trop Med Hyg* **95**: 225-232.

Felsenfeld, G., Boyes, J., Chung, J., Clark, D., and Studitsky, V. (1996) Chromatin structure and gene expression. *Proc Natl Acad Sci U S A* **93**: 9384-9388.

Fenton, B., Clark, J.T., Khan, C.M., Robinson, J.V., Walliker, D., Ridley, R., Scaife, J.G., and McBride, J.S. (1991) Structural and antigenic polymorphism of the 35- to 48-kilodalton merozoite surface antigen (MSA-2) of the malaria parasite Plasmodium falciparum. *Mol Cell Biol* **11**: 963-971.

Fernandez, V., Treutiger, C.J., Nash, G.B., and Wahlgren, M. (1998) Multiple adhesive phenotypes linked to rosetting binding of erythrocytes in Plasmodium falciparum malaria. *Infect Immun* **66**: 2969-2975.

Fernandez, V., Hommel, M., Chen, Q., Hagblom, P., and Wahlgren, M. (1999) Small, clonally variant antigens expressed on the surface of the Plasmodium falciparum-infected erythrocyte are encoded by the rif gene family and are the target of human immune responses. *J Exp Med* **190**: 1393-1404.

Fernandez, V., Chen, Q., Sundstrom, A., Scherf, A., Hagblom, P., and Wahlgren, M. (2002) Mosaic-like transcription of var genes in single Plasmodium falciparum parasites. *Mol Biochem Parasitol* **121**: 195-203.

Feuk, L., Carson, A.R., and Scherer, S.W. (2006) Structural variation in the human genome. *Nat Rev Genet* **7**: 85-97.

Figueiredo, L., and Scherf, A. (2005) Plasmodium telomeres and telomerase: the usual actors in an unusual scenario. *Chromosome Res* **13**: 517-524.

Figueiredo, L.M., Pirrit, L.A., and Scherf, A. (2000) Genomic organisation and chromatin structure of Plasmodium falciparum chromosome ends. *Mol Biochem Parasitol* **106**: 169-174.

Figueiredo, L.M., Freitas-Junior, L.H., Bottius, E., Olivo-Marin, J.C., and Scherf, A. (2002) A central role for Plasmodium falciparum subtelomeric regions in spatial positioning and telomere length regulation. *Embo J* **21**: 815-824.

Flick, K., Scholander, C., Chen, Q., Fernandez, V., Pouvelle, B., Gysin, J., and Wahlgren, M. (2001) Role of nonimmune IgG bound to PfEMP1 in placental malaria. *Science* **293**: 2098-2100.

Florens, L., Washburn, M.P., Raine, J.D., Anthony, R.M., Grainger, M., Haynes, J.D., Moch, J.K., Muster, N., Sacci, J.B., Tabb, D.L., Witney, A.A., Wolters, D., Wu, Y., Gardner, M.J., Holder, A.A., Sinden, R.E., Yates, J.R., and Carucci, D.J. (2002) A proteomic view of the Plasmodium falciparum life cycle. *Nature* **419**: 520-526.

Florens, L., Liu, X., Wang, Y., Yang, S., Schwartz, O., Peglar, M., Carucci, D.J., Yates, J.R., 3rd, and Wub, Y. (2004) Proteomics approach reveals novel proteins on the surface of malaria-infected erythrocytes. *Mol Biochem Parasitol* **135**: 1-11.

Foote, S.J., Thompson, J.K., Cowman, A.F., and Kemp, D.J. (1989) Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of P. falciparum. *Cell* **57**: 921-930.

Foote, S.J., Kyle, D.E., Martin, R.K., Oduola, A.M., Forsyth, K., Kemp, D.J., and Cowman, A.F. (1990) Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in Plasmodium falciparum. *Nature* **345**: 255-258.

Forsyth, K.P., Philip, G., Smith, T., Kum, E., Southwell, B., and Brown, G.V. (1989) Diversity of antigens expressed on the surface of erythrocytes infected with mature Plasmodium falciparum parasites in Papua New Guinea. *Am J Trop Med Hyg* **41**: 259-265.

Frank, M., Dzikowski, R., Costantini, D., Amulic, B., Berdougo, E., and Deitsch, K. (2006) Strict pairing of var promoters and introns is required for var gene silencing in the malaria parasite Plasmodium falciparum. *J Biol Chem* **281**: 9942-9952.

Frank, M., Dzikowski, R., Amulic, B., and Deitsch, K. (2007) Variable switching rates of malaria virulence genes are associated with chromosomal position. *Mol Microbiol* **64**: 1486-1498.

Freeman, J.L., Perry, G.H., Feuk, L., Redon, R., McCarroll, S.A., Altshuler, D.M., Aburatani, H., Jones, K.W., Tyler-Smith, C., Hurles, M.E., Carter, N.P., Scherer, S.W., and Lee, C. (2006) Copy number variation: new insights in genome diversity. *Genome Res* **16**: 949-961.

Freitas-Junior, L.H., Bottius, E., Pirrit, L.A., Deitsch, K.W., Scheidig, C., Guinet, F., Nehrbass, U., Wellems, T.E., and Scherf, A. (2000) Frequent ectopic recombination of virulence factor genes in telomeric chromosome clusters of P. falciparum. *Nature* **407**: 1018-1022.

Freitas-Junior, L.H., Hernandez-Rivas, R., Ralph, S.A., Montiel-Condado, D., Ruvalcaba-Salazar, O.K., Rojas-Meza, A.P., Mancio-Silva, L., Leal-Silvestre, R.J., Gontijo, A.M., Shorte, S., and Scherf, A. (2005) Telomeric heterochromatin propagation and histone acetylation control mutually exclusive expression of antigenic variation genes in malaria parasites. *Cell* **121**: 25-36.

Fried, M., and Duffy, P.E. (1996) Adherence of Plasmodium falciparum to chondroitin sulfate A in the human placenta. *Science* **272**: 1502-1504.

Gamain, B., Trimnell, A.R., Scheidig, C., Scherf, A., Miller, L.H., and Smith, J.D. (2005) Identification of multiple chondroitin sulfate A (CSA)-binding domains in the var2CSA gene transcribed in CSA-binding parasites. *J Infect Dis* **191**: 1010-1013.

Gannoun-Zaki, L., Jost, A., Mu, J., Deitsch, K.W., and Wellems, T.E. (2005) A silenced Plasmodium falciparum var promoter can be activated in vivo through spontaneous deletion of a silencing element in the intron. *Eukaryot Cell* **4**: 490-492.

Gardiner, D.L., Holt, D.C., Thomas, E.A., Kemp, D.J., and Trenholme, K.R. (2000) Inhibition of Plasmodium falciparum clag9 gene function by antisense RNA. *Mol Biochem Parasitol* **110**: 33-41.

Gardner, M.J., Tettelin, H., Carucci, D.J., Cummings, L.M., Smith, H.O., Fraser, C.M., Venter, J.C., and Hoffman, S.L. (1999) The malaria genome sequencing project: complete sequence of Plasmodium falciparum chromosome 2. *Parassitologia* **41**: 69-75.

Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., Carlton, J.M., Pain, A., Nelson, K.E., Bowman, S., Paulsen, I.T., James, K., Eisen, J.A., Rutherford, K., Salzberg, S.L., Craig, A., Kyes, S., Chan, M.S., Nene, V., Shallom, S.J., Suh, B., Peterson, J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haft, D., Mather, M.W., Vaidya, A.B., Martin, D.M., Fairlamb, A.H., Fraunholz, M.J., Roos, D.S., Ralph, S.A., McFadden, G.I., Cummings, L.M., Subramanian, G.M., Mungall, C., Venter, J.C., Carucci, D.J., Hoffman, S.L., Newbold, C., Davis, R.W., Fraser, C.M., and Barrell, B. (2002) Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature* **419**: 498-511.

Gartenberg, M.R. (2000) The Sir proteins of Saccharomyces cerevisiae: mediators of transcriptional silencing and much more. *Curr Opin Microbiol* **3**: 132-137.

Gatton, M.L., Peters, J.M., Fowler, E.V., and Cheng, Q. (2003) Switching rates of Plasmodium falciparum var genes: faster than we thought? *Trends Parasitol* **19**: 202-208.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki,

G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y., and Zhang, J. (2004) Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* **5**: R80.

Grant, S.F., Steinlicht, S., Nentwich, U., Kern, R., Burwinkel, B., and Tolle, R. (2002) SNP genotyping on a genome-wide amplified DOP-PCR template. *Nucleic Acids Res* **30**: e125.

Gunasekera, A.M., Myrick, A., Militello, K.T., Sims, J.S., Dong, C.K., Gierahn, T., Le Roch, K., Winzeler, E., and Wirth, D.F. (2007) Regulatory motifs uncovered among gene expression clusters in Plasmodium falciparum. *Mol Biochem Parasitol* **153**: 19-30.

Gupta, S., Snow, R.W., Donnelly, C.A., Marsh, K., and Newbold, C. (1999) Immunity to non-cerebral severe malaria is acquired after one or two infections. *Nat Med* **5**: 340-343.

Haeggstrom, M., Kironde, F., Berzins, K., Chen, Q., Wahlgren, M., and Fernandez, V. (2004) Common trafficking pathway for variant antigens destined for the surface of the Plasmodium falciparum-infected erythrocyte. *Mol Biochem Parasitol* **133**: 1-14.

Hall, N., Karras, M., Raine, J.D., Carlton, J.M., Kooij, T.W., Berriman, M., Florens, L., Janssen, C.S., Pain, A., Christophides, G.K., James, K., Rutherford, K., Harris, B., Harris, D., Churcher, C., Quail, M.A., Ormond, D., Doggett, J., Trueman, H.E., Mendoza, J., Bidwell, S.L., Rajandream, M.A., Carucci, D.J., Yates, J.R., 3rd, Kafatos, F.C., Janse, C.J., Barrell, B., Turner, C.M., Waters, A.P., and Sinden, R.E. (2005) A comprehensive survey of the Plasmodium life cycle by genomic, transcriptomic, and proteomic analyses. *Science* **307**: 82-86.

Handunnetti, S.M., van Schravendijk, M.R., Hasler, T., Barnwell, J.W., Greenwalt, D.E., and Howard, R.J. (1992) Involvement of CD36 on erythrocytes as a rosetting receptor for Plasmodium falciparum-infected erythrocytes. *Blood* **80**: 2097-2104.

Hayward, R.E., Derisi, J.L., Alfadhli, S., Kaslow, D.C., Brown, P.O., and Rathod, P.K. (2000) Shotgun DNA microarrays and stage-specific gene expression in Plasmodium falciparum malaria. *Mol Microbiol* **35**: 6-14.

Heddini, A., Pettersson, F., Kai, O., Shafi, J., Obiero, J., Chen, Q., Barragan, A., Wahlgren, M., and Marsh, K. (2001) Fresh isolates from children with severe Plasmodium falciparum malaria bind to multiple receptors. *Infect Immun* **69**: 5849-5856.

Hiller, N.L., Bhattacharjee, S., van Ooij, C., Liolios, K., Harrison, T., Lopez-Estrano, C., and Haldar, K. (2004) A host-targeting signal in virulence proteins reveals a secretome in malarial infection. *Science* **306**: 1934-1937.

Holt, D.C., Gardiner, D.L., Thomas, E.A., Mayo, M., Bourke, P.F., Sutherland, C.J., Carter, R., Myers, G., Kemp, D.J., and Trenholme, K.R. (1999) The cytoadherence linked asexual gene family of Plasmodium falciparum: are there roles other than cytoadherence? *Int J Parasitol* **29**: 939-944.

Holt, D.C., Fischer, K., Tchavtchitch, M., Wilson, D.W., Hauquitz, N.E., Hawthorne, P.L., Gardiner, D.L., Trenholme, K.R., and Kemp, D.J. (2001) Clags in Plasmodium falciparum and other species of Plasmodium. *Mol Biochem Parasitol* **118**: 259-263.

Hommel, M., David, P.H., and Oligino, L.D. (1983) Surface alterations of erythrocytes in Plasmodium falciparum malaria. Antigenic variation, antigenic diversity, and the role of the spleen. *J Exp Med* **157**: 1137-1148.

Horrocks, P., Pinches, R., Christodoulou, Z., Kyes, S.A., and Newbold, C.I. (2004) Variable var transition rates underlie antigenic variation in malaria. *Proc Natl Acad Sci U S A* **101**: 11129-11134.

Horrocks, P., Pinches, R.A., Chakravorty, S.J., Papakrivos, J., Christodoulou, Z., Kyes, S.A., Urban, B.C., Ferguson, D.J., and Newbold, C.I. (2005) PfEMP1 expression is reduced on the surface of knobless Plasmodium falciparum infected erythrocytes. *J Cell Sci* **118**: 2507-2518.

Howard, R.J., Lyon, J.A., Uni, S., Saul, A.J., Aley, S.B., Klotz, F., Panton, L.J., Sherwood, J.A., Marsh, K., Aikawa, M., and et al. (1987) Transport of an Mr approximately 300,000 Plasmodium falciparum protein (Pf EMP 2) from the intraerythrocytic asexual parasite to the cytoplasmic face of the host cell membrane. *J Cell Biol* **104**: 1269-1280.

Howard, R.J., Barnwell, J.W., Rock, E.P., Neequaye, J., Ofori-Adjei, D., Maloy, W.L., Lyon, J.A., and Saul, A. (1988) Two approximately 300 kilodalton Plasmodium falciparum proteins at the surface membrane of infected erythrocytes. *Mol Biochem Parasitol* **27**: 207-223.

Hughes, A.L., and Verra, F. (2001) Very large long-term effective population size in the virulent human malaria parasite Plasmodium falciparum. *Proc Biol Sci* **268**: 1855-1860.

Iafrate, A.J., Feuk, L., Rivera, M.N., Listewnik, M.L., Donahoe, P.K., Qi, Y., Scherer, S.W., and Lee, C. (2004) Detection of large-scale variation in the human genome. *Nat Genet* **36**: 949-951.

Jafari, S., Le Bras, J., Bouchaud, O., and Durand, R. (2004) Plasmodium falciparum clonal population dynamics during malaria treatment. *J Infect Dis* **189**: 195-203.

Janssen, C.S., Barrett, M.P., Turner, C.M., and Phillips, R.S. (2002) A large gene family for putative variant antigens shared by human and rodent malaria parasites. *Proc Biol Sci* **269**: 431-436.

Janssen, C.S., Phillips, R.S., Turner, C.M., and Barrett, M.P. (2004) Plasmodium interspersed repeats: the major multigene superfamily of malaria parasites. *Nucleic Acids Res* **32**: 5712-5720.

Jeffares, D.C., Pain, A., Berry, A., Cox, A.V., Stalker, J., Ingle, C.E., Thomas, A., Quail, M.A., Siebenthall, K., Uhlemann, A.C., Kyes, S., Krishna, S., Newbold, C., Dermitzakis, E.T., and Berriman, M. (2007) Genome variation and evolution of the malaria parasite Plasmodium falciparum. *Nat Genet* **39**: 120-125.

Jensen, J.B., Capps, T.C., and Carlin, J.M. (1981) Clinical drug-resistant falciparum malaria acquired from cultured parasites. *Am J Trop Med Hyg* **30**: 523-525.

Joy, D.A., Feng, X., Mu, J., Furuya, T., Chotivanich, K., Krettli, A.U., Ho, M., Wang, A., White, N.J., Suh, E., Beerli, P., and Su, X.Z. (2003) Early origin and recent expansion of Plasmodium falciparum. *Science* **300**: 318-321.

Kaestli, M., Cortes, A., Lagog, M., Ott, M., and Beck, H.P. (2004) Longitudinal assessment of Plasmodium falciparum var gene transcription in naturally infected asymptomatic children in Papua New Guinea. *J Infect Dis* **189**: 1942-1951.

Kaneko, O., Soubes, S.C., and Miller, L.H. (1999) Plasmodium falciparum: invasion of Aotus monkey red blood cells and adaptation to Aotus monkeys. *Exp Parasitol* **93**: 116-119.

Kaneko, O., Mu, J., Tsuboi, T., Su, X., and Torii, M. (2002) Gene structure and expression of a Plasmodium falciparum 220-kDa protein homologous to the Plasmodium vivax reticulocyte binding proteins. *Mol Biochem Parasitol* **121**: 275-278.

Kaviratne, M., Khan, S.M., Jarra, W., and Preiser, P.R. (2002) Small variant STEVOR antigen is uniquely located within Maurer's clefts in Plasmodium falciparum-infected red blood cells. *Eukaryot Cell* **1**: 926-935.

Kaviratne, M., Fernandez, V., Jarra, W., Cunningham, D., Galinski, M.R., Wahlgren, M., and Preiser, P.R. (2003) Antigenic variation in *Plasmodium falciparum* and other *Plasmodium* species. In *Antigenic Variation*. Craig, A. and Scherf, A. (eds). London: Academic Press, pp. 291-317.

Kelly, J.M., McRobert, L., and Baker, D.A. (2006) Evidence on the chromosomal location of centromeric DNA in Plasmodium falciparum from etoposide-mediated topoisomerase-II cleavage. *Proc Natl Acad Sci U S A* **103**: 6706-6711.

Kemp, D.J., Cowman, A.F., and Walliker, D. (1990) Genetic diversity in Plasmodium falciparum. *Adv Parasitol* **29**: 75-149.

Kerr, M.K. (2003) Design considerations for efficient and effective microarray studies. *Biometrics* **59**: 822-828.

Kidgell, C., Volkman, S.K., Daily, J., Borevitz, J.O., Plouffe, D., Zhou, Y., Johnson, J.R., Le Roch, K., Sarr, O., Ndir, O., Mboup, S., Batalov, S., Wirth, D.F., and Winzeler, E.A. (2006) A systematic map of genetic variation in *Plasmodium falciparum*. *PLoS Pathog* **2**: e57.

Kilejian, A. (1979) Characterization of a protein correlated with the production of knob-like protrusions on membranes of erythrocytes infected with Plasmodium falciparum. *Proc Natl Acad Sci U S A* **76**: 4650-4653.

Kissinger, J.C., Brunk, B.P., Crabtree, J., Fraunholz, M.J., Gajria, B., Milgram, A.J., Pearson, D.S., Schug, J., Bahl, A., Diskin, S.J., Ginsburg, H., Grant, G.R., Gupta, D., Labo, P., Li, L., Mailman, M.D., McWeeney, S.K., Whetzel, P., Stoeckert, C.J., and Roos, D.S. (2002) The Plasmodium genome database. *Nature* **419**: 490-492.

Kooij, T.W., Carlton, J.M., Bidwell, S.L., Hall, N., Ramesar, J., Janse, C.J., and Waters, A.P. (2005) A Plasmodium whole-genome synteny map: indels and synteny breakpoints as foci for species-specific genes. *PLoS Pathog* **1**: e44.

Kraemer, S.M., and Smith, J.D. (2003) Evidence for the importance of genetic structuring to the structural and functional specialization of the Plasmodium falciparum var gene family. *Mol Microbiol* **50**: 1527-1538.

Kyes, S., Pinches, R., and Newbold, C. (2000) A simple RNA analysis method shows var and rif multigene family expression patterns in Plasmodium falciparum. *Mol Biochem Parasitol* **105**: 311-315.

Kyes, S., Horrocks, P., and Newbold, C. (2001) Antigenic variation at the infected red cell surface in malaria. *Annu Rev Microbiol* **55**: 673-707.

Kyes, S.A., Rowe, J.A., Kriek, N., and Newbold, C.I. (1999) Rifins: a second family of clonally variant proteins expressed on the surface of red cells infected with Plasmodium falciparum. *Proc Natl Acad Sci U S A* **96**: 9333-9338.

Kyes, S.A., Christodoulou, Z., Raza, A., Horrocks, P., Pinches, R., Rowe, J.A., and Newbold, C.I. (2003) A well-conserved Plasmodium falciparum var gene shows an unusual stage-specific transcript pattern. *Mol Microbiol* **48**: 1339-1348.

Lambros, C., and Vanderberg, J.P. (1979) Synchronization of Plasmodium falciparum erythrocytic stages in culture. *J Parasitol* **65**: 418-420.

Lasonder, E., Ishihama, Y., Andersen, J.S., Vermunt, A.M., Pain, A., Sauerwein, R.W., Eling, W.M., Hall, N., Waters, A.P., Stunnenberg, H.G., and Mann, M. (2002) Analysis of the Plasmodium falciparum proteome by high-accuracy mass spectrometry. *Nature* **419**: 537-542.

Lavazec, C., Sanyal, S., and Templeton, T.J. (2006) Hypervariability within the Rifin, Stevor and Pfmc-2TM superfamilies in Plasmodium falciparum. *Nucleic Acids Res* **34**: 6696-6707.

Lavazec, C., Sanyal, S., and Templeton, T.J. (2007) Expression switching in the stevor and Pfmc-2TM superfamilies in Plasmodium falciparum. *Mol Microbiol* **64**: 1621-1634.

Lavstsen, T., Salanti, A., Jensen, A.T., Arnot, D.E., and Theander, T.G. (2003) Sub-grouping of Plasmodium falciparum 3D7 var genes based on sequence analysis of coding and non-coding regions. *Malar J* **2**: 27.

Lavstsen, T., Magistrado, P., Hermsen, C.C., Salanti, A., Jensen, A.T., Sauerwein, R., Hviid, L., Theander, T.G., and Staalsoe, T. (2005) Expression of *Plasmodium falciparum* erythrocyte membrane protein 1 in experimentally infected humans. *Malar J* **4**: 21.

Le Roch, K.G., Zhou, Y., Blair, P.L., Grainger, M., Moch, J.K., Haynes, J.D., De La Vega, P., Holder, A.A., Batalov, S., Carucci, D.J., and Winzeler, E.A. (2003) Discovery of gene function by expression profiling of the malaria parasite life cycle. *Science* **301**: 1503-1508.

Leech, J.H., Barnwell, J.W., Miller, L.H., and Howard, R.J. (1984) Identification of a strain-specific malarial antigen exposed on the surface of Plasmodium falciparum-infected erythrocytes. *J Exp Med* **159**: 1567-1575.

Levine, N.D. (1988) Progress in taxonomy of the Apicomplexan protozoa. *J Protozool* **35**: 518-520.

Li, L., Stoeckert, C.J., Jr., and Roos, D.S. (2003) OrthoMCL: identification of ortholog groups for eukaryotic genomes. *Genome Res* **13**: 2178-2189.

Linardopoulou, E.V., Williams, E.M., Fan, Y., Friedman, C., Young, J.M., and Trask, B.J. (2005) Human subtelomeres are hot spots of interchromosomal recombination and segmental duplication. *Nature* **437**: 94-100.

Ling, I.T., Florens, L., Dluzewski, A.R., Kaneko, O., Grainger, M., Yim Lim, B.Y., Tsuboi, T., Hopkins, J.M., Johnson, J.R., Torii, M., Bannister, L.H., Yates, J.R. 3rd, Holder, A.A., Mattei, D. (2004) The *Plasmodium falciparum* clag9 gene encodes a rhoptry protein that is transferred to the host erythrocyte upon invasion. *Mol Microbiol.* **52** :107-18.

Liu, J., Gluzman, I.Y., Drew, M.E., and Goldberg, D.E. (2005) The role of Plasmodium falciparum food vacuole plasmepsins. *J Biol Chem* **280**: 1432-1437.

Ljungström, I., Perlmann, H., Schichtherle, M., Scherf, A., and Wahlgen, M. (2004) Methods in Malaria Research (http://www.mr4.org/MR4_Protocols.html). 4th Ed. *Malaria Research and Reference Reagent Resource Center.*

Llinas, M., Bozdech, Z., Wong, E.D., Adai, A.T., and DeRisi, J.L. (2006) Comparative whole genome transcriptome analysis of three Plasmodium falciparum strains. *Nucleic Acids Res* **34**: 1166-1173.

Lopez-Estrano, C., Bhattacharjee, S., Harrison, T., and Haldar, K. (2003) Cooperative domains define a unique host cell-targeting signal in Plasmodium falciparum-infected erythrocytes. *Proc Natl Acad Sci U S A* **100**: 12402-12407.

Lu, Y., Hall, T., Gay, L.S., and Donelson, J.E. (1993) Point mutations are associated with a gene duplication leading to the bloodstream reexpression of a trypanosome metacyclic VSG. *Cell* **72**: 397-406.

Luse, S.A., and Miller, L.H. (1971) Plasmodium falciparum malaria. Ultrastructure of parasitized erythrocytes in cardiac vessels. *Am J Trop Med Hyg* **20**: 655-660.

MacPherson, G.G., Warrell, M.J., White, N.J., Looareesuwan, S., and Warrell, D.A. (1985) Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. *Am J Pathol* **119**: 385-401.

Magowan, C., Wollish, W., Anderson, L., and Leech, J. (1988) Cytoadherence by Plasmodium falciparum-infected erythrocytes is correlated with the expression of a family of variable proteins on infected erythrocytes. *J Exp Med* **168**: 1307-1320.

Maier, A.G., Rug, M., O'Neill, M.T., Beeson, J.G., Marti, M., Reeder, J., and Cowman, A.F. (2007) Skeleton-binding protein 1 functions at the parasitophorous vacuole membrane to traffic PfEMP1 to the Plasmodium falciparum-infected erythrocyte surface. *Blood* **109**: 1289-1297.

Manski-Nankervis, J.A., Gardiner, D.L., Hawthorne, P., Holt, D.C., Edwards, M., Kemp, D.J., and Trenholme, K.R. (2000) The sequence of clag 9, a subtelomeric gene of plasmodium falciparum is highly conserved. *Mol Biochem Parasitol* **111**: 437-440.

Marsh, K., and Howard, R.J. (1986) Antigens induced on erythrocytes by P. falciparum: expression of diverse and conserved determinants. *Science* **231**: 150-153.

Marsh, K., Otoo, L., Hayes, R.J., Carson, D.C., and Greenwood, B.M. (1989) Antibodies to blood stage antigens of Plasmodium falciparum in rural Gambians and their relation to protection against infection. *Trans R Soc Trop Med Hyg* **83**: 293-303.

Marti, M., Good, R.T., Rug, M., Knuepfer, E., and Cowman, A.F. (2004) Targeting malaria virulence and remodeling proteins to the host erythrocyte. *Science* **306**: 1930-1933.

McKenzie, F.E., and Bossert, W.H. (2005) An integrated model of Plasmodium falciparum dynamics. *J Theor Biol* **232**: 411-426.

McRobert, L., Preiser, P., Sharp, S., Jarra, W., Kaviratne, M., Taylor, M.C., Renia, L., and Sutherland, C.J. (2004) Distinct trafficking and localization of STEVOR proteins in three stages of the Plasmodium falciparum life cycle. *Infect Immun* **72**: 6597-6602.

Mefford, H.C., and Trask, B.J. (2002) The complex structure and dynamic evolution of human subtelomeres. *Nat Rev Genet* **3**: 91-102.

Merino, E.F., Fernandez-Becerra, C., Durham, A.M., Ferreira, J.E., Tumilasci, V.F., d'Arc-Neves, J., da Silva-Nunes, M., Ferreira, M.U., Wickramarachchi, T., Udagama-Randeniya, P., Handunnetti, S.M., Del Portillo, H.A. (2006) Multi-character population study of the vir subtelomeric multigene superfamily of Plasmodium vivax, a major human malaria parasite.*Mol Biochem Parasitol.* **149**:10-6

Miller, L.H., Roberts, T., Shahabuddin, M., and McCutchan, T.F. (1993) Analysis of sequence diversity in the Plasmodium falciparum merozoite surface protein-1 (MSP-1). *Mol Biochem Parasitol* **59**: 1-14.

Miller, L.H., Good, M.F., and Milon, G. (1994) Malaria pathogenesis. *Science* **264**: 1878-1883.

Mok, B.W., Ribacke, U., Winter, G., Yip, B.H., Tan, C.S., Fernandez, V., Chen, Q., Nilsson, P., and Wahlgren, M. (2007) Comparative transcriptomal analysis of isogenic *Plasmodium falciparum* clones of distinct antigenic and adhesive phenotypes. *Mol Biochem Parasitol* **151**: 184-192.

Mu, J., Duan, J., Makova, K.D., Joy, D.A., Huynh, C.Q., Branch, O.H., Li, W.H., and Su, X.Z. (2002) Chromosome-wide SNPs reveal an ancient origin for Plasmodium falciparum. *Nature* **418**: 323-326.

Mu, J., Awadalla, P., Duan, J., McGee, K.M., Joy, D.A., McVean, G.A., and Su, X.Z. (2005) Recombination hotspots and population structure in Plasmodium falciparum. *PLoS Biol* **3**: e335.

Mu, J., Awadalla, P., Duan, J., McGee, K.M., Keebler, J., Seydel, K., McVean, G.A., and Su, X.Z. (2007) Genome-wide variation and identification of vaccine targets in the Plasmodium falciparum genome. *Nat Genet* **39**: 126-130.

Munasinghe, A., Patankar, S., Cook, B.P., Madden, S.L., Martin, R.K., Kyle, D.E., Shoaibi, A., Cummings, L.M., and Wirth, D.F. (2001) Serial analysis of gene expression (SAGE) in Plasmodium falciparum: application of the technique to A-T rich genomes. *Mol Biochem Parasitol* **113**: 23-34.

Murthy, K.K., Mahboubi, V.S., Santiago, A., Barragan, M.T., Knoll, R., Schultheiss, H.P., O'Connor, D.T., Schork, N.J., and Rana, B.K. (2005) Assessment of multiple displacement amplification for polymorphism discovery and haplotype determination at a highly polymorphic locus, MC1R. *Hum Mutat* **26**: 145-152.

Nair, S., Williams, J.T., Brockman, A., Paiphun, L., Mayxay, M., Newton, P.N., Guthmann, J.P., Smithuis, F.M., Hien, T.T., White, N.J., Nosten, F., and Anderson, T.J. (2003) A selective sweep driven by pyrimethamine treatment in southeast asian malaria parasites. *Mol Biol Evol* **20**: 1526-1536.

Navarro, M., and Gull, K. (2001) A pol I transcriptional body associated with VSG mono-allelic expression in Trypanosoma brucei. *Nature* **414**: 759-763.

Noviyanti, R., Brown, G.V., Wickham, M.E., Duffy, M.F., Cowman, A.F., and Reeder, J.C. (2001) Multiple var gene transcripts are expressed in Plasmodium falciparum infected erythrocytes selected for adhesion. *Mol Biochem Parasitol* **114**: 227-237.

Nunes, M.C., Goldring, J.P., Doerig, C., and Scherf, A. (2007) A novel protein kinase family in Plasmodium falciparum is differentially transcribed and secreted to various cellular compartments of the host cell. *Mol Microbiol* **63**: 391-403.

O'Donnell, R.A., Freitas-Junior, L.H., Preiser, P.R., Williamson, D.H., Duraisingh, M., McElwain, T.F., Scherf, A., Cowman, A.F., and Crabb, B.S. (2002) A genetic screen for improved plasmid segregation reveals a role for Rep20 in the interaction of Plasmodium falciparum chromosomes. *Embo J* **21**: 1231-1239.

Oakley, M.S., Kumar, S., Anantharaman, V., Zheng, H., Mahajan, B., Haynes, J.D., Moch, J.K., Fairhurst, R., McCutchan, T.F., and Aravind, L. (2007) Molecular factors and biochemical pathways induced by febrile temperature in intraerythrocytic Plasmodium falciparum parasites. *Infect Immun* **75**: 2012-2025.

Ockenhouse, C.F., Tegoshi, T., Maeno, Y., Benjamin, C., Ho, M., Kan, K.E., Thway, Y., Win, K., Aikawa, M., and Lobb, R.R. (1992) Human vascular endothelial cell adhesion receptors for Plasmodium falciparum-infected erythrocytes: roles for endothelial leukocyte adhesion molecule 1 and vascular cell adhesion molecule 1. *J Exp Med* **176**: 1183-1189.

Pain, A., Ferguson, D.J., Kai, O., Urban, B.C., Lowe, B., Marsh, K., and Roberts, D.J. (2001) Platelet-mediated clumping of Plasmodium falciparum-infected erythrocytes is a common adhesive phenotype and is associated with severe malaria. *Proc Natl Acad Sci U S A* **98**: 1805-1810.

Pasloske, B.L., Baruch, D.I., van Schravendijk, M.R., Handunnetti, S.M., Aikawa, M., Fujioka, H., Taraschi, T.F., Gormley, J.A., and Howard, R.J. (1993) Cloning and characterization of a Plasmodium falciparum gene encoding a novel high-molecular weight host membrane-associated protein, PfEMP3. *Mol Biochem Parasitol* **59**: 59-72.

Pasloske, B.L., Baruch, D.I., Ma, C., Taraschi, T.F., Gormley, J.A., and Howard, R.J. (1994) PfEMP3 and HRP1: co-expressed genes localized to chromosome 2 of Plasmodium falciparum. *Gene* **144**: 131-136.

Patankar, S., Munasinghe, A., Shoaibi, A., Cummings, L.M., and Wirth, D.F. (2001) Serial analysis of gene expression in Plasmodium falciparum reveals the global expression profile of erythrocytic stages and the presence of anti-sense transcripts in the malarial parasite. *Mol Biol Cell* **12**: 3114-3125.

Peterson, D.S., Miller, L.H., and Wellems, T.E. (1995) Isolation of multiple sequences from the Plasmodium falciparum genome that encode conserved domains homologous to those in erythrocyte-binding proteins. *Proc Natl Acad Sci U S A* **92**: 7100-7104.

Petter, M., Haeggström, M., Khattab, A., Fernandez, V., Klinkert, M.Q., Wahlgren, M. (2007) Variant proteins of the *Plasmodium falciparum* RIFIN family show distinct subcellular localization and developmental expression patterns. *Mol Biochem Parasitol.* (in press)

Ploner, A., Calza, S., Gusnanto, A., and Pawitan, Y. (2005) Multidimensional local false discovery rate for microarray studies. *Bioinformatics* **22**: 556-565.

Pologe, L.G., Pavlovec, A., Shio, H., and Ravetch, J.V. (1987) Primary structure and subcellular localization of the knob-associated histidine-rich protein of Plasmodium falciparum. *Proc Natl Acad Sci U S A* **84**: 7139-7143.

Polson, H.E., and Blackman, M.J. (2005) A role for poly(dA)poly(dT) tracts in directing activity of the Plasmodium falciparum calmodulin gene promoter. *Mol Biochem Parasitol* **141**: 179-189.

Preiser, P.R., Jarra, W., Capiod, T., and Snounou, G. (1999) A rhoptry-protein-associated mechanism of clonal phenotypic variation in rodent malaria. *Nature* **398**: 618-622.

Price, R.N., Uhlemann, A.C., Brockman, A., McGready, R., Ashley, E., Phaipun, L., Patel, R., Laing, K., Looareesuwan, S., White, N.J., Nosten, F., and Krishna, S. (2004) Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number. *Lancet* **364**: 438-447.

Qazi, K.R., Wikman, M., Vasconcelos, N.M., Berzins, K., Stahl, S., and Fernandez, C. (2005) Enhancement of DNA vaccine potency by linkage of Plasmodium falciparum malarial antigen gene fused with a fragment of HSP70 gene. *Vaccine* **23**: 1114-1125.

Qin, L.X., and Kerr, K.F. (2004) Empirical evaluation of data transformations and ranking statistics for microarray analysis. *Nucleic Acids Res* **32**: 5471-5479.

R Development Core Team. R: A language and environment for statistical computing [http://www.R-project.org/].

Ralph, S.A., Scheidig-Benatar, C., and Scherf, A. (2005) Antigenic variation in Plasmodium falciparum is associated with movement of var loci between subnuclear locations. *Proc Natl Acad Sci U S A* **102**: 5414-5419.

Rasti, N., Wahlgren, M., Chen, Q. (2004) Molecular aspects of malaria pathogenesis.*FEMS Immunol Med Microbiol.* **41**:9-26.

Rasti, N., Namusoke, F., Chene, A., Chen, Q., Staalsoe, T., Klinkert, M.Q., Mirembe, F., Kironde, F., and Wahlgren, M. (2006) Nonimmune immunoglobulin binding and multiple adhesion characterize Plasmodium falciparum-infected erythrocytes of placental origin. *Proc Natl Acad Sci U S A* **103**: 13795-13800.

Rayner, J.C., Galinski, M.R., Ingravallo, P., and Barnwell, J.W. (2000) Two Plasmodium falciparum genes express merozoite proteins that are related to Plasmodium vivax and Plasmodium yoelii adhesive proteins involved in host cell selection and invasion. *Proc Natl Acad Sci U S A* **97**: 9648-9653.

Rayner, J.C., Vargas-Serrato, E., Huber, C.S., Galinski, M.R., and Barnwell, J.W. (2001) A Plasmodium falciparum homologue of Plasmodium vivax reticulocyte binding protein (PvRBP1) defines a trypsin-resistant erythrocyte invasion pathway. *J Exp Med* **194**: 1571-1581.

Recker, M., Nee, S., Bull, P.C., Kinyanjui, S., Marsh, K., Newbold, C., and Gupta, S. (2004) Transient cross-reactive immune responses can orchestrate antigenic variation in malaria. *Nature* **429**: 555-558.

Redon, R., Ishikawa, S., Fitch, K.R., Feuk, L., Perry, G.H., Andrews, T.D., Fiegler, H., Shapero, M.H., Carson, A.R., Chen, W., Cho, E.K., Dallaire, S., Freeman, J.L., Gonzalez, J.R., Gratacos, M., Huang, J., Kalaitzopoulos, D., Komura, D., MacDonald, J.R., Marshall, C.R., Mei, R., Montgomery, L., Nishimura, K., Okamura, K., Shen, F., Somerville, M.J., Tchinda, J., Valsesia, A., Woodwark, C., Yang, F., Zhang, J., Zerjal, T., Zhang, J., Armengol, L., Conrad, D.F., Estivill, X., Tyler-Smith, C., Carter, N.P., Aburatani, H., Lee, C., Jones, K.W., Scherer, S.W., and Hurles, M.E. (2006) Global variation in copy number in the human genome. *Nature* **444**: 444-454.

Reeder, J.C., Cowman, A.F., Davern, K.M., Beeson, J.G., Thompson, J.K., Rogerson, S.J., and Brown, G.V. (1999) The adhesion of Plasmodium falciparum-infected erythrocytes to chondroitin sulfate A is mediated by P. falciparum erythrocyte membrane protein 1. *Proc Natl Acad Sci U S A* **96**: 5198-5202.

Ribacke, U., Mok, B.W., Wirta, V., Normark, J., Lundeberg, J., Kironde, F., Egwang, T.G., Nilsson, P., and Wahlgren, M. (2007) Genome wide gene amplifications and deletions in *Plasmodium falciparum*. *Mol Biochem Parasitol* **155**: 33-44.

Rich, S.M., Hudson, R.R., and Ayala, F.J. (1997) Plasmodium falciparum antigenic diversity: evidence of clonal population structure. *Proc Natl Acad Sci U S A* **94**: 13040-13045.

Ridley, R.G., Takacs, B., Etlinger, H., and Scaife, J.G. (1990) A rhoptry antigen of Plasmodium falciparum is protective in Saimiri monkeys. *Parasitology* **101 Pt 2**: 187-192.

Robert, C., Pouvelle, B., Meyer, P., Muanza, K., Fujioka, H., Aikawa, M., Scherf, A., and Gysin, J. (1995) Chondroitin-4-sulphate (proteoglycan), a receptor for Plasmodium falciparum-infected erythrocyte adherence on brain microvascular endothelial cells. *Res Immunol* **146**: 383-393.

Roberts, D.D., Sherwood, J.A., Spitalnik, S.L., Panton, L.J., Howard, R.J., Dixit, V.M., Frazier, W.A., Miller, L.H., and Ginsburg, V. (1985) Thrombospondin binds falciparum malaria parasitized erythrocytes and may mediate cytoadherence. *Nature* **318**: 64-66.

Roberts, D.J., Craig, A.G., Berendt, A.R., Pinches, R., Nash, G., Marsh, K., and Newbold, C.I. (1992) Rapid switching to multiple antigenic and adhesive phenotypes in malaria. *Nature* **357**: 689-692.

Robson, K.J.H., Walliker, D., Creasey, A., McBride, J., Beale, G., and Wilson, R.J.M. (1992) Cross-contamination of Plasmodium Cultures. *Parasitology Today* **8**: 38-39.

Rogerson, S.J., Chaiyaroj, S.C., Ng, K., Reeder, J.C., and Brown, G.V. (1995) Chondroitin sulfate A is a cell surface receptor for Plasmodium falciparum-infected erythrocytes. *J Exp Med* **182**: 15-20.

Rooney, A.P., and Ward, T.J. (2005) Evolution of a large ribosomal RNA multigene family in filamentous fungi: birth and death of a concerted evolution paradigm. *Proc Natl Acad Sci U S A* **102**: 5084-5089.

Rosario, V. (1981) Cloning of naturally occurring mixed infections of malaria parasites. *Science* **212**: 1037-1038.

Rosenthal, P.J. (2004) Cysteine proteases of malaria parasites. *Int J Parasitol* **34**: 1489-1499.

Rottmann, M., Lavstsen, T., Mugasa, J.P., Kaestli, M., Jensen, A.T., Muller, D., Theander, T., and Beck, H.P. (2006) Differential expression of var gene groups is associated with morbidity caused by Plasmodium falciparum infection in Tanzanian children. *Infect Immun* **74**: 3904-3911.

Rowe, J.A., Moulds, J.M., Newbold, C.I., and Miller, L.H. (1997) P. falciparum rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. *Nature* **388**: 292-295.

Ruangjirachuporn, W., Afzelius, B.A., Paulie, S., Wahlgren, M., Berzins, K., and Perlmann, P. (1991) Cytoadherence of knobby and knobless Plasmodium falciparum-infected erythrocytes. *Parasitology* **102 Pt 3**: 325-334.

Ruangjirachuporn, W., Afzelius, B.A., Helmby, H., Hill, A.V., Greenwood, B.M., Carlson, J., Berzins, K., Perlmann, P., and Wahlgren, M. (1992) Ultrastructural analysis of fresh Plasmodium falciparum-infected erythrocytes and their cytoadherence to human leukocytes. *Am J Trop Med Hyg* **46**: 511-519.

Sachs, J., and Malaney, P. (2002) The economic and social burden of malaria. *Nature* **415**: 680-685.

Saint, R.B., Coppel, R.L., Cowman, A.F., Brown, G.V., Shi, P.T., Barzaga, N., Kemp, D.J., and Anders, R.F. (1987) Changes in repeat number, sequence, and reading frame in S-antigen genes of Plasmodium falciparum. *Mol Cell Biol* **7**: 2968-2973.

Salanti, A., Jensen, A.T., Zornig, H.D., Staalsoe, T., Joergensen, L., Nielsen, M.A., Khattab, A., Arnot, D.E., Klinkert, M.Q., Hviid, L., and Theander, T.G. (2002) A sub-family of common and highly conserved Plasmodium falciparum var genes. *Mol Biochem Parasitol* **122**: 111-115.

Salanti, A., Staalsoe, T., Lavstsen, T., Jensen, A.T., Sowa, M.P., Arnot, D.E., Hviid, L., and Theander, T.G. (2003) Selective upregulation of a single distinctly structured *var* gene in chondroitin sulphate A-adhering *Plasmodium falciparum* involved in pregnancy-associated malaria. *Mol Microbiol* **49**: 179-191.

Salanti, A., Dahlback, M., Turner, L., Nielsen, M.A., Barfod, L., Magistrado, P., Jensen, A.T., Lavstsen, T., Ofori, M.F., Marsh, K., Hviid, L., and Theander, T.G. (2004) Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. *J Exp Med* **200**: 1197-1203.

Sam-Yellowe, T.Y., Florens, L., Johnson, J.R., Wang, T., Drazba, J.A., Le Roch, K.G., Zhou, Y., Batalov, S., Carucci, D.J., Winzeler, E.A., and Yates, J.R., 3rd (2004) A Plasmodium gene family encoding Maurer's cleft membrane proteins: structural properties and expression profiling. *Genome Res* **14**: 1052-1059.

Samonte, R.V., and Eichler, E.E. (2002) Segmental duplications and the evolution of the primate genome. *Nat Rev Genet* **3**: 65-72.

Sargeant, T.J., Marti, M., Caler, E., Carlton, J.M., Simpson, K., Speed, T.P., and Cowman, A.F. (2006) Lineage-specific expansion of proteins exported to erythrocytes in malaria parasites. *Genome Biol* **7**: R12.

Saul, A., Cooper, J., Hauquitz, D., Irving, D., Cheng, Q., Stowers, A., and Limpaiboon, T. (1992) The 42-kilodalton rhoptry-associated protein of *Plasmodium falciparum*. *Mol Biochem Parasitol* **50**: 139-149.

Semblat, J.P., Raza, A., Kyes, S.A., Rowe, J.A. (2006) Identification of *Plasmodium falciparum* var1CSA and var2CSA domains that bind IgM natural antibodies. *Mol Biochem Parasitol.* **146**: 192-197

Scherf, A., Hernandez-Rivas, R., Buffet, P., Bottius, E., Benatar, C., Pouvelle, B., Gysin, J., and Lanzer, M. (1998) Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of *var* genes during intra-erythrocytic development in *Plasmodium falciparum*. *Embo J* **17**: 5418-5426.

Scherf, A., Figueiredo, L.M., and Freitas-Junior, L.H. (2001) Plasmodium telomeres: a pathogen's perspective. *Curr Opin Microbiol* **4**: 409-414.

Schneider, A.G., and Mercereau-Puijalon, O. (2005) A new Apicomplexa-specific protein kinase family: multiple members in *Plasmodium falciparum*, all with an export signature. *BMC Genomics* **6**: 30.

Scholander, C., Treutiger, C.J., Hultenby, K., Wahlgren, M. (1996) Novel fibrillar structure confers adhesive property to malaria-infected erythrocytes. *Nat Med.* **2**: 204-8.

Sebat, J., Lakshmi, B., Troge, J., Alexander, J., Young, J., Lundin, P., Maner, S., Massa, H., Walker, M., Chi, M., Navin, N., Lucito, R., Healy, J., Hicks, J., Ye, K., Reiner, A., Gilliam, T.C., Trask, B., Patterson, N., Zetterberg, A., and Wigler, M. (2004) Large-scale copy number polymorphism in the human genome. *Science* **305**: 525-528.

Shankaranarayana, G.D., Motamedi, M.R., Moazed, D., and Grewal, S.I. (2003) Sir2 regulates histone H3 lysine 9 methylation and heterochromatin assembly in fission yeast. *Curr Biol* **13**: 1240-1246.

Sharp, S., Lavstsen, T., Fivelman, Q.L., Saeed, M., McRobert, L., Templeton, T.J., Jensen, A.T., Baker, D.A., Theander, T.G., and Sutherland, C.J. (2006) Programmed transcription of the var gene family, but not of stevor, in Plasmodium falciparum gametocytes. *Eukaryot Cell* **5**: 1206-1214.

Shirley, M.W., Biggs, B.A., Forsyth, K.P., Brown, H.J., Thompson, J.K., Brown, G.V., and Kemp, D.J. (1990) Chromosome 9 from independent clones and isolates of Plasmodium falciparum undergoes subtelomeric deletions with similar breakpoints in vitro. *Mol Biochem Parasitol* **40**: 137-145.

Silvestrini, F., Bozdech, Z., Lanfrancotti, A., Di Giulio, E., Bultrini, E., Picci, L., Derisi, J.L., Pizzi, E., and Alano, P. (2005) Genome-wide identification of genes upregulated at the onset of gametocytogenesis in Plasmodium falciparum. *Mol Biochem Parasitol* **143**: 100-110.

Smith, J.D., Chitnis, C.E., Craig, A.G., Roberts, D.J., Hudson-Taylor, D.E., Peterson, D.S., Pinches, R., Newbold, C.I., and Miller, L.H. (1995) Switches in expression of *Plasmodium falciparum var* genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell* **82**: 101-110.

Smith, J.D., Craig, A.G., Kriek, N., Hudson-Taylor, D., Kyes, S., Fagan, T., Pinches, R., Baruch, D.I., Newbold, C.I., and Miller, L.H. (2000a) Identification of a Plasmodium falciparum intercellular adhesion molecule-1 binding domain: a parasite adhesion trait implicated in cerebral malaria. *Proc Natl Acad Sci U S A* **97**: 1766-1771.

Smith, J.D., Subramanian, G., Gamain, B., Baruch, D.I., and Miller, L.H. (2000b) Classification of adhesive domains in the *Plasmodium falciparum* erythrocyte membrane protein 1 family. *Mol Biochem Parasitol* **110**: 293-310.

Smyth, G.K. (2005) Limma: linear models for microarray data. In *Bioinformatics and Computational Biology Solutions using R and Bioconductor.* Gentleman, R., Carey, V., Dudoit, S., Irizarry, R. and Huber, W. (eds). New York: Springer, pp. 397-420.

Smyth, G.K., Michaud, J., and Scott, H. (2005) The use of within-array replicate spots for assessing differential expression in microarray experiments. *Bioinformatics* **21**: 2067-2075.

Smythe, J.A., Coppel, R.L., Day, K.P., Martin, R.K., Oduola, A.M., Kemp, D.J., and Anders, R.F. (1991) Structural diversity in the Plasmodium falciparum merozoite surface antigen 2. *Proc Natl Acad Sci U S A* **88**: 1751-1755.

Snounou, G. (2002) Genotyping of Plasmodium spp. Nested PCR. *Methods Mol Med* **72**: 103-116.

Snow, R.W., Guerra, C.A., Noor, A.M., Myint, H.Y., and Hay, S.I. (2005) The global distribution of clinical episodes of Plasmodium falciparum malaria. *Nature* **434**: 214-217.

Spielmann, T., Fergusen, D.J., and Beck, H.P. (2003) *etramps*, a new *Plasmodium falciparum* gene family coding for developmentally regulated and highly charged membrane proteins located at the parasite-host cell interface. *Mol Biol Cell* **14**: 1529-1544.

Staines, H.M., Alkhalil, A., Allen, R.J., De Jonge, H.R., Derbyshire, E., Egee, S., Ginsburg, H., Hill, D.A., Huber, S.M., Kirk, K., Lang, F., Lisk, G., Oteng, E., Pillai, A.D., Rayavara, K., Rouhani, S., Saliba, K.J., Shen, C., Solomon, T., Thomas, S.L., Verloo, P., and Desai, S.A. (2007) Electrophysiological studies of malaria parasite-infected erythrocytes: current status. *Int J Parasitol* **37**: 475-482.

Stranger, B.E., Forrest, M.S., Dunning, M., Ingle, C.E., Beazley, C., Thorne, N., Redon, R., Bird, C.P., de Grassi, A., Lee, C., Tyler-Smith, C., Carter, N., Scherer, S.W., Tavare, S., Deloukas, P., Hurles, M.E., and Dermitzakis, E.T. (2007) Relative impact of nucleotide and copy number variation on gene expression phenotypes. *Science* **315**: 848-853.

Su, X.Z., Heatwole, V.M., Wertheimer, S.P., Guinet, F., Herrfeldt, J.A., Peterson, D.S., Ravetch, J.A., and Wellems, T.E. (1995) The large diverse gene family *var* encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythrocytes. *Cell* **82**: 89-100.

Sutherland, C.J. (2001) Stevor transcripts from Plasmodium falciparum gametocytes encode truncated polypeptides. *Mol Biochem Parasitol* **113**: 331-335.

Tachado, S.D., Gerold, P., McConville, M.J., Baldwin, T., Quilici, D., Schwarz, R.T., and Schofield, L. (1996) Glycosylphosphatidylinositol toxin of Plasmodium induces nitric oxide synthase expression in macrophages and vascular endothelial cells by a protein tyrosine kinase-dependent and protein kinase C-dependent signaling pathway. *J Immunol* **156**: 1897-1907.

Tanabe, K., Mackay, M., Goman, M., and Scaife, J.G. (1987) Allelic dimorphism in a surface antigen gene of the malaria parasite Plasmodium falciparum. *J Mol Biol* **195**: 273-287.

Taylor, H.M., Kyes, S.A., Harris, D., Kriek, N., and Newbold, C.I. (2000a) A study of *var* gene transcription in vitro using universal *var* gene primers. *Mol Biochem Parasitol* **105**: 13-23.

Taylor, H.M., Kyes, S.A., and Newbold, C.I. (2000b) *Var* gene diversity in *Plasmodium falciparum* is generated by frequent recombination events. *Mol Biochem Parasitol* **110**: 391-397.

Taylor, H.M., Triglia, T., Thompson, J., Sajid, M., Fowler, R., Wickham, M.E., Cowman, A.F., and Holder, A.A. (2001) *Plasmodium falciparum* homologue of the genes for *Plasmodium vivax* and *Plasmodium yoelii* adhesive proteins, which is transcribed but not translated. *Infect Immun* **69**: 3635-3645.

Trager, W., Rudzinska, M.A., and Bradbury, P.C. (1966) The fine structure of Plasmodium falciparum and its host erythrocytes in natural malarial infections in man. *Bull World Health Organ* **35**: 883-885.

Trager, W., and Jensen, J.B. (1976) Human malaria parasites in continuous culture. *Science* **193**: 673-675.

Trask, B.J., Massa, H., Brand-Arpon, V., Chan, K., Friedman, C., Nguyen, O.T., Eichler, E., van den Engh, G., Rouquier, S., Shizuya, H., and Giorgi, D. (1998) Large multi-chromosomal duplications encompass many members of the olfactory receptor gene family in the human genome. *Hum Mol Genet* **7**: 2007-2020.

Trenholme, K.R., Gardiner, D.L., Holt, D.C., Thomas, E.A., Cowman, A.F., and Kemp, D.J. (2000) clag9: A cytoadherence gene in Plasmodium falciparum essential for binding of parasitized erythrocytes to CD36. *Proc Natl Acad Sci U S A* **97**: 4029-4033.

Treutiger, C.J., Heddini, A., Fernandez, V., Muller, W.A., and Wahlgren, M. (1997) PECAM-1/CD31, an endothelial receptor for binding Plasmodium falciparum-infected erythrocytes. *Nat Med* **3**: 1405-1408.

Treutiger, C.J., Scholander, C., Carlson, J., McAdam, K.P., Raynes, J.G., Falksveden, L., and Wahlgren, M. (1999) Rouleaux-forming serum proteins are involved in the rosetting of Plasmodium falciparum-infected erythrocytes. *Exp Parasitol* **93**: 215-224.

Triglia, T., Thompson, J., Caruana, S.R., Delorenzi, M., Speed, T., and Cowman, A.F. (2001) Identification of proteins from *Plasmodium falciparum* that are homologous to reticulocyte binding proteins in *Plasmodium vivax*. *Infect Immun* **69**: 1084-1092.

Triglia, T., Duraisingh, M.T., Good, R.T., and Cowman, A.F. (2005) Reticulocyte-binding protein homologue 1 is required for sialic acid-dependent invasion into human erythrocytes by *Plasmodium falciparum*. *Mol Microbiol* **55**: 162-174.

Trimnell, A.R., Kraemer, S.M., Mukherjee, S., Phippard, D.J., Janes, J.H., Flamoe, E., Su, X.Z., Awadalla, P., and Smith, J.D. (2006) Global genetic diversity and evolution of *var* genes associated with placental and severe childhood malaria. *Mol Biochem Parasitol* **148**: 169-180.

Udomsangpetch, R., Aikawa, M., Berzins, K., Wahlgren, M., and Perlmann, P. (1989a) Cytoadherence of knobless *Plasmodium falciparum*-infected erythrocytes and its inhibition by a human monoclonal antibody. *Nature* **338**: 763-765.

Udomsangpetch, R., Wahlin, B., Carlson, J., Berzins, K., Torii, M., Aikawa, M., Perlmann, P., and Wahlgren, M. (1989b) *Plasmodium falciparum*-infected erythrocytes form spontaneous erythrocyte rosettes. *J Exp Med* **169**: 1835-1840.

Udomsangpetch, R., Reinhardt, P.H., Schollaardt, T., Elliott, J.F., Kubes, P., and Ho, M. (1997) Promiscuity of clinical Plasmodium falciparum isolates for multiple adhesion molecules under flow conditions. *J Immunol* **158**: 4358-4364.

van Schravendijk, M.R., Handunnetti, S.M., Barnwell, J.W., and Howard, R.J. (1992) Normal human erythrocytes express CD36, an adhesion molecule of monocytes, platelets, and endothelial cells. *Blood* **80**: 2105-2114.

Vazquez-Macias, A., Martinez-Cruz, P., Castaneda-Patlan, M.C., Scheidig, C., Gysin, J., Scherf, A., and Hernandez-Rivas, R. (2002) A distinct 5' flanking var gene region regulates Plasmodium falciparum variant erythrocyte surface antigen expression in placental malaria. *Mol Microbiol* **45**: 155-167.

Viebig, N.K., Gamain, B., Scheidig, C., Lepolard, C., Przyborski, J., Lanzer, M., Gysin, J., and Scherf, A. (2005) A single member of the *Plasmodium falciparum* var multigene family determines cytoadhesion to the placental receptor chondroitin sulphate A. *EMBO Rep* **6**: 775-781.

Vincensini, L., Richert, S., Blisnick, T., Van Dorsselaer, A., Leize-Wagner, E., Rabilloud, T., and Braun Breton, C. (2005) Proteomic analysis identifies novel proteins of the Maurer's clefts, a secretory compartment delivering Plasmodium falciparum proteins to the surface of its host cell. *Mol Cell Proteomics* **4**: 582-593.

Vogt, A.M., Barragan, A., Chen, Q., Kironde, F., Spillmann, D., and Wahlgren, M. (2003) Heparan sulfate on endothelial cells mediates the binding of Plasmodium falciparum-infected erythrocytes via the DBL1alpha domain of PfEMP1. *Blood* **101**: 2405-2411.

Vogt, A.M., Winter, G., Wahlgren, M., and Spillmann, D. (2004) Heparan sulphate identified on human erythrocytes: a Plasmodium falciparum receptor. *Biochem J* **381**: 593-597.

Volkman, S.K., Barry, A.E., Lyons, E.J., Nielsen, K.M., Thomas, S.M., Choi, M., Thakore, S.S., Day, K.P., Wirth, D.F., and Hartl, D.L. (2001) Recent origin of Plasmodium falciparum from a single progenitor. *Science* **293**: 482-484.

Volkman, S.K., Hartl, D.L., Wirth, D.F., Nielsen, K.M., Choi, M., Batalov, S., Zhou, Y., Plouffe, D., Le Roch, K.G., Abagyan, R., and Winzeler, E.A. (2002) Excess polymorphisms in genes for membrane proteins in Plasmodium falciparum. *Science* **298**: 216-218.

Volkman, S.K., Sabeti, P.C., DeCaprio, D., Neafsey, D.E., Schaffner, S.F., Milner, D.A., Jr., Daily, J.P., Sarr, O., Ndiaye, D., Ndir, O., Mboup, S., Duraisingh, M.T., Lukens, A., Derr, A., Stange-Thomann, N., Waggoner, S., Onofrio,

R., Ziaugra, L., Mauceli, E., Gnerre, S., Jaffe, D.B., Zainoun, J., Wiegand, R.C., Birren, B.W., Hartl, D.L., Galagan, J.E., Lander, E.S., and Wirth, D.F. (2007) A genome-wide map of diversity in *Plasmodium falciparum*. *Nat Genet* **39**: 113-119.

Voss, T.S., Thompson, J.K., Waterkeyn, J., Felger, I., Weiss, N., Cowman, A.F., and Beck, H.P. (2000) Genomic distribution and functional characterisation of two distinct and conserved Plasmodium falciparum var gene 5' flanking sequences. *Mol Biochem Parasitol* **107**: 103-115.

Voss, T.S., Kaestli, M., Vogel, D., Bopp, S., and Beck, H.P. (2003) Identification of nuclear proteins that interact differentially with *Plasmodium falciparum* var gene promoters. *Mol Microbiol* **48**: 1593-1607.

Voss, T.S., Healer, J., Marty, A.J., Duffy, M.F., Thompson, J.K., Beeson, J.G., Reeder, J.C., Crabb, B.S., and Cowman, A.F. (2006) A var gene promoter controls allelic exclusion of virulence genes in *Plasmodium falciparum* malaria. *Nature* **439**: 1004-1008.

Wahlgren, M., Abrams, J.S., Fernandez, V., Bejarano, M.T., Azuma, M., Torii, M., Aikawa, M., Howard, R.J. (1995) Adhesion of *Plasmodium falciparum*-infected erythrocytes to human cells and secretion of cytokines (IL-1-beta, IL-1RA, IL-6, IL-8, IL-10, TGF beta, TNF alpha, G-CSF, GM-CSF. *Scand J Immunol.* **42**:626-36.

Wahlgren, M., Fernandez, V., Chen, Q., Svard, S., and Hagblom, P. (1999) Waves of malarial var-iations. *Cell* **96**: 603-606.

Warrell, D.A., Molyneux, M.E., and Beales, P.E. (1990) Severe and complicated malaria. World Health Organization, Division of Control of Tropical Diseases. *Trans R Soc Trop Med Hyg* **84 Suppl 2**: 1-65.

Watanabe, J., Wakaguri, H., Sasaki, M., Suzuki, Y., and Sugano, S. (2007) Comparasite: a database for comparative study of transcriptomes of parasites defined by full-length cDNAs. *Nucleic Acids Res* **35**: D431-438.

Weber, J.L. (1988) Interspersed repetitive DNA from Plasmodium falciparum. *Mol Biochem Parasitol* **29**: 117-124.

WHO (2005) World Malaria Report

Winograd, E., and Sherman, I.W. (1989) Characterization of a modified red cell membrane protein expressed on erythrocytes infected with the human malaria parasite Plasmodium falciparum: possible role as a cytoadherent mediating protein. *J Cell Biol* **108**: 23-30.

Winter, G., Chen, Q., Flick, K., Kremsner, P., Fernandez, V., and Wahlgren, M. (2003) The 3D7var5.2 (var COMMON) type var gene family is commonly expressed in non-placental *Plasmodium falciparum* malaria. *Mol Biochem Parasitol* **127**: 179-191.

Winter, G., Kawai, S., Haeggstrom, M., Kaneko, O., von Euler, A., Kawazu, S., Palm, D., Fernandez, V., and Wahlgren, M. (2005) SURFIN is a polymorphic antigen expressed on *Plasmodium falciparum* merozoites and infected erythrocytes. *J Exp Med* **201**: 1853-1863.

Wirta, V. (2005) KTH-package for microarray data analysis.

Wooden, J., Kyes, S., and Sibley, C.H. (1993) PCR and strain identification in Plasmodium falciparum. *Parasitol Today* **9**: 303-305.

Wootton, J.C., Feng, X., Ferdig, M.T., Cooper, R.A., Mu, J., Baruch, D.I., Magill, A.J., and Su, X.Z. (2002) Genetic diversity and chloroquine selective sweeps in Plasmodium falciparum. *Nature* **418**: 320-323.

Yang, Y.H., Dudoit, S., Luu, P., Lin, D.M., Peng, V., Ngai, J., and Speed, T.P. (2002) Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Res* **30**: e15.

Young, J.A., Fivelman, Q.L., Blair, P.L., de la Vega, P., Le Roch, K.G., Zhou, Y., Carucci, D.J., Baker, D.A., and Winzeler, E.A. (2005) The Plasmodium falciparum sexual development transcriptome: a microarray analysis using ontology-based pattern identification. *Mol Biochem Parasitol* **143**: 67-79.

Yue, Y., and Haaf, T. (2006) 7E olfactory receptor gene clusters and evolutionary chromosome rearrangements. *Cytogenet Genome Res* **112**: 6-10.

Zhang, L., Lu, H.H., Chung, W.Y., Yang, J., and Li, W.H. (2005) Patterns of segmental duplication in the human genome. *Mol Biol Evol* **22**: 135-141.

Zhang, Z., and Dietrich, F.S. (2005) Identification and characterization of upstream open reading frames (uORF) in the 5' untranslated regions (UTR) of genes in *Saccharomyces cerevisiae*. *Curr Genet* **48**: 77-87.