

**Exiting the Erythrocyte:
Functional and Temporal Analysis
of a Malarial Subtilase**

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Abstract

Plasmodium falciparum is an obligate intracellular parasite, which causes 95% of worldwide malaria cases annually. Malarial symptoms occur during replication of parasites inside erythrocytes. Multiple cycles of host cell invasion, replication inside a parasitophorous vacuole (PV) and escape from the host cell result in gradually increasing parasitaemia. Escape from the host cell (egress) is regulated by proteases and may involve perforin-like proteins. PfSUB1, a subtilisin-like serine protease, is essential to *P. falciparum* blood stage development and egress. Just before cell rupture, the protease is discharged into the PV, where it processes multiple parasite surface proteins and PV proteins.

The main aim of this project was to analyse the function of PfSUB1 by three approaches which relied on *in vitro* biochemical analyses and *P. falciparum* transfections. Firstly, a conditional knockdown approach was used to analyse the function of PfSUB1 using the FKBP regulatable system. Two complementary strategies were used: down-regulation of PfSUB1 levels using a C-terminal FKBP domain and inhibition of PfSUB1 activity using an N-terminal FKBP fusion with the PfSUB1 prodomain (a potent inhibitor of recombinant PfSUB1). Expression of recombinant PfSUB1-FKBP in Sf9 insect cells demonstrated that FKBP does not interfere with PfSUB1 activity, FKBP was successfully integrated into the endogenous *pfsu1* gene. In the second approach, *in vitro* studies showed that recombinant *E. coli*-derived FKBP-prodomain fusion protein inhibits recombinant PfSUB1. Strong evidence was obtained which indicates that episomal expression of a non-regulatable prodomain in *P. falciparum* is not tolerated by the parasite.

Secondly, to further characterise the enzyme, an *in silico* approach was used to predict new SUB1 substrates, and a proteomic approach was taken to validate substrates *in vitro*. Several putative new substrates were identified, which suggest that PfSUB1 is a multifunctional enzyme with numerous roles in invasion and egress.

Finally, attempts were made to establish a PfSUB1-sensitive FRET-based system to monitor PfSUB1 activity *in vivo*. A recombinant FRET reporter was expressed in *E. coli*; this was shown to exhibit FRET and to be PfSUB1-sensitive *in vitro*. Preliminary *in vivo* data are presented, which suggest that protease-sensitive FRET is possible in *P. falciparum*.

I, Natalie Clare Silmon de Monerri, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Preface

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List of Abbreviations

1

6xHis Hexahistidine

A

AMA1 Apical membrane antigen 1

ACT Artemisinin combination therapy

C

Calcium Ca^{2+}

CAD Conditional aggregation domain

cADPR Cyclic ADP-ribose

CDC Cholesterol-dependent cytolysin

cGMP Cyclic GMP

Clp Caseinolytic protease

CM Cerebral malaria

CSP Circumsporozoite protein

D

DBL Duffy binding-like protein

DDT Dichlorodiphenyltrichloroethane

DG Dense granules

DMSO Dimethyl sulphoxide

DTT α -dithiothreitol

E

EBA-175 Erythrocyte binding antigen 175

EC Erythrocyte cytosol

ECP1 Egress cysteine protease 1

EM Electron microscopy

EPM Erythrocyte plasma membrane

ER Endoplasmic reticulum

F

FKBP	FK506-binding protein
FRET	Förster resonance energy transfer
FV	Food vacuole

G

GDP	Gross domestic product
GSHA	S-linked glutathione agarose

H

HAP	Histoaspartic protease
HRP	Horse radish peroxidase
HSP	Heparan sulphate proteoglycans

I

IFA	Immunofluorescence assay
Immuno-EM	Immunoelectron microscopy
IRS	Indoor residual spraying
ITN	Insecticide-treated bed nets

L

LB	Luria-Bertani
LC/MS/MS	Liquid chromatography mass spectrometry

M

MACPF	Membrane attack complex/perforin
MIC	Secreted microneme protein
MSP	Merozoite surface protein
MSP1	Merozoite surface protein 1
MS/MS	Tandem mass spectrometry
MSRP	MSP7-like related protein

N

NEB	New England Biolabs
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P

p31	PfSUB1 prodomain
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PBS	Phosphate buffered saline
PbSUB1	<i>P. berghei</i> subtilisin-like protease 1
PCR	Polymerase chain reaction
PEXEL	<i>Plasmodium</i> export element
PfEMP1	<i>P. falciparum</i> erythrocyte membrane protein 1
PfRH	<i>P. falciparum</i> reticulocyte binding homologues
PfSUB1	<i>P. falciparum</i> subtilisin-like protease 1
PfSUB2	<i>P. falciparum</i> subtilisin-like protease 2
PFP	Pore-forming protein
PfPPLP	<i>P. falciparum</i> perforin-like protein
PKG	cGMP-dependent protein kinase
PM	Plasmepsin
PoPS	Prediction of protease specificity
PP1	Protein phosphatase 1
PPLP	<i>Plasmodium</i> perforin-like protein
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
R	
RESA	Ring-infected erythrocyte surface antigen
RAP1	Rhoptry-associated protein 1
RAMA	Ring-associated membrane antigen
RIMA	Ring membrane antigen
RP-HPLC	Gradient elution reversed phase high pressure liquid chromatography
ROM1	Rhomboid protease 1
RON	Rhoptry neck protein
rp31	Recombinant PfSUB1 prodomain
rFKBP-p31	Recombinant FKBP-PfSUB1 prodomain fusion protein
rPfSUB1	Recombinant PfSUB1
rPfSUB1-FKBP	Recombinant PfSUB1-FKBP fusion

S

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERA	Serine repeat antigen
SSC	Saline-sodium citrate buffer

T

Tet	Tetracycline-inducible
TetR	Tetracycline repressor protein
TgPLP1	<i>T. gondii</i> perforin-like protein 1
TLCK	N- α -p-tosyl-L-lysine chloromethyl ketone
TPCK	L-1-tosylamide-2-phenylethylchloromethyl ketone
TRAP	Thrombospondin-related anonymous protein
TVN	Tubovesicular network

U

UIS3	Upregulated in sporozoites 3
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W

WHO	World Health Organisation
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1. Introduction

1.1. *Malaria: a devastating worldwide burden*

Malaria is a mosquito-borne disease, which kills up to three million people per year. Around 500 million non-fatal cases of malaria are presented annually (Snow et al., 2005, Breman, 2001), though this figure may be a vast underestimate due to difficulties in monitoring the disease. Malaria is caused by protozoan parasites of the genus *Plasmodium*, which have an intricate life cycle involving replication inside erythrocytes, resulting in gradually increasing parasitaemia, which can be fatal. Around 95% of cases are caused by *P. falciparum* (World Health Organisation, 2009), which is the only species able to cause cerebral malaria due to its ability to sequester in the brain. It is therefore considered the most dangerous species of *Plasmodium*. Malaria is most prevalent in sub-Saharan Africa and South East Asia. In those areas, children under the age of five and pregnant women are particularly at risk. Importantly, malaria is both preventable and treatable with antimalarial drugs. However, owing to widespread drug resistance and continued obstacles in vaccine development, as well as resistance of the mosquito vector to insecticides, it remains a global health burden. Malaria causes significant morbidity and mortality across the world and is a hindrance to socioeconomic development in countries where it is prevalent. New drugs, vaccines and insecticides are urgently required to combat this devastating disease.

1.1.1. **Malaria throughout history**

Malarious symptoms were described as early as the 5th century BC by Hippocrates (Cox, 2002). Malaria is identifiable in his writings by the clinical symptom of periodic fever and the use of cinchona powder as a cure; the active ingredient of this is quinine, which is still used as an antimalarial drug today. Early remedies for malaria also included alcohol and opium. In the 1600s, an apothecary apprentice developed the first antimalarial formulation which was, in essence, a white wine infusion of cinchona powder (Reiter, 2000). The name malaria is derived from the mediaeval Italian term “mala aria,” literally meaning bad air, since the cause of malaria was thought to be strong-smelling products of anaerobic bacteria in saline mud in marshes (Reiter, 2000). In England, malaria was known as “ague,” meaning marsh

fever, and is described in many literary works and throughout history. The author Geoffrey Chaucer describes a periodic fever in Nun's Priest's tale, and William Shakespeare mentions ague in eight of his plays. The Italian poet Dante, who died of malaria, also refers to ague in *The Inferno* (Reiter, 2000). Malaria plagued the political leader Oliver Cromwell, who is thought to have died from it in 1658; also, by DNA analysis, it was recently discovered that the Egyptian pharaoh Tutankhamun was infected with *P. falciparum* when he died in 1323 BC (Hawass et al., 2010). Despite its historical prevalence, a scientific understanding of malaria only came about towards the end of the 19th century. In a hospital in Algeria, Alphonse Laveran discovered black pigment in a patient's blood (Coluzzi, 1999). The black pigment is now known to be haemozoin, a biocrystallised by-product of haemoglobin digestion by malaria parasites. Laveran later identified gametocytes, the transmissible form of the parasite, in human blood. He was the first physician to hypothesise that mosquitoes in marsh areas transmitted the disease. Ronald Ross, a doctor, later established that mosquitoes do indeed transmit malaria (Coluzzi, 1999). Ross pioneered early efforts to control malaria and later received the Nobel Prize in Physiology or Medicine in 1902.

1.1.2. The complexities of the Apicomplexa

Malaria is caused by obligate intracellular protozoa of the genus *Plasmodium*, in the phylum Apicomplexa. There are over 5,000 apicomplexan genera, many of which are pathogenic. They have complex life cycles, including sexual and asexual replicative stages. Seven apicomplexan genera cause disease in humans: *Plasmodium*, *Babesia*, *Cryptosporidium*, *Isospora*, *Cyclospora*, *Sarcocystis* and *Toxoplasma*. Species of the genera *Eimeria* and *Theileria* cause poultry and bovine diseases respectively. *Toxoplasma gondii* infects almost all warm-blooded animals, but occasionally causes severe infections in immunocompromised humans and unborn babies. This parasite is readily amenable to genetic manipulation, transient and stable transfection methods are of high efficiency and it can be studied with relative ease by microscopy (Kim & Weiss, 2004, Soldati & Boothroyd, 1993). Hence, it is a useful model for several aspects of cell biology and host cell invasion by intracellular pathogens.

Across the phylum, there is a conserved mechanism of host cell invasion. The invasive form of these parasites, termed a "zoite", binds to the host cell non-

specifically, then reorients so that its apical end directly faces the host cell surface. The zoite enters the host cell in an active, parasite-driven invasion step, whereby the parasite and host cell membranes form a moving junction through which the parasite enters the host cell. In most cases, as the zoite enters, a PV is formed, inside which it replicates. Notably, *Theileria* and *Babesia spp.* initially form a PV but it is not maintained; they replicate freely inside the host cell cytoplasm (Potgieter & Els, 1977, Shaw, 1997). Genome sequencing has revealed many similarities across the Apicomplexa phylum, and further studies have indicated that homologues of several important invasion molecules from different genera can be used to complement *T. gondii* proteins (Di Cristina *et al.*, 1999, O'Connor *et al.*, 2003), suggesting that the invasion machinery is conserved.

A defining characteristic of apicomplexan zoites is a unique set of organelles, some of which secrete their contents in a temporal manner and contain functionally distinct sets of proteins (examples include: (Kafsack *et al.*, 2008, Bannister *et al.*, 2003, Kadota *et al.*, 2004, Kaiser *et al.*, 2004, Harris *et al.*, 2005, Mercier *et al.*, 2005, Sam-Yellowe *et al.*, 2004)). Of these organelles, micronemes, rhoptries and dense granules (DGs) are important for host cell invasion. The cigar-shaped micronemes vary in number and are 120 nm in length. In some studies in *P. falciparum*, micronemes appear to be attached to the end of a rhoptry (Bannister & Mitchell, 1989). Micronemes and rhoptries are located at the apical end of the zoite and release proteins that form the moving junction. In *T. gondii*, when release of proteins from micronemes is specifically inhibited, parasites cannot invade (Carruthers *et al.*, 1999), suggesting that microneme proteins are crucial for parasite-host recognition.

The club-shaped rhoptries are the largest apical organelles and the best studied. There are two distinct rhoptry subcompartments: the rhoptry neck and rhoptry bulb; the rhoptry bulb appears as a dense granular mass by transmission electron microscopy and with electron-lucent patches are visible in the neck area (Bannister *et al.*, 2000). *P. falciparum* merozoites have two rhoptries, which are implicated in PV formation and parasite-host recognition (Stewart *et al.*, 1986, Sam-Yellowe *et al.*, 1988, Etzion *et al.*, 1991, Bradley *et al.*, 2005, Nichols *et al.*, 1983, Bannister, 1986 #2705). Many *P. falciparum* rhoptry proteins have no homologues in *T. gondii*; this perhaps is indicative of key differences between the PVs of *T. gondii* and *P. falciparum*.

DGs are scattered throughout the cytoplasm and evidence suggests that they release proteins involved in host cell modification (Mills *et al.*, 2007). DGs were first identified in the apicomplexan parasite *Sarcocystis tenella*, so named as they are electron-dense in transmission electron micrographs (Dubremetz & Dissous, 1980). Extensive work on DGs has been carried out in *T. gondii*, where a range of different functions have been discovered for DG proteins. For example, DG proteins GRA1 and GRA2 are involved in biogenesis of the *T. gondii* PV nanotubular network (Mercier *et al.*, 2002). In comparison, relatively little work on DGs has been carried out in *P. falciparum*. In *P. falciparum*, they are spherical and have a diameter of around 100 nm. To date, the only known markers of DGs in *Plasmodium* are the proteins ring-infected erythrocyte surface antigen (RESA) and ring infected membrane antigen (RIMA) (Aikawa *et al.*, 1990, Trager *et al.*, 1992). RESA is involved in increasing the heat stability and rigidity of the erythrocyte (Maier *et al.*, 2008, Silva *et al.*, 2005, Da Silva *et al.*, 1994), while RIMA is observed in the plasma membrane of newly invaded ring-stage parasites and is thought to be involved in preventing multiple invasions (Trager *et al.*, 1992). Originally, *P. falciparum* subtilisin-like protease 1 (PfSUB1) was thought to localise to DGs (Blackman *et al.*, 1998), however more recent immunoelectron microscopic (immuno-EM) studies showed that PfSUB1 does not colocalise with RESA, but is present in tear drop-shaped organelles named “exonemes” (Yeoh *et al.*, 2007). Currently, PfSUB1 is the only known marker of *P. falciparum* exonemes. Whether exonemes are a conserved feature of apicomplexan parasites has yet to be determined. Another new subset of DGs called mononemes was also recently identified, to which the authors localise rhomboid protease 1 (ROM1) (Singh *et al.*, 2007). However, the resolution of immunofluorescence assay (IFA) images presented by Singh *et al* is not high enough to permit detailed analysis of the location of ROM1. Furthermore, EM evidence for mononemes is lacking. Since this study, Srinivasan *et al* and Brossier *et al* have provided evidence by EM that in the mouse model species *P. berghei* and *T. gondii*, ROM1 is almost certainly micronemal (Srinivasan *et al.*, 2009, Brossier *et al.*, 2008). Therefore, it is unclear whether mononemes exist particularly as, to date, no other proteins have been localised to them.

Other organelles, which are non-secretory, are also found among apicomplexan parasites. Some species have a conoid, an complex responsible for

sustaining the microtubular cytoskeleton (Morrissette & Sibley, 2002); this is absent in *Plasmodium spp.* Most apicomplexans possess an apicoplast, an ancient non-photosynthetic plastid thought to have been acquired by secondary endosymbiosis (Marechal & Cesbron-Delauw, 2001). The apicoplast appears to be essential and is implicated in haem, isoprenoid and neutral lipid synthesis (Marechal & Cesbron-Delauw, 2001, Seeber & Soldati-Favre, 2010).

1.1.3. Introducing the *Plasmodium* genus

Species of *Plasmodium* cause malaria in a wide range of vertebrate hosts, from snakes and birds to mice and humans. A wealth of information has been obtained by the use of animal models of malaria as they offer the possibility of analysing the mechanism of disease progression *in vivo*. *P. berghei*, *P. yoelii* and *P. chabaudi* are commonly used mouse malaria species, each of which has different characteristics in terms of infectivity and is therefore used for different types of study. Murine malaria species can also be genetically manipulated; transfection technology exists for *P. berghei* (de Koning-Ward et al., 2000, Janse et al., 2006), *P. yoelii* (Mota et al., 2001) and *P. chabaudi*; (Reece & Thompson, 2008). Recently, a mouse model of *P. falciparum* was developed by use of nonmyelodepleted mice which were engrafted with human erythrocytes and successfully used to analyse therapeutic effects of antimalarials (Jimenez-Diaz et al., 2009), though this has yet to be widely used.

Five species of *Plasmodium spp.* cause disease in humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*. In the cases of *P. vivax* and *P. ovale*, dormant hypnozoites are produced, which remain in the liver until they are reactivated, sometimes after many years. Several studies highlight the potential for cross-species infections by Plasmodia, which is concerning in terms of malaria control as such a reservoir would be very difficult to manage. It was only in 2008 that it was widely accepted that *P. knowlesi* can cause widespread human infections (Cox-Singh et al., 2008); prior to this it was considered principally a monkey malaria parasite, though the first infection in humans were observed some time ago (Chin et al., 1965). Recently, there have also been reports of human species infecting primates. Hayakawa et al observed the presence of *P. malariae* in chimpanzees, 30 years after they were exported from Africa to Japan (Hayakawa et al., 2009). Similarly, *P. ovale* has been observed in chimpanzees in Africa (Duval et

al., 2009). Whether these are random occurrences or a real threat has yet to be determined.

1.1.4. Clinical symptoms and pathology

Patients with malaria present with a high fever associated with the rupture of infected erythrocytes every 48 or 72 hours (depending on the species of *Plasmodium*). This is often accompanied by vomiting, diarrhoea and severe anaemia. The severity of infection varies with species and from individual to individual, indicating that the interaction of pathogen and host is a vital determinant in development of malaria. In severe cases, malaria can cause liver failure and fits, resulting in central nervous system and brain complications. These complications are associated with cerebral malaria (CM), which occurs when parasites sequester in the brain microvasculature, causing blood vessel blockage and sometimes resulting in coma (Adams et al., 2002b). CM causes death in 40% of cases and up to 20% of CM patients develop neurological sequelae (World Health Organisation, 2009). Sequestration of parasites can occur in organs such as the kidneys, which can lead to organ failure. Primigravidae pregnant women are especially susceptible to primary infection and repeated episodes of malaria, possibly due to suppressed immunity. If severe anaemia arises in pregnant women, babies can have low birthweights which leads to numerous health problems. Furthermore, patients developing severe infections may require frequent blood transfusions, increasing their risk of HIV infection as much of the blood used in malaria-endemic areas is unscreened.

1.1.5. Geographic prevalence and epidemiological studies

Malaria is endemic in 108 countries. In 2008, 243 million cases were reported and 863,000 deaths, 90% of which occurred in Africa (World Health Organisation, 2009). Of those deaths, 85% were children under the age of 5 (The All-Party Parliamentary Group on Malaria and Neglected Tropical Diseases, 2010). Across the world, the risk of malaria varies widely. Residents of sub-Saharan Africa and India are at greatest risk as those areas currently have the highest number of annual global deaths and transmission rates, while areas such as South America and China are considered low risk areas, as are Mexico and countries in the Middle

East, which have very few annual cases (World Health Organisation, 2009). The global burden is thought to be highly underestimated due to the general difficulties of monitoring diseases in developing countries (Cibulskis et al., 2007). Monitoring relies on population-based surveys and routine surveys in health facilities. However, many people do not use formal health systems, due to living in rural areas or having a lack of confidence in diagnostics. At the national level, monitoring of malaria is particularly difficult because the transmission intensity and seasons vary across geographical areas and from year to year (Cibulskis et al., 2007). Effective malaria control requires accurate monitoring to assess the risk of malaria contraction and the effectiveness of intervention techniques.

1.1.6. The socioeconomic burden of malaria

Malaria is a debilitating disease in terms of health, but it has also had a profound influence on socioeconomic development. It is a massive burden on health systems, accounting for 40% of public health expenditure worldwide (World Health Organisation, 2009). Malaria particularly affects the poor, who cannot afford drugs and have limited access to healthcare. Indeed, a decrease in household savings due to malaria has been reported (Nur, 1993). As malaria is widespread in developing countries which have poor infrastructure and limited means of transportation, the cost of seeking care is in itself a problem. Poor economic management, political unrest and widespread corruption have played a major role in maintaining and exacerbating the poor infrastructure in those countries (Breman, 2001).

As malaria particularly affects children, it is responsible for an overall reduction in universal education. Studies indicate that children miss on average 15% of school days per year due to malaria in Kenya (Brooker *et al.*, 2000). It is equally concerning that malaria is linked to changes in cognitive ability and academic achievement in parasitaemic schoolchildren (Al Serouri *et al.*, 2000). Leading on from this, family members are prevented from working because they must look after the ill children, resulting in a decrease in productivity.

Hence, economically, malaria is a major hindrance to the progression of countries where it is endemic. From 1965 to 1990, the gross domestic product (GDP) of countries in which *P. falciparum* is prevalent rose annually by 0.4%, compared to 2.3% in malaria-free countries (Gallup & Sachs, 2001) (It is important

to note, however, that malaria is just one factor amongst many that contribute to a low rise in GDP; other parasitic, bacterial and viral diseases as well as malnutrition have similarly hindered economic development in these countries). There is also a lack of foreign investment in malarious zones for fear of workers contracting the disease. This concern is not unreasonable; as an example of this, Billiton, a UK mining company, built an aluminium smelter in Mozambique and was subsequently faced with 7,000 cases of malaria and 13 related deaths among expatriate employees (Sachs & Malaney, 2002). A lack of foreign investment has further contributed to the slow progression of the economy in malarious areas (Gallup & Sachs, 2001).

1.2. *Malaria combat and control strategies*

Malaria was eradicated from Europe and Northern America in the first half of the twentieth century: swamps were drained, the insecticide dichlorodiphenyltrichloroethane (DDT) was widely used to kill mosquitoes, and cases diminished (Kitron & Spielman, 1989). However, a widespread ban of DDT in the 1970s, due to its health risks as well as increasing mosquito resistance, left the rest of the world marooned as the number of malaria cases continued to increase. Thus, malaria remains a global burden. With the long term goal of eliminating malaria, several international public and private partnerships and organisations including Medicines for Malaria Venture, the Bill and Melinda Gates foundation and the Global Fund to Fight Aids, Tuberculosis and Malaria are pushing for research into vaccine and drug development as well as vector control. Here, I discuss current vector control methods, prevention and treatment strategies.

1.2.1. Vector control

In light of the failures of DDT to control malaria vectors, alternative control measures are currently being implemented. These include insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS) which involves spraying insecticide indoors. Of the 108 endemic regions, 68 are currently distributing free ITNs and IRS is carried out in 44 countries (World Health Organisation, 2009). Unfortunately, though simple preventatives, there are fundamental problems with ITN and IRS control measures. For example, regular washing of ITNs results in the loss of

insecticide potency, therefore frequent reimpregnation with insecticide is required. Holes can easily form in the net and must be repaired for the nets to be effective protection. Moreover, some mosquitoes, especially the main malaria vectors in South East Asia, feed in the early evening rather than late at night. In cultures where groups often remain outside until late at night, ITNs are ineffective. In such cases, IRS has been similarly unsuccessful, particularly in Asia where mosquitoes tend to be exophilic. More effective control measures taking these concerns into consideration must be developed if malaria is to be eradicated.

1.2.2. Vaccine stumbling blocks

Vaccine development is a field of extensive research, but to date no vaccine has been implemented in a clinical setting. After repeated exposure to malaria, patients develop immunity against severe disease (Dubois & Pereira da Silva, 1995), giving hope that a vaccine might be achievable. However, there are many obstacles to overcome on the way to developing an effective vaccine.

The first subunit vaccine against malaria, SPf66, was developed in 1987 (Patarroyo *et al.*, 1987), with promising efficacy results; however, later studies deemed it ineffective as efficacy fell to 35% (Valero *et al.*, 1996, Beck *et al.*, 1997). The first multistage and multicomponent vaccine, NYVAC-Pf7, was developed in the 90s (Tine *et al.*, 1996). Though immune responses and a delay in parasite growth were observed in all experimental infections, complete protection only occurred in 1 of 35 vaccinated individuals (Ockenhouse *et al.*, 1998). On a more optimistic note, the most promising vaccine against *P. falciparum* to date is RTS,S, which has now reached phase 3 clinical trials (Casares *et al.*, 2010), though it is not yet in clinical use. RTS,S comprises regions of the circumsporozoite protein (CSP), found on the surface of sporozoites, the form of the parasite which replicates in the liver. To specifically target CSP to the liver, CSP is packaged inside a hepatitis B virus particle (Stoute *et al.*, 1997). This raises an antibody and cellular response, preventing invasion of hepatocytes by sporozoites. This is the first malaria vaccine that has reached phase 3 clinical trials, however, it only provides 53 % protection (Bejon *et al.*, 2008), highlighting the difficulty in obtaining high efficacy and protection rates for a malaria vaccine.

There are several reasons why development of a high efficacy vaccine is such a challenge. In general, the immunological response to malaria is complex and

poorly understood. The identification of possible protective host immune responses is challenging. Due to the number of genes present in the parasite genome (there are over 5,000), it is difficult to dissect the immune response to the parasite. Many different types of immune cell are activated during infection and it is unclear which of these is responsible for clearing the parasite or which is a “side effect” of infection. Some consider malaria to be the result of a hyperimmune response to infection, as the immune response directly contributes to pathogenesis (reviewed by Artavanis-Tsakonas and colleagues (Artavanis-Tsakonas *et al.*, 2003)). What is clear is that naturally acquired immunity to malaria involves an antibody-mediated response, targeting parasite surface proteins, erythrocyte-binding antigens and variant proteins (Bull & Marsh, 2002). Antibodies from naturally immune individuals are also capable of opsonising infected erythrocytes for phagocytosis (Groux & Gysin, 1990). Cell-mediated immunity on the other hand is poorly understood, though a CD4+ T-cell response is essential for protective immunity in a mouse model of malaria (Langhorne *et al.*, 1990). Moreover, *Plasmodium spp.* has evolved to evade the human immune system very efficiently. As an obligate intracellular parasite, its main advantage is that the parasite is only ever exposed to the immune system during stages where it needs to exit and invade new cells. For example, during asexual development, the parasite reproduces solely inside erythrocytes until it reaches the end of its development, when it exits in order to invade new erythrocytes. Newly formed parasites are released, but rapidly reinvade in a matter of seconds, thereby effectively minimising exposure to the host. Furthermore, during invasion, essential ligands are protected as they are only exposed when they are released from the apical organelles. Also, unlike other types of cell, the erythrocyte does not express major histocompatibility complex molecules on its surface (which are used to display internal peptides to the immune system). Parasites are therefore able to replicate relatively undetected, hidden away from contact with circulating antibodies.

Another complication is that since the parasite has shown the ability to develop drug resistance, it is clear that it is capable of rapid evolutionary change. Any vaccine has to take this into account – highly polymorphic proteins, for example, are probably poor components of a vaccine as the immune system would select for one polymorphism. Secondly, several variant antigen families and highly polymorphic proteins are found across the genus. An example of this is erythrocyte

membrane protein 1 (PfEMP1), a large 200-350 kDa variable protein encoded by the *var* gene family, of which there are approximately 60 members in *P. falciparum*. PfEMP1 is expressed on the surface of infected erythrocytes, where it is involved in cytoadherence (Barnes *et al.*, 1994). During the course of infection, different *var* genes are expressed. The switch in PfEMP1 gene expression is thought to be controlled by immune pressure, resulting in recrudescence peaks of infection as the parasite alters expression to avoid antibodies (Marsh & Howard, 1986). There is evidence that the acquisition of immunity against severe disease correlates with the presence of antibodies to PfEMP1 variants (Bull *et al.*, 1998; Warimwe, 2009 #2806).

Currently, new vaccines are being developed. Some of these are composed of single, highly immunogenic antigens. Examples of these are important parasite surface proteins MSP1 and AMA1 are the foci of many blood stage vaccines because blocking these proteins would prevent parasites from invading erythrocytes, thereby limiting the amplification of parasites in the bloodstream (Lazarou *et al.*, 2009). Alternatively, vaccines targeting liver stages are particularly attractive since liver stage development occurs before the onset of symptoms caused by erythrocytic development. Recently, 25% protection against experimental human infection was obtained using a chimpanzee adenoviral vector encapsulating a hybrid form of TRAP, a sporozoite protein (Duncan, 2009). On the other hand, studies looking into whole parasite vaccines are looking promising, for example, the use of irradiated sporozoites or live, genetically attenuated strains. Early studies by Nussenzweig and Kramer showed that immunisation of mice with irradiated sporozoites confers partial protection to subsequent challenge (Nussenzweig *et al.*, 1967; Kramer, 1975 #2646), and immunisation of humans with irradiated *P. falciparum* sporozoites results in 92% protection (Hoffman *et al.*, 2002). However, there are concerns about the safety of such vaccines in terms of the dose of radiation required to sufficiently attenuate the parasites (Silvie *et al.*, 2002). As an alternative to irradiation, genetically attenuated parasites are an attractive, potentially safe vaccine development strategy and have shown promising results in mouse strains and *P. falciparum*. Vaccination of mice with UIS3 (upregulated in sporozoite 3) knockout sporozoite lines results in 100% protection against further infections (Mueller *et al.*, 2005). A genetically attenuated *P. falciparum* strain with no abnormalities in the life cycle aside from an arrest in liver stage development was also recently generated by knocking out sporozoite proteins P52 and P36

(VanBuskirk *et al.*, 2009). This strain confers protective immunity in a mouse model with grafted human hepatocytes. The risk, however, with genetically attenuated strains is that whether reversion to wild type strains can occur is unknown, hence the safety of such strains as vaccines is unclear.

The Malaria Vaccine Technology Roadmap, a global strategy set up in 2006, is a list of objectives for the development of a vaccine by 2025. By this date, the goal is to have an affordable vaccine with over 80% protection against severe disease and malaria-related death, with a minimum of 4 years (preferably lifelong) protection. In addition to this, it must be protective after only a few doses, and protective against *all* stages of *all* strains of malaria parasite. Such requirements are ambitious, particularly when considering the biological problems of producing a vaccine against such a complex organism.

1.2.3. Antimalarial drugs and resistance

In combination with vector control, antimalarial therapeutics are used for prophylaxis and treatment. Quinine is still considered to be the best drug for treatment of complicated malaria, though it has severe side-effects (Padmaja *et al.*, 1999). Its synthetic 4-aminoquinoline derivative, chloroquine, is the most widely used antimalarial drug because it is currently the cheapest and most effective drug available. Antifolate-based therapies, which target folate metabolism in the parasite, are also widely used; these include proguanil, sulphadoxine-pyrimethamine and dihydrofolate reductase inhibitors. However, the fastest acting antimalarial developed to date is artemisinin (and its derivatives) (White, 1997), which is the first line treatment for severe cases of malaria. Derived from the sweet wormwood plant *Quinchaosu*, it has been used in Chinese traditional medicine for over 2000 years. Low yields are however obtained by extraction from the plant source, so the drug is relatively expensive meaning that it cannot be widely distributed. Companies such as The Artemisinin Enterprise are, on the other hand, exploring alternative sources of artemisinin to improve production and lower costs.

Despite the wide availability of antimalarial treatments, a major hindrance to the control of malaria is the emergence of drug-resistant parasite strains, which can survive or multiply despite administration of antimalarials (World Health Organisation, 1973). The first reports of chloroquine resistance were in Colombia in the 1960s (Young & Moore, 1961). *P. falciparum* and *P. vivax* strains exhibiting

resistance to proguanil were observed in West Africa in 1949 (Seaton & Adams, 1949, Seaton & Lourie, 1949) and resistance against sulphadoxine-pyrimethimine combination drugs was first evident in the 1980s (Hurwitz *et al.*, 1981). On the contrary, there have been relatively few reports of quinine resistance (Bjorkman, 1991). The causes of resistance are numerous. Endemic countries and high transmission areas are particularly at risk of drug resistance (Bloland, 2001). Counterfeit and substandard drugs are rife in many malarious zones while underdosing or use of less active drugs can lead to resistance as in these cases, the parasite is not sufficiently cleared from the system (Bloland, 2001). It is imperative that indiscriminate and irresponsible use of antimalarial drugs is banned.

Resistance arises through several different mechanisms, which are poorly understood. A well-studied example is chloroquine resistance. Resistance to chloroquine has been mapped to, among others, mutations in the chloroquine resistance transporter gene, which encodes a food vacuole integral membrane protein (Fidock *et al.*, 2000). These mutations were later shown to cause the transporter to be “leaky,” allowing free transport of chloroquine out of the food vacuole (Martin *et al.*, 2009), thereby preventing chloroquine from accumulating inside the parasite.

In an effort to thwart development of drug resistance, the World Health Organisation (WHO) recommends combination drug therapy (World Health Organisation, 2009). For resistance to arise simultaneously to two drugs which target separate pathways and have different modes of action, mutations in both pathways or a mutation that blocks both drugs at the same time would be required, which is unlikely to occur. For this reason, the WHO advises the use of artemisinin in combination therapy (ACTs) rather than monotherapy. Despite this approach, there are worrying reports of failure of ACTs on the Thai-Cambodian border (Dondorp *et al.*, 2009), suggesting emergence of local resistance to artemisinin. Problems are thought to be caused in part by ACTs taking a longer time to clear from the system compared to chloroquine-based therapies. Therefore, parasites are exposed to drugs for longer; hence, there is a higher likelihood that resistance will develop. Despite the emergence of ACT resistance, combination therapy is widely replacing single therapy and has had successes elsewhere with ACT and other combination drugs (World Health Organisation, 2009). Better controls for dosing and safety control mechanisms are being implemented by the WHO, as well as a

clampdown on counterfeits (World Health Organisation, 2009). The mass treatment of populations regardless of disease status is also being considered by the WHO and British government (World Health Organisation, 2009; The All-Party Parliamentary Group on Malaria and Neglected Tropical Diseases, 2010 #2475). As mentioned earlier, use of ITNs and IRS may result in fewer infections and thereby less resistance, if they are widely implemented. In combination with ITNs and IRS, new drugs must be developed to provide alternative treatments in patients with multidrug-resistant infections. Two recent multidisciplinary efforts to identify new lead compounds for antimalarial drug development have marked a global effort to combat malaria. Gamo *et al* and Guigemde *et al* recently presented promising studies on combinatorial compound libraries and identified novel promising lead compounds which are specific to *Plasmodium spp.* and of high potency (Gamo *et al.*, 2010, Guiguemde *et al.*, 2010). These newly identified compounds could pave the way for a new generation of antimalarial drugs with alternative modes of action. Drug resistance has led to an increase in the cost and complexity of development of new anti-parasite drugs. Biological targets must be even more carefully considered before embarking on drug discovery programs. Resistance remains an important threat, further supporting the urgent need for a vaccine, identification of novel drug targets and development of new antimalarial therapeutics.

1.3. The life cycle of the malaria parasite

Plasmodium spp. have a complex life cycle involving several rounds of invasion of different types of cell, in the vector and vertebrate host (Figure 1). *Anopheles gambiae sensu stricto* is the main vector responsible for human malaria parasite transmission in sub-Saharan Africa (Kiszewski *et al.*, 2004). Gravid female mosquitoes rely on human blood to feed their developing eggs and become infected by biting a human carrying malaria parasites. In turn, the mosquitoes transmit further infections by depositing invasive, highly motile *Plasmodium spp.* sporozoites into the dermis during a blood meal.

Once in the skin, sporozoites can remain there for several hours (Yamauchi *et al.*, 2007). Some sporozoites use their motility to travel through to the lymph organs (Amino *et al.*, 2006), while others glide across epithelial cells to the nearest blood vessel and are thereby transferred to the liver. As few as 10 sporozoites, in the case of *P. vivax*, can initiate a productive malaria infection (Ungureanu *et al.*,

1976). In the liver, sporozoites are sequestered by binding to heparan sulphate proteoglycans (HSPs) on the surface of epithelial cells. HSPs are recognised by two sporozoite surface proteins, circumsporozoite protein (CSP) and thrombospondin-related anonymous protein (TRAP) (Muller *et al.*, 1993, Ancsin & Kisilevsky, 2004). Once sequestered, sporozoites must breach the sinusoidal cell layer to gain access to hepatocytes. Two secreted proteins called SPECT and SPECT2 are essential for this process; parasites deficient in SPECT or SPECT2 cannot traverse the sinusoidal cell layer (Ishino *et al.*, 2004; Ishino, 2005 #2292). SPECT2 is a perforin-like protein which probably mediates wounding of sinusoidal cell membranes by forming large multimeric pores in the cell membrane. Subsequently, sporozoites migrate through several hepatocytes before invading a final hepatocyte. The purpose of migration is unclear and is poorly understood, but studies have shown that migration results in an increase in secretion of TRAP onto the parasite surface (Mota *et al.*, 2002). It is possible that a minimum level of TRAP is required for the establishment of hepatocyte infection. Upon invasion, CSP is proteolytically processed by a cysteine protease; this is essential for infection of hepatocytes *in vitro* and *in vivo* (Coppi *et al.*, 2005). Inside the hepatocyte, sporozoites differentiate and replicate. Within 6-14 days, the infected hepatocyte has developed into a hepatic schizont, filled with thousands of merozoites, the form required for infection of erythrocytes. Parasites induce non-apoptotic death of the hepatocyte (Sturm *et al.*, 2006) and parasite-filled vesicles called merosomes bud off and are released into the sinusoid lumen (Sturm *et al.*, 2006). Release of merozoites from merosomes marks the beginning of blood stage development, essential for the propagation of parasites in the host.

Merozoites invade and replicate asexually inside erythrocytes, eventually bursting out and reinvading new cells (Figure 2). Initially, the newly-invaded parasite forms a ring-like structure in the erythrocyte cytosol (EC), termed a ring-stage parasite. After 24 hours, in the case of *P. falciparum*, this develops into a trophozoite, the main metabolically active stage of the parasite in blood stages. During this stage, the parasite increases in size and extends into the EC. The trophozoite stages are succeeded by the start of nuclear division, which occurs by schizogony: the formation of a multinucleated syncytium and subsequent cytokinesis. This is the final stage of the asexual cycle. 48 hours after merozoite invasion, the infected erythrocyte ruptures, releasing 16 to 32 new merozoites into

the blood stream, which go on to repeat the cycle.

A small percentage of ring-stage parasites are committed to developing into gametocytes (Alano, 2007), which are precursor cells required for sexual development. Gametocytes are taken up by mosquitoes during a blood meal. Early studies by William MacCallum in 1897 indicated that there are two types of gamete which are morphologically distinct (Maccallum, 1897); these are male and female gametocytes. Upon being taken up by the mosquito, gametocytes are activated to form extracellular gametes. Activation is induced by xanthurenic acid and temperature change in the mosquito midgut (Billker *et al.*, 1998). The female gamete is fertilised by the male gamete, resulting in the formation of a zygote. Following this, the zygote develops into a motile ookinete, which invades midgut epithelial cells and develops into an oocyst. The parasite divides by sporogony, producing sporozoites, which are eventually released and migrate to the mosquito salivary glands. The glands are reached by sporozoites traversing the salivary gland wall; sporozoites are then transmitted to another human host during a subsequent blood meal, thus the life cycle continues.

1.4. Malariology and genetic manipulation in the post-genomic era

Species of *Plasmodium* have a 23-26 Mb, AT-rich genome which encodes around 5,500 genes (Gardner *et al.*, 2002, Pollack *et al.*, 1982). The *P. falciparum* genome was sequenced in 2002 (Gardner *et al.*, 2002) and annotation is ongoing (Kalume *et al.*, 2005). Completion and annotation of the genomes of a number of other *Plasmodium* species, as well as the human, mouse and *Anopheles gambiae* genomes (Lander *et al.*, 2001, Venter *et al.*, 2001, Church *et al.*, 2009, Holt *et al.*, 2002, Kalume *et al.*, 2005) has further provided a wealth of information to this field. In particular, the genome projects have opened the door for genetic manipulation of malaria parasites, especially *P. falciparum* (Wu *et al.*, 1996, Crabb & Cowman, 1996). There has been an enormous leap in terms of functional characterisation of parasite proteins, meaning that it is possible to analyse any gene, rather than studying only highly abundant and/or immunogenic proteins.

1.5. Transfection of asexual *P. falciparum* parasites

The asexual stages of the *P. falciparum* life cycle can be cultured *in vitro* in human blood (Bass & Johns, 1912). There is evidence suggesting that *in vivo*, the progression of asexual development is regulated by fluctuations in host melatonin levels, which relates to circadian rhythm (Hotta et al., 2000). In culture, synchronisation is achieved manually by isolating schizonts by density centrifugation on colloidal silica (or other methods) followed by sorbitol treatment, which destroys late-stage parasites (Trager & Jensen, 1976; Lambros, 1979 #2699). It is perhaps difficult to imagine that it has only been 15 years since transfection and genetic manipulation of *P. falciparum* blood stages were made possible (Wu et al., 1996, Crabb et al., 1997, Crabb & Cowman, 1996), considering the myriad of studies which have been published since then, making use of gene knockouts and epitope tags as well as other genetic modifications.

Parasites are able to maintain circular plasmid vectors in the form of large, stable concatamers, which results in the need to cycle parasites on and off drug to select for integrants (Crabb & Cowman, 1996). Episomal expression is similarly difficult because *Plasmodium* promoters are poorly characterised and plasmid segregation is poor (O'Donnell et al., 2002). In addition, genetically modified lines are slow to obtain as transfection methods have an estimated efficiency of only 1×10^{-6} (O'Donnell et al., 2002). This is generally attributed to the physical barriers to transfection, in that input DNA must cross the erythrocyte plasma membrane (EPM), parasitophorous vacuole membrane (PVM) and parasite plasma membrane, before reaching the nuclear envelope and entering the nucleus. In *P. berghei*, increased transfection efficiency is observed with the transfection of schizonts with linear constructs; *P. falciparum* schizonts on the other hand do not survive electroporation, therefore ring stage parasites must be transfected. Since transfection of rings is less efficient compared to schizonts, circular rather than linearised vectors must be used as they are more stable (Iwanaga et al., 2010), resulting in long periods of selection to obtain integrants.

The development of a negative selection system using herpes simplex virus-derived thymidine kinase (Duraisingh et al., 2002) has greatly improved the selection of double homologous recombination integrants (Duraisingh et al., 2003). However, use of the drug ganciclovir for negative selection can also kill parasites which are thymidine-kinase negative, i.e. where double homologous recombination has occurred (Duraisingh et al., 2002). Negative selection using a *Saccharomyces*

cerevisiae cytosine deaminase and uracil phosphoribosyl transferase chimeric gene has also been developed (Maier *et al.*, 2006, Maier *et al.*, 2009b), however this method has yet to be widely used . A high efficiency transposon-mediated integration system was also developed in 2005 (Balu *et al.*, 2005). Transposons can be used to epitope tag or fuse GFP to proteins, trap promoters or knock out function (Damasceno *et al.*, 2010). However, the transposable element used inserts at the sequence TTAA (Balu & Adams, 2006), therefore it is more applicable to mutagenesis studies than for the integration of constructs into specific loci.

1.6. Conditional knockdown and inducible systems in *P. falciparum*

Since the parasite is haploid during asexual development, it is not possible to obtain parasites where genes that are essential to parasite viability have been knocked out or modified to be deleterious to growth. For this reason, conditional knockdown approaches and inducible systems are being increasingly relied on for the analysis of function of such essential genes. However, few conditional knockdown systems have been used with any success in *P. falciparum*. Several publications purport to have ablated gene expression using RNA interference (Malhotra *et al.*, 2002, Dasaradhi *et al.*, 2005); however the lack of genes encoding key enzymes for RNA-based silencing of gene expression indicates that it is unlikely to occur in *P. falciparum* (Baum *et al.*, 2009). Therefore, it is likely that the effects observed in the aforementioned studies are results of toxicity. It is also possible that silencing occurs by an unknown mechanism that is distinct from canonical RNA interference. A novel approach to gene regulation using autocatalytic RNA, which uses ribozymes to downregulate mRNA, was recently reported (Agop-Nersesian *et al.*, 2008). Downregulation of ribozyme-regulated genes was achieved in *T. gondii*, but unfortunately was not functional in *P. falciparum*.

The tetracycline-inducible (Tet) system, first developed for mammalian systems (Gossen & Bujard, 1992, Gossen *et al.*, 1995), was adapted for *T. gondii*, then *P. falciparum* in 2005 (Meissner *et al.*, 2001, Meissner *et al.*, 2005). Two systems exist; one where transcription is turned on by use of the drug tetracycline (Tet^{ON}) or prevented (Tet^{OFF}). To date, only Tet^{ON} systems have been developed for *T. gondii* and *P. falciparum*. The gene of interest is placed under the control of a minimal, truncated promoter which leads to low levels of transcription in the

absence of a transactivating protein (TA). Regulation of transcription is achieved by expression of a fusion protein between tetracycline repressor protein (TetR), which binds tetracycline, and TA which drives high levels of transcription when bound to the tetracycline operator. This fusion protein (TetR-TA) is generally expressed under a stage-specific promoter. In the presence of tetracycline, the TetR-TA fusion protein binds tetracycline and is prevented from binding the tetracycline operator, resulting in low levels of expression. In the absence of tetracycline, the TetR-TA fusion protein is expressed and binds to the tetracycline operator, leading to high levels of expression of the gene of interest. Though successful conditional knockouts have been obtained in *T. gondii* (Mital *et al.*, 2005, Brossier *et al.*, 2008) using this system, it has had less success in *P. falciparum* (Koussis, unpublished data). The major problem with this system is that large vectors are used, which encode three gene cassettes. Constructs tend therefore to be unstable in *E. coli* and parasites, being prone to rearrangements.

Recently, a simple conditional system for the expression of transgenes from an episomal construct was devised (Epp *et al.*, 2008). Using a bidirectional promoter, levels of a transgene and a selectable marker (blastidicin S deaminase) can be regulated by altering the concentration of blastidicin S in the culture medium. High concentrations of drug are used to select for parasites carrying large concatamers with multiple copies of the transgene, thereby resulting in increased protein production (Epp *et al.*, 2008). A major advantage of this is that constructs are much smaller and more stable than traditional transfection constructs. This method is amenable for conditional regulation of second copies of proteins and could potentially be used to complement double crossover knockouts. However, whether sufficient levels of protein can be expressed using this system in order to rescue the knockout parasites is unknown. Another limitation with this system is that levels of protein are only slowly modified over the course of several weeks which is not ideal for phenotypic or complementation studies.

In 2006, a conditional system using a “destabilisation domain” for rapid downregulation of target protein levels was developed in mammalian cells (Banaszynski *et al.*, 2006), which was subsequently adapted for use in *P. falciparum* and *T. gondii* (Armstrong & Goldberg, 2007, Herm-Gotz *et al.*, 2007). Mutants of the human FK506-binding protein (FKBP), F36V or L106P, have a destabilising effect on target proteins when fused to the N- or C-terminus

(Banaszynski et al., 2006). Destabilisation is prevented by proteasome inhibitors (Banaszynski et al., 2006), therefore it is assumed that fusion proteins are targeted to the proteasome for degradation. Rapid downregulation of protein levels is achievable in mammalian cells and protection against FKBP-mediated degradation is achieved by the addition of a stabilising ligand, which is a rapamycin derivative called Shield-1. This method has been adapted for the regulation of FKBP fusion proteins in *P. falciparum* and *T. gondii* (Armstrong & Goldberg, 2007, Herm-Gotz et al., 2007). In *P. falciparum*, examples include regulation of episomally-expressed YFP levels and downregulation of endogenous falcipain-2, *P. falciparum* calpain and calcium (Ca^{2+})-dependent kinase CDPK5 (Russo et al., 2009b, Armstrong & Goldberg, 2007, Dvorin et al., 2010). The system has been exploited in *T. gondii* and has been particularly successful for dominant negative studies (Agop-Nersesian et al., 2009, Breinich et al., 2009, van Dooren et al., 2009). *P. falciparum* studies wherein dominant-negative FKBP fusions have been used have yet to be published. A major limitation of this system, however, is that some proteins are not functional as FKBP fusions, as is the case for TgMyoA (Herm-Gotz et al., 2007). It may therefore be of interest to establish whether proteins retain their intrinsic function *in vitro*. The degree of degradation also varies widely among proteins (Dvorin et al., 2010, Armstrong & Goldberg, 2007, Russo et al., 2009b) and parasites. In *T. gondii*, degradation is very rapid, occurring within 8 h of removal of Shield-1, whereas in *P. falciparum* it is much slower (approximately 24 h) (Armstrong & Goldberg, 2007), restricting the applicability of this system in *P. falciparum*. Since these studies, new mutants of FKBP have been obtained, which have a greater destabilising effect when fused to the C-terminus of the protein of interest (Chu et al., 2008). This may resolve the problem of inefficient degradation of FKBP fusion proteins in *P. falciparum*.

Other mutants of FKBP, when fused to a protein of interest, induce aggregation of fusion protein in the endoplasmic reticulum (ER) when fused to a protein of interest. Aggregation can be reversed by addition of another rapamycin derivative (Wandless, 2000, Rivera et al., 2000). These conditional aggregation domains (CAD) were successfully used to regulate expression of proteins encoded by episomal constructs, for trafficking studies on PfSBP1, an exported protein (Saridaki et al., 2008). A concern with this approach is toxicity, due to the amount of aggregated protein in the ER. This may be more of a problem when attempting to

regulate endogenous genes in long term culture. As yet, whether it is possible to use this system to regulate endogenous genes in *P. falciparum* is unknown. Also, as with the FKBP system, the drawback here is that fusion of CAD domains may be deleterious to protein function, particularly as 2-4 12 kDa domains must be fused to the protein for effective aggregation (Rivera et al., 2000).

1.7. The asexual erythrocytic cycle

The asexual erythrocytic cycle accounts for all of the symptoms associated with malaria: fever, resulting in part from the release of haemozoin into the blood; anaemia, a consequence of erythrocyte destruction and dyserythropoiesis; and splenomegaly, caused by infected erythrocytes being targeted to the spleen for destruction. During the asexual cycle, merozoites invade erythrocytes, modify the host cell extensively and divide to form daughter merozoites, which eventually exit, going on to invade fresh erythrocytes and continue the cycle.

1.7.1. Invasion of erythrocytes by merozoites

The merozoite is an ovoid cell, 1.6 μm long and 1 μm wide (Langreth et al., 1978), with a highly organised ultrastructure (Figure 3). Merozoites recognise, attach to and enter erythrocytes in a very short time frame, as has been shown by live and fixed microscopy (Dvorak et al., 1975; Glushakova, 2005 #773, Gilson & Crabb, 2009); following release from schizonts, merozoites can invade in as fast as 3 seconds (Gilson & Crabb, 2009). This highly efficient process has probably emerged to limit the exposure of essential invasion proteins to the immune system. Invasion itself occurs in essentially four stages: (i) primary, reversible attachment, (ii) reorientation and erythrocyte deformation, and (iii) irreversible attachment and tight junction formation, which is succeeded by (iv) formation of the PV.

1.7.1.1. Primary, reversible attachment

Fascinating time lapse microscopy by Gilson et al shows that soon after contact with the erythrocyte, the parasite is able to “fling and wave” the erythrocyte around (Gilson & Crabb, 2009). This primary attachment is short-lasting and reversible (Bannister & Dluzewski, 1990), involving key proteins on the merozoite surface. In EM studies, the merozoite surface appears as a thick fibrillar coat; its composition is

probably crucial as it is the first point of contact with the host cell. The merozoite surface is composed of integral and peripheral membrane proteins, generally called merozoite surface proteins (MSPs). MSPs are generally either GPI-anchored or peripherally associated with GPI-anchored proteins. They are thought to mediate weak, transient interactions with host cells during invasion. Antibodies raised against several of the MSPs block invasion, which highlights their important function in merozoite invasion (for example: (Lazarou et al., 2009, Pirson & Perkins, 1985, Woehlbier et al., 2010)). Various MSPs appear to have roles in different events during invasion as localisation studies of these proteins indicate that they are not evenly distributed on the merozoite surface (Sanders et al., 2005). The GPI-anchored proteins Pf41, Pf38, Pf12 and Pf92 localise to the merozoite surface (Sanders et al., 2005), and Pf92 is refractory to genetic deletion, suggesting that it is an important molecule. Pf41, Pf38 and Pf12 all have conserved erythrocyte binding domains (Garcia et al., 2009), supporting a role for these proteins in erythrocyte invasion. Two large scale proteomic studies have since identified more GPI-anchored proteins (Sanders et al., 2006, Gilson et al., 2006) suggesting that there might be MSPs that as yet remain uncharacterised.

Of all the MSPs, Merozoite surface protein-1 (MSP1) is the most intensively studied as it is an important vaccine candidate. It covers the entire surface of the parasite and is thought to be essential for adhesion to erythrocytes (Holder & Freeman, 1982). MSP1 cannot be knocked out in blood stages (O'Donnell *et al.*, 2000), supporting its important role in this stage of the life cycle. There is evidence that MSP1 binds band 3, a major erythrocyte component (Goel *et al.*, 2003), and heparin-like molecules which naturally occur on the erythrocyte surface (Boyle *et al.*, 2010). For these reasons, it is the best candidate for mediating initial contact to the host cell. This notion is supported by the finding that many anti-MSP1 antibodies are invasion-inhibitory (Siddiqui *et al.*, 1987). A role in merozoite development in liver stages was also recently shown (Combe *et al.*, 2009). MSP1 forms a complex with at least two other proteins, MSP6 and MSP7 (Trucco *et al.*, 2001, Stafford *et al.*, 1994, Stafford *et al.*, 1996). However, the function of this complex in invasion remains unknown.

1.7.1.2. *Reorientation and erythrocyte deformation*

Following attachment, the parasite reorients itself so that the apical organelles are

juxtaposed to the erythrocyte surface. Reorientation is likely to be caused by a gradient of adhesive proteins, which increase in concentration towards the apical end of the merozoite (Lew & Tiffert, 2007). This gradient possibly results from the release of adhesion proteins from the micronemes at the apical tip, from which they redistribute across the surface from anterior to posterior. As the parasite reorients, the RBC appears to undergo rapid and extensive deformation (Dvorak et al., 1975), which is postulated to be a result of cytoskeletal rearrangements induced by contact with the merozoite (Gilson & Crabb, 2009). This may be related to an influx of Ca^{2+} during invasion (Lew & Tiffert, 2007). Whether membrane transformation is actually triggered by Ca^{2+} influx and whether secretion of apical organelles is involved is unknown. Several seconds after deformation, the erythrocyte appears to return to its usual biconcave shape (Gilson & Crabb, 2009, Dvorak et al., 1975). Gilson et al speculate that secretion of apical organelles may occur at this point, preceding erythrocyte penetration (Gilson & Crabb, 2009).

1.7.1.3. *Irreversible attachment and tight junction formation*

The weak interactions mediated by the MSPs precede the stronger, irreversible interactions which occur after merozoite reorientation. These interactions are mediated by proteins stored in micronemes and rhoptries. Secretion of these organelles may occur in response to reorientation of the parasite, which is thought to cause fluctuations in potassium and Ca^{2+} levels (Singh et al., 2010). Prime candidates for mediating these stronger attachments are the PfRH and Duffy binding-like proteins (DBLs). The DBLs are characterised by a cysteine-rich domain, originally identified as an essential *P. vivax* receptor for Duffy antigen on the surface of erythrocytes (Wertheimer & Barnwell, 1989, Fang et al., 1991). The DBL domain is conserved in many *Plasmodium* species; DBLs are the major sialic acid-binding ligands expressed by the parasite. Sialic acid is an important invasion receptor on the erythrocyte surface; early studies using neuraminidase-treated erythrocytes demonstrated a reduction in invasion of those cells (Miller et al., 1977, Mitchell et al., 1986). An essential DBL, EBA-175, was identified as an erythrocyte binding component in parasite culture supernatant (Camus & Hadley, 1985). EBA-175 binds a key erythrocyte component, glycophorin A (Orlandi et al., 1992); invasion efficiency is dramatically reduced in erythrocytes deficient in glycophorin A (Miller et al., 1977). Similarly, EBA-140 is an important receptor for glycophorin C

and its function is conserved despite it being highly polymorphic (Maier et al., 2003, Maier et al., 2009a).

The PfRH proteins, on the other hand, are important for host cell recognition and use of alternative invasion pathways. *P. vivax*, which only invades reticulocytes, relies on two apically located reticulocyte-binding proteins for the selection of erythrocytes (Galinski *et al.*, 1992). Homologues of these proteins, the PfRH proteins, were later studied in *P. falciparum*. PfRH1 is essential for sialic acid-dependent invasion (Rayner *et al.*, 2001), while knock outs of PfRH2a and PfRH2b in the sialic acid-dependent strain W2Mef results in a switch to sialic acid-independent invasion (Desimone *et al.*, 2009). These findings suggest that the PfRH proteins are important for sialic acid binding. The ability to use of multiple invasion pathways is presumably a mechanism for immune response evasion and coping with polymorphisms in erythrocyte receptors.

As the aforementioned proteins form an irreversible interaction with the erythrocyte surface, a ring of close contact called the “tight junction” forms between the parasite and erythrocyte membranes; this was initially observed by EM studies (Aikawa *et al.*, 1978). The junction moves rearward as the parasite is propelled into the erythrocyte, driven by an actin-myosin motor complex (Baum *et al.*, 2006). AMA1 is an essential, highly abundant micronemal protein which is crucial for this complex. The functional conservation of AMA1 across the *Plasmodium* genus and Apicomplexa phylum indicates an important role for this molecule in cell entry (Triglia *et al.*, 2000, Baum *et al.*, 2006, Hehl *et al.*, 2000). Around the time of invasion, AMA1 is secreted onto the surface of merozoites, where it is anchored by a transmembrane domain (Triglia *et al.*, 2000) and associates with rhoptry neck proteins (RONs) to form an essential part of the tight junction (Richard *et al.*, 2010, Collins *et al.*, 2009). Studies using an invasion-inhibitory antibody suggest that if AMA1 is prevented from binding the RONs, parasite invasion is inhibited (Collins *et al.*, 2009). Richard *et al* propose that AMA1-RON association occurs before rhoptry secretion and is important for the release of proteins from this organelle (Richard *et al.*, 2010).

EM studies on *P. knowlesi* show that, as the parasite enters the red blood cell, the fuzzy merozoite coat is shed (Aikawa *et al.*, 1978). Shedding of MSP1 was observed in 1991 (Blackman *et al.*, 1991) and it was later discovered that a Ca²⁺-dependent membrane bound “sheddase” was accountable (Blackman & Holder,

1992). A similar MSP1 processing pattern occurs in *P. knowlesi* (Blackman *et al.*, 1996), suggesting that the function and importance of MSP1 shedding is conserved across the genus. The sheddase was identified by Harris *et al* in 2005 as a micronemal subtilisin-like protease called PfSUB2 (Harris *et al.*, 2005). PfSUB2 removes ligands thought to be crucial for invasion interactions, also including AMA1, which it sheds from the merozoite surface (Harris *et al.*, 2005). Rhomboid protease ROM4 also sheds AMA1 during invasion (Baker *et al.*, 2006). The function of shedding by either of these enzymes is unclear, but it may be important for the disruption of strong interactions between the erythrocyte and the parasite, to allow the merozoite to continue entering the erythrocyte. It could also be important in terms of immunopathology by releasing many highly immunogenic proteins into the extracellular milieu.

1.7.1.4. *Formation of the PV*

As the parasite enters the erythrocyte, rhoptries secrete proteins which form the PV (Stewart *et al.*, 1986, Sam-Yellowe *et al.*, 1988, Etzion *et al.*, 1991, Bradley *et al.*, 2005). It is well established that material from the rhoptries is released during invasion and later expansion of the PV (Nichols *et al.*, 1983, Bannister *et al.*, 1986). The PV is a non-fusogenic vacuole which provides a hospitable environment for the parasite to reproduce. The membrane is particularly important for transport of nutrients, and the export of proteins into the EC. By labelling of erythrocyte membranes with fluorescent lipophilic probes and following their fate by fluorescence microscopy, Ward *et al* revealed that host cell lipids are incorporated into the PVM during invasion, but not erythrocyte surface proteins (Ward *et al.*, 1993). Furthermore, rhoptry components are present in the newly-formed membrane (Sam-Yellowe *et al.*, 1988). Completion of invasion is marked by the sealing of the erythrocyte membrane around the merozoite.

1.7.2. **Development after invasion: trophozoite stages**

Soon after invasion, the merozoite loses its apical organelles (Aikawa *et al.*, 1978), and proteins which modify the host cell are thought to be secreted from DGs (Torii *et al.*, 1989, Culvenor *et al.*, 1991). In the trophozoite stages, the parasite enlarges in size and DNA replication begins. Haemoglobin, a rich source of amino acids for

protein production, is imported from the erythrocyte host and is trafficked to a food vacuole (FV) in the developing parasite. There, it is hydrolysed by specialised enzymes which break down the haemoglobin into large fragments, and then short peptides and haem (reviewed in (Francis et al., 1997)). Early studies using purified FVs showed that haemoglobin digestion is an ordered process, initiated by aspartic proteases (Goldberg et al., 1990, Gluzman et al., 1994). As haem is a toxic by-product, it is neutralised by hydrolysis to haemozoin (Fitch & Kanjananggulpan, 1987). As well as being a source of nutrition for the parasite, degradation of haemoglobin may be important for maintaining osmotic stability inside the erythrocyte (Lew et al., 2004). During development, parasites undergo major ultrastructural changes. DG secretion occurs just before finger-like projections of the PVM begin to extend into the host cell cytosol (Torii et al., 1989), forming the tubovesicular network (TVN), Maurer's clefts and knobs. Since the erythrocyte has no organelles, the TVN is considered to be the parasite equivalent of the trans-Golgi network in mammalian cells. The function of the Maurer's clefts is unclear, but several variant antigens localise to them, including STEVOR and rifin proteins (Petter et al., 2007). This suggests that the Maurer's clefts play a role in trafficking and export of variant antigens, thereby implicating them in immune evasion. Knobs are protrusions of the erythrocyte membrane; since they are mostly composed of cytoadherence ligand PfEMP1, it is possible that they are involved in anchoring the parasite during cytoadherence (which is mediated by PfEMP1). Recently, evidence has come to light suggesting that Maurer's clefts are in fact secretory organelles which concentrate proteins before trafficking to the erythrocyte (Bhattacharjee et al., 2008).

In order to carry out these important changes to the erythrocyte, many parasite proteins are exported to the erythrocyte (Marti *et al.*, 2004)(Hiller *et al.*, 2004, Maier et al., 2008). Several exported proteins are involved in antigen presentation or cytoadherence; others are involved in cytoskeletal remodelling and formation of the Maurer's clefts (Petter et al., 2007, Maier et al., 2008). Export is essential for survival and virulence. PfEMP1, for example, is important for antigenic switching and cytoadherence (Pouvelle *et al.*, 2000); other proteins, such as RESA, described earlier, have a role in altering the structural integrity of the erythrocyte. Exported proteins must travel through the parasite and across the PVM to reach the host cell. The default pathway for proteins with signal peptides is the PV (Adisa *et*

al., 2003), but for export into the erythrocyte, an extra N-terminal targeting element termed the *Plasmodium* export element (PEXEL) is required (van Ooij *et al.*, 2008, Marti *et al.*, 2004). 400 proteins in the *P. falciparum* predicted proteome contain PEXEL sequences and are therefore hypothesised to be exported to the erythrocyte (Marti *et al.*, 2004). The PEXEL motif is removed in the ER by an aspartyl protease, Plasmeprin V (Boddey *et al.*, 2009, Russo *et al.*, 2009a). Proteins are exported through the PVM by a complex of proteins which form the export “machine” (de Koning-Ward *et al.*, 2009). EXP2, a transmembrane protein, forms the core of this complex and may be the pore through which proteins are fed, though this has yet to be determined. The heat shock protein HSP101 is implicated in driving ATP-dependent unfolding which is known to be essential for proteins to cross the PVM (Gehde *et al.*, 2008, de Koning-Ward *et al.*, 2009). It is, however, likely that proteins are exported to the host cell by other non-PEXEL mechanisms, since the parasite also exports PEXEL-negative proteins to the erythrocyte (Spielmann *et al.*, 2006b).

1.7.3. Schizogony: formation of 16-32 merozoites

By late schizogony, the nucleus has divided 4 times and up to 32 merozoites are observed per schizont. In late stage schizonts, merozoites are visible as distinct entities, where they have budded from the residual body of the schizont, which contains the haemozoin-packed FV. At this stage, the haemoglobin inside the erythrocyte is almost completely degraded and begins to form a compact, dense mass (Jamjoom, 1988). Surrounding the merozoites is the PV, which is filled with several different proteins, including serine repeat antigens (SERA), thought to be important for release of merozoites, and S-antigens, highly polymorphic and heat stable proteins (Delplace *et al.*, 1988, Coppel *et al.*, 1988, Mattei *et al.*, 1988). Finally, the PVM and EM rupture and merozoites are released in a poorly understood process called “egress.”

1.7.3.1. Merozoite egress

Egress involves a complex, protease- and kinase-regulated pathway, which has yet to be unravelled. It is an essential step in the life cycle, whereby parasites are released into the blood stream, ready to invade new erythrocytes. This process is characterised by several phenomena, which have been observed in intricate

videomicroscopy and EM studies.

1.7.3.1.1. Evidence from live imaging and ultrastructural studies

P. falciparum merozoites become mobile immediately before egress (Glushakova et al., 2005, Gilson & Crabb, 2009); *P. knowlesi* merozoites behave similarly (Dvorak et al., 1975). This activation of motility could be due to an influx of intracellular Ca^{2+} as occurs in *T. gondii* (Arrizabalaga & Boothroyd, 2004, Endo et al., 1982, Caldas et al., 2010) (though merozoites have not been shown to exhibit gliding motility as is observed in *T. gondii* tachyzoites). This may be a response to degradation of the erythrocyte cytoskeleton, or rhoptry release, as concentric membranes extruding from merozoite rhoptries were observed in late schizonts by electron microscopy (EM) (Bannister et al., 1986). It is possible that the increased movement is related to the breakdown of the PVM, a process which is essential for egress (Langreth et al., 1978).

During egress, *T. gondii* parasites appear to push their way out of the host cell (Endo et al., 1982). In *P. falciparum* there is evidence that a membranous bleb forms on the surface of the infected erythrocyte just before egress (Glushakova et al., 2005, Gilson & Crabb, 2009), which could be merozoites attempting to physically escape the host cell in a similar manner. This phenomenon was also observed in *P. knowlesi* (Dvorak et al., 1975). Following this, an increase in intracellular pressure is thought to occur just before rupture, as schizonts seem to become slightly enlarged (Dvorak et al., 1975, Glushakova et al., 2005, Gilson & Crabb, 2009). Finally, parasites appear to form a pre-rupture “flower” form, before rupturing in an explosive fashion, dispersing merozoites and the residual body (Glushakova et al., 2005).

1.7.3.1.2. Schizonts become porated prior to egress

It has been known for several years that the *T. gondii* host cell plasma membrane and PVM become permeabilised preceding egress, allowing antibodies access to the host cytoplasm and parasite plasma membrane (Black et al., 2000), which has been attributed to a perforin-like protein TgPLP1 (Kafsack et al., 2008) Recent evidence suggests that late *P. falciparum* schizonts also appear to be porated just

before egress (Glushakova et al., 2010). In this study, by incubating parasites with phalloidin (a fluorescent actin-binding molecule), labelling of intracellular structures was observed in late schizonts, but not in immature schizonts or trophozoites. This apparent “poration” was blocked by the use of poloxamine, a non-ionic surfactant previously shown to seal radiopermeabilised biological membranes (Hannig et al., 1999). The molecules responsible for poration are unknown, and the purpose of such an event is unclear. The authors speculate that it could be mediated by a perforin-like parasite protein called PfPPLP2, but this remains to be investigated.

1.7.3.1.3. Signalling cascades during egress

Egress is controlled (at least in part) by signalling molecules and phosphorylation-dependent signalling cascades. Conditional knockdown of the Ca^{2+} -dependent protein kinase CDPK5 results in a blockage in egress; parasites are still viable when mechanically released from schizonts (Dvorin et al., 2010), implying that CDPK5 is involved in the final stages of egress, just before merozoite release. Another kinase, CDPK1, is also implicated in egress, since treatment of parasites with a specific CDPK1 inhibitor blocks rupture (Kato et al., 2008). As these data imply that kinases are important during egress, it is perhaps reasonable to assume that phosphatases will be involved in the same pathway, conceivably as negative regulators. The phosphatase inhibitor okadaic acid prevents invasion (Dluzewski & Garcia, 1996), implying that phosphatases are involved in asexual stages. Furthermore, there is evidence that protein phosphatase 1 (PP1) plays a role in *P. falciparum* egress as when parasites are treated with a PP1 inhibitor, egress is prevented (Blisnick et al., 2006). The authors attribute this effect to hyperphosphorylation of PfSBP1, a Maurer’s cleft protein; however, there could be other PP1 substrates that were not identified in this study, which require dephosphorylation during egress. The importance of phosphatases in merozoite egress remains poorly understood.

Several signalling molecules are used by apicomplexan parasites, some of which are important for invasion and egress. In *T. gondii*, there is direct evidence for a role of Ca^{2+} in egress as treatment of parasites with the Ca^{2+} ionophore A23187 induces premature egress (Caldas et al., 2010, Endo et al., 1982), while egress is prevented by chelation of intracellular Ca^{2+} (Mondragon & Frixione, 1996). In *P. falciparum*, few studies have investigated the importance of Ca^{2+} in egress, though

several proteins involved in egress require Ca^{2+} as a cofactor (such as CDPK5 mentioned above). An example of is calpain, a Ca^{2+} -dependent erythrocyte protease which is essential for egress (Chandramohanadas *et al.*, 2009). As the erythrocyte has no Ca^{2+} stores, it is possible that Ca^{2+} is released from the parasite and activates calpain. Other signalling molecules are involved in parasite egress, such as abscisic acid, a plant-like hormone, which is a regulator of cyclic ADP ribose (cADPR) levels. Abscisic acid regulates cADPR production in *T. gondii* and is involved in development and egress (Nagamune *et al.*, 2008). Whether a similar molecule is involved in *P. falciparum* egress is unknown. Cyclic guanosine monophosphate (cGMP) appears to be involved in the final stages of schizogony; Taylor *et al* identified a cGMP-dependent protein kinase (PKG) which is essential in blood stages (Taylor *et al.*, 2009). Treatment with a PKG inhibitor, compound 1, results in a block in schizont rupture (which does not occur when transgenic parasites with a gatekeeper mutation are treated with compound 1). Interestingly, in contrast to CDPK5 knockdown parasites, compound 1-treated merozoites were not viable when schizonts were mechanically ruptured (Dvorin *et al.*, 2010), suggesting that PKG acts upstream of CDPK5. This, and whether PKG acts directly on CDPK5, has yet to be confirmed.

1.7.3.1.4. Breakdown of the PVM and EPM

For parasites to escape the schizont, this requires rupture of both the PVM and EPM. Evidence for egress via a non-explosive fusion of the PVM and EPM was presented by Winograd *et al* using live video microscopy; merozoites were released through a single site in the erythrocyte membrane (Winograd *et al.*, 1999). This model is supported by early EM studies of schizont egress where it appears that a residual membrane is left behind (Dvorak *et al.*, 1975), now known as the residual body. Contrary to this, in a study by Glushakova *et al*, fluorescence and differential interference contrast microscopy was used to analyse live schizonts (Glushakova *et al.*, 2005). Analysis of membranes remaining after egress ruled out a membrane fusion event, since parasite- and erythrocyte-derived membranes were segregated. Treatment with positive-curvature amphiphiles, which inhibit rupture of cell membranes, did not prevent this process, again indicating that membrane fusion probably does not occur during egress.

Ultrastructural evidence indicates that in late schizonts, the PVM is absent

(Langreth *et al.*, 1978) and PV-localised proteins appear to flow into the erythrocyte cytosol before merozoites are released (Wickham *et al.*, 2003). Using fluorescent proteins targeted to the PV and EC, Wickham *et al.* provided the first evidence that egress is a 2-step mechanism involving breakdown of both of these membranes. The order in which the PVM and EPM rupture has been a subject of much debate. Soni and colleagues treated parasites with cysteine protease inhibitor E64 (which prevents egress) and used IFA to determine whether the PVM or EPM ruptures first (Soni *et al.*, 2005). In this study, staining of very late E64-treated schizonts revealed clusters of PVM-associated merozoites, which did not co-localise with erythrocyte band 3, thus suggesting that the EPM had already ruptured and the PVM remained. Analysis of similar E64-induced merozoite clusters by confocal laser microscopy indicated that EXP1, a PVM protein, localises to the periphery of these clusters (Gelhaus *et al.*, 2005), confirming Soni and colleagues' findings. Gelhaus *et al.* also showed that the fluorescent cysteine protease inhibitor bADA blocks egress by inhibition of EPM rupture, not PVM rupture, as confirmed by IFA using anti-EXP2 (a PVM marker) and anti-glycophorin (an EPM marker) antibodies.

On the contrary, other studies indicate that the PVM probably ruptures first, followed by rupture of the EPM. Salmon and colleagues treated schizonts with E64 and observed an inhibition of PVM rupture, not EPM, supporting the notion that the PVM must rupture before the EPM (Salmon *et al.*, 2001). It is possible that the discrepancies relating to the effect of E64 between the aforementioned studies and Salmon *et al.* relate to the timing of treatment. Salmon *et al.* found that E64 only blocks rupture when applied to immature schizonts (Li *et al.*, 2002), not late stages (Salmon *et al.*, 2001), suggesting that the cysteine protease target is active in early egress-related events. Glushakova *et al.* later categorically showed by live videomicroscopy and EM studies that E64 acts during the last few minutes before egress, and blocks EPM rupture, not PVM rupture, in a reversible manner (Glushakova *et al.*, 2008). Recent studies indicate that host-derived cysteine protease calpain-1 is essential for egress (Chandramohanadas *et al.*, 2009); calpain-1 could be the target of E64. Combining these data, it is highly likely that PVM rupture precedes EPM rupture in a 2-step process, which is mediated by one or more cysteine proteases. It is possible that two cysteine proteases are involved in egress, one in PVM breakdown, which must act early on in egress, and one in EPM breakdown, such as the papain-like SERAs and calpain. To date, only calpain

has been proven to have a role in merozoite egress (Chandramohanadas et al., 2009).

1.8. Proteases have diverse functions

Proteases are enzymes, which catalyse the cleavage of peptide bonds by hydrolysis, the addition of a water molecule. Catalysis is mediated by nucleophilic attack of the carbonyl carbon of a peptide by a crucial residue in the active site of the enzyme. Hydrolytic enzymes tend to have catalytic triads or dyads, where two or three key amino acid residues interact to catalyse cleavage. Proteases are grouped into 6 main mechanistic types, depending on the active site residue required for catalytic activity: serine, glutamic acid, cysteine, aspartic, metallo- or threonine protease (Rawlings & Barrett, 1993, Rawlings et al., 2010). Within a group, enzymes tend to have conserved catalytic triads but they may vary in other active site or peripheral residues. Proteases are found across all kingdoms of life, with functions ranging from the degradation of proteins into their constituent components to the specific processing of proteins at conserved motifs.

Some proteases degrade proteins non-specifically into short peptides and amino acids. This is important for the turnover of proteins that have fulfilled their function. The proteasome, a large multisubunit protease complex degrades 80 to 90% of cellular proteins; it is also necessary for supplying internal peptides to major histocompatibility complexes, which display internal peptide antigens to circulating immune cells (Rock *et al.*, 1994). Other examples of degradative proteases include those present in the mammalian gut, such as chymotrypsin or elastase, which degrade ingested proteins so that nutrients can be absorbed by gut endothelial cells. Similarly, some bacteria secrete digestive enzymes into the extracellular environment to degrade proteins and thereby facilitate the uptake of nutrients.

While some proteases cleave with no discernible specificity, other proteases process their substrates in a highly specific manner at conserved sequences, with a defined function. Residues within cleavage sites are named according to Schechter and Berger nomenclature (Schechter & Berger, 1967), whereby cleavage occurs between subsites P1 and P1'; subsites either side of the scissile bond are termed P2, P3, P4 or P2', P3', P4', etc. Prohormone convertases remove a regulatory prodomain from hormone precursors at a specific sequence (Julius *et al.*, 1984); another example is the regulation of extracellular matrix components by secretion of

proteases, including those which degrade collagen, into the extracellular milieu. Collagenases cleave collagen at specific sequences, in order to maintain structural integrity but allowing for flexibility (Alberts, 2002).

Proteolysis may be required for protein “activation.” Most proteases are synthesised as zymogens, inactive precursors, which require proteolysis in order to be able to catalyse biological reactions. Examples of this are caspases, mediators of programmed cell death, which are present in the cell as proenzymes and some are activated by other caspases upon pro-apoptotic signalling (Alberts, 2002). Caspases disrupt the apoptotic cell by cleaving nuclear laminins, degrading DNase inhibitors and cytosolic proteins. Premature activation of caspases would result in uncontrolled cell death, therefore they are synthesised as inactive precursors and only activated in response to proapoptotic signalling. Similarly, some degradative proteases such as chymotrypsin are produced as inactive precursors, to prevent degradation of the cells in which the enzyme is synthesised. Chymotrypsin is firstly cleaved by trypsin and then self-activates. For some proteases, their activity is regulated by a change in pH, or exposure to a new environment including new chemicals or presence of other proteases. Others, such as caspase 9, are activated by dimerisation (Renuis *et al.*, 2001).

Proteolysis can also expose binding sites for protein-protein interactions or induce structural changes resulting in an alteration of function. HIV protease specifically processes viral components so that they can reassemble into infectious virions, an essential process for HIV to infect subsequent cells (Kohl *et al.*, 1988). Proteases are important in the NFκB signalling pathway, where in response to a signal, the inhibitor IκB is degraded. This exposes a nuclear localisation signal, and NFκB is transported to the nucleus to fulfil its function as a transcription factor (Alberts, 2002).

1.8.1. The role of proteases in the *Plasmodium spp.* life cycle

Several major proteolytic and post-translational modifications have been documented in schizont stages (Holder & Freeman, 1982, Delplace *et al.*, 1987, Perkins, 1988, Foth *et al.*, 2008). Proteases involved in the *Plasmodium spp.* life cycle have long been seen as potential drug and vaccine targets for malaria due to the inhibitory effect of broad-spectrum protease inhibitors on invasion of erythrocytes (Banyal *et al.*, 1981, Hadley *et al.*, 1983). There are 92 predicted

proteases in the *P. falciparum* genome (Wu et al., 2003), many of which have unknown functions in the malarial life cycle. The role of some of these proteins is summarised in Table 1.

1.8.1.1. *Protease inhibitors block different stages of the parasite life cycle*

It is evident from a wide range of protease inhibitor studies that proteases are involved in many stages of the *Plasmodium* spp. life cycle. Treatment of trophozoites with leupeptin and chymotrypsin leads to an accumulation of undigested haemoglobin in the FV and prevents further parasite development (Dluzewski et al., 1986), suggesting that haemoglobin digestion is vital to the progression of the asexual cycle. Proteasome inhibitors also prevent *P. falciparum* development, which is attributed to a family of threonine proteases encoded by the genome (Gantt et al., 1998, Wu et al., 2003).

As mentioned, earlier, merozoite egress in blood stages involves a proteolytic cascade. Treatment of asexual parasites with cysteine and serine protease inhibitors results in blockage of egress (Glushakova et al., 2005, Salmon et al., 2001, Soni et al., 2005, Dluzewski et al., 1986, Hadley et al., 1983, Glushakova et al., 2008). Protease inhibitors also selectively inhibit PVM and EPM rupture, as described in 1.7.3.1.4. Interesting data examining release of merozoites from liver stage schizonts shows that destruction of PVM of liver schizonts and subsequent release of merozoites is prevented by treatment with E64 (Sturm et al., 2006), suggesting that cysteine proteases are involved in both of these processes. It is now evident that gametocyte egress from erythrocytes involves similar mechanisms and is prevented by several broad specificity protease inhibitors (Gabriele Pradel, unpublished data). Furthermore, incubation of gametocytes with E64 results in an almost complete block in oocyst production (Eksi *et al.*, 2007), suggesting that proteases are involved in sexual development within the mosquito.

Turning to invasion, proteases are implicated in this process as in the presence of protease inhibitors, invasion is abrogated. Early studies on *P. knowlesi* indicated that treatment with a broad range of protease inhibitors prevents invasion of erythrocytes; these include chymostatin and leupeptin (reversible serine and cysteine protease inhibitors), N- α -p-tosyl-L-lysine chloromethyl ketone (TLCK, an irreversible inhibitor of trypsin-like serine proteases) and L-1-tosylamide-2-

phenylethylchloromethyl ketone (TPCK, an irreversible inhibitor of chymotrypsin-like serine proteases) (Banyal et al., 1981, Hadley et al., 1983, Dejkriengkraikhul & Wilairat, 1983) . The same studies showed that incubation with pepstatin and elastatinal (aspartic and serine protease inhibitors respectively) causes a 50% reduction in invasion, suggesting that an aspartyl protease and serine protease are, in part, involved in invasion; furthermore, the metalloprotease inhibitor phosphoramidon reduces invasion by 30%. Intriguingly, in these studies, pretreatment of erythrocytes with these inhibitors did not block invasion, implying that the proteolytic activity essential for invasion is parasite-derived. On the other hand, when chymostatin or leupeptin are introduced into permeabilised and resealed erythrocytes, invasion is prevented (Dluzewski et al., 1986), which is suggestive of an erythrocyte cysteine or serine protease being involved this process.

1.8.2. Metalloproteases: poorly understood in *P. falciparum*

Metalloproteases require metal ion cofactors for catalytic activity. The two major groups are endo- or exopeptidases. Treatment of parasites with metal chelation compounds reduces invasion but not egress, suggesting that metalloproteases are involved in invasion (Kitjaroentham et al., 2006). The molecular identity of such proteases remains unknown. The *P. falciparum* genome encodes 20 genes for metalloproteases (Wu et al., 2003), some of which have been characterised at the molecular level. In particular, falcilysin, a member of the M16 family of metalloproteases (characterised by a requirement for a zinc cofactor for catalysis), is an intriguing protease as it is localised to the FV as well as vesicular structures within the parasite plasma membrane (Murata & Goldberg, 2003, Eggleston et al., 1999). Falcilysin carries out haemoglobin digestion and cleaves transit peptides for targeting of proteins to the apicoplast in two distinct subcompartments of the parasite (Ponpuak et al., 2007).

1.8.3. Aspartic proteases in *P. falciparum*

1.8.3.1. *The Plasmeprin family of aspartic proteases*

Several aspartic proteases are expressed by the malaria parasite, the most well characterised being the plasmepsin (PM) family. The family comprises ten

enzymes. PM VI, VII and VIII are expressed in the exoerythrocytic stages (Banerjee et al., 2002). Four of the ten PM colocalise with haemozoin (Banerjee et al., 2002) and are thought to be haemoglobinases. These include PM I, II, IV and histoaspartic protease (HAP), which have been studied most thoroughly. PM I, II, IV and HAP have a conserved domain structure consisting of an N-terminal pro-region containing a transmembrane domain and a mature catalytic domain (Banerjee et al., 2002). After trafficking to the FV, they are released from the membrane by non-aspartic proteolysis (Banerjee et al., 2003). More recently, proteolytic maturation has been attributed to parasite cysteine protease called falcipains (see section 1.8.4.1)(Drew et al., 2008). PM I and II are thought to initiate haemoglobin digestion by cleaving it in the highly conserved hinge region, causing unravelling of the protein inside the FV (Gluzman et al., 1994, Goldberg et al., 1991). Omara-Opyene and colleagues published a study describing the systematic deletion of PM I, II, IV and HAP in asexual blood stages, resulting in reduced growth in PMI and PMIV knockout parasites (Omara-Opyene et al., 2004). Double knockouts of PMI and PMII or PMII and HAP were also successfully obtained (Bonilla et al., 2007b). Simultaneous deletion of all four PMs resulted in slowed development and malformation of the FV (Bonilla et al., 2007a). Together, these studies indicate that the FV PMs are not essential for blood stage development, but are very important for formation of the FV.

Other PMs are unlikely to be involved in haemoglobin digestion as they do not localise to the FV. PM V resides in the ER (Klemba & Goldberg, 2005) and was recently shown to cleave PEXEL motifs on proteins destined for export to the EC (Boddey et al., 2009, Russo et al., 2009a). It is an essential protease (Boddey et al., 2009) and considering its unique function is an attractive enzyme for drug development. On the other hand, PM IX and X are expressed during schizogony (Le Roch *et al.*, 2004, Florens *et al.*, 2002); it is therefore possible that they are involved in egress or invasion. Furthermore, PM II may be involved in egress as well as haemoglobin digestion as it is able to degrade erythrocytic spectrin, band 4.1 and actin *in vitro* (Le Bonniec *et al.*, 1999). Conversely, PMs are conserved in *T. gondii*, where PM I appears to have a likely role in cell division (Shea *et al.*, 2007), raising the possibility that *P. falciparum* PMs have functions which have yet to be identified.

1.8.3.2. *Malarial signal peptidases: potential antimalarial drug targets?*

N-terminal signal peptides are used by eukaryotic cells to direct proteins to the secretory transport system. These signal sequences are proteolytically removed by signal peptidases which reside in the ER. There are two subtypes of signal peptidase, which have either an aspartic acid or serine residue in the active site; two serine-type signal peptidases are encoded by the *P. falciparum* genome (Wu et al., 2003). Treatment of infected human and mouse hepatocytes with a general signal peptidase inhibitor results in a blockage in parasite development (Parvanova et al., 2009); additionally, incubation of asexual stage parasites with the mammalian signal peptidase inhibitors (Z-LL)2-ketone and L-685,458 hinders parasite growth (Li et al., 2009). These inhibitor studies suggest that signal peptidases are important for blood stage development; furthermore, PfSPP, an aspartyl-type signal peptidase, was shown by repeated attempts at genetic disruption to be essential for blood stage development (Li et al., 2009). Signal peptidases are potentially important antimalarial drug targets as they are important for many different protein functions.

1.8.4. Cysteine proteases are important for asexual stages

1.8.4.1. *Falcipains: haemoglobinases and more*

Falcipains are malarial papain-like cysteine proteases, several of which localise to the FV. In *P. falciparum*, there are 4 falcipain enzymes: falcipain 1, falcipain 2, falcipain 2' and falcipain 3. Falcipain 1 is encoded on chromosome 1, while the other three genes are present in a 12 kb region on chromosome 14 (Rosenthal, 2004). Falcipains 2 and 2' share 99% identity in their catalytic regions, and have very similar biochemistry (Singh et al., 2006). Knockout of falcipain-2 results in a swollen FV (Sijwali & Rosenthal, 2004), which can be rescued by a conditional expression of a second copy (Armstrong & Goldberg, 2007), indicating that falcipain-2 is likely to be involved in haemoglobin digestion. Falcipain 2-knockout parasites have no growth defect, but are more sensitive to protease inhibitors (Sijwali et al., 2006). Surprisingly, despite this falcipain 2' is not upregulated in falcipain 2 knockout parasites, ruling out a compensatory mechanism by falcipain 2'

(Sijwali & Rosenthal, 2004). Instead, falcipain 3 appears to be upregulated, which is essential for blood stage development (Sijwali et al., 2006). Though falcipain 3 is able to hydrolyse haemoglobin *in vitro* (Sijwali et al., 2001), in contrast to the other falcipains, falcipain 3 is upregulated later in the life cycle, implying that its major function is not as a haemoglobinase. Its main function remains unknown, but to date it has not been knocked out, suggesting that whatever the function might be, it is important for blood stage development. The function of falcipain 2 is also unclear, as it cleaves erythrocytic ankyrin and band 4.1 *in vitro* and there is evidence that it is responsible for the processing of those proteins in late blood stages (Dua et al., 2001, Hanspal et al., 2002). This suggests that falcipain 2 may be involved in egress or remodelling of the host cell during late schizogony.

Falcipain 1 was originally thought to be involved in invasion by use of an activity-based probe YA29 (Greenbaum et al., 2002). However, later studies showed that it is not essential in blood stages, and YA29 inhibited invasion in the absence of falcipain 1 (Sijwali et al., 2004). Upregulation of other falcipains to compensate for loss of falcipain 1 was not ruled out by this study. The role of the other falcipains in invasion and egress remains unclear, therefore further investigation is required.

1.8.4.2. *Calpain proteases are important for parasite development*

Calpain is a Ca^{2+} -dependent, multifunctional cysteine protease with roles in cytoskeletal remodelling and the cell cycle. In 1991, Olaya et al showed that addition of calpain inhibitors to culture medium of *P. falciparum* results in a substantial decrease in erythrocyte invasion (Olaya & Wasserman, 1991). Leading on from this, a conditional knockdown of *P. falciparum* calpain, which is essential in blood stages, was shown to result in a defect in pre-S-phase development with no effect on invasion efficiency (Russo et al., 2009b). Erythrocytes also harbour a calpain called calpain-1; egress is prevented in calpain-1-depleted human erythrocytes and invasion is dramatically reduced (Chandramohanadas et al., 2009).

1.8.4.3. *The elusive serine repeat antigens (SERA) are implicated in egress*

The SERA family is a family of papain-like proteins which are highly conserved across *Plasmodium* species yet are absent in other apicomplexan parasites (Rosenthal, 2004). The *P. falciparum* genome encodes 9 SERA genes, all of which are located on chromosome 2 apart from SERA9 (McCoubrie et al., 2007). The SERAs appear to have evolved from genetic duplication events, resulting in two groups of genes; one encodes papain-like proteins with a conserved cysteine residue in the putative active site, and the other where the cysteine is mutated to a serine residue (Hodder et al., 2003). This is mirrored in the *P. berghei* genome, though there are only 5 SERA genes (Putrianti et al., 2009). The identification of cysteine- and serine-type SERAs has prompted speculation that the cysteine-type SERAs are true papain-like proteases, and the serine-type have evolved to have a different function. One possibility is a role in transesterification; replacement of the catalytic cysteine of cathepsin L (a papain-like enzyme) with a serine residue results in loss of proteolytic function but gain of silica-condensing activity (Fairhead et al., 2008).

Several studies indicate that the SERAs may all localise to the PV. SERA5 localises to the PV in late *P. falciparum* schizonts (Knapp et al., 1989, Delplace et al., 1988). To date, the subcellular locations of the other SERAs remain unclear. Early IFA studies claim that SERA3, SERA4 and SERA6 are located in the PV (Aoki et al., 2002), though the resolution of these images is insufficient to enable determination of the exact location of these proteins, this study also lacks EM evidence for PV localisation. Early EM evidence for PV localisation of SERA6 was published by Knapp et al however, the antibody used appears to have high background, therefore it is difficult to decipher a specific signal for SERA6 (Knapp et al., 1991). It is, however, likely that they do localise to this compartment because they all have predicted secretory signal peptides (Yeoh et al., 2007, Knapp et al., 1991). PfSERA4, PfSERA5 and PfSERA6 are all proteolytically processed prior to egress of blood stage schizonts. PfSERA5, the best characterised SERA, is cleaved into 3 fragments of 47 kDa, 56 kDa, and 17 kDa (Li et al., 2002, Delplace et al., 1988). In the final stages of egress, the 56 kDa fragment is further processed to 50 kDa, an event which is inhibited by leupeptin (Delplace et al., 1987, Delplace et al., 1988, Debrabant & Delplace, 1989). PfSERA4 and PfSERA6 are probably similarly processed (Miller et al., 2002, Yeoh et al., 2007). There is also evidence that PbSERA3 (homologue of PfSERA6) is cleaved in *P. berghei* liver stages

(Schmidt-Christensen *et al.*, 2008). In 2007, Yeoh and colleagues showed that PfSUB1 is directly responsible for the processing of PfSERA4, PfSERA5 and PfSERA6 (Yeoh *et al.*, 2007). The same study indicates that inhibition of PfSUB1 with a specific compound blocks egress. p50 and p56 are commonly thought to be the functional forms of all SERAs due to homology with papain. Structural studies show that the papain-like domain and p56 are similar in 3D structure to papain, though there are some structural anomalies (Hodder *et al.*, 2003, Hodder *et al.*, 2009). In 2003, Hodder and colleagues published a report suggesting that recombinant SERA5 has chymotrypsin-like activity (Hodder *et al.*, 2003); though this could not be confirmed by our group (Stallmach, unpublished data). As yet any enzymatic function of any SERA family members has yet to be firmly established.

PfSERA5 appears to be refractory to genetic deletion in *in vitro* culture of *P. falciparum* (Miller *et al.*, 2002, McCoubrie *et al.*, 2007), but a recent study in *P. berghei* convincingly showed complete genetic ablation of PbSERA1 and PbSERA2, homologues of PfSERA4 and PfSERA5 respectively (Putrianti *et al.*, 2009), with no obvious phenotype in all life cycle stages. This suggests that PfSERA4 and PfSERA5 may also be non-essential genes, in turn suggesting that PfSERA6, homologous to PbSERA3, may be the only essential SERA in *P. falciparum* asexual blood stages. It is, however, difficult to know which of these proteins are actually orthologous in function as complementation studies have yet to be attempted. These findings do not exclude a role for the SERAs in the immunopathology of malaria.

There is evidence that the SERAs play an important role in invasion and egress in asexual stages. Antibodies against SERA5 are invasion-inhibitory (Pang *et al.*, 1999), which could be related to evidence that the N- and C-termini bind of SERA5 to merozoites (Li *et al.*, 2002, Puentes *et al.*, 2000). Knockout of *P. berghei* SERA8 (ECP-1, egress cysteine protease 1), results in prevention of egress from oocysts (Aly & Matuschewski, 2005). When the SERA8-knockout oocysts were mechanically disrupted, sporozoites remained motile and infectious. In summary, the SERAs are expressed during schizogony and have a suitable subcellular location to be involved in egress, and they are proteolytically processed by PfSUB1, which is essential for egress (see section 1.9.5).

1.9. Serine proteases are found throughout nature and have

diverse functions

Serine proteases are widespread throughout nature and are very diverse in function. There are four main clans of serine protease, grouped according to structural homology. The two largest are the chymotrypsin- and subtilisin-like proteases, which have very similar catalytic triads but can be distinguished by their very different protein scaffolds, illustrating that these clans are an example of divergent evolution. The chymotrypsin-like clan comprises the well-studied digestive proteases chymotrypsin, trypsin and elastase. Though similar in structure, they have very different substrate specificities. Chymotrypsin cleaves peptides after a bulky, hydrophobic residue; while trypsin requires a positively charged amino acid residue and elastase a small neutral residue. Rhomboids are unusual serine proteases, capable of intramembrane proteolysis i.e. cleavage of proteins within a phospholipid bilayer. Initially discovered as playing a role in embryogenesis in *Drosophila melanogaster* (Urban et al., 2001), rhomboids have now been identified in nearly every organism sequenced to date. Rhomboids have a preference for small residues at subsite P1 and a proline at P1', among other sequence requirements (Strisovsky et al., 2009); but the most important requirement for proteolysis is that the cleavage site lies in the transmembrane domain of the substrate. Another type of serine protease, caseinolytic proteases (Clp), tend to associate into proteasome-like multimeric complexes with ATPases (Chandu & Nandi, 2004) and are involved in ATP-dependent degradation of intracellular proteins. They tend to cleave sequences between methionine or leucine and alanine, and tend to require magnesium for catalysis.

1.9.1. The importance of serine proteases in *P. falciparum*

The genome encodes 16 serine proteases (Wu et al., 2003), including Clp proteases, rhomboid intramembrane proteases and subtilisin-like proteases. Surprisingly, genes encoding chymotrypsin-like enzymes are lacking from the genome. Serine protease activity is known to be important for *P. falciparum* blood stage development, as treatment of parasites with broad specificity serine protease inhibitors blocks parasite development (Banyal et al., 1981, Dejkriengkraikhul & Wilairat, 1983, Hadley et al., 1983, Dluzewski et al., 1986, Delplace et al., 1988, Arastu-Kapur et al., 2008). Five genes encoding Clp-like enzymes are found in the

P. falciparum genome (Wu et al., 2003). Of these, only one has been characterised at the molecular level. PfClpP was localised to the nucleus, where the authors suggest it is involved in degradation of transcription factors (Lin et al., 2009). The *P. falciparum* genome encodes nine genes for rhomboid-like proteins (Dowse & Soldati, 2005) and that of *T. gondii* also has 6 genes encoding rhomboid-like proteins (Brossier et al., 2008). Like *P. falciparum*, *T. gondii* secretes proteins from the micronemes onto its surface during invasion. Several secreted micronemal proteins (MICs) are subjected to proteolysis during invasion, and this is mostly mediated by rhomboids on the tachyzoite surface. Conditional downregulation of TgROM4 in *T. gondii* results in parasites that are unable to form a tight junction and have retarded surface protein shedding (Buguliskis et al., 2010). In *P. falciparum*, PfROM4 has been shown to cleave AMA1 *in vitro* (Baker et al., 2006), in a manner similar to rhomboid cleavage of *T. gondii* MICs (Triglia et al., 2009). PfROM1 acts similarly to TgROM4 and PfROM4 by shedding EBA-175 during invasion (Baker et al., 2006).

1.9.2. Subtilisin-like proteases share a common catalytic mechanism

Subtilisin-like proteases were originally identified in prokaryotes but were later shown to be present in viruses and eukaryotes. There are over 200 subtilisins, which are subdivided into 6 families by sequence homology: subtilisin, thermitase, proteinase K, lantibiotic peptidase, kexin and pyrolysins (Siezen & Leunissen, 1997). All of these enzymes require Ca^{2+} for stability and activity. Subtilisin E, the classical subtilisin, was isolated from *Bacillus subtilis*. Bacterial subtilisins generally have wide specificity and are degradative, secreted enzymes. Mammalian subtilisins, on the other hand, are mostly highly specific in their activity, and are responsible for maturation of their substrates. One example is furin, also known as a prohormone convertase. Furin specifically cleaves its substrates, such as proalbumin, at a dibasic processing site with the consensus sequence Arg-X-(Arg/Lys)-Arg (Molloy et al., 1992). Several pathogens are dependent on furin or furin-like proteases for entry into or exit from host cells, e.g. some HIV or *Bacillus anthracis* proteins must be proteolytically activated by furin (Molloy et al., 1992).

It is to the protein engineering field that molecular biologists owe their vast knowledge of subtilisins, since subtilisin-like proteases have been widely exploited

in industry (mostly as additives to biological washing powders) and have been extensively manipulated in protein engineering applications. Since the 1980s, subtilisin E, subtilisin BPN' and savinase have used as model enzymes for protein engineering studies. In fact, to date over half of the amino acids of subtilisin BPN' have been mutated (reviewed in (Bryan, 2000)). From these extensive studies, it became clear that all subtilisins use a conserved mechanism for catalysis of peptide bond cleavage. The active site consists of a catalytic triad of aspartic acid, histidine and serine residues, which use a charge relay for catalysis. The significance of the catalytic residues was dissected in 1988 by replacement of each residue in *Bacillus amyloliquefaciens* subtilisin with an alanine residue (Carter & Wells, 1988); mutation of the serine and histidine active site residues were found to have the greatest effect on catalysis. Interestingly, mutation of the catalytic serine to a cysteine residue in subtilisin Novo results in loss of proteolytic activity but gain of esterification activity (Philipp *et al.*, 1979). The hydroxyl group of the serine acts as a nucleophile, attacking the carbonyl carbon of the peptide bond. The carboxyl group of the aspartic acid residue forms a hydrogen bond with one of the nitrogens in the histidine imidazole ring, making it electronegative. The other nitrogen in the imidazole ring is then able to accept hydrogen from the serine hydroxyl group in order to coordinate attack of the peptide bond. Additionally, glycine and serine residues contribute to the stability of the reaction by creating an oxyanion hole, where glycine and serine donate hydrogens for hydrogen bonding. Chymotrypsin-like enzymes, despite having a different scaffold, perform enzymatic catalysis in an identical manner.

1.9.3. Subtilisins across the Apicomplexa phylum

Subtilisins have been identified in several Apicomplexa species. The *T. gondii* genome encodes 12 subtilisin-like serine proteases, only some of which have been functionally characterised. Of these, *T. gondii* subtilisin-like protease 1 (TgSUB1) is GPI-anchored and micronemal and is cleaved upon release of micronemes into small fragments (Miller *et al.*, 2001). It was recently shown that *T. gondii* SUB1 removes adhesive complexes of secreted microneme proteins from the tachyzoite surface (Lagal *et al.*, 2010). *N. caninum* SUB1 is also found in micronemes (Louie *et al.*, 2002); *C. parvum* SUB1 is also located at the apical end of the parasite (Wanyiri *et al.*, 2009), though to which organelles it localises is as yet unknown.

Since treatment of parasites with serine and subtilisin protease inhibitors blocks parasite infection *in vitro* (Feng et al., 2007), it is likely that *C. parvum* SUB1 is involved in invasion of host cells. *B. divergens* SUB1 also appears to be important for merozoite invasion as invasion is prevented in the presence of anti-SUB1 antibodies (Montero et al., 2006). These studies suggest that subtilisins have a conserved role in apicomplexan asexual stages, in proteolysis during host cell invasion.

1.9.4. *P. falciparum* subtilisin-like serine proteases

The *P. falciparum* genome encodes 3 subtilisin-like serine proteases: PfSUB1, PfSUB2 and PfSUB3 (Blackman et al., 1998, Hackett et al., 1999, Aurrecochea et al., 2009). The catalytic domains of all three are most closely related to bacterial subtilisin family A or pyrolysine-like family F subclass (Withers-Martinez et al., 2004, Siezen & Leunissen, 1997). The *pfsb1* and *pfsb2* genes are refractory to genetic disruption in blood stages (Yeoh et al., 2007, Hackett et al., 1999), and the gene encoding PfSUB3 can be knocked out in blood stages with no obvious phenotype, indicating that it does not play an essential role *in vitro* during this part of the life cycle (O'Donnell and Blackman, unpublished data). Little is known about PfSUB3 other than that it is expressed, and is not restricted to expression during erythrocytic stages (Bozdech et al., 2003, Le Roch et al., 2004). PfSUB2 was mentioned earlier, as the micronemal sheddase responsible for removal of merozoite surface ligands during invasion (Harris et al., 2005). Its *P. berghei* homologue is also essential for invasion (Uzureau et al., 2004). It is significantly larger in size than PfSUB3 and PfSUB1 due to the presence of a transmembrane region and cytoplasmic domain (Harris et al., 2005).

1.9.5. PfSUB1 expression, localisation and processing

Blackman et al identified PfSUB1 in 1998 using a PCR screen for subtilisin-like proteases in *P. falciparum* cDNA, using oligonucleotides based on conserved regions across the subtilisin family (Blackman et al., 1998). Sequencing of the resulting PCR product revealed it to have significant homology to subtilisin-like serine proteases. TgSUB1 is the closest apicomplexan relative to PfSUB1 in terms of amino acid similarity (51% identity), and interestingly was first identified by use of

cross-reactive anti-PfSUB1 antibodies (Miller et al, 2001). However, TgSUB1 and PfSUB1 are not orthologues; TgSUB1 shares more similarity with PfSUB2 as they are both micronemal (Miller et al., 2001; Barale, 1999 #2428) and important for shedding of parasite invasion ligands (Harris et al., 2005; Lagal, 2010 #2813).

PfSUB1 is encoded by a single-copy gene, *pfsub1*, and is synthesised as a 78 kDa pre-pro-protein (Blackman et al., 1998). The signal peptide is removed during secretory transport through the endoplasmic reticulum, and the prodomain by autocatalytic cleavage after folding is complete. As with most subtilisins, the prodomain acts as an intramolecular chaperone, evident from the fact that attempts to express recombinant PfSUB1 without it results in unfolded PfSUB1 (Withers-Martinez *et al.*, 1999). Again, like other subtilisins, the prodomain of PfSUB1 is also a nanomolar inhibitor of the mature PfSUB1 protease (Jean *et al.*, 2003); indeed, it has higher inhibitory potency than the best small molecule inhibitor of PfSUB1 known to date (Janse & Waters, 2007).

In vivo, removal of the PfSUB1 prodomain by autocatalytic cleavage at the internal sequence $_{215}\text{LVASD}_{\downarrow}\text{NIDIS}_{224}$ releases a 54 kDa active protein (Figure 4) (Sajid *et al.*, 2000). It is unknown when the prodomain and PfSUB1 dissociate. PfSUB1 is then further processed to a 47 kDa species (Figure 4), as a result of a further Ca^{2+} -dependent cleavage event at $_{247}\text{EVEND}_{\downarrow}\text{AEDYD}_{256}$. The importance of this cleavage event is unclear as both p54 and p47 have catalytic activity when expressed *in vitro* (Sajid *et al.*, 2000). Conversion of p54 to p47 may be regulated by dipeptidyl peptidase 3 (DPAP3), since chemical inhibition of this enzyme, prevents production of p47 (Arastu-Kapur *et al.*, 2008) though how this occurs remains unclear. This study does not account for a previous observation that p54 to p47 conversion occurs during insect cell expression *in vitro* (Sajid *et al.*, 2000), indicating that processing could be sporadic or an intrinsic activity of PfSUB1.

1.9.6. The function of PfSUB1 in *P. falciparum*

Several studies have highlighted the importance of PfSUB1 in asexual stages. Using antibodies raised against *E. coli*-derived recombinant PfSUB1, the parasite protein was localised to merozoite exoemes in late schizonts (Figure 5) (Blackman *et al.*, 1998, Yeoh *et al.*, 2007). IFA studies and analysis of the processing of its substrates indicates that PfSUB1 is released into the PV just prior to egress (Figure 5) (Yeoh *et al.*, 2007). Attempts at knocking out PfSUB1 have failed, suggesting

that it is essential for maintenance of the asexual blood stage life cycle. Since *pfsub1* is thought to be an essential gene, it could be important for drug discovery. Yeoh et al demonstrated that treatment of asexual parasites with a PfSUB1-specific inhibitor, MRT12113, results in arrest of exit and hindered invasion, implying that PfSUB1 is important for parasite release and priming merozoites for invasion (Yeoh et al., 2007, Koussis et al., 2009). Furthermore, merozoites released from schizonts in the presence of this compound are not invasion-competent (Koussis et al., 2009). This work represents one of the first indications that there is a link between invasion and egress in *P. falciparum*, that in the final stages of egress the parasites are being prepared for the next task. A higher concentration of MRT12113 is required to block egress compared to invasion, suggesting that PfSUB1 is more important for egress (Koussis et al., 2009, Yeoh et al., 2007). PfSUB1 is, therefore, thought to be a key player in erythrocyte exit by merozoites.

Preceding merozoite release, PfSUB1 is responsible for the proteolytic maturation of a small repertoire of substrates within the PV (Yeoh et al., 2007). This includes SERA5, the most abundant *P. falciparum* protein. PfSUB1 also processes MSP1, MSP6, MSP7 (Koussis et al., 2009). Proteolytic maturation of MSP1 and SERA5 is also prevented when compound 1 is used (Dvorin et al., 2010), suggesting that the regulation of exoneme secretion and the release of PfSUB1 into the PV is PKG-dependent. PfSUB1 is thought to be involved in a proteolytic pathway which leads to egress. Little is known about the timing of its activity, though it is speculated that it is active in the PV just prior to breakdown of the PVM. This is because SERA5 is processed just prior to egress (Delplace et al., 1988) and PfSUB1 is observed being released from schizonts in the process of egress (Yeoh et al., 2007). Thorough analysis of its function in merozoite egress and parasite survival is necessary before PfSUB1 can be considered as a therapeutic target.

PfSUB1 can be expressed in a soluble, recombinant form in Sf9 insect cells and (Withers-Martinez *et al.*, 2002). The availability of recombinant enzyme has permitted analysis of its activity and identification of novel substrates. Characterisation of PfSUB1 specificity via examination of validated and peptide substrates has given an insight into the selective nature of PfSUB1 (Koussis et al., 2009, Blackman *et al.*, 2002). For example, there is a tendency for polar residues in the P' positions and a requirement for glycine or alanine at the P2 position.

1.10. Egress via pore-forming proteins

Intracellular pathogens use a wide array of mechanisms to get out of their host cells. Notably, *Leishmania* spp. and *T. gondii* both use a pore-forming protein to escape the PV and host cell (Almeida-Campos & Horta, 2000, Kafsack et al., 2008). It is possible that *P. falciparum*, which expresses 5 perforin-like proteins, also egresses by use of a pore-forming protein. Pore-forming proteins (PFPs) have a diverse range of functions in many different organisms. Some form ion channels or transport channels, others are important in attack by pathogenic organisms. Several organisms express pore-forming toxins which enable destruction of host cells in order for the pathogens to exit the host.

1.10.1. MACPF are conserved across all kingdoms of life

MACPFs (membrane attack complex/ perforin domain containing proteins) are soluble proteins which form a membrane pore complex in response to a physiological change, such as increased concentration of Ca^{2+} ions or proteolytic maturation. MACPFs are found in virtually every organism, from plants to mammals to protozoa. Perforin is a 67 kDa MACPF domain-containing protein in storage granules of cytotoxic T-lymphocytes and natural killer cells, and is released during immune responses into the extracellular milieu. There, high levels of extracellular Ca^{2+} ions are thought to induce oligomerisation of perforin molecules into around 15 nm polyperforin pores, resulting in activation of apoptosis. In mammals, perforin is key to cytotoxic immunity e.g. against bacterial infection (Kagi et al., 1994a, Kagi et al., 1994b). Several members of the complement protein family have MACPF domains. Complement proteins are secreted into the extracellular milieu and are involved in a protein cascade which destroys antibody-labelled cells such as bacteria. Complement factors C6 to C9 all have MACPF domains. The complement pathway terminates with assembly of a so-called membrane attack complex (MAC) consisting of factors C6 to C9, which form a 10 nm pore in the lipid bilayer. The pore allows flooding of the cell with extracellular fluids, thereby inducing lysis by increased osmotic pressure. C9 oligomerisation is inhibited by the protein CD59, which is thought to directly target the amphipathic helices important for membrane insertion (Huang et al., 2006).

In 2007, the structure of perfringolysin, a cholesterol-dependent cytolysin

(CDC) expressed by *Clostridium perfringens*, revealed that the MACPF domain is used in defence as well as attack (Rossjohn *et al.*, 1997). This protein, however, appears to be non-lytic and its mechanism of activity remains unknown. Since then, other cholesterol-dependent cytolysins have been identified in intracellular pathogens, implicating MACPFs in membrane destruction from the interior as well as exterior in the immune system. Domain IV of CDCs is important for recognition of target surfaces for pore insertion (Rossjohn *et al.*, 1997). All CDCs analysed to date have C-terminal immunoglobulin-like folds implicated in protein or lipid binding. Perfringolysin O C-terminus also has a cholesterol-binding domain essential for membrane binding (Shimada *et al.*, 2002). *Leishmania amazonensis* expresses leishporin, a cholesterol-independent cytolysin, which is lytic to erythrocytes and nucleated cells (Noronha *et al.*, 1996, Castro-Gomes *et al.*, 2009).

Not all MACPF-domain containing proteins have a lytic function. Astrotactin, a mammalian glial cell MACPF is important for neural cell migration (Adams *et al.*, 2002a). Some of the complement factors which harbour MACPF domains do not contain the amphipathic helices required for membrane insertion including complement C6. Other MACPFs include *Drosophila* torso-like protein, and apextrin from *Helicoidaris erythrogramma*. Both of these proteins are important in development of *Drosophila* and the sea anemone; to date, no lytic function has been demonstrated for these proteins. Pore-forming toxins are also used by pathogens for delivery of proteins into the host cytoplasm. For example, *Bacillus anthracis* uses the PFP protective antigen to transport lethal factor and oedema factor toxins, a Ca^{2+} -dependent adenylate cyclase and a metalloprotease respectively (Leppla, 1982).

1.10.2. Important features of the MACPF domain

The MACPF domain is a 20-30 kDa domain, mainly alpha-helical in structure, which harbours two key amphipathic helices implicated in membrane insertion by conversion to beta strands (Rosado *et al.*, 2007). A classical cysteine motif is conserved among MACPFs, as each domain forms several disulphide bonds. Some MACPFs, including human perforin-1, also have conserved Arg213 and Glu343 residues which are important for intermolecular interactions leading to pore assembly (Baran *et al.*, 2009). Flanking the MACPF domain are variable N- and C-terminal domains implicated in receptor binding. Perforin, for example, has a C-

terminal C2 Ca^{2+} -binding domain which is responsible for perforin oligomerisation (Voskoboinik et al., 2005), and an EGF-like domain which lies N-terminal to the C2 domain. Complement factors have N-terminal L2 lipid-binding motifs as well as C-terminal thrombospondin-like repeats and EGF-like domains; all of these are thought to be important for pore formation at the end of the complement cascade (Musingarimi et al., 2002). (Scibek et al., 2002).

1.10.3. Activation of MACPF pores

How MACPF-containing proteins assemble into pores is unclear; assembly most likely occurs by a variety of different mechanisms. Most bacterial pore-forming toxins are activated by host proteases, which induce oligomerisation and an “insertion-competent” pre-pore state (Bravo et al., 2007). Membrane insertion is then triggered by a decrease in pH. *L. amazonensis* Leishporin is thought to be activated by a cytosolic serine protease (Almeida-Campos & Horta, 2000). *Vibrio cholerae* similarly secretes a haemolytic toxin, which is activated by several different proteases, including haemagglutinin/protease, a major secreted protease of *V. cholerae* (Nagamune et al., 1996). *Bacillus thuringiensis* infects lepidopteran insects, where it lyses midgut epithelial cells by secretion of Cry3 toxins (Rausell et al., 2004). Cry3 toxins are processed at the N-terminus by a range of different host proteases; the cleavage is thought to result in exposure of a hydrophobic patch essential for binding of the toxin to target membranes. The activation of trialysin, a pore-forming protein in hematophagous insects, is also prevented by use of serine protease inhibitors (Allary et al., 2002). Similarly, *B. anthracis* protective antigen is proteolytically activated by furin-like proteases in order to facilitate its binding to other anthrax toxins (Singh et al., 1989, Klimpel et al., 1992, Molloy et al., 1992). Proteolysis results in protective antigen being able to associate with either lethal or oedema factor, resulting in the formation of ion-selective pores.

1.10.4. Perforin-like proteins (PLPs) expressed in apicomplexan parasites

Several PLPs harbouring MACPF domains are conserved across the Apicomplexa phylum. The *Babesia* and *Theileria* spp. genomes encode 6 PLPs, though of these only one *Babesia* spp. PLP and three *Theileria* spp. PLPs are expressed at the

mRNA level (Kafsack & Carruthers, 2010). *Eimeria* spp. encodes two PLPs, and *Neospora* spp. three. No PLPs have been identified in *Cryptosporidium* spp. The *T. gondii* genome encodes two PLPs, though only one appears to be expressed at the protein level in tachyzoites (Kafsack et al., 2008). Genetic ablation of *T. gondii* perforin-like protein 1 (TgPLP1) revealed an essential role in egress from host cells through pore-mediated disruption of the PVM and possibly the host PM. Though the TgPLP1 gene can be deleted, parasite egress is severely delayed in its absence. A 5-fold reduction in virulence was also observed in mice (Kafsack et al., 2008). Secretion of TgPLP1 was furthermore shown to be Ca²⁺-dependent. Interestingly, TgPLP1 is N-terminally processed by TgSUB1 (Lagal et al., 2010). Whether this is important for its function has yet to be determined, but it does suggest interplay between proteases and pore-forming proteins in the Apicomplexa.

Interestingly, *in silico* studies on apicomplexan PLPs indicate that there are several key features, which distance these proteins from their mammalian homologues. MACPF-domain containing proteins tend to have a signature motif Y/W-G-T/S-H-F/Y-X₆-G-G which is highly conserved (Slade *et al.*, 2008). Across the Apicomplexa, this motif is W-X₂-F/L-F/I-X₂-F/Y-G-T-H-X₇-G-G (Kafsack & Carruthers, 2010). Humans with perforin deficiency, where a disease called type 2 familial haemophagocytic lymphohistiocytosis develops, have a 50% tendency to have missense mutations in the MACPF signature motif (Baran et al., 2009), suggesting that this motif is crucial for function. Furthermore, though the C-terminal sequences of apicomplexan PLPs differ in length and sequence, they have a conserved repetitive pleated β -sheet motif (Kafsack & Carruthers, 2010). The N-termini of the apicomplexan PLPs are not conserved, nor do they show any homology to known proteins; therefore the PLP N-termini may be important for the unique function of each PLP.

1.10.5. *Plasmodium* spp. perforin-like proteins (PPLPs)

Five genes encoding PPLPs are conserved in all species of *Plasmodium* spp., information about which is summarised in Table 2. The nomenclature of PPLPs (PPLP1-PPLP5) applies to all homologues across the *Plasmodium* genus, the *P. falciparum* PPLPs are termed PfPPLPs. Strikingly, none of them have the conserved Arg213 or Glu343 residues mentioned earlier (Baran et al., 2009), suggesting a difference in pore-forming mechanism compared to canonical

perforins. The closest *P. falciparum* relative of TgPLP1 is PfPPLP1, known as SPECT2 (Kafsack & Carruthers, 2010). SPECT2 is a micronemal protein secreted from sporozoites into the extracellular milieu, responsible for wounding of sinusoidal cells in order to allow sporozoite passage through the cell layer, preceding hepatocyte infection (Ishino et al., 2005). SPECT2 knockout parasites cannot traverse the sinusoidal layer and are thereby prevented from establishing a liver stage infection. *P. berghei* PPLP3 and PPLP5 were additionally found to be important for ookinete invasion of the mosquito midgut (Kadota et al., 2004, Ishino et al., 2005). Since similar phenotypes were observed for PPLP3 and PPLP5 knockouts, the authors suggest that they have complementary functions and may interact to form a pore structure. However, no further evidence has been obtained to support these theories.

The functions of PPLP2 and PPLP4 remain unknown. Unpublished studies indicate that these genes are essential in *P. berghei* blood stages; PPLP2 was refractory to three attempts at genetic deletion (Ecker, Personal communication), PPLP4 knockout parasites could not be cloned, suggesting that PPLP4 has a crucial role in parasite survival (Ecker *et al.*, 2008). Though PPLP2 and PPLP4 appear to be important in blood stages, at least in a mouse model, the precise role of these proteins during the erythrocytic cycle remains to be revealed.

1.11. Thesis aims

The main aims of this project were to analyse the function of PfSUB1 and two perforin-like proteins PPLP2 and PPLP4 in the asexual stages of *P. falciparum*. This relied on *in vitro* biochemical analyses and *in vivo* studies of cultured *P. falciparum* parasites.

- Conditional knockdown of PfSUB1

Analysis of PfSUB1 was carried out using three different techniques. Firstly, as it is an essential protease, I attempted to generate a conditional knockdown line in order to elucidate the function of PfSUB1 *in vivo*. The FKBP destabilisation domain system, recently applied to *P. falciparum* and *T. gondii* (Armstrong & Goldberg, 2007, Herm-Gotz et al., 2007), was used. A PfSUB1-FKBP fusion was expressed in a heterologous expression system which confirmed that it is catalytically active. A non-clonal *P. falciparum* line, where FKBP was integrated into the *pfsu1* locus was obtained. As an alternative approach to conditional regulation of PfSUB1 activity,

the PfSUB1 prodomain, known to be a potent inhibitor of recombinant PfSUB1, was used to inhibit the protease *in vivo*. Using the FKBP system again, the prodomain (p31) was used a molecular switch for PfSUB1. The advantage to this compared to the previous approach is that the endogenous PfSUB1 was not be modified. *In vitro* testing of recombinant FKBP-prodomain (FKBP-p31) against recombinant PfSUB1 confirmed that the prodomain retained its inhibitory capacity when fused to FKBP. Following *in vitro* studies, the FKBP-p31 was expressed on an episome in *P. falciparum* with the aim of inhibiting PfSUB1 activity *in vivo*.

- Identification of novel PfSUB1 substrates

Second, I attempted to identify novel PfSUB1 substrates using a protease specificity modelling program PoPS (Boyd et al., 2004, Boyd et al., 2005) and by applying stringencies according to characteristics of known PfSUB1 substrates. Several putative substrates were identified. This provided the foundations for a collaborative large scale proteomics analysis of PfSUB1 substrates, by use of recombinant enzyme and schizont lysate. Specific putative substrates were then analysed by western blot and compared to physiological processing. Peptides based on cleavage sites predicted by bioinformatics were incubated with PfSUB1 and analysed by reversed phase high pressure liquid chromatography, to ascertain whether they are true PfSUB1 cleavage sites.

- Analysis of the spatiotemporal activity of PfSUB1

Third, the spatiotemporal activity of PfSUB1 was analysed. This involved *in vitro* expression and validation of a PfSUB1-sensitive fluorescent reporter containing a well-characterised PfSUB1 cleavage site present in SERA5. The reporter was shown to be PfSUB1-sensitive and to exhibit fluorescence resonance energy transfer (FRET). Following this, parasites were transfected with an episomal vector for expression of this reporter in the PV.

Figure 1. The complexities of the malaria life cycle

With the bite of an infected mosquito, 1-10 sporozoites are injected into the human host (A). The majority of sporozoites travel to the liver, and subsequently invade and replicate inside hepatocytes (B). 48 hours later, the sporozoite has divided by schizogony, resulting in production of tens of thousands of merozoites. When released from the hepatocyte, merozoites invade and replicate asexually inside erythrocytes (C). This leads to a gradual rise in parasitaemia, resulting in the onset of malarial symptoms. During erythrocytic development, a small percentage of parasites develop into gametocytes (D). These cells are important for sexual development, which occurs inside the mosquito. Male and female gametocytes are taken up by the mosquito during a blood meal, and develop into gametes inside the mosquito midgut (E). Fusion of the gametes results in a zygote, which develops into an ookinete (E). The ookinete is able to invade the midgut epithelium and develops by sporogony, forming an oocyst, filled with sporozoites (F). Once mature, the oocyst ruptures, releasing sporozoites into the midgut, which migrate to the salivary gland in preparation for another blood meal.

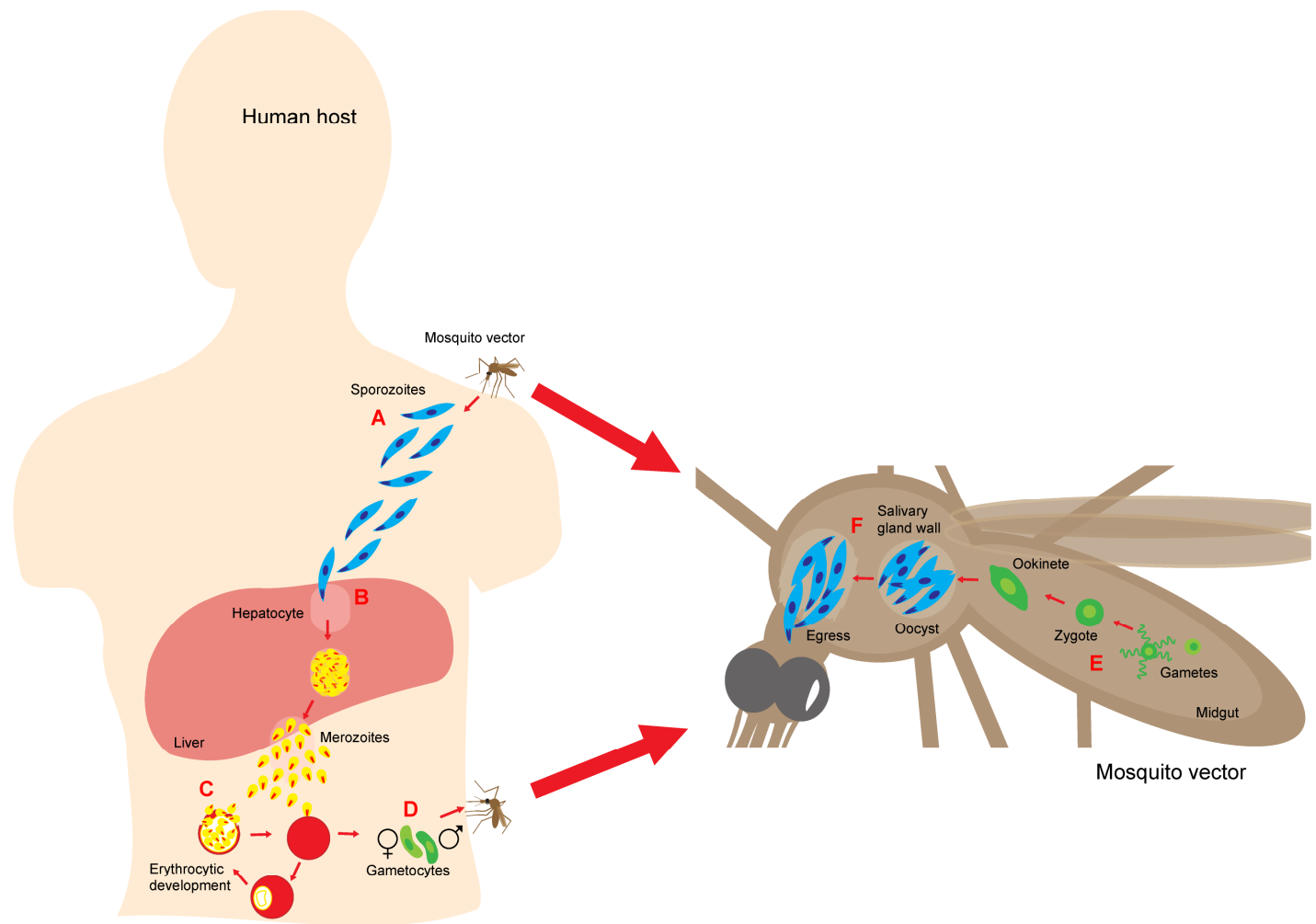


Figure 2. The asexual erythrocytic cycle of *Plasmodium* spp.

Merozoites bind to and invade erythrocytes, where they reproduce inside a PV. The ring stage develops into a trophozoite, where DNA replication and growth occurs. Finally, the trophozoite differentiates into a multinucleated syncytium called a schizont. Division by schizogony results in the formation of 16-32 merozoites. In a process called egress, merozoites are released and continue the cycle. Importantly, egress and invasion is protease inhibitor-sensitive.

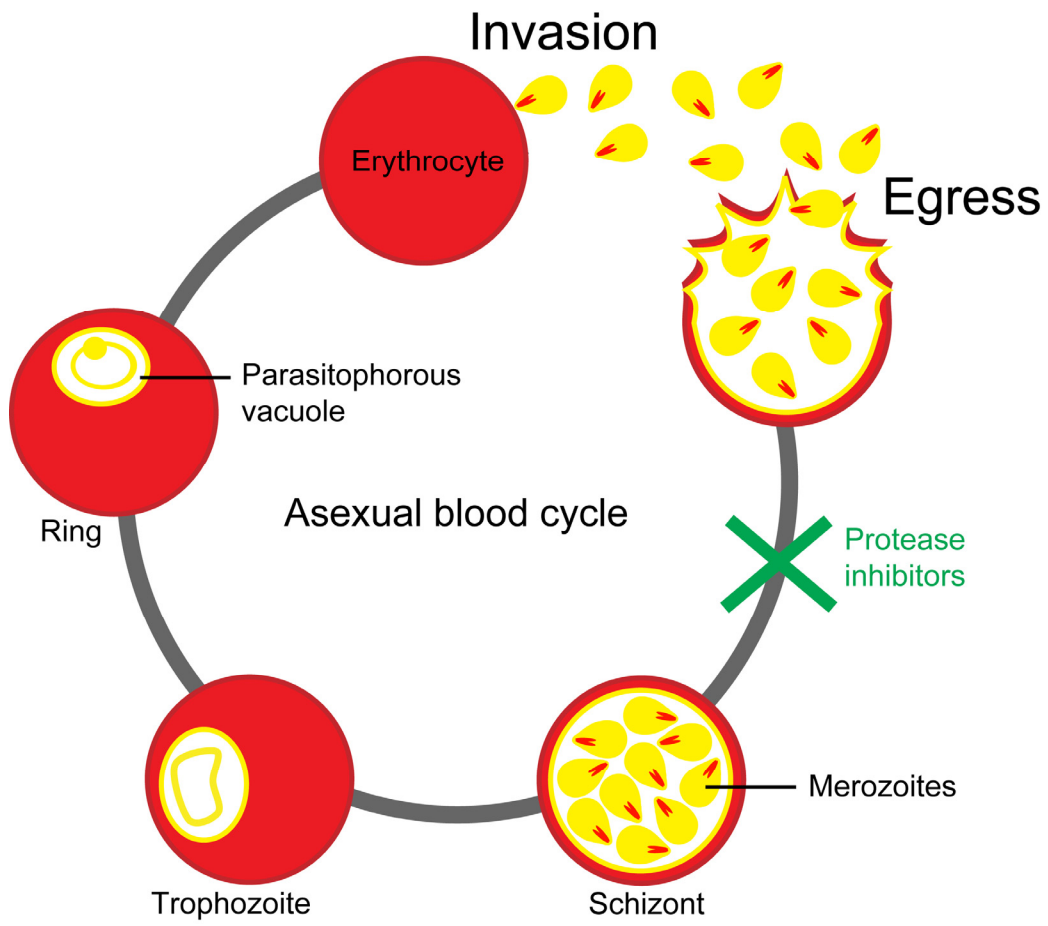


Figure 3. The ultrastructure of a *P. falciparum* merozoite.

Merozoites, the specialised zoite of the malaria blood stage cycle, have highly specialised organelles for invading erythrocytes. 2-3 microtubules are present in *P. falciparum* merozoites. Several types of secretory organelle are present, including rhoptries and micronemes, essential for invasion; dense granules, involved in host cell modification, exonemes, which store subtilisin-like protease PfSUB1. The apical tip of the parasite is capped with polar rings, composed of microtubules. The pellicular cisternae, which lie underneath the plasma membrane of the merozoite, anchor to the polar rings. The merozoite also has an apicoplast organelle, which is essential to parasite survival and important in metabolism. The merozoite surface is covered with coat proteins including merozoite surface protein (MSP) 1, 6 and 7.

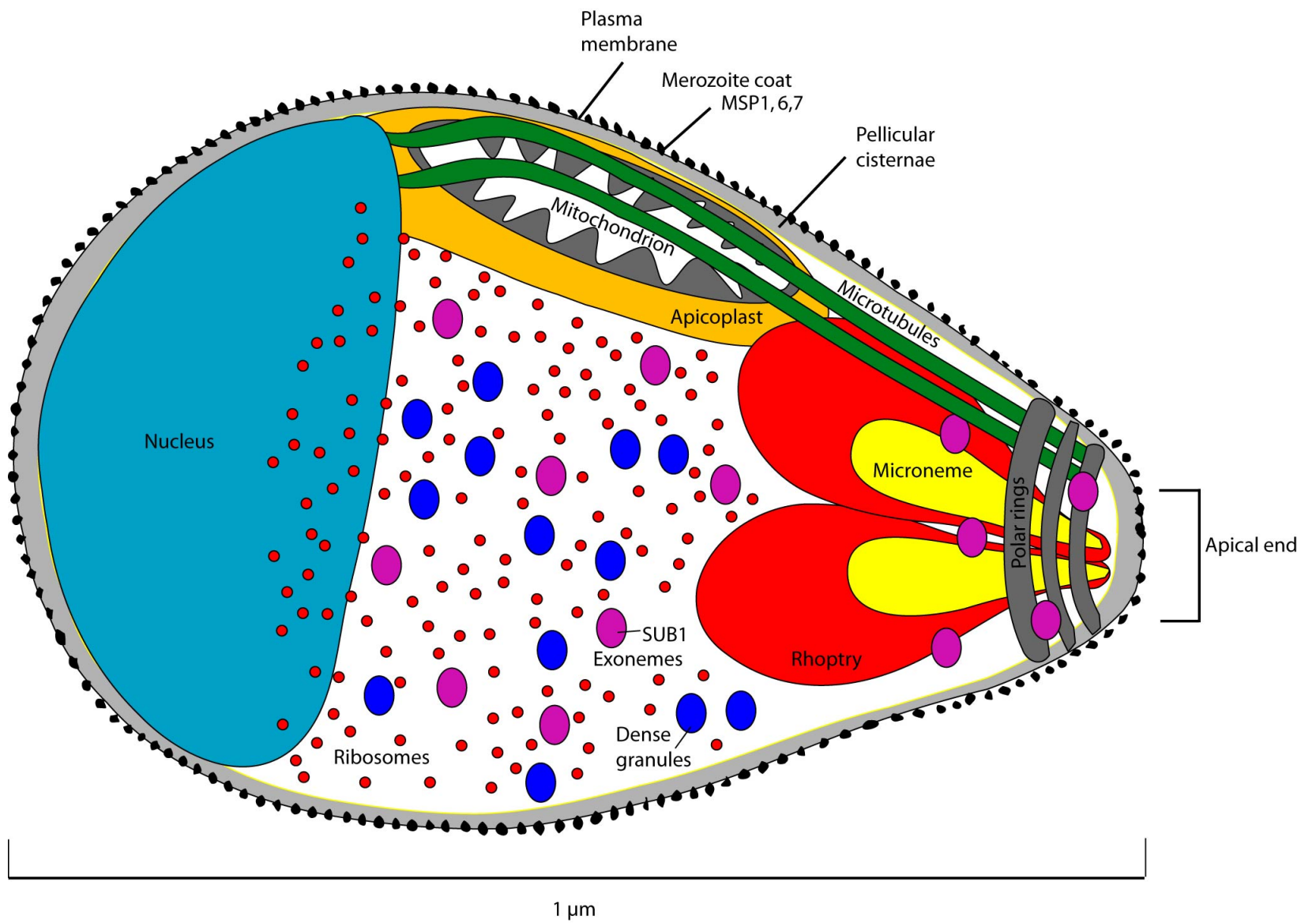


Table 1. Proteases in the *P. falciparum* genome

The *P. falciparum* genome encodes 92 proteases (Wu et al., 2003), many of which have unknown functions in the malarial life cycle. This table summarises information about some of the proteases encoded by the parasite genome. These encompass the metallo-, aspartic, cysteine and serine protease families, and illustrate the diverse nature of malarial proteases.

Protease type	Family	Name	Expressed in asexual stages?	Location	Function	Essential? (blood stages)	References
Metallo	M16 metallo-protease	Falcilysin	Yes	FV	Haemoglobin digestion and cleavage of apicoplast transit peptides	-	(Ponpuak et al., 2007, Murata & Goldberg, 2003)
Aspartic	Plasmepsin	PMI	Yes	FV	Haemoglobin digestion	No	(Omara-Opyene et al., 2004)
		PMII	Yes	FV	Haemoglobin digestion	No	(Omara-Opyene et al., 2004)
		HAP	Yes	FV	Haemoglobin digestion	No	(Omara-Opyene et al., 2004)
		PMIV	Yes	FV	Haemoglobin digestion	No	(Omara-Opyene et al., 2004)
		PMV	Yes	ER	PEXELase	Yes	(Klemba & Goldberg, 2005, Boddey et al., 2009, Russo et al., 2009a)
		PMVI	No	-	-	-	(Le Roch et al., 2004, Florens et al., 2002)
		PMVII	No	-	-	-	(Le Roch et al., 2004, Florens et al., 2002)
		PMVIII	No	-	-	-	(Le Roch et al., 2004,

						Florens et al., 2002)	
		PMIX	Yes	EC	Invasion/egress?	-	(Le Roch et al., 2004, Florens et al., 2002)
		PMX	Yes	EC	Invasion/egress?	-	(Le Roch et al., 2004, Florens et al., 2002)
	Signal peptidase	SPP	Yes	ER	Cleavage of N-terminal signal peptides	Yes?	(Li et al., 2009)
Cysteine	Falcipain	Falcipain 1	Yes	FV	Haemoglobin digestion and invasion?	No	(Greenbaum et al., 2002, Sijwali et al., 2004)
		Falcipain 2	Yes	FV	Haemoglobin digestion and egress?	No	(Sijwali & Rosenthal, 2004, Sijwali et al., 2006, Dua et al., 2001, Hanspal et al., 2002)
		Falcipain 2'	Yes	FV	Haemoglobin digestion	No	(Sijwali et al., 2004)
		Falcipain 3	Yes	FV	Haemoglobin digestion and egress?	Yes	(Sijwali et al., 2001, Sijwali et al., 2004)
	Calpain	PfCalpain 1	Yes	Parasite	Transition into pre-S-phase development	Yes	(Russo et al., 2009b)
	Calpain	Erythrocyte calpain 1	Yes	EC	Invasion and egress	Yes	(Chandramohanadas et al., 2009)
	SERA	SERA1, 2,	?	PV?	Egress?	No	(Aoki et al., 2002)

3, 7

		SERA4	Yes	PV?	Egress?	No	(Aoki et al., 2002, Yeoh et al., 2007)
		SERA5	Yes	PV	Egress?	Yes	(Miller et al., 2002, McCoubrie et al., 2007, Delplace et al., 1987, Delplace et al., 1988, Debrabant & Delplace, 1989, Li et al., 2002, Aoki et al., 2002, Yeoh et al., 2007)
		SERA6	Yes	PV?	Egress?	Yes	(Aoki et al., 2002, Schmidt-Christensen et al., 2008, Yeoh et al., 2007)
		SERA8	No			Yes (in oocysts)	(Aly & Matuschewski, 2005)
Serine	Clp	ClpP	Yes	Nucleus	Degradation of transcription factors		(Lin et al., 2009)
	Rhomboid	ROM4	Yes	Parasite surface	Parasite replication and shedding of AMA1	Yes	(Baker et al., 2006).

	ROM1	Yes	Mononeme	Shedding of EBA175	Yes	(Singh et al., 2007)
Subtilisin-like	SUB1	Yes	Exoneme	Processing of PV and MSP proteins	Yes	(Blackman et al., 1998, Hackett et al., 1999, Aurrecochea et al., 2009)
	SUB2	Yes	Microneme	Merozoite sheddase	Yes	(Blackman et al., 1998, Hackett et al., 1999, Aurrecochea et al., 2009)
	SUB3	Yes	-	-	No	(Blackman et al., 1998, Hackett et al., 1999, Aurrecochea et al., 2009)

Figure 4. Synthesis and processing of mature PfSUB1.

A. PfSUB1 is synthesised as a pre-pro-protein with a classical signal peptide, regulatory prodomain and a catalytic domain. B. During secretory transport, the 83 kDa precursor is proteolytically processed at the N-terminus to remove the signal peptide. It then undergoes autocatalytic cleavage at the site LVSAD₂₁₉NIDIS to release the prodomain (p31) and the active 54 kDa catalytic domain (p54). During late schizogony, p54 undergoes further processing by an unknown protease at the N-terminal site EVEND₂₅₁AEDYD, resulting in a 47 kDa fragment (p47).

A)



B)

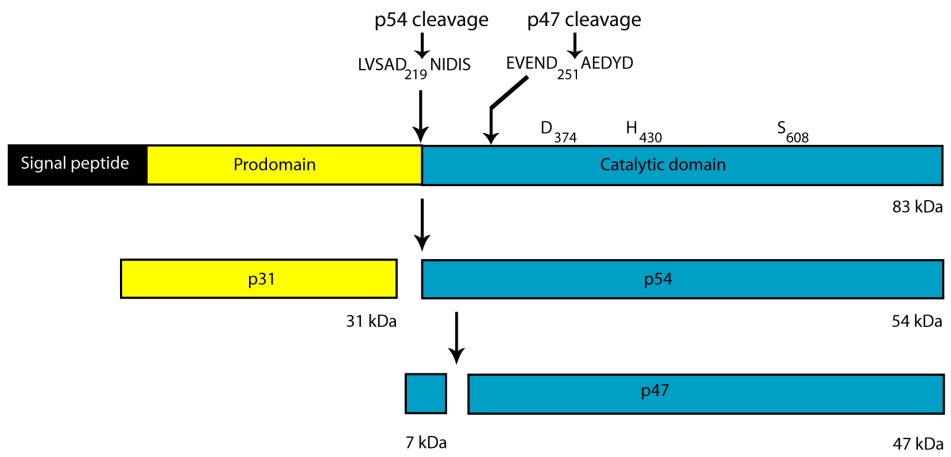
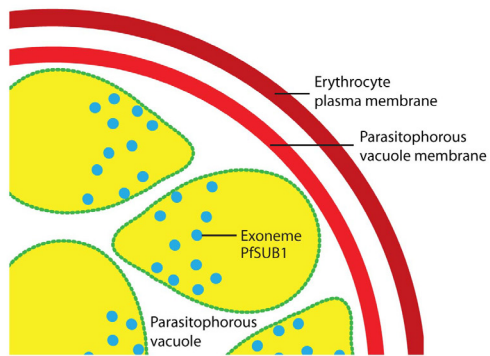


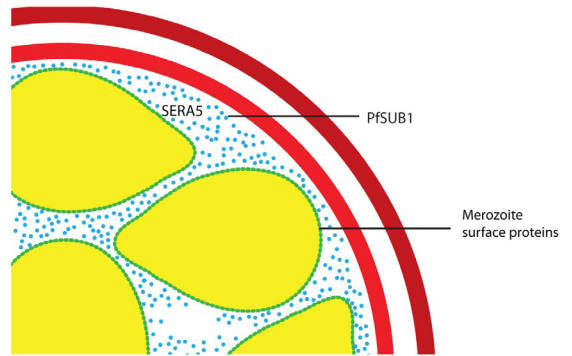
Figure 5. Release of PfSUB1 into the PV preceding egress.

In late schizogony, PfSUB1 is trafficked to dense granule-like organelles called (A). Just before schizont rupture, exonemes are thought to discharge their contents into the PV (B), where PfSUB1 (blue spots) comes into contact with its substrates, including SERA5 and merozoite surface proteins. This precedes breakdown of the PVM and EPM during egress (C).

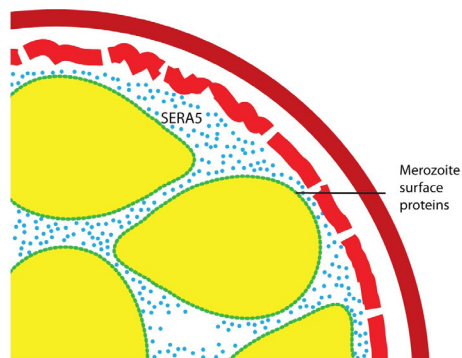
A) Late segmented schizont: PFSUB1 is stored in exonemes



B) Prior to schizont rupture: PFSUB1 is released into the PV



C) Egress: breakdown of the PVM



D) Egress: breakdown of the EPM

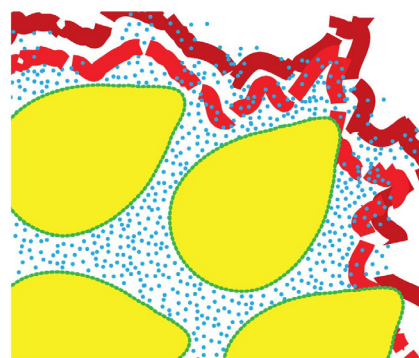


Table 2. *Plasmodium* spp. PPLPs

Five genes encoding PPLPs are conserved across the *Plasmodium* genus. The nomenclature from *P. yoelii* is used for PPLP homologues in other *Plasmodium* species. *P. berghei* has two copies of PPLP3. PPLP1 is important for sporozoite traversal of the sinusoidal cell layer, while PPLP3 and PPLP5 appear to affect ookinete invasion of the mosquito midgut, perhaps synergistically. Of these proteins, three are dispensable for blood stage growth in *P. yoelii* and *P. berghei*. The other two, PPLP2 and PPLP4, cannot be knocked out in *P. berghei* blood stages. *T. gondii* PLP1, of which the closest Plasmodial relative is PPLP1, is important for *T. gondii* egress from its host cell.

Name	<i>P. falciparum</i>	<i>P. yoelii</i>	<i>P. berghei</i>	Other names	Gene disrupted (<i>P. berghei</i>)	Function
PPLP1	PFD0430c	PY00454	PB000252.01.0	SPECT2	Yes	Sporozoite breaching of the liver sinusoidal cell layer prior to hepatocyte infection (Ishino et al., 2005)
PPLP2	PFL0805w	PY00181	PB000619.01.0	-	No (Ecker, Personal communication)	Unknown
PPLP3	PF11145w	PY05180	PB301406.00.0, PB000936.01.0	MOAP	Yes	Ookinete invasion of the mosquito midgut (Kadota et al., 2004)
PPLP4	PF08_0050	PY03076	PB000100.01.0	-	No (Ecker et al., 2008)	Unknown
PPLP5	PF08_0052	PY03943	PB000511.01.0	-	Yes	Ookinete invasion of the mosquito midgut (Ecker et al., 2007)
TgPLP1	-	-	-	-	Yes (tachyzoite)	<i>T. gondii</i> egress (Kafsack et al., 2008)

2. Methods

2.1. *Bioinformatic approaches*

2.1.1. Sequence analysis tools

ProtParam was used to compute physicochemical parameters of proteins (expasy.org/tools/protparam.html). SignalP (www.cbs.dtu.dk/services/SignalP/) was used to predict classical signal peptides. Nucleotide and protein alignments were generated using ClustalW (www.ebi.ac.uk/Tools/clustalw2/index.html). To predict transmembrane domains, the online tool TMPred was used (www.ch.embnet.org/software/TMPRED_form.html). Secondary structure predictions were performed using JPred (www.compbio.dundee.ac.uk/~www-jpred/). NCBI Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/blast/Blast.cgi) was used to identify similarity to other proteins or DNA sequences. Interpro was used to identify regions of significant homology to known domains (www.ebi.ac.uk/interpro/). To assign putative functions to proteins of unknown function, BLAST and Interpro predictions were used. PlasmoDB was the source of information for *Plasmodium* spp. genes and proteins (plasmodb.org/plasmo/).

2.1.2. *In silico* identification of PfSUB1 substrates

A PfSUB1 specificity model was created in the online application Prediction of Protease Specificity (PoPS) (pops.csse.monash.edu.au/pops.html) (Boyd et al., 2004, Boyd et al., 2005), by combining information from previous analysis of PfSUB1 specificity using peptide substrates and known PfSUB1 cleavage sites in validated substrates (Withers-Martinez et al., 2002, Sajid et al., 2000, Koussis et al., 2009, Yeoh et al., 2007). The entire *P. falciparum* predicted proteome was downloaded from PlasmoDB (www.plasmoDB.org)(Wellcome Trust Sanger Institute, EuPathDB) in FASTA format and uploaded into PoPS, then analysed using the PfSUB1 specificity model. The resulting list of proteins containing predicted PfSUB1 cleavage sites was then delimited according to the following stringencies: presence of a classical signal peptide or anchor (SignalP), known or predicted subcellular location, known or predicted function (Interpro), timing of expression (mRNA or protein)(Le Roch et al., 2004, Hall et al., 2005, Florens et al.,

2002), size (ProtParam) and number of predicted transmembrane domains (TMPred).

2.2. Molecular biology techniques

2.2.1. *E. coli* strains and transformation

Subcloning Efficiency™ DH5α™ and MAX Efficiency® DH5α™ (Invitrogen™) competent *E. coli* were used for propagation of DNA and subcloning in conjunction with calcium chloride. BL21-DE3 Gold competent *E. coli* (Stratagene) or SHuffle™ competent *E. coli* (New England Biolabs (NEB)) were used for protein expression. Transformations were carried out according to manufacturer's instructions. For plasmids carrying an ampicillin resistance gene, cells were mixed with plasmid DNA and incubated for 5 min on ice. For plasmids carrying a kanamycin resistance gene, cells were mixed with plasmid DNA and incubated on ice for 30 min, before heat shocking at 37°C or 42°C for 30 seconds and incubating with Luria-Bertani media (LB) (Bertani, 1951) for 1 hour at 37°C. Cells were then plated out onto agar plates containing the appropriate antibiotics.

2.2.2. Preparation of plasmid DNA

DNA for cloning, sequencing or transfection was purified using a Miniprep or Maxiprep kit (Qiagen), according to manufacturer's instructions. DNA yields were estimated by electrophoresis on a 0.7% agarose gel, and comparison to the quantitative DNA ladder, SmartLadder (Eurogentec) or quantified using a Nanodrop spectrophotometer (Thermo Scientific).

2.2.3. Nucleotide sequencing

DNA sequencing was carried out by Beckman Coulter Genomics.

2.2.4. DNA-modifying enzymes

Restriction endonucleases were purchased from Roche and NEB, and digests were carried out according to the manufacturer's instructions. For purification of DNA fragments after digestion, either the QIAquick® PCR purification kit (Qiagen) or the QIAquick® gel extraction kit (Qiagen) was used. Klenow enzyme (Roche) or T4

DNA polymerase (NEB) were used to generate blunt ends. Antarctic phosphatase (NEB) was used to remove 5' phosphate groups from DNA fragments. DNA fragments were ligated using the Rapid DNA ligation kit (Roche).

2.2.5. Polymerase chain reaction (PCR)

Platinum® Taq High fidelity DNA polymerase (Invitrogen™) or Pfu Turbo® (Stratagene) were used to amplify gene fragments for vector construction, as instructed by the manufacturers. For reactions where proofreading was not required, Thermoprime Taq DNA polymerase (Thermo Scientific) was used. Reactions were carried out in a ThermoHybaid Omn-E PCR machine. QIAquick® PCR purification kit (Qiagen) was used to purify DNA from PCR reactions

2.2.6. Primers

Oligonucleotide primers were synthesised by Eurogentec or Sigma-Aldrich®. Primers were diluted to a 100 µM stock in double distilled water (ddH₂O) and stored at -20°C. For PCR reactions, a 10 µM stock was used. All oligonucleotides used for plasmid construction, diagnostic PCR analysis and reverse transcription PCR are listed in Table 3.

2.3. Vector construction

All vectors used in this work are listed in Table 4.

2.3.1. Constructs for recombinant protein expression

2.3.1.1. Construction of *pIB-SUB1-FKBP*

pIB-SUB1-FKBP was designed for the expression of a recodonised PfSUB1 gene with a C-terminal FKBP domain in Sf9 insect cells. To construct this vector, an intermediate construct, *pBlueScriptKS+PfSUB1-FKBP* (*pBSKS+SUB1-FKBP*) was first made by fusing DNA encoding the synthetic *pfsb1* gene (*SUB1synth*) from *pBSKS+SUB1synth* (a kind gift from Kostas Koussis, NIMR) to the DNA sequence encoding FKBP (Figure 6). The FKBP sequence was obtained from *pHH1-SUB2-FKBP* (a kind gift from Matthew Child, NIMR). The sequence encoding *SUB1-FKBP* was removed from *pBSKS+SUB1-FKBP* by digestion with *Spe* I, blunting and

subsequent digestion with Hind III. pIB-SUB1 (a kind gift from Kostas Koussis, NIMR) was cut with EcoR I, blunted using Klenow fragment, and cut with Hind III. SUB1-FKBP was then ligated into pIB-SUB1, forming pIB-SUB1-FKBP (Figure 7).

2.3.1.2. Construction of *pET30-Xa/LiC-FKBP-p31*

pET30-Xa/LiC-p31 was previously used to express recombinant p31 in *E. coli* (Jean et al., 2003). This vector was modified by cloning in DNA sequence encoding FKBP upstream of the sequence encoding p31. The resulting vector (pET30-Xa/LiC-FKBP-p31) was used to express recombinant FKBP-p31 in the same system. pET30-Xa/LiC-FKBP-p31 was cloned by amplifying FKBP by PCR from pDONR221-FKBP (a kind gift from Daniel Goldberg, Washington University of St Louis) (Armstrong & Goldberg, 2007) using primers 3F and 3R (Table 3). The resulting PCR product was digested with Kpn I and Nde I and ligated into pET30-Xa/LiC-p31 (Figure 8), forming pET30-Xa/LiC-FKBP-p31.

2.3.1.3. Construction of *pRSFRET-SERA5*

A construct designed for the expression of recombinant 6xHis-tagged PfSUB1-sensitive FRET reporter protein (rFRET-SERA5) was cloned by replacement of DNA encoding an elastase-sensitive linker in pRSFRET-ELA (a kind gift from Richard Bayliss, Institute of Cancer Research), with DNA encoding a PfSUB1-sensitive linker in the form of the SERA5 site 1 cleavage site. Forward and reverse complementary DNA fragments encoding the linker (oligonucleotides EIKAETEDDD_F and EIKAETEDDD_R, see Table 3) were diluted to 1 pmol/ μ l in ddH₂O and annealed by mixing at a 1:1 molar ratio, heating to 95°C for 20 min and slowly cooling to 21°C. This insert was ligated into Bgl II and EcoR I sites of pRSFRET-ELA, resulting in the construct pRSFRET-SERA5 (Figure 9).

2.3.1.4. Construction of *pGEX6.1-L1-His*, *pGEX6.1-L2-His*, *pGEX6.1-L3-His*, *pGEX6.1-L4-His*, *pGEX6.1-501-His*, *pGEX6.1-502-His*, *pGEX6.1-503-His*, *pGEX6.1-504-His*

Constructs for the expression of N-terminal GST and C-terminal hexahistidine (6xHis) tagged PPLP2 and PPLP4 domains in *E. coli* were generated by amplifying protein-coding regions of *pfpplp2* and *pfpplp4* genes by PCR from *P. falciparum*

DNA and cloning these fragments into pGEX6.1 (GE Healthcare). The primer pairs used for amplification are specified in Figure 10 and their sequences in Table 3. Primers included 5' BamH I restriction sites and 3' sequence encoding 6xHis tags with Xho I restriction sites. PCR products and pGEX6.1 were digested with these enzymes and ligated (Figure 10).

2.3.2. Constructs for transfection of *P. falciparum*

All constructs used in this study for transfection of *P. falciparum* include a human dihydrofolate reductase (hDHFR) cassette, which confers resistance to WR99210, and a 3' untranslated region (3' UTR) from *P. berghei* dihydrofolate reductase thymidylate synthase (DHFR-TS).

2.3.2.1. Construction of *pHH1-PfSUB1-FKBP*

A construct designed to integrate an FKBP destabilisation domain into the 3' end of the coding sequence of *pfsub1* was cloned by insertion of FKBP-encoding DNA into pHH1-SUB1-HA3 (Figure 11). FKBP was amplified from pDONR3P3-FKBP (a kind gift from Dan Goldberg, Washington University in St Louis) using primers 30F and 30R which included Xho I and Nco I sites (Table 3). The PCR product was digested with Xho I and Nco I and ligated into pHH1-SUB1-HA3.

2.3.2.2. Construction of *pHH4-p31*

A construct for the overexpression of p31 in the PV using the AMA1 promoter and signal peptide sequence of EBA-175 was generated by cloning sequence encoding p31 into pHH4-AMA1-EBA175SS-GFP (a kind gift from Ellen Knüpfer) to generate pHH4-p31. DNA encoding p31 was amplified from pET30Xa/LIC-p31 (Jean et al., 2003) using primers 28F and 23R (Table 3). The resulting PCR product was purified and ligated into Zero PCR Blunt vector (Invitrogen™), then cloned into pHH4-AMA1-EBA175SS-GFP using Xma I and Sal I sites (Figure 12).

2.3.2.3. Construction of *pHH4-FKBP-p31*

A construct for the regulatable expression of p31 by fusion to FKBP in late schizonts was generated by cloning into pHH4-AMA1-EBA175SS-GFP. FKBP-p31 sequence was amplified from pET30Xa/LIC-FKBP-p31 using primers 28F and 20R

(Table 3). Subsequently, the PCR product was ligated into Zero PCR Blunt vector (Invitrogen™), then cloned into pHH4-AMA1-EBA175SS-GFP using Xma I and Sal I sites (Figure 13).

2.3.2.4. Construction of pHH4-FRET-SERA5

A construct for the episomal expression of FRET-SERA5 in *P. falciparum* was generated by replacement of DNA coding for GFP in pHH4-AMA1pro-EBA175SS-GFP with sequence encoding FRET-SERA5. FRET-SERA5 was amplified from pRS-FRET-SERA5 by PCR (using primers 21F and 21R, Table 3). The PCR product was purified and ligated into Zero PCR Blunt vector (Invitrogen™) prior to sequencing. PCR Blunt was cut with Xma I and Spe I and DNA was inserted into pHH4-AMA1pro-EBA175SS-GFP ligated into pHH4-AMA1pro-EBA175SS-GFP yielding the construct pHH4-FRET-SERA5 (Figure 14).

2.3.2.5. Construction of pHH1-PPLP2HA3, pHH1-PPLP2STOPHA3, pHH1-PPLP4HA3 and pHH1-PPLP4STOPHA3

Constructs designed to integrate by single crossover into the *pfpplp2* and *pfpplp4* genes were constructed by cloning targeting regions of *pfpplp2* and *pfpplp4* into pHH1-SUB1-HA3 (Figure 15) (Yeoh et al., 2007). Regions of *pfpplp2* or *pfpplp4* were amplified by PCR from *P. falciparum* genomic DNA. The primer pairs used were 46F and 46R (pHH1-PPLP2HA3), 45F and 45R (pHH1-PPLP4HA3), 46F and 52R (pHH1-PPLP2STOPHA3), 45F and 53R (pHH1-PPLP4STOPHA3, 58F and 58R (pHH1-PPLP2Δ) and 59F and 59R (pHH1-PPLP4Δ) (Table 3). Primers included restriction enzyme sites for cloning, therefore PCR products were digested with either Hpa I and Xho I (pHH1-PPLP2HA3 or pHH1-PPLP2STOPHA3), or Bgl II and Xho I (pHH1-PPLP4HA3 or pHH1-PPLP4STOPHA3) and cloned into pHH1-SUB1-HA3, which was pre-cut with the same enzymes (Figure 15).

2.3.2.6. Construction of pHTK-PPLP2 and pHTK-PPLP4

Constructs designed to delete *pfpplp2* and *pfpplp4* by double homologous recombination using the thymidine kinase negative selection system (Duraisingh et al., 2002) was generated by cloning in regions flanking *pfpplp2* and *pfpplp4* protein

coding sequence (Figure 16). Regions of *pfpplp2* or *pfpplp4* were amplified by PCR from *P. falciparum* genomic DNA, using primers 56F and 56R for the amplification of pHTK-PPLP2 flank 1 and 57CF and 57R for flank 2; pHTK-PPLP4 flanking regions were amplified using primers 54F and 54R for flank 1 and 55F and 55R for flank 2 (Table 3). Flank 1 regions were cloned into Sac II and Bgl II of pHTK and clones were sequenced. Flank 2 regions were then cloned into EcoR I and Avr II in correct clones, after which they were sequenced. This generated pHTK-PPLP2 and pHTK-PPLP4.

2.4. Immunochemical and biochemical methods

2.4.1. N-terminal sequencing

N-terminal sequencing analysis was carried out by the Protein and Nucleic Acid Chemistry Facility (University of Cambridge) according to their instructions.

2.4.2. Antibodies

Antibodies and the dilutions used in this thesis are summarised in Table 5. For use in Western blot, antibodies were diluted in PBS containing 1% bovine serine albumin (BSA) and 0.02% NaN₃. Horseradish peroxidase (HRP) conjugated secondary antibodies were used.

2.4.3. Immunofluorescence assays (IFA)

Thin films of parasites were air-dried, fixed in ice-cold dry acetone for 30 seconds, and then washed in PBS with Triton X-100. Slides were blocked overnight at 4°C with PBS containing 3% BSA. Samples were circled using an Immunopen (Calbiochem). Incubations and washes were carried out in the dark. Smears were incubated with primary antibody for 30 min at 37°C, washed in PBS, and then incubated with secondary fluorescence-labelled antibody for another 30 min at 37°C, before washing again in PBS. Samples were stained with 4,6-diamidino-2-phenylindol (DAPI) for 10 seconds for visualisation of parasite nuclei, and washed in PBS. One drop of glycerol was added to each circle, and a coverslip placed over the slide and fixed in place using nail polish. Samples were viewed using a Zeiss Axioplan 2 imaging system.

2.4.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to standard methods (Sambrook J., 1989). Resolving gel (8-5% 30:1 acrylamide/ bisacrylamide, 0.1% SDS, 375 mM tris-HCl, pH 8.8) and stacking gel (5% acrylamide, 0.1% SDS, 200 mM tris-HCl, pH 6.8) solutions were polymerised using 0.04-0.1% N,N,N',N'-tetramethyl-1-,2-diaminomethane and 0.1% ammonium persulphate. Novex® Sharp Pre-stained protein standard (Invitrogen™) or Low Molecular Weight Marker (Pharmacia) was used. 2x SDS sample buffer was used for all SDS-PAGE experiments. Non-reducing sample buffer contained 1.51% Tris-HCl, 20% glycerol, 4.6% SDS, 1×10⁻²% bromophenol blue. Reducing sample buffer was made up just before use by the addition of α -dithiothreitol (DTT) to a final concentration of 100 mM.

2.4.5. Fixing and staining SDS-PAGE gels

SDS-PAGE gels were fixed and stained with Coomassie brilliant blue (0.1% (w/v) Coomassie Brilliant Blue R, 4500 ml methanol, 4500 ml ddH₂O, 900 ml acetic acid) for 10 min and destained by use of multiple washes in 5% (w/v) methanol, 10% (w/v) acetic acid.

2.4.6. Western blot

Proteins were separated by SDS-PAGE and were transferred to Hybond-C Extra nitrocellulose (Amersham Biosciences) overnight in an AppletonWood Wet blotter in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol). Nitrocellulose blots were blocked for 30 min in 5% (w/v) milk powder (Premier International Foods) in PBS (137 mM NaCl, 3 mM KCl, 8 mM Na₂PO₄, 1.5 mM KH₂PO₄, pH 7.2) containing 0.05% (w/v) Tween-20 (PBST) and washed 3 x 5 min with PBST. Blots were incubated with primary antibodies for 1 hour, washed for 3 x 5 min with PBST and incubated with secondary antibodies in PBST for 1 hour at room temperature, then further washed for 3 x 20 min with PBST. 1 ml Enhanced Chemiluminescent solution (Pierce) was incubated with blots for 5 min preceding exposure to BioMax™ MR X-ray Film (Kodak®).

2.5. *Expression of recombinant proteins*

2.5.1. Production of recombinant proteins in *Spodoptera frugiperda* Sf9 insect cells

2.5.1.1. Transient expression of recombinant PfSUB1 (rPfSUB1) and recombinant PfSUB1-FKBP (rPfSUB1-FKBP)

Sf9 cells were grown to 80-90% confluency, and 1-3 x 10⁵ cells were plated in growth medium and incubated at 27°C overnight. 100 µl medium was pipetted into a tube, and 3 µl GeneJuice transfection reagent (Merck4Biosciences) added dropwise, before mixing and incubation for 5 min at room temperature. 1 µg plasmid DNA (pMIB-SUB1 or pMIB-SUB1-FKBP, encoding rPfSUB1 and rSUB1-FKBP) was added, mixed and incubated at room temperature for 15 min. The medium from the cells was aspirated and replaced by the transfection mixture, which was added dropwise. The plate was rocked to ensure even distribution and incubated at room temperature for 1 hour. Tunicamycin (Sigma) was added to a concentration of 62.5 ng/ ml and 1 µM or 1.5 µM Shield-1 (Cheminpharma) was added. Transfected cells were incubated for 72 hours before the culture supernatants were harvested and analysed by Western blot.

2.5.1.2. Large scale production and purification of recombinant PfSUB1 (rPfSUB1)

rPfSUB1 was expressed in Sf9 cells using the Baculovirus system (Invitrogen) and purified as described previously (Withers-Martinez et al., 2002).

NB: rPfSUB1 was not quantified in terms of protein concentration as it was not sufficiently pure following purification.

2.5.2. Expression of recombinant proteins in *E. coli*

Protease-deficient BL21 (DE3) Gold *E. coli* (Stratagene) were transformed according to section 2.2.1. 20 ml LB containing antibiotic was inoculated with one bacterial colony and incubated overnight at 37°C. 500 ml LB containing antibiotic was inoculated with 10 ml overnight culture and grown to an optical density of 0.6 (at 600 nm). Protein expression was induced at 37°C for 4 hours using 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). For increased solubility of insoluble proteins, SHuffle™ cells were used which were cultured at 30°C and protein

expression was induced with 1 mM IPTG at 16°C overnight.

2.5.2.1. *Production and purification of recombinant PfSUB1 prodomain (rp31)*

rp31 was expressed in *E. coli* and purified as previously described (Jean et al., 2003).

2.5.2.2. *Production and purification of rFKBP-p31*

Induced bacterial pellets were lysed in Bugbuster (Novagen) according to manufacturer's instructions. The supernatant of the resulting bacterial lysate was mixed with 25x EDTA-free protease inhibitor cocktail (Roche Diagnostics) and purified by anion exchange followed by gel filtration. The supernatant was sterile filtered using a 0.22 µm filter system (Corning) and applied to a Hi Trap Q-Sepharose 5 ml column (GE Healthcare) using a peristaltic pump running at 6 ml/min. The column was washed with 150 mM NaCl 200 mM Tris-HCl pH 8.2. rFKBP-p31 was eluted using a Fast Protein Liquid Chromatography (FPLC) pump (GE Healthcare) on a 100 ml gradient of 150 mM NaCl to 500 mM NaCl, running at 1 ml /min and taking 2.5ml fractions. Fractions containing the major protein peak (identified by Western blot using anti-FKBP antibodies (Affinity Bioreagents) were pooled and concentrated in an Amicon Ultra centrifugal device with a 10 kDa cut-off (Millipore). Concentrated fractions were applied to a Superdex 200 pg column (GE Healthcare) connected to an FPLC pump (GE Healthcare). Fractions containing the major protein peak (detected by Western blot using anti-FKBP antibodies) were pooled and concentrated in an Amicon Ultra centrifugal device with a 10 kDa cut-off (Millipore).

2.5.2.3. *Production and purification of recombinant FRET-ELA (rFRET-ELA) and FRET-SERA5 (rFRET-SERA5)*

Induced bacterial pellets were lysed in 2 ml Bugbuster® (Novagen) according to the manufacturer's instructions. Bacterial lysate was incubated with LiquiChip Nickel-NTA beads (Qiagen) at room temperature for 1 min, centrifuged at 13,000 rpm for 1 min, and the supernatant discarded. The beads were washed 4 times by repeated resuspension in 20 mM tris-HCl, 100 mM NaCl, 20 mM imidazole, 0.05% triton X-

100. rFRET-ELA and rFRET-SERA5 were eluted by incubating with 20 mM Tris-HCl, 100 mM NaCl, 150 mM Imidazole, 0.05% Triton X-100 and centrifuging at 13,000 rpm for 1 min.

2.5.2.4. Production and purification of GST fusion proteins

500 ml induced SHuffle™ *E. coli* culture was pelleted by centrifugation and lysed using Bugbuster (Novagen). Inclusion bodies were resuspended in 30 ml sodium chloride tris-EDTA (STE) buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1mM EDTA) and DTT was added to a final concentration of 5 mM. Lysis was achieved by the addition of 1.5% N-laurylsarcosine and sonicated for 1 second pulses for 1 min with a Vibracell sonicating microprobe (Sonics & Materials). The lysate was clarified by centrifugation at 10,000 x g for 5 min at 4°C and adjusted to 4% Triton X-100 preceding incubation at 4°C with shaking for 15 min. Recombinant GST fusion proteins were purified from lysates using S-linked glutathione agarose (GSHA)(Sigma). Lysate was incubated with GSHA for 15 min at 4°C on a rotating wheel. The GSHA was washed 5 times with ice cold PBS by repeated centrifugation at low speed. Protein was eluted with 1 M reduced glutathione in PBS.

2.6. Peptide assays

2.6.1. Peptides

All peptides used during this study were N-terminally acetylated. Peptides were synthesised by Biomatik and HPLC-purified, and provided at at least 95% purity. Peptides were dissolved at a concentration of 100 mM in dimethyl sulphoxide (DMSO) and stored at -20°C.

2.6.2. Peptide cleavage assays

Peptide stocks were diluted to 5 mM in 25 mM HEPES pH 7.4, 12 mM CaCl₂, 25 mM CHAPS in a volume of 100 µl. This was split into two tubes, each containing 50 µl diluted peptide. To one tube, 5 µl recombinant PfSUB1 was added. Both tubes were incubated at 37°C for 2 hours. Cleavage of peptides was assessed using gradient elution reversed phase high pressure liquid chromatography (RP-HPLC). 20 µl samples of digested or undigested peptides were fractionated on a Vydac C₁₈ column and eluted at 1 ml/ min on a 0-45% (v/ v) gradient of acetonitrile in 0.1%

trifluoroacetic acid over 35 min. Digested peptides were identified by electrospray mass spectrometry (ESI-MS) as previously described (Blackman et al., 2002, Withers-Martinez et al., 2002).

2.6.3. FRET reporter assays

2.6.3.1. In vitro cleavage assay

rFRET-ELA and rFRET-SERA5 were diluted 1:10 in 20 mM tris pH 8.2, 100 mM NaCl and 0.05% triton X-100. 1 µl PfSUB1 was added to 40 µl of FRET reporter, and incubated at 37°C for 2 hours. Enzyme activity in each sample was inhibited by addition of 10 µl 2x reducing SDS sample buffer and incubated at 95 °C for 5 min. Samples were analysed by SDS-PAGE and Coomassie staining.

2.6.3.2. Observation of FRET by fluorimetry

rFRET-ELA and rFRET-SERA5 were diluted 1:40 in 20 mM tris-HCl pH 8.2, 100 mM NaCl and 0.05% triton X-100. 100 µl FRET reporter was added per well of a 96-well plate (NUNC) and 1 µl rPfSUB1 or 1 µl rPfSUB1 and 1 µl rp31 added immediately before assaying in a fluorescence spectrophotometer (Varian). Readings were made at 1 min intervals with the following settings: excitation: 435 nm and 475 nm, slit width: 5.0 nm; and emission: 485 nm and 528 nm, slit width: 2.5 nm.

2.7. Assaying PfSUB1 activity by fluorimetry

PfSUB1 activity was assessed using a rhodamine-labelled peptide substrate SERAst1F-6R, which has the sequence Ac-CIKAETEDDC-OH (tetramethylrhodamine substitution at both cysteine side-chains using 6-iodoacetamidotetramethylrhodamine) as previously described (Blackman et al., 2002), in the presence of purified rp31 or rFKBP-p31. Purified rPfSUB1 was diluted 1:30 in cold sterile-filtered digestion buffer (50 mM tris-HCl pH 8.2, 12 mM CaCl₂, 0.05% v/v NP40) just prior to use. An additional well was set up with 50 µl buffer only. This was mixed well, and 50 µl added per well of a white FluoroNunc 96-well plate (NUNC). 0.5 µl rp31 and partially purified rFKBP-p31 were added to test wells. SERAst1F-6R was diluted from a 40 µM DMSO stock solution 1:100 in digestion buffer, and 50 µl was added to each well. The contents of each well was mixed by

pipetting, and then transferred to a fluorescence spectrophotometer (Varian). The kinetics programme was started, and set up to blank on the well containing no protease. Readings were made at 5-15 min intervals with the following settings: excitation: 552 nm, slit width: 5.0 nm; and emission: 580 nm, slit width: 2.5 nm.

2.8. Identification of novel PfSUB1 substrates

2.8.1. Identification of membrane-associated PfSUB1 substrates

Schizonts were purified, treated with a cocktail of protease inhibitors (Table 6) and saponin lysed as described previously (Koussis et al., 2009). Schizonts were stored at -80°C until used. 150 µl schizonts (2 x 10⁹) were thawed into 1.2 ml ice-cold 25 mM HEPES pH 7.4 12 mM CaCl₂ with supplementary protease inhibitors (10 µM E64, 1 µM pepstatin A, 10 µg/ml leupeptin and 10 µg/ml antipain). The schizonts were washed twice by centrifugation and resuspended in 400 µl 25 mM HEPES pH 7.4 12 mM CaCl₂ and divided into two aliquots. To one aliquot, 30 µl rPfSUB1 was added (sample PT+), and to the other 20 µl rp31 was added (sample PT-). Both samples were incubated at 37°C for 2 h. Subsequently, samples were solubilised in 1.6 ml 8 M urea, 25 mM CHAPS, 20 mM DTT in 10 mM tris HCl pH 8.2 and mixed at room temperature for 45 min. The samples were clarified by centrifugation and filtering (Nanosep MF GHP, 0.45 µm, PALL Life Sciences). Immediately before RP-HPLC analysis, samples were acidified by the addition of 3.2 µl trifluoroacetic acid to a final concentration of 0.2% v/v. Alternatively, for analysis by western blot, after incubation at 37°C, proteins were solubilised in SDS loading buffer and subjected to SDS-PAGE.

2.8.2. Identification of non-membrane-associated PfSUB1 substrates

Approximately 4 x 10⁹ purified schizonts were snap frozen (without protease inhibitor treatment) and thawed in 1.6 ml ice cold 25 mM HEPES pH 7.4 12 mM CaCl₂ containing the same inhibitors as described above. Lysed parasites were clarified by centrifugation and the supernatant retained. Two equal aliquots were made, to which 30 µl rPfSUB1 or 60 µl rp31 was added (ST+ and ST-, respectively). These samples were incubated for 1 h at 37°C and acidified as above. Alternatively, for analysis by western blot, after incubation at 37°C, proteins were solubilised in

SDS loading buffer and subjected to SDS-PAGE.

2.8.3. RP-HPLC resolution of treated schizont lysates

Samples treated as described in 2.8.1 and 2.8.2 were resolved by RP-HPLC using a Vydac 4.6 mm x 150 mm 214TP C4 column at a flow rate of 1 ml/ min on a 0-18 % v/v acetonitrile (in 0.1% v/v TFA) gradient running for 20 min, then 18-63% acetonitrile for a further 40 minutes. 65 1 ml eluate fractions were collected per run, which were then dried in a SpeedVac and resuspended in 40 µl reducing SDS-PAGE sample buffer. SDS-PAGE was performed as described in 2.4.4 on an 8-16% linear gradient gel (Invitrogen). The gel was stained with InstantBlue (Generon). Gel slices were prepared for analysis using a Janus liquid handling system (PerkinElmer) by placing the excised protein gel slices in wells of a 96-well microtitre plate (NUNC) and destaining with 50% v/v acetonitrile and 50 mM ammonium bicarbonate, before reducing with 10 mM DTT and alkylating with 55 mM iodoacetamide. Proteins were then digested overnight at 37°C with 6 ng/ µl trypsin enzyme (Promega), resulting in peptides which were extracted using 1% v/v formic acid and 2% acetonitrile. Peptides were analysed using nano-scale capillary LC/MS/MS using a nanoAcquity UPLC (Waters) flowing at 300 nl / ml. Peptides were trapped using a C18 Symmetry Precolumn (5 µm, 180 µm x 20 mm, Waters) before separation on a C18 BEH130 analytical UPLC column (1.7 µm 75 µm x 250 mm, Waters) and elution on a gradient of acetonitrile. The outlet of the analytical column contained a Triversa nanomate microfluidic chip for mass spectrometric analysis (Advion), from which information was obtained using an orthogonal acceleration quadrupole time of flight mass spectrometer (SYNAPT-HDMS, Waters). Automatic MS/ MS was acquired on the eight most intense, multiply-charged precursor ions (in the m/z range 400-1500), and MS/MS data were acquired for the m/z range 50-1995. LC/MS/MS data were compared to the UniProt KB (release 15.5) protein database using the Mascot search engine programme (Matrix Science).

2.9. Culture and transfection of *P. falciparum*

2.9.1. Maintenance and synchronisation

P. falciparum parasites of the clone 3D7 were used for all experiments. Parasites

were cultured in plastic tissue culture flasks (Nunc™) at 2-4% haematocrit in RPMI with Albumax medium (Invitrogen™ GIBCO®), supplemented with 10% L-glutamine in human blood (Trager & Jensen, 1976). Thin blood smears were fixed with 100% methanol, and stained with 10% Giemsa stain (VWR International) for 10 min. Parasite stage was determined by light microscopy. Mature stage parasites were isolated on a 70% (v/v) Percoll (Amersham Pharmacia) density gradient as described (Dluzewski et al., 1984, Rivadeneira et al., 1983). Further synchronisation using 5% D-sorbitol was performed as previously described (Lambros & Vanderberg, 1979, Trager & Jensen, 1976).

2.9.2. Preparation of merozoites, schizonts and culture supernatant for Western blots

Schizonts were purified as described in 2.9.1 and frozen at -80°C until use. For isolation of merozoites, late schizonts were incubated at 37°C for 3-5 hours without erythrocytes until all schizonts had ruptured. Merozoites were collected by centrifugation at 2000 rpm for 3 min. For preparation of culture supernatant samples, late schizonts were incubated with RPMI without Albumax for 3-5 hours, and the culture supernatant was collected by centrifugation at 2000 rpm for 3 min. One tablet of 25 x EDTA-free protease inhibitors was added to culture supernatant prior to concentration using a concentrated in an Amicon Ultra centrifugal device with a 10 kDa molecular weight cut-off.

2.9.3. Extraction of genomic DNA

DNA from transfected or non-transfected parasites was obtained by lysis of pelleted 5% trophozoites in 0.15% saponin (BDH Laboratory Supplies) in PBS and extraction using a DNeasy Blood & Tissue Kit (Qiagen).

2.9.4. Reverse transcription PCR (RT-PCR)

RNA was extracted from asynchronous parasites (where schizonts and trophozoites were overrepresented and rings were underrepresented) using RNeasy® (Qiagen) and QIAshredder® (Qiagen) kits according to the manufacturer's instructions. RNA quality was assessed by analysing on a 1% agarose gel made with RNase-free ddH₂O (Sigma) and concentration was determined using a Nanodrop

spectrophotometer (Thermo Scientific). DNA was removed by treatment with Turbo DNase (Ambion, Inc) and RT-PCR was performed using specific primers (Table 3), using Reverse Transcription System (Promega) according to the manufacturer's instructions.

2.9.5. Transfection of *P. falciparum*

100 µg ethanol-precipitated plasmid DNA was resuspended in 30 µl sterile Tris-EDTA buffer and 170 µl incomplete cytomix (120 mM KCl, 0.15 mM CaCl₂, 2 mM EDTA, 5 mM MgCl₂, 10 mM K₂HPO₄/KH₂PO₄, 25 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] pH7.6) (Wu et al., 1995, Wu et al., 1996). 200 µl 10-20% ring-stage parasites were added and mixed, then transferred to a 2 mm cuvette (Biorad)(Crabb et al., 1997, Crabb & Cowman, 1996, Fidock & Wellems, 1997). Parasites were electroporated at 310 V, 950 uF with ∞ resistance using an Electrocell Manipulator® 600 (BTX). Transfected parasites were transferred to small plastic tissue culture flasks (Nunc™) containing 200 µl human red blood cells and 10 ml RPMI with Albumax. After 24 hours, the media was aspirated and replaced with 10 ml RPMI containing Albumax and either 2.5 nM or 10 nM WR 99210 (Jacobus Pharmaceuticals). The culture media was subsequently exchanged every day for 4 days to remove cell debris which accumulates during electroporation and then twice a week until parasites were detected by Giemsa smear. Parasites were generally detectable in blood smears 2-3 weeks post transfection. After this, parasite stocks (at around 5 % ring parasitaemia) were frozen in liquid nitrogen and genomic DNA was prepared for parasites containing integration vectors. For parasites transfected with episomal vectors, drug selection was maintained throughout culturing. For parasites transfected with integration vectors, integrants were selected by drug cycling. Drug was removed from the media and parasites cultured in its absence for 3-4 weeks, after which the drug was added back and the media changed daily for 2 days. Once parasitaemia was re-established, parasites were frozen in liquid nitrogen and genomic DNA was prepared. The above process was repeated until integration was established. Integration was confirmed by integration PCR and southern hybridisation.

2.9.6. Plasmid rescue

5-10 µl genomic DNA extracted from transfected *P. falciparum* parasites was transformed into *E. coli* DH5α. Colonies were picked and incubated in LB containing appropriate antibiotic overnight at 37°C, before Miniprep DNA preparation using a QIAquick® Spin Miniprep Kit (Qiagen).

2.9.7. Southern hybridisation

2.9.7.1. DNA preparation

DNA extracted from parasites (see 2.9.3) was digested with restriction enzymes to provide suitable sized DNA fragments for analysis. Digested DNA was separated on a 0.7% agarose gel (Biorad laboratories) containing 1:20,000 dilution of SYBR® Safe DNA gel stain (Invitrogen). DNA was nicked in order to increase transfer efficiency by exposure to UV on a transilluminator (UVP – Bio Doc-It). The gel was incubated at room temperature with gentle agitation for 1 hour in denaturing buffer (0.5 M NaOH, 0.75 M NaCl), rinsed in ddH₂O and incubated for 1 hour in neutralizing buffer (0.5 M Tris-HCl, pH 7.4, 0.75 M NaCl). The DNA was transferred onto a Hybond N+ membrane (Amersham Biosciences) over night by capillary action transfer (Sambrook J., 1989).

2.9.7.2. Hybridisation

The membrane was incubated with hybridisation buffer (6x saline-sodium citrate buffer (SSC) (1x SSC is 150 mM NaCl, 15 mM Sodium citrate pH 7) 5x Denhardt's solution (0.1% BSA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone), 0.5% SDS, 0.01 mg ml⁻¹ sonicated salmon sperm DNA (Stratagene) for 20 min at 62°C. The DNA probe was amplified from genomic DNA from untransfected *P. falciparum* by PCR using specific primers (Table 3) and purified using QIAquick® PCR purification kit (Qiagen). The probe was labelled with α-[³²P] adenosine triphosphate (Amersham Biosciences) by random priming (Feinberg & Vogelstein, 1983) using a Prime-It® Random Prime Labelling kit (Stratagene) according to manufacturer's instructions. Purification of the probe and removal of unincorporated nucleotides was achieved using ProbeQuant™ G-50 Micro Columns according to the manufacturer's instructions (Amersham Biosciences). The labelled probe was added to the hybridisation buffer and incubated with the membrane overnight at 62°C. The probe was poured off and the membrane washed three times in 2 x SSC for 20 min at

62°C. The southern blot was visualised by exposure to BioMax™ MR film (Kodak®) at -80°C.

2.9.8. Integration PCR

To assess whether constructs had integrated into *P. falciparum*, PCR was performed (as described in 2.2.5) using extracted genomic DNA (see 2.9.3).

Table 3. List of oligonucleotides used in this thesis

Oligonucleotides used for vector construction (A), RT-PCR (B), production of probes for Southern blot analysis (C) and integration PCR (D) are listed here. Primers annealing to the sense strands of DNA are labelled F and primers annealing to the antisense strands are labelled R.

A Vector construction

Primer name	Sequence (5' to 3')
3F	CAAAAAGCAGGCCATATGGGAGTGCAGG
3R	GTACAAGAAAGCTGGGGTACCTTCTTCCGG
20R	CCAGAGGAGAGTACTAGTCTTAATCAGC
21F	GGAGACCCGGGATGGGCAGCAGC
21R	CCAGACTCGAGGGTACTAGTGTCCAGC
28F	GGCTCCGGTATTGAGCCCGGAAGGAGG
23R	CCAGAGGAGAGTGCAGCCTTAATCAGC
30F	CGTTTCTCGTTCAACTTTCTTGTACAAACTCGAGTTCGG
30R	CGCGGCGCGCCGGCCCCATGGTCATTCC
32F	GCACTAGCTCAAAATATGGGATCCTTAAATTATGC
32R	CATTATCTATTCCGTACAACCTCGAGGTGGTGGTGGTGGTGGTGGTATTATTA TAAAATAATAAAGCC
33F	CGTTATTTCAAGAGGATCCCATTAATGTAGATGG
33R	CGATCCAGATTTAAAGATCTCGAGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT TATTCC
34F	GCCGAATTTAAGAATGCTGGATCCAAATTTAAAAGTAC
34R	CCAAATAATTGTAATCCATACTCGAGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT ATAAAATATTAAAGC
35F	GGTACACATGTTGCATATGAAGGATCCTTAGGTGG
35R	CCTAAATATATTTTCATATGCCTCGAGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT TACC
45F	CGTTTACGACAGATCTGAGAAATTTAAAACTAATAAAAAGTAAGGACCCC
45R	CATATGACATTTTTTCTCGAGTTTATATGTAACTTTCTATTTCGTGTTC C
46F	GCTGGTCCACCACCTGGGTAAACAACATGTCCTATAGG
46R	CGTATTAACCTACTCGAGTTCCACCAAATTTGTTTGCCCCGTGGG
49F	GTGGATCCAATCATAAAAATGATGATAATCATATG
49R	GTGTTCTGGTATAACCTCGAGCTTCATCGAC
50F	CCGGATCCTATATTCATGATGATACTATACAAAATG
50R	GGTATTATATGAAAACACTCGAGATATTTGGATTG
51F	GGAGATATACCCCGGGGAGTGC
52R	CGTATTAACCTACTCGAGTTACACCAAATTTGTTTGCCCCGTGGG
53R	CATATGACATTTTTTCTCGAGTTTATATGTAACTTTCTATTTCGTGTTC C

54F	CGTATGTTACCGCGGTATCTATATAGGTTCG
54R	GCTTTTATATGACTAGTACGCTGAAAATGG
55F	GGACAATATGAATTCAGAAATTCGGGG
55R	CCGTTTTTACTTGTCCCTAGGTTATTATCG
56F	GGTCAATGATGAAAAAGACCGCGGAAATAACCTGG
56R	CCTCTTCCTTATGGTTACTAGTAATTCCTTCC
57CF	CCACCTGATCGATTAACATGTCCTATAGG
57R	GGGATCGACCCTAGGCAATGATTGC
58F	GGTAATCCTGAAGGAGATCTTACATTGAACG
58R	CCACCTAAATATATTTCCCTCGAGAACATGTGTACC
59F	CGTATGTTACTTGTTTAGATCTATAGGTTCG
59R	CGATCCAGATTTCTCGAGATTTAAATATGGG
EIKAETEDDD_F	GATCTGAAATTAAAGCTGAAACTGAAGATGACGATG
EIKAETEDDD_R	AATTCATCGTCATCTTCAGTTTCAGCTTTAATTTCA

B Southern blot probes

Primer name	Sequence (5' to 3')
61F	GGTAAATATCATTTCATGATGAATTTTCG
61R	CCTGAATTATTTACAAAATTAGCACC

C RT-PCR primers

Primer name	Sequence (5' to 3')
PfPPLP1_F	CGTGGTCTTTATATTGTTGTATTTATATG
PfPPLP1_R	CAATTAATGAATCAGCCTCTCCTAAAGG
PfPPLP2_F	GTATAAGGGAACGCATGGCACAAAATTTTGC
PfPPLP2_R	CCTGTCATACATTCTTCTTCTTATGCCCTTCC
PfPPLP3_F	GGTTATGATTTTATATTTGGGAACCCAATAGGTGACCC
PfPPLP3_R	GTGACACTCTTCCCACATTTTCACTTTTGCG
PfPPLP4_F	GAAATATTCAGCAAATATTTGGGAAAAGGC
PfPPLP4_R	CATGGTATTAAATGACATACCACCAAAAATGGG
PfPPLP5_F	CAATGTAACGCCTAAATGTAAAAACGGCG
PfPPLP5_R	CCGAAGGAATATAATCAATTGCGAAACCTGC

D Integration PCRs

Primer name	Sequence (5' to 3')
35F	GGTACACATGTTGCATATGAAGGATCCTTAGGTGG
67R	CCCATGGCATAGTCCGGGACGTC
68F	GGTCATATGAATAAAGGAAGGGGC
69F	CGTAACAATTTTACACTATGTGC
33F	CGTTATTTCAAGAGGATCCCATTAATGTAGATGG
70F	CGTAATAATGTTTTGTCATGTCCC
70R	CGTATTAACTTACAATCATTCCACC
71F	CCATCTTAAACTATTTGTGACAAGG
71R	GGTTAACAAAGAAGAAGCTCAGAG

Table 4. List of constructs used in this thesis

Constructs used for are listed here with their key features and purposes.

Name	Features	Purpose	Source
pBSKS+SUB1synth-FKBP	SUB1 synthetic gene fused to FKBP	Intermediate construct for cloning of pIB-SUB1synth-FKBP	This work
pDONR221-FKBP	FKBP domain for N-terminal tagging	Invitrogen™ gateway system for generation of FKBP constructs. Used for amplification of FKBP for pET-30-Xa/LIC-FKBPSUB1pro vector construction	Daniel Goldberg (Armstrong & Goldberg, 2007)
pIB-SUB1-synth	PfSUB1 sequence recodonised for yeast expression	Expression of recombinant PfSUB1 in Sf9 insect cells	Kostas Koussis (NIMR, UK)
pIBSUB1FKBP	PfSUB1 sequence recodonised for yeast expression C-terminal FKBP domain	Expression of recombinant PfSUB1-FKBP in Sf9 insect cells	This work
pET-30-Xa/LIC-p31	Gene encoding the PfSUB1 propeptide with N-terminal 6xHis and S-tag	Expression of recombinant p31 in <i>E. coli</i>	Mike Blackman, NIMR (Jean et al., 2003)
pET-30-Xa/LIC-FKBP-p31	Gene encoding FKBP fused to p31	Expression of recombinant FKBP-tagged p31 in <i>E. coli</i>	This work
pRS-FRET-SERA5	N-terminal 6xHis tag SERA5 site 1 linker	Expression of recombinant 6xHis-tagged FRET reporter with a SERA5 site 1 linker in <i>E. coli</i>	This work

pRS-FRET-ELA	N-terminal 6xHis tag Elastase-sensitive linker	Expression of recombinant 6xHis-tagged FRET reporter with an elastase-sensitive linker in <i>E. coli</i>	Richard Bayliss (CRUK, UK)
pGEX6.1-L1-His	N-terminal GST tag C-terminal 6xHis tag	Expression of a GST and 6xHis-tagged PPLP2 domain (amino acids 576-788) in <i>E. coli</i>	This work
pGEX6.1-L2-His	N-terminal GST tag C-terminal 6xHis tag	Expression of a GST and 6xHis-tagged PPLP2 domain (amino acids 576-661) in <i>E. coli</i>	This work
pGEX6.1-L3-His	N-terminal GST tag C-terminal 6xHis tag	Expression of a GST and 6xHis-tagged PPLP2 domain (amino acids 669-788) in <i>E. coli</i>	This work
pGEX6.1-L4-His	N-terminal GST tag C-terminal 6xHis tag	Expression of a GST and 6xHis-tagged PPLP2 domain (amino acids 447-521) in <i>E. coli</i>	This work
pGEX6.1-501-His	N-terminal GST tag C-terminal 6xHis tag	Expression of a GST and 6xHis-tagged PPLP4 domain (amino acids 150-372) in <i>E. coli</i>	This work
pGEX6.1-502-His	N-terminal GST tag C-terminal 6xHis tag	Expression of a GST and 6xHis-tagged PPLP4 domain (amino acids 150-254) in <i>E. coli</i>	This work
pGEX6.1-503-His	N-terminal GST tag C-terminal 6xHis tag	Expression of a GST and 6xHis-tagged PPLP4 domain (amino acids 269-372) in <i>E. coli</i>	This work
pGEX6.1-504-His	N-terminal GST tag C-terminal 6xHis tag	Expression of a GST and 6xHis-tagged PPLP4 domain (amino acids 571-621) in <i>E. coli</i>	This work

pHH1-SUB1-FKBP	3' targeting region from	<i>pfsub1</i>	Integration of FKBP into the 3' locus of <i>pfsub1</i> gene in <i>P. falciparum</i>	This work
pHTK-PPLP2	Thymidine kinase gene for negative selection		Double homologous recombination for attempting to disrupt the <i>pplp2</i> gene in <i>P. falciparum</i>	This work
pHTK-PPLP4	Thymidine kinase gene for negative selection		Double homologous recombination for attempting to disrupt the <i>pplp4</i> gene in <i>P. falciparum</i>	This work
pHH1-PPLP2HA3	3' targeting region from	<i>pplp2</i>	Single homologous recombination for integration of an HA3 tag into the <i>pplp2</i> locus in <i>P. falciparum</i>	This work
pHH1-PPLP4HA3	3' targeting region from	<i>pplp4</i>	Single homologous recombination for integration of an HA3 tag into the <i>pplp4</i> locus in <i>P. falciparum</i>	This work
pHH1-PPLP2STOPHA3	3' targeting region from	<i>pplp2</i>	Single homologous recombination for integration of the <i>P. berghei</i> DHFR-TS 3' UTR into the <i>pplp2</i> locus in <i>P. falciparum</i>	This work
pHH1-PPLP4STOPHA3	3' targeting region from	<i>pplp4</i>	Single homologous recombination for integration of the <i>P. berghei</i> DHFR-TS 3' UTR into the <i>pplp4</i> locus in <i>P. falciparum</i>	This work
pHH1-PPLP2Δ	5' targeting region from	<i>pfpplp2</i>	Single homologous recombination for integration of a truncated <i>pfpplp2</i> gene into the <i>pfpplp2</i> locus in <i>P. falciparum</i>	This work

pHH1-PPLP4Δ	5' targeting region from <i>pfpplp2</i>	Single homologous recombination for integration of a truncated <i>pfpplp4</i> gene into the <i>pfpplp4</i> locus in <i>P. falciparum</i>	This work
pHH4-p31	AMA1 promoter EBA175 signal peptide p31 sequence	Episomal expression of p31 in <i>P. falciparum</i>	This work
pHH4-FKBP-p31	AMA1 promoter EBA175 signal peptide FKBP-tagged p31 sequence	Episomal expression of regulatable PfSUB1 prodomain in <i>P. falciparum</i>	This work
pHH4-GFP	AMA1 promoter EBA175 signal peptide GFP	Episomal expression of the GFP in <i>P. falciparum</i>	Ellen Knuepfer (NIMR, UK)
pHH4-FRET-SERA5	AMA1 promoter EBA175 signal peptide FRET-SERA5	Episomal expression of FRET-SERA5 in <i>P. falciparum</i>	This work

Figure 6. Construction of pBSKS+SUB1synth-FKBP

pIB-PfSUB1synth-FKBP was designed for the expression of rPfSUB1-FKBP in Sf9 cells. To construct this vector, the intermediate construct pBSKS+-Xho I-SUB1synth-FKBP was generated. To remove the Xho I site, pBSKS+ was digested with Xho I, blunted and religated (1), resulting in the construct pBSKS+-Xho I. pBSKS+-Xho I was digested with Pst I and Spe I (2). pBSKS+SUB1synth was digested with Pst I and Spe I (3) to remove SUB1synth, which was ligated into pBSKS+-Xho I (4), generating pBSKS+-Xho I SUB1synth. pBSKS+-Xho I was digested with Xho I (present in the SUB1synth sequence) (5). To obtain sequence encoding FKBP, pHH1-SUB1-FKBP was digested with Xho I (6). FKBP sequence was ligated into pBSKS+-Xho I SUB1synth (7), which generated pBSKS+SUB1synthFKBP. The orientation of FKBP was determined by restriction analysis with Nde I and Bsg I. This diagram is not drawn to scale.

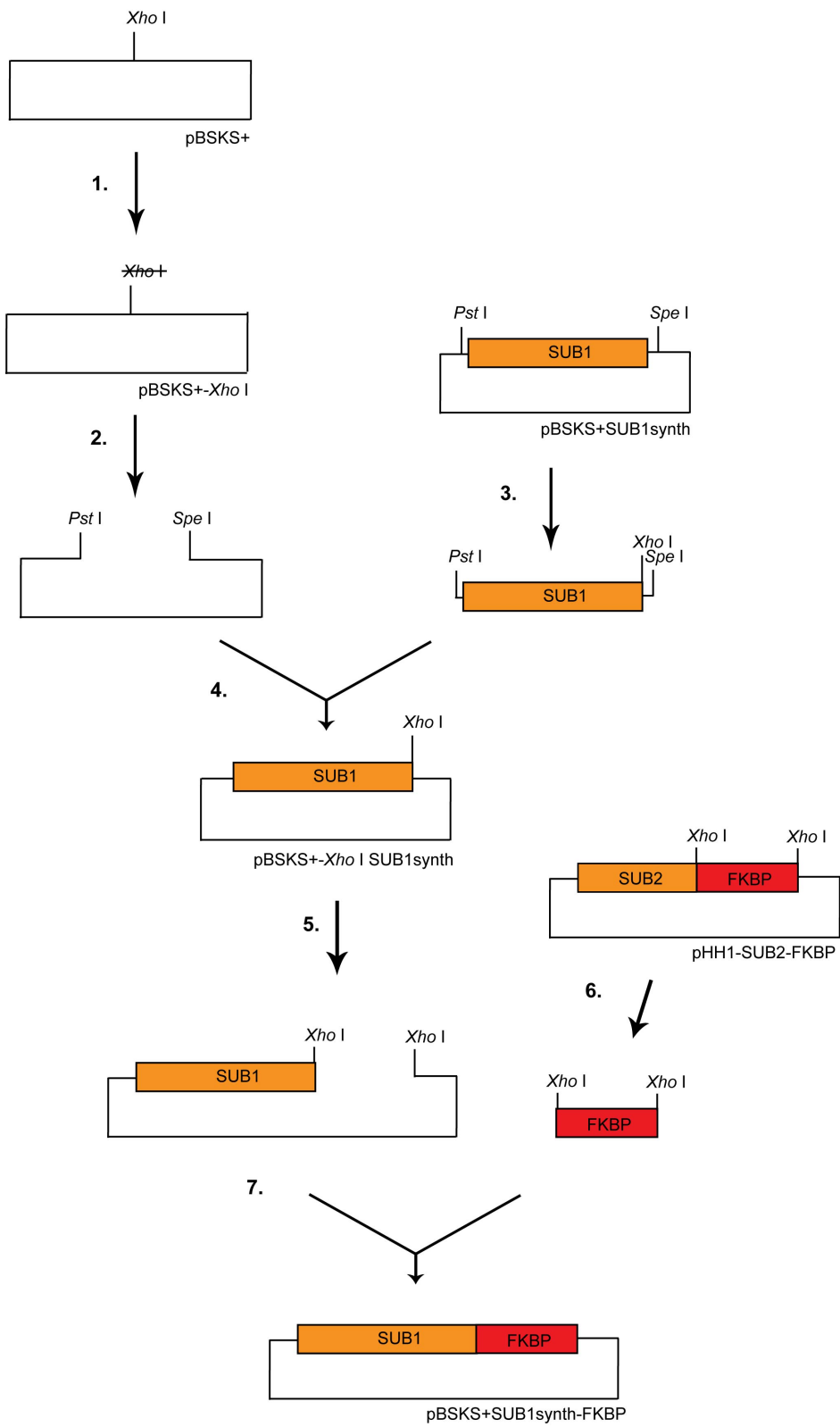


Figure 7. Construction of pIB-SUB1-FKBP

To construct pIB-SUB1-FKBP for expression of recombinant SUB1-FKBP in Sf9 cells, the pBSKS+SUB1synth-FKBP (described in Figure 6) was digested with Spe I, blunted and digested with Hind III to obtain the fragment SUB1synth-FKBP (1). pIB-SUB1synth was digested with Spe I, blunted and digested with Hind III (2), and SUB1synth-FKBP was ligated into the backbone (3), generating pIB-SUB1-FKBP. This diagram is not drawn to scale.

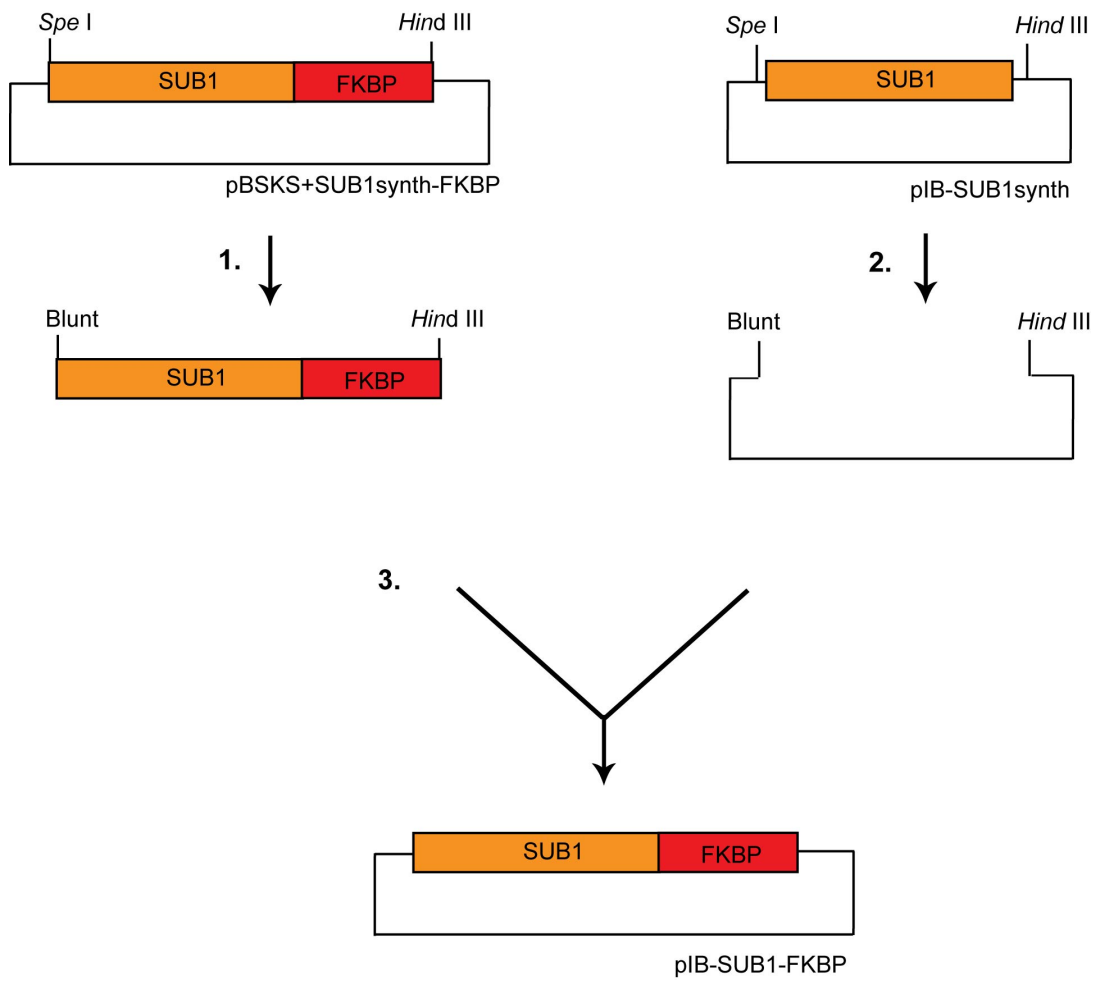


Figure 8. Construction of pET30-Xa/LiC-FKBP-p31

pET30-Xa/LiC-FKBP-p31 was designed for expression of FKBP-rp31 in *E. coli*. pET30-Xa/LiC-FKBP-p31 was made in several steps, by inserting DNA encoding FKBP into the 5' end of the sequence encoding p31 and replacing sequence encoding S- and 6xHis purification tags (tag). pET30-Xa/LiC-p31 was digested with Kpn I and Nde I (1). FKBP was amplified from pDONR221-FKBP by PCR using primers 3F and 3R which contained Kpn I and Nde I restriction sites, which it was then digested with (2). FKBP was ligated into pET30-Xa/LiC-p31 to generate pET30-Xa/LiC-FKBP-p31 (3). This diagram is not drawn to scale.

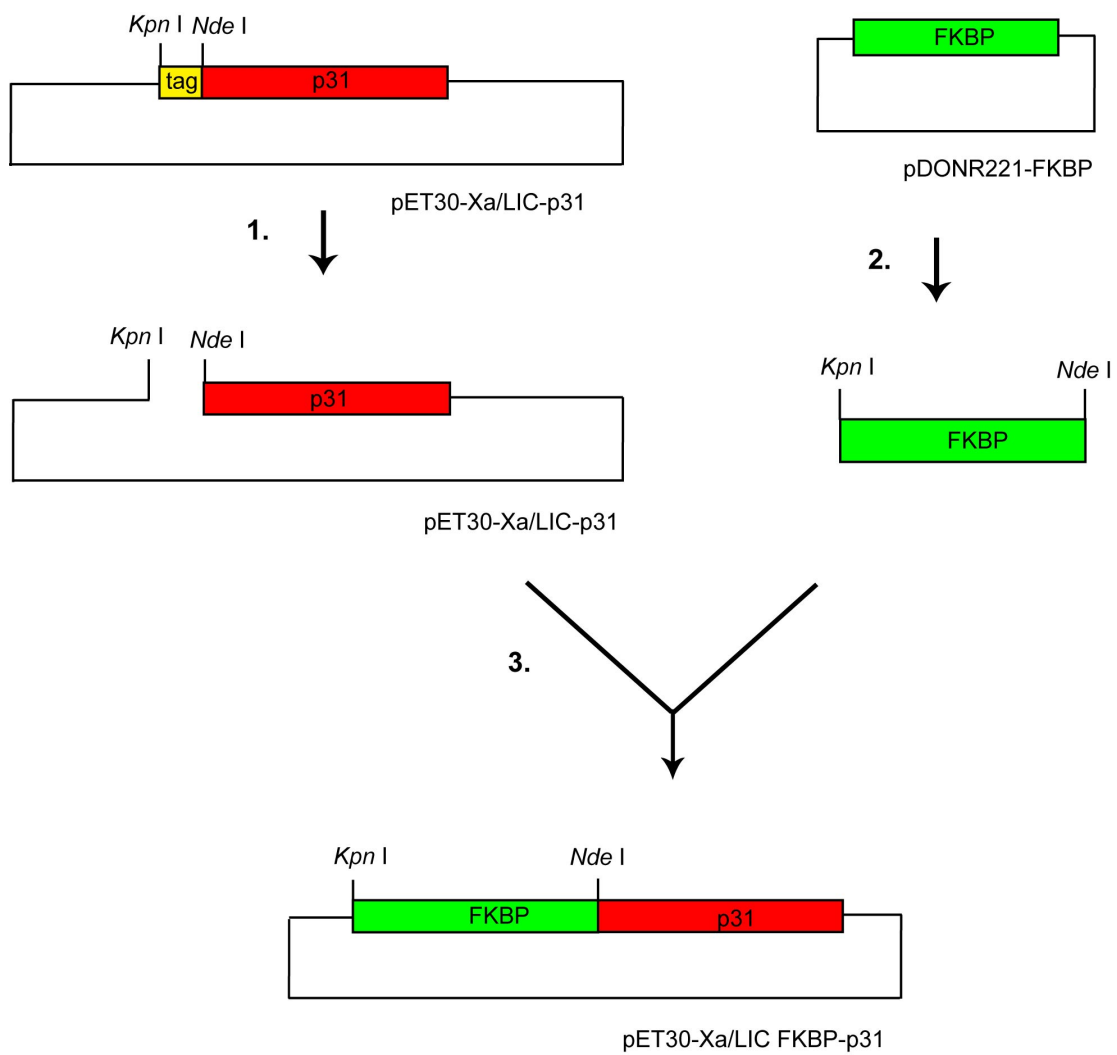
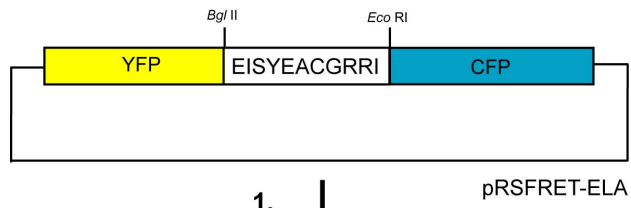
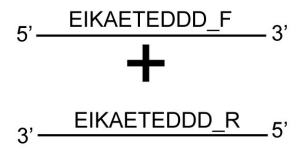
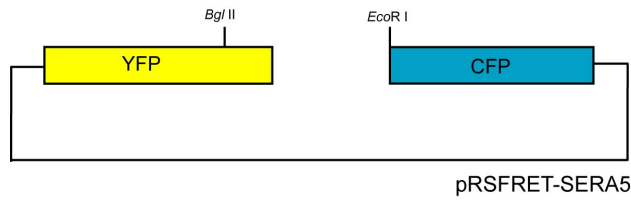


Figure 9. Construction of pRSFRET-SERA5

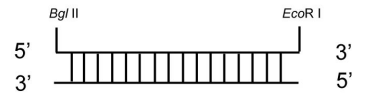
pRSFRET-SERA5 was cloned by replacing the sequence encoding EISYEACGRRI with that encoding EIKAETEDDD. Oligonucleotides EIKAETEDDD_F and EIKAETEDDD_R were annealed, forming Bgl II and EcoR I sites at the 5' and 3' ends respectively (1). pRSFRET-ELA was digested with Bgl II and EcoR I (2). The new linker sequence was ligated into pRSFRET-ELA, which resulted in pRSFRET-SERA5 (3). This diagram is not drawn to scale.



1. ↓



2. ↓



3.

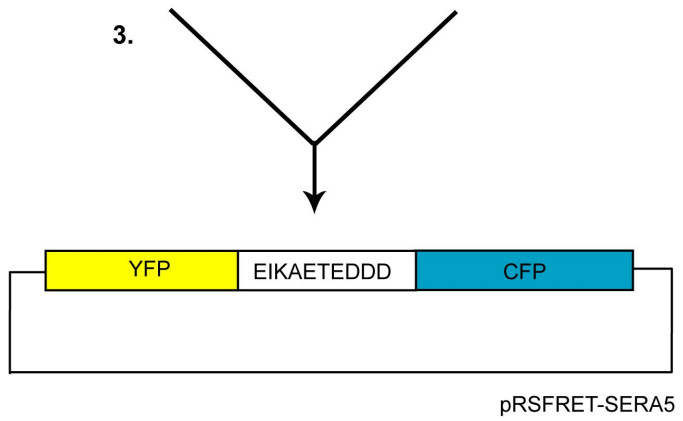


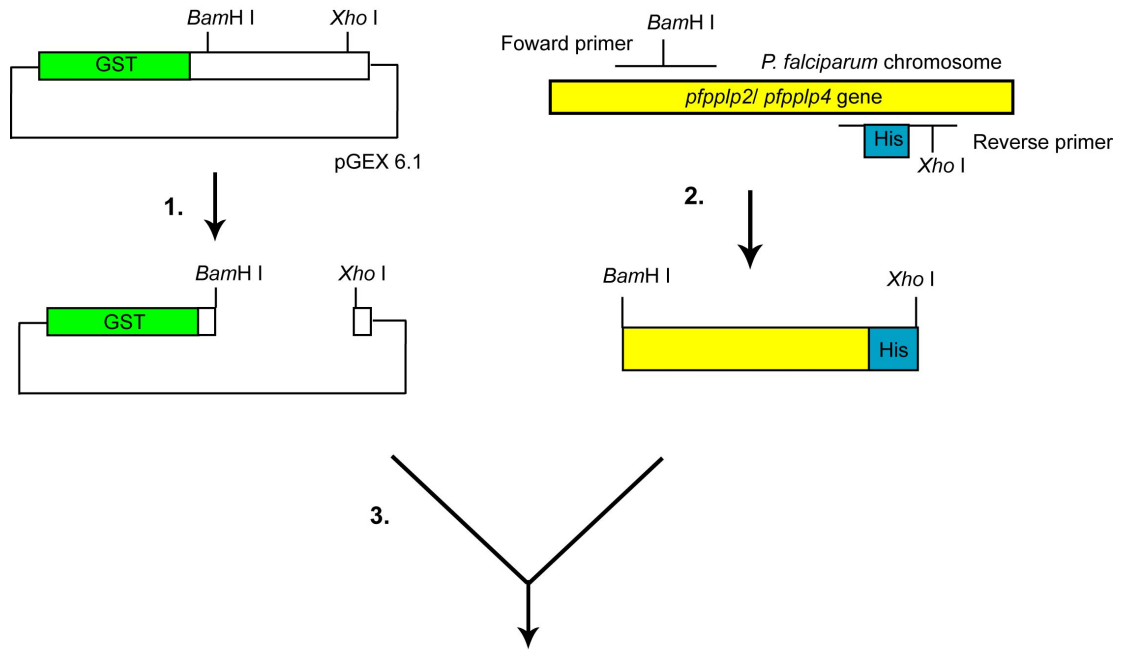
Figure 10. Construction of pGEX6.1 vectors for expression of PfPPLP2 and PfPPLP4 domains

Eight constructs were made for the expression of different recombinant PfPPLP2 and PfPPLP4 proteins, which are N-terminal GST fusion proteins with C-terminal 6xHis tags (His). pGEX6.1 was digested with BamH I and Xho I (1). Several regions of *pfpplp2* and *pfpplp4* were amplified by PCR using primers which inserted 5' BamH I sites and 3' sequence encoding 6xHis tags followed by 3' Xho I sites (2). The resulting PCR fragments were digested with BamH I and Xho I. Ligation of the digested *pfpplp2* and *pfpplp4* PCR products into pGEX6.1 yielded eight constructs encoding GST- and His-tagged PfPPLP domains (3). This diagram is not drawn to scale.

A

Insert	Amino acids to be expressed	Primers
L1	576-788	34F/34R
L2	576-661	34F/35R
L3	669-788	35F/34R
L4	447-521	49F/49R
501	150-372	32F/32R
502	150-254	32F/33R
503	269-372	33F/32R
504	571-621	50F/50R

B



Constructs:

pGEX 6.1-GST-PfPPLP2_1-His
pGEX 6.1-GST-PfPPLP2_2-His
pGEX 6.1-GST-PfPPLP2_3-His
pGEX 6.1-GST-PfPPLP2_4-His
pGEX 6.1-GST-PfPPLP4_1-His
pGEX 6.1-GST-PfPPLP4_2-His
pGEX 6.1-GST-PfPPLP4_3-His
pGEX 6.1-GST-PfPPLP4_4-His

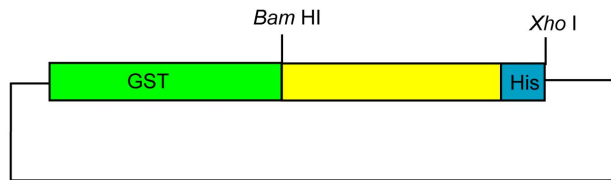


Figure 11. Construction of pHH1-PfSUB1-FKBP

pHH1-PfSUB1-FKBP was designed to integrate a HA3 tag into the 3' end of the PfSUB1 coding sequence by single homologous recombination. pHH1-PfSUB1-FKBP was cloned by replacement of the sequence encoding the HA3 tag with DNA encoding FKBP. pHH1-PfSUB1-HA3 was digested with Xho I and Nco I (1). FKBP was amplified from pDONR3P3-FKBP and digested with Xho I and Nco I (2). FKBP was ligated into pHH1-PfSUB-HA3 to form pHH1-PfSUB1-FKBP (3). This diagram is not drawn to scale.

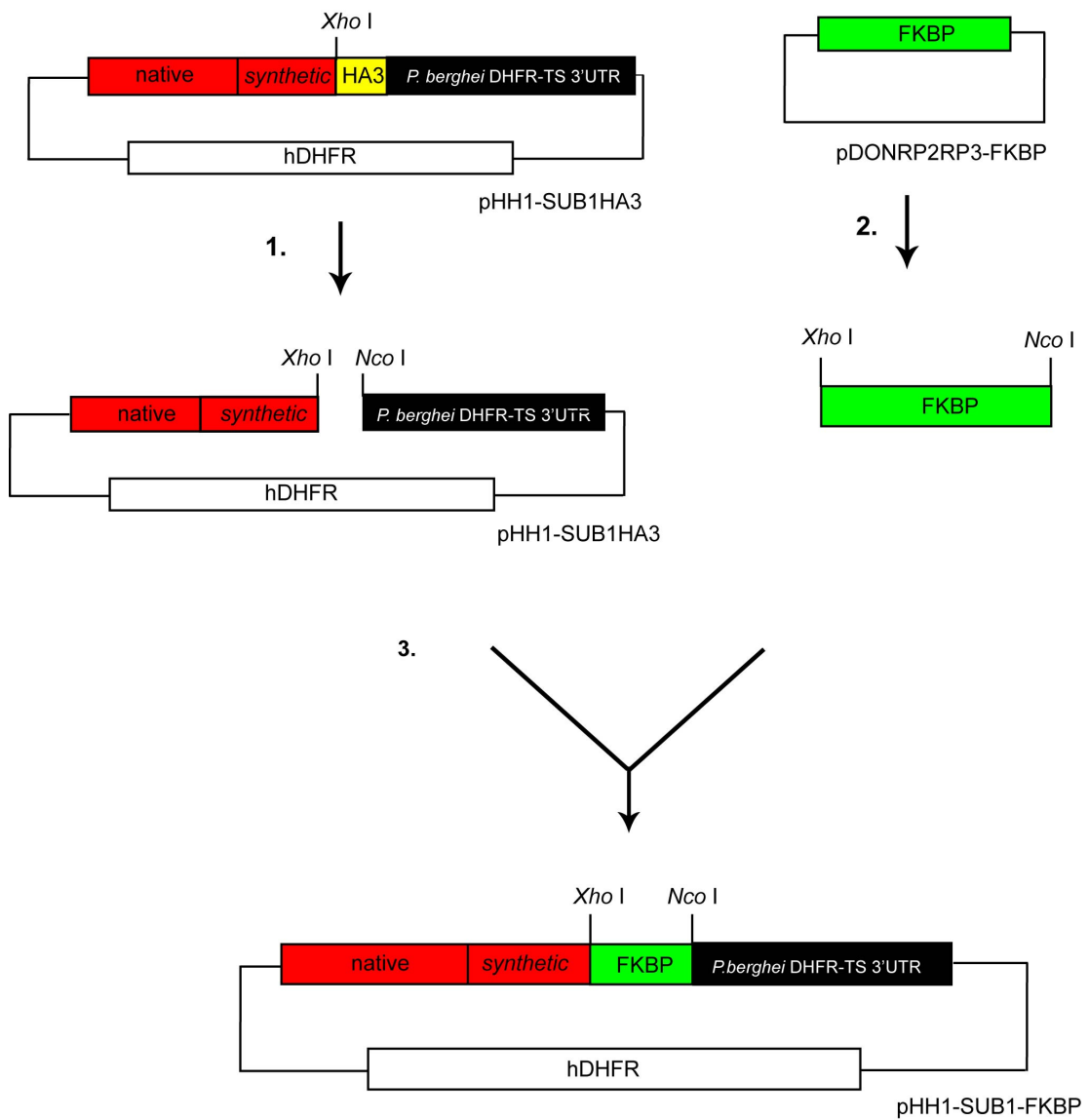


Figure 12. Construction of pHH4-p31

pHH4-p31 was constructed with the aim of expressing p31 in late schizonts in the PV. For this purpose, the AMA1 promoter (AMA1pro) was used to drive expression of p31 late in the cycle, and the EBA175 signal peptide (EBA175SS) was used to target p31 to the PV. pHH4-p31 was constructed by replacement of DNA encoding GFP in pHH4-AMA1pro-EBA175-SS-GFP with sequence encoding p31. DNA encoding p31 was amplified from pET30 Xa/LiC-p31 using primers including Xma I and Sal I sites (1) and cloned into pPCRBlunt-p31 for sequencing (2). pPCRBlunt-p31 was digested with Xma I and Sal I (3) and pHH4-AMA1pro-EBA175-SS-GFP was digested with the same enzymes (4). To generate pHH4-p31, these two fragments were ligated (5). This diagram is not drawn to scale.

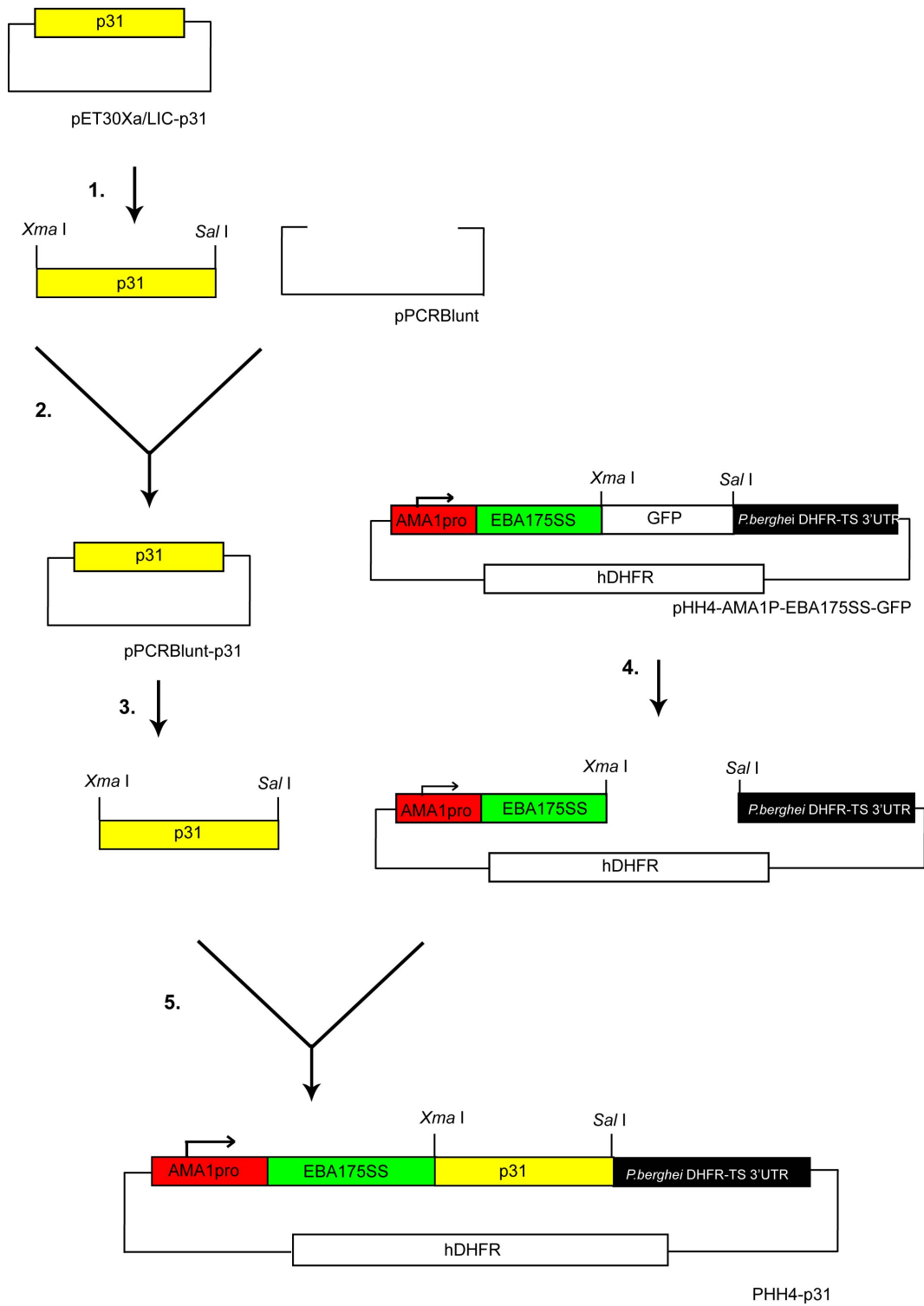


Figure 13. Construction of pHH4-FKBP-p31

pHH4-FKBP-p31 was designed for episomal expression of regulatable rp31, in late schizonts in the PV. For this reason, the AMA1 promoter (AMA1pro) was used to drive expression in late schizonts, and the EBA175 signal peptide (EBA175-SS) was used to target FKBP-p31 to the PV. To generate pHH4-FKBP-rp31, sequence encoding GFP was replaced with DNA encoding FKBP-p31. FKBP-p31 was amplified from pET30 Xa/LiC-FKBP-p31 using primers which included Xma I and Sal I restriction sites (1). The resulting PCR product was cloned into pPCRBlunt for sequencing (2). FKBP-p31 sequence was removed by digestion with Xma I and Sal I (3). pHH4-AMA1pro-EBA175-SS-GFP was digested with Xma I and Sal I to remove sequence encoding GFP (4) which was replaced with FKBP-prodomain by ligation (5). This diagram is not drawn to scale.

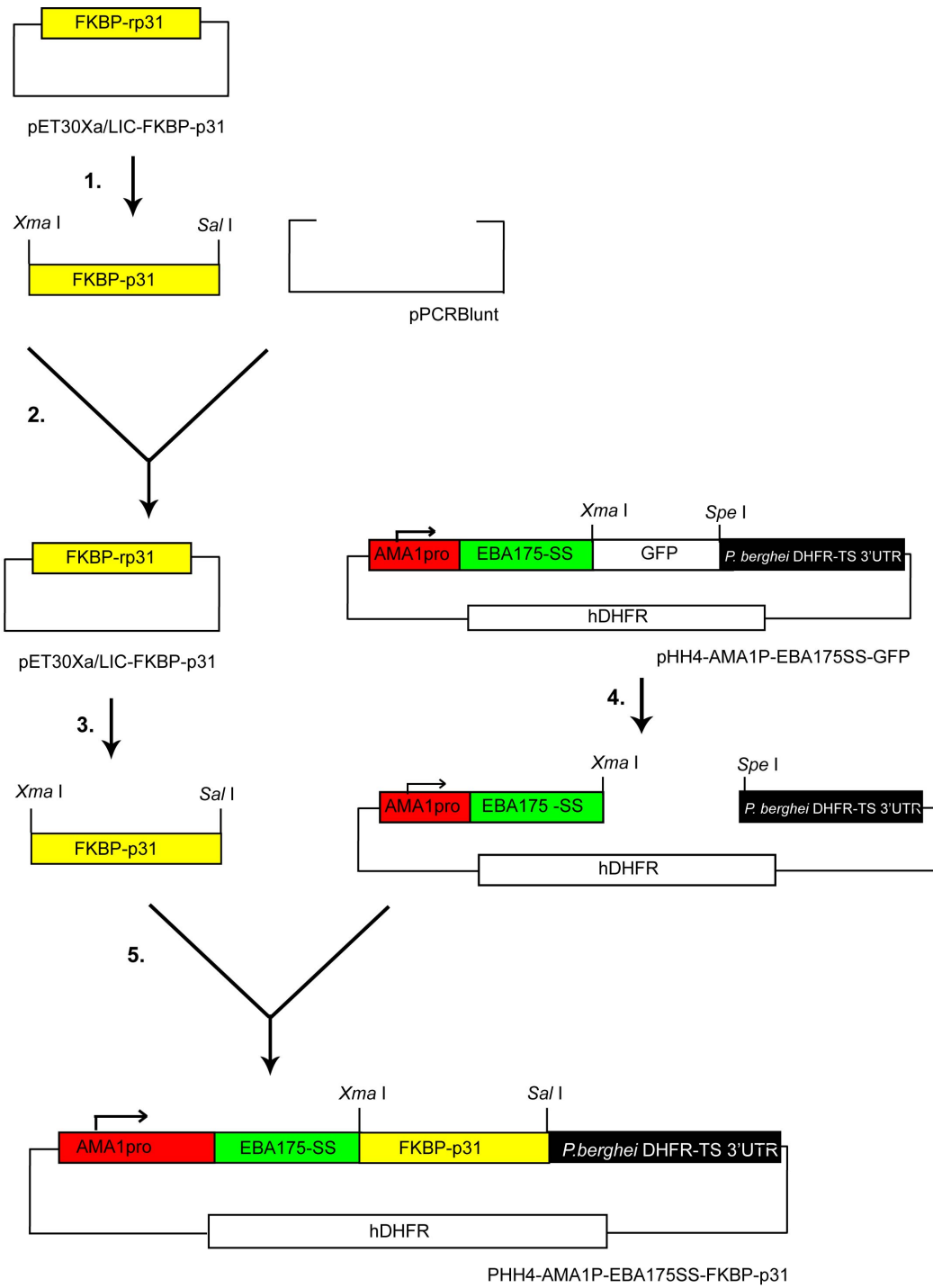


Figure 14. Construction of pHH4-FRET-SERA5

pHH4-FRET-SERA5 was constructed by replacing GFP in pHH4-AMA1pro-EBA175-SS-GFP with sequence encoding FRET-SERA5. Sequence encoding FRET-SERA5 from pRS-FRET-SERA5 was amplified by PCR (1) and cloned into pPCRBlunt, generating pPCRBlunt-FRET-SERA5 (2). pPCRBlunt-FRET-SERA5 was digested with Xma I and Spe I (3), pHH4-AMA1pro-EBA175-SS-GFP was digested with Xma I and Spe I (4). FRET-SERA5 sequence was ligated into pHH4-AMA1pro-EBA175-SS-GFP (5), resulting in pHH4-FRET-SERA5. This diagram is not drawn to scale.

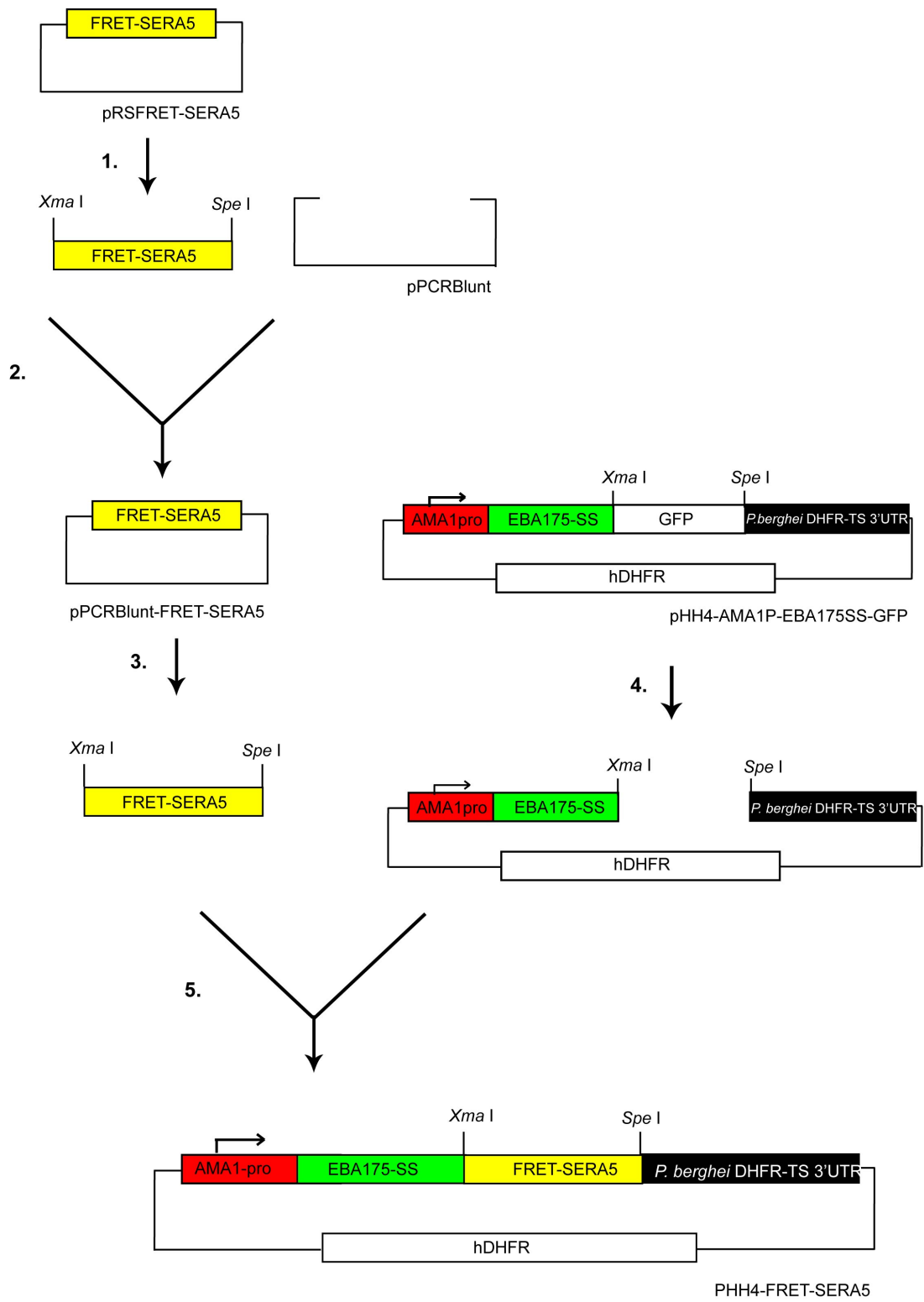
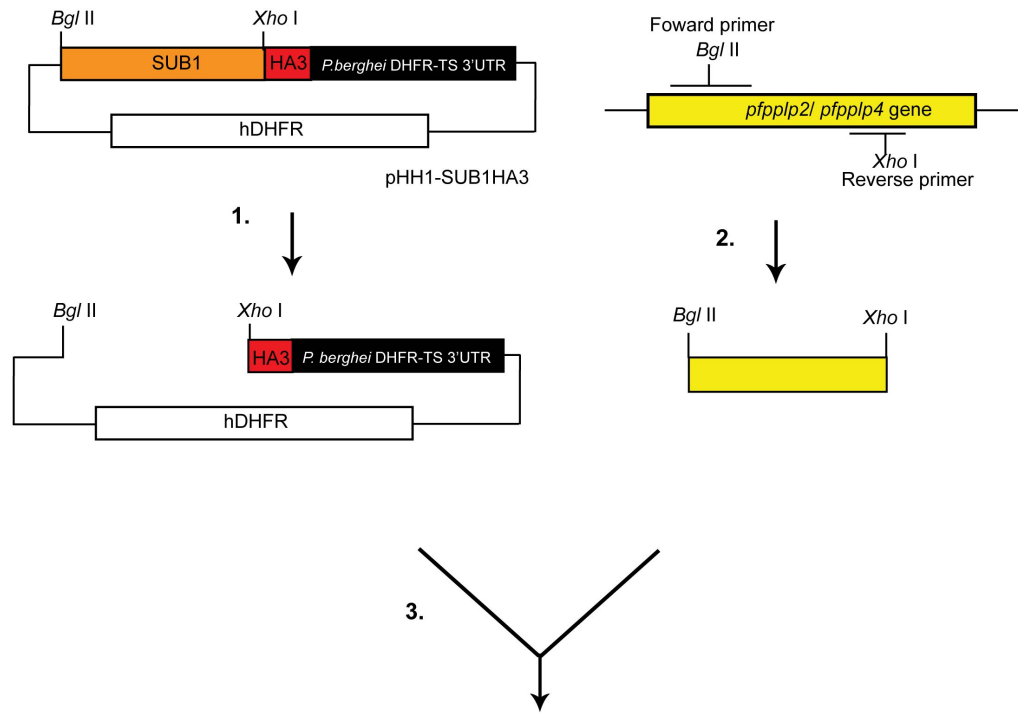


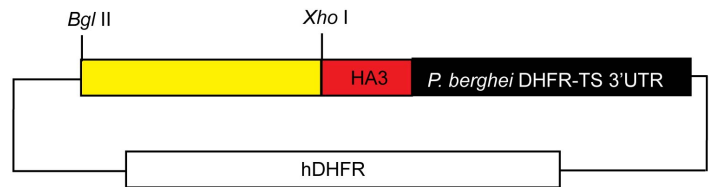
Figure 15. Construction of pHH1-PPLP2HA3, pHH1-PPLP2STOPHA3, pHH1-PPLP2Δ, pHH1-PPLP4HA3, pHH1-PPLP4STOPHA3 and pHH1-PPLP2Δ

pHH1-PPLP2HA3, pHH1-PPLP2STOPHA3, pHH1-PPLP4HA3 and pHH1-PPLP4STOPHA3 were all based on the pHH1 vector previously used to integrate a HA3 tag into the 3' end of the *pfsu1* coding sequence by single crossover integration (Yeoh et al., 2007). pHH1-PPLP2Δ and pHH1-PPLP4Δ were used to attempt to functionally knock out PfPPLP2 and PfPPLP4 function by truncating the genes encoding these proteins, based on the original pHH1 single homologous integration vector. The backbone of these constructs is pHH1-SUB1-HA3 which was previously used to integrate sequence encoding HA3 into the 3' end of the coding region of *pfsu1*. pHH1-SUB1-HA3 was digested with Hpa I or Bgl II and Xho I (1). Cloning of all of the vectors firstly involved PCR amplification of targeting regions of *pfpplp2* and *pfpplp4* using primers which incorporated Hpa I and Xho I (for vectors targeting *pfpplp2*) or Bgl II and Xho I (for vectors targeting *pfpplp4*), with which these regions were then digested (2). To generate pHH1-PPLP2STOPHA3, pHH1-PPLP2Δ, pHH1-PPLP4STOPHA3 and pHH1-PPLP4Δ, reverse primers included TAA stop codons (STOP). Digested *pfpplp2* or *pfpplp4* targeting sequences were ligated into the backbone (3), generating 6 constructs (A., B., C.). This diagram is not drawn to scale.



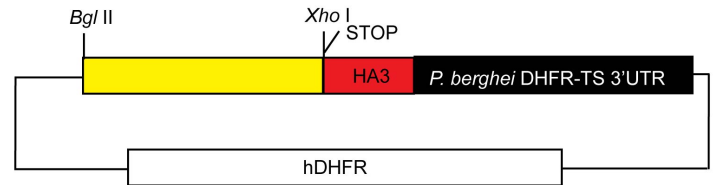
A. Integration of HA3 tag

pHH1-PPLP2HA3
pHH1-PPLP4HA3



B. Integration of *P. berghei* DHFR-TS 3' UTR

pHH1-PPLP2STOPHA3
pHH1-PPLP4STOPHA3



C. Integration of truncated *pfpplp2* or *pfpplp4* sequence

pHH1-PPLP2Δ
pHH1-PPLP4Δ

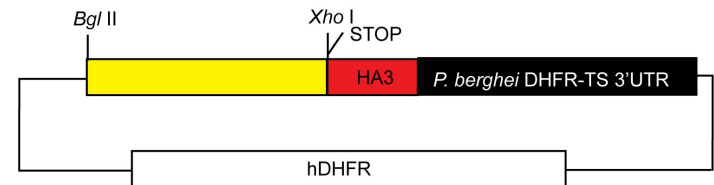


Figure 16. Construction of pHTK-PPLP2 and pHTK-PPLP4

pHTK-PPLP2 and pHTK-PPLP4 were used to attempt to disrupt the *pfpp/p2* and *pfpp/p4* genetic loci by double homologous recombination, using the thymidine kinase vector system. The vectors comprise two regions of DNA (flank 1 and flank 2) which flank the protein coding sequences of *pfpp/p2* and *pfpp/p4*. Between these two regions is the human dihydrofolate reductase (hDHFR) cassette which confers resistance to WR 99210; if double homologous recombination at the two flanking regions occurs, the hDHFR cassette would replace the protein coding sequences of *pfpp/p2* and *pfpp/p4*. For negative selection, the vector contains a gene encoding thymidine kinase, which causes parasites to be susceptible to Ganciclovir, thereby causing double homologous integration to be favoured as through this, the thymidine kinase gene is removed. To clone pHTK-PPLP2 and pHTK-PPLP4 constructs, flank 1 sequences were amplified by PCR from genomic DNA using primers containing Sac II and Bgl II and digested with those enzymes (1). pHTK was digested with Sac II and Bgl II (2) and flank 1 was ligated into pHTK (3), generating pHTK-flank 1. Flank 2 sequence was amplified from genomic DNA using primers containing EcoR I and Avr II sites, and the PCR product was digested with those enzymes (4). pHTK-flank 1 was digested with EcoR I and Avr II (5) and flank 2 was ligated into pHTK (6), resulting in pHTK-PPLP2 and pHTK-PPLP4. This diagram is not drawn to scale.

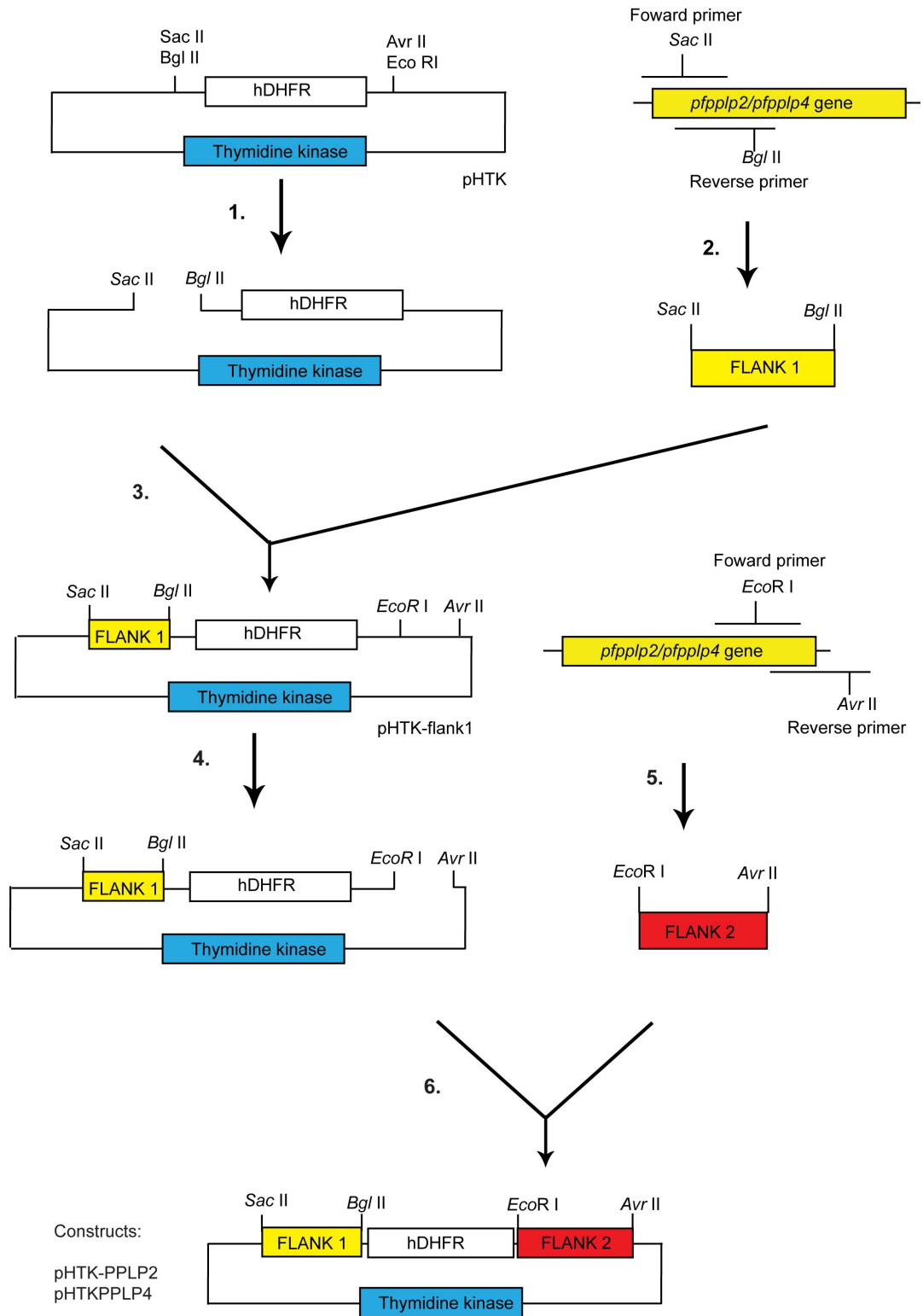


Table 5. Antibodies used in this work.

Antibodies used in this work are listed here with the species in which the antibodies were raised, whether they are polyclonal or monoclonal and their source.

Antibody	Polyclonal/ Monoclonal	Species	Working concentration	Source
α-PfSUB1GST	Polyclonal	Rabbit	1/1000	Mike Blackman (NIMR)
α-FKBP12	Polyclonal	Rabbit	1/1000	Affinity Bioreagents
α-PPLP2	Polyclonal	Mouse	1/100	This work
α-PPLP4	Polyclonal	Mouse	1/100	This work
α-RAP1 2.29	Monoclonal	Mouse	1/1000	Jana McBride (University of Edinburgh)
α-RhopH3	Polyclonal	Rabbit	1/100	Irene Ling (NIMR)
α-MSRP2	Polyclonal	Mouse	1/1000	Madhu Kaddekoppola (NIMR)
α-AMA1	Monoclonal	Mouse	1/1000	Christine Collins (NIMR)
α-MSP1 X509	Monoclonal	Mouse	1/10000	Mike Blackman (NIMR)
α-SERA5 24C6.1F1	Polyclonal	Rabbit	1/1000	Robert Stallmach (NIMR)
α-PfSUB1 prodomain 4B4.1F6.B10	Monoclonal	Mouse	1/1000	Malcolm Strath (NIMR)
α-GFP	Monoclonal	Mouse	1/500	Roche
α-Rabbit HRP	Polyclonal	Goat	1/10000	Sigma
α-Mouse HRP	Polyclonal	Goat	1/10000	Sigma

Table 6. Protease inhibitors used in PfSUB1 processing assays

Protease inhibitors used for PfSUB1 processing assays described in section 2.8 are listed here with the concentrations at which they were used.

Inhibitor	Target Protease Class	Concentration
AEBSF	Irreversible serine inhibitor	1 mM
Antipain	Reversible cysteine/serine inhibitor	10 $\mu\text{g mL}^{-1}$
DCI	Irreversible serine inhibitor	10 μM
E64	Irreversible cysteine inhibitor	10 μM
EDTA	Calcium chelator	5 mM
EGTA	Calcium chelator	5 mM
Leupeptin	Reversible cysteine/serine inhibitor	10 $\mu\text{g mL}^{-1}$
Pepstatin	Reversible aspartic inhibitor	1 μM
pHMB	Irreversible serine inhibitor	1 mM
PMSF	Irreversible serine inhibitor	1 mM
TLCK	Irreversible serine inhibitor (trypsin-like serine proteases)	10 μM
TPCK	Irreversible serine inhibitor (chymotrypsin-like serine proteases)	10 μM

3. Results chapter 1: conditional knockdown of PfSUB1

3.1. Introduction

Previous work has shown that the *pfsub1* gene cannot be knocked out in asexual blood stages, suggesting that it is essential (Yeoh et al., 2007). To date, therefore, most studies analysing the function of PfSUB1 have relied on recombinant, purified enzyme (Withers-Martinez et al., 2002) for *in vitro* experiments and a PfSUB1 inhibitor for use *in vivo* to inhibit invasion or egress, which is of low potency and therefore must be used at high concentrations, which increases the likelihood of off-target effects (Koussis et al., 2009, Yeoh et al., 2007). To dissect the role of PfSUB1 *in vivo*, a conditional knockdown system would therefore be a very useful tool. Previous attempts by members of our laboratory to conditionally regulate PfSUB1 using the tetracycline regulatory system were not successful due to problems with plasmid rearrangements and the failure to regulate PfSUB1 expression levels (Koussis, unpublished data).

Recently, a conditional regulatory system based on fusion of “destabilisation domains” to proteins of interest to control protein levels was adapted for use in *T. gondii* and *P. falciparum* (Herm-Gotz et al., 2007, Armstrong & Goldberg, 2007). The destabilisation domains used are mutants of FK-506 binding protein 12 (FKBP), which when fused to the protein of interest cause proteasome-dependent degradation of the protein (Figure 17). This can be reversed by the addition of a stabilising ligand called Shield-1, which binds to FKBP and prevents its degradation. In *P. falciparum*, this system has been used successfully to regulate both endogenous and episomally expressed proteins (Dvorin et al., 2010, Armstrong & Goldberg, 2007).

Here, I attempted to conditionally knock down PfSUB1 using two complementary approaches (Figure 17) which exploit the FKBP system. The first approach, approach A, involved the regulation of endogenous PfSUB1 protein levels by generation of a PfSUB1-FKBP fusion parasite line, while approach B attempted to inhibit PfSUB1 activity *in vivo* using a regulatable FKBP-tagged PfSUB1 prodomain expressed from an episomal construct.

3.2. Results

3.2.1. Approach A: Conditional regulation of endogenous PfSUB1 levels

The first approach aimed to regulate endogenous PfSUB1 levels directly by fusing FKBP to the C-terminus of endogenous PfSUB1. This was performed using single-crossover homologous integration to fuse the sequence encoding FKBP to the 3' end of the coding sequence in the *pfsb1* gene (Figure 17). N-terminal fusions are more efficiently degraded in the absence of Shield-1 than C-terminal fusions (Armstrong & Goldberg, 2007), so this would have been preferred as the best way to produce a regulatable PfSUB1 derivative. However, the prodomain of PfSUB1 is thought to be released from the protein soon after translation; therefore, if FKBP was fused to the N-terminus of PfSUB1, it is likely that it would be rapidly removed. Furthermore, if it were possible to fuse FKBP to the N-terminus, integration would involve N-terminal modification of the *pfsb1* locus. This would require a long targeting region, which would be unlikely to integrate at the extreme N-terminus, particularly if it had a negative effect on PfSUB1 activity.

3.2.1.1. A fusion of FKBP and PfSUB1 is catalytically active when expressed in Sf9 insect cells

Histidine or triple haemagglutinin epitope (HA3) tags can be fused to the C-terminus of PfSUB1 without interfering with catalytic activity (Yeoh et al., 2007, Withers-Martinez et al., 2002). However, these tags are relatively small modifications, and constructs designed to integrate a GFP tag (27 kDa) into the 3' end of the coding sequence of *pfsb1* have failed to integrate into the *P. falciparum* genome (Yeoh et al., 2007). This suggests that the C-terminal region of PfSUB1 is important for its activity. Thus whether a C-terminal fusion of FKBP, which is 12 kDa in size, would interfere with PfSUB1 activity was unknown. It was therefore important to demonstrate that such a fusion did not abolish the intrinsic catalytic activity of PfSUB1 before attempting to modify the endogenous *pfsb1* gene in *P. falciparum*. PfSUB1 can be expressed and purified in an active, recombinant form (rPfSUB1) in a heterologous expression system using Sf9 insect cells (Withers-Martinez et al., 2002), so this was an ideal system in which to test whether fusion to FKBP interferes with rPfSUB1 activity. Insect cells were transiently transfected with the plasmid pMIB-PfSUB1-FKBP for the expression and secretion of a C-terminal FKBP

fusion of recombinant PfSUB1 (rPfSUB1-FKBP) into the insect cell culture supernatant. rPfSUB1 does not undergo autocatalytic processing when glycosylated, therefore tunicamycin was used in the insect cell cultures to inhibit N-glycosylation (Withers-Martinez et al., 2002). Culture supernatants were analysed 48 h after transfection by Western blot using anti-PfSUB1 antibodies (Figure 18). rPfSUB1-FKBP was detected as a 120 kDa precursor which is converted to a 65 kDa fragment in the presence of tunicamycin. This size shift indicates that rPfSUB1-FKBP is able to undergo autocatalytic processing, in turn suggesting that the fusion protein is catalytically active. As a control, insect cells were transfected with plasmid pMIB-PfSUB1 in order to express unmodified recombinant PfSUB1 (rPfSUB1), which was secreted as the expected 50 kDa band in the presence of tunicamycin. The 15 kDa size difference between processed rPfSUB1 and rPfSUB1-FKBP is attributed to the presence of the FKBP domain at the C-terminus of rPfSUB1-FKBP. From this experiment, it was concluded that FKBP does not interfere with PfSUB1 catalytic activity when fused to the C-terminus of the protease.

3.2.1.2. *Integration of FKBP into the 3' end of the pfsub1 coding region*

Following on from these encouraging *in vitro* studies, it was decided to attempt to modify the endogenous *pfsub1* gene in a similar manner. In previous work, to introduce a HA3 tag into the *pfsub1* gene, a targeting vector pHH1-SUB1-HA3 was used which comprised a fusion between native and recodonised *pfsub1* sequence to force integration upstream of mutations in the synthetic sequence (Yeoh et al., 2007). Here, with the aim of introducing FKBP into the 3' end of the protein coding sequence of the *pfsub1* gene, a similar vector was used, whereby the HA3 tag in pHH1-SUB1-HA3 was replaced with FKBP, pHH1-SUB1-FKBP. Parasites were transfected with this construct and cycled on and off drug to select against parasites harbouring non-integrated input plasmids (see section 1.5). During transfection, drug cycling, routine growth and synchronisation, parasites were cultured in medium containing 0.5 μ M Shield-1. After 4 drug-cycles, the non-clonal parasites were analysed by Southern blot. Non-integrated episome was detected at the expected size of 7 kb (Figure 19). A band at 5.5 kb was observed in cycles 2 and 4. This is close to the expected size of the integrant locus (4.8 kb), but is slightly larger. This band could therefore either represent integrant or rearranged episome.

The ratio of this band to wild type band intensities did not diminish by cycle 4 and the episome band intensity also did not decrease, indicating that cycling had not enriched for parasites with the 5.5 kb band. It is possible that FKBP might interfere with PfSUB1 activity because both the wild type and episome populations are maintained. Cloning was embarked upon, but due to problems with erythrocyte lysis it could not be completed due to time constraints. In the absence of a clonal line, the non-clonal parasites were analysed to determine whether PfSUB1-FKBP is expressed at the protein level. Parasites from cycle 1 (in which there was no indication that integration of the plasmid had taken place) and cycle 4 were synchronised and cultured for an entire erythrocytic growth cycle (48 h) in the presence or absence of Shield-1. Purified schizonts from these cultures were then analysed by Western blot using anti-PfSUB1 antibodies (Figure 20). A signal was detected in all samples at the same size as PfSUB1 in an untransfected control sample of purified late schizonts. A faint band was observed in cycle 1, cycle 4 and wild type parasites at around 80 kDa which is likely to correspond to immature PfSUB1. From this, it was concluded that PfSUB1-FKBP was not expressed at detectable levels.

3.2.2. Approach B: Conditional inhibition of PfSUB1 using p31

Subtilisin prodomains are generally potent and highly selective inhibitors of their cognate proteases (Fugere et al., 2002, Li et al., 1995). Studies on the prodomain of PfSUB1 (p31) have shown that, in accord with the above, it is a potent inhibitor of both recombinant and parasite-derived PfSUB1 (Jean et al., 2003), but does not inhibit other subtilisin-like serine proteases including BPN' and subtilisin Carlsberg (Jean et al., 2003). As a second approach to PfSUB1 knockdown, I attempted to exploit the high potency and selectivity of p31 by using it as an endogenous inhibitor of PfSUB1 activity in the parasite. Since our current model suggests that PfSUB1 exerts its physiological activity in the PV, it was decided to attempt to target an FKBP-tagged p31 to the PV. Regulating the stability of the PfSUB1-FKBP fusion with Shield-1 would thus act as a molecular switch for PfSUB1 activity (Figure 17), inhibiting PfSUB1 only in the presence of Shield-1. This approach was considered to have the advantage that the endogenous *pfsu1* gene would not be modified. It also avoids continuous culture of parasites in the presence of Shield-1, which is a concern as toxicity has been observed when culturing long-term with this compound

(Tobin A., Leicester University, unpublished data).

Structural studies of prodomain-subtilisin complexes have shown that the C-terminus of the bound prodomain lies in the active site of the cognate enzymes (Bryan *et al.*, 1995). For this reason, it was decided that the C-terminus of p31 could not be tagged using FKBP as it would likely interfere with the inhibitory activity of p31. On the other hand, N-terminal fusion to a hexahistidine tag (6xHis) and S-tag has little effect on the binding of recombinant p31 (rp31) to mature PfSUB1 (Jean *et al.*, 2003). Therefore, FKBP was fused to the N-terminus of p31.

3.2.2.1. *rFKBP-p31 inhibits rPfSUB1 in vitro*

To confirm that p31 retains its inhibitory capacity when fused to FKBP, initial experiments focused on expressing an FKBP-p31 fusion protein in a recombinant form, which was then tested against rPfSUB1 *in vitro* to assess its inhibitory activity. The FKBP-p31 fusion protein (rFKBP-p31) was expressed in *E. coli* and purified by ion exchange and gel filtration. rFKBP-p31 was recognised by both anti-prodomain and anti-FKBP12 antibodies by Western blot (Figure 21). rFKBP-p31 was not quantified because it was not pure following purification. The inhibitory effect of rFKBP-p31 was assessed using a fluorogenic peptide substrate based on the PfSUB1 cleavage site 1 in SERA5 (Blackman *et al.*, 2002, Yeoh *et al.*, 2007). rFKBP-p31 and wild type rp31 (Jean *et al.*, 2003) were incubated with rPfSUB1, and the activity of rPfSUB1 was measured by spectrofluorimetry (Figure 22). Encouragingly, these results indicated that the rFKBP-p31 is inhibitory to rPfSUB1 activity.

3.2.2.2. *Overexpression of p31 in P. falciparum is not tolerated*

Having demonstrated that N-terminal fusion of FKBP to rp31 does not ablate its inhibitory activity, attempts to regulate PfSUB1 activity *in vivo* using the modified p31 were embarked upon. Before attempting to express regulatable p31, it was decided to ascertain whether overexpression of p31 in the PV is tolerated by the parasite. If overexpression of p31 were tolerated, it would not be possible to use p31 to conditionally regulate PfSUB1 activity. Since p31 is a potent inhibitor of rPfSUB1 *in vitro*, it was thought to be likely that expression of p31 would not be tolerated in parasites. GFP has been previously targeted to the PV during late

schizogony using a construct (pHH4-GFP) containing the *ama1* promoter, which drives strong, late stage expression, and the EBA175 signal peptide (Knuepfer, unpublished data). For our purposes, a similar vector (pHH4-p31) was used wherein GFP was replaced with p31 (Figure 23); parasites were transfected with pHH4-p31 and pHH4-GFP as a control. Upon selection in the presence of WR99210, drug-resistant parasites were expanded from the transfected cultures, initially suggesting that the construct was not deleterious to parasite growth, as was expected. However, upon further examination of the construct by plasmid rescue and diagnostic restriction digestion of the DNA, it was evident that the pHH4-p31 plasmid had rearranged, resulting in loss of a segment of the AMA1 promoter (Figure 23). This had occurred in four parasite lines, which were transfected at different times. Different rearrangements were observed in different transfected lines, but all were modifications to the AMA1 promoter. In contrast, no rearrangements were observed in pHH4-GFP plasmid rescued from parasites transfected with that construct (Figure 23). Since plasmid rearrangement was not observed with this control construct, rearrangement is likely to be related to expression of p31 in the PV. From this, it was concluded that drug treatment had selected for parasites carrying rearranged pHH4-p31. The reason for this is unknown, but since the promoter is affected, it is likely that p31 is not expressed or that expression levels of p31 are low enough to have little effect on parasite development. These experiments imply that overexpression of p31 in the PV is deleterious to *P. falciparum*, presumably by inhibiting PfSUB1 or that it is toxic in another way. This was an important finding as it suggests that if PfSUB1 is inhibited during egress, parasites do not survive. It also, essentially, validates the use of p31 as a regulatable inhibitor of PfSUB1 *in vivo*.

3.2.2.3. *Expression of regulatable p31 in P. falciparum*

These exciting results led us to attempt to obtain regulated expression of p31 in the PV. Parasites were transfected with a similar construct expressing an FKBP-p31 (pHH4-FKBP-p31). As previously, this was under control of the *ama1* promoter and directed to parasite secretory system by means of an EBA175 secretory signal peptide. Following drug selection, schizonts from the transfected culture were cultured with or without Shield-1 for 48 h, and then treated with the detergent saponin, which lyses the PVM and EPM, allowing the separation of membrane-

associated and soluble PV and erythrocyte cytosolic proteins. The resulting parasite lysates were analysed by Western blot using anti-p31 monoclonal antibodies (Figure 24). The transgene-derived FKBP-p31 was expected to be present in the soluble fraction since it was expected to be trafficked to the PV. Unexpectedly, no signal was detected in parasites, indicating that the FKBP-p31 was not expressed in the presence of Shield-1 (or expressed at undetectable levels). Since it was possible that the transfection construct had rearranged in a similar manner to pHH4-p31, plasmid rescue was performed and the DNA analysed by diagnostic restriction digests. Digestion with Cla I and Xma I, which flank the AMA1 promoter region, showed that the promoter had not undergone detectable rearrangements since no difference between transfected and rescued plasmids was observed (Figure 25). Digestion with EcoR I, Cla I and Nhe I also resulted in fragments of the expected sizes indicating that the backbone had not rearranged. It was concluded that drug treatment had not selected for parasites carrying rearranged parasites, as observed with pHH4-p31 (Figure 23). These experiments imply that pHH4-FKBP-p31 is tolerated by parasites but that it is not expressed at detectable levels.

3.3. Discussion

The role of PfSUB1 *in vivo* is only partly understood. The aim of this project was to obtain a conditional knockdown of PfSUB1 in order to address some of the many questions we have about this enzyme. Inhibitor studies suggest that PfSUB1 is essential for egress and invasion (Yeoh et al., 2007), but it is important to confirm these results with a genetic approach because it is possible that the inhibitor used has unknown off-target effects, as it is of low potency and therefore must be used at high concentrations. By conditionally knocking down PfSUB1, this would provide a means of analysing the essential role of this protein in asexual stages.

In this chapter, conditional knockdown was attempted by two strategies, which both used the FKBP destabilisation domain system, involving either direct downregulation of the gene product or inhibition of PfSUB1 activity using its own prodomain. Both of these approaches rely on FKBP fusion proteins being degraded, a process which appears to be proteasome-specific (Banaszynski et al., 2006). Whether soluble proteins secreted into the PV can be efficiently regulated using the FKBP system is unknown, though there is evidence that proteins which enter the secretory system can be regulated, as is the case for falcipain 2 (Armstrong &

Goldberg, 2007). Very little is known about how protein levels are regulated outside of the parasite, for example in the PV, making the likelihood of downregulation of soluble PfSUB1-FKBP and FKBP-p31 questionable. However, an FKBP fusion of PfSUB 2, which is released from micronemes during invasion, appears to be degraded in the absence of Shield-1 (Matthew Child and Mike Blackman, NIMR, unpublished) giving hope that PfSUB1-FKBP might be regulatable. Furthermore, downregulation of CDPK5 using FKBP is thought to occur before the enzyme reaches its membrane-associated location (which is likely to be cytosolic) (Dvorin et al., 2010). A similar scenario could occur with PfSUB1, which is targeted to the exonemes and thought to be present as membrane-associated aggregates before it is released into the PV (Kostas Koussis and Mike Blackman, NIMR, unpublished). However, the success of the FKBP system in *P. falciparum* and *T. gondii* appears to vary from protein to protein (Armstrong & Goldberg, 2007, Russo et al., 2009b, Herm-Gotz et al., 2007), therefore, despite these concerns, the project was attempted.

To validate the first approach to PfSUB1 knockdown whereby FKBP was fused directly to endogenous PfSUB1, PfSUB1-FKBP was expressed in insect cells. This showed that FKBP does not interfere with the autocatalytic processing of PfSUB1 when fused to the C-terminus. It was concluded from this that PfSUB1-FKBP is catalytically active. To next analyse the function of PfSUB1 *in vivo*, attempts were made to fuse FKBP to PfSUB1 by homologous recombination to the *pfsub1* gene. Southern blot revealed a population of parasites appearing after multiple cycles wherein either integration of the vector had occurred or the targeting vector had rearranged. It was not possible to distinguish these two events in this Southern blot. In the future, whether integration or plasmid rearrangement has occurred will be determined by analysing the vector by plasmid rescue and diagnostic digests and Southern blots. Analysis of non-clonal parasites with an anti-PfSUB1 antibody also suggested that PfSUB1-FKBP is expressed at very low levels or not at all. This could be due to a possible rearrangement of the targeting construct, leading to no integration and therefore no expression of PfSUB1-FKBP. Alternatively, there may be poor expression levels of PfSUB1-FKBP. It is possible that in the samples analysed, only a small percentage of the parasites are mutants - perhaps, by the time parasites had been cultured and synchronised to prepare material for Western blot analysis, episome-carrying wild type parasites may have

outgrown the integrant population. One explanation for this is that FKBP may somehow interfere with PfSUB1 activity and integration of pHH1-SUB1-FKBP is not a desirable modification of PfSUB1, resulting in parasites that have a delayed blood stage cycle. Therefore synchronisation would select for parasites which are more advanced than pHH1-SUB1-FKBP integrants. It was assumed that rPfSUB1-FKBP expressed in insect cells is fully catalytically active but this was not tested quantitatively. To evaluate the effect of fusion on enzymatic activity, rPfSUB1-FKBP would need to be expressed at high levels, purified, and analysed kinetically in comparison to wild type rPfSUB1. It is also possible that fusion to FKBP may affect important non-catalytic properties of PfSUB1, such as interaction with its macromolecular substrates in the PV and at the merozoite surface. On the other hand, failure to detect PfSUB1-FKBP may also be due to clipping of FKBP from the C-terminus of PfSUB1 by another enzyme or during secretory transport. To analyse these transgenic parasites in further detail, the parasites now need to be cloned so that a single population can be analysed.

The aim of the second approach to PfSUB1 knockdown was based on the known highly specific inhibitory properties of the p31. A recombinant FKBP-p31 fusion protein was first shown to be PfSUB1-inhibitory *in vitro*. Importantly, overexpression of p31 in the PV was not tolerated by parasites, strongly suggesting that p31 can be deleterious to the parasite when targeted to the PV. This validates the approach, since if p31 were tolerated by parasites, it could not be used for conditional knockdown of PfSUB1 activity. Therefore, these findings indicate that p31 could be used for conditional inhibition of PfSUB1 activity *in vivo*. Following this, a line carrying a construct for the expression of FKBP-regulatable p31 in the PV was generated. However, upon analysis by Western blot of drug-selected parasites, no expression of FKBP-p31 was detected. The reason for the failure to detect FKBP-p31 is unclear, but presumably reflects very low levels of expression from the episome. It is important to note that while anti-p31 antibodies recognise rp31 (Malcolm Strath and Mike Blackman, unpublished), whether they recognise parasite-derived p31 is unknown since they have never been used to unambiguously detect the free prodomain in the parasite. The pHH4-FKBP-p31 construct was designed to express the FKBP-p31 at high levels, driven by the AMA1 promoter (which is a strong, late-stage promoter). The negative Western blot data suggest that either the FKBP-p31 is not expressed or the antibodies cannot

detect it. The lack of expression could perhaps be confirmed using an anti-FKBP12 antibody, however it was found that this antibody only reliably detected purified recombinant proteins (data not shown). Aside from problems with antibody sensitivity, if the parasites are not expressing FKBP-p31, there are several explanations for this. It is possible that degradation of the FKBP-p31 is very efficient and requires higher concentrations of Shield-1 for stabilisation than are commonly used (in this study 0.5 μ M was used, which was sufficient to stabilise YFP in a previous study (Armstrong & Goldberg, 2007)). Future experiments could analyse the effect of higher concentrations of Shield-1. On the other hand, FKBP-p31 may be being degraded in the secretory transport system and it may have an intrinsic signal for degradation after removal from PfSUB1 catalytic domain. However, the fact that the non-regulatable p31 construct is not tolerated argues against this. Plasmid rearrangements could also cause a lack of expression. Plasmid rescues from pHH4-FKBP-pro transfected parasites indicated that no major rearrangements had occurred, however subtle rearrangements which are difficult to visualise by diagnostic digestion could have taken place. These could be identified by DNA sequencing. Alternatively, if the plasmid is intact, low expression levels may result from low copy number. Increasing the concentration of blasticidin in the culture medium with parasites transfected with episomal constructs with blasticidin-selectable markers has been shown to select for higher copy number (Mamoun *et al.*, 1999, Epp *et al.*, 2008). There is no evidence that the same effect occurs with WR99210, however this could be determined experimentally and might provide a solution to low copy number. Alternatively, the construct could be integrated into a redundant genomic locus, thereby avoiding problems with copy number.

If a conditional knockdown line is obtained in the future, several further experiments will be carried out to pursue the questions under investigation here. These will include analysis of conditional knockdown parasites using proteomics to analyse the global processing of proteins during egress with and without PfSUB1, which might result in the identification of new PfSUB1 substrates. The current model is that inhibition of PfSUB1 prevents schizonts from rupturing. This may be because breakdown of the PVM is blocked, though this has not been confirmed experimentally. To examine this, EM studies could be carried out on conditional knockdown parasites to determine whether the PVM remains intact upon schizont maturation in the absence of PfSUB1. Furthermore, since the merozoite surface is

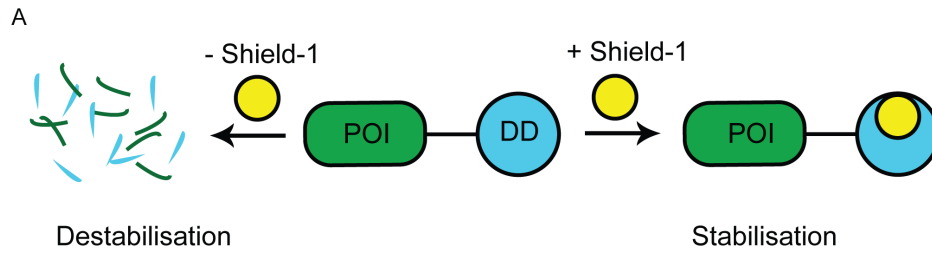
modified by PfSUB1, this will be similarly analysed by EM and might reveal detectable differences in surface structure in the presence or absence of PfSUB1. Parasites could also thereby be analysed for unexpected subcellular deformities resulting from PfSUB1 knockdown, in the event that PfSUB1 is involved in currently unknown pathways. In addition to these questions, a conditional knockdown line will be used to analyse PfSUB1 function in more detail. Since deleterious mutations cannot be integrated into the endogenous locus, episomal expression of mutated *pfsub1* genes could be used to attempt to complement PfSUB1 knockdown. These could include catalytic triad or active site mutations to alter the specificity of PfSUB1, or changes in the promoter region to analyse how PfSUB1 is trafficked to exonemes. Also, PfSUB1 cannot be used to complement *P. berghei* SUB1 (PbSUB1), as was attempted by double crossover integration into *P. berghei* (Sharon Yeoh, Mike Blackman and Rita Tewari, NIMR, unpublished data). Why PfSUB1 cannot complement PbSUB1 is unclear; this could be addressed using the conditional knockdown line, by attempting to complement PfSUB1 knockdown with PbSUB1 or other orthologues. If obtained in the future, the two conditional knockdown lines described in 3.2.1 and 3.2.2 could be analysed side by side as there should be no phenotypic differences between the parasites in which PfSUB1 is downregulated by destabilisation mediated by fusion to FKBP, or inhibited by overexpression of p31 in the PV. In all cases, growth assays, invasion and egress assays will be used to establish phenotypic differences where PfSUB1 is knocked down. Live imaging will be used to assess whether parasites can invade and egress in the absence of PfSUB1. It would also be interesting to culture PfSUB1-FKBP parasites in the absence of Shield-1, to see whether parasites are able to revert to being Shield-1 independent as Dvorin and colleagues found with CDPK5-FKBP knockdown parasites (Dvorin et al., 2010). Further experiments could also include mechanical disruption of SUB1-FKBP schizonts to determine whether merozoites in which PfSUB1 is knocked down are invasion-competent. As PfSUB1 processes important proteins on the merozoite surface, and is thereby thought to “prime” merozoites for invasion, knockdown parasites are likely to be non-invasive.

The importance of PfSUB1 in egress and invasion is unclear. PfSUB1 is responsible for the proteolytic maturation of several merozoite surface and PV proteins; though whether any of these processes are the reason why PfSUB1 is essential to parasite survival is unknown. In conclusion, a conditional knockdown of

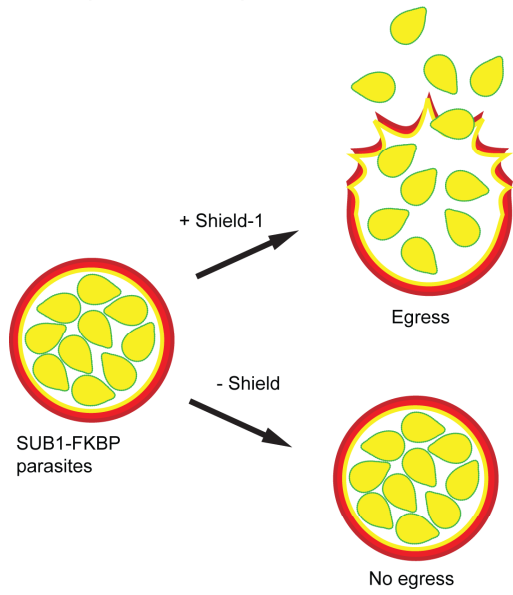
PfSUB1 would be hugely informative to analyse the function of this protein *in vivo*. The experiments presented here show that p31 and PfSUB1 retain their intrinsic activities when expressed in heterologous systems as FKBP fusion proteins. However, translating these studies into cultured *P. falciparum* parasites has been and remains a difficult challenge that must be overcome to analyse the function of this important protease in the asexual blood stages of *P. falciparum*.

Figure 17. Conditional knockdown of PfSUB1 using the FKBP destabilisation domain system

A. The FKBP destabilisation domain system relies on fusion of FKBP (DD) to the protein of interest, which results in the protein being degraded in the absence of stabilising small-molecule ligand Shield-1. In the presence of Shield-1, proteins are stabilised and are not degraded. Attempts to conditionally regulate PfSUB1 *in vivo* comprised two different approaches. B. In the first approach, PfSUB1 would be directly regulated by a C-terminal fusion of the endogenous protein to FKBP (PfSUB1-FKBP parasites). PfSUB1-FKBP parasites were expected to have a wild type egress phenotype in the presence of Shield-1, but be unable to egress in its absence. C. In the second strategy, parasites expressing an episomal copy of the p31 fused N-terminally to FKBP were generated (FKBP-p31 parasites). These were expected to have a defect in egress in the presence of Shield-1, and appear wild type in its absence.



B Regulatable endogenous PfSUB1



C Regulatable PfSUB1 prodomain

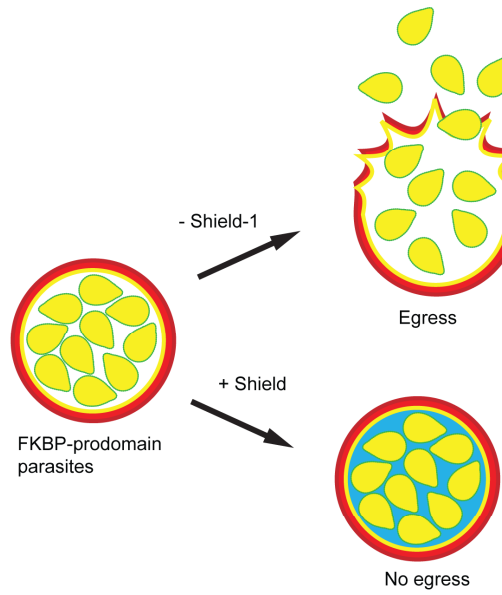


Figure 18. PfSUB1-FKBP expressed in Sf9 insect cells is catalytically active

Sf9 insect cells were transiently transfected with pMIB-PfSUB1-FKBP and pMIB-PfSUB1, in the presence (TN+) or absence (TN-) of tunicamycin. 1 or 1.5 μ M Shield-1 were used to stabilise expression. In the absence of tunicamycin, anti-FKBP12 antibodies (Affinity Bioreagents) recognise a 120 kDa full length precursor. In the absence of tunicamycin, PfSUB1-FKBP is again observed as a 120 kDa full length (presumably N-glycosylated) precursor. In the presence of tunicamycin and two different concentrations of Shield-1, full conversion to a smaller 70 kDa protein is observed, presumably due to autocatalytic removal of p31. Under the same conditions, wild type PfSUB1 (rPfSUB1) is observed as a processed 50 kDa band.

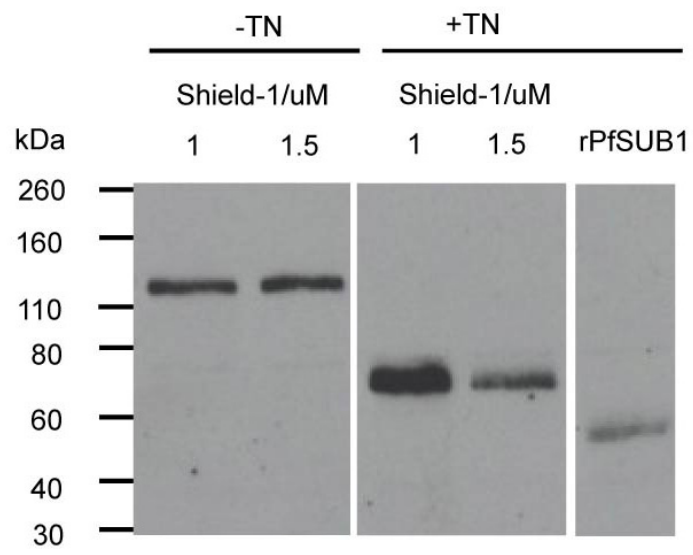
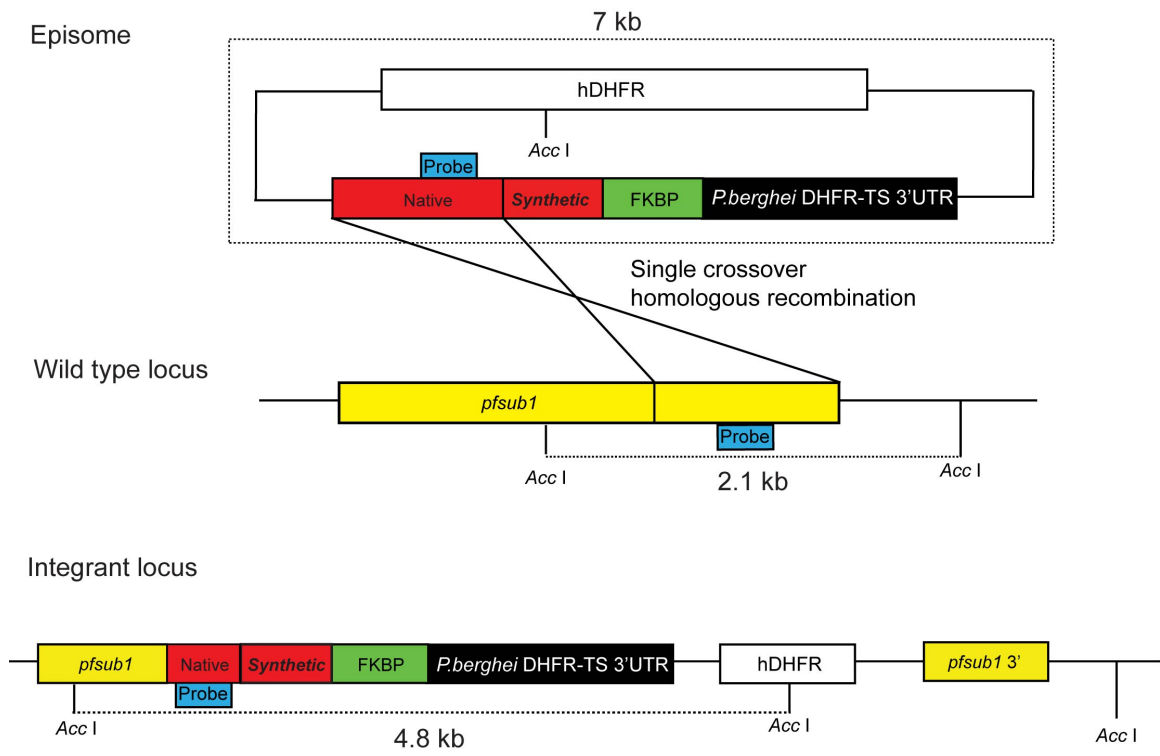


Figure 19. FKBP may be integrated into the *pfsub1* locus in *P. falciparum*

A. Plasmid pHH1-PfSUB1-FKBP contains 345 bp of synthetic recodonised sequence (synthetic) encoding the C-terminal 115 residues of PfSUB1 fused in frame to sequence encoding FKBP. This was fused in-frame to 598 bp of upstream native *pfsub1* sequence (native), to form the targeting sequence (target), homologous to a target region in the *pfsub1* locus. The predicted structure of the *pfsub1* genomic locus using the restriction enzyme Acc I following integration of pHH1-PfSUB1-FKBP is shown. B. Southern blot of genomic DNA extracted from the parental 3D7 strain and non-cloned transfected pHH1-PfSUB1-FKBP parasites from drug cycle 1 (c=1), cycle 2 (c=2) and cycle 4 (c=4), digested with Acc I. The blot was probed with a [³²P]-labelled 800 bp *pfsub1* fragment (probe). DNA from untransfected 3D7 *P. falciparum* was used as a control (3D7). A band at the predicted size of 2.1 kb for the wild type locus is observed in all digests. Episome is detected in all cycles as a 7 kb band. A 5.5 kb band is observed in c=2 and c=4 DNA which is absent from the 3D7 parental line and differs from the episome band, which is either indicative of plasmid rearrangement or of integration of the plasmid into the *pfsub1* locus, since the expected size for integration is 4.8 kb.

A



B

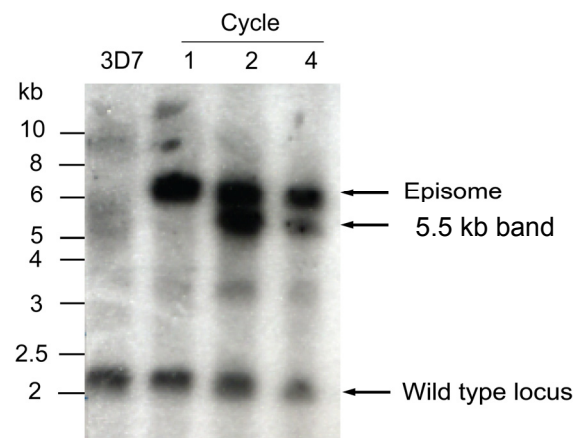


Figure 20. PfSUB1-FKBP is not detected in a non-clonal line of parasites transfected with pHH1-SUB1-FKBP

pHH1-SUB1-FKBP-transfected parasites from drug cycle 1 (c=1) and cycle 4 (c=4) were synchronised and cultured in the presence (+) or absence (-) of Shield-1 for one cycle. Schizonts were isolated and solubilised directly into SDS sample buffer, and separated by SDS-PAGE alongside untransfected *P. falciparum* 3D7 schizonts (3D7) and recombinant PfSUB1 (rPfSUB1). These samples were probed by Western blot using anti-PfSUB1 antibodies. PfSUB1 is detected in all lanes at the same sizes as in the 3D7 control sample. A faint band at 80 kDa is present in some of the PfSUB1-FKBP schizonts (arrowed) which is likely to be unprocessed PfSUB1 precursor.

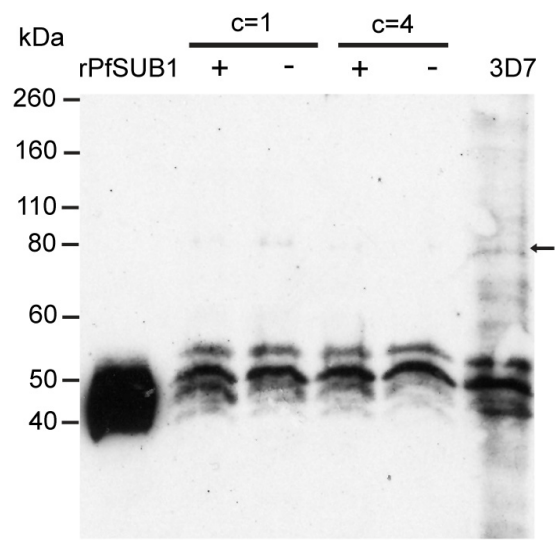
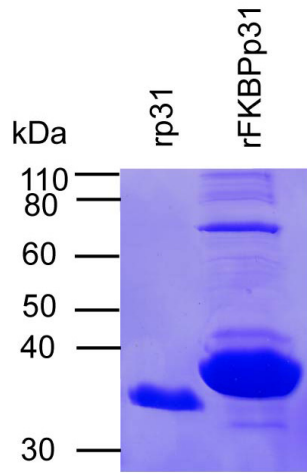


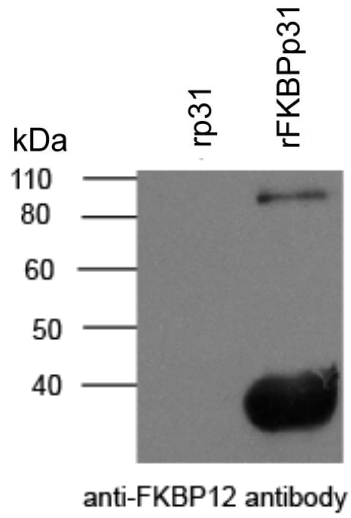
Figure 21. Expression and purification of rFKBP-p31

rFKBP-p31 was expressed in *E. coli* and purified by anion exchange and gel filtration, then subjected to SDS-PAGE and Coomassie staining along with rp31 (A). rFKBP-p31 was not completely pure and therefore it was not quantified in terms of protein concentration. rFKBP-p31 was detected using anti-FKBP12 (B) or anti-p31 antibodies (C). Both antibodies recognise a 42 kDa band in the FKBP-p31 tracks, plus a 110 kDa band which is probably an SDS-resistant aggregate of rp31.

A



B



C

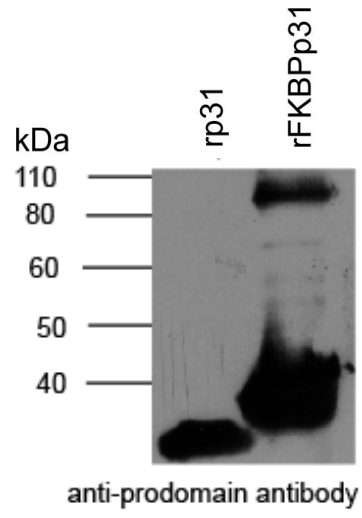


Figure 22 Inhibition of recombinant PfSUB1 with recombinant FKBP-p31

Recombinant PfSUB1 was incubated with a rhodamine-labelled SERA5 site 1 peptide in PfSUB1 digestion buffer, and the increase in fluorescence intensity measured by spectrofluorimetry. After 25 minutes, purified rFKBP-p31 or rp31 was added to each well and measurements were continued. As controls, two wells were set up where no p31 was added (positive control), or no PfSUB1 (negative control). Upon addition of p31 or FKBP-p31, no further increase in activity is observed.

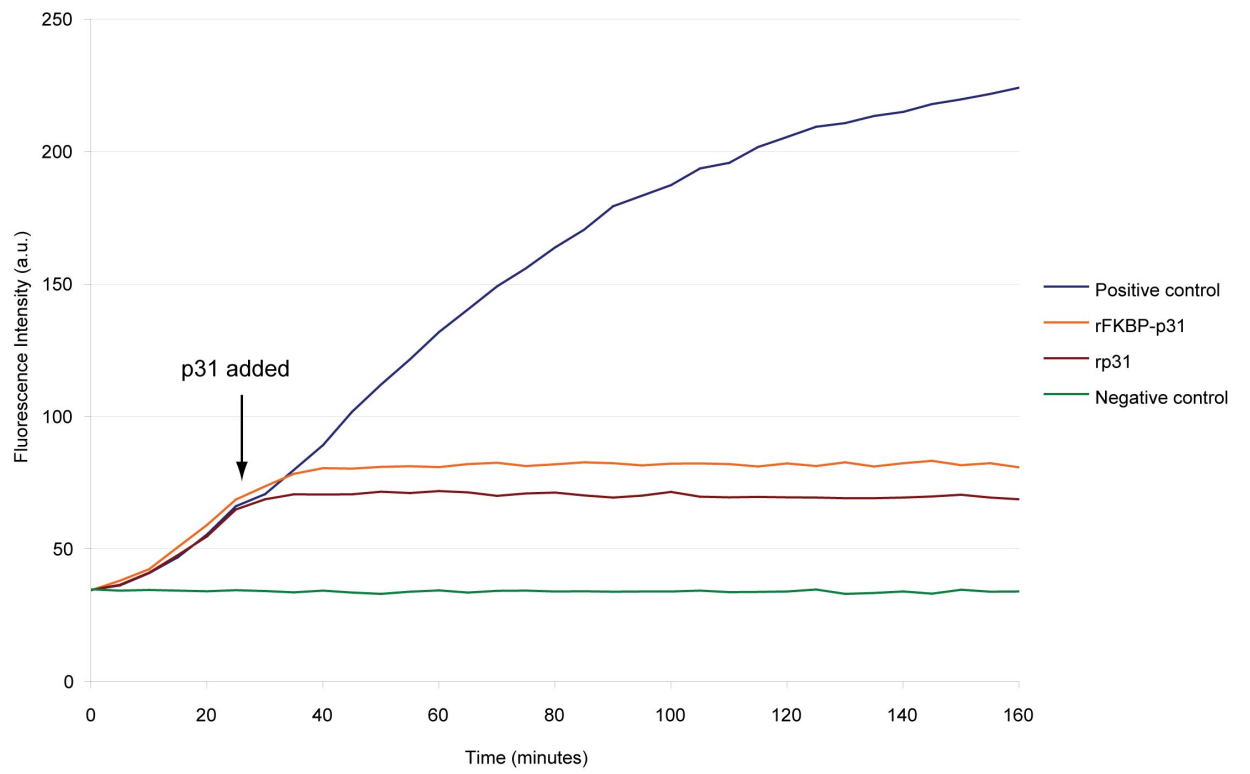
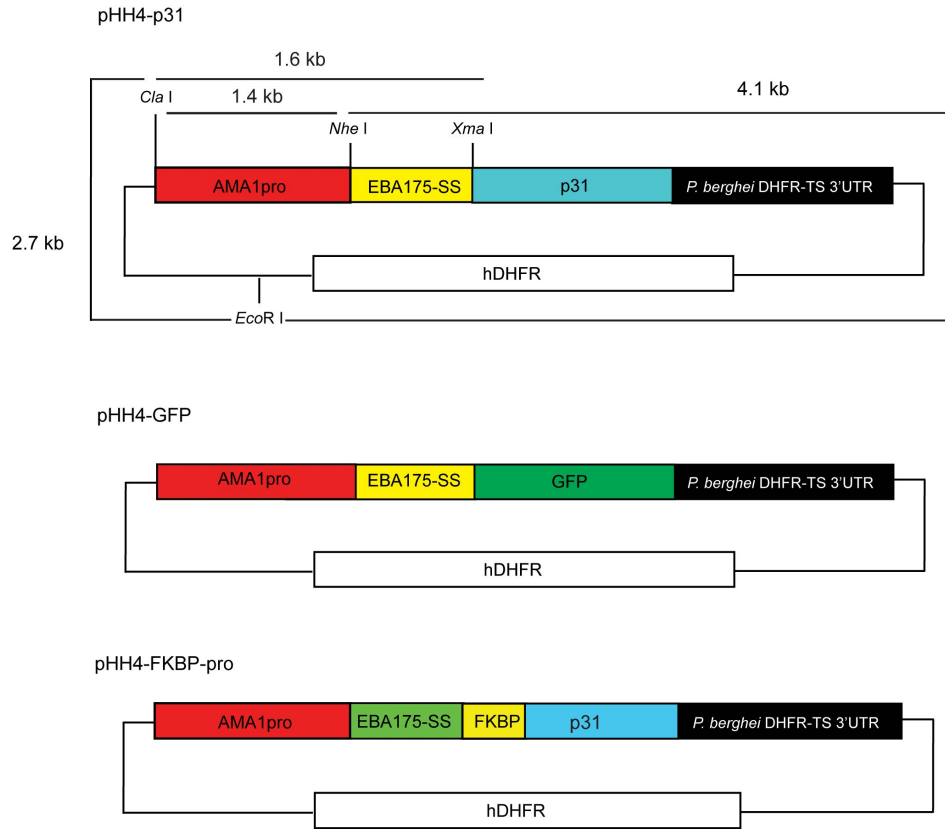


Figure 23. Constitutive expression of p31 in *P. falciparum* is not tolerated: plasmid rescue attempts show selection for drug-resistant parasites harbouring plasmid with a rearranged promoter

A Parasites were transfected with one of each of 3 plasmids designed for transgenic expression of either unmodified p31 (pHH4-p31), GFP (pHH4-GFP) or FKBP-p31 (pHH4-FKBP-p31). Expression was driven by the AMA1 promoter and in each case the protein was N-terminally fused to the secretory signal peptide of EBA175, which has previously been used to target proteins to the PV (Ellen Knuepfer and Tony Holder, NIMR, unpublished). Parasites carrying these plasmids were selected by use of the human dihydrofolate reductase cassette (hDHFR) which confers resistance to WR 99210. Unique restriction enzyme sites are shown in these schematics and the expected sizes from digestion with these enzymes. These schematics are not drawn to scale. B. Clones from plasmid rescues (PR) from parasites transfected with pHH4-pro and pHH4-GFP were analysed by restriction digest using Cla I and Xma I to screen for rearrangements in these constructs. pHH4-p31 DNA samples were extracted from 4 independent transfections (PR1-4). Digests were compared to digests of Maxiprep DNA (MP) used for transfections. Digestion of DNA extracted from parasites transfected with pHH4-GFP indicated that the promoter was intact and ran at the same size as MP DNA. Digestion of PR from pHH4-p31 parasites using Cla I and Xma I resulted in smaller promoter fragments compared to MP DNA. These fragments are not the same size in all PRs.

A



B

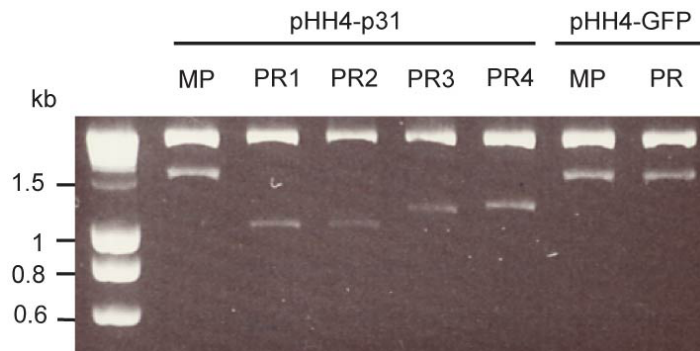
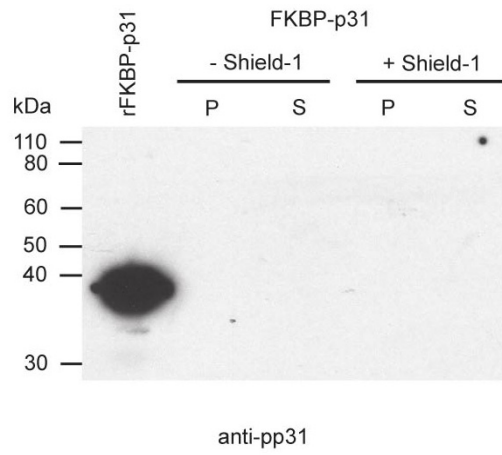


Figure 24. Expression of transgenic FKBP-p31 is not detected in parasites transfected with plasmid pHH4-FKBP-p31

pHH4-FKBP-p31-transfected parasites expanded under drug selection were synchronised and cultured for one cycle in the presence or absence of 0.5 μ M Shield-1. Schizonts were isolated on a Percoll gradient, then lysed with saponin and centrifuged to separate soluble RBC, exported or PV proteins (S) and membrane-associated proteins or parasite soluble proteins (P). In panel A, samples were probed with anti-p31 monoclonal antibody 4B4.1F6.B10. rFKBP-p31 was used as a positive control. No signal was observed in these samples. B. To confirm that late schizonts expressing AMA1 had been purified, the same samples were probed with anti-AMA1 antibody CRC3 R2.

A



B

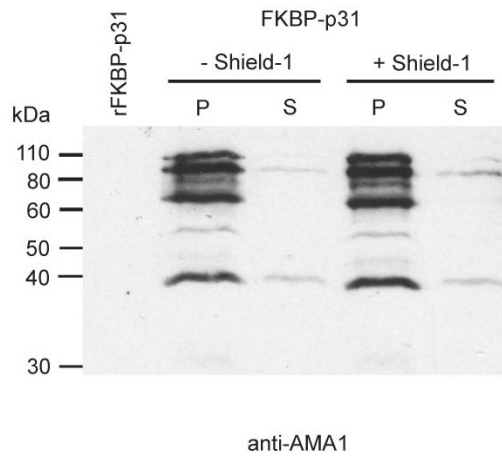
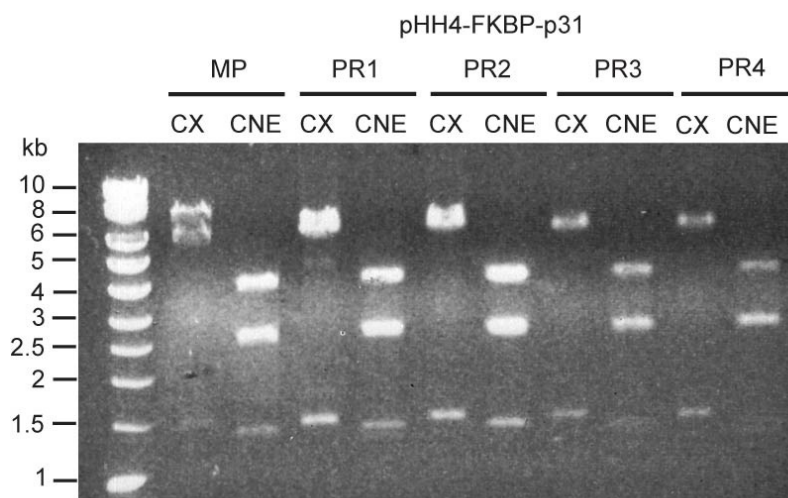


Figure 25. Diagnostic digests of rescued FKBP-p31 plasmids indicate that the plasmid has not rearranged

Plasmid rescue from parasites transfected with pHH4-FKBP-p31 yielded 4 plasmid clones (PR1, PR2, PR3, PR4). These were digested with either Cla I and Xma I (CX) or Cla I, Nhe I and EcoR I (CNE) and compared to digestion patterns of the pHH4-FKBP-p31 input transfection DNA (MP). Expected sizes are indicated in Figure 23. Compared to the digestion pattern observed with pHH4-FKBP-p31 MP, PR1-4 are similar. There is an additional band at 6 kb in the MP sample, which is likely to be supercoiled DNA.



4. Results chapter 2: spatiotemporal analysis of PfSUB1 activity

4.1. Introduction

PfSUB1 is thought to be active in the PV just prior to or during egress but the timing of its activity is poorly understood. Egress occurs over a period of just minutes (Dvorak et al., 1975, Glushakova et al., 2005, Gilson & Crabb, 2009), therefore determining at which point in the egress process PfSUB1 becomes active is a considerable challenge. This is because it is difficult to synchronise parasites with a tight enough window to be able to discriminate between pre-egress and later stages by Western blot or IFA. Such studies are further complicated by the lack of complete understanding of egress itself. Analysing live cells by live microscopy would avoid some of these problems as a single schizont could be monitored over time. This would be aided by the use of a visible reporter of PfSUB1 activity which could be used in live cells e.g. a PfSUB1 substrate which is designed to report when PfSUB1 is active. This would enable the visualisation of PfSUB1 activity by live fluorescence microscopy combined with simultaneous brightfield microscopy to monitor when egress occurs.

Förster resonance energy transfer (FRET) effect is the non-radiative transfer of energy from a donor to an acceptor fluorophore (e.g. cyan and yellow fluorescent proteins (CFP, YFP)). Typically, for FRET to occur, the two fluorophores must have overlapping absorption and emission spectra and be spatially adjacent, within 80-100Å of one another. The efficiency of the transfer is dependent on the relative orientation of the donor and acceptor dipole moments, the spectral overlap of the donor and acceptor, and the distance between the fluorophores (Förster, 1948). FRET is used for a variety of applications including examining protein-protein interactions or conformational changes within proteins and, importantly, to monitor protease activity, and have been widely applied in live cells. Protease activity can be monitored by using FRET partners that are linked using flexible polypeptides containing protease-specific cleavage sites; thus protease activity is indicated by a decrease in acceptor fluorophore emission (a loss of FRET) (Figure 26). The first protease-sensitive FRET reporter was developed by Mitra *et al*, who demonstrated that blue and red fluorescent proteins connected by a Factor Xa-sensitive linker

could be used to detect Factor Xa activity *in vitro* (Mitra *et al.*, 1996). Since then, protease-sensitive FRET reporters have been expressed in various cell types for real time experiments *in vivo*, monitoring protease activity spatially and temporally using live fluorescence microscopy. Examples of this include studies on caspase 3 activity during apoptosis using a caspase 3-sensitive FRET reporter in HeLa cells (Luo *et al.*, 2001, Rehm *et al.*, 2002, Takemoto *et al.*, 2003) and in live *Drosophila melanogaster* salivary glands (Takemoto *et al.*, 2007). Similarly, analysis of β -secretase activity was mediated using a FRET reporter anchored to the cell surface (Lu *et al.*, 2007).

To date, protease-sensitive FRET reporters have not been used in *P. falciparum*. The aim of the work described in this chapter was to use PfSUB1-sensitive FRET to monitor PfSUB1 spatiotemporal activity in *P. falciparum* in real time. The PfSUB1-sensitive reporter was targeted to the PV in late schizonts, so that when PfSUB1 is released into the PV during the final stages of egress, it would come into contact with the FRET reporter. Cleavage of the reporter by PfSUB1 was anticipated to result in a change in YFP to CFP emission (Figure 26). The feasibility of PfSUB1-sensitive FRET was tested *in vitro* using recombinant enzyme and a recombinant FRET reporter, before embarking on studies in cultured *P. falciparum* parasites.

4.2. Results

4.2.1. A recombinant PfSUB1-sensitive FRET reporter is cleaved by PfSUB1 *in vitro*

To determine whether a PfSUB1-sensitive FRET reporter could be used to detect PfSUB1 activity, the system was first validated *in vitro* using recombinant enzyme and reporter. Two reporters were used, both comprising YFP and CFP connected via an 11-residue linker containing a protease cleavage site, with an N-terminal hexahistidine tag (6xHis) attached to YFP for purification purposes. One reporter (FRET-ELA) contained an elastase (a serine protease) cleavage site (EISYEACGRR), which PfSUB1 was expected not to cleave; this therefore acted as the negative control. The other reporter (FRET-SERA5) contained a linker that included the SERA5 site 1 cleavage site (EIKAETEDDDF), previously shown to be an efficiently-cleaved PfSUB1 substrate (Yeoh *et al.*, 2007, Koussis *et al.*, 2009). To

generate a construct for the expression of recombinant FRET-SERA5 (rFRET-SERA5), the plasmid pRSFRET-ELA, which encodes recombinant FRET-ELA (rFRET-ELA), was modified, replacing sequence encoding the elastase cleavage site with sequence encoding the SERA5 site 1 site, to make the construct pRSFRET-SERA5. Cloning of this construct was extremely challenging, probably because YFP and CFP are almost identical in DNA sequence. Several strategies were explored to produce pRSFRET-SERA5. First, site-directed mutagenesis was performed to mutate the linker sequence. However, this resulted in truncated gene products when attempting to express proteins from this construct in *E. coli*. Second, the CFP gene was amplified by PCR using primers encoding the new linker region, but this resulted in truncated DNA sequences (despite many attempts at PCR optimisation). Finally, the construct was made by ligating long oligonucleotides encoding the linker region into the vector; extensive screening was carried out to identify correct clones.

Once the construct had been made, both reporters were expressed in *E. coli* and purified using nickel-histidine chelation. The resulting purified proteins are shown in Figure 27; comparable amounts of both proteins are present in these samples. The sensitivity of the reporters to PfSUB1 was assessed by incubating equal amounts of FRET-ELA and FRET-SERA5 with recombinant PfSUB1 (rPfSUB1) and analysing by SDS-PAGE and spectrofluorimetry. Firstly, the reporters were incubated with rPfSUB1, or rPfSUB1 plus recombinant PfSUB1 prodomain (rp31; a highly potent inhibitor of PfSUB1), or buffer alone for 2 hours at 37°C, and then separated by SDS-PAGE. Coomassie staining revealed that rFRET-SERA5 was cleaved by rPfSUB1 whilst the rFRET-ELA was not, as was expected (Figure 27). Cleavage was inhibited in the presence of rp31, indicating that cleavage was specific to rPfSUB1. Next, to determine whether cleavage results in a decrease in FRET effect over time, the experiment was repeated and the fluorescence intensity of the reporters was measured in a spectrofluorimeter by exciting at 435 nm (the peak excitation wavelength of CFP) and detecting emission at 485 nm (the peak emission wavelength of CFP) or 528 nm (the peak emission wavelength of YFP). This confirmed that the reporters both exhibit FRET, and that cleavage of rFRET-SERA5 resulted in a loss of FRET as expected (Figure 28). This loss of FRET did not occur in the presence of rp31, confirming that the effect is due to cleavage by rPfSUB1. These data provide evidence that FRET-SERA5 can act

as a reporter for PfSUB1 activity.

In order to determine whether rFRET-SERA5 had been cleaved at the predicted site, larger amounts of the reporter were incubated with rPfSUB1 to generate the two cleavage products, with the aim of determining the N-terminal sequence of the monomeric CFP. For this reason, CFP had to be separated from the YFP. The 6xHis at the N-terminus of YFP was therefore used to deplete 6xHis-tagged YFP by incubating the mixture with NiNTA agarose beads (Figure 29). The unbound cleavage product containing CFP (which has no purification tags) was concentrated and subjected to N-terminal sequencing. This confirmed its N-terminal sequence as TEDDDF, indicating that the reporter was indeed cleaved within the SERA5 site 1 linker at the predicted EIKAE↓TEDDDF bond (Figure 29). These encouraging results confirmed that the FRET-SERA5 reporter is specifically cleaved by PfSUB1 at the expected site.

4.2.2. Expression of a PfSUB1-sensitive FRET reporter in *P. falciparum*

Following on from these promising results, attempts were made to generate a parasite line expressing FRET-SERA5, with the ultimate aim of monitoring PfSUB1 activity *in vivo* by live fluorescence microscopy. Construct pHH4FRET-SERA5 was designed to obtain expression of FRET-SERA5 driven by the AMA1 promoter (which drives strong, late expression of AMA1 in schizonts). FRET-SERA5 was targeted to the PV using the secretory signal peptide of the microneme protein EBA-175, which has previously been used in combination with the AMA1 promoter to direct GFP to the PV (Ellen Knuepfer and Tony Holder, NIMR, unpublished data). The construct was transfected into *P. falciparum* and parasites harbouring the plasmid selected under drug pressure. To confirm that FRET-SERA5 was expressed in the *P. falciparum* line and in the soluble fraction (which would be consistent with localisation to the PV), pHH4FRET-SERA5 parasites were synchronised and lysed using saponin to separate the membrane-associated and parasite proteins (pellet) from the soluble EC and PV proteins (supernatant). Analysis of these samples by Western blot using anti-GFP antibodies detected a 60 kDa band in the pellet and 60 kDa and 30 kDa bands in the pellet and supernatant (Figure 30). These sizes correlate with full length and cleaved reporter respectively, suggesting that FRET-SERA5 is expressed as a soluble protein and may be

cleaved by PfSUB1. Unfortunately, these samples degraded during storage at -20°C and further experiments could not be carried out with them. Attempts to resynchronise parasites and repeat the experiment resulted in the failure to detect any signal with anti-GFP antibodies, suggesting that the parasites had stopped expressing FRET-SERA5. Parasites were therefore transfected again and a second transfectant line was established. Western blot analysis of schizont extracts from this line, however, (Figure 30) detected a 60 kDa band which is the same size as full length rFRET-SERA5. However, no signal at the same size as recombinant CFP was detected, perhaps due to the low levels of FRET-SERA5 in the supernatant or the absence of cleavage. Samples were reprobbed using anti-SERA5 antibodies which indicated that lysis of the parasites was incomplete (SERA5 is a PV protein which should only be detected in the supernatant of the samples if lysis is complete). However, this does not explain the difficulty in detecting the reporter by Western blot as it would therefore be expected to be detectable in the saponin pellet. Since expression of FRET-SERA5 from pHH4-FRET-SERA5 is driven by the AMA1 promoter, expression of AMA1 could be used to confirm that parasites of a stage where the AMA1 promoter is active, i.e. late schizonts, were present in these samples. AMA1 is membrane-associated and would therefore not be expected to be present in the saponin supernatant. To this end, the saponin pellets and supernatants were probed with anti-AMA1 antibodies, which detected AMA1 in the membrane-associated fraction (Figure 30). Hence, this confirms that the parasite preparations used to produce the extracts contained late schizonts. FRET-SERA5 could not be detected by live fluorescence imaging or by IFA using anti-GFP antibodies, in either of the parasite lines examined. These data suggest that a PfSUB1-sensitive reporter can be expressed in *P. falciparum*, but that low expression levels are a challenge to be overcome in future work.

4.3. Discussion

The aim of this project was to follow PfSUB1 activity in the parasite in real time. To this end, the feasibility of PfSUB1-sensitive FRET was examined *in vitro*, and the same reporter was expressed episomally in *P. falciparum*. A recombinant FRET reporter exhibiting PfSUB1-sensitivity was successfully developed to detect PfSUB1 activity *in vitro*. The finding that a PfSUB1-sensitive reporter could be engineered *in vitro* is in itself is very interesting – the reporter could be exploited further *in vitro*.

Since it is a fluorescent, proteinaceous PfSUB1 substrate, it offers advantages over synthetic peptides in that it more closely represents a real PfSUB1 substrate. This reporter could be further used to characterise PfSUB1 activity by, for example, inserting randomised linker regions and assaying cleavage by spectrofluorimetry, as has been carried out with a hepatitis cysteine protease 3C(pro)-sensitive reporter (Huitema & Eltis, 2010). The recombinant reporter could also be used to identify inhibitors of PfSUB1 using spectrofluorimetric assays.

Despite the promising results obtained in *in vitro* studies, the project stumbled at expression of the PfSUB1-sensitive FRET reporter in *P. falciparum*. Establishing and maintaining high levels of FRET-SERA5 expression was a major difficulty. The reason for this is unclear. Several studies have reported expression of GFP in *P. falciparum* (examples include (Klemba *et al.*, 2004a, Treeck *et al.*, 2009, Tonkin *et al.*, 2004)), so expression of a fusion between two fluorescent proteins was not expected to be problematic. In the first instance, no signal was observed by live fluorescence microscopy nor by live-cell IFA, suggesting that the reporter was not expressed at very high levels. It was also difficult to detect FRET-SERA5 by Western blot. Poor expression levels may be due to the fact that the AMA1 promoter drives expression in a short time window, therefore there is insufficient time for the reporter to accumulate at high levels in the PV before schizont rupture. For unknown reasons, the promoter may also be unable to drive high expression levels of this protein. In future work the AMA1 promoter could perhaps be replaced with a constitutive promoter, which may drive higher expression levels. However, the risk of this is that excessive levels of FRET reporter might be toxic to parasites. Transgenic parasites appeared to lose expression of FRET-SERA5 after continued culture, which suggests that expression of the reporter was somehow deleterious to the parasite; therefore, using a constitutive promoter might result in similar problems. Alternatively, low expression levels could be caused by the selection of parasites carrying episomes with low copy numbers. This could perhaps be resolved by inclusion of the rep20 segregation sequence into the plasmid (O'Donnell *et al.*, 2002). This might enable better plasmid segregation, so that more parasites are carrying more episomal copies per cell, thereby leading to higher levels of expression. Alternatively, the reporter could be integrated into a redundant genomic locus and a clonal line established, so that expression is maintained. Future work may involve transfection of cultured *P. knowlesi* parasites with pHH4-

FRET-SERA5, with the aim of integrating the construct into the genome (collaboration with Robert Moon, NIMR). *P. knowlesi* is very amenable to genetic manipulation; linear constructs can be transfected which results in a higher transfection efficiency and more rapid homologous recombination compared to that of cultured *P. falciparum* parasites (van der Wel *et al.*, 1997, Kocken *et al.*, 2002, Wel *et al.*, 2004). *P. knowlesi* parasites are also larger in size than *P. falciparum*, therefore there are larger PV-filled spaces in between parasites, which might be easier to analyse by microscopy compared to *P. falciparum*. However, it has not been confirmed that *P. knowlesi* SUB1 (PkSUB1) cleaves the SERA proteins, and if it does, it is unknown whether it will cleave the *P. falciparum* SERA5 linker sequence in the reporter. One way to evaluate this would be to test the sensitivity of rFRET-SERA5 to PkSUB1 *in vitro* using recombinant PkSUB1 (if this can be expressed *in vitro*). Homology modelling indicates that the architecture of the PkSUB1 active site groove is very similar to that of PfSUB1 (Christlaine Withers-Martinez and Mike Blackman, NIMR, unpublished), so it is possible that rFRET-SERA5 is PkSUB1-sensitive.

Once a parasite line expressing the FRET reporter is established, the system will need to be validated in several steps. Firstly, it will be important to show that FRET-SERA5 is indeed targeted to the lumen of the PV. This could be confirmed by differential streptolysin O (which ruptures the EPM and not the PVM) and saponin fractionation; here, saponin fractionation confirmed that FRET-SERA5 is in the soluble fraction of the PV or RBC but streptolysin O treatment could exclude that FRET-SERA5 is exported to the RBC. Colocalisation of the FRET reporter by IFA with a well-established PV marker such as SERA5 would confirm this. Secondly, a time course following cleavage of the reporter by Western blot and IFA during egress would confirm that, if a loss of FRET is observed during egress by live microscopy, this could be correlated with the reporter being cleaved. It will also be important to show that the reporter is being specifically cleaved by PfSUB1. This could be demonstrated by two complementary methods. Firstly, purified schizonts could be incubated with rPfSUB1 *in vitro*, using an assay which was previously used to identify PfSUB1 substrates (Koussis *et al.*, 2009). Briefly, a schizont lysate is obtained and incubated with recombinant PfSUB1, then subjected to analysis by Western blot using anti-GFP antibodies to monitor cleavage. Secondly, it will be important to confirm that FRET-SERA5, if cleaved during

egress, is cleaved by PfSUB1. FRET reporters tend to be highly resistant to non-specific proteolysis (Bokman & Ward, 1981, Felber *et al.*, 2004); GFP can be expressed in *P. falciparum* without being degraded, therefore the likelihood of FRET-SERA5 being cleaved by another protease is low. However, the FRET linker region is highly flexible, which might make it susceptible to proteolysis by enzymes other than PfSUB1; therefore non-specific proteolysis would need to be confirmed. To this end, the two fragments of the reporter could be pulled down using anti-GFP antibodies and the C-terminal portion could then be purified by depleting His-YFP (as was carried out with recombinant protein in this chapter). Subsequently, N-terminal sequencing could be used to confirm whether the linker was cleaved at the correct position. This, again, would rely on high levels of FRET-SERA5 expression in parasites.

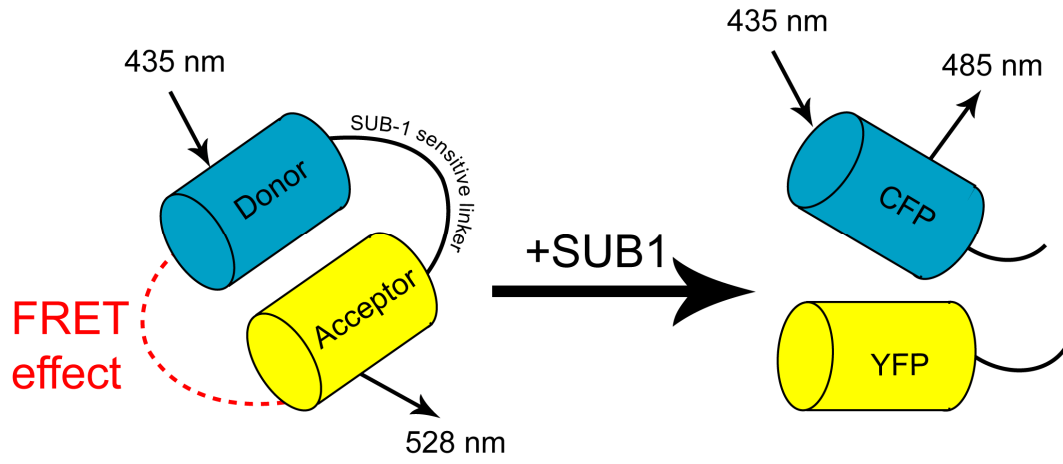
If these validation studies provide encouraging results, live imaging and IFA time courses could be carried out to analyse when PfSUB1 is active in the PV during the course of the egress pathway. Future experiments could look at the influence of protease inhibitors or other egress-inhibitory compounds on PfSUB1 activity to determine when it is active in relation to other proteases or events in egress. For example, E64, leupeptin and antipain block egress, but not PfSUB1 activity (Withers-Martinez *et al.*, 2002, Hadley *et al.*, 1983, Dluzewski *et al.*, 1986, Glushakova *et al.*, 2008, Glushakova *et al.*, 2005), but whether PfSUB1 is active in the PV of parasites treated with these inhibitors is unclear. Treatment of parasites expressing FRET-SERA5 with the aforementioned inhibitors would indicate whether PfSUB1 is regulated by these inhibitors. Another interesting compound to use for treatment of FRET-SERA5 parasites would be compound 1, which inhibits the GMP-dependent protein kinase, PKG (Taylor *et al.*, 2009), and prevents processing of SERA5 and MSP1 (Dvorin *et al.*, 2010). It would be of interest to determine whether PfSUB1 is active in compound 1-treated parasites because mechanical disruption of compound-1 treated parasites are not viable (Taylor *et al.*, 2009), which could be related to PfSUB1 being inactive. Furthermore, current data from our lab (Christine Collins and Mike Blackman, NIMR, unpublished) suggests that PKG may be responsible for the release of PfSUB1 from exonemes, which could explain the potent inhibition of egress observed in the presence of compound 1. The PfSUB1-sensitive FRET system could be applied to observe whether PfSUB1 is indeed released and active in the PV in the presence of compound 1.

The studies presented here show that it is possible to generate a PfSUB1-sensitive FRET reporter which is cleaved *in vitro*, and may be cleaved by PfSUB1 *in vivo*. A future challenge will be to establish high enough expression levels to be able to visualise PfSUB1 activity in live parasites. If successful, this system could facilitate very interesting studies on the spatiotemporal activity of PfSUB1 prior to and during egress.

Figure 26. PfSUB1-sensitive Fluorescence Resonance Energy Transfer

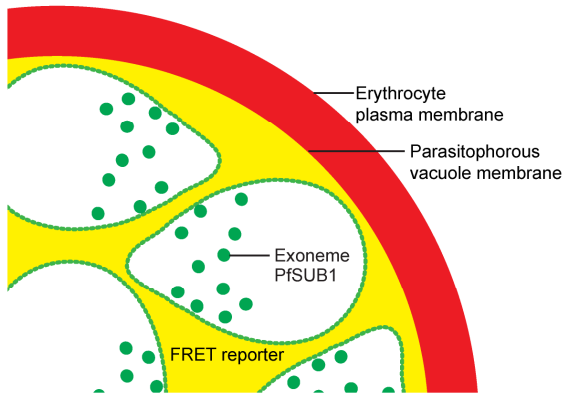
A. FRET is the non-radiative transfer of energy between two fluorophores with overlapping absorption and emission spectra. The donor fluorophore e.g. CFP, is excited by energy at a peak wavelength of 435 nm and emits energy at a maximum wavelength of 485 nm wavelength. This wavelength is able to excite the acceptor fluorophore, which emits at a peak wavelength of 528 nm. The transfer of energy between the fluorophores resulting in emission from the acceptor is termed the “FRET effect.” In a PfSUB1-sensitive FRET reporter, the two fluorophores are kept in close contact via a PfSUB1-sensitive linker. Cleavage by PfSUB1 releases two free fluorophores, CFP and YFP. When these are excited at 435 nm, emission at 485 nm occurs but not at 528 nm as the acceptor is no longer excited. B. Schematic depicting PfSUB1-sensitive FRET being used to monitor PfSUB1 spatiotemporal activity *in vivo* in cultured *P. falciparum*. The PfSUB1-sensitive reporter is expressed in the PV in late schizonts. When PfSUB1 is released from exonemes into the PV, a change from YFP (yellow) to CFP (cyan) signal is expected as the reporter is cleaved by PfSUB1.

A



B

1) Late segmented schizont: FRET reporter is uncleaved in the PV



2) Prior to schizont rupture: PfSUB1 is released into the PV

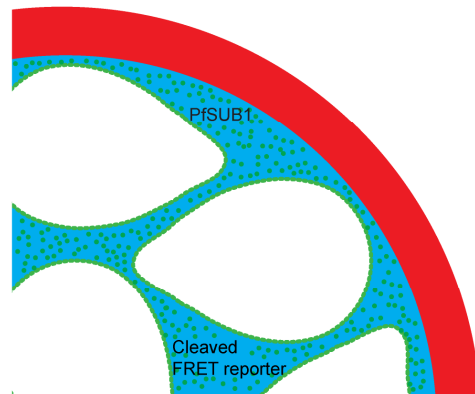
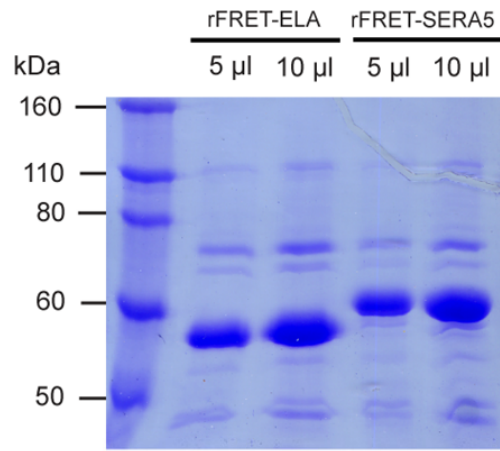


Figure 27. Purified recombinant FRET-SERA5 is cleaved by rPfSUB1

A. rFRET-SERA5 and rFRET-ELA were expressed in *E. coli* and purified by nickel-histidine chelation chromatography. Eluted proteins were separated by SDS-PAGE; 5 μ l or 10 μ l of each sample was run so that the relative amounts of each reporter could be compared, showing that similar amounts of protein are present in each preparation. B. The recombinant proteins were incubated for 2 hours at 37°C with buffer only (-), with rPfSUB1 (+) or with rPfSUB1 plus the PfSUB1 prodomain, rp31 (++). rp31 is present as a prominent band in ++ samples and is indicated. Only rFRET-SERA5 was cleaved in the presence of PfSUB1, and this was blocked by the presence of rp31. The full length reporters are indicated, as are the monomers of YFP and CFP which are released after cleavage (which migrate as a closely-spaced doublet on SDS PAGE).

A



B

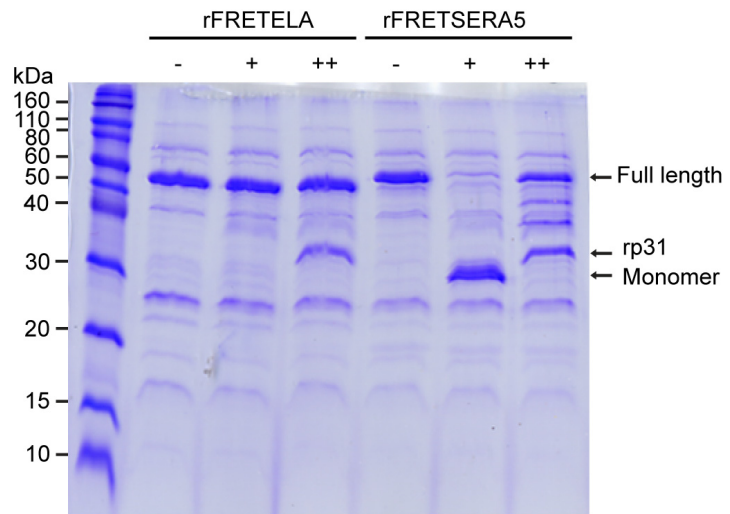


Figure 28. rFRET-SERA5 exhibits FRET, which is abolished upon cleavage by PfSUB1

rFRET-SERA5 and rFRET-ELA were incubated with buffer, with rPfSUB1 or with rPfSUB1 plus rp31, and analysed by spectrofluorimetry. Samples were excited at 435 nm. Emitted fluorescence (in arbitrary units; a.u.) was measured at 485 nm (CFP) and 528 nm (YFP) simultaneously. A reduction in YFP emission (indicating a loss of FRET) was only observed when rFRET-SERA5 was incubated with rPfSUB1, confirming that the FRET effect is lost in the presence of rPfSUB1.

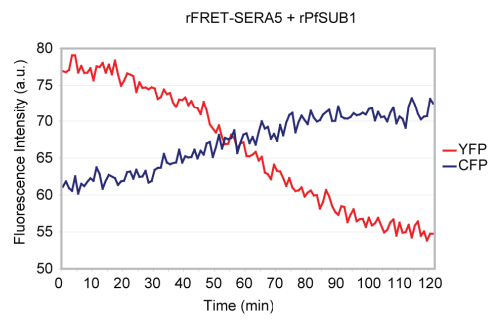
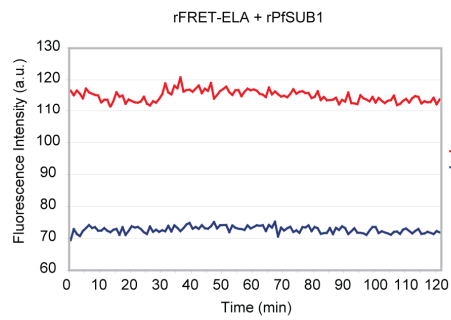
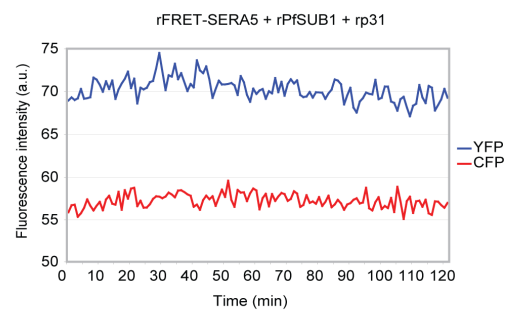
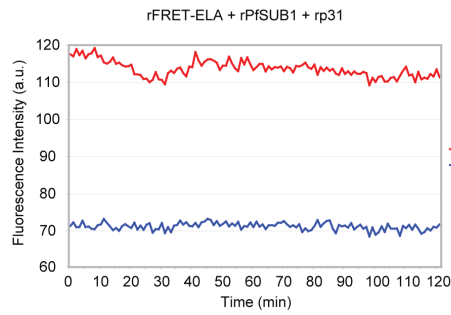
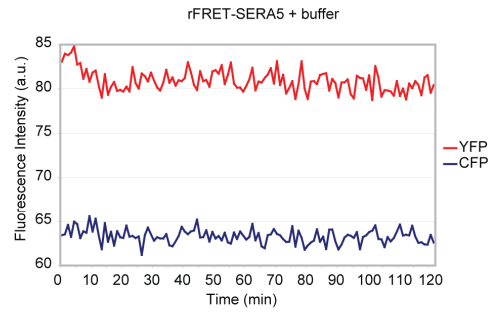
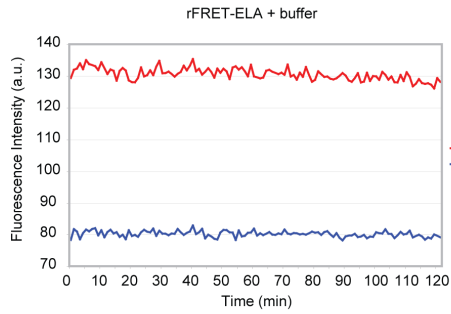
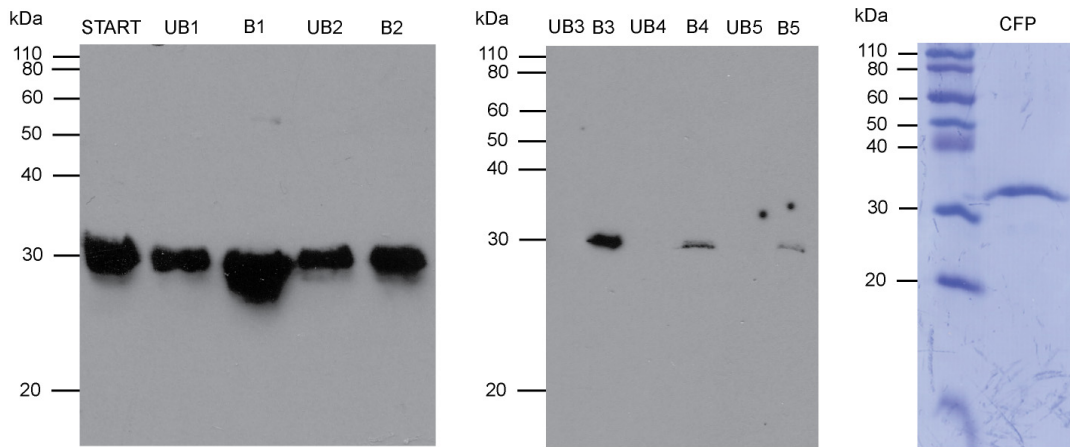


Figure 29. rFRET-SERA5 is cleaved at the expected site by rPfSUB1

To determine the site of PfSUB1 cleavage, rFRET-SERA5 was incubated with rPfSUB1 and the cleaved CFP portion was purified by repeatedly incubating protease-treated rFRET-SERA5 with NiNTA agarose beads and centrifuging to deplete His-YFP. A sample of the protein mixture before depletion was taken (START) and after each step samples of unbound proteins (UB) were taken, and the NiNTA agarose beads (B) were solubilised in SDS buffer. Samples from each step (e.g. UB1, B1) were probed by Western blot using anti-His antibodies (Sigma). In the left hand panel, 20 μ l each protein sample was run on SDS-PAGE; in the middle panel, 5 μ l protein sample was analysed. A lower loading volume allowed differences in signal intensity between the UB and B samples to be detected more accurately. The corresponding decrease in sensitivity of the assay for detecting contaminant His-YFP was not significant as CFP was required to be only partially pure for N-terminal sequencing. Consecutive rounds of depletion with NiNTA agarose beads resulted in purified CFP (right hand panel) which did not react strongly with anti-histidine antibodies (Panel B, UB5). The purified CFP was N-terminally sequenced by Edman degradation (performed at PNAC, University of Cambridge), which indicated that its N-terminal sequence was TEDDDF. It was concluded that the reporter is cleaved by rPfSUB1 at EIKAE↓TEDDDF (B).

A



B

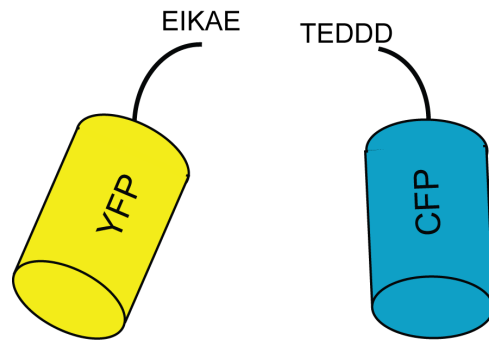
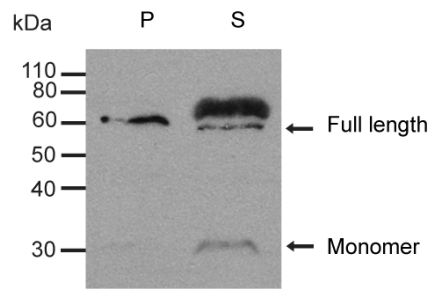
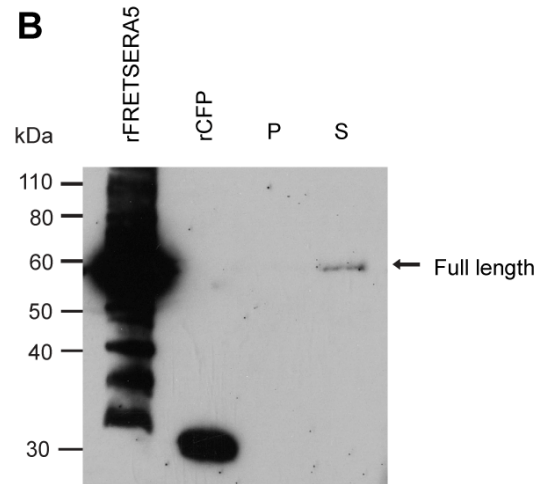


Figure 30. FRET-SERA5 is expressed in a soluble form in *P. falciparum*

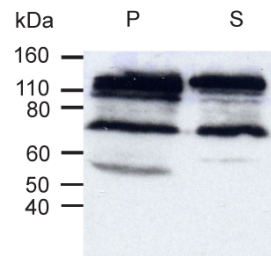
Schizonts from parasites harbouring pHH4-FRET-SERA5 were purified and treated with saponin to separate soluble PV and soluble RBC proteins, and PVM, EPM and soluble parasite cytosolic proteins. Resulting pellet (P) and supernatant (S) samples were separated by SDS-PAGE and probed using anti-GFP monoclonal antibodies (Roche) (A). A band at 62 kDa was detected in the pellet and three bands at 65 kDa, 60 kDa and 30 kDa were present in the supernatant. Since the upper 65 kDa band is absent from the pellet sample, this could be a contaminant. The 62 kDa and 60 kDa bands are likely therefore to correlate to full length reporter with and without the secretory signal peptide respectively. The 30 kDa band thus probably relates to cleaved CFP and YFP (monomeric). Full length and monomeric reporter are indicated with arrows. Schizonts from a second transfected line were purified and saponin lysed again, probed with anti-GFP antibodies alongside full length rFRET-SERA5 and purified cleaved recombinant CFP (rCFP) (B). A weak signal in parasite supernatant (S) at 60 kDa was observed, which presumably is full length FRET reporter. The same samples as used in panel B were reprobbed with anti-SERA5 antibodies (C) or anti-AMA1 antibodies (CRC3-R2) (D); SERA5 is a PV protein, therefore probing with these antibodies reveals that saponin lysis was incomplete as SERA5 should be present in only the supernatant (S), not the pellet (P). AMA1 was used as an indicator of late schizogony and should not be released by saponin lysis as it is membrane-associated. That AMA1 is detected confirms that late schizonts were present in these samples.

A

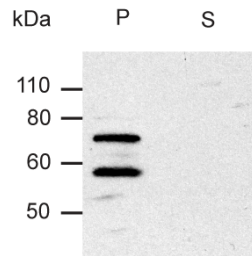
anti-GFP

B

anti-GFP

C

anti-SERA5

D

anti-AMA1

5. Results chapter 3: identification of novel PfSUB1 substrates

5.1. Introduction

During or just prior to egress, PfSUB1 is responsible for the proteolytic maturation of a number of PV-resident substrates, including the putative papain-like proteases SERA4, SERA5, and SERA6, and the merozoite surface proteins MSP1, MSP6 and MSP7 (Koussis et al., 2009, Yeoh et al., 2007). PfSUB1 processes SERA5 at two positions called site 1 and site 2, and SERA4 and SERA6 are probably processed in the same manner (Yeoh et al., 2007). PfSUB1 also cleaves SERA5 at a third, allele-specific site near the N-terminus (Li et al., 2002). Alignment of all eight *P. falciparum* SERA family proteins expressed during the asexual blood stages of the parasite life cycle indicates that the sequences flanking cleavage sites 1 and 2 are conserved across the family (Yeoh et al., 2007). PfSUB1 processes MSP1 at three positions and MSP6 and MSP7 at one (Koussis et al., 2009). The identification of conserved sites in SERA family members and the presence of cleavage sites in multiple MSP proteins suggests that PfSUB1 may cleave other MSP or SERA proteins. It is also possible that PfSUB1 processes other proteins on the merozoite surface or in the PV during egress. Therefore, the objective of this part of the project was to ask the question: does PfSUB1 have other substrates in addition to these?

To address this question, we took advantage of findings from previous studies where PfSUB1 specificity was characterised using purified recombinant PfSUB1 (rPfSUB1) and synthetic peptides. Analysis of the autocatalytic cleavage site within PfSUB1 (Withers-Martinez et al., 2002, Sajid et al., 2000), of synthetic peptide substrates (Withers-Martinez et al., 2002) and of the SERA5 and MSP cleavage sequences, led to the assembly of the putative PfSUB1 consensus recognition sequence Ile/Leu/Val/Thr/Phe-Xaa-Gly/Ala-Paa(not Leu)↓Xaa (where Xaa is any amino acid residue and Paa tends to be a polar residue) (Yeoh et al., 2007). Further alanine scanning experiments supported this consensus motif (Koussis et al., 2009). In addition, there is a general tendency for charged or acidic residues in the P' subsites (Figure 31). To date, only Gly or Ala have been observed at P2 in validated cleavage sites; homology modelling of PfSUB1 suggests that this

is due to a restricted S2 pocket (Withers-Martinez et al., 2002). Furthermore, cleavage is blocked or greatly reduced by the presence of leucine at P1 (Withers-Martinez et al., 2002), and Leu is not observed at P1 in validated cleavage sites (Figure 31).

PoPS (Prediction of Protease Specificity) is a computational application, which enables modelling of protease specificity and *in silico* prediction of potential protease substrates. This approach has been used in several studies to identify novel substrates of caspase 8, dust mite Derp 1 protease and membrane type 1 matrix metalloprotease, among others (Golubkov *et al.*, 2005, Furmonaviciene *et al.*, 2007, Scott *et al.*, 2008). To predict new PfSUB1 substrates *in silico* in this study, PoPS was used to model PfSUB1 specificity by incorporating the information described above. This led to the identification of several new putative PfSUB1 substrates. An *in vitro* proteomic approach was then used to experimentally identify PfSUB1 substrates. Several hits were investigated in further detail by Western blot using specific antibodies, as well as by examining *in vitro* cleavage of acetylated peptides based on predicted PfSUB1 cleavage sites using recombinant PfSUB1. Through a combination of the predictive and experimental approaches, it was found that two proteins, MSP7-like protein 2 (MSRP2) and RAP1 are likely to be new physiological PfSUB1 substrates.

5.2. Results

5.2.1. Modelling PfSUB1 specificity

Generation of a protease substrate specificity model involves permitting or excluding residues at each subsite of the cleavage site, based on whether they are known to allow or prevent cleavage. Using the information about PfSUB1 specificity discussed above, a PoPS PfSUB1 specificity model was assembled. In the first step, 15 validated PfSUB1 cleavage sites (from the previously confirmed physiological substrates described above) were aligned, and each amino acid residue was given a score for each subsite within the cleavage site, related to the frequency of the residue across the validated sequences. This is graphically represented in a WebLogo image (Figure 32). Scoring in a PoPS protease specificity model allows the user to rank cleavage sites in order of how likely they are to be cleaved. However, this feature was not used here, because although a

higher frequency of a certain residue at a given position could indicate a preference for PfSUB1, there is insufficient information about what constitutes a “better” PfSUB1 cleavage site. In order to increase the specificity of the model, residues which are absent from a certain subsite in the validated cleavage sites, or are known to prevent cleavage when at a specific subsite, were blocked based on information obtained from the cleavage of acetylated peptides by rPfSUB1 *in vitro* (Koussis et al., 2009, Withers-Martinez et al., 2002, Sajid et al., 2000).

5.2.2. *In silico* prediction of *P. falciparum* PfSUB1 substrates

The model described in 5.2 was used to computationally scan the entire predicted proteome of *P. falciparum* (5,679 proteins) (available at <http://www.PlasmoDB.org>), resulting in a list of 2,086 proteins with putative PfSUB1 sites (36.9% of the predicted proteome). This primary list of potential substrates generated by this step was then delimited by several factors common to all previously identified substrates (Figure 33). Firstly, the subcellular localisation of each substrate was considered. PfSUB1 is thought to mediate its physiological activity upon release into the PV (Yeoh et al., 2007), therefore, putative substrates were considered more likely to be true substrates if they localise to that compartment. The majority of established PV, PVM and MSP proteins have N-terminal secretory signals, and transport to the PV is considered the default pathway for proteins with classical signal peptides (Adisa et al., 2003). For that reason, each of the 2,086 putative substrates was analysed using the algorithm SignalP, which predicts the presence of signal peptides in a user-provided protein sequence. Each putative substrate was then included or excluded accordingly. This resulted in the elimination of 77% of the primary protein set, leaving 480 proteins with predicted secretory signal peptides for further analysis (Figure 33).

In a second filter step, the predicted or known function of the putative substrates was used as a selection criterion. Since PfSUB1 is involved in invasion and/or egress, we were interested in proteins involved in the same processes. Proteins predicted or known to be involved in other pathways and processes such as metabolism or protein translation were therefore excluded from the putative substrate list. Where no function had been assigned, homology searches were carried out using NCBI-BLAST to identify closely related proteins, and conserved functional domains were identified using InterPro. Proteins were then assigned a

putative function, which was used for selection or elimination. The functional distribution of the 480 proteins predicted to be secreted is shown in Figure 34. Considering their putative or known functions, many proteins are unlikely to be PfSUB1 substrates. Substrates were then included or excluded accordingly. This may have resulted in the removal of some true PfSUB1 substrates; however, in order to narrow down the list, which probably contained many false positives, it was considered essential. Hypothetical proteins with no significant homology to other proteins were retained in the filtered set as there was no basis for their exclusion.

Finally, in a third filter, substrates were only selected if they are at least known to be transcribed, if not translated during asexual blood-stage schizogony. Again, this was because PfSUB1 is active during schizogony; putative substrates must be expressed at the same time as PfSUB1 for PfSUB1-mediated processing to be a possibility. Information from several large scale analyses of protein and mRNA expression across the life cycle (Le Roch et al., 2004, Florens et al., 2002, Hall et al., 2005) was used to determine whether the expression of potential substrates coincides with PfSUB1 expression. Hits with a molecular weight of above 200 kDa were eliminated since all known PfSUB1 substrates are below this size, and these would also be technically difficult to work with if further validation was required. Proteins with more than one predicted transmembrane domain (aside from predicted signal peptides) were also excluded as all known PfSUB1 substrates are soluble (though because some MSPs are GPI-anchored, predicted GPI-anchored proteins were retained).

Another option available in PoPS software is to include secondary structure predictions of predicted protease cleavage sites. With few exceptions, proteases recognise their substrates in an extended β -strand conformation (Tyndall *et al.*, 2005). Few *Plasmodium spp.* proteins have been characterised at the structural level, but secondary structural features can be predicted *in silico*. To assess whether secondary structure could be incorporated into PfSUB1 substrate prediction, the secondary structure of the 20 amino acids around the scissile bond of each known PfSUB1 cleavage site was analysed using JPred (a secondary structure prediction algorithm). Secondary structure predictions are presented in Figure 35. Several cleavage sites, including the MSP7, SERA4 site 2 and SERA6 site 1 sites, occur in what are predicted to be completely disordered regions. In most others, the scissile bond tends to lie in a disordered region but other residues

forming part of the putative recognition sequence are within an α -helix or β -strand; examples of these are the SUB1 autocatalytic cleavage site, SERA5 site 1, SERA5 site 2, SERA5 site 3, SERA6 site 2 and SERA4 site 2. Since these data indicate a high degree of variation across the established cleavage sites, secondary structure predictions were not used for delimitation in the screen for new substrates.

Application of the three filters described above resulted in a list of 77 “most likely” putative PfSUB1 substrates, summarised in Table 7. This corresponds to 1.4% of predicted *P. falciparum* proteins. As anticipated, this list included known substrates SERA4, SERA5, SERA6 and MSP1, MSP6 and MSP7 (these are not included in Table 7), confirming that the use of the model and stringencies selects for proteins likely to be PfSUB1 substrates. Several new candidates were identified. Of note, twenty-four new putative substrates are hypothetical proteins with no known function or homology to other known proteins. Other candidates included MSP7-like family proteins MSRP1 and MSRP2, MSP3 (an MSP6-like protein), and Pf92 and Pf12, all of which are PV proteins or associated with the merozoite surface; S-antigen, an abundant PV protein; the PVM proteins exported protein 1 (EXP1) and early transcribed membrane proteins (ETRAMPs); rhoptry proteins RhopH2, RhopH3, ring-associated membrane antigen (RAMA) and rhoptry-associated protein (RAP1); several cytoadherence-linked antigens (CLAGs), which are known to associate with RhopH2 and RhopH3 (Kaneko *et al.*, 2005); apical sushi protein (ASP), an invasion-related microneme protein (O’Keeffe *et al.*, 2005); and merozoite thrombospondin-related anonymous protein (MTRAP), which is also important for invasion (Baum *et al.*, 2006). In addition to these proteins, 4 perforin-like proteins were identified, about which little is known. These are particularly of interest as PfSUB1 substrates as they are thought to be involved in membrane disruption, which is an important process during egress as the PVM and EPM must be breached in order to allow merozoites out of the erythrocyte. Interestingly, several of the putative substrates identified using PoPS undergo proteolytic maturation *in vivo* (Table 7). These data present several new putative PfSUB1 substrates, which could be followed up in further detail to determine whether they are cleaved by PfSUB1 *in vitro* and *in vivo*.

5.2.3. *In silico* prediction of potential erythrocyte PfSUB1 substrates

Above, putative PfSUB1 substrates expressed by *P. falciparum* were identified. On the other hand, whether PfSUB1 is involved in proteolysis of erythrocyte components during schizogony is unknown. PfSUB1 is not known to be exported into the erythrocyte. However, the contents of the PV are released into the erythrocyte compartment after PVM breakdown during egress (Wickham et al., 2003), so it is predicted that PfSUB1 would have access to erythrocyte components during egress and could thereby potentially contribute to EPM destabilisation. To investigate whether PfSUB1 could process erythrocyte proteins, the PfSUB1 specificity model was used to identify putative PfSUB1 cleavage sites in a human erythrocyte proteome data set (Kakhniashvili et al., 2004) wherein Kakhniashvili et al used ion trap tandem mass spectrometry and liquid chromatography to identify 182 membrane-associated and soluble cytoplasmic erythrocyte proteins (other, more extensive studies have identified larger numbers of erythrocyte proteins; however these data could not be accessed in the present study as the accession numbers of these proteins appeared to be obsolete.). Using the PoPS model, PfSUB1 cleavage sites were identified in 40 human erythrocyte cytoplasmic proteins and 47 membrane-associated proteins in this erythrocyte proteome (Table 8). Unlike the prediction of *P. falciparum* PfSUB1 substrates hits in the erythrocyte proteome could not be further delimited, because it is not known whether PfSUB1 cleaves erythrocyte components, nor what the function of PfSUB1-mediated proteolysis might be in this case. The hits included the cytoskeletal components ankyrin, band 3, and α - and β -spectrin, which are of particular interest since these proteins associate with one another (Bennett & Stenbuck, 1979) and have an important role in the fluidity and structural integrity of the EPM (reviewed in (Bennett & Gilligan, 1993)). Proteolytic processing of several of these components is known to occur physiologically in infected erythrocytes (Dua et al., 2001, Raphael et al., 2000, Roggwiler et al., 1996), supporting the notion that their degradation may be important for egress. It was concluded from this analysis that several erythrocyte proteins have putative PfSUB1 sites, but these must be investigated in further detail to determine whether they are physiological substrates of PfSUB1.

5.2.4. Proteomic identification of novel PfSUB1 substrates

Following on from these interesting predictions, we attempted to identify novel substrates experimentally, by using a modification of an *in vitro* assay, which was

previously developed and used for the identification of MSP1, MSP6 and MSP7 as PfSUB1 substrates (Koussis et al., 2009). In this method, endogenous proteolytic activity in the parasite material is blocked with a set of protease inhibitors (including inhibitors of PfSUB1) and then incubated with exogenously-added rPfSUB1. For our purposes, in order to analyse the global effect of rPfSUB1 on a parasite lysate, a large scale assay was performed. Importantly, the original protocol used by Koussis et al was modified to include incubation of non-PfSUB1-treated samples with rp31 (which is a nanomolar inhibitor of rPfSUB1 and endogenous PfSUB1 (Jean et al., 2003)) in order to inhibit any residual PfSUB1 activity not blocked by use of the broad-spectrum inhibitors in the initial step. The workflow of this approach is described in Figure 36. To analyse membrane-associated proteins, purified schizonts were treated with protease inhibitors, then lysed in the detergent saponin, which permeabilises the PVM and EPM, thereby releasing soluble EC and PV proteins. These proteins and the residual protease inhibitors were then washed away by centrifugation, resulting in a preparation containing membrane-associated PVM, EPM, and merozoite proteins (PT). Soluble proteins (ST) were released by freeze-thawing purified schizonts into PfSUB1 digestion buffer containing protease inhibitors, and the supernatant was clarified by centrifugation (a different method was used to prepare soluble proteins as it would not have been possible to remove excess inhibitors from the protein preparations easily and saponin may interfere with rPfSUB1 activity.) PT and ST protein mixtures were then exposed to rPfSUB1 (PT+/ST+) or rPfSUB1 with rPfSUB1pro (PT-/ST-) and incubated at 37°C. Analysis of treated protein preparations by Western blot using anti-MSP1 and anti-SERA5 antibodies showed conversion of the precursor molecules to smaller fragments, which were indistinguishable from physiologically processed proteins (Figure 37).

Proteins which were cleaved in the presence of rPfSUB1 were then identified by liquid chromatography mass spectrometry (LC/MS/MS). Preliminary attempts at resolving proteins by direct 1-dimensional SDS-PAGE and Coomassie staining prior to tandem mass spectrometry (MS/MS) were unsuccessful (Figure 37), presumably because the preparation of proteins was highly complex and many proteins co-migrated. Therefore, to reduce sample complexity, it was necessary to resolve proteins before identification by LC/MS/MS. Gradient elution reversed phase high pressure liquid chromatography (RP-HPLC) separates proteins on the basis of hydrophobicity. RP-HPLC has the major advantage, compared to other

separation techniques such as 2-dimensional electrophoresis, that it enables rapid and high resolution of proteins. It also effectively concentrates proteins as they are eluted from the column. Silica, the most common particle material in RP-HPLC columns, has a high binding capacity, allowing large sample volumes to be loaded; silica is also relatively insensitive to the high urea and detergent concentrations used here (see Material and Methods). Furthermore, elution is performed using volatile liquids, which can easily be removed by freeze-drying. To reduce the complexity of our samples, RP-HPLC was used prior to LC/MS/MS. PT+/PT- samples were solubilised in 8 M urea and 25 mM CHAPS before being loaded onto the column, while ST+/ST- proteins could be loaded directly without further treatment. Collected fractions were then resolved by SDS-PAGE and stained; equivalent PT+/PT- and ST+/ST- fractions were resolved in adjacent lanes. Stained polypeptide bands, which were modified in rPfSUB1-treated samples compared to control samples (Figure 38), were cut out, subjected to in-gel trypsin digestion, and digests analysed by LC/MS/MS. Peptides were then matched back to protein sequences using the MASCOT search engine (Figure 38 and Table 9).

Strikingly, many of the proteins which shifted in response to rPfSUB1 treatment were identified as fragments of SERA4, SERA5 and MSP1, all of which are previously-established PfSUB1 substrates. The fact that known substrates were identified suggests that our method is valid as these proteins act as internal positive controls. In addition, not all proteins were cleaved in the presence of rPfSUB1, limiting the possibility of false positives. Using this approach, 26 proteins were identified as PfSUB1 substrates (Table 9), some of which were predicted substrates (58%) (Table 7, Table 8). Substrates identified by proteomics were also cross-referenced to the initial predictions generated by PoPS, before subcellular location, timing of expression, etc. were taken into account as described in 5.2.2, in the event that application of stringencies had resulted in false negatives. This revealed that 88% of substrates identified by proteomics were initially predicted by PoPS to be putative PfSUB1 substrates before consideration of other factors. Novel putative substrates identified by the mass-spectrometric analysis included PVM proteins such as EXP1, merozoite proteins and erythrocyte structural proteins α - and β -spectrin. Unexpectedly, Alba, a DNA- and RNA-binding protein was identified. Alba has a predicted PfSUB1 cleavage site, but was removed from the PoPS-based predictions using the delimitation steps, as it does not have a signal peptide and

was not thought likely to be involved in egress or invasion. Similarly, elongation initiation factor 1 α (EIF1 α) appears to be processed in the presence of rPfSUB1, but was eliminated from the PoPS-based predictions due to the lack of a signal peptide and again, being unlikely to be involved in invasion or egress. Though these proteins may be true physiologically relevant substrates, they may also be false positives, which are cleaved by PfSUB1 *in vitro* but would not encounter the enzyme *in vivo*. This analysis provided us with a set of putative PfSUB1 substrates, which were processed in the presence of rPfSUB1. Several of these proteins have predicted PfSUB1 cleavage sites and some are known to be processed physiologically. Others were unexpected findings, which will need to be validated in the future.

5.2.5. Further analysis of putative substrates

To verify some of the findings in 5.2.4, it was decided to analyse several individual proteins in further detail by probing PfSUB1-treated schizont lysates by Western blot using specific antibodies against putative substrates. Intact merozoite, intact schizonts and culture supernatants were used to compare artificially processed protein fractions to physiological processing. Importantly, the PoPS search had not only provided predictions of putative PfSUB1 substrates; it also provided us with predicted cleavage sites. This permitted prediction of the sizes of protein fragments after cleavage by PfSUB1, and provided putative sequences for design of peptide substrates to test with rPfSUB1. The *in silico* data could thus be used to support or rule out PfSUB1 being responsible for a putative processing event. RAP1, MSRP2 and RhopH3, as well as erythrocytic α - and β -spectrin were chosen for this more detailed analysis, as laid out below.

5.2.5.1. Validation of RAP1 as a PfSUB1 substrate

RAP1 is an extensively-studied rhoptry protein which is a component of the low molecular weight rhoptry complex (Ridley et al., 1990). During schizogony, RAP1 is known to be processed at $_{190}IVGA\downarrow DEEA_{195}$, generating a 67 kDa protein called p67 (Ridley et al., 1991, Howard et al., 1998). The PoPS-based *in silico* analysis predicted RAP1 to be a PfSUB1 substrate (Table 7) and this was confirmed by our proteomic study (Table 9). To further investigate these predictions, a RAP1-specific

monoclonal antibody, 2.29 (Clark et al., 1987), was used to probe schizont lysates prepared for proteomic analyses of membrane-associated proteins (Figure 39). This confirmed that RAP1 is indeed processed in the presence of rPfSUB1. Two overlapping cleavage sites were predicted by PoPS to be present in RAP1 (Table 7), $_{190}\text{IVGA}\downarrow\text{DEE}_{193}$ and $_{189}\text{VGAD}\downarrow\text{EEA}_{192}$. N-acetylated decameric peptides based on both sites were incubated with or without rPfSUB1 and analysed by RP-HPLC. The RP-HPLC elution profiles are shown in (Figure 39). Only the peptide GIVGADEEAP, relating to the first predicted cleavage site, was cleaved by rPfSUB1. The two cleavage products were collected and analysed by ESI-MS by Steve Howell (NIMR, UK). This confirmed that the peptide is cleaved at $_{1}\text{GIVGA}\downarrow\text{DEEAP}_{10}$; (the downward-pointing arrow indicates the site of cleavage) confirming that if PfSUB1 does process RAP1, it would occur at $_{186}\text{IVGA}\downarrow\text{DEE}_{193}$, not $_{189}\text{VGAD}\downarrow\text{EEA}_{192}$. This finding is consistent with PfSUB1 being responsible for RAP1 processing.

To assess the conservation of this cleavage site across RAP1 homologues, RAP1 sequences from *P. vivax*, *P. knowlesi*, *P. chabaudi* and *P. berghei* were obtained from PlasmoDB and analysed using the PoPS model. Surprisingly, only the *P. falciparum* and *P. chabaudi* RAP1 sequence possesses a predicted PfSUB1 cleavage site. Furthermore, alignment of the sequences of the RAP1 homologues indicated that the *P. falciparum* and *P. chabaudi* sites do not align with one another (Figure 40). This suggests that either the PfSUB1 orthologues in these species have different substrate specificities and therefore their substrates cannot be identified using the PfSUB1 specificity model, or that processing of RAP1 is not an important modification of RAP1 (at least in those species). In conclusion, RAP1 appears to be processed by PfSUB1 in *P. falciparum*, but the biological significance of this cleavage is unclear.

5.2.5.2. Validation of MSRP2 as a PfSUB1 substrate

Processing of MSP7 is well-characterised and occurs at a single cleavage site (Pachebat et al., 2007, Koussis et al., 2009). MSRP2 is a member of the MSP7-like multigene family on chromosome 13 (Mello et al., 2002). All of the MSP7-like family genes can be knocked out in blood stages; and only MSRP2 appears to be expressed at the protein level in this stage (Kadekoppala et al., 2010). MSRP2 has

a predicted PfSUB1 cleavage site (Table 8.), but was not identified by proteomics (Table 9). This is probably because it is a low abundance protein. To further investigate the possibility of MSRP2 being a PfSUB1 substrate, schizont lysates treated with rPfSUB1 were probed using anti-MSRP2 antibodies (a kind gift from Madhu Kadekoppala, NIMR)(Kadekoppala et al., 2010). This indicated that MSRP2 undergoes processing in the presence of rPfSUB1, being converted to a protein of a similar size to that observed in culture supernatant (Figure 41). Similar to the case of RAP1, two PfSUB1 cleavage sites were predicted by PoPS in the MSRP2 sequence; ⁶⁹IIGQ↓GIF₇₅, ⁸⁹LKGE↓SED₉₇ (Table 7). To assess whether these sequences were sensitive to cleavage by PfSUB1, N-acetylated decamer peptides based on these cleavage sites were incubated with PfSUB1 and examined by RP-HPLC; the resulting RP-HPLC traces are presented in (Figure 41). The more hydrophobic peptide DIIGQGIFSL was not cleaved by rPfSUB1, but the second peptide was. Analysis of cleaved Ac-SLKGESEDNT by ESI-MS confirmed that the peptide is cleaved at ⁵SLKGE↓SEDNT₁₀ as was predicted in 5.2.2. To determine whether PfSUB1-mediated cleavage of MSRP2 is conserved across *Plasmodium* spp., the amino acid sequences of each MSRP2 homologue were scanned using the PfSUB1 specificity model. All of the MSRP2 homologues are predicted to be PfSUB1 substrates, indicating that processing may be a conserved and important feature of MSRP2 (Figure 42).

5.2.5.3. Validation of RhopH3 as a PfSUB1 substrate

RhopH3, a 110 kDa rhoptry protein, forms a complex with RhopH1 and RhopH2 (Lustigman et al., 1988). It undergoes processing at the C-terminus in late schizogony (Sam-Yellowe et al., 1988). By PoPS analysis, two PfSUB1 processing sites were predicted in RhopH3, ⁷⁹³TSAA↓STS₇₉₉ and ⁸⁰²ISGS↓EGP₈₀₈ (Table 7). Western blot analysis of rPfSUB1-treated schizont lysates with an anti-RhopH3 antibody indicated that RhopH3 is processed by rPfSUB1 *in vitro* (Figure 43) however RhopH3 in the rPfSUB1-treated sample does not appear to be the same size as that in culture supernatant. To analyse whether the predicted processing sites could be cleaved by rPfSUB1, two N-acetylated peptides based on the predicted PfSUB1 cleavage sites were incubated with rPfSUB1. This revealed that they are not cleaved by rPfSUB1 (Figure 43). This is surprising, considering RhopH3 appears to be processed in proteolysed samples; however this could be an

artefact of rPfSUB1 treatment. Together, these data suggest that RhopH3 is not a physiological substrate of PfSUB1.

5.2.5.4. *Validation of erythrocytic α - and β -spectrin as PfSUB1 substrates*

Erythrocyte components were among the possible substrates identified by bioinformatics (Table 8) and proteomics (Table 9). This is of particular interest as the parasite must destabilise the host cytoskeleton in order to escape the red blood cell, and several erythrocyte components are known to be processed during the parasite life cycle (Blackman, 2008, Dua et al., 2001, Hanspal et al., 2002, Le Bonniec et al., 1999). In our proteomics approach, α - and β -spectrin were cleaved in the presence of rPfSUB1. They also have multiple predicted PfSUB1 processing sites (Table 8). The two chains of spectrin form a characteristic doublet on SDS-PAGE at 260 kDa, as observed in the proteomic analysis (Table 9). To assess the possibility of PfSUB1-mediated cleavage of α - and β -spectrin *in vitro*, erythrocyte ghosts were incubated with either rPfSUB1 or rPfSUB1 and rp31 and samples taken every 30 minutes subjected to SDS-PAGE and Coomassie blue staining. Degradation of α - and β -spectrin by PfSUB1 could not be detected (Figure 44) suggesting that PfSUB1 is not directly responsible for the cleavage of these proteins.

5.2.5.5. *Perforin-like proteins – a role in PfSUB1-mediated egress?*

It was of particular interest that 4 PfPPLPs were predicted by the PoPS model to contain potential PfSUB1 cleavage sites, since these proteins are involved in membrane destabilisation and some pore-forming proteins require proteolytic activation (Uellner et al., 1997, Olson & Gouaux, 2005). Studies of PPLPs in *P. berghei* and *P. yoelii* have revealed that these proteins have unique functions in breaching membranes in multiple stages of the malaria life cycle (Kaiser et al., 2004, Kadota et al., 2004, Ishino et al., 2005, Ecker et al., 2007). The PoPS predictions led to the question of whether PfSUB1 could be involved in proteolytically activating PfPPLPs in order to destabilise the PVM. By analogy to *T. gondii*, which uses the micronemal protein TgPLP1 to mediate egress from its host

cell by disrupting the PVM (Kafsack et al., 2008), it is possible that *Plasmodium* spp. also uses PPLPs for egress. TgPLP1 may be processed by TgSUB1 (Carruthers, personal communication), supporting the idea that PfSUB1 might play a role in processing PfPPLPs. Five perforin-like proteins are conserved across the *Plasmodium* genus (Kaiser et al., 2004, Kafsack & Carruthers, 2010); here, the *P. falciparum* homologues are referred to as PfPPLP1-PfPPLP5 (their respective genes are named *pfpplp1*, *pfpplp2*, etc.). Perforin-like proteins contain membrane attack/ perforin (MACPF) domains which are important for oligomerisation and insertion of pores into membranes. In 5.2.2, putative PfSUB1 cleavage sites identified in four of the PfPPLPs, which are mapped in Figure 45 (the reason for a lack of a predicted cleavage site in PfPPLP5 could be because it may not be expressed in blood stages; therefore it would not come into contact with PfSUB1). In PfPPLP1, PfPPLP2 and PfPPLP4, cleavage sites occur outside of the MACPF domain. Whether there is a link between PfSUB1 and PfPPLPs or membrane destabilisation *in vivo* is unknown.

5.2.5.5.1. Five genes encoding PfPPLPs are transcribed during asexual stages

Nothing is known about the function of these proteins in blood stages. Large scale transcriptional data suggest that all *pfpplps* are transcribed in blood stages (Le Roch et al., 2004), but this contradicts previous RT-PCR and oligonucleotide microarray analysis on cDNA from asexual stage parasites by Kappe et al (Kappe et al., 2004). Florens et al provided MS/MS evidence that PfPPLP1 and PfPPLP2 are expressed at the protein level in blood stages (Florens et al., 2002), and this was confirmed by Khan and colleagues (Khan, unpublished work)(available on PlasmoDB.org). The aforementioned studies, however, only identified single peptides derived from PfPPLP1 and PfPPLP2, suggesting that if they are expressed, it is at very low levels. There is conflicting data on transcription of the *pfpplps* during asexual stages (Kaiser et al., 2004, Le Roch et al., 2004).

With the aim of confirming that *pfpplps* are transcribed during asexual stages, RT-PCR was performed on mRNA extracted from asynchronous *P. falciparum* parasites (predominantly trophozoites and schizonts). RT-PCR products of the expected sizes were obtained for all *pfpplps* (Figure 46). For *pfpplp1*, the

PCR product spans regions containing introns. As expected, a larger product was obtained for genomic DNA, and a smaller PCR product was observed the reverse transcriptase-treated mRNA sample (+RT), confirming that genomic DNA was absent from +RT. In conclusion, these results show that all *pfpplps* are transcribed during asexual stages.

5.2.5.5.2. Raising antibodies and generation of transgenic parasites

Through studies in *P. berghei*, it is apparent that *P. berghei* pplp2 is refractory to genetic deletion in asexual stages (Ecker, Personal communication), and while disruption of the gene encoding *P. berghei* PPLP4 was confirmed by PCR, a clonal knockout line could not be established (Ecker et al., 2008). These findings suggest that both pplp2 and pplp4 are important for blood stage development. Since pplp2 and pplp4 appear to be important for *P. berghei* blood stage development, the *P. falciparum* homologues *pfppl2* and *pfppl4* were focussed on. Having confirmed that the *pfpplps* are transcribed, it is of interest to determine whether PfPPLP2 and PfPPLP4 are expressed at the protein level in asexual blood stages. MS/MS evidence from Florens et al suggests that PfPPLP2 is expressed at the protein level in merozoites (Florens et al., 2002) but this has not been confirmed using specific antibodies. The same study suggests that PfPPLP4 is expressed in ookinetes, but not in asexual stages. It was therefore decided to raise antibodies against PfPPLP2 and PfPPLP4; this work is ongoing. Since it was unknown whether either of these proteins undergoes proteolytic maturation, it was decided to produce antibodies against multiple regions of PfPPLP2 and PfPPLP4. These regions are summarised in Figure 47. Regions of the MACPF domain were expressed, which is the central and presumably functional part of these molecules. Three different recombinant proteins based on the MACPF domain of PfPPLP2 and PfPPLP4 were made, as it was unclear which of these would be most soluble. Segments of the PfPPLP2 and PfPPLP4 coding sequences were therefore amplified from *P. falciparum* genomic DNA and expressed as recombinant GST- and His- fusion proteins. All of these proteins were insoluble as GST-fusions. The program Protean was then used to identify soluble, highly antigenic regions of PfPPLP2 and PfPPLP4. Based on this, a C-terminal domain of PfPPLP2 and an N-terminal domain of PfPPLP4 were

expressed. Unfortunately, once again neither of these domains were soluble, therefore all of the recombinant proteins made in *E. coli* were purified from inclusion bodies and refolded as described by Frangioni and Neel (Frangioni & Neel, 1993) before purification using GSH agarose (GSHA). Attempts to raise antisera to detect PfPPLP2 and PfPPLP4 are ongoing.

As an alternative approach to raising antibodies and to determine the importance of these genes, parasites were transfected with several constructs designed to integrate into and to modify the *pfpplp2* and *pfpplp4* genetic loci. The expected gene products resulting from successful integration of these constructs are depicted in Figure 48. Firstly, constructs were cloned for the purpose of integrating HA3 tags into the *pfpplp2* and *pfpplp4* genetic loci in *P. falciparum*. Epitope tagging can be used as an alternative to raising antibodies against proteins, especially proteins that may be poorly immunogenic. Therefore, it was decided that epitope tagging could aid detection of PfPPLP2 and PfPPLP4 in parasites. Epitope-tagged lines could be used in combination with or to validate antibodies against recombinant domains of PfPPLP2 and PfPPLP4. Constructs pHH1-PPLP2-HA3 and pHH1-PPLP4-HA3 were designed to integrate sequences encoding HA3 epitope tags into the 3' ends of the *pfpplp2* and *pfpplp4* coding regions, by single cross-over homologous recombination. The constructs include a targeting region followed by the *P. berghei* dihydrofolate reductase thymidylate kinase (*P. berghei* DHFR-TS 3' UTR) 3' untranslated region (3' UTR). To determine whether *pfpplp2* and *pfpplp4* genetic loci are accessible for genetic modification, parasites were also transfected with control constructs containing the same targeting regions but with the inclusion of stop codons just upstream of the sequence encoding the HA3 tag. These constructs were expected to integrate as they do not alter the gene product, thereby acting as an important control to show that the genetic loci are accessible to genetic manipulation.

The homologues of *pfpplp2* and *pfpplp4* cannot be knocked out in *P. berghei* asexual stages. It is therefore likely that they are also essential in *P. falciparum*, but this has not been addressed. To investigate the importance of PfPPLP2 and PfPPLP4 in *P. falciparum* asexual stages, constructs were designed to truncate the *pfpplp2* and *pfpplp4* genes by single crossover homologous recombination. If the MACPF domain is important for the function of these proteins and they are essential, the constructs were expected not to integrate. The constructs included an

800 bp targeting region from the 5' end of the coding regions of *pfpplp2* and *pfpplp4* followed by a stop codon. Integration would essentially truncate both genes in the middle of the sequence encoding the MACPF domain of *pfpplp2* and *pfpplp4* (constructs pHH1-PPLP2 Δ and pHH1-PPLP4 Δ), generating functional knockouts of PfPPLP2 and PfPPLP4 (Figure 48). Considering that *pfpplp2* and *pfpplp4* cannot be knocked out in *P. berghei*, it was thought to be unlikely that these constructs would integrate.

Since it is unknown whether the truncation constructs would integrate, to investigate whether complete deletions of *pfpplp2* and *pfpplp4* were possible, parasites were transfected with vectors to delete the genes by double crossover homologous recombination using the thymidine kinase vector system (Duraisingh et al., 2002). This involves two targeting regions, at the 5' (flank 1) and 3' (flank 2) ends of the gene, and if double cross-over homologous recombination occurs, the gene of interest would be replaced with the human dihydrofolate reductase cassette (hDHFR). Parasites are selected using the negative selection drug ganciclovir, which is toxic to parasites carrying the thymidine kinase (TK) gene i.e. parasites where the construct has not integrated by double cross-over homologous recombination retain the TK gene and therefore are sensitive to ganciclovir.

All of the aforementioned constructs are being used for ongoing experiments to attempt to generate transgenic parasites. It is of much interest whether PfPPLPs are expressed, and whether they are involved in PfSUB1-dependent release of merozoites.

5.3. Discussion

Previous work has shown that PfSUB1 proteolytically modifies several MSP and PV proteins during egress. Here, using information about the specificity of PfSUB1 obtained from previous experimental data, 77 *P. falciparum* putative PfSUB1 substrates and 87 erythrocyte putative PfSUB1 substrates were identified *in silico*. By combining these predictions with an *in vitro* proteomic approach to substrate identification, 23 novel putative PfSUB1 substrates were identified experimentally. Several of these proteins were analysed in further detail by SDS-PAGE and Western blot using available antibodies, and by cleaving peptides based on the predicted PfSUB1 sites with rPfSUB1. The results presented here suggest that the role of PfSUB1 extends to processing proteins in the rhoptries, PVM and, indirectly,

the erythrocyte.

5.3.1. Predicting PfSUB1 substrates *in silico*

Numerous studies have identified putative protease substrates *in silico*. Most computational methods have been applied to granzyme B and caspases since their substrate preference is fairly well described. Substrates for these enzymes have been identified using several different computational methods (Piippo et al., 2010, Wee et al., 2006, Yang, 2005). Recently, a new method was developed which incorporates true and false positive peptide sequences as well as secondary or tertiary structural features so that the protease model is trained on predefined structural features (Barkan et al., 2010). Here, the computational application PoPS was used, which uses a position-specific scoring matrix to define protease specificity (Boyd et al., 2005, Boyd et al., 2004). Using information about validated PfSUB1 substrates, a matrix was assembled and substrates were predicted by scanning the *P. falciparum* predicted proteome and an erythrocyte proteome. The model was based on 15 sequences from previously-established substrates; therefore the substrates predicted here only include those whose cleavage sites share similarity with the previously validated substrates. For example, all SERAs known to be processed by PfSUB1 have at least two PfSUB1 cleavage sites, in some cases three (Andrea Ruecker, Michael Shea and Mike Blackman, NIMR, unpublished data)(Li et al., 2002, Ruecker, unpublished data, Yeoh et al., 2007). However, SERA7 only has 1 predicted site using our model, whereas when the primary sequence was analysed by Yeoh et al, it was evident that SERA7 has a second sequence which is closely related to other PfSUB1 cleavage sites in the SERAs (Yeoh et al., 2007). This was not predicted to be a PfSUB1 cleavage site since the PoPS model does not permit Phe residues in the P4 subsite (Figure 32), as it has not been confirmed that SERA7 is processed by PfSUB1. Therefore, it is possible that there are many false negatives from this bioinformatic approach, questioning the sensitivity of this model. It was difficult to obtain a balance between high specificity and sensitivity; since our knowledge of PfSUB1 specificity is incomplete, the model was also promiscuous and this led to a large percentage of the *P. falciparum* proteome being predicted to be PfSUB1 substrates (36.7%). Where prediction of PfSUB1 substrates was performed using the *P. falciparum* predicted proteome, several stringencies were applied, taking into account

information available about validated PfSUB1 substrates, including timing of expression and subcellular location. These characteristics were largely based on predictions where experimental evidence was not available; therefore some proteins that could be true substrates, such as Alba and EIF α , will inevitably have been discarded using these criteria. In the case of erythrocyte proteins, it was not possible to use the same stringencies as used for prediction of substrates in the *P. falciparum* proteome; therefore more of these proteins are likely to be false positives. Using the PfSUB1 specificity model, 48% of the erythrocyte proteins were predicted to be cleaved by PfSUB1. This figure could not be reduced by delimitation. Whether PfSUB1 cleaves erythrocyte proteins at all is unknown. Since the erythrocyte lacks compartmentalisation, PfSUB1 would probably have access to many of these proteins after PVM breakdown.

The PoPS model could potentially be improved by incorporating non-cleavable sequences which were discovered by analysing peptides based on predicted PfSUB1 sites, of which several are not cleaved by PfSUB1 despite being predicted cleavage site sequences (Figure 39, Figure 41, Figure 43). Incorporation of these sequences would increase the specificity of the PfSUB1 model by limiting the number of false positives. Also, as new substrates are identified *in vitro*, new sites which do not fit the specificity model, such as perhaps SERA7 site 1, could also be incorporated into the model to further improve the prediction of substrates.

5.3.2. Identifying protease substrates by proteomics

Over the past decade, proteomic techniques have been used increasingly to identify protease substrates. These include chemically and enzymatically engineered peptide libraries, which have been used to identify amino acid sequence preferences (Schilling & Overall, 2008). However, an obvious limitation with peptide-based methods is that they do not account for the influence of higher order protein structure on cleavage. Alternatively, several methods are used to analyse proteolysis of complex protein mixtures. One example is PROTOMAP, which is a relatively new technique, where proteolysed and control samples are separated by SDS-PAGE and the gel is sliced into bands (Dix et al., 2008). The bands are trypsinised and analysed by LC/MS/MS and spectral counting. The major advantage of this technique is that an infinite number of samples can be compared. However, PROTOMAP is difficult to apply to limited proteolysis in a high

background of unmodified proteins since it does not enrich proteolysed proteins and there is a preferential selection of high abundance proteins. Furthermore, because of this, sample analysis requires long periods of MS/MS or LC/MS/MS instrument time for small sample volumes.

Here, we took advantage of the specific activity of rPfSUB1 to mimic physiological processing in a parasite lysate containing (mostly) full-length proteins. As with many approaches to the identification of protease substrates *in vitro*, a major difficulty lies in mimicking physiological conditions of protease-mediated degradation. Purified rPfSUB1 was used to treat a lysate of schizont proteins, some of which would not come into contact with PfSUB1 under normal physiological conditions since they are present in different subcellular compartments. Under assay conditions, cytosolic proteins, nuclear, host cell proteins are all potentially accessible to the added rPfSUB1, leading to potential for false positives. However, from the fact that most proteins, when analysed by SDS-PAGE after RP-HPLC fractionation, were not modified in rPfSUB1-treated samples (Figure 37), it appears that there are probably very few false positives. Furthermore, there is evidence that several of the proteins which were identified by proteomics are processed physiologically during the asexual life cycle. In addition to this, many of these proteins were predicted to be PfSUB1 substrates in our PoPS-based analysis. RhopH3 may be a false positive, since the artificially processed protein does not appear to migrate at the same size as processed RhopH3 *in vivo*. However, due to a lack of available antibody, this could not be fully confirmed. Furthermore, it is possible that peptides based on the putative RhopH3 processing site may not be cleaved as they are in the wrong conformation for cleavage, or they are false positives from the PoPS-based prediction of PfSUB1 substrates.

The results indicate that rPfSUB1 processes several proteins, which are similar in size to the predicted sizes according to PoPS, and similar in size to proteins in untreated 3D7 parasites, however they do not confirm that PfSUB1 is responsible (directly or indirectly) for their processing *in vivo*, nor do they prove that PfSUB1 processing is essential for their function.

5.3.3. New substrates: RAP1 and MSRP2

Two proteins were chosen for further analysis. RAP1 was identified as a substrate by the PoPS-based screen and by proteomics, and MSRP2 by PoPS alone. RAP1

has been extensively studied, but the identity of the protease which cleaves p82 to p67 (Ridley et al., 1991) has remained unknown until now. Here, rPfSUB1 was shown to mediate the conversion of p82 to p67 *in vitro*. Furthermore, a peptide based on the known RAP1 cleavage site was cleaved by rPfSUB1 at the correct site. In conclusion, RAP1 appears to be a PfSUB1 substrate. Previous work by others has suggested that RAP1 is involved in egress and/or invasion. Parasite invasion is inhibited by use of the anti-RAP1 monoclonal antibody 2.29 as well as several other RAP1 monoclonal antibodies which bind in the vicinity of the PfSUB1 cleavage site (Harnyuttanakorn et al., 1992). This suggests that these antibodies may inhibit invasion by interfering with PfSUB1 processing. Arguing against an important role for RAP1 in invasion or egress, however, is evidence that the rap1 gene can be truncated in asexual stages (Baldi et al., 2000), implying that RAP1 is not essential for blood stage growth. On the other hand, a complete knockout of RAP1 is lacking and so it is possible that the N-terminus of RAP1 is essential, not the C-terminus. RAP1 may be involved in PV establishment as it is transferred to the PV during invasion (Baldi et al., 2000), though this has yet to be confirmed. RAP1 forms a complex with RAP2, RAP3 and ring-associated membrane antigen (RAMA), an association which is thought to be important for trafficking to the rhoptries during rhoptry biogenesis (Richard et al., 2009, Baldi et al., 2000, Schofield et al., 1986, Clark et al., 1987, Bushell et al., 1988). RAMA is processed upon reaching the rhoptries, which abolishes its association with RAP1 (Richard et al., 2009). RAMA also has a predicted PfSUB1 site (Table 7) and was confirmed to be cleaved by rPfSUB1 by the proteomic analysis (Table 9). It is possible that PfSUB1 processing might be important for RAMA and RAP1 to dissociate upon reaching the rhoptries (Richard et al., 2009). RAMA has an N-terminal prodomain which is removed in the rhoptries; cleavage occurs between 477L and 478Q (Richard et al., 2009). The predicted PfSUB1 cleavage site is very close to this at $_{401}\text{LQGD}\downarrow\text{SDD}_{405}$ (Table 8.), but is not identical, making it unlikely that PfSUB1 is responsible for this event.

MSRP2, on the other hand, is likely to be a physiological substrate of PfSUB1. MSRP2 is processed physiologically at the time of egress (Kadekoppala et al., 2010), and was identified as a PfSUB1 substrate both by the PoPS analysis and by the western blot-based experiments. The absence of MSRP2 in the proteomic analysis is attributed to it being of low abundance. The importance of PfSUB1-

mediated MSRP2 processing is unclear. Several merozoite surface proteins are processed by PfSUB1 during egress, including MSP7, which is of the same family as MSRP2 (Koussis et al., 2009). MSP7 is a membrane-associated protein which forms a complex with MSP6 and MSP1 which is found on the surface of merozoites (Pachebat et al., 2007, Holder *et al.*, 1985, Stafford et al., 1996, Trucco et al., 2001, Kauth *et al.*, 2006, Kauth *et al.*, 2003). Each of these proteins is processed by PfSUB1 (Koussis et al., 2009). Unlike MSP7, however, MSRP2 is a soluble PV protein and is not detected on the merozoite surface (Kadekoppala et al., 2010). Therefore, the importance of MSRP2 processing cannot be directly compared to that of MSP7.

Preliminary experiments were also performed to investigate the role of PfPPLPs in PfSUB1-mediated egress. RT-PCR confirmed all five genes encoding PfPPLPs are transcribed, a finding which supports data from Hall *et al* (Hall et al., 2005) and contradicts that of Kaiser *et al* (Kaiser et al., 2004). Future analysis should include a time course to determine when maximum transcription of these genes occurs during the asexual stages of the life cycle. There are ongoing attempts to raise antibodies against recombinant domains of PfPPLP2 and PfPPLP4 to determine whether these proteins are expressed during asexual stages. Antibodies will be very useful tools for analysing the timing of expression and localisation of these proteins. It would, furthermore, be possible to carry out immuno-EM studies in order to pin down the precise location of these proteins in parasites. Aside from localisation studies, the antibodies and epitope tags could also be used for pull-down experiments from parasite lysates, to validate the antibodies and to identify binding partners of PfPPLP2 and PfPPLP4. Similarly, it might be possible to carry out pulse chase experiments to follow protein synthesis and potential proteolytic processing. If processing of PfPPLP2 and PfPPLP4 is observed a similar assay to that described by Koussis *et al* (Koussis et al., 2009) and in Chapter 2, could be used to determine whether processing is PfSUB1-mediated. This method involves incubation of rPfSUB1 with protease inhibitor-treated schizont lysates and assessment of processing using specific antibodies.

Evidence from work in *P. berghei* indicates that *pplp2* and *pplp4* are important for blood stages; to determine whether these genes are also important in *P. falciparum*, the genetic loci were targeted using a series of different constructs for epitope tagging and disruption; all of these studies are ongoing. Whether

PfPPLP2 and PfPPLP4 are involved in PfSUB1-mediated egress remains unknown, but the transcription of genes encoding perforin-like proteins suggests, intriguingly, that there might be a role for PfPPLPs in asexual stages.

5.3.4. New functions of PfSUB1

The identification of PfSUB1 substrates that localise to the rhoptries implies that PfSUB1 has access to rhoptry proteins. There are three possible explanations for this. Firstly, PfSUB1 could have direct access to rhoptry proteins once it has been released into the PV, because once fusion of the apical duct has occurred, the rhoptry membrane is probably continuous with the merozoite PM (Bannister et al., 2000). The rhoptry compartment may therefore become accessible to PV-located proteins. Secondly, since nothing is known about exoneme secretion or how PfSUB1 reaches the PV, it is possible that exonemes fuse directly to the cytoplasmic face of the rhoptries and that PfSUB1 makes its way to the PV via the rhoptry compartment. Localisation studies on PfSUB1 argue against this, as they do not show localisation in the rhoptries (Yeoh et al., 2007); on the other hand, the majority of exonemes may fuse to the PM while a small subset releases its contents into the rhoptries, at levels difficult to detect by immuno-EM. Alternatively, rhoptries may secrete their contents in the late stages of schizogony (Bannister et al., 1986), as occurs with micronemes in *P. falciparum* and *T. gondii* (Waters et al., 1990, Kafsack et al., 2008).

The identification of PVM substrates in this study is particularly intriguing. During egress, the PVM is ruptured, prior to breakdown of the EPM. Since the molecules directly mediating membrane breakdown have yet to be identified, it is of particular interest that several PVM proteins possess predicted PfSUB1 cleavage sites, and that one in particular, EXP1, was identified in our proteomics search. EXP1 is an essential PVM protein with an unknown function (Maier et al., 2008), though it appears to form homo-oligomeric complexes which Spielmann *et al.* suggest are membrane pores (Spielmann *et al.*, 2006a). Several ETRAMPs, also PVM proteins, have predicted PfSUB1 sites but were not detected in the proteomic analysis, perhaps as they are low abundance proteins. Like EXP1, they are essential in asexual stages and form homo-oligomeric complexes (Spielmann et al., 2006a). Proteolysis of these proteins by PfSUB1 could render them unstable, resulting in membrane collapse. To date, there is no evidence that either EXP1 or

ETRAMPs are physiologically processed. However, if PVM rupture occurs immediately prior to egress, which occurs very rapidly (Gilson & Crabb, 2009, Glushakova et al., 2008, Glushakova et al., 2005), it might be difficult to detect smaller fragments of these proteins in late schizonts, though they may be detectable in culture supernatant.

Considering that the EPM also ruptures during egress, the appearance of α - and β -spectrin in both the PoPS-based and proteomic searches for substrates is particularly interesting. However, a direct role of PfSUB1 in spectrin degradation is uncertain, since cleavage of spectrin was not observed when erythrocyte ghosts were incubated with rPfSUB1. One explanation for these observations might be that PfSUB1 is involved in a proteolytic cascade, whereby it may activate other proteases such as members of the putative papain-like protease family, the SERAs. If a cascade is activated, it is also likely to take place in our *in vitro* assay. If proteases like the SERAs are present in the schizont lysates as inactive precursors, treatment using protease inhibitors and removal of residual inhibitors by centrifugation would not block their subsequent activation and proteolytic activity following rPfSUB1-treatment. On the other hand, the failure to detect PfSUB1-mediated digestion of spectrin could simply be difficult to observe in the absence of anti-spectrin antibodies. Physiologically, spectrin is cleaved by an erythrocytic cysteine protease called calpain 1 (Boivin *et al.*, 1990). Calpain 1 may also be involved in remodelling of the erythrocyte cytoskeleton by *P. falciparum* to enable egress to proceed (Chandramohanadas et al., 2009), though whether it cleaves spectrin during egress is unknown. It is possible that if PfSUB1 is involved in a proteolytic cascade, calpain could be activated indirectly by PfSUB1. Degradation of spectrin in cultured *P. falciparum* has not been reported, but it does occur in a murine model of cerebral malaria and may be mediated by calpain as elevated levels of erythrocytic calpain are observed in the cerebellum and cerebral cortex (Shukla *et al.*, 2006).

Proteases are thought to play an important role in degradation of the erythrocyte during schizogony. Using a biotinylated cysteine protease probe, Gelhaus *et al* showed that cysteine protease activity occurs inside the EC in schizonts (Gelhaus et al., 2005). Falcipain 2 is located in the FV, PV and EC, and cleaves anykrin and band 4.1 during late stages of egress (Dua et al., 2001, Hanspal et al., 2002). Falcipain 2 cleaves band 4.1 within a spectrin-actin binding

domain, which is thought to mediate destabilisation of the erythrocyte membrane as this is a key structural interaction. There is also evidence that recombinant plasmepsin II degrades spectrin, though whether this occurs *in vivo* is unknown (Le Bonniec et al., 1999). PfM18APP, an M18 aminopeptidase expressed in asexual stages, binds spectrin and other erythrocyte cytoskeletal components *in vitro*, which may indicate that it has a role in cytoskeletal modification (Lauterbach & Coetzer, 2008). Degradation of cytoskeletal components could result in membrane destabilisation by rendering the erythrocyte unable to contain the densely-packed merozoites.

In terms of proteomics, it would be interesting to carry out further studies using these samples. In particular, it would be of interest to identify where these proteins are being cleaved. The RP-HPLC purified fractions of rPfSUB1-treated schizont lysates could be further analysed by subjecting individual bands to N-terminal sequencing to confirm whether PfSUB1 is cleaving these proteins at the predicted sites. This analysis would also provide cleavage sites of any proteases acting downstream of PfSUB1. This could be combined with analysis of proteolytic processing during invasion and egress, to ascertain whether these processing events also occur physiologically.

The importance of maturation of any of its substrates by PfSUB1 remains unclear. Antibodies which interfere with MSP1 processing result in blockage of invasion (Lazarou et al., 2009), which suggests that PfSUB1-mediated processing is important. Monoclonal antibody 43E5, which recognises the N-terminus of the SERA5 p47 fragment, is invasion-inhibitory (Fox *et al.*, 1997), though whether this antibody interrupts SERA5 processing to p47 is unknown. The next step in this project is to investigate the importance of PfSUB1-mediated processing, a task that is somewhat challenging, considering that several PfSUB1 substrates are essential to parasite growth *in vitro*. Surprisingly, mutagenesis of the PfSERA5 site 2 to render it non-cleavable can be achieved without affecting parasite viability in *P. falciparum* (Christine Collins and Sharon Yeoh, NIMR, unpublished data), suggesting that blockage of PfSUB1 cleavage of PfSERA5, at least at site 2, is tolerated by the parasite. MSRP2 and RAP1 are not essential to the parasite life cycle (Baldi et al., 2000, Kadekoppala et al., 2010). By studying the processing of these proteins, it might be possible to gain valuable information about the importance of PfSUB1-mediated processing.

In conclusion, the data presented in this chapter indicate that PfSUB1 is a multifunctional enzyme, responsible for the processing of rhoptry, PV, PVM and may be indirectly responsible for processing of erythrocyte components. Further work must be carried out to ascertain the physiological relevance of these findings.

Figure 31. PfSUB1 displays sequence preferences at cleavage site subsites
Alignment of all validated PfSUB1 cleavage sites indicates that PfSUB1 has trends in specificity at subsites within cleavage sites. Residues are labelled according to the single letter amino acid code. Sub-sites are described according to Schechter and Berger notation of sub-sites surrounding the scissile bond at P1-P1' (Schechter & Berger, 1967). P4 residues tend to be hydrophobic and only Gly or Ala are present at P2. A polar residue always precedes the scissile bond in the P1 subsite. P2'-P5' exhibit an acidic tendency in positions. Adapted from Yeoh et al (Yeoh et al., 2007) and expanded.

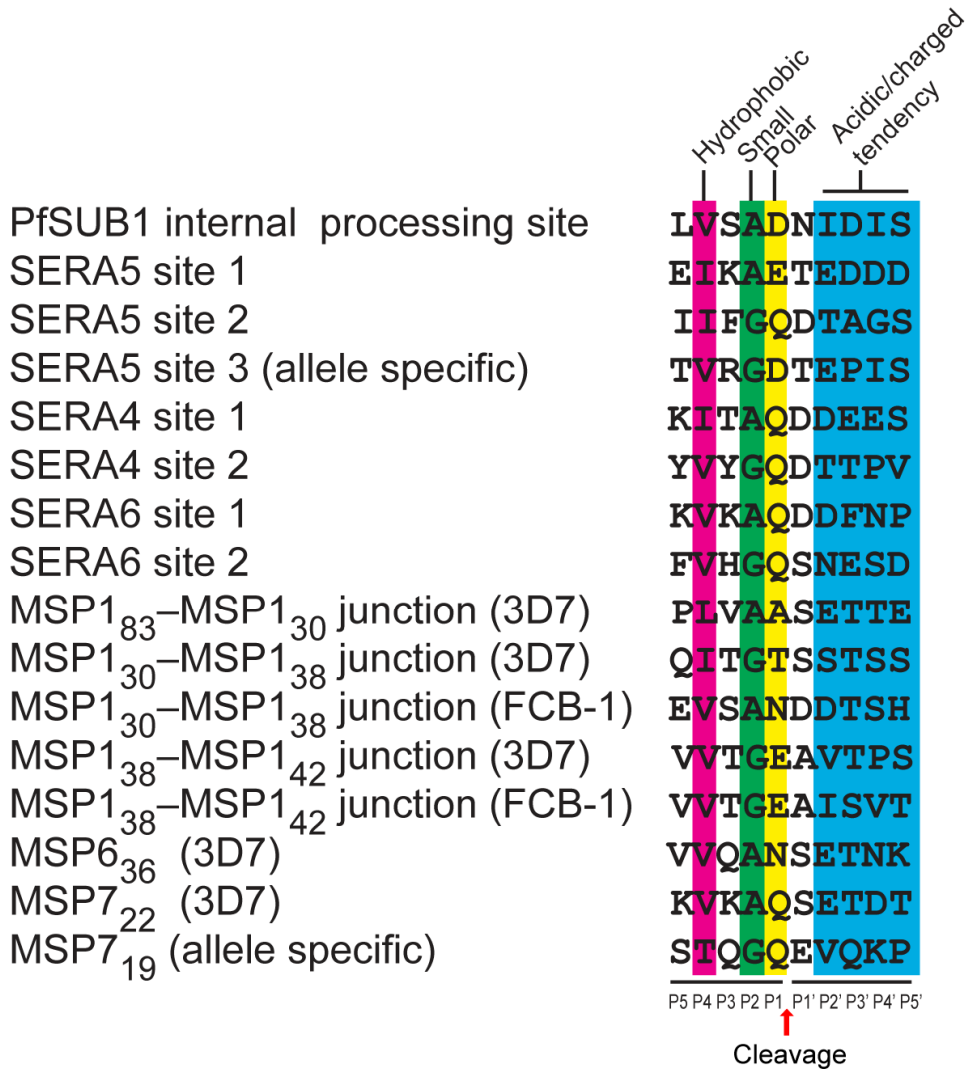
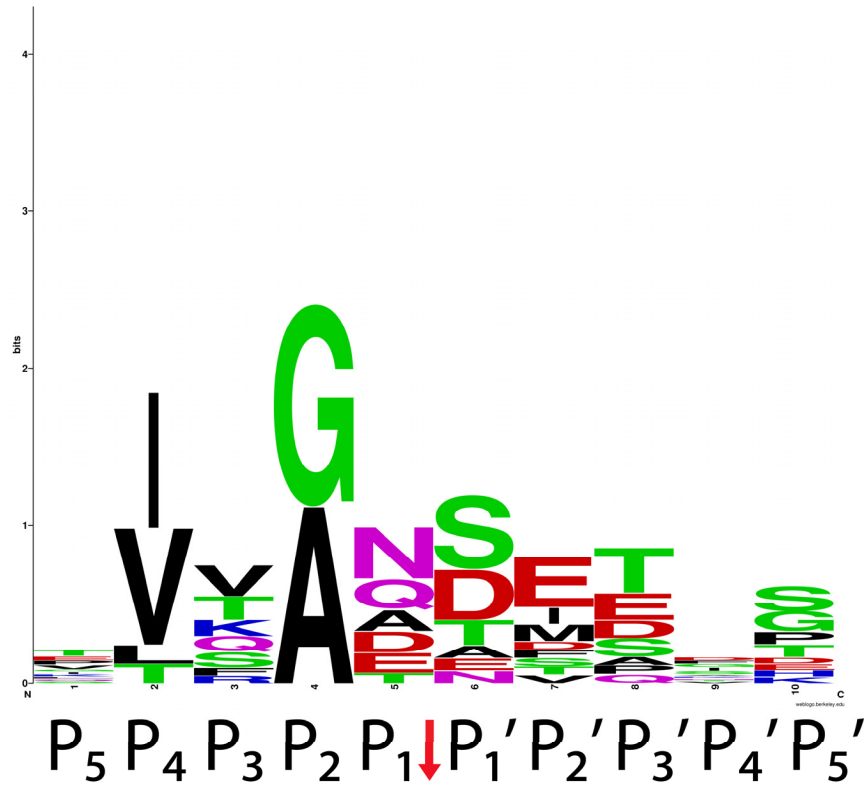


Figure 32. Generation of a PfSUB1 specificity model

To generate a consensus motif for PfSUB1, 16 cleavage sites from previously-established PfSUB1 substrates were aligned and converted into a WebLogo graphic (A). The graphic represents a 10-mer peptide annotated according to Schechter and Berger (Schechter & Berger, 1967). Residues are labelled with the single letter amino acid code. The height of the letter at a given position reflects the frequency of the residue at that position. These cleavage sites were used to assemble a PfSUB1 specificity model for use in PoPS (B). Each amino acid residue was given a score for each sub-site within the cleavage site according to its frequency at a certain site in the validated sequences. Residues which are not present in a certain sub-site in those sequences, or which are known to inhibit cleavage, were blocked (#) in order to decrease the sensitivity of the model.

A



B

Residues	Subsites						
	P4	P3	P2	P1	P1'	P2'	P3'
Ala	#	0.0	5.0	4.0	2.0	0.0	0.0
Arg	#	0.0	#	#	#	#	#
Asn	#	0.0	#	4.0	2.0	3.0	0.0
Asp	#	0.0	#	4.0	4.0	3.0	3.0
Cys	#	0.0	#	#	#	0.0	#
Gln	#	0.0	#	4.0	#	0.0	#
Glu	#	0.0	#	4.0	2.0	4.0	3.0
Gly	#	0.0	5.0	#	0.0	0.0	#
His	#	0.0	#	#	#	#	#
Ile	5.0	0.0	#	#	#	0.0	#
Leu	3.0	0.0	#	#	#	0.0	#
Lys	#	0.0	#	#	#	#	#
Met	#	0.0	#	#	#	0.0	#
Phe	#	0.0	#	#	#	4.0	3.0
Pro	#	#	#	#	#	#	0.0
Ser	#	0.0	#	0.0	4.0	0.0	2.0
Thr	3.0	0.0	#	2.0	3.0	0.0	3.0
Trp	#	0.0	#	#	#	0.0	#
Tyr	#	0.0	#	#	#	0.0	#
Val	5.0	0.0	#	#	#	0.0	#

Figure 33. Workflow for the prediction of PfSUB1 substrates

The PoPS PfSUB1 specificity model was used to scan 5,679 *P. falciparum* predicted proteins. Of these proteins, 2,086 harboured putative PfSUB1 cleavage sites. To select “most likely” substrates, several delimiting criteria were used. PfSUB1 is a secreted protein which is active in the PV, therefore proteins with N-terminal secretory signal peptides were selected (n=480). Proteins which are expressed at the transcriptional or protein level, with 1 transmembrane domain (TM), a molecular mass of less than 200 kDa and a putative function in invasion or egress were selected. Hypothetical proteins with no known function were also retained. Application of these stringencies resulted in a shortlist of 77 novel putative PfSUB1 substrates.

Alignment of PfSUB1-cleavable sequences



WebLogo graphic



PoPS model assembly



5,679 *P. falciparum* predicted proteins

PoPS model scan



2,086 proteins with putative PfSUB1 sites

Signal peptide prediction



480 secreted proteins

Refinement:

mRNA expression in asexual stages

1 TMD

MW \leq 200 kDa

Putative function in invasion/egress

Hypothetical proteins



77 novel putative PfSUB1 substrates

Figure 34. Distribution of putative and known functions of predicted PfSUB1 substrates

Bar graph showing the distribution of predicted and known functions of predicted PfSUB1 substrates (480 secreted proteins). Many of these proteins are involved in processes where PfSUB1 is unlikely to play a role, for example transcription and translation (11.2%). Nearly 50% of predicted PfSUB1 substrates are hypothetical. In particular, proteases and peptidases make up 1.9% of the predicted PfSUB1 substrate repertoire (proteases make up 1.6% of the *P. falciparum* predicted proteome), while 1.2% of predicted substrates are merozoite surface proteins.

Distribution of putative substrates by function

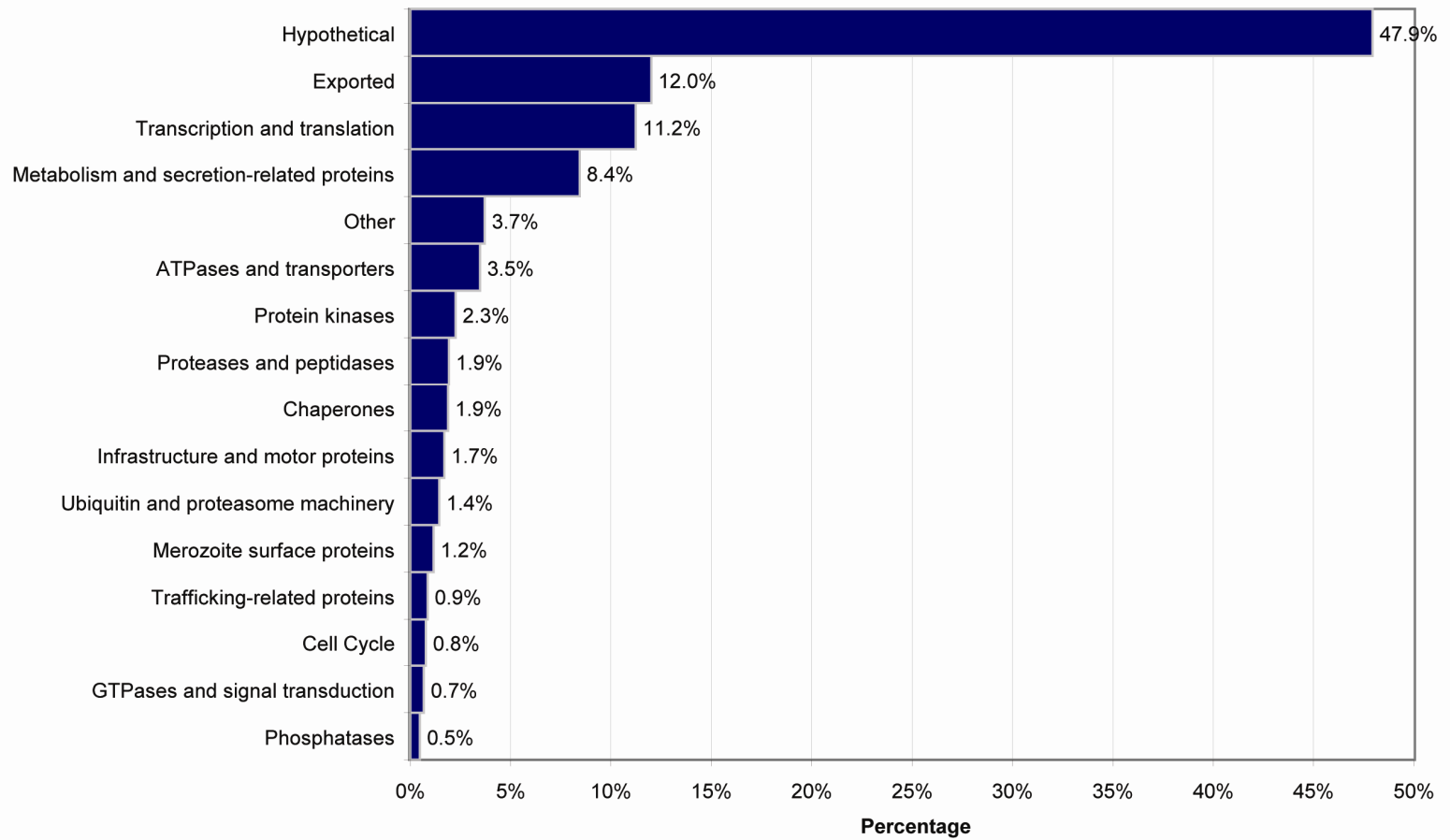


Figure 35. The predicted secondary structure of validated PfSUB1 cleavage sites is variable.

Secondary structure predictions for all cleavage sites occurring in the 3D7 cloned line of *P. falciparum* were obtained using JPred. The final secondary structure prediction is shown (Jnet). Most cleavage sites occur in disordered regions (-) and others occur within predicted α -helices (H) or β -strands (E).

SUB1	IKILEEKGALIESDKLVSADNIDISGIKDAIRRGEENIDV
Jnet	HHHHHH-----EEEEHHHHHH-----
MSP1 ₈₃₋₃₀	MLKKEQAVLSSITQPLVAASETTEDGGHSTHTLSQSGETE
Jnet	---HHHHHHHHHHHHHHHHHH---HHH-----E
MSP1 ₃₀₋₃₈	LKEENHIKKLLEEKQKITGTSSTSSPGNTTVNTAQSATHS
Jnet	H---HHHHHHH-----
MSP1 ₃₈₋₄₂	PIFGESEDNDEYLDQVVTGEAISVTMDNILSGFENEYDVI
Jnet	EEE-----EEE-----EE
MSP6 ₃₆	KGANGLTGATENITQVVQANSETNKNPTSHSNSTTTSLNN
Jnet	-----HHHHHHH-----
MSP7 ₁₉	PLFQNLGLFGKNVLSKVKAQSETDTQSKNEQEISTQGQEV
Jnet	-----HHH
SERA5 site 1	VCYKYLSEDIVSKFKEIKAETEDDDDDDYTEYKLTESIDN
Jnet	-----HHHHHHHH-----HHHHHHHH
SERA5 site 2	EKEDNENNKKLGNNYIIFGQDTAGSGQSGKESNTALESAG
Jnet	HHHHHH-----EEEE-----E-----
SERA5 site 3	LPSNGTTGEQGSSTGTVRGDTEPISDSSSSSSSSSSSSSS
Jnet	EEEE-----EEEEEEEE-----EEEE-EE-----
SERA6 site 1	FKYVSSEMKKKMNEIKVKAQDDFNPNEYKLIESIDNILSK
Jnet	-----HHHH-----HHHHHHHHHHHHHH
SERA6 site 2	ETSQDFESENDYDNAFVHGQSNESEDTNKEGKNVHNSVEK
Jnet	-----EEEE-----EEEEEEEEEEEE-----
SERA4 site 1	KYVSSNQKELIKKQLKITAQDDEESSEYHLSESIKLLKN
Jnet	-----H-HHHHHHH-----HHHHHHHHHHHH
SERA4 site 2	TFNSNKEEKSMNKNSYVYGQDTPVENEAPRSGVQKPTL
Jnet	-----EEEE-----

Table 7. "Most likely" PfSUB1 substrates

Application of the stringencies described in section 5.2.2 resulted in the identification of 77 proteins with at least one putative PfSUB1 cleavage site, which are secreted and expressed at least at the mRNA level in schizogony. Some of these proteins have homologues in other *Plasmodium* spp. species, others do not. Cleavage sites span subsites P4-P3' around the scissile bond. There is evidence that some of these proteins are processed physiologically.

a Downward arrow denotes the scissile bond

b *Pb*, *P. berghei*; *Pc*, *P. chabaudi*; *Pk*, *P. knowlesi*; *Py*, *P. yoelii*; *Pv*, *P. vivax*

PlasmoDB Accession number	Gene product	No. of predicted cleavage sites	Sequence at cleavage site(s) (P4-P3) ^a	Homologues ^b	Evidence of processing
Invasion- or egress-related proteins					
PF10_0345	MSP3	1	ITGN↓DFS	None	(Pearce <i>et al.</i> , 2004)
PF10_0347	H101 (MSP3-like)	2	IIGD↓DFS, IDGE↓SGT	None	(Pearce <i>et al.</i> , 2005)
PF10_0348	DBLMSP (MSP3-like)	3	INAN↓DLE, IVGQ↓DVP, LEGN↓SID	None	(Wickramarachchi <i>et al.</i> , 2009)
PF10_0355	MSP3-like	2	ITGN↓DSN, IDAN↓NQN	None	
PF10_0350	MSP3-like	2	TYGE↓NLN, TEGD↓GSS	None	
MAL13P1.174	MSRP2 (MSP7-like)	2	IIGQ↓GIF, LKGE↓SED	<i>Pc, Pk, Pv, Py</i>	(Kadekoppala <i>et al.</i> , 2008)
PF13_0193	MSRP3 (MSP7-like)	1	VTGQ↓NVD	<i>Pb, Pc, Pk, Pv, Py</i>	
MAL13P1.173	MSRP4 (MSP7-like)	2	IKGN↓SEE, IKGQ↓GFF	<i>Pc, Pk, Pv, Py</i>	
PF13_0194	MSRP5 (MSP7-like)	3	LSAA↓TND, LRGT↓SQN, TQGS↓ENN	None	
MAL13P1.56	M1-aminopeptidase	1	VEGE↓EYT	<i>Pb, Pc, Pk, Pv, Py</i>	(Florent <i>et al.</i> , 1998, Allary <i>et al.</i> , 2002)
PF10_0281	MTRAP	1	IEGD↓NIT	<i>Pb, Pc, Pv, Pk</i>	(Baker <i>et al.</i> , 2006)
PFD0295c	ASP	1	LIAE↓NEA	<i>Pb, Pc, Pk, Pv, Py</i>	(O'Keeffe <i>et al.</i> , 2005)
MAL7P1.208	RAMA	1	LQGD↓SDD	<i>Pb, Pc, Pk, Pv, Py</i>	(Topolska <i>et al.</i> , 2004, Richard <i>et al.</i> , 2009)
PF14_0102	RAP1	1	IVGA↓DEE,	<i>Pb, Pc, Pk, Pv, Py</i>	(Howard <i>et al.</i> , 1998, Ridley <i>et al.</i> , 1991)
PFE0080c	RAP2	1	LVGT↓NNN	<i>Pb, Pc, Pk, Pv, Py</i>	
PFB0360c	SERA1	3	IKAE↓AED, VMAT↓NCF, TRGE↓EDD	<i>Pb, Pc, Pk, Pv, Py</i>	
PFB0355c	SERA2	2	IWQQ↓ETT, TKGE↓DDA	<i>Pb, Pc, Pk, Pv, Py</i>	
PFB0350c	SERA3	3	VKAA↓SVD, LYGQ↓EES, TFGQ↓NSN	<i>Pb, Pc, Pk, Pv, Py</i>	
PFB0330c	SERA7	1	ISAQ↓DEP	<i>Pb, Pc, Pk, Pv, Py</i>	
PFI0135c	SERA9	3	VHQ↓SGE, ISGQ↓SSS, TTAD↓AFN	<i>Pb, Pc, Pk, Pv, Py</i>	
PFI1445w	RhopH2	2	INAD↓DVS, LYGN↓NNN	<i>Pb, Pc, Pk, Pv, Py</i>	
PFI0265c	RhopH3	2	TSAA↓STS, ISGS↓EGP	<i>Pb, Pc, Pk, Pv, Py</i>	(Sam-Yellowe <i>et al.</i> , 1988)
PFC0110w	CLAG 3.2	2	ITGN↓SVN, TDAD↓DET	<i>Pb, Pc, Pk, Pv, Py</i>	
PFC0120w	CLAG 3.1	2	ITGN↓SVN, TDAD↓DET	<i>Pb, Pc, Pk, Pv, Py</i>	

MAL7P1.229	CLAG	2	ILGN↓SVN, LNGD↓SNP	<i>Pb, Pc, Pk, Pv, Py</i>	
PFB0935w	CLAG	1	TSGS↓SME	<i>Pb, Pc, Pk, Pv, Py</i>	
PFI1730w	CLAG9	2	LNGN↓NNA, LNAD↓GAE	<i>Pb, Pc, Pk, Pv, Py</i>	
PF11_0174	DPAP1	3	VYAN↓TAS, LRGQ↓NFS, TCGS↓SQP	<i>Pb, Pc, Pk, Pv, Py</i>	(Klemba <i>et al.</i> , 2004b)
PF11_0162	Falcipain-3	2	IAAS↓DDF, TTGA↓EEN	<i>Pb, Pc, Pk, Pv, Py</i>	
PF10_0159	GBP130	1	ILAE↓GED	None	(Ravetch <i>et al.</i> , 1985)
Other proteins					
PF14_0281	Plasmepsin IX	1	ILGN↓ATF	<i>Pb, Pc, Pk, Pv, Py</i>	
PF14_0075	Plasmepsin IV	1	TDAD↓DLE	<i>Pb, Pc, Pk, Pv, Py</i>	
PFF0615c	Pf12 6-cysteine protein	2	VIGS↓SMF, INGS↓NGN	<i>Pb, Pc, Pk, Pv, Py</i>	
PF13_0338	Pf92 6-cysteine protein	3	VIGT↓NTT, TNAQ↓NLN, TEGT↓AME	<i>Pk, Pv</i>	
PF14_0201	Pf113 6-cysteine protein	5	IMGN↓SFD, VNAN↓DDE, IEAS↓ESS, TDAE↓AID LQGS↓EQS	<i>Pb, Pc, Pk, Pv, Py</i>	
PF10_0356	LSA1	1	IKGQ↓DEN	<i>Pb, Pc, Pk, Pv, Py</i>	
PFA0280w	Pfa35-2	3	VKGQ↓DQF, IKGE↓EEN, LHAQ↓SGD	<i>Pb, Pc, Pk, Pv, Py</i>	
PF11_0224	EXP1	1	VTAQ↓DVT	<i>Pb, Pc, Pk, Pv, Py</i>	
PFE1590w	ETRAMP5	1	VVGN↓SSS	<i>Pk, Pv</i>	
PF10_0323	ETRAMP10.2	1	TQGN↓DSF	<i>Pk, Pv</i>	
MAL8P1.6	ETRAMP8	1	TLGE↓NYE	<i>Pk, Pv</i>	
PFA0675w	RESA-like protein	2	VKGD↓AEE, LNGE↓DIT	None	
PF11_0509	RESA	1	LDGS↓EDE	None	
PFD0430c	PPLP1	2	TNAD↓TVT, TAGS↓SSE	<i>Pb, Pc, Pk, Pv, Py</i>	
PFL0805w	PPLP2	1	THGT↓NFA	<i>Pb, Pc, Pk, Pv, Py</i>	
PFI1145w	PPLP3	2	IGGT↓TIF, VKGS↓TNT	<i>Pb, Pc, Pk, Pv, Py</i>	
PF08_0050	PPLP4	1	TEGN↓TIA	<i>Pb, Pc, Pk, Pv, Py</i>	
PFD1215w	PHISTa	1	TKAN↓AND	None	
MAL13P1.475	PHISTb	1	IFGD↓DSF	None	
PFD0095c	PHISTb	1	LKGD↓NNN	None	
PF10_0025	Pf70	4	TSAD↓NNT, LFAN↓NLT, LLAQNLE, LKGS↓SGS	<i>Pk, Pv</i>	
PF10_0343	S-antigen	1	THGS↓EDE	None	
Hypothetical proteins					
PF10_0342	Unknown protein	1	VHGA↓TED	None	

PF11_0069	Unknown protein	1	IKGD↓SMT	<i>Pb, Pc, Pk, Pv, Py</i>
PFC0282w	Unknown protein	1	IQGD↓SME	<i>Pb, Pc, Pk, Pv, Py</i>
MAL13P1.107	Unknown protein	2	VEAE↓SIN, IKGN↓NVE	<i>Pv</i>
PFC0571c	Unknown protein	1	VKGT↓SSF	<i>Pb, Pc, Pk, Pv, Py</i>
PFA0210c	Unknown protein	1	VQAQ↓AQA	<i>Pb, Pc, Pk, Pv, Py</i>
PFF0090w	Unknown protein	1	VYAS↓ANA	<i>Pk, Pv</i>
PF14_0693	Unknown protein	1	ISAS↓NND	<i>Pb, Pc, Pk, Pv, Py</i>
PF14_0293	Unknown protein	2	LEGD↓SME, TMAS↓AAS	<i>Pb, Pc, Pk, Pv, Py</i>
PF11_0400	Unknown protein	1	LYGN↓TFN	<i>Pb, Pc, Pk, Pv, Py</i>
MAL13P1.268	Unknown protein	1	LEGE↓TQD	<i>Pk, Pv</i>
PF14_0045	Unknown protein	1	LRGS↓NFN	<i>Pk, Pv</i>
PFE0365c	Unknown protein	1	VFGS↓STF	<i>Pb, Pc, Pv, Pk</i>
PFI1463w	Unknown protein	1	IFGS↓SCF	<i>Pk, Pv, Py</i>
PF08_0091	Unknown protein	1	IAGS↓AFS	<i>Pb, Pc, Pk, Pv, Py</i>
PFL1015w	Unknown protein	1	LNAN↓DEN	<i>Pb, Pc, Pv, Pk</i>
PF08_0081	Unknown protein	1	TDAN↓SNN	<i>Pb, Pc, Pk, Pv, Py</i>
PFA0445w	Unknown protein	1	LIGN↓SCN	<i>Pb, Pc, Pk, Pv, Py</i>
PFL2515c	Unknown protein	1	LSGE↓SGP	<i>Pv</i>
PFD1130w	Unknown protein	1	LKGE↓EDE	<i>Pk, Pv</i>
PFF1465w	Unknown protein	1	LEGD↓EDF	<i>Pb, Pc, Pv, Pk</i>
MAL8P1.66	Unknown protein	1	TLGE↓NYE	<i>Pb, Pc, Pk, Pv, Py</i>
PF14_0344	Unknown protein	4	VHAN↓DDA, VDAN↓NNE, LFGE↓NEE, LKGA↓GVN	<i>Pb, Pc, Pk, Pv, Py</i>
MAL7P1.170	Unknown protein	1	TSGA↓NGN	None
PFL1065c	Unknown protein	1	LVGS↓STT	<i>Pb, Pv, Pk</i>

Table 8. Candidate Human Erythrocytic PfSUB1 Substrates

A human erythrocyte proteome dataset (Kakhniashvili et al., 2004) was scanned using the same PfSUB1 specificity model used to predict substrates in the *P. falciparum* predicted proteome. Of the 182 proteins in this proteome, 48% harbour putative PfSUB1 cleavage sites.

Accession number (GI)	Gene product	No. of sites	Predicted sites (P3-P4')
Membrane-associated proteins			
229674	Aldolase A	4	ILAA↓DES, TNGE↓TTT, LAAD↓EST, LAGT↓NGE
107446	Protein 4.2	3	IQAE↓SWD, LTAQ↓TGE, IKGS↓GMD
4506413	Ras-related protein Rap-1A precursor	2	ITAQ↓STF, TAGT↓EQF
7661678	Ras-related protein Rap-1B precursor	2	ITAQ↓STF, TAGT↓EQF
105337	Ankyrin 1, erythrocyte splice form 2	4	VTAE↓DSS, IGGT↓DQA, VYAN↓ECA, LSGT↓EQA
2224621	KIAA0340	2	IHGE↓SSS, LQAT↓DLP
10947036	Ankyrin 1 isoform 4	4	VTAE↓DSS, IGGT↓DQA, VYAN↓ECA, LSGT↓EQA
11245444	ATP-binding cassette half-transporter	2	IDGQ↓DIS, VEAA↓AQA
13645508	Similar to ankyrin 1, isoform 1; ankyrin-1, erythrocytic; ankyrin-R	4	VTAE↓DSS, IGGT↓DQA, VYAN↓ECA, LSGT↓EQA
1174412	Spectrin alpha chain, erythrocyte	5	ILGE↓TAE, VCAQ↓GED, INAD↓ELP, TLAA↓TQS, TNGN↓GAD
7512338	Cell surface glycoprotein CD44	2	ICAA↓NNT, THGS↓ESD
14763181	Similar to Creatine kinase, M chain (M-CK)	2	VAGD↓EES, LLAS↓GMA
17476989	Similar to beta-spectrin	8	VFGE↓TDA, IQGQ↓TDP, VEAA↓EID, LVAQ↓DNF, LLAA↓SYD, TSAT↓EFE, LDAS↓TAE
1082624	Myc far upstream element-binding protein	1	IGGD↓AGT
1103842	Band 7.2b stomatin	2	VIAA↓EGE, TNAD↓SAT
10947042	Ankyrin 1 isoform 2	3	IGGT↓DQA, VYAN↓ECA, LSGT↓EQA
12698027	KIAA1741 protein	5	VLGQ↓EQP, VSGA↓GFS, TQGE↓DYS, LAGQ↓ESP, TWGS↓SAA
14286105	Plasma membrane calcium-transporting ATPase 4	4	VTGD↓NIN, IAGT↓DVA, VTGD↓GTN, TMGN↓ATA
18586430	Hypothetical protein	1	VFGQ↓EAA
18588504	Hypothetical protein	5	VGAA↓AGA, TRAQ↓AGP, TLGQ↓EIA, LRAE↓GAA, TKAT↓GGD
106140	Glycophorin A	1	ISAS↓STT
4505237	Erythrocyte membrane protein isoform 1	2	INGT↓NVT, LKAS↓EGE
2765839	Rhesus D category VI type III protein	1	ISGS↓SLA
5902116	Zona pellucida binding protein	1	IVGS↓TSF
10800054	Rh blood D group antigen polypeptide	1	ISGS↓SLA
13623437	EPB49 protein	1	LSAE↓DFS
17489129	Erythroblast membrane-associated protein	1	ILGS↓EYF
2134798	37202B-CAM protein	1	TRGQ↓SGT

12644231	Erythrocyte adducin subunit alpha	1	TTGD↓DSD
18589892	Similar to Lutheran blood group (Auberger b antigen included)	1	TRGQ↓SGT
115206	Tetrahydrofolate synthase, cytoplasmic	2	VVAT↓GQP, LVAA↓AID
18558481	Hypothetical protein	2	VSGS↓AMP, TCGS↓GLN
1314306	Channel-like integral membrane protein	1	LDAD↓DIN
4502177	Aquaporin 1	1	LDAD↓DIN
5803145	RalA-binding protein 1	1	LLAQ↓ENE
14916944	Protein 4.1	3	LTAQ↓TIT, TAAE↓GDN, LKAS↓NGD
13277550	FLOT2 protein	1	LLAQ↓AEA
18587629	Flotillin 2	1	LLAQ↓AEA
4507021	Band 3 anion transport protein	2	THAN↓ALT, TEAT↓ATD
23830903	Ribosome biogenesis protein BOP1	4	LWAQ↓EDP, LVAA↓AVE, LEAS↓EEE, LFAS↓GSD
11433346	RAP2B, member of RAS oncogene family	1	TAGT↓EQF
17472555	Hypothetical protein	1	TMGS↓SSF
481515	Actin beta' chain - human	1	TAAS↓SSS
18572484	Similar to hypothetical protein DKFZp434M131.1 - human (fragment)	3	THAQ↓GIS, THAQ↓GIS, LGGS↓GGS
87863	Heavy chain V-V region (Ab2022)	1	LKAS↓DTA
1730569	Phosphatidylinositol-4-phosphate 5-kinase type III (1-phosphatidylinositol-4-phosphate kinase)	1	LKGS↓TVA
4505611	poly(A)-specific ribonuclease PARN isoform 1	1	IMAS↓TQP
Cytoplasmic Proteins			
18548268	Hypothetical protein	2	IQGD↓SDS, LTGT↓EEE
1172607	Proteasome subunit beta type-5 precursor	1	ISGA↓TFS
7019579	Zinc finger protein 180	1	VCAQ↓DSF
18595882	Similar to discs, large (Drosophila) homolog 3 (neuroendocrine-dlg)	4	VGGE↓DGE, VQGD↓SLE, INAS↓DDE, LKQQ↓EDA
4557014	Catalase	4	VAGE↓SGS, VNAN↓GEA, LVGN↓NTP, TTGA↓GNP
4506925	SH3 domain-binding glutamic acid-rich-like protein	1	IAAN↓EEN
4503291	D-dopachrome decarboxylase	3	VVGT↓AED, LCAA↓AAS, LSGS↓TEP
13640732	Similar to Acylamino-acid-releasing enzyme (Acyl-peptide hydrolase) (APH) (Acylaminoacyl-peptidase)	1	VSAS↓DDE
33695074	DNA-damage inducible protein 2	1	VIGT↓TGS
14770762	Similar to Protein-glutamine gamma-glutamyltransferase	1	VEGA↓GLT

4506215	Proteasome (prosome, macropain) 26S subunit, ATPase	1	VKAT↓NGP
8924260	TIP120 protein; KIAA0829 protein	3	VLGE↓GVP, ISGS↓SYA, LKAA↓DID
14774921	ATP citrate lyase	2	IIAE↓GIP, LNAS↓GST
18571465	Hypothetical protein	1	IRAQ↓GLP
5729877	Heat shock cognate 71 kDa protein isoform 1	1	LGGE↓DFD
123678	Heat shock protein HSP 90-alpha	2	LEGD↓DDT, TSAS↓GDE
6005942	Vasolin-containing protein	1	LAGE↓SES
15082580	Similar to valosin-containing protein	1	LAGE↓SES
4826964	UV excision repair protein RAD23 homolog A	2	VTGS↓EYE, TEAA↓GEN
5730023	RuvB-like 2	2	IAGS↓EIF, LKGE↓TMD
11434723	Diacylglycerol kinase, gamma (90kD)	1	TEGA↓SNS
12803567	Transgelin 2	1	IQAS↓TMA
5453607	T-complex protein 1 subunit eta isoform a	2	LTAA↓SEA, TAAS↓EAA
4502419	Flavin reductase	1	LQAA↓DVD
13650577	Early endosome antigen 1	3	LEAD↓SLE, LRAT↓ELS, LSAS↓ETS
14784166	Ubiquitin isopeptidase T	3	LSAD↓SAS, TSAA↓ADP, LRAT↓NNS
4506195	Proteasome subunit beta type-2	1	TAAA↓NFT
4885389	Hydroxacyl glutathione hydrolase	1	TLAE↓EFT
18583537	Similar to OLFACTORY RECEPTOR-LIKE PROTEIN F6	1	TAGE↓SGN
1616766	Cationic trypsinogen	1	LEGN↓EQF
4502203	ADP-ribosylation factor 3	1	TCAT↓SGD
14758460	Ubiquitin-activating enzyme E1	1	LRAE↓NYD
18676716	FLJ00257 protein	1	LIAQ↓NST
5031857	L-lactate dehydrogenase A chain isoform 1	1	VIGS↓GCN
13786847	Chain A, Human Heart L-Lactate Dehydrogenase H	1	VIGS↓GCN
14745882	Aldehyde dehydrogenase 1 family	1	TAGA↓AIS
2098448	Human Carbonic Anhydrase	1	LDGQ↓GSE
4557395	Carbonic anhydrase 2	1	LDGQ↓GSE
11514573	Chain A, Site-Specific Mutant (His64 Replaced With Ala) Of Human Carbonic Anhydrase	1	LDGQ↓GSE
13528948	Proteasome (prosome, macropain) subunit, alpha type, 3	1	LSAS↓TFS

Figure 36. Workflow for the experimental identification of PfSUB1 substrates

For analysis of membrane-associated proteins, purified schizonts were incubated with a cocktail of protease inhibitors and permeabilised using the detergent saponin. The saponin lysate was centrifuged and the released soluble proteins removed by washing. For analysis of soluble proteins, parasites were freeze-thawed and the supernatant clarified by centrifugation. The resulting pellet of membrane-associated proteins or supernatant containing soluble proteins was incubated with or without rPfSUB1. Treated samples were analysed by SDS-PAGE and Western blot with available antibodies, or further resolved by solubilisation in 8 M urea, 25 mM CHAPS and RP-HPLC (membrane-associated proteins) fractionation on a C4 column. Equivalent rPfSUB1-treated or untreated RP-HPLC fractions were resolved side by side on SDS-PAGE preceding tryptic digestion and LC/MS/MS peptide mapping of shifted bands.

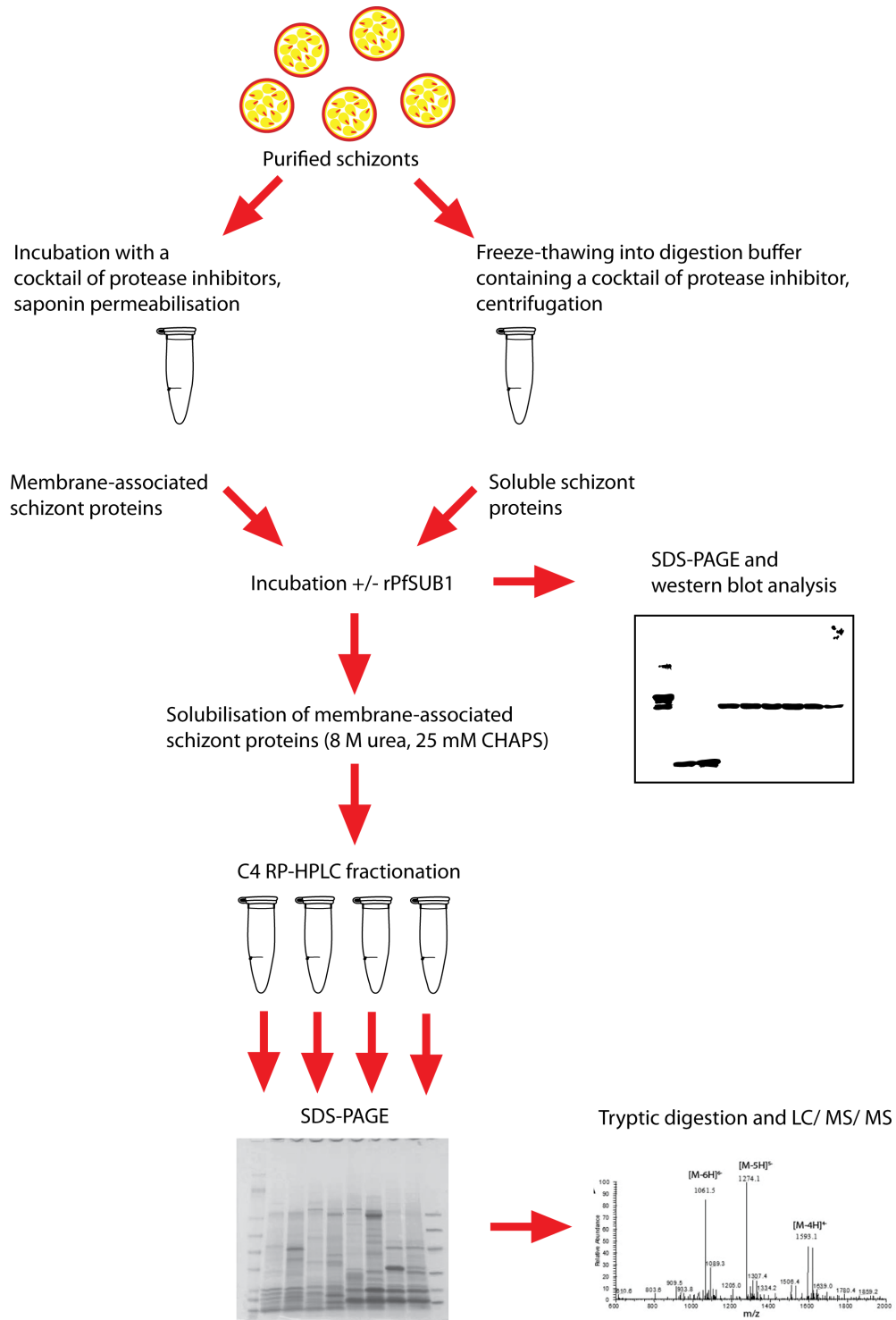
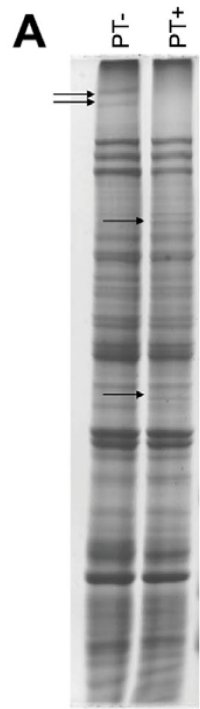


Figure 37. Mimicry of physiological processing by incubation of schizont proteins with rPfSUB1

Treatment of schizont proteins with rPfSUB1 or with rPfSUB1 and rPfSUB1pro for 1 hour at 37C°, followed by separation by SDS-PAGE indicates that very few proteins shift in size in response to rPfSUB1 (indicated with arrows) (**A**). Near complete processing of MSP1 (detected with monoclonal antibody X509) was observed in schizont membrane associated protein preparation in the presence of rPfSUB1 (PT+) (**B**), which was not observed in the presence of rPfSUB1pro (PT-). A similar result was obtained for SERA5 (detected with monoclonal antibody 24C6.1F1) in a preparation of rPfSUB1-treated soluble schizont proteins (ST+) (**C**), where processing of the SERA5 precursor p126 to p73 and p56 is almost complete. Conversion of SERA5 does not occur in the presence of rPfSUB1pro (ST-).



Coomassie-stained
SDS-PAGE

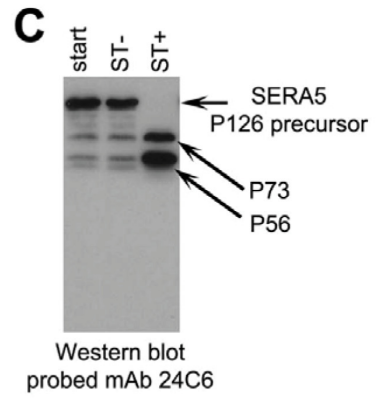
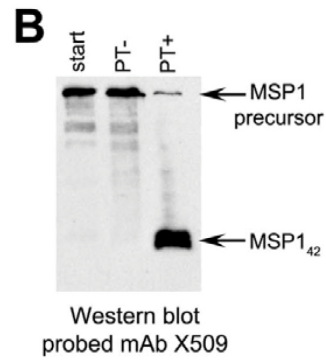
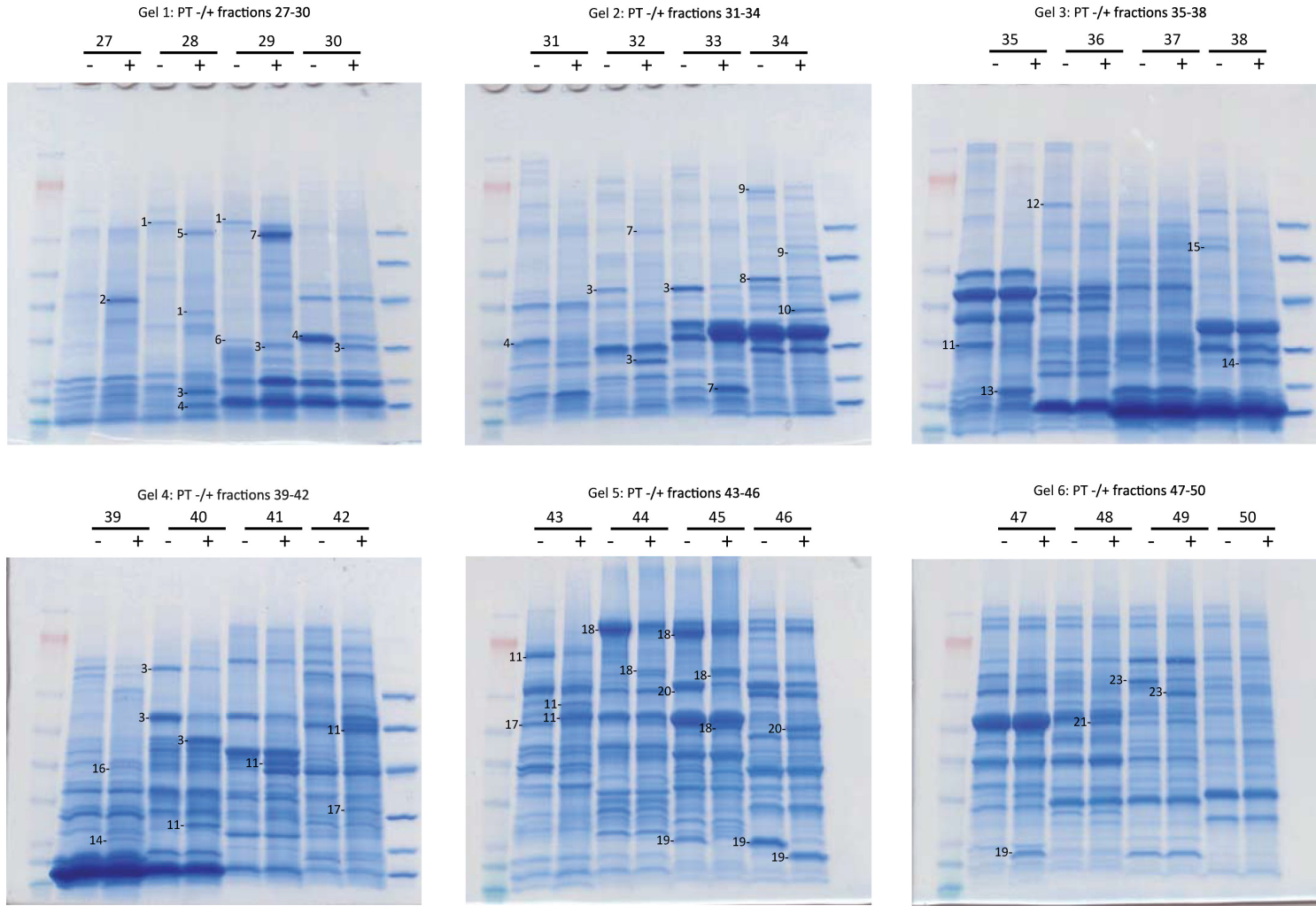


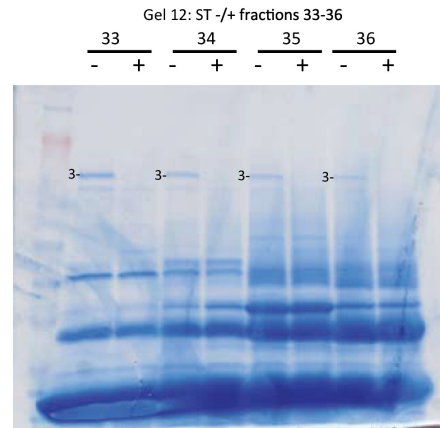
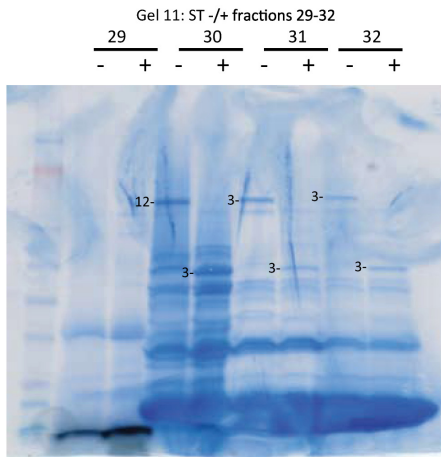
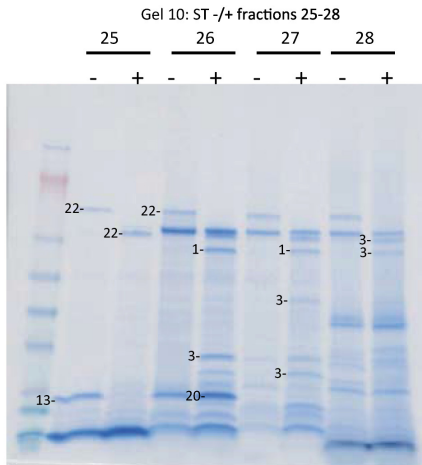
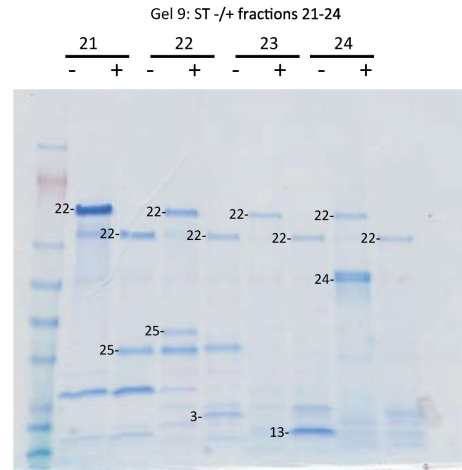
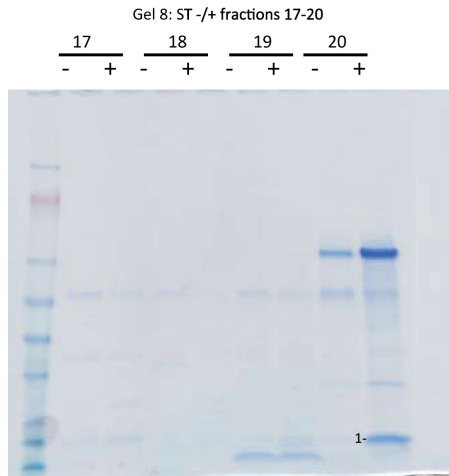
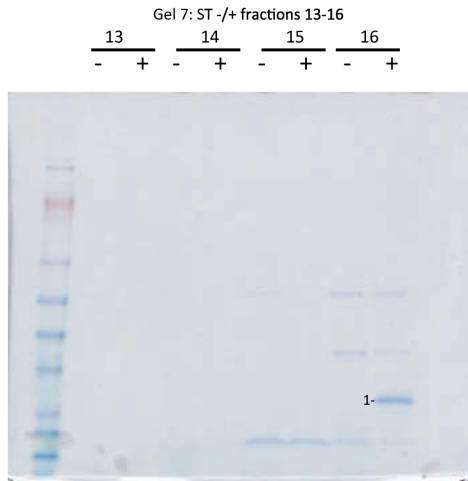
Figure 38. SDS-PAGE resolution of RP-HPLC fractions

A, B, C. Fractions of PfSUB1+ or PfSUB1- protein preparations were resolved by SDS-PAGE and stained with InstantBlue. Bands which visibly shifted in size between equivalent PfSUB1+ and PfSUB1- fractions were cut out and subjected to trypsinisation preceding LC/MS/MS analysis. See Table 9 for labelling of bands.

A.



B.



C.

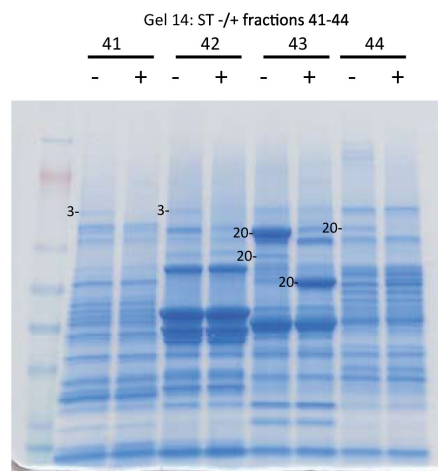
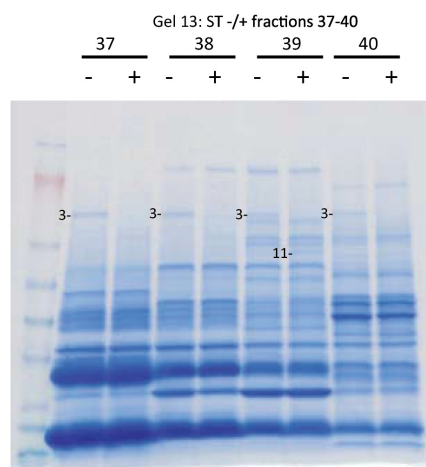


Table 9. PfSUB1 substrates identified by proteomics

Analysis of PfSUB1+ and PfSUB1- samples by RP-HPLC and SDS-PAGE followed by LC/MS/MS led to the identification of 23 proteins which are processed in the presence of rPfSUB1. The ID number relates to bands in Table 9. Substrates were cross-referenced to predictions in Table 7 and Table 8. Cross-referencing to the non-delimited list of PfSUB1 substrates (PoPS prediction) was carried out if hits had been eliminated due to predicted characteristics. Some of these proteins are processed physiologically.

ID number (Figure 33)	PlasmoDB (or NCBI) accession number	Gene product	Fraction		Predicted site (PoPS)	Predicted PfsUB1 substrate? (Table 7, Table 8)	Known or probable sub-cellular location	Published evidence of processing
			ST (soluble)	PT (membrane)				
1	PF10_0159	GBP130	✓	✓	✓	✓	PV	(Ravetch <i>et al.</i> , 1985)
2	PF10_0361	Unknown protein	×	✓	✓	×	ND	
3	PFB0340c	SERA5	✓	✓	✓	✓	PV	(Debrabant <i>et al.</i> , 1992; Delplace <i>et al.</i> , 1987; Delplace <i>et al.</i> , 1988)(Li <i>et al.</i> , 2002)(Yeoh <i>et al.</i> , 2007)
4	PF08_0074	DNA/RNA binding protein Alba	×	✓	✓	×	ND	
5	PF11_0111	Asparagine rich antigen	×	✓	✓	×	ND	
6	PFE0845c	60S ribosomal protein L8	×	✓	✓	×	ND	
7	PFE0040c	MESA/PfEMP2	✓	×	×	×	MS	
8	PFL0105w	Unknown protein	×	✓	×	×	ND	
9	PF14_0344	Unknown protein	×	✓	✓	✓	ND	
10	PF13_0304	Elongation initiation factor 1a	✓	✓	✓	×	ND	
11	PFI1475w	MSP1	✓	✓	✓	✓	MS	(Blackman <i>et al.</i> , 1991)
12	PFB0345c	SERA4	✓	✓	✓	✓	PV	(Li <i>et al.</i> , 2002)(Yeoh <i>et al.</i> , 2007)
13	PF13_0197	MSP7	✓	✓	✓	✓	MS	(Pachebat <i>et al.</i> , 2007)
14	PF10_0268	MCP1	×	✓	✓	✓	Cytosol	
15	PF14_0700	DNAJ protein	×	✓	✓	×	ND	
16	PFL0280c	Histone binding protein	×	✓	×	×	ND	
17	PF08_0054	HSP70	✓	✓	✓	×	PV	
18	Q5VYL1	Human α -spectrin	×	✓	✓	✓	Erythrocyte	
18	Q59FP5	Human β -spectrin	×	✓	✓	✓	Erythrocyte	
19	PF11_0224	EXP1	×	✓	✓	✓	PVM	
20	PFL1385c	MSP9/ABRA	✓	✓	×	×	PV	
21	PF14_0102	RAP1	✓	✓	✓	✓	Rhoptry	(Howard <i>et al.</i> , 1998; Ridley <i>et al.</i> , 1991)
22	PF10_0343	S-antigen	✓	×	✓	✓	PV	
23	PFI0265c	RhopH3	×	✓	✓	✓	Rhoptry	(Sam-Yellowe <i>et al.</i> , 1988)
24	P04264	Human keratin	✓	×	-	-	-	
25	MAL7P1.208	RAMA	✓	×	✓	✓	Rhoptry	(Richard <i>et al.</i> , 2009; Topolska <i>et al.</i> , 2004)

Figure 39. RAP1 is processed by rPfSUB1

A. RAP1 undergoes processing from p82 to p67 at $_{186}\text{GIVGA}\downarrow\text{DEEAP}_{195}$; this was hypothesised to be mediated by PfSUB1. The small arrow indicates the processing site (N.B. diagram is not to scale). **B.** Incubation of a schizont lysate with rPfSUB1 for 2 hours at 37°C resulted in conversion of full length RAP1 to a 70 kDa species (+), detected by Western blot using anti-RAP1 monoclonal antibodies. Low-level background processing at time zero (START) and in a mock-treated sample incubated with rp31 (-) was observed. A 70 kDa protein running at the same size as the PfSUB1-treated sample was observed in merozoite and schizont lysates. **C.** RP-HPLC traces of two acetylated peptide based on predicted PfSUB1 processing sites, incubated with or without rPfSUB1. Cleavage of Ac-GIVGADEEAP resulted in two peptides which were identified by ESI-MS while Ac-IVGADEEAPP was not cleaved.

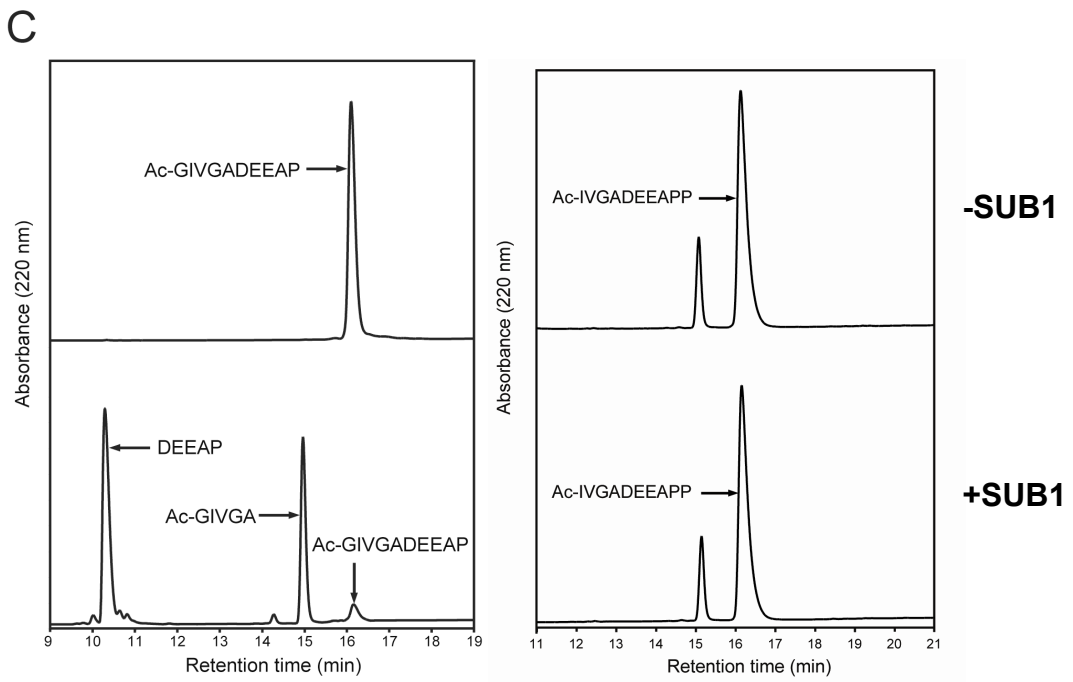
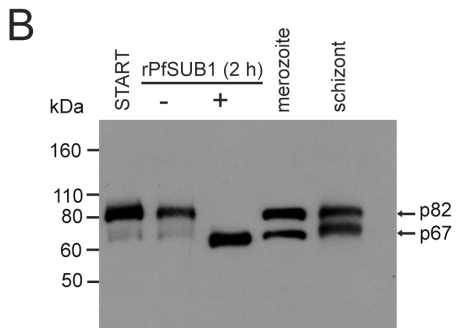
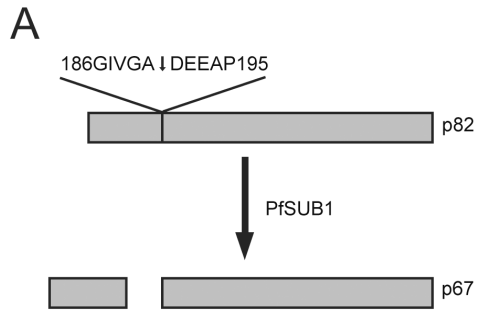


Figure 40. Conservation of PfSUB1 cleavage sites in RAP1 orthologues

Amino acid sequences *P. knowlesi*, *P. vivax*, *P. berghei* and *P. chabaudi* RAP1 orthologues were scanned using the PoPS PfSUB1 specificity model. Predicted PfSUB1 cleavage sites are highlighted. Using this model, only *P. chabaudi* RAP1 is a predicted PfSUB1 substrate.

PvRAP1 MITCVSLLVLFYALYQNVSSGIPINGERKIDNHKEPEEFNPDDINSWMRLDDANFLNTW 60
PyRAP1 MITCVSLLVLFYALYQNVSSGIPINGERKIDNHKEPEEFNPDDINSWMRLDDANFLNTW 60
PfRAP1 MSFYLGSLVLIIFHVLFRNVADGINVNGDNNYKTIINNDNFDDYNYWTPINKKEFLNSY 60
PbRAP1 -----
PkRAP1 -----
PcRAP1 MFTKIVSLFILSRLLLQDCSVAFNVRDSNVIS-----SYSHGYNSPSIKNEELGDYNYF 54

PvRAP1 TKNVSDISFVESKASKETG-----SENGDASSTGSGK-----DSYGDW 98
PyRAP1 TKNVSDISFVESKASKETG-----SENGDASSTGSGK-----DSYGDW 98
PfRAP1 EDKFSSESFLENKSSVDDGNINLTDSTSNKSSKKGHRSRVRSASAAAILEEDDSKDDM 120
PbRAP1 -----
PkRAP1 -----
PcRAP1 KKMVPKVSFLQEENDGNND----- 73

PvRAP1 NFMTNQNEAGKPGKPKSNSGESNASSSDGKSSASAKSGSKSGSKYGGSSYSDY--SAYDS 156
PyRAP1 NFMTNQNEAGKPGKPKSNSGESNASSSDGKSSASAKSGSKSGSKYGGSSYSDY--SAYDS 156
PfRAP1 EFKASPS-VVKTSTPSGTQTSGLKSSSPSTKSSSPSNVKSASPHGESNSSEE--STTKS 177
PbRAP1 -----
PkRAP1 -----
PcRAP1 -----KNESDSNAQPNLPETNEPLPADATNQNDTASNENTENKENP 115

PvRAP1 GSASS-----VGSRE-----FENEMYEFALQHPMEKLTKEMDILKNDYT 195
PyRAP1 GSASS-----VGSRE-----FENEMYEFALQHPMEKLTKEMDILKNDYT 195
PfRAP1 SKRSASVAGIVGADEEAPPAPKNTLTPLEELYPTNVNLFNYKYSLNMEENINILKNEGD 237
PbRAP1 -----
PkRAP1 -----
PcRAP1 ENK----- 118

PvRAP1 KVKEEEGKILDEEHKEIEEKRKEERLKMLAEGDVEKNKGDEEINFIKHDTDRIRGGFT 255
PyRAP1 KVKEEEGKILDEEHKEIEEKRKEERLKMLAEGDVEKNKGDEEINFIKHDTDRIRGGFT 255
PfRAP1 LVAQKEEFYDENMEKAKQDKKALEKIGKESDEEPPFMFSENK-FLENQVKERNVAGSFS 296
PbRAP1 -----
PkRAP1 -----
PcRAP1 ---ENTENKENIEKKEKKGKKEKPKRELKAQGDPELLKDRDYTLIGENTINSLKHAEE 175

PvRAP1 EFLSNLNPFFKKEIKPMKKEISLITYIPDKIVNKEKIMRDLGISHKYEPYQQSILYTCPNS 315
PyRAP1 EFLSNLNPFFKKEIKPMKKEISLITYIPDKIVNKEKIMRDLGISHKYEPYQQSILYTCPNS 315
PfRAP1 RFFSKLNPFFKDEKIEKTEVSKKTFSGIGFNLTEKEAKVLGVGVTYQEYPETMLYNCPPN 356
PbRAP1 -----MHTLGYDKEFKLAELTTIQSCPND 24
PkRAP1 -----MHTLGYDKEFKLAELTTIQSCPND 24
PcRAP1 SNDETIEEEETEYKVDENNQPLIKYTPDYTERLKKAMHKLGYDKEFKLAELRQVQSCPND 235

: * . : : .***.

PvRAP1 VFFFDSMENLRKELDKNHEKEAITNKILDHNKECLKNFGLDFELPDNKTCLGNVIGSIG 375
PyRAP1 VFFFDSMENLRKELDKNHEKEAITNKILDHNKECLKNFGLDFELPDNKTCLGNVIGSIG 375
PfRAP1 SNLFDTIESLQGRVIDIKKRESMISTTFEQQKECLKNMGVLDLELNDTQCKFGTCIGSFG 416
PbRAP1 NFLFDIFPQAIQKFQENDMKY--IQTQGDQYVECIKHKHLVGSNDQLKLNFGNSVNTFG 82
PkRAP1 NFLFDIFPQAIQKFQENDMKY--IQTQGDQYVECIKHKHLVGSNDQLKLNFGNSVNTFG 82
PcRAP1 NFLFDAYPESIEEFKKNDFRR--MDAIETRFIACLRHNLIRTNGWDTRLKFGNSVNTFG 293
: ** : . : * : : * : : * : : * : : *

PvRAP1 EYHVRLYEIENDLLKYQPSLDYMTLADDYKLVKNDVNTLENVNFCLLNPKTLEDFLKKKE 435
PyRAP1 EYHVRLYEIENDLLKYQPSLDYMTLADDYKLVKNDVNTLENVNFCLLNPKTLEDFLKKKE 435
PfRAP1 EHHRLRYEFENDLLKFPNIDYLTLDAGYKQKNDIYELSHVNFCLLNPKTLEEFLLKKKE 476
PbRAP1 PYKIPQKMITFDLIRLPSNITPVNLANDYYLSESEFPNLHKLNYCLLHPAKLEKLLKRRK 142
PkRAP1 PYKIPQKMITFDLIRLPSNITPVNLANDYYLSESEFPNLHKLNYCLLHPAKLEKLLKRRK 142
PcRAP1 PYALQNDLGVYDLTNLPSKVYINVVNDYVIPSESEFPNLRLKLNQCLLNPGKLEKLLKQKN 353
: : ** . . : : : : * : : : * : : * : : * : : *

PvRAP1 IMELMG-EDPIAYEEKFTKYMEESINCHLESLIYEDLDS-----SQDTKIVLKNVSKS 487
PyRAP1 IMELMG-EDPIAYEEKFTKYMEESINCHLESLIYEDLDS-----SQDTKIVLKNVSKS 487
PfRAP1 IKDLMGGDDLIKYKENFDNFMISITICHIESLIYDDIEA-----SQDIAAVLKIASKS 529
PbRAP1 IKSYINNTESGSYDNFFKKAMNESIECHVENTLHMILSKLTLFMFFNVNPKDSKNILKQ 202
PkRAP1 IKSYINNTESGSYDNFFKKAMNESIECHVENTLHMILSKLTLFMFFNVNPKDSKNILKQ 202
PcRAP1 IKSYITDIDKGSYDEFFKNALNESIRCHIEYLLYELLEQDRFRKYKQVPVDLEKDLKK 413
* . : : * . : * : : . ** * : * : : . . : * . : * . :

PvRAP1 LYLQNGLTYSKSKLINKLFNEIQKNPEPIFEKLTWIYENMYHLKRDYTFLAFKTVCDKY 547
PyRAP1 LYLQNGLTYSKSKLINKLFNEIQKNPEPIFEKLTWIYENMYHLKRDYTFLAFKTVCDKY 547
PfRAP1 LHVITSGLSYKARKLVYKIYSEIQKNPEDELYEKLTWIYDNIYMIKRYTAYALEGVCS-Y 588
PbRAP1 LYI IKSGLSYRSRKYVDNAYKKVINNFKDYENKIKLIDSNLENITSYAAHAFGNLCNTY 262
PkRAP1 LYI IKSGLSYRSRKYVDNAYKKVINNFKDYENKIKLIDSNLENITSYAAHAFGNLCNTY 262
PcRAP1 LYLKSGLSYRSRRHVDNIFKEIVEDIDYHEQQFRLEKNMVKITMYYSGLSGLDSCIEY 473
* : : . * : * : : : : : : : : . : : : : * : : . * : : * * :

PvRAP1 VSHN--SIYTSLQGMTSYIIIEYTRLYGACFKNITIIYNAVISGIHEQMKNLMLMPRSGLL 605
PyRAP1 VSHN--SIYTSLQGMTSYIIIEYTRLYGACFKNITIIYNAVISGIHEQMKNLMLMPRSGLL 605
PfRAP1 LEHDKSQMYTELH-IYNKIVDSVRYSSCFKNVIVYNAIISGIHEKIKHFLKLVPRHNFL 647
PbRAP1 MEKD--NIYEANAYLYEHIAPSIKIFSSCIKHLTIYNYIISNLLGQVKHLSYTPRKPIL 320
PkRAP1 MEKD--NIYEANAYLYEHIAPSIKIFSSCIKHLTIYNYIISNLLGQVKHLSYTPRKPIL 320
PcRAP1 FEKD--NIHEAHAYLDHFAKPIRLFSSCIKNMTIYNNVMSSHVSRMKQLLTHTPRKPIL 531
. . : : : : : : : : : * : * : * : * : * : * : * : *

PvRAP1 SDVHFEALLHKENKKITRDTYVLNDYDPSVKAYALTQVERLPMVSVINSFFEAKKALS 665
PyRAP1 SDVHFEALLHKENKKITRDTYVLNDYDPSVKAYALTQVERLPMVSVINSFFEAKKALS 665
PfRAP1 LDYHFNSIFEKEIKPAKYSTSHIYFDPTVASAYYNLDRRTMVTIINDYFEAKKELTV 707
PbRAP1 KDIHFKALLNKFKPKCN----ELPYDPTVKSFALGELTREP IHGLIHSYFEYKDKDLD 376
PkRAP1 KDIHFKALLNKFKPKCN----ELPYDPTVKSFALGELTREP IHGLIHSYFEYKDKDLD 376
PcRAP1 KEIHFVLLNKFKPKQNKD---HLPYHPTVKSFALGELTREP IYGFNHAFFYKDKQVLD 588
: * : : : * * . : : * : * : * : * : * : * : * : * : * : *

PvRAP1 MLAQMKLDLFTLTNEDLKIPNDKGANSKLTAKLISIIYKAEIKKYFKEMRDDYVFLIKARY 725
 PyRAP1 MLAQMKLDLFTLTNEDLKIPNDKGANSKLTAKLISIIYKAEIKKYFKEMRDDYVFLIKARY 725
 PfRAP1 IVSRMKTDMLSLQNEESKIPNDKSANSKLATRLMKKFKAEIRDFFKEMRIQYAKLINIRY 767
 PbRAP1 IMQKLLDIFSLANKDLKFPADLPDYKLFKDIVNKYKKEIKILFQEMNSEYVVKLFKMRI 436
 PkRAP1 IMQKLLDIFSLANKDLKFPADLPDYKLFKDIVNKYKKEIKILFQEMNSEYVVKLFKMRI 436
 PcRAP1 IMYKIKLDVFSLVRKGDGLELAPENNELYEQLLNKYKKELRALLQEMNYEYVVKLFEMRL 648
 :: :* *:::* .: . . : :* ::. :* *:: ::**. :*. *:: *

PvRAP1 KGHYKKNYLLYKRLE 740
 PyRAP1 KGHYKKNYLLYKRLE 740
 PfRAP1 RSHLKKNYFAFKRLD 782
 PbRAP1 SAFYQKDFFIYDRVF 451
 PkRAP1 SAFYQKDFFIYDRVF 451
 PcRAP1 SAFYQKDLMTYGRLF 663
 .. :* : : *

Figure 41. MSRP2 is processed by rPfSUB1

A. Antibodies against MSRP2 detect a 20 kDa protein in rPfSUB1-treated schizont lysate (+), separated by SDS-PAGE on a 15% polyacrylamide gel. This protein was not observed at time zero (START) or in a mock-treated sample incubated with rp31 (-). A 25 kDa band was also observed in a culture supernatant sample (CS) but not observed in merozoite (Mer) or schizont (Sch) lysates. Asterisks indicate non-specific bands detected by the anti-MSRP2 antibody (Kadekoppala et al., 2010). **B.** RP-HPLC traces of acetylated peptides based on the two predicted PfSUB1 processing sites, incubated with (lower panel) or without (upper panel) rPfSUB1. Ac-SLKGESEDNT was cleaved by rPfSUB1 and the resulting peak was identified by ESI-MS as SLKGE (Steve Howell, NIMR). The second, highly hydrophobic peptide Ac-DIIGQGIFSL (right upper and lower panels) was not cleaved in the presence of rPfSUB1 (lower panel). **C.** We propose this model for MSRP2 processing by PfSUB1. MSRP2 undergoes two processing events, resulting in conversion of the 35 kDa protein (MSRP₂₃₅) to a 28 kDa intermediate product (MSRP₂₂₈) observed in trophozoites, and a 25 kDa product (MSRP₂₂₅) observed in late schizonts and culture supernatant (Kadekoppala et al., 2010). Cleavage of MSRP2 within the predicted PfSUB1 cleavage site ${}_{90}\text{SLKGE}\downarrow\text{SEDNT}_{99}$ results in conversion of MSRP₂₂₈ to MSRP₂₂₅.

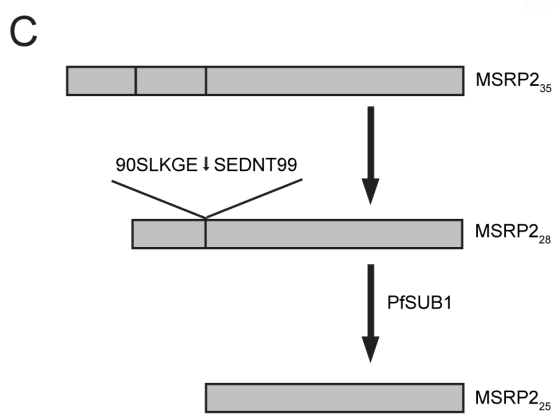
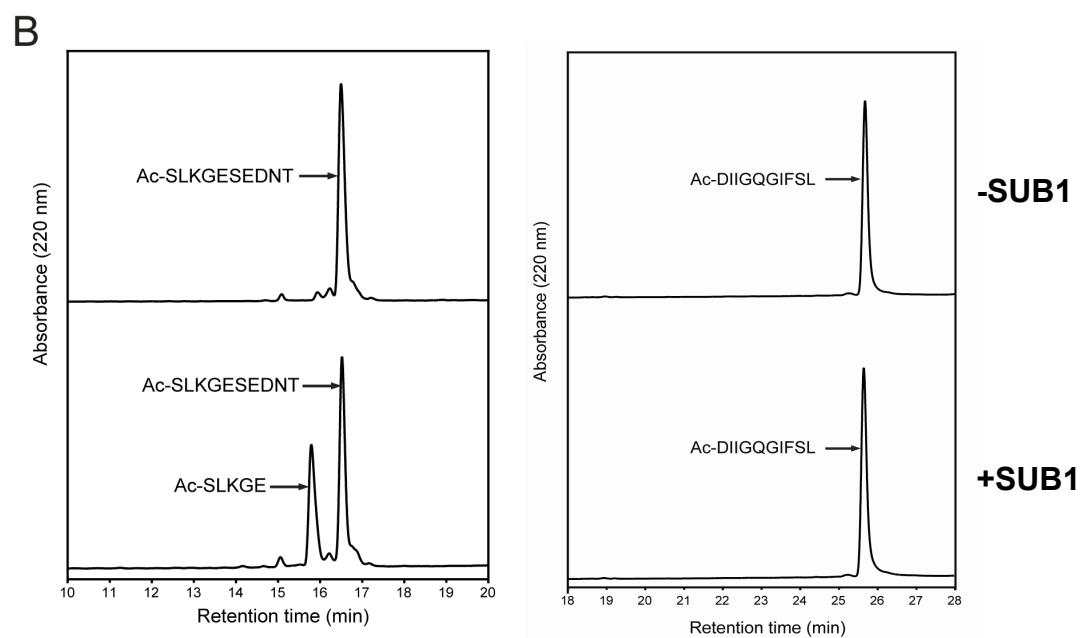
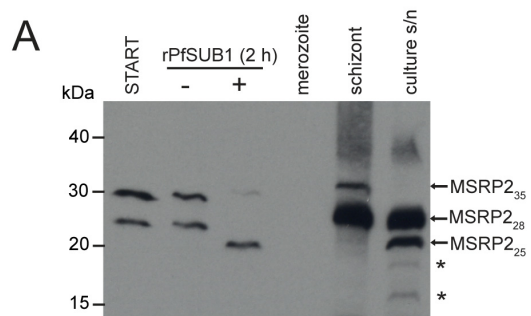


Figure 42. Conservation of PfSUB1 cleavage sites in MSRP2 orthologues

The amino acid sequences of the *P. knowlesi*, *P. vivax*, *P. berghei* and *P. chabaudi* MSRP2 orthologues were scanned using the PoPS PfSUB1 specificity model. All MSRP2 orthologues are predicted to be PfSUB1 substrates, but as the multiple sequence alignment shows (ClustalW), the sites do not align. Predicted PfSUB1 sites are highlighted.

```

PkMSRP2      MKKSIAIFGSLFVLLSHSIVSSEKVICQKKKNLEQDAMHMLMKKLESYKLSATDNSEV 60
PvMSRP2      MKKKIVLFGSLFVLLSCSTVSSSEKLGIQKKKNLEQDATHALMKKLESYKLSATDNSEI 60
PbMSRP2      MMAYKKLCFLAILALSILKAVSANDYSNNDDNVDESISDVINIFKNKNHDLVNNPEYISD 60
PcMSRP2      MAAYKKLYFLAILLGLFFKAVSADNFSNDD---DENMSDVINIFKNKNHDLVNNPNYISD 57
PfMSRP2      -MKGQAIYFVFLYFLNLCVGCNSKNNKR-----TSYNKKKENLKNFDN-IVLDD 49
      :      ..      . *...:      :.      * :.      .      .

PkMSRP2      FVKEIEALKKQIEQLQHGQGGVNEGVTLGHALENEA-----ANESTKKTIFGVDEDDL 114
PvMSRP2      FNKEIESLKKQIDQLHQHGGENEGLGHLLESEA-----ANESTKKTIFGVDEDDL 114
PbMSRP2      IKKKYQLLKNQIDQMNKYEKGMSSGDIANILEEY-----DESDNDKIVLGMSEDDL 115
PcMSRP2      IKKKFQLLKKQIDQMNKYEGMTSGDIGNILEEEAEDEEENEDKVAFGMSEEDLDN 117
PfMSRP2      FYSAFDSQDNYESKLLKNEDDIIGQIFSLISKKN-----QEKEKSLKGESEDNTKL 101
      : . : :. :...:      . : :...:      .. * * *...: .

PkMSRP2      YDVDFTGQSKGKIKGQSSKYQKKVAGNDEPNVEASGVGTTGTGDSGTESPPGTTGTQNAQ 174
PvMSRP2      YDGDFTGQSKGKFKGHSFKAQKKVEGNDE---NIGGVVPTGNSASNSQS-TGGSGSQNAS 170
PbMSRP2      YDDDFWQGSANKLTPVQMGSDANGATLVEAN----- 146
PcMSRP2      YDDDFWQGAKKAVPVPKDDDAAGAAVVEGN----- 148
PfMSRP2      QVTKVQGAQVDQAVEPLEKSPEDEN----- 126
      .. *      :

PkMSRP2      SAENSVSQSTQSTVSSSVSTGESSGSGNTGASSESSQSSSGDGSQKNDEVVPLQNNGEV 234
PvMSRP2      PPQGSPSDSAQ---GSQVTN--STGSTVT-LNAPSSSHSTGQ-PQQSAGVSLPTGTAEV 223
PbMSRP2      -----VESQASERNQNGETVRNGQNANDGVQLGS 176
PcMSRP2      -----ADPQGSQETDGGQVTR-----TDGTSLNG 173
PfMSRP2      -----

PkMSRP2      TSDAAQSSQA-APEGPAAPAGQASHAPVPEVKYLDKLYDEVLKGEDGKNGIHIPEFHSK 293
PvMSRP2      ASNTAQTSPAGSPGGQAATSGQPESGRVNVKYLKLYDEVLKTDAKDEIHVPPFHSK 283
PbMSRP2      SGQGNVQNRSDGIQGGNNEVQAGSESIIEKNAFLGTIFDEILNEQNHNQVHTTQYHSK 236
PcMSRP2      -----QTQVAGGTPGAG-TPAAVVPVPGKTAFLGTVFDEMLKAQDHAKVHTTNEYHSK 226
PfMSRP2      -----KEIPTLDSPQNGNPTSYSNLSTPPLKRMDEVFDDVLKHLNKEDKVVTDENK 180
      :      :. :...: * : . :      :.*

PkMSRP2      YNDFRKKYE-LTMNEQEYQMMKFLDAFFKKGESTN--AVCPLEFFKVLNNSLQEEFD 350
PvMSRP2      YNDFRKKYE-FTMNEREQIVKNLDAFFKKGDNPS--PADAVSFFKMLNDPNVQKEFD 340
PbMSRP2      YNSLKSECD-FAMNLEEYAIAKKIISSYFKSGTAEN--PIYLYDILIKSLNDEEYKHKFK 293
PcMSRP2      YNALKGECDFAMSLEEYIAKKLISYFN-GTTEN--PIHLYDILIKAITDEEYKHKFK 282
PfMSRP2      YNEFKKEFDIFTMNVSEYEIMKNLLITFSKKIDENNQIQTKIENIFNKALKDNKYKEQFK 240
      ** : : : : :.*      ** : *...: : :      .      : : * : : . : :.*

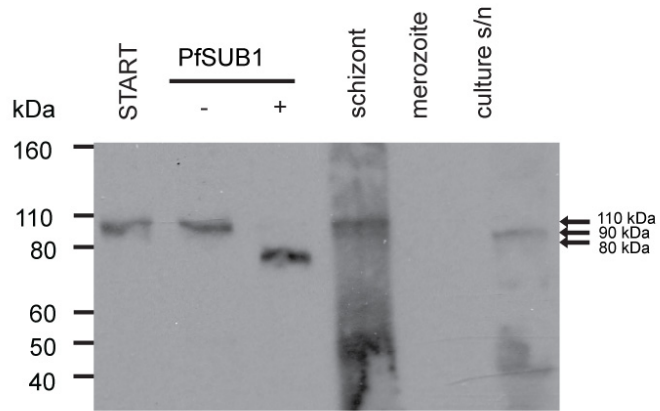
PkMSRP2      NFQHGGLYGFAKRHNYLRGEKTTNEKLYHDLLKNIINLLNTIEMK 394
PvMSRP2      NFVHGLYGFAKRHNYLRGERMTDKLYDELKLVNLLNTIEVK 384
PbMSRP2      NFIYGVYFAKKNYLSSESRLSEE--NNNFIASVLKALATVDLK 335
PcMSRP2      NFIYGIYFAKKNYLSSTARLAE--NSQFISNVLNVLATVDLK 324
PfMSRP2      NFIYGLYFAKRNHLYLVNKTNDTTLHKDLFENALNLINTI--- 281
      ** : * : . * : : * : : : : : : : : : * :

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Figure 43. RhopH3 is processed by rPfSUB1 *in vitro*, but not in *P. falciparum*

A. Antibodies against RhopH3 recognise a 110 kDa protein at time zero (START) and a smaller fragment at 80 kDa in PfSUB1-treated sample (+), separated by SDS-PAGE on a 7.5% polyacrylamide gel. In schizonts, RhopH3 is 110 kDa in size; in culture supernatant a lower band at 90 kDa is present. This does not appear to be the same size as is observed in the PfSUB1-treated sample. **B.** RP-HPLC traces of acetylated peptides based on the two predicted PfSUB1 processing sites, incubated with (lower panel) or without (upper panel) rPfSUB1. Neither of these peptides were cleaved by rPfSUB1.

A



B

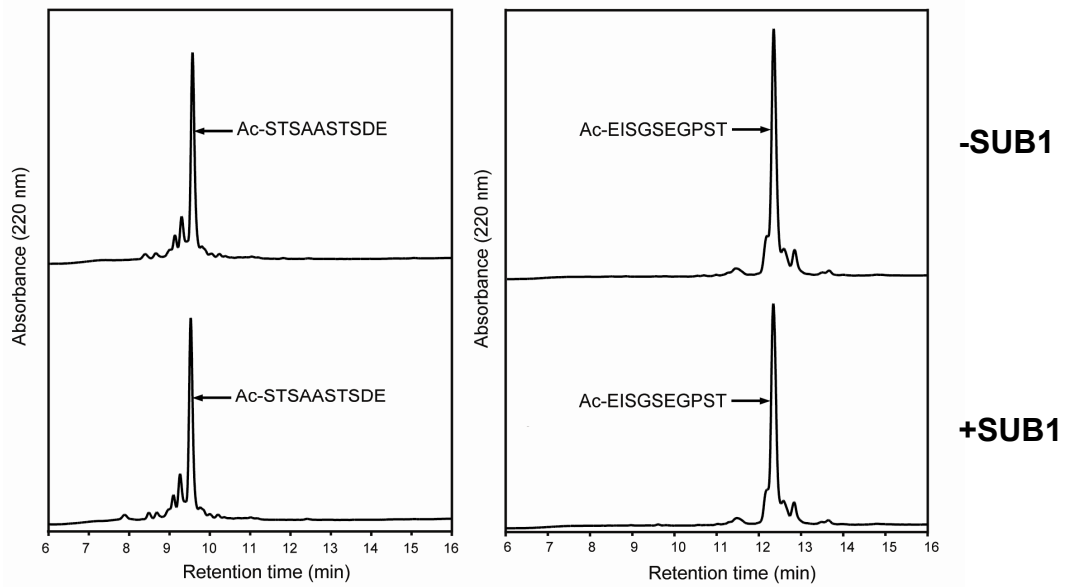


Figure 44. Human erythrocytic α and β -spectrin in erythrocyte ghosts are not cleaved by rPfSUB1

Erythrocyte ghosts were incubated with (+) or without (-) rPfSUB1 for 1 hour. Samples were taken every 30 minutes and separated by SDS-PAGE. α - and β -spectrin are observed as a doublet at around 260 kDa which does not change in intensity when incubated with rPfSUB1. Furthermore, there is no evidence of lower bands appearing or disappearing, suggesting that rPfSUB1 does not process erythrocyte ghost components.

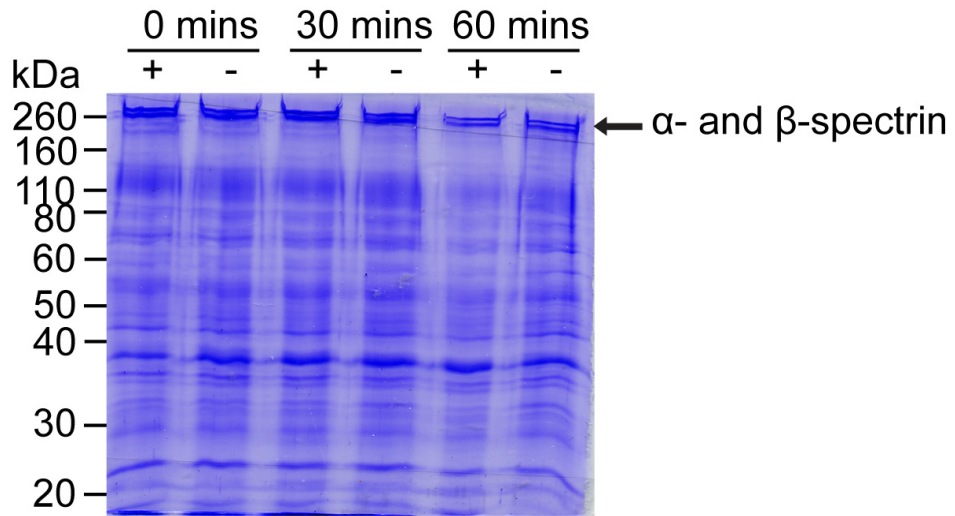


Figure 45. PfSUB1 processing sites present in PfPPLPs

PfPPLP1, PfPPLP2, PfPPLP3 and PfPPLP4 were all predicted to be PfSUB1 substrates in chapter 2 of this thesis. The putative cleavage sites (arrows) and their relative positions in the proteins (subscript) are indicated in this schematic. Each of the proteins has a secretory signal peptide (SP) and a central MACPF domain (MACPF). In PfPPLP1, PfPPLP2 and PfPPLP4, the cleavage sites flank the MACPF domain. In PfPPLP3, the two cleavage sites are within the MACPF domain. PfPPLP5 does not have a predicted PfSUB1 site.

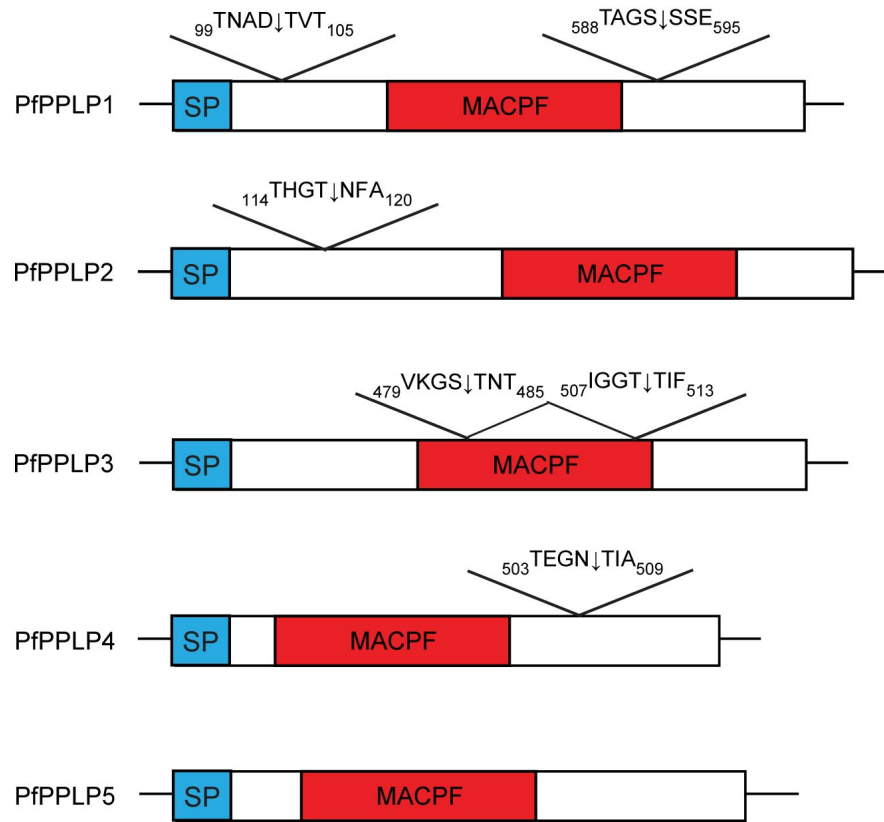
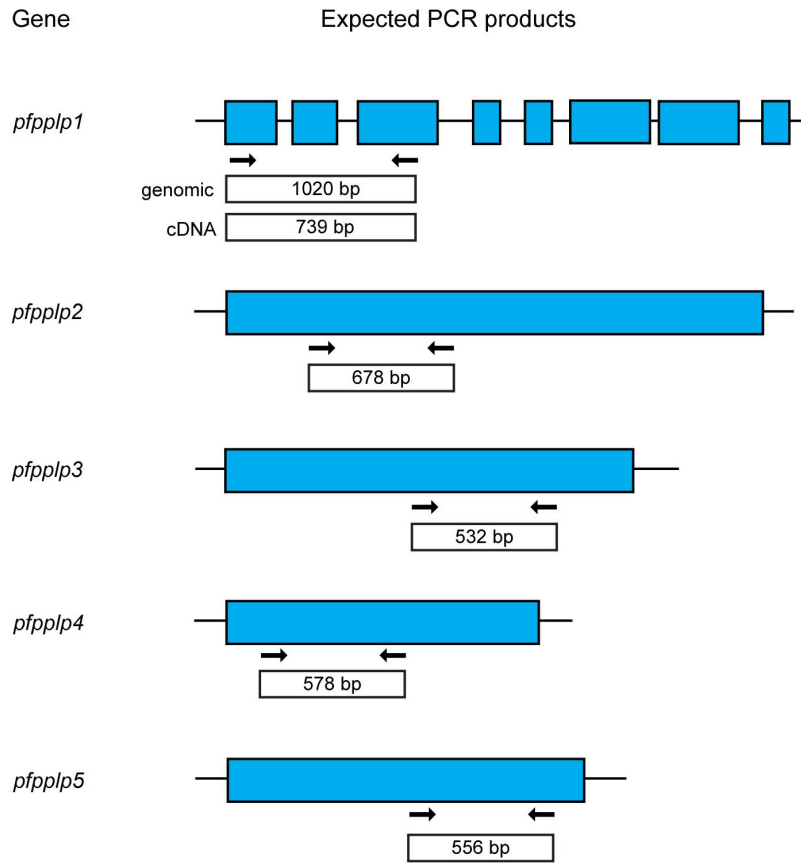


Figure 46. All *pfpplps* are transcribed during asexual development

RT-PCR using primers (position indicated by arrows) specific for each *pfpplp* gene was performed on mRNA from asynchronous parasites comprised predominantly of trophozoites and schizonts (A). Since the region amplified from the *pfpplp1* gene contains introns, a 1020 bp PCR product was expected from genomic DNA, and a smaller 739 bp PCR product when mRNA was analysed as the introns would be removed by splicing. RT-PCR products are shown in B. Products were amplified from untransfected *P. falciparum* genomic DNA (G), and reverse transcriptase-treated mRNA (RT+) or non-treated mRNA (RT-). PCR products at the expected sizes were obtained in +RT samples using primers to amplify regions of *pfpplp1-5* cDNA. Primers for the detection of *pfpplp1* cDNA detected a 1020 bp product in genomic DNA, and several smaller bands. These bands were of less intensity and therefore are likely to be non-specific products. This analysis confirms that all *pfpplps* are transcribed during asexual stages.

A



B

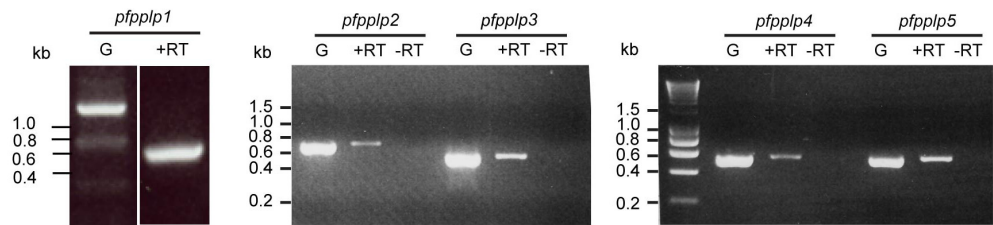


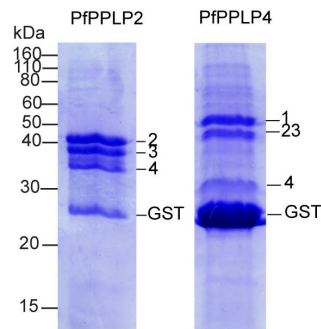
Figure 47. Recombinant expression and purification of PfPPLP2 and PfPPLP4 domains in *E. coli* for antibody production.

Several recombinant domains of PfPPLP2 and PfPPLP4 were expressed in *E. coli*. A shows regions of PfPPLP2 and PfPPLP4 which were expressed and their relative predicted molecular weights. Antibodies raised in mice against single recombinant domains did not detect a signal in merozoites or schizonts lysates. Pooled recombinant domains separated by SDS-PAGE and stained with Coomassie blue are shown in B. Free GST, a result of degradation during expression, is indicated (arrow). Single recombinant protein bands are indicated (1, 2, 3, 4). Insufficient quantities of PfPPLP2 protein 1 could be made in *E. coli* so this was not included in the PfPPLP2 mixture for immunisation. C. Serum from mice immunised with PfPPLP2 proteins (anti-PfPPLP2) and PfPPLP4 proteins (anti-PfPPLP4) were used to probe a sample of the antigens which were immunised (A), purified merozoites (M) and purified schizonts (S). Pre-immune sera was used as a control, however, this reacted non-specifically with schizonts. The expected sizes of PfPPLP2 and PfPPLP4 are 125 kDa and 75 kDa respectively. PfPPLP2 antibodies appeared to recognise the immunogen, however they reacted with the entire blot, suggesting that binding was not specific. PfPPLP4 antibodies reacted strongly with the immunogen and recognised a 75 kDa band in merozoites and schizonts. A lower band was observed at 60 kDa.

A

PfPPLP2			PfPPLP4		
Protein	Region (amino acids)	Predicted molecular weight (kDa)	Protein	Region (amino acids)	Predicted molecular weight (kDa)
1	576-788	52	1	150-372	54
2	576-661	38	2	150-254	40
3	669-788	41	3	269-372	40
4	447-521	35	4	571-621	33

B



C

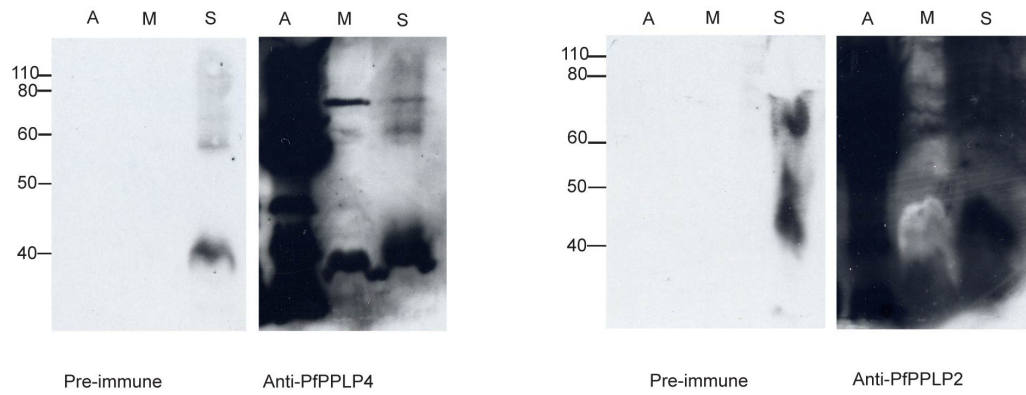
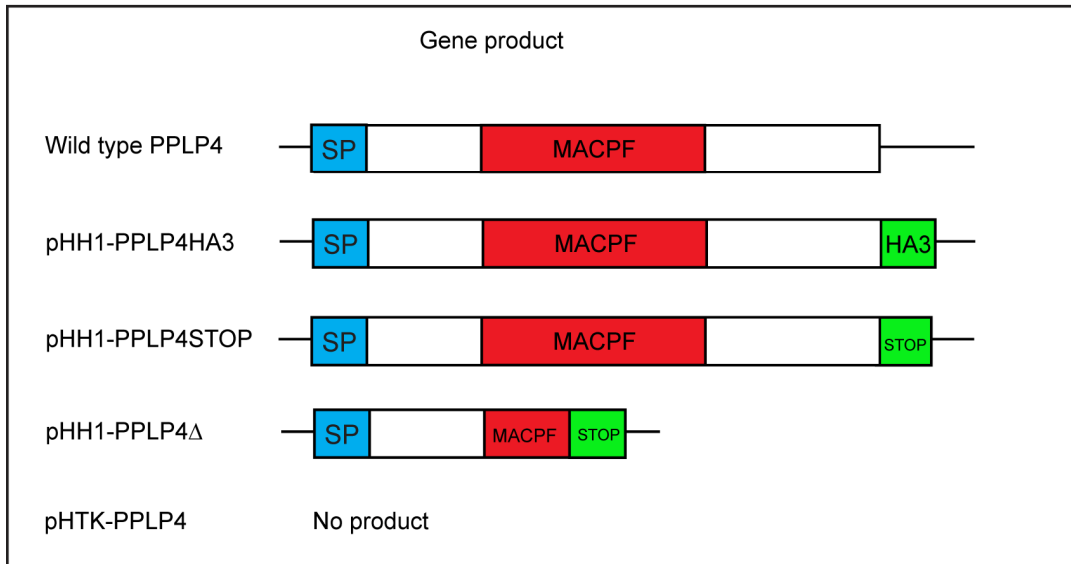
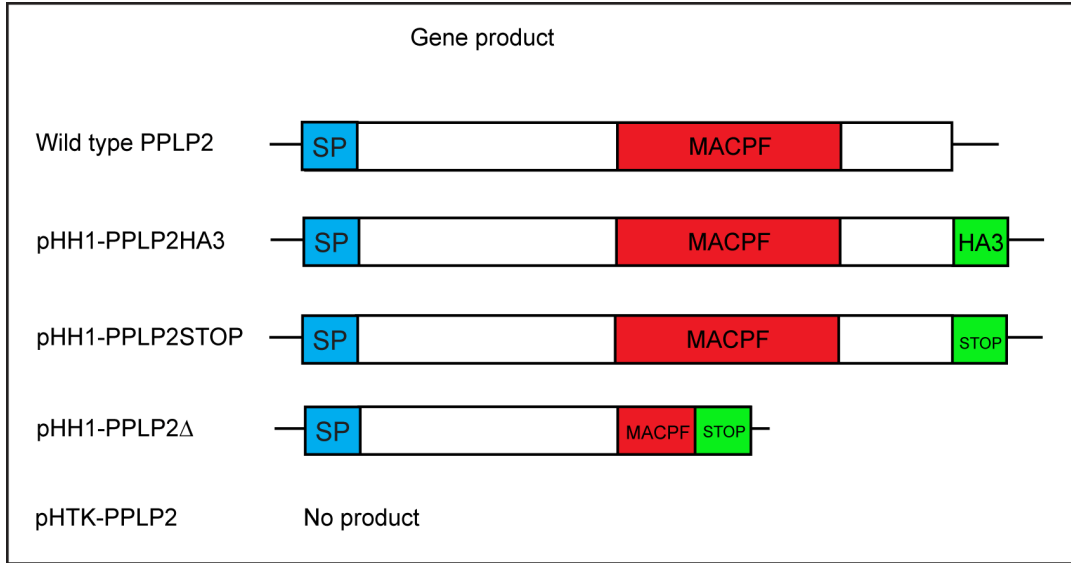


Figure 48. Modification of *pfpplp2* and *pfpplp4* genetic loci: constructs and expected gene products resulting from successful integration

Parasites were transfected with constructs to modify the *pfpplp2* and *pfpplp4* genetic loci. The wild type *pfpplp2* and *pfpplp4* genes encode full length proteins with N-terminal secretory signal peptides (SP) and central MACPF domains (MACPF). The expected gene products resulting from integration of 4 different constructs are illustrated; *pfpplp2* modifications are shown in the top panel, and *pfpplp4* modifications in the bottom panel. To integrate HA3 tags into *pfpplp2* and *pfpplp4* by single cross-over homologous recombination, pHH1-PPLP2HA3 and pHH1-PPLP4HA3 were constructed. In pHH1-PPLP2STOP and pHH1-PPLP4STOP, the same targeting sequence as pHH1-PPLP2HA3 and pHH1-PPLP4HA3 was used but the HA3 is replaced with a stop codon (STOP); these constructs act as controls to show that the *pfpplp2* and *pfpplp4* loci can be modified using this construct. No change is expected at the protein level. pHH1-PPLP2 Δ and pHH1-PPLP4 Δ are designed to integrate a HA3 tag into the middle of the MACPF domain in *pfpplp2* and *pfpplp4*, resulting in expression of truncated gene products, acting as functional knockouts. Finally, pHTK-PPLP2 and pHTK-PPLP4 were designed to delete the *pfpplp2* and *pfpplp4* genes by double cross-over homologous recombination; if genetic disruption is successful, PfPPLP2 and PfPPLP4 proteins are not expected to be made.



6. Discussion: Signalling, proteases and membrane disruption

Egress of *P. falciparum* merozoites from erythrocytes requires a combination of protease activity, signalling events and membrane disruption. The work presented in this thesis focuses on the roles of PfSUB1 and PfPPLPs in the asexual intraerythrocytic cycle of *P. falciparum* and provides a basis for further examination of the function and importance of these proteins during blood stage development. PfSUB1 and PfPPLPs are only a small, but important part of a very complex process (Figure 49. E), which has many unanswered questions.

Egress occurs in several steps, which PfSUB1 and PfPPLPs may be involved in. As *Plasmodium spp.* are obligate intracellular parasites, invasion and egress are fundamentally linked because the parasite spends very little time outside of the erythrocyte host. Both processes must therefore be highly efficient. The merozoite surface is extensively modified in late schizonts, which appears to be an important preparatory event for invasion. MSP1, MSP6 and MSP7 are proteolytically processed by PfSUB1 and inhibition of this processing results in severely reduced invasion efficiency (Koussis et al., 2009). The N- and C-terminal domains of SERA5 generated by PfSUB1-mediated proteolysis also bind to the merozoite surface (Li et al., 2002). Antibodies against the N-terminus of SERA5 are invasion-inhibitory (Pang et al., 1999, Li et al., 2002), suggesting that the N-terminus is important for invasion. The identification of possible other PfSUB1 substrates which are present on the merozoite surface and in the rhoptries (Results chapter 2: identification of novel PfSUB1 substrates) further suggests that PfSUB1 plays a major role in priming the merozoite for invasion.

During egress the PVM is ruptured, preceding EPM breakdown. The PVM contains integral and peripheral membrane proteins, which are likely to be important for the structural integrity of the vacuole membrane. To date, there is no published evidence that PVM proteins are acted upon by proteases during egress. There is conflicting data surrounding the effects of broad specificity protease inhibitors on PVM and EPM breakdown (as discussed in the introduction). However, one could speculate that since there is evidence suggesting that PVM rupture may be inhibited by serine and cysteine protease inhibitors (Salmon et al., 2001, Wickham et al., 2003, Soni et al., 2005, Gelhaus et al., 2005), breakdown of the membrane is

likely to be (directly or indirectly) mediated by proteases. The integral PVM proteins, EXP1 and ETRAMPs, are thought to form pores in the PVM (Spielmann et al., 2006a). EXP1, ETRAMP5, ETRAMP10.2 and ETRAMP8 were identified as possible substrates of PfSUB1 by bioinformatics and proteomics (Results chapter 2: identification of novel PfSUB1 substrates). If these proteins are crucial for the structural integrity of the PVM, proteolysis could result in conformational changes in their structures, causing destabilisation of the PVM by disrupting the pores. Alternatively, since the SERA proteins are putative papain-like enzymes, their proteolytic maturation by PfSUB1 could result in the SERAs acting on PVM membrane proteins to mediate membrane rupture. However, whether the SERAs are enzymes has yet to be formally demonstrated. Another possibility is that PfPPLPs are involved in membrane rupture. PfPPLPs may be secreted from micronemes during egress, as occurs in *T. gondii* (Kafsack et al., 2008). They could directly act on the PVM by forming large pores in the membrane, resulting in rupture due to osmotic stress, which has previously been implicated in egress (Glushakova et al., 2005). PfPPLP-mediated membrane rupture could also be PfSUB1-dependent, since PfSUB1 cleavage sites were identified *in silico* in four of the five PfPPLPs (Results chapter 2: identification of novel PfSUB1 substrates).

PVM rupture is followed by EPM rupture, which is the final step of egress, resulting in explosive release of merozoites (Glushakova et al., 2005). Rupture of the EPM is inhibited by E64 (Glushakova et al., 2008), indicating that it is cysteine protease-dependent. There is also evidence for proteolytic maturation of erythrocyte components during the asexual life cycle (Le Bonniec et al., 1999, Dua et al., 2001, Raphael et al., 2000, Shenai *et al.*, 2000, Hanspal et al., 2002). Erythrocyte calpain, falcipain-2, plasmepsin II and PfSUB1 have potential roles in direct or indirect modification of the erythrocyte cytoskeleton (Chandramohanadas et al., 2009, Dua et al., 2001, Le Bonniec et al., 1999, Hanspal et al., 2002, Hatanaka *et al.*, 1984, Boivin et al., 1990)(Results chapter 2: identification of novel PfSUB1 substrates). EPM rupture may be cysteine protease-dependent (Glushakova et al., 2008) and PfSUB1 probably does not directly cleave spectrin (Results chapter 2: identification of novel PfSUB1 substrates). Based on this finding, it would be unlikely that PfSUB1 is directly responsible for EPM rupture and it may act through downstream mediators, such as the SERA proteins. Whether any of the SERAs are able to cleave erythrocyte components is unknown and as aforementioned, whether they

are enzymes remains unproven. On the other hand, it is possible that erythrocyte calpain-1 is the essential mediator of membrane rupture as it is essential for egress (Chandramohanadas et al., 2009) and is known to be an important modifier of the erythrocyte cytoskeleton (Hatanaka et al., 1984, Boivin et al., 1990). It is possible that all of the proteases mentioned above act synergistically on the erythrocyte cytoskeleton in order to break up its complex structure. This could lead to membrane destabilisation as the membrane would no longer be supported by the cytoskeleton. Alternatively, there is evidence that the infected erythrocyte becomes porated in late schizonts preceding egress, suggesting a role for pore-forming proteins in the final stages of egress (Glushakova et al., 2010). The identity of these pore-forming proteins is unknown, but PfPPLPs could be involved by disrupting the EPM directly. It is likely that EPM rupture is mediated by a combination of all of these factors.

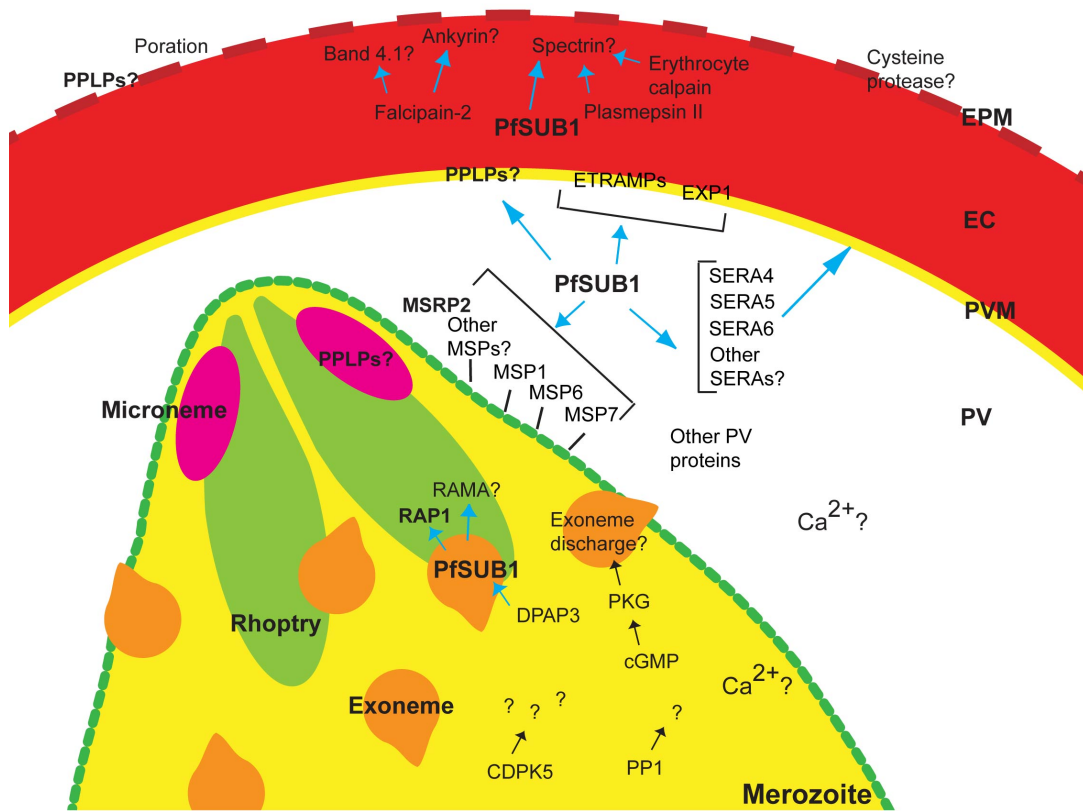
It is likely that there is interplay between signalling pathways and proteases in mediating egress, since treatment of parasites with inhibitors of kinases (Taylor et al., 2009), proteases (see Introduction) and poration (Glushakova et al., 2010) all result in a block in egress. Knockdown of the Ca^{2+} -dependent kinase CDPK5 results in a block in egress (Dvorin et al., 2010). Analysis of CDPK5-deficient schizonts indicated that, although egress is blocked, SERA5 and MSP1 are processed normally, and when the stalled schizonts were mechanically disrupted, the resulting merozoites were viable. This shows that PfSUB1 is active in the absence of CDPK5, but that a lack of CDPK5 blocks egress. It is possible that activation of the PfSUB1 pathway results in activation of kinases, which then trigger final egress events. Whether PfSUB1 is able to activate a kinase-signalling pathway is unknown. On the other hand, when the cGMP-dependent protein kinase PKG is inhibited, merozoites from mechanically disrupted schizonts are not viable (Dvorin et al., 2010). Together with the CDPK5 findings, these data suggests that PKG acts upstream of CDPK5 and PfSUB1. Very recent evidence from our lab (Christine Collins and Mike Blackman, NIMR, unpublished) suggests that PKG is involved in mediating exoneme release This is the first piece of evidence linking secondary messengers, kinases and proteases in egress. Ca^{2+} is probably also a key signal for egress activation. *T. gondii* egress is stimulated by a rise in intracellular Ca^{2+} (Arrizabalaga & Boothroyd, 2004) and *P. falciparum* CDPKs and subtilisin-like proteases involved in egress are Ca^{2+} -dependent. PfPPLPs could also be activated

by a rise in Ca^{2+} levels, as is the case with human perforin-1 (Voskoboinik et al., 2005) and as has been proposed for *T. gondii* (Kafsack et al., 2008). Cascades of signalling molecules, enzymes and PfPPLPs are likely to act in combination with PfSUB1 in regulating egress.

In conclusion, many questions remain as to how egress is regulated and mediated. What is clear is that egress is a highly organised, tightly regulated process essential to parasite survival. Targeting egress for antimalarial drug development is as important as ever, as blocking this step would prevent parasites from replicating in the human host. Considering the global impact of malaria and the worrying rise of antimalarial resistance, it is essential to continue to identify new drug targets to fight this devastating disease.

Figure 49. Egress involves signalling molecules, kinases and phosphatases, proteases and possibly PfPPLPs

Schizont egress involves many different pathways which may intersect. Signalling by cGMP and the cGMP-dependent protein kinase PKG results in the discharge of exosomes containing PfSUB1 into the rhoptries, where it processes RAP1 and possibly RAMA, and also into the PV. In the PV, PfSUB1 modifies merozoite surface proteins (MSP) thought to be important for priming the merozoite for invasion. PfSUB1 also processes putative papain-like enzymes SERA4, SERA5 and SERA6 and may cleave other SERA proteins. Evidence in this thesis suggests that PfSUB1 may also process PVM proteins EXP1 and ETRAMPs, which is possibly required for PVM rupture. Alternatively, activation of the SERAs may lead to PVM breakdown. PVM rupture could also be caused by PfPPLPs, which may be micronemal and released during egress. PfSUB1 may activate these pore-forming proteins to enable them to insert into membranes. To destabilise the erythrocyte cytoskeleton, PfSUB1, falcipain-2, calpain and plasmepsin II may be involved in proteolysis of cytoskeletal components spectrin, ankyrin and band 4.1. Finally, EPM rupture occurs in a cysteine protease-dependent manner, which may be mediated by calpain, and may involve PPLPs. Interestingly, schizonts appear to be perforated just before egress, which could be a result of insertion of PfPPLPs into the EPM. Ca^{2+} levels are likely to be elevated during egress, and Ca^{2+} is likely to be a key activator of egress as PfSUB1 and CDPK5 in particular are Ca^{2+} -dependent enzymes, and it is thought that PKG may cause Ca^{2+} release. Ca^{2+} -dependent protein kinase CDPK5 is thought to act downstream of PfSUB1, though its substrates have yet to be identified. Protein phosphatase PP1 is also thought to be involved in egress, though its substrates are unknown. Blue arrows indicate proteolytic activity, black arrows indicate signalling mechanisms. Question marks indicate unknowns.



List of references

- Adams, N. C., T. Tomoda, M. Cooper, G. Dietz & M. E. Hatten, (2002a) Mice that lack astrotactin have slowed neuronal migration. *Development* **129**: 965-972.
- Adams, S., H. Brown & G. Turner, (2002b) Breaking down the blood-brain barrier: signaling a path to cerebral malaria? *Trends Parasitol* **18**: 360-366.
- Adisa, A., M. Rug, N. Klonis, M. Foley, A. F. Cowman & L. Tilley, (2003) The signal sequence of exported protein-1 directs the green fluorescent protein to the parasitophorous vacuole of transfected malaria parasites. *J Biol Chem* **278**: 6532-6542.
- Agop-Nersesian, C., B. Naissant, F. Ben Rached, M. Rauch, A. Kretzschmar, S. Thiberge, R. Menard, D. J. Ferguson, M. Meissner & G. Langsley, (2009) Rab11A-controlled assembly of the inner membrane complex is required for completion of apicomplexan cytokinesis. *PLoS Pathog* **5**: e1000270.
- Agop-Nersesian, C., J. Pfahler, M. Lanzer & M. Meissner, (2008) Functional expression of ribozymes in Apicomplexa: towards exogenous control of gene expression by inducible RNA-cleavage. *International journal for parasitology* **38**: 673-681.
- Aikawa, M., L. H. Miller, J. Johnson & J. Rabbege, (1978) Erythrocyte entry by malarial parasites. A moving junction between erythrocyte and parasite. *J Cell Biol* **77**: 72-82.
- Aikawa, M., M. Torii, A. Sjolander, K. Berzins, P. Perlmann & L. H. Miller, (1990) Pf155/RESA antigen is localized in dense granules of *Plasmodium falciparum* merozoites. *Exp Parasitol* **71**: 326-329.
- Al Serouri, A. W., S. M. Grantham-McGregor, B. Greenwood & A. Costello, (2000) Impact of asymptomatic malaria parasitaemia on cognitive function and school achievement of schoolchildren in the Yemen Republic. *Parasitology* **121** (Pt 4): 337-345.
- Alano, P., (2007) *Plasmodium falciparum* gametocytes: still many secrets of a hidden life. *Molecular microbiology* **66**: 291-302.
- Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., (2002) *Molecular Biology of the Cell*. Garland Science, New York.
- Allary, M., J. Schrevel & I. Florent, (2002) Properties, stage-dependent expression and localization of *Plasmodium falciparum* M1 family zinc-aminopeptidase. *Parasitology* **125**: 1-10.
- Almeida-Campos, F. R. & M. F. Horta, (2000) Proteolytic activation of leishporin: evidence that *Leishmania amazonensis* and *Leishmania guyanensis* have distinct inactive forms. *Mol Biochem Parasitol* **111**: 363-375.
- Aly, A. S. & K. Matuschewski, (2005) A malarial cysteine protease is necessary for *Plasmodium* sporozoite egress from oocysts. *J Exp Med* **202**: 225-230.
- Amino, R., S. Thiberge, B. Martin, S. Celli, S. Shorte, F. Frischknecht & R. Menard, (2006) Quantitative imaging of *Plasmodium* transmission from mosquito to mammal. *Nat Med* **12**: 220-224.
- Ancsin, J. B. & R. Kisilevsky, (2004) A binding site for highly sulfated heparan sulfate is identified in the N terminus of the circumsporozoite protein: significance for malarial sporozoite attachment to hepatocytes. *J Biol Chem* **279**: 21824-21832.
- Aoki, S., J. Li, S. Itagaki, B. A. Okech, T. G. Egwang, H. Matsuoka, N. M. Palacpac, T. Mitamura & T. Horii, (2002) Serine repeat antigen (SERA5) is predominantly expressed among the SERA multigene family of *Plasmodium falciparum*, and the acquired antibody titers correlate with serum inhibition of the parasite growth. *J Biol Chem* **277**: 47533-47540.
- Arastu-Kapur, S., E. L. Ponder, U. P. Fonovic, S. Yeoh, F. Yuan, M. Fonovic, M. Grainger, C. I. Phillips, J. C. Powers & M. Bogyo, (2008) Identification of proteases that regulate erythrocyte rupture by the malaria parasite *Plasmodium falciparum*. *Nat Chem Biol* **4**: 203-213.
- Armstrong, C. M. & D. E. Goldberg, (2007) An FKBP destabilization domain modulates protein levels in *Plasmodium falciparum*. *Nat Methods*.
- Arrizabalaga, G. & J. C. Boothroyd, (2004) Role of calcium during *Toxoplasma gondii* invasion and egress. *International journal for parasitology* **34**: 361-368.

Artavanis-Tsakonas, K., J. E. Tongren & E. M. Riley, (2003) The war between the malaria parasite and the immune system: immunity, immunoregulation and immunopathology. *Clin Exp Immunol* **133**: 145-152.

Aurrecochea, C., J. Brestelli, B. P. Brunk, J. Dommer, S. Fischer, B. Gajria, X. Gao, A. Gingle, G. Grant, O. S. Harb, M. Heiges, F. Innamorato, J. Iodice, J. C. Kissinger, E. Kraemer, W. Li, J. A. Miller, V. Nayak, C. Pennington, D. F. Pinney, D. S. Roos, C. Ross, C. J. Stoeckert, Jr., C. Treatman & H. Wang, (2009) PlasmoDB: a functional genomic database for malaria parasites. *Nucleic Acids Res* **37**: D539-543.

Baker, R. P., R. Wijetilaka & S. Urban, (2006) Two *Plasmodium* rhomboid proteases preferentially cleave different adhesins implicated in all invasive stages of malaria. *PLoS Pathog* **2**: e113.

Baldi, D. L., K. T. Andrews, R. F. Waller, D. S. Roos, R. F. Howard, B. S. Crabb & A. F. Cowman, (2000) RAP1 controls rhoptry targeting of RAP2 in the malaria parasite *Plasmodium falciparum*. *The EMBO journal* **19**: 2435-2443.

Balu, B. & J. H. Adams, (2006) Functional genomics of *Plasmodium falciparum* through transposon-mediated mutagenesis. *Cell Microbiol* **8**: 1529-1536.

Balu, B., D. A. Shoue, M. J. Fraser, Jr. & J. H. Adams, (2005) High-efficiency transformation of *Plasmodium falciparum* by the lepidopteran transposable element piggyBac. *Proc Natl Acad Sci U S A* **102**: 16391-16396.

Banaszynski, L. A., L. C. Chen, L. A. Maynard-Smith, A. G. Ooi & T. J. Wandless, (2006) A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. *Cell* **126**: 995-1004.

Banerjee, R., S. E. Francis & D. E. Goldberg, (2003) Food vacuole plasmepsins are processed at a conserved site by an acidic convertase activity in *Plasmodium falciparum*. *Mol Biochem Parasitol* **129**: 157-165.

Banerjee, R., J. Liu, W. Beatty, L. Pelosof, M. Klemba & D. E. Goldberg, (2002) Four plasmepsins are active in the *Plasmodium falciparum* food vacuole, including a protease with an active-site histidine. *Proc Natl Acad Sci U S A* **99**: 990-995.

Bannister, L. H. & A. R. Dluzewski, (1990) The ultrastructure of red cell invasion in malaria infections: a review. *Blood Cells* **16**: 257-292; discussion 293-257.

Bannister, L. H., J. M. Hopkins, A. R. Dluzewski, G. Margos, I. T. Williams, M. J. Blackman, C. H. Kocken, A. W. Thomas & G. H. Mitchell, (2003) *Plasmodium falciparum* apical membrane antigen 1 (PfAMA-1) is translocated within micronemes along subpellicular microtubules during merozoite development. *J Cell Sci* **116**: 3825-3834.

Bannister, L. H., J. M. Hopkins, R. E. Fowler, S. Krishna & G. H. Mitchell, (2000) Ultrastructure of rhoptry development in *Plasmodium falciparum* erythrocytic schizonts. *Parasitology* **121** (Pt 3): 273-287.

Bannister, L. H. & G. H. Mitchell, (1989) The fine structure of secretion by *Plasmodium knowlesi* merozoites during red cell invasion. *J Protozool* **36**: 362-367.

Bannister, L. H., G. H. Mitchell, G. A. Butcher & E. D. Dennis, (1986) Lamellar membranes associated with rhoptries in erythrocytic merozoites of *Plasmodium knowlesi*: a clue to the mechanism of invasion. *Parasitology* **92** (Pt 2): 291-303.

Banyal, H. S., G. C. Misra, C. M. Gupta & G. P. Dutta, (1981) Involvement of malarial proteases in the interaction between the parasite and host erythrocyte in *Plasmodium knowlesi* infections. *J Parasitol* **67**: 623-626.

Baran, K., M. Dunstone, J. Chia, A. Ciccone, K. A. Browne, C. J. Clarke, N. Lukyanova, H. Saibil, J. C. Whisstock, I. Voskoboinik & J. A. Trapani, (2009) The molecular basis for perforin oligomerization and transmembrane pore assembly. *Immunity* **30**: 684-695.

Barkan, D. T., D. R. Hostetter, S. Mahrus, U. Pieper, J. A. Wells, C. S. Craik & A. Sali, (2010) Prediction of protease substrates using sequence and structure features. *Bioinformatics* **26**: 1714-1722.

Barnes, D. A., J. Thompson, T. Triglia, K. Day & D. J. Kemp, (1994) Mapping the genetic locus implicated in cytoadherence of *Plasmodium falciparum* to melanoma cells. *Mol Biochem Parasitol* **66**: 21-29.

Bass, C. C. & F. M. Johns, (1912) The Cultivation of Malarial Plasmodia (*Plasmodium Vivax* and *Plasmodium Falciparum*) in Vitro. *J Exp Med* **16**: 567-579.

Baum, J., A. T. Papenfuss, G. R. Mair, C. J. Janse, D. Vlachou, A. P. Waters, A. F. Cowman, B. S. Crabb & T. F. de Koning-Ward, (2009) Molecular genetics and comparative genomics reveal RNAi is not functional in malaria parasites. *Nucleic Acids Res* **37**: 3788-3798.

Baum, J., D. Richard, J. Healer, M. Rug, Z. Krnajski, T. W. Gilberger, J. L. Green, A. A. Holder & A. F. Cowman, (2006) A conserved molecular motor drives cell invasion and gliding motility across malaria life cycle stages and other apicomplexan parasites. *J Biol Chem* **281**: 5197-5208.

Beck, H. P., I. Felger, W. Huber, S. Steiger, T. Smith, N. Weiss, P. Alonso & M. Tanner, (1997) Analysis of multiple *Plasmodium falciparum* infections in Tanzanian children during the phase III trial of the malaria vaccine SPf66. *J Infect Dis* **175**: 921-926.

Bejon, P., J. Lusingu, A. Olotu, A. Leach, M. Lievens, J. Vekemans, S. Mshamu, T. Lang, J. Gould, M. C. Dubois, M. A. Demoitie, J. F. Stallaert, P. Vansadia, T. Carter, P. Njuguna, K. O. Awuondo, A. Malabeja, O. Abdul, S. Gesase, N. Mturi, C. J. Drakeley, B. Savarese, T. Villafana, W. R. Ballou, J. Cohen, E. M. Riley, M. M. Lemnge, K. Marsh & L. von Seidlein, (2008) Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age. *N Engl J Med* **359**: 2521-2532.

Bennett, V. & D. M. Gilligan, (1993) The spectrin-based membrane skeleton and micron-scale organization of the plasma membrane. *Annu Rev Cell Biol* **9**: 27-66.

Bennett, V. & P. J. Stenbuck, (1979) The membrane attachment protein for spectrin is associated with band 3 in human erythrocyte membranes. *Nature* **280**: 468-473.

Bertani, G., (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* **62**: 293-300.

Bhattacharjee, S., C. van Ooij, B. Balu, J. H. Adams & K. Haldar, (2008) Maurer's clefts of *Plasmodium falciparum* are secretory organelles that concentrate virulence protein reporters for delivery to the host erythrocyte. *Blood* **111**: 2418-2426.

Billker, O., V. Lindo, M. Panico, A. E. Etienne, T. Paxton, A. Dell, M. Rogers, R. E. Sinden & H. R. Morris, (1998) Identification of xanthurenic acid as the putative inducer of malaria development in the mosquito. *Nature* **392**: 289-292.

Bjorkman, A., (1991) Quinine-resistant malaria in Africa. *Trans R Soc Trop Med Hyg* **85**: 138-139.

Black, M. W., G. Arrizabalaga & J. C. Boothroyd, (2000) Ionophore-resistant mutants of *Toxoplasma gondii* reveal host cell permeabilization as an early event in egress. *Mol Cell Biol* **20**: 9399-9408.

Blackman, M. J., (2008) Malarial proteases and host cell egress: an 'emerging' cascade. *Cell Microbiol* **10**: 1925-1934.

Blackman, M. J., J. E. Corrie, J. C. Croney, G. Kelly, J. F. Eccleston & D. M. Jameson, (2002) Structural and biochemical characterization of a fluorogenic rhodamine-labeled malarial protease substrate. *Biochemistry* **41**: 12244-12252.

Blackman, M. J., E. D. Dennis, E. M. Hirst, C. H. Kocken, T. J. Scott-Finnigan & A. W. Thomas, (1996) *Plasmodium* knowlesi: secondary processing of the malaria merozoite surface protein-1. *Exp Parasitol* **83**: 229-239.

Blackman, M. J., H. Fujioka, W. H. Stafford, M. Sajid, B. Clough, S. L. Fleck, M. Aikawa, M. Grainger & F. Hackett, (1998) A subtilisin-like protein in secretory organelles of *Plasmodium falciparum* merozoites. *J Biol Chem* **273**: 23398-23409.

Blackman, M. J. & A. A. Holder, (1992) Secondary processing of the *Plasmodium falciparum* merozoite surface protein-1 (MSP1) by a calcium-dependent membrane-bound serine protease: shedding of MSP133 as a noncovalently associated complex with other fragments of the MSP1. *Mol Biochem Parasitol* **50**: 307-315.

Blackman, M. J., H. Whittle & A. A. Holder, (1991) Processing of the *Plasmodium falciparum* major merozoite surface protein-1: identification of a 33-kilodalton secondary processing product which is shed prior to erythrocyte invasion. *Mol Biochem Parasitol* **49**: 35-44.

Blisnick, T., L. Vincensini, G. Fall & C. Braun-Breton, (2006) Protein phosphatase 1, a *Plasmodium falciparum* essential enzyme, is exported to the host cell and implicated in the release of infectious merozoites. *Cell Microbiol* **8**: 591-601.

Bioland, P., (2001) Drug Resistance in Malaria. In: WHO (ed). pp.

Boddey, J. A., A. N. Hodder, S. Gunther, P. R. Gilson, H. Patsiouras, E. A. Kapp, J. A. Pearce, T. F. de Koning-Ward, R. J. Simpson, B. S. Crabb & A. F. Cowman, (2009) An aspartyl protease directs malaria effector proteins to the host cell. *Nature* **463**: 627-631.

Boivin, P., C. Galand & D. Dhermy, (1990) In vitro digestion of spectrin, protein 4.1 and ankyrin by erythrocyte calcium dependent neutral protease (calpain I). *Int J Biochem* **22**: 1479-1489.

Bokman, S. H. & W. W. Ward, (1981) Renaturation of green-fluorescent protein. *Biochemical and Biophysical Research Communications* **101**: 1372-1380.

Bonilla, J. A., T. D. Bonilla, C. A. Yowell, H. Fujioka & J. B. Dame, (2007a) Critical roles for the digestive vacuole plasmepsins of *Plasmodium falciparum* in vacuolar function. *Molecular microbiology* **65**: 64-75.

Bonilla, J. A., P. A. Moura, T. D. Bonilla, C. A. Yowell, D. A. Fidock & J. B. Dame, (2007b) Effects on growth, hemoglobin metabolism and paralogous gene expression resulting from disruption of genes encoding the digestive vacuole plasmepsins of *Plasmodium falciparum*. *International journal for parasitology* **37**: 317-327.

Boyd, S. E., M. Garcia de la Banda, R. N. Pike, J. C. Whisstock & G. B. Rudy, (2004) PoPS: a computational tool for modeling and predicting protease specificity. *Proc IEEE Comput Syst Bioinform Conf*: 372-381.

Boyd, S. E., R. N. Pike, G. B. Rudy, J. C. Whisstock & M. Garcia de la Banda, (2005) PoPS: a computational tool for modeling and predicting protease specificity. *J Bioinform Comput Biol* **3**: 551-585.

Boyle, M. J., J. S. Richards, P. R. Gilson, W. Chai & J. G. Beeson, (2010) Interactions with heparin-like molecules during erythrocyte invasion by *Plasmodium falciparum* merozoites. *Blood* **115**: 4559-4568.

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

Bradley, P. J., C. Ward, S. J. Cheng, D. L. Alexander, S. Coller, G. H. Coombs, J. D. Dunn, D. J. Ferguson, S. J. Sanderson, J. M. Wastling & J. C. Boothroyd, (2005) Proteomic analysis of rhoptry organelles reveals many novel constituents for host-parasite interactions in *Toxoplasma gondii*. *J Biol Chem* **280**: 34245-34258.

Bravo, A., S. S. Gill & M. Soberon, (2007) Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon* **49**: 423-435.

Breinich, M. S., D. J. Ferguson, B. J. Foth, G. G. van Dooren, M. Lebrun, D. V. Quon, B. Striepen, P. J. Bradley, F. Frischknecht, V. B. Carruthers & M. Meissner, (2009) A dynamin is required for the biogenesis of secretory organelles in *Toxoplasma gondii*. *Curr Biol* **19**: 277-286.

Breman, J. G., (2001) The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. *Am J Trop Med Hyg* **64**: 1-11.

Brooker, S., H. Guyatt, J. Omumbo, R. Shretta, L. Drake & J. Ouma, (2000) Situation analysis of malaria in school-aged children in Kenya - what can be done? *Parasitol Today* **16**: 183-186.

Brossier, F., G. L. Starnes, W. L. Beatty & L. D. Sibley, (2008) Microneme rhomboid protease TgROM1 is required for efficient intracellular growth of *Toxoplasma gondii*. *Eukaryotic cell* **7**: 664-674.

Bryan, P., L. Wang, J. Hoskins, S. Ruvinov, S. Strausberg, P. Alexander, O. Almog, G. Gilliland & T. Gallagher, (1995) Catalysis of a protein folding reaction: mechanistic implications of the 2.0 Å structure of the subtilisin-prodomain complex. *Biochemistry* **34**: 10310-10318.

Bryan, P. N., (2000) Protein engineering of subtilisin. *Biochim Biophys Acta* **1543**: 203-222.

Buguliskis, J. S., F. Brossier, J. Shuman & L. D. Sibley, (2010) Rhomboid 4 (ROM4) affects the processing of surface adhesins and facilitates host cell invasion by *Toxoplasma gondii*. *PLoS Pathog* **6**: e1000858.

Bull, P. C., B. S. Lowe, M. Kortok, C. S. Molyneux, C. I. Newbold & K. Marsh, (1998) Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. *Nat Med* **4**: 358-360.

Bull, P. C. & K. Marsh, (2002) The role of antibodies to *Plasmodium falciparum*-infected-erythrocyte surface antigens in naturally acquired immunity to malaria. *Trends Microbiol* **10**: 55-58.

Bushell, G. R., L. T. Ingram, C. A. Fardoulis & J. A. Cooper, (1988) An antigenic complex in the rhoptries of *Plasmodium falciparum*. *Mol Biochem Parasitol* **28**: 105-112.

Caldas, L. A., W. de Souza & M. Attias, (2010) Microscopic analysis of calcium ionophore activated egress of *Toxoplasma gondii* from the host cell. *Vet Parasitol* **167**: 8-18.

Camus, D. & T. J. Hadley, (1985) A *Plasmodium falciparum* antigen that binds to host erythrocytes and merozoites. *Science* **230**: 553-556.

Carruthers, V. B., O. K. Giddings & L. D. Sibley, (1999) Secretion of micronemal proteins is associated with toxoplasma invasion of host cells. *Cell Microbiol* **1**: 225-235.

Carter, P. & J. A. Wells, (1988) Dissecting the catalytic triad of a serine protease. *Nature* **332**: 564-568.

Casares, S., T. D. Brumeanu & T. L. Richie, (2010) The RTS,S malaria vaccine. *Vaccine* **28**: 4880-4894.

Castro-Gomes, T., F. R. Almeida-Campos, C. E. Calzavara-Silva, R. A. da Silva, F. Frezard & M. F. Horta, (2009) Membrane binding requirements for the cytolytic activity of *Leishmania amazonensis* leishporin. *FEBS Lett* **583**: 3209-3214.

Chandramohanadas, R., P. H. Davis, D. P. Beiting, M. B. Harbut, C. Darling, G. Velmourougane, M. Y. Lee, P. A. Greer, D. S. Roos & D. C. Greenbaum, (2009) Apicomplexan Parasites Co-Opt Host Calpains to Facilitate Their Escape from Infected Cells. *Science*.

Chandu, D. & D. Nandi, (2004) Comparative genomics and functional roles of the ATP-dependent proteases Lon and Clp during cytosolic protein degradation. *Res Microbiol* **155**: 710-719.

Chin, W., P. G. Contacos, G. R. Coatney & H. R. Kimball, (1965) A Naturally Acquired Quotidian-Type Malaria in Man Transferable to Monkeys. *Science* **149**: 865.

Chu, B. W., L. A. Banaszynski, L. C. Chen & T. J. Wandless, (2008) Recent progress with FKBP-derived destabilizing domains. *Bioorg Med Chem Lett* **18**: 5941-5944.

Church, D. M., L. Goodstadt, L. W. Hillier, M. C. Zody, S. Goldstein, X. She, C. J. Bult, R. Agarwala, J. L. Cherry, M. DiCuccio, W. Hlavina, Y. Kapustin, P. Meric, D. Maglott, Z. Birtle, A. C. Marques, T. Graves, S. Zhou, B. Teague, K. Potamouisis, C. Churas, M. Place, J. Herschleb, R. Runnheim, D. Forrest, J. Amos-Landgraf, D. C. Schwartz, Z. Cheng, K. Lindblad-Toh, E. E. Eichler & C. P. Ponting, (2009) Lineage-specific biology revealed by a finished genome assembly of the mouse. *PLoS Biol* **7**: e1000112.

Cibulskis, R. E., D. Bell, E. M. Christophel, J. Hii, C. Delacollette, N. Bakayita & M. W. Aregawi, (2007) Estimating trends in the burden of malaria at country level. *Am J Trop Med Hyg* **77**: 133-137.

Clark, J. T., R. Anand, T. Akoglu & J. S. McBride, (1987) Identification and characterisation of proteins associated with the rhoptry organelles of *Plasmodium falciparum* merozoites. *Parasitol Res* **73**: 425-434.

Collins, C. R., C. Withers-Martinez, F. Hackett & M. J. Blackman, (2009) An inhibitory antibody blocks interactions between components of the malarial invasion machinery. *PLoS Pathog* **5**: e1000273.

Coluzzi, M., Bradley, D., (1999) The Malaria Challenge After One Hundred Years of Malariology. In: Parassitologia. Rome: La Sapienza, University of Rome, pp.

Combe, A., D. Giovannini, T. G. Carvalho, S. Spath, B. Boisson, C. Loussert, S. Thiberge, C. Lacroix, P. Gueirard & R. Menard, (2009) Clonal conditional mutagenesis in malaria parasites. *Cell Host Microbe* **5**: 386-396.

Coppel, R. L., P. E. Crewther, J. G. Culvenor, L. H. Perrin, G. V. Brown, D. J. Kemp & R. F. Anders, (1988) Variation in p126, a parasitophorous vacuole antigen of *Plasmodium falciparum*. *Mol Biol Med* **5**: 155-166.

Coppi, A., C. Pinzon-Ortiz, C. Hutter & P. Sinnis, (2005) The *Plasmodium* circumsporozoite protein is proteolytically processed during cell invasion. *J Exp Med* **201**: 27-33.

Cox-Singh, J., T. M. Davis, K. S. Lee, S. S. Shamsul, A. Matusop, S. Ratnam, H. A. Rahman, D. J. Conway & B. Singh, (2008) *Plasmodium knowlesi* malaria in humans is

widely distributed and potentially life threatening. *Clin Infect Dis* **46**: 165-171.

Cox, F. E., (2002) History of human parasitology. *Clin Microbiol Rev* **15**: 595-612.

Crabb, B. S. & A. F. Cowman, (1996) Characterization of promoters and stable transfection by homologous and nonhomologous recombination in *Plasmodium falciparum*. *Proc Natl Acad Sci U S A* **93**: 7289-7294.

Crabb, B. S., T. Triglia, J. G. Waterkeyn & A. F. Cowman, (1997) Stable transgene expression in *Plasmodium falciparum*. *Mol Biochem Parasitol* **90**: 131-144.

Culvenor, J. G., K. P. Day & R. F. Anders, (1991) *Plasmodium falciparum* ring-infected erythrocyte surface antigen is released from merozoite dense granules after erythrocyte invasion. *Infect Immun* **59**: 1183-1187.

Da Silva, E., M. Foley, A. R. Dluzewski, L. J. Murray, R. F. Anders & L. Tilley, (1994) The *Plasmodium falciparum* protein RESA interacts with the erythrocyte cytoskeleton and modifies erythrocyte thermal stability. *Mol Biochem Parasitol* **66**: 59-69.

Damasceno, J. D., S. M. Beverley & L. R. Tosi, (2010) A transposon toolkit for gene transfer and mutagenesis in protozoan parasites. *Genetica* **138**: 301-311.

Dasaradhi, P. V., A. Mohammed, A. Kumar, M. J. Hossain, R. K. Bhatnagar, V. S. Chauhan & P. Malhotra, (2005) A role of falcipain-2, principal cysteine proteases of *Plasmodium falciparum* in merozoite egression. *Biochem Biophys Res Commun* **336**: 1062-1068.

de Koning-Ward, T. F., D. A. Fidock, V. Thathy, R. Menard, R. M. van Spaendonk, A. P. Waters & C. J. Janse, (2000) The selectable marker human dihydrofolate reductase enables sequential genetic manipulation of the *Plasmodium berghei* genome. *Mol Biochem Parasitol* **106**: 199-212.

de Koning-Ward, T. F., P. R. Gilson, J. A. Boddey, M. Rug, B. J. Smith, A. T. Papenfuss, P. R. Sanders, R. J. Lundie, A. G. Maier, A. F. Cowman & B. S. Crabb, (2009) A newly discovered protein export machine in malaria parasites. *Nature* **459**: 945-949.

Debrabant, A. & P. Delplace, (1989) Leupeptin alters the proteolytic processing of P126, the major parasitophorous vacuole antigen of *Plasmodium falciparum*. *Mol Biochem Parasitol* **33**: 151-158.

Dejkriengkraikhul, P. & P. Wilairat, (1983) Requirement of malarial protease in the invasion of human red cells by merozoites of *Plasmodium falciparum*. *Z Parasitenkd* **69**: 313-317.

Delplace, P., A. Bhatia, M. Cagnard, D. Camus, G. Colombet, A. Debrabant, J. F. Dubremetz, N. Dubreuil, G. Prensier, B. Fortier & et al., (1988) Protein p126: a parasitophorous vacuole antigen associated with the release of *Plasmodium falciparum* merozoites. *Biology of the cell / under the auspices of the European Cell Biology Organization* **64**: 215-221.

Delplace, P., B. Fortier, G. Tronchin, J. F. Dubremetz & A. Vernes, (1987) Localization, biosynthesis, processing and isolation of a major 126 kDa antigen of the parasitophorous vacuole of *Plasmodium falciparum*. *Mol Biochem Parasitol* **23**: 193-201.

Desimone, T. M., C. V. Jennings, A. K. Bei, C. Comeaux, B. I. Coleman, P. Refour, T. Triglia, J. Stubbs, A. F. Cowman & M. T. Duraisingh, (2009) Cooperativity between *Plasmodium falciparum* adhesive proteins for invasion into erythrocytes. *Molecular microbiology*.

Di Cristina, M., F. Ghouze, C. H. Kocken, S. Naitza, P. Cellini, D. Soldati, A. W. Thomas & A. Crisanti, (1999) Transformed *Toxoplasma gondii* tachyzoites expressing the circumsporozoite protein of *Plasmodium knowlesi* elicit a specific immune response in rhesus monkeys. *Infect Immun* **67**: 1677-1682.

Dix, M. M., G. M. Simon & B. F. Cravatt, (2008) Global mapping of the topography and magnitude of proteolytic events in apoptosis. *Cell* **134**: 679-691.

Dluzewski, A. R. & C. R. Garcia, (1996) Inhibition of invasion and intraerythrocytic development of *Plasmodium falciparum* by kinase inhibitors. *Experientia* **52**: 621-623.

Dluzewski, A. R., I. T. Ling, K. Rangachari, P. A. Bates & R. J. Wilson, (1984) A simple method for isolating viable mature parasites of *Plasmodium falciparum* from cultures. *Trans R Soc Trop Med Hyg* **78**: 622-624.

Dluzewski, A. R., K. Rangachari, R. J. Wilson & W. B. Gratzer, (1986) *Plasmodium falciparum*: protease inhibitors and inhibition of erythrocyte invasion. *Exp Parasitol* **62**: 416-422.

Dondorp, A. M., F. Nosten, P. Yi, D. Das, A. P. Phyto, J. Tarning, K. M. Lwin, F. Ariey, W. Hanpithakpong, S. J. Lee, P. Ringwald, K. Silamut, M. Imwong, K. Chotivanich, P. Lim, T. Herdman, S. S. An, S. Yeung, P. Singhasivanon, N. P. Day, N. Lindegardh, D. Socheat & N. J. White, (2009) Artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med* **361**: 455-467.

Dowse, T. J. & D. Soldati, (2005) Rhomboid-like proteins in Apicomplexa: phylogeny and nomenclature. *Trends Parasitol* **21**: 254-258.

Drew, M. E., R. Banerjee, E. W. Uffman, S. Gilbertson, P. J. Rosenthal & D. E. Goldberg, (2008) *Plasmodium* food vacuole plasmepsins are activated by falcipains. *J Biol Chem* **283**: 12870-12876.

Dua, M., P. Raphael, P. S. Sijwali, P. J. Rosenthal & M. Hanspal, (2001) Recombinant falcipain-2 cleaves erythrocyte membrane ankyrin and protein 4.1. *Mol Biochem Parasitol* **116**: 95-99.

Dubois, P. & L. Pereira da Silva, (1995) Towards a vaccine against asexual blood stage infection by *Plasmodium falciparum*. *Res Immunol* **146**: 263-275.

Dubremetz, J. F. & C. Dissous, (1980) Characteristic proteins of micronemes and dense granules from *Sarcocystis tenella* zoites (Protozoa, Coccidia). *Mol Biochem Parasitol* **1**: 279-289.

Duncan, C., (2009). In.: University of Oxford, pp.

Duraisingh, M. T., T. Triglia & A. F. Cowman, (2002) Negative selection of *Plasmodium falciparum* reveals targeted gene deletion by double crossover recombination. *International journal for parasitology* **32**: 81-89.

Duraisingh, M. T., T. Triglia, S. A. Ralph, J. C. Rayner, J. W. Barnwell, G. I. McFadden & A. F. Cowman, (2003) Phenotypic variation of *Plasmodium falciparum* merozoite proteins directs receptor targeting for invasion of human erythrocytes. *The EMBO journal* **22**: 1047-1057.

Duval, L., E. Nerrienet, D. Rousset, S. A. Sadeuh Mba, S. Houze, M. Fourment, J. Le Bras, V. Robert & F. Ariey, (2009) Chimpanzee malaria parasites related to *Plasmodium ovale* in Africa. *PLoS One* **4**: e5520.

Dvorak, J. A., L. H. Miller, W. C. Whitehouse & T. Shiroishi, (1975) Invasion of erythrocytes by malaria merozoites. *Science* **187**: 748-750.

Dvorin, J. D., D. C. Martyn, S. D. Patel, J. S. Grimley, C. R. Collins, C. S. Hopp, A. T. Bright, S. Westenberger, E. Winzeler, M. J. Blackman, D. A. Baker, T. J. Wandless & M. T. Duraisingh, (2010) A plant-like kinase in *Plasmodium falciparum* regulates parasite egress from erythrocytes. *Science* **328**: 910-912.

Ecker, A., E. S. Bushell, R. Tewari & R. E. Sinden, (2008) Reverse genetics screen identifies six proteins important for malaria development in the mosquito. *Molecular microbiology* **70**: 209-220.

Ecker, A., S. B. Pinto, K. W. Baker, F. C. Kafatos & R. E. Sinden, (2007) *Plasmodium berghei*: plasmodium perforin-like protein 5 is required for mosquito midgut invasion in *Anopheles stephensi*. *Exp Parasitol* **116**: 504-508.

Ecker, A., Tewari, R., (Personal communication). In., pp.

Eggleston, K. K., K. L. Duffin & D. E. Goldberg, (1999) Identification and characterization of falcilysin, a metallopeptidase involved in hemoglobin catabolism within the malaria parasite *Plasmodium falciparum*. *J Biol Chem* **274**: 32411-32417.

Eksi, S., B. Czesny, G. J. van Gemert, R. W. Sauerwein, W. Eling & K. C. Williamson, (2007) Inhibition of *Plasmodium falciparum* oocyst production by membrane-permeant cysteine protease inhibitor E64d. *Antimicrob Agents Chemother* **51**: 1064-1070.

Endo, T., K. K. Sethi & G. Piekarski, (1982) *Toxoplasma gondii*: calcium ionophore A23187-mediated exit of trophozoites from infected murine macrophages. *Exp Parasitol* **53**: 179-188.

Epp, C., D. Raskolnikov & K. W. Deitsch, (2008) A regulatable transgene expression system for cultured *Plasmodium falciparum* parasites. *Malar J* **7**: 86.

Etzion, Z., M. C. Murray & M. E. Perkins, (1991) Isolation and characterization of rhoptries of *Plasmodium falciparum*. *Mol Biochem Parasitol* **47**: 51-61.

Fairhead, M., K. A. Johnson, T. Kowatz, S. A. McMahon, L. G. Carter, M. Oke, H. Liu, J. H. Naismith & C. F. van der Walle, (2008) Crystal structure and silica condensing activities of

silicatein alpha-cathepsin L chimeras. *Chem Commun (Camb)*: 1765-1767.

Fang, X. D., D. C. Kaslow, J. H. Adams & L. H. Miller, (1991) Cloning of the *Plasmodium vivax* Duffy receptor. *Mol Biochem Parasitol* **44**: 125-132.

Feinberg, A. P. & B. Vogelstein, (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132**: 6-13.

Felber, L. M., S. M. Cloutier, C. Kundig, T. Kishi, V. Brossard, P. Jichlinski, H. J. Leisinger & D. Deperthes, (2004) Evaluation of the CFP-substrate-YFP system for protease studies: advantages and limitations. *Biotechniques* **36**: 878-885.

Feng, X., D. E. Akiyoshi, G. Widmer & S. Tzipori, (2007) Characterization of subtilase protease in *Cryptosporidium parvum* and *C. hominis*. *J Parasitol* **93**: 619-626.

Fidock, D. A., T. Nomura, A. K. Talley, R. A. Cooper, S. M. Dzekunov, M. T. Ferdig, L. M. Ursos, A. B. Sidhu, B. Naude, K. W. Deitsch, X. Z. Su, J. C. Wootton, P. D. Roepe & T. E. Wellems, (2000) Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol Cell* **6**: 861-871.

Fidock, D. A. & T. E. Wellems, (1997) Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. *Proc Natl Acad Sci U S A* **94**: 10931-10936.

Fitch, C. D. & P. Kanjanangulpan, (1987) The state of ferriprotoporphyrin IX in malaria pigment. *J Biol Chem* **262**: 15552-15555.

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

Florent, I., Z. Derhy, M. Allary, M. Monsigny, R. Mayer & J. Schrevel, (1998) A *Plasmodium falciparum* aminopeptidase gene belonging to the M1 family of zinc-metalloproteinases is expressed in erythrocytic stages. *Mol Biochem Parasitol* **97**: 149-160.

Förster, T., (1948) Intermolecular energy migration and fluorescence. *Ann Physik (Leipzig)* **2**: 55-75.

Foth, B. J., N. Zhang, S. Mok, P. R. Preiser & Z. Bozdech, (2008) Quantitative protein expression profiling reveals extensive post-transcriptional regulation and post-translational modifications in schizont-stage malaria parasites. *Genome Biol* **9**: R177.

Fox, B. A., P. Xing-Li, K. Suzue, T. Horii & D. J. Bzik, (1997) *Plasmodium falciparum*: an epitope within a highly conserved region of the 47-kDa amino-terminal domain of the serine repeat antigen is a target of parasite-inhibitory antibodies. *Exp Parasitol* **85**: 121-134.

Francis, S. E., D. J. Sullivan, Jr. & D. E. Goldberg, (1997) Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. *Annu Rev Microbiol* **51**: 97-123.

Frangioni, J. V. & B. G. Neel, (1993) Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. *Anal Biochem* **210**: 179-187.

Fugere, M., P. C. Limperis, V. Beaulieu-Audy, F. Gagnon, P. Lavigne, K. Klarskov, R. Leduc & R. Day, (2002) Inhibitory potency and specificity of subtilase-like pro-protein convertase (SPC) prodomains. *J Biol Chem* **277**: 7648-7656.

Furmonaviciene, R., A. M. Ghaemmaghami, S. E. Boyd, N. S. Jones, K. Bailey, A. C. Willis, H. F. Sewell, D. A. Mitchell & F. Shakib, (2007) The protease allergen Der p 1 cleaves cell surface DC-SIGN and DC-SIGNR: experimental analysis of in silico substrate identification and implications in allergic responses. *Clin Exp Allergy* **37**: 231-242.

Galinski, M. R., C. C. Medina, P. Ingravallo & J. W. Barnwell, (1992) A reticulocyte-binding protein complex of *Plasmodium vivax* merozoites. *Cell* **69**: 1213-1226.

Gallup, J. L. & J. D. Sachs, (2001) The economic burden of malaria. *Am J Trop Med Hyg* **64**: 85-96.

Gamo, F. J., L. M. Sanz, J. Vidal, C. de Cozar, E. Alvarez, J. L. Lavandera, D. E. Vanderwall, D. V. Green, V. Kumar, S. Hasan, J. R. Brown, C. E. Peishoff, L. R. Cardon & J. F. Garcia-Bustos, (2010) Thousands of chemical starting points for antimalarial lead identification. *Nature* **465**: 305-310.

Gantt, S. M., J. M. Myung, M. R. Briones, W. D. Li, E. J. Corey, S. Omura, V. Nussenzweig & P. Sinnis, (1998) Proteasome inhibitors block development of *Plasmodium* spp. *Antimicrob Agents Chemother* **42**: 2731-2738.

Garcia, J., H. Curtidor, C. G. Pinzon, M. Vanegas, A. Moreno & M. E. Patarroyo, (2009) Identification of conserved erythrocyte binding regions in members of the *Plasmodium falciparum* Cys6 lipid raft-associated protein family. *Vaccine* **27**: 3953-3962.

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

Gehde, N., C. Hinrichs, I. Montilla, S. Charpian, K. Lingelbach & J. M. Przyborski, (2008) Protein unfolding is an essential requirement for transport across the parasitophorous vacuolar membrane of *Plasmodium falciparum*. *Molecular microbiology*.

Gelhaus, C., R. Vicik, T. Schirmeister & M. Leippe, (2005) Blocking effect of a biotinylated protease inhibitor on the egress of *Plasmodium falciparum* merozoites from infected red blood cells. *Biol Chem* **386**: 499-502.

Gilson, P. R. & B. S. Crabb, (2009) Morphology and kinetics of the three distinct phases of red blood cell invasion by *Plasmodium falciparum* merozoites. *International journal for parasitology* **39**: 91-96.

Gilson, P. R., T. Nebl, D. Vukcevic, R. L. Moritz, T. Sargeant, T. P. Speed, L. Schofield & B. S. Crabb, (2006) Identification and stoichiometry of glycosylphosphatidylinositol-anchored membrane proteins of the human malaria parasite *Plasmodium falciparum*. *Mol Cell Proteomics* **5**: 1286-1299.

Glushakova, S., G. Humphrey, E. Leikina, A. Balaban, J. Miller & J. Zimmerberg, (2010) New Stages in the Program of Malaria Parasite Egress Imaged in Normal and Sickle Erythrocytes. *Curr Biol*.

Glushakova, S., J. Mazar, M. F. Hohmann-Marriott, E. Hama & J. Zimmerberg, (2008) Irreversible effect of cysteine protease inhibitors on the release of malaria parasites from infected erythrocytes. *Cell Microbiol*.

Glushakova, S., D. Yin, T. Li & J. Zimmerberg, (2005) Membrane transformation during malaria parasite release from human red blood cells. *Curr Biol* **15**: 1645-1650.

Gluzman, I. Y., S. E. Francis, A. Oksman, C. E. Smith, K. L. Duffin & D. E. Goldberg, (1994) Order and specificity of the *Plasmodium falciparum* hemoglobin degradation pathway. *J Clin Invest* **93**: 1602-1608.

Goel, V. K., X. Li, H. Chen, S. C. Liu, A. H. Chishti & S. S. Oh, (2003) Band 3 is a host receptor binding merozoite surface protein 1 during the *Plasmodium falciparum* invasion of erythrocytes. *Proc Natl Acad Sci U S A* **100**: 5164-5169.

Goldberg, D. E., A. F. Slater, R. Beavis, B. Chait, A. Cerami & G. B. Henderson, (1991) Hemoglobin degradation in the human malaria pathogen *Plasmodium falciparum*: a catabolic pathway initiated by a specific aspartic protease. *J Exp Med* **173**: 961-969.

Goldberg, D. E., A. F. Slater, A. Cerami & G. B. Henderson, (1990) Hemoglobin degradation in the malaria parasite *Plasmodium falciparum*: an ordered process in a unique organelle. *Proc Natl Acad Sci U S A* **87**: 2931-2935.

Golubkov, V. S., S. Boyd, A. Y. Savinov, A. V. Chekanov, A. L. Osterman, A. Remacle, D. V. Rozanov, S. J. Doxsey & A. Y. Strongin, (2005) Membrane type-1 matrix metalloproteinase (MT1-MMP) exhibits an important intracellular cleavage function and causes chromosome instability. *J Biol Chem* **280**: 25079-25086.

Gossen, M. & H. Bujard, (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A* **89**: 5547-5551.

Gossen, M., S. Freundlieb, G. Bender, G. Muller, W. Hillen & H. Bujard, (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science* **268**: 1766-1769.

Greenbaum, D. C., A. Baruch, M. Grainger, Z. Bozdech, K. F. Medzihradzsky, J. Engel, J. DeRisi, A. A. Holder & M. Bogoy, (2002) A role for the protease falcipain 1 in host cell invasion by the human malaria parasite. *Science* **298**: 2002-2006.

Groux, H. & J. Gysin, (1990) Opsonization as an effector mechanism in human protection

against asexual blood stages of *Plasmodium falciparum*: functional role of IgG subclasses. *Res Immunol* **141**: 529-542.

Guiguemde, W. A., A. A. Shelat, D. Bouck, S. Duffy, G. J. Crowther, P. H. Davis, D. C. Smithson, M. Connelly, J. Clark, F. Zhu, M. B. Jimenez-Diaz, M. S. Martinez, E. B. Wilson, A. K. Tripathi, J. Gut, E. R. Sharlow, I. Bathurst, F. El Mazouni, J. W. Fowble, I. Forquer, P. L. McGinley, S. Castro, I. Angulo-Barturen, S. Ferrer, P. J. Rosenthal, J. L. Derisi, D. J. Sullivan, J. S. Lazo, D. S. Roos, M. K. Riscoe, M. A. Phillips, P. K. Rathod, W. C. Van Voorhis, V. M. Avery & R. K. Guy, (2010) Chemical genetics of *Plasmodium falciparum*. *Nature* **465**: 311-315.

Hackett, F., M. Sajid, C. Withers-Martinez, M. Grainger & M. J. Blackman, (1999) PfSUB-2: a second subtilisin-like protein in *Plasmodium falciparum* merozoites. *Mol Biochem Parasitol* **103**: 183-195.

Hadley, T., M. Aikawa & L. H. Miller, (1983) *Plasmodium knowlesi*: studies on invasion of rhesus erythrocytes by merozoites in the presence of protease inhibitors. *Exp Parasitol* **55**: 306-311.

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

Hannig, J., J. Yu, M. Beckett, R. Weichselbaum & R. C. Lee, (1999) Poloxamine 1107 sealing of radiopermeabilized erythrocyte membranes. *Int J Radiat Biol* **75**: 379-385.

Hanspal, M., M. Dua, Y. Takakuwa, A. H. Chishti & A. Mizuno, (2002) *Plasmodium falciparum* cysteine protease falcipain-2 cleaves erythrocyte membrane skeletal proteins at late stages of parasite development. *Blood* **100**: 1048-1054.

Harnyuttanakorn, P., J. S. McBride, S. Donachie, H. G. Heidrich & R. G. Ridley, (1992) Inhibitory monoclonal antibodies recognise epitopes adjacent to a proteolytic cleavage site on the RAP-1 protein of *Plasmodium falciparum*. *Mol Biochem Parasitol* **55**: 177-186.

Harris, P. K., S. Yeoh, A. R. Dluzewski, R. A. O'Donnell, C. Withers-Martinez, F. Hackett, L. H. Bannister, G. H. Mitchell & M. J. Blackman, (2005) Molecular identification of a malaria merozoite surface sheddase. *PLoS Pathog* **1**: 241-251.

Hatanaka, M., N. Yoshimura, T. Murakami, R. Kannagi & T. Murachi, (1984) Evidence for membrane-associated calpain I in human erythrocytes. Detection by an immunoelectrophoretic blotting method using monospecific antibody. *Biochemistry* **23**: 3272-3276.

Hawass, Z., Y. Z. Gad, S. Ismail, R. Khairat, D. Fathalla, N. Hasan, A. Ahmed, H. Elleithy, M. Ball, F. Gaballah, S. Wasef, M. Fateen, H. Amer, P. Gostner, A. Selim, A. Zink & C. M. Pusch, (2010) Ancestry and pathology in King Tutankhamun's family. *JAMA* **303**: 638-647.

Hayakawa, T., N. Arisue, T. Udono, H. Hirai, J. Sattabongkot, T. Toyama, T. Tsuboi, T. Horii & K. Tanabe, (2009) Identification of *Plasmodium malariae*, a human malaria parasite, in imported chimpanzees. *PLoS One* **4**: e7412.

Hehl, A. B., C. Lekutis, M. E. Grigg, P. J. Bradley, J. F. Dubremetz, E. Ortega-Barria & J. C. Boothroyd, (2000) Toxoplasma gondii homologue of plasmodium apical membrane antigen 1 is involved in invasion of host cells. *Infect Immun* **68**: 7078-7086.

Herm-Gotz, A., C. Agop-Nersesian, S. Munter, J. S. Grimley, T. J. Wandless, F. Frischknecht & M. Meissner, (2007) Rapid control of protein level in the apicomplexan Toxoplasma gondii. *Nat Methods*.

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

Hodder, A. N., D. R. Drew, V. C. Epa, M. Delorenzi, R. Bourgon, S. K. Miller, R. L. Moritz, D. F. Frecklington, R. J. Simpson, T. P. Speed, R. N. Pike & B. S. Crabb, (2003) Enzymic, phylogenetic, and structural characterization of the unusual papain-like protease domain of *Plasmodium falciparum* SERA5. *J Biol Chem* **278**: 48169-48177.

Hodder, A. N., R. L. Malby, O. B. Clarke, W. D. Fairlie, P. M. Colman, B. S. Crabb & B. J.

Smith, (2009) Structural Insights into the Protease-like Antigen *Plasmodium falciparum* SERA5 and Its Noncanonical Active-Site Serine. *J Mol Biol.*

Hoffman, S. L., L. M. Goh, T. C. Luke, I. Schneider, T. P. Le, D. L. Doolan, J. Sacci, P. de la Vega, M. Dowler, C. Paul, D. M. Gordon, J. A. Stoute, L. W. Church, M. Sedegah, D. G. Heppner, W. R. Ballou & T. L. Richie, (2002) Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. *J Infect Dis* **185**: 1155-1164.

Holder, A. A. & R. R. Freeman, (1982) Biosynthesis and processing of a *Plasmodium falciparum* schizont antigen recognized by immune serum and a monoclonal antibody. *J Exp Med* **156**: 1528-1538.

Holder, A. A., M. J. Lockyer, K. G. Odink, J. S. Sandhu, V. Riveros-Moreno, S. C. Nicholls, Y. Hillman, L. S. Davey, M. L. Tizard, R. T. Schwarz & et al., (1985) Primary structure of the precursor to the three major surface antigens of *Plasmodium falciparum* merozoites. *Nature* **317**: 270-273.

Holt, R. A., G. M. Subramanian, A. Halpern, G. G. Sutton, R. Charlab, D. R. Nusskern, P. Wincker, A. G. Clark, J. M. Ribeiro, R. Wides, S. L. Salzberg, B. Loftus, M. Yandell, W. H. Majoros, D. B. Rusch, Z. Lai, C. L. Kraft, J. F. Abril, V. Anthouard, P. Arensburger, P. W. Atkinson, H. Baden, V. de Berardinis, D. Baldwin, V. Benes, J. Biedler, C. Blass, R. Bolanos, D. Boscus, M. Barnstead, S. Cai, A. Center, K. Chaturverdi, G. K. Christophides, M. A. Chrystal, M. Clamp, A. Cravchik, V. Curwen, A. Dana, A. Delcher, I. Dew, C. A. Evans, M. Flanigan, A. Grundschober-Freimoser, L. Friedli, Z. Gu, P. Guan, R. Guigo, M. E. Hillenmeyer, S. L. Hladun, J. R. Hogan, Y. S. Hong, J. Hoover, O. Jaillon, Z. Ke, C. Kodira, E. Kokoza, A. Koutsos, I. Letunic, A. Levitsky, Y. Liang, J. J. Lin, N. F. Lobo, J. R. Lopez, J. A. Malek, T. C. McIntosh, S. Meister, J. Miller, C. Mobarry, E. Mongin, S. D. Murphy, D. A. O'Brochta, C. Pfannkoch, R. Qi, M. A. Regier, K. Remington, H. Shao, M. V. Sharakhova, C. D. Sitter, J. Shetty, T. J. Smith, R. Strong, J. Sun, D. Thomasova, L. Q. Ton, P. Topalis, Z. Tu, M. F. Unger, B. Walenz, A. Wang, J. Wang, M. Wang, X. Wang, K. J. Woodford, J. R. Wortman, M. Wu, A. Yao, E. M. Zdobnov, H. Zhang, Q. Zhao, et al., (2002) The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science* **298**: 129-149.

Hotta, C. T., M. L. Gazarini, F. H. Beraldo, F. P. Varotti, C. Lopes, R. P. Markus, T. Pozzan & C. R. Garcia, (2000) Calcium-dependent modulation by melatonin of the circadian rhythm in malarial parasites. *Nat Cell Biol* **2**: 466-468.

Howard, R. F., D. L. Narum, M. Blackman & J. Thurman, (1998) Analysis of the processing of *Plasmodium falciparum* rhoptry-associated protein 1 and localization of Pr86 to schizont rhoptries and p67 to free merozoites. *Mol Biochem Parasitol* **92**: 111-122.

Huang, Y., F. Qiao, R. Abagyan, S. Hazard & S. Tomlinson, (2006) Defining the CD59-C9 binding interaction. *J Biol Chem* **281**: 27398-27404.

Huitema, C. & L. D. Eltis, (2010) A fluorescent protein-based biological screen of proteinase activity. *J Biomol Screen* **15**: 224-229.

Hurwitz, E. S., D. Johnson & C. C. Campbell, (1981) Resistance of *Plasmodium falciparum* malaria to sulfadoxine-pyrimethamine ('Fansidar') in a refugee camp in Thailand. *Lancet* **1**: 1068-1070.

Ishino, T., Y. Chinzei & M. Yuda, (2005) A *Plasmodium* sporozoite protein with a membrane attack complex domain is required for breaching the liver sinusoidal cell layer prior to hepatocyte infection. *Cell Microbiol* **7**: 199-208.

Ishino, T., K. Yano, Y. Chinzei & M. Yuda, (2004) Cell-passage activity is required for the malarial parasite to cross the liver sinusoidal cell layer. *PLoS Biol* **2**: E4.

Iwanaga, S., S. M. Khan, I. Kaneko, Z. Christodoulou, C. Newbold, M. Yuda, C. J. Janse & A. P. Waters, (2010) Functional identification of the *Plasmodium* centromere and generation of a *Plasmodium* artificial chromosome. *Cell Host Microbe* **7**: 245-255.

Jamjoom, G. A., (1988) Formation and role of malaria pigment. *Rev Infect Dis* **10**: 1029-1034.

Janse, C. J., J. Ramesar & A. P. Waters, (2006) High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite *Plasmodium berghei*. *Nat Protoc* **1**: 346-356.

Janse, C. J. & A. P. Waters, (2007) The exoneme helps malaria parasites to break out of

blood cells. *Cell* **131**: 1036-1038.

Jean, L., F. Hackett, S. R. Martin & M. J. Blackman, (2003) Functional characterization of the propeptide of *Plasmodium falciparum* subtilisin-like protease-1. *J Biol Chem* **278**: 28572-28579.

Jimenez-Diaz, M. B., T. Mulet, S. Viera, V. Gomez, H. Garuti, J. Ibanez, A. Alvarez-Doval, L. D. Shultz, A. Martinez, D. Gargallo-Viola & I. Angulo-Barturen, (2009) Improved murine model of malaria using *Plasmodium falciparum* competent strains and non-myelodepleted NOD-scid IL2Rgammanull mice engrafted with human erythrocytes. *Antimicrob Agents Chemother* **53**: 4533-4536.

Julius, D., A. Brake, L. Blair, R. Kunisawa & J. Thorner, (1984) Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-alpha-factor. *Cell* **37**: 1075-1089.

Kadekoppala, M., R. A. O'Donnell, M. Grainger, B. S. Crabb & A. A. Holder, (2008) Deletion of the *Plasmodium falciparum* merozoite surface protein 7 gene impairs parasite invasion of erythrocytes. *Eukaryotic cell* **7**: 2123-2132.

Kadekoppala, M., S. A. Ogun, S. Howell, R. S. Gunaratne & A. A. Holder, (2010) Systematic genetic analysis of the *Plasmodium falciparum* MSP7-like family reveals differences in protein expression, location, and importance in asexual growth of the blood-stage parasite. *Eukaryotic cell* **9**: 1064-1074.

Kadota, K., T. Ishino, T. Matsuyama, Y. Chinzei & M. Yuda, (2004) Essential role of membrane-attack protein in malarial transmission to mosquito host. *Proc Natl Acad Sci U S A* **101**: 16310-16315.

Kafsack, B. F. & V. B. Carruthers, (2010) Apicomplexan perforin-like proteins. *Commun Integr Biol* **3**: 18-23.

Kafsack, B. F., J. D. Pena, I. Coppens, S. Ravindran, J. C. Boothroyd & V. B. Carruthers, (2008) Rapid Membrane Disruption by a Perforin-Like Protein Facilitates Parasite Exit from Host Cells. *Science*.

Kagi, D., B. Ledermann, K. Burki, H. Hengartner & R. M. Zinkernagel, (1994a) CD8+ T cell-mediated protection against an intracellular bacterium by perforin-dependent cytotoxicity. *Eur J Immunol* **24**: 3068-3072.

Kagi, D., B. Ledermann, K. Burki, P. Seiler, B. Odermatt, K. J. Olsen, E. R. Podack, R. M. Zinkernagel & H. Hengartner, (1994b) Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* **369**: 31-37.

Kaiser, K., N. Camargo, I. Coppens, J. M. Morrissey, A. B. Vaidya & S. H. Kappe, (2004) A member of a conserved *Plasmodium* protein family with membrane-attack complex/perforin (MACPF)-like domains localizes to the micronemes of sporozoites. *Mol Biochem Parasitol* **133**: 15-26.

Kakhniashvili, D. G., L. A. Bulla, Jr. & S. R. Goodman, (2004) The human erythrocyte proteome: analysis by ion trap mass spectrometry. *Mol Cell Proteomics* **3**: 501-509.

Kalume, D. E., S. Peri, R. Reddy, J. Zhong, M. Okulate, N. Kumar & A. Pandey, (2005) Genome annotation of *Anopheles gambiae* using mass spectrometry-derived data. *BMC Genomics* **6**: 128.

Kaneko, O., B. Y. Yim Lim, H. Iriko, I. T. Ling, H. Otsuki, M. Grainger, T. Tsuboi, J. H. Adams, D. Mattei, A. A. Holder & M. Torii, (2005) Apical expression of three RhopH1/Clag proteins as components of the *Plasmodium falciparum* RhopH complex. *Mol Biochem Parasitol* **143**: 20-28.

Kappe, S. H., C. A. Buscaglia & V. Nussenzweig, (2004) *Plasmodium* sporozoite molecular cell biology. *Annu Rev Cell Dev Biol* **20**: 29-59.

Kato, N., T. Sakata, G. Breton, K. G. Le Roch, A. Nagle, C. Andersen, B. Bursulaya, K. Henson, J. Johnson, K. A. Kumar, F. Marr, D. Mason, C. McNamara, D. Plouffe, V. Ramachandran, M. Spooner, T. Tuntland, Y. Zhou, E. C. Peters, A. Chatterjee, P. G. Schultz, G. E. Ward, N. Gray, J. Harper & E. A. Winzeler, (2008) Gene expression signatures and small-molecule compounds link a protein kinase to *Plasmodium falciparum* motility. *Nat Chem Biol* **4**: 347-356.

Kauth, C. W., C. Epp, H. Bujard & R. Lutz, (2003) The merozoite surface protein 1 complex of human malaria parasite *Plasmodium falciparum*: interactions and arrangements of

subunits. *J Biol Chem* **278**: 22257-22264.

Kauth, C. W., U. Woehlbier, M. Kern, Z. Mekonnen, R. Lutz, N. Mucke, J. Langowski & H. Bujard, (2006) Interactions between merozoite surface proteins 1, 6, and 7 of the malaria parasite *Plasmodium falciparum*. *J Biol Chem* **281**: 31517-31527.

Khan, S., (unpublished work). In.: Leiden Malaria Group, pp.

Kim, K. & L. M. Weiss, (2004) *Toxoplasma gondii*: the model apicomplexan. *International journal for parasitology* **34**: 423-432.

Kiszewski, A., A. Mellinger, A. Spielman, P. Malaney, S. E. Sachs & J. Sachs, (2004) A global index representing the stability of malaria transmission. *Am J Trop Med Hyg* **70**: 486-498.

Kitjaroentham, A., T. Suthiphongchai & P. Wilairat, (2006) Effect of metalloprotease inhibitors on invasion of red blood cell by *Plasmodium falciparum*. *Acta Trop* **97**: 5-9.

Kitron, U. & A. Spielman, (1989) Suppression of transmission of malaria through source reduction: antianopheline measures applied in Israel, the United States, and Italy. *Rev Infect Dis* **11**: 391-406.

Klemba, M., W. Beatty, I. Gluzman & D. E. Goldberg, (2004a) Trafficking of plasmepsin II to the food vacuole of the malaria parasite *Plasmodium falciparum*. *J Cell Biol* **164**: 47-56.

Klemba, M., I. Gluzman & D. E. Goldberg, (2004b) A *Plasmodium falciparum* dipeptidyl aminopeptidase I participates in vacuolar hemoglobin degradation. *J Biol Chem* **279**: 43000-43007.

Klemba, M. & D. E. Goldberg, (2005) Characterization of plasmepsin V, a membrane-bound aspartic protease homolog in the endoplasmic reticulum of *Plasmodium falciparum*. *Mol Biochem Parasitol* **143**: 183-191.

Klimpel, K. R., S. S. Molloy, G. Thomas & S. H. Leppla, (1992) Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. *Proc Natl Acad Sci U S A* **89**: 10277-10281.

Knapp, B., E. Hundt, U. Nau & H. A. Kupper, (1989) Molecular cloning, genomic structure and localization in a blood stage antigen of *Plasmodium falciparum* characterized by a serine stretch. *Mol Biochem Parasitol* **32**: 73-83.

Knapp, B., U. Nau, E. Hundt & H. A. Kupper, (1991) A new blood stage antigen of *Plasmodium falciparum* highly homologous to the serine-stretch protein SERP. *Mol Biochem Parasitol* **44**: 1-13.

Knuepfer, E., Holder, A., (unpublished data). In.: The National Institute for Medical Research, pp.

Kocken, C. H., H. Ozwara, A. van der Wel, A. L. Beetsma, J. M. Mwenda & A. W. Thomas, (2002) *Plasmodium knowlesi* provides a rapid *in vitro* and *in vivo* transfection system that enables double-crossover gene knockout studies. *Infect Immun* **70**: 655-660.

Kohl, N. E., E. A. Emini, W. A. Schleif, L. J. Davis, J. C. Heimbach, R. A. Dixon, E. M. Scolnick & I. S. Sigal, (1988) Active human immunodeficiency virus protease is required for viral infectivity. *Proc Natl Acad Sci U S A* **85**: 4686-4690.

Koussis, K., Blackman, M.J., (unpublished data). In.: The National Institute for Medical Research, pp.

Koussis, K., C. Withers-Martinez, S. Yeoh, M. Child, F. Hackett, E. Knuepfer, L. Juliano, U. Woehlbier, H. Bujard & M. J. Blackman, (2009) A multifunctional serine protease primes the malaria parasite for red blood cell invasion. *The EMBO journal*.

Lagal, V., E. M. Binder, M. H. Huynh, B. F. Kafsack, P. K. Harris, R. Diez, D. Chen, R. N. Cole, V. B. Carruthers & K. Kim, (2010) *Toxoplasma gondii* Protease TgSUB1 is Required for Cell Surface Processing of Micronemal Adhesive Complexes and Efficient Adhesion of Tachyzoites. *Cell Microbiol*.

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

Lander, E. S., L. M. Linton, B. Birren, C. Nusbaum, M. C. Zody, J. Baldwin, K. Devon, K. Dewar, M. Doyle, W. FitzHugh, R. Funke, D. Gage, K. Harris, A. Heaford, J. Howland, L. Kann, J. Lehoczký, R. LeVine, P. McEwan, K. McKernan, J. Meldrim, J. P. Mesirov, C. Miranda, W. Morris, J. Naylor, C. Raymond, M. Rosetti, R. Santos, A. Sheridan, C. Sougnez, N. Stange-Thomann, N. Stojanovic, A. Subramanian, D. Wyman, J. Rogers, J.

Sulston, R. Ainscough, S. Beck, D. Bentley, J. Burton, C. Clee, N. Carter, A. Coulson, R. Deadman, P. Deloukas, A. Dunham, I. Dunham, R. Durbin, L. French, D. Grafham, S. Gregory, T. Hubbard, S. Humphray, A. Hunt, M. Jones, C. Lloyd, A. McMurray, L. Matthews, S. Mercer, S. Milne, J. C. Mullikin, A. Mungall, R. Plumb, M. Ross, R. Shownkeen, S. Sims, R. H. Waterston, R. K. Wilson, L. W. Hillier, J. D. McPherson, M. A. Marra, E. R. Mardis, L. A. Fulton, A. T. Chinwalla, K. H. Pepin, W. R. Gish, S. L. Chisoe, M. C. Wendl, K. D. Delehaunty, T. L. Miner, A. Delehaunty, J. B. Kramer, L. L. Cook, R. S. Fulton, D. L. Johnson, P. J. Minx, S. W. Clifton, T. Hawkins, E. Branscomb, P. Predki, P. Richardson, S. Wenning, T. Slezak, N. Doggett, J. F. Cheng, A. Olsen, S. Lucas, C. Elkin, E. Uberbacher, M. Frazier, et al., (2001) Initial sequencing and analysis of the human genome. *Nature* **409**: 860-921.

Langhorne, J., B. Simon-Haarhaus & S. J. Meding, (1990) The role of CD4+ T cells in the protective immune response to *Plasmodium chabaudi* *in vivo*. *Immunol Lett* **25**: 101-107.

Langreth, S. G., J. B. Jensen, R. T. Reese & W. Trager, (1978) Fine structure of human malaria *in vitro*. *J Protozool* **25**: 443-452.

Lauterbach, S. B. & T. L. Coetzer, (2008) The M18 aspartyl aminopeptidase of *Plasmodium falciparum* binds to human erythrocyte spectrin *in vitro*. *Malar J* **7**: 161.

Lazarou, M., J. A. Patino, R. M. Jennings, R. S. McIntosh, J. Shi, S. Howell, E. Cullen, T. Jones, J. R. Adame-Gallegos, J. A. Chappel, J. S. McBride, M. J. Blackman, A. A. Holder & R. J. Pleass, (2009) Inhibition of erythrocyte invasion and *Plasmodium falciparum* merozoite surface protein 1 processing by human immunoglobulin G1 (IgG1) and IgG3 antibodies. *Infect Immun* **77**: 5659-5667.

Le Bonniec, S., C. Deregnaucourt, V. Redeker, R. Banerjee, P. Grellier, D. E. Goldberg & J. Schrevel, (1999) Plasmepsin II, an acidic hemoglobinase from the *Plasmodium falciparum* food vacuole, is active at neutral pH on the host erythrocyte membrane skeleton. *J Biol Chem* **274**: 14218-14223.

Le Roch, K. G., J. R. Johnson, L. Florens, Y. Zhou, A. Santrosyan, M. Grainger, S. F. Yan, K. C. Williamson, A. A. Holder, D. J. Carucci, J. R. Yates, 3rd & E. A. Winzeler, (2004) Global analysis of transcript and protein levels across the *Plasmodium falciparum* life cycle. *Genome Res* **14**: 2308-2318.

Leppa, S. H., (1982) Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. *Proc Natl Acad Sci U S A* **79**: 3162-3166.

Lew, V. L., L. Macdonald, H. Ginsburg, M. Krugliak & T. Tiffert, (2004) Excess haemoglobin digestion by malaria parasites: a strategy to prevent premature host cell lysis. *Blood Cells Mol Dis* **32**: 353-359.

Lew, V. L. & T. Tiffert, (2007) Is invasion efficiency in malaria controlled by pre-invasion events? *Trends Parasitol* **23**: 481-484.

Li, J., T. Mitamura, B. A. Fox, D. J. Bzik & T. Horii, (2002) Differential localization of processed fragments of *Plasmodium falciparum* serine repeat antigen and further processing of its N-terminal 47 kDa fragment. *Parasitol Int* **51**: 343-352.

Li, X., H. Chen, N. Bahamontes-Rosa, J. F. Kun, B. Traore, P. D. Crompton & A. H. Chishti, (2009) *Plasmodium falciparum* signal peptide peptidase is a promising drug target against blood stage malaria. *Biochem Biophys Res Commun* **380**: 454-459.

Li, Y., Z. Hu, F. Jordan & M. Inouye, (1995) Functional analysis of the propeptide of subtilisin E as an intramolecular chaperone for protein folding. Refolding and inhibitory abilities of propeptide mutants. *J Biol Chem* **270**: 25127-25132.

Lin, W., M. Chan & T. S. Sim, (2009) Atypical caseinolytic protease homolog from *Plasmodium falciparum* possesses unusual substrate preference and a functional nuclear localization signal. *Parasitol Res* **105**: 1715-1722.

Louie, K., R. Nordhausen, T. W. Robinson, B. C. Barr & P. A. Conrad, (2002) Characterization of *Neospora caninum* protease, NcSUB1 (NC-P65), with rabbit anti-N54. *J Parasitol* **88**: 1113-1119.

Lu, J., Z. Zhang, J. Yang, J. Chu, P. Li, S. Zeng & Q. Luo, (2007) Visualization of beta-secretase cleavage in living cells using a genetically encoded surface-displayed FRET probe. *Biochem Biophys Res Commun* **362**: 25-30.

Luo, K. Q., V. C. Yu, Y. Pu & D. C. Chang, (2001) Application of the fluorescence resonance energy transfer method for studying the dynamics of caspase-3 activation during UV-induced apoptosis in living HeLa cells. *Biochem Biophys Res Commun* **283**: 1054-1060.

Lustigman, S., R. F. Anders, G. V. Brown & R. L. Coppel, (1988) A component of an antigenic rhoptry complex of *Plasmodium falciparum* is modified after merozoite invasion. *Mol Biochem Parasitol* **30**: 217-224.

Maccallum, M. D., (1897) On the Flagellated Form of the Malaria Parasite. *Lancet* **150**: 1240-1241.

Maier, A. G., J. Baum, B. Smith, D. J. Conway & A. F. Cowman, (2009a) Polymorphisms in erythrocyte binding antigens 140 and 181 affect function and binding but not receptor specificity in *Plasmodium falciparum*. *Infect Immun* **77**: 1689-1699.

Maier, A. G., J. A. Braks, A. P. Waters & A. F. Cowman, (2006) Negative selection using yeast cytosine deaminase/uracil phosphoribosyl transferase in *Plasmodium falciparum* for targeted gene deletion by double crossover recombination. *Mol Biochem Parasitol* **150**: 118-121.

Maier, A. G., B. M. Cooke, A. F. Cowman & L. Tilley, (2009b) Malaria parasite proteins that remodel the host erythrocyte. *Nat Rev Microbiol* **7**: 341-354.

Maier, A. G., M. T. Duraisingh, J. C. Reeder, S. S. Patel, J. W. Kazura, P. A. Zimmerman & A. F. Cowman, (2003) *Plasmodium falciparum* erythrocyte invasion through glycophorin C and selection for Gerbich negativity in human populations. *Nat Med* **9**: 87-92.

Maier, A. G., M. Rug, M. T. O'Neill, M. Brown, S. Chakravorty, T. Szeszak, J. Chesson, Y. Wu, K. Hughes, R. L. Coppel, C. Newbold, J. G. Beeson, A. Craig, B. S. Crabb & A. F. Cowman, (2008) Exported proteins required for virulence and rigidity of *Plasmodium falciparum*-infected human erythrocytes. *Cell* **134**: 48-61.

Malhotra, P., P. V. Dasaradhi, A. Kumar, A. Mohammed, N. Agrawal, R. K. Bhatnagar & V. S. Chauhan, (2002) Double-stranded RNA-mediated gene silencing of cysteine proteases (falcipain-1 and -2) of *Plasmodium falciparum*. *Molecular microbiology* **45**: 1245-1254.

Mamoun, C. B., I. Y. Gluzman, S. Goyard, S. M. Beverley & D. E. Goldberg, (1999) A set of independent selectable markers for transfection of the human malaria parasite *Plasmodium falciparum*. *Proc Natl Acad Sci U S A* **96**: 8716-8720.

Marechal, E. & M. F. Cesbron-Delauw, (2001) The apicoplast: a new member of the plastid family. *Trends Plant Sci* **6**: 200-205.

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

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

Martin, R. E., R. V. Marchetti, A. I. Cowan, S. M. Howitt, S. Broer & K. Kirk, (2009) Chloroquine transport via the malaria parasite's chloroquine resistance transporter. *Science* **325**: 1680-1682.

Mattei, D., G. Langsley, C. Braun-Breton, M. Guillotte, J. F. Dubremetz & O. Mercereau-Pujalon, (1988) The S-antigen of *Plasmodium falciparum* Palo Alto represents a new S-antigen serotype. *Mol Biochem Parasitol* **27**: 171-180.

McCoubrie, J. E., S. K. Miller, T. Sargeant, R. T. Good, A. N. Hodder, T. P. Speed, T. F. de Koning-Ward & B. S. Crabb, (2007) Evidence for a common role for the serine-type *Plasmodium falciparum* serine repeat antigen proteases: implications for vaccine and drug design. *Infect Immun* **75**: 5565-5574.

Meissner, M., S. Brecht, H. Bujard & D. Soldati, (2001) Modulation of myosin A expression by a newly established tetracycline repressor-based inducible system in *Toxoplasma gondii*. *Nucleic Acids Res* **29**: E115.

Meissner, M., E. Krejany, P. R. Gilson, T. F. de Koning-Ward, D. Soldati & B. S. Crabb, (2005) Tetracycline analogue-regulated transgene expression in *Plasmodium falciparum* blood stages using *Toxoplasma gondii* transactivators. *Proc Natl Acad Sci U S A* **102**: 2980-2985.

Mello, K., T. M. Daly, J. Morrissey, A. B. Vaidya, C. A. Long & L. W. Bergman, (2002) A multigene family that interacts with the amino terminus of plasmodium MSP-1 identified using the yeast two-hybrid system. *Eukaryotic cell* **1**: 915-925.

Mercier, C., K. D. Adjogble, W. Daubener & M. F. Delauw, (2005) Dense granules: are they key organelles to help understand the parasitophorous vacuole of all apicomplexa parasites? *International journal for parasitology* **35**: 829-849.

Mercier, C., J. F. Dubremetz, B. Rauscher, L. Lecordier, L. D. Sibley & M. F. Cesbron-Delauw, (2002) Biogenesis of nanotubular network in *Toxoplasma* parasitophorous vacuole induced by parasite proteins. *Mol Biol Cell* **13**: 2397-2409.

Miller, L. H., J. D. Haynes, F. M. McAuliffe, T. Shiroishi, J. R. Durocher & M. H. McGinniss, (1977) Evidence for differences in erythrocyte surface receptors for the malarial parasites, *Plasmodium falciparum* and *Plasmodium knowlesi*. *J Exp Med* **146**: 277-281.

Miller, S. A., E. M. Binder, M. J. Blackman, V. B. Carruthers & K. Kim, (2001) A conserved subtilisin-like protein TgSUB1 in microneme organelles of *Toxoplasma gondii*. *J Biol Chem* **276**: 45341-45348.

Miller, S. K., R. T. Good, D. R. Drew, M. Delorenzi, P. R. Sanders, A. N. Hodder, T. P. Speed, A. F. Cowman, T. F. de Koning-Ward & B. S. Crabb, (2002) A subset of *Plasmodium falciparum* SERA genes are expressed and appear to play an important role in the erythrocytic cycle. *J Biol Chem* **277**: 47524-47532.

Mills, J. P., M. Diez-Silva, D. J. Quinn, M. Dao, M. J. Lang, K. S. Tan, C. T. Lim, G. Milon, P. H. David, O. Mercereau-Puijalon, S. Bonnefoy & S. Suresh, (2007) Effect of plasmodial RESA protein on deformability of human red blood cells harboring *Plasmodium falciparum*. *Proc Natl Acad Sci U S A* **104**: 9213-9217.

Mital, J., M. Meissner, D. Soldati & G. E. Ward, (2005) Conditional expression of *Toxoplasma gondii* apical membrane antigen-1 (TgAMA1) demonstrates that TgAMA1 plays a critical role in host cell invasion. *Mol Biol Cell* **16**: 4341-4349.

Mitchell, G. H., T. J. Hadley, M. H. McGinniss, F. W. Klotz & L. H. Miller, (1986) Invasion of erythrocytes by *Plasmodium falciparum* malaria parasites: evidence for receptor heterogeneity and two receptors. *Blood* **67**: 1519-1521.

Mitra, R. D., C. M. Silva & D. C. Youvan, (1996) Fluorescence resonance energy transfer between blue-emitting and red-shifted excitation derivatives of the green fluorescent protein. *Gene* **173**: 13-17.

Molloy, S. S., P. A. Bresnahan, S. H. Leppla, K. R. Klimpel & G. Thomas, (1992) Human furin is a calcium-dependent serine endoprotease that recognizes the sequence Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen. *J Biol Chem* **267**: 16396-16402.

Mondragon, R. & E. Frixione, (1996) Ca²⁺-dependence of conoid extrusion in *Toxoplasma gondii* tachyzoites. *J Eukaryot Microbiol* **43**: 120-127.

Montero, E. M., L. M. Gonzalez, M. Rodriguez, Y. Oksov, M. J. Blackman & C. A. Lobo, (2006) A conserved subtilisin protease identified in *Babesia divergens* merozoites. *J Biol Chem*.

Morrisette, N. S. & L. D. Sibley, (2002) Cytoskeleton of apicomplexan parasites. *Microbiol Mol Biol Rev* **66**: 21-38; table of contents.

Mota, M. M., J. C. Hafalla & A. Rodriguez, (2002) Migration through host cells activates *Plasmodium* sporozoites for infection. *Nat Med* **8**: 1318-1322.

Mota, M. M., V. Thathy, R. S. Nussenzweig & V. Nussenzweig, (2001) Gene targeting in the rodent malaria parasite *Plasmodium yoelii*. *Mol Biochem Parasitol* **113**: 271-278.

Mueller, A. K., M. Labaied, S. H. Kappe & K. Matuschewski, (2005) Genetically modified *Plasmodium* parasites as a protective experimental malaria vaccine. *Nature* **433**: 164-167.

Muller, H. M., I. Reckmann, M. R. Hollingdale, H. Bujard, K. J. Robson & A. Crisanti, (1993) Thrombospondin related anonymous protein (TRAP) of *Plasmodium falciparum* binds specifically to sulfated glycoconjugates and to HepG2 hepatoma cells suggesting a role for this molecule in sporozoite invasion of hepatocytes. *The EMBO journal* **12**: 2881-2889.

Murata, C. E. & D. E. Goldberg, (2003) *Plasmodium falciparum* falcilysin: a metalloprotease with dual specificity. *J Biol Chem* **278**: 38022-38028.

Musingarimi, P., M. E. Plumb & J. M. Sodetz, (2002) Interaction between the C8 alpha-gamma and C8 beta subunits of human complement C8: role of the C8 beta N-terminal thrombospondin type 1 module and membrane attack complex/perforin domain. *Biochemistry* **41**: 11255-11260.

Nagamune, K., L. M. Hicks, B. Fux, F. Brossier, E. N. Chini & L. D. Sibley, (2008) Abscisic

acid controls calcium-dependent egress and development in *Toxoplasma gondii*. *Nature* **451**: 207-210.

Nagamune, K., K. Yamamoto, A. Naka, J. Matsuyama, T. Miwatani & T. Honda, (1996) In vitro proteolytic processing and activation of the recombinant precursor of El Tor cytolysin/hemolysin (pro-HlyA) of *Vibrio cholerae* by soluble hemagglutinin/protease of *V. cholerae*, trypsin, and other proteases. *Infect Immun* **64**: 4655-4658.

Nichols, B. A., M. L. Chiappino & G. R. O'Connor, (1983) Secretion from the rhoptries of *Toxoplasma gondii* during host-cell invasion. *J Ultrastruct Res* **83**: 85-98.

Noronha, F. S., F. J. Ramalho-Pinto & M. F. Horta, (1996) Cytolytic activity in the genus *Leishmania*: involvement of a putative pore-forming protein. *Infect Immun* **64**: 3975-3982.

Nur, E. T., (1993) The impact of malaria on labour use and efficiency in the Sudan. *Soc Sci Med* **37**: 1115-1119.

Nussenzweig, R. S., J. Vanderberg, H. Most & C. Orton, (1967) Protective immunity produced by the injection of x-irradiated sporozoites of *Plasmodium berghei*. *Nature* **216**: 160-162.

O'Connor, R. M., K. Kim, F. Khan & H. D. Ward, (2003) Expression of Cpgp40/15 in *Toxoplasma gondii*: a surrogate system for the study of *Cryptosporidium* glycoprotein antigens. *Infect Immun* **71**: 6027-6034.

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

O'Donnell, R. A., A. Saul, A. F. Cowman & B. S. Crabb, (2000) Functional conservation of the malaria vaccine antigen MSP-119 across distantly related *Plasmodium* species. *Nat Med* **6**: 91-95.

O'Keefe, A. H., J. L. Green, M. Grainger & A. A. Holder, (2005) A novel Sushi domain-containing protein of *Plasmodium falciparum*. *Mol Biochem Parasitol* **140**: 61-68.

Ockenhouse, C. F., P. F. Sun, D. E. Lanar, B. T. Wellde, B. T. Hall, K. Kester, J. A. Stoute, A. Magill, U. Krzych, L. Farley, R. A. Wirtz, J. C. Sadoff, D. C. Kaslow, S. Kumar, L. W. Church, J. M. Crutcher, B. Wizel, S. Hoffman, A. Lavani, A. V. Hill, J. A. Tine, K. P. Guito, C. de Taisne, R. Anders, W. R. Ballou & et al., (1998) Phase I/IIa safety, immunogenicity, and efficacy trial of NYVAC-Pf7, a pox-vectored, multiantigen, multistage vaccine candidate for *Plasmodium falciparum* malaria. *J Infect Dis* **177**: 1664-1673.

Olaya, P. & M. Wasserman, (1991) Effect of calpain inhibitors on the invasion of human erythrocytes by the parasite *Plasmodium falciparum*. *Biochim Biophys Acta* **1096**: 217-221.

Olson, R. & E. Gouaux, (2005) Crystal structure of the *Vibrio cholerae* cytolysin (VCC) protoxin and its assembly into a heptameric transmembrane pore. *J Mol Biol* **350**: 997-1016.

Omara-Opyene, A. L., P. A. Moura, C. R. Sulsona, J. A. Bonilla, C. A. Yowell, H. Fujioka, D. A. Fidock & J. B. Dame, (2004) Genetic disruption of the *Plasmodium falciparum* digestive vacuole plasmepsins demonstrates their functional redundancy. *J Biol Chem* **279**: 54088-54096.

Orlandi, P. A., F. W. Klotz & J. D. Haynes, (1992) A malaria invasion receptor, the 175-kilodalton erythrocyte binding antigen of *Plasmodium falciparum* recognizes the terminal Neu5Ac(alpha 2-3)Gal- sequences of glycophorin A. *J Cell Biol* **116**: 901-909.

Pachebat, J. A., M. Kadekoppala, M. Grainger, A. R. Dluzewski, R. S. Gunaratne, T. J. Scott-Finnigan, S. A. Ogun, I. T. Ling, L. H. Bannister, H. M. Taylor, G. H. Mitchell & A. A. Holder, (2007) Extensive proteolytic processing of the malaria parasite merozoite surface protein 7 during biosynthesis and parasite release from erythrocytes. *Mol Biochem Parasitol* **151**: 59-69.

Padmaja, U. K., P. Adhikari & P. Periera, (1999) Experience with quinine in falciparum malaria. *Indian J Med Sci* **53**: 153-157.

Pang, X. L., T. Mitamura & T. Horii, (1999) Antibodies reactive with the N-terminal domain of *Plasmodium falciparum* serine repeat antigen inhibit cell proliferation by agglutinating merozoites and schizonts. *Infect Immun* **67**: 1821-1827.

Parvanova, I., S. Epiphonio, A. Fauq, T. E. Golde, M. Prudencio & M. M. Mota, (2009) A small molecule inhibitor of signal peptide peptidase inhibits *Plasmodium* development in the

liver and decreases malaria severity. *PLoS One* **4**: e5078.

Patarroyo, M. E., P. Romero, M. L. Torres, P. Clavijo, A. Moreno, A. Martinez, R. Rodriguez, F. Guzman & E. Cabezas, (1987) Induction of protective immunity against experimental infection with malaria using synthetic peptides. *Nature* **328**: 629-632.

Pearce, J. A., A. N. Hodder & R. F. Anders, (2004) The alanine-rich heptad repeats are intact in the processed form of *Plasmodium falciparum* MSP3. *Exp Parasitol* **108**: 186-189.

Pearce, J. A., K. Mills, T. Triglia, A. F. Cowman & R. F. Anders, (2005) Characterisation of two novel proteins from the asexual stage of *Plasmodium falciparum*, H101 and H103. *Mol Biochem Parasitol* **139**: 141-151.

Perkins, M., (1988) Stage-dependent processing and localization of a *Plasmodium falciparum* protein of 130,000 molecular weight. *Exp Parasitol* **65**: 61-68.

Petter, M., M. Haeggstrom, A. Khattab, V. Fernandez, M. Q. Klinkert & M. Wahlgren, (2007) Variant proteins of the *Plasmodium falciparum* RIFIN family show distinct subcellular localization and developmental expression patterns. *Mol Biochem Parasitol* **156**: 51-61.

Philipp, M., I. H. Tsai & M. L. Bender, (1979) Comparison of the kinetic specificity of subtilisin and thiolsubtilisin toward n-alkyl p-nitrophenyl esters. *Biochemistry* **18**: 3769-3773.

Piippo, M., N. Lietzen, O. S. Nevalainen, J. Salmi & T. A. Nyman, (2010) Pripper: prediction of caspase cleavage sites from whole proteomes. *BMC Bioinformatics* **11**: 320.

Pirson, P. J. & M. E. Perkins, (1985) Characterization with monoclonal antibodies of a surface antigen of *Plasmodium falciparum* merozoites. *J Immunol* **134**: 1946-1951.

Pollack, Y., A. L. Katzen, D. T. Spira & J. Golenser, (1982) The genome of *Plasmodium falciparum*. I: DNA base composition. *Nucleic Acids Res* **10**: 539-546.

Ponpuak, M., M. Klemba, M. Park, I. Y. Gluzman, G. K. Lamppa & D. E. Goldberg, (2007) A role for falcilysin in transit peptide degradation in the *Plasmodium falciparum* apicoplast. *Molecular microbiology* **63**: 314-334.

Potgieter, F. T. & H. J. Els, (1977) The fine structure of intra-erythrocytic stages of *Babesia bigemina*. *Onderstepoort J Vet Res* **44**: 157-168.

Pouvelle, B., P. A. Buffet, C. Lepolard, A. Scherf & J. Gysin, (2000) Cytoadhesion of *Plasmodium falciparum* ring-stage-infected erythrocytes. *Nat Med* **6**: 1264-1268.

Puentes, A., J. Garcia, R. Vera, Q. R. Lopez, M. Urquiza, M. Vanegas, L. M. Salazar & M. E. Patarroyo, (2000) Serine repeat antigen peptides which bind specifically to red blood cells. *Parasitol Int* **49**: 105-117.

Putrianti, E. D., A. Schmidt-Christensen, I. Arnold, V. T. Heussler, K. Matuschewski & O. Silvie, (2009) The *Plasmodium* serine-type SERA proteases display distinct expression patterns and non-essential *in vivo* roles during life cycle progression of the malaria parasite. *Cell Microbiol*.

Raphael, P., Y. Takakuwa, S. Manno, S. C. Liu, A. H. Chishti & M. Hanspal, (2000) A cysteine protease activity from *Plasmodium falciparum* cleaves human erythrocyte ankyrin. *Mol Biochem Parasitol* **110**: 259-272.

Rausell, C., I. Garcia-Robles, J. Sanchez, C. Munoz-Garay, A. C. Martinez-Ramirez, M. D. Real & A. Bravo, (2004) Role of toxin activation on binding and pore formation activity of the *Bacillus thuringiensis* Cry3 toxins in membranes of *Leptinotarsa decemlineata* (Say). *Biochim Biophys Acta* **1660**: 99-105.

Ravetch, J. V., J. Kochan & M. Perkins, (1985) Isolation of the gene for a glycoprotein-binding protein implicated in erythrocyte invasion by a malaria parasite. *Science* **227**: 1593-1597.

Rawlings, N. D. & A. J. Barrett, (1993) Evolutionary families of peptidases. *Biochem J* **290** (Pt 1): 205-218.

Rawlings, N. D., A. J. Barrett & A. Bateman, (2010) MEROPS: the peptidase database. *Nucleic Acids Res* **38**: D227-233.

Rayner, J. C., E. Vargas-Serrato, C. S. Huber, M. R. Galinski & J. W. Barnwell, (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.

Reece, S. E. & J. Thompson, (2008) Transformation of the rodent malaria parasite *Plasmodium chabaudi* and generation of a stable fluorescent line PcGFPCON. *Malar J* **7**:

183.

Rehm, M., H. Dussmann, R. U. Janicke, J. M. Tavare, D. Kogel & J. H. Prehn, (2002) Single-cell fluorescence resonance energy transfer analysis demonstrates that caspase activation during apoptosis is a rapid process. Role of caspase-3. *J Biol Chem* **277**: 24506-24514.

Reiter, P., (2000) From Shakespeare to Defoe: malaria in England in the Little Ice Age. *Emerg Infect Dis* **6**: 1-11.

Renatus, M., H. R. Stennicke, F. L. Scott, R. C. Liddington & G. S. Salvesen, (2001) Dimer formation drives the activation of the cell death protease caspase 9. *Proc Natl Acad Sci U S A* **98**: 14250-14255.

Richard, D., L. M. Kats, C. Langer, C. G. Black, K. Mitri, J. A. Boddey, A. F. Cowman & R. L. Coppel, (2009) Identification of rhoptry trafficking determinants and evidence for a novel sorting mechanism in the malaria parasite *Plasmodium falciparum*. *PLoS Pathog* **5**: e1000328.

Richard, D., C. A. MacRaid, D. T. Riglar, J. A. Chan, M. Foley, J. Baum, S. A. Ralph, R. S. Norton & A. F. Cowman, (2010) Interaction between *Plasmodium falciparum* apical membrane antigen 1 and the rhoptry neck protein complex defines a key step in the erythrocyte invasion process of malaria parasites. *J Biol Chem* **285**: 14815-14822.

Ridley, R. G., H. W. Lahm, B. Takacs & J. G. Scaife, (1991) Genetic and structural relationships between components of a protective rhoptry antigen complex from *Plasmodium falciparum*. *Mol Biochem Parasitol* **47**: 245-246.

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

Rivadeneira, E. M., M. Wasserman & C. T. Espinal, (1983) Separation and concentration of schizonts of *Plasmodium falciparum* by Percoll gradients. *J Protozool* **30**: 367-370.

Rivera, V. M., X. Wang, S. Wardwell, N. L. Courage, A. Volchuk, T. Keenan, D. A. Holt, M. Gilman, L. Orci, F. Cerasoli, Jr., J. E. Rothman & T. Clackson, (2000) Regulation of protein secretion through controlled aggregation in the endoplasmic reticulum. *Science* **287**: 826-830.

Rock, K. L., C. Gramm, L. Rothstein, K. Clark, R. Stein, L. Dick, D. Hwang & A. L. Goldberg, (1994) Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* **78**: 761-771.

Roggwiller, E., M. E. Betoulle, T. Blisnick & C. Braun Breton, (1996) A role for erythrocyte band 3 degradation by the parasite gp76 serine protease in the formation of the parasitophorous vacuole during invasion of erythrocytes by *Plasmodium falciparum*. *Mol Biochem Parasitol* **82**: 13-24.

Rosado, C. J., A. M. Buckle, R. H. Law, R. E. Butcher, W. T. Kan, C. H. Bird, K. Ung, K. A. Browne, K. Baran, T. A. Bashtannyk-Puhlovich, N. G. Faux, W. Wong, C. J. Porter, R. N. Pike, A. M. Ellisdon, M. C. Pearce, S. P. Bottomley, J. Emsley, A. I. Smith, J. Rossjohn, E. L. Hartland, I. Voskoboinik, J. A. Trapani, P. I. Bird, M. A. Dunstone & J. C. Whisstock, (2007) A common fold mediates vertebrate defense and bacterial attack. *Science* **317**: 1548-1551.

Rosenthal, P. J., (2004) Cysteine proteases of malaria parasites. *International journal for parasitology* **34**: 1489-1499.

Rossjohn, J., S. C. Feil, W. J. McKinstry, R. K. Tweten & M. W. Parker, (1997) Structure of a cholesterol-binding, thiol-activated cytolysin and a model of its membrane form. *Cell* **89**: 685-692.

Ruecker, A., Shea, M., Blackman, M.J., (unpublished data). In.: The National Institute for Medical Research, pp.

Russo, I., S. Babbitt, V. Muralidharan, T. Butler, A. Oksman & D. E. Goldberg, (2009a) Plasmepsin V licenses *Plasmodium* proteins for export into the host erythrocyte. *Nature* **463**: 632-636.

Russo, I., A. Oksman, B. Vaupel & D. E. Goldberg, (2009b) A calpain unique to alveolates is essential in *Plasmodium falciparum* and its knockdown reveals an involvement in pre-S-phase development. *Proc Natl Acad Sci U S A*.

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

680-685.

Sajid, M., C. Withers-Martinez & M. J. Blackman, (2000) Maturation and specificity of *Plasmodium falciparum* subtilisin-like protease-1, a malaria merozoite subtilisin-like serine protease. *J Biol Chem* **275**: 631-641.

Salmon, B. L., A. Oksman & D. E. Goldberg, (2001) Malaria parasite exit from the host erythrocyte: a two-step process requiring extraerythrocytic proteolysis. *Proc Natl Acad Sci U S A* **98**: 271-276.

Sam-Yellowe, T. Y., L. Florens, T. Wang, J. D. Raine, D. J. Carucci, R. Sinden & J. R. Yates, 3rd, (2004) Proteome analysis of rhoptry-enriched fractions isolated from *Plasmodium* merozoites. *J Proteome Res* **3**: 995-1001.

Sam-Yellowe, T. Y., H. Shio & M. E. Perkins, (1988) Secretion of *Plasmodium falciparum* rhoptry protein into the plasma membrane of host erythrocytes. *J Cell Biol* **106**: 1507-1513.

Sambrook J., G., (1989) Molecular Cloning: A Laboratory Manual. *Cold Spring Harbor Laboratory Press*.

Sanders, P. R., P. R. Gilson, G. T. Cantin, D. C. Greenbaum, T. Nebl, D. J. Carucci, M. J. McConville, L. Schofield, A. N. Hodder, J. R. Yates, 3rd & B. S. Crabb, (2005) Distinct protein classes including novel merozoite surface antigens in Raft-like membranes of *Plasmodium falciparum*. *J Biol Chem* **280**: 40169-40176.

Sanders, P. R., L. M. Kats, D. R. Drew, R. A. O'Donnell, M. O'Neill, A. G. Maier, R. L. Coppel & B. S. Crabb, (2006) A set of glycosylphosphatidyl inositol-anchored membrane proteins of *Plasmodium falciparum* is refractory to genetic deletion. *Infect Immun* **74**: 4330-4338.

Saridaki, T., C. P. Sanchez, J. Pfahler & M. Lanzer, (2008) A conditional export system provides new insights into protein export in *Plasmodium falciparum*-infected erythrocytes. *Cell Microbiol* **10**: 2483-2495.

Schechter, I. & A. Berger, (1967) On the size of the active site in proteases. I. Papain. *Biochem Biophys Res Commun* **27**: 157-162.

Schilling, O. & C. M. Overall, (2008) Proteome-derived, database-searchable peptide libraries for identifying protease cleavage sites. *Nat Biotechnol* **26**: 685-694.

Schmidt-Christensen, A., A. Sturm, S. Horstmann & V. T. Heussler, (2008) Expression and processing of *Plasmodium berghei* SERA3 during liver stages. *Cell Microbiol* **10**: 1723-1734.

Schofield, L., G. R. Bushell, J. A. Cooper, A. J. Saul, J. A. Upcroft & C. Kidson, (1986) A rhoptry antigen of *Plasmodium falciparum* contains conserved and variable epitopes recognized by inhibitory monoclonal antibodies. *Mol Biochem Parasitol* **18**: 183-195.

Scibek, J. J., M. E. Plumb & J. M. Sodetz, (2002) Binding of human complement C8 to C9: role of the N-terminal modules in the C8 alpha subunit. *Biochemistry* **41**: 14546-14551.

Scott, F. L., G. J. Fuchs, S. E. Boyd, J. B. Denault, C. J. Hawkins, F. Dequiedt & G. S. Salvesen, (2008) Caspase-8 cleaves histone deacetylase 7 and abolishes its transcription repressor function. *J Biol Chem* **283**: 19499-19510.

Seaton, D. R. & A. R. Adams, (1949) Acquired resistance to proguanil in *Plasmodium falciparum*. *Lancet* **2**: 323.

Seaton, D. R. & E. M. Lourie, (1949) Acquired resistance to proguanil in *Plasmodium vivax*. *Lancet* **1**: 394.

Seeber, F. & D. Soldati-Favre, (2010) Metabolic pathways in the apicoplast of apicomplexa. *Int Rev Cell Mol Biol* **281**: 161-228.

Shaw, M. K., (1997) The same but different: the biology of Theileria sporozoite entry into bovine cells. *International journal for parasitology* **27**: 457-474.

Shea, M., U. Jakle, Q. Liu, C. Berry, K. A. Joiner & D. Soldati-Favre, (2007) A family of aspartic proteases and a novel, dynamic and cell-cycle-dependent protease localization in the secretory pathway of *Toxoplasma gondii*. *Traffic* **8**: 1018-1034.

Shenai, B. R., P. S. Sijwali, A. Singh & P. J. Rosenthal, (2000) Characterization of native and recombinant falcipain-2, a principal trophozoite cysteine protease and essential hemoglobinase of *Plasmodium falciparum*. *J Biol Chem* **275**: 29000-29010.

Shimada, Y., M. Maruya, S. Iwashita & Y. Ohno-Iwashita, (2002) The C-terminal domain of perfringolysin O is an essential cholesterol-binding unit targeting to cholesterol-rich microdomains. *Eur J Biochem* **269**: 6195-6203.

Shukla, M., Y. Rajgopal & P. P. Babu, (2006) Activation of calpains, calpastatin and spectrin cleavage in the brain during the pathology of fatal murine cerebral malaria. *Neurochem Int* **48**: 108-113.

Siddiqui, W. A., L. Q. Tam, K. J. Kramer, G. S. Hui, S. E. Case, K. M. Yamaga, S. P. Chang, E. B. Chan & S. C. Kan, (1987) Merozoite surface coat precursor protein completely protects Aotus monkeys against *Plasmodium falciparum* malaria. *Proc Natl Acad Sci U S A* **84**: 3014-3018.

Siezen, R. J. & J. A. Leunissen, (1997) Subtilases: the superfamily of subtilisin-like serine proteases. *Protein Sci* **6**: 501-523.

Sijwali, P. S., K. Kato, K. B. Seydel, J. Gut, J. Lehman, M. Klemba, D. E. Goldberg, L. H. Miller & P. J. Rosenthal, (2004) *Plasmodium falciparum* cysteine protease falcipain-1 is not essential in erythrocytic stage malaria parasites. *Proc Natl Acad Sci U S A* **101**: 8721-8726.

Sijwali, P. S., J. Koo, N. Singh & P. J. Rosenthal, (2006) Gene disruptions demonstrate independent roles for the four falcipain cysteine proteases of *Plasmodium falciparum*. *Mol Biochem Parasitol* **150**: 96-106.

Sijwali, P. S. & P. J. Rosenthal, (2004) Gene disruption confirms a critical role for the cysteine protease falcipain-2 in hemoglobin hydrolysis by *Plasmodium falciparum*. *Proc Natl Acad Sci U S A* **101**: 4384-4389.

Sijwali, P. S., B. R. Shenai, J. Gut, A. Singh & P. J. Rosenthal, (2001) Expression and characterization of the *Plasmodium falciparum* haemoglobinase falcipain-3. *Biochem J* **360**: 481-489.

Silva, M. D., B. M. Cooke, M. Guillotte, D. W. Buckingham, J. P. Sauzet, C. Le Scanf, H. Contamin, P. David, O. Mercereau-Puijalon & S. Bonnefoy, (2005) A role for the *Plasmodium falciparum* RESA protein in resistance against heat shock demonstrated using gene disruption. *Molecular microbiology* **56**: 990-1003.

Silvie, O., J. P. Semblat, J. F. Franetich, L. Hannoun, W. Eling & D. Mazier, (2002) Effects of irradiation on *Plasmodium falciparum* sporozoite hepatic development: implications for the design of pre-erythrocytic malaria vaccines. *Parasite immunology* **24**: 221-223.

Singh, N., P. S. Sijwali, K. C. Pandey & P. J. Rosenthal, (2006) *Plasmodium falciparum*: biochemical characterization of the cysteine protease falcipain-2'. *Exp Parasitol* **112**: 187-192.

Singh, S., M. M. Alam, I. Pal-Bhowmick, J. A. Brzostowski & C. E. Chitnis, (2010) Distinct External Signals Trigger Sequential Release of Apical Organelles during Erythrocyte Invasion by Malaria Parasites. *PLoS Pathog* **6**: e1000746.

Singh, S., M. Plassmeyer, D. Gaur & L. H. Miller, (2007) Mononeme: a new secretory organelle in *Plasmodium falciparum* merozoites identified by localization of rhomboid-1 protease. *Proc Natl Acad Sci U S A* **104**: 20043-20048.

Singh, Y., V. K. Chaudhary & S. H. Leppla, (1989) A deleted variant of Bacillus anthracis protective antigen is non-toxic and blocks anthrax toxin action *in vivo*. *J Biol Chem* **264**: 19103-19107.

Slade, D. J., L. L. Lovelace, M. Chruszcz, W. Minor, L. Lebioda & J. M. Sodetz, (2008) Crystal structure of the MACPF domain of human complement protein C8 alpha in complex with the C8 gamma subunit. *J Mol Biol* **379**: 331-342.

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

Soldati, D. & J. C. Boothroyd, (1993) Transient transfection and expression in the obligate intracellular parasite *Toxoplasma gondii*. *Science* **260**: 349-352.

Soni, S., S. Dhawan, K. M. Rosen, M. Chafel, A. H. Chishti & M. Hanspal, (2005) Characterization of events preceding the release of malaria parasite from the host red blood cell. *Blood Cells Mol Dis* **35**: 201-211.

Spielmann, T., D. L. Gardiner, H. P. Beck, K. R. Trenholme & D. J. Kemp, (2006a) Organization of ETRAMPs and EXP-1 at the parasite-host cell interface of malaria parasites. *Molecular microbiology* **59**: 779-794.

Spielmann, T., P. L. Hawthorne, M. W. Dixon, M. Hannemann, K. Klotz, D. J. Kemp, N. Klonis, L. Tilley, K. R. Trenholme & D. L. Gardiner, (2006b) A cluster of ring stage-specific genes linked to a locus implicated in cytoadherence in *Plasmodium falciparum* codes for

PEXEL-negative and PEXEL-positive proteins exported into the host cell. *Mol Biol Cell* **17**: 3613-3624.

Srinivasan, P., I. Coppens & M. Jacobs-Lorena, (2009) Distinct roles of *Plasmodium* rhomboid 1 in parasite development and malaria pathogenesis. *PLoS Pathog* **5**: e1000262.

Stafford, W. H., M. J. Blackman, A. Harris, S. Shai, M. Grainger & A. A. Holder, (1994) N-terminal amino acid sequence of the *Plasmodium falciparum* merozoite surface protein-1 polypeptides. *Mol Biochem Parasitol* **66**: 157-160.

Stafford, W. H., B. Gunder, A. Harris, H. G. Heidrich, A. A. Holder & M. J. Blackman, (1996) A 22 kDa protein associated with the *Plasmodium falciparum* merozoite surface protein-1 complex. *Mol Biochem Parasitol* **80**: 159-169.

Stallmach, R., Blackman, M.J., (unpublished data). In.: The National Institute for Medical Research, pp.

Stewart, M. J., S. Schulman & J. P. Vanderberg, (1986) Rhoptry secretion of membranous whorls by *Plasmodium falciparum* merozoites. *Am J Trop Med Hyg* **35**: 37-44.

Stoute, J. A., M. Slaoui, D. G. Heppner, P. Momin, K. E. Kester, P. Desmons, B. T. Wellde, N. Garcon, U. Krzych & M. Marchand, (1997) A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmodium falciparum* malaria. RTS,S Malaria Vaccine Evaluation Group. *N Engl J Med* **336**: 86-91.

Strisovsky, K., H. J. Sharpe & M. Freeman, (2009) Sequence-specific intramembrane proteolysis: identification of a recognition motif in rhomboid substrates. *Mol Cell* **36**: 1048-1059.

Sturm, A., R. Amino, C. van de Sand, T. Regen, S. Retzlaff, A. Rennenberg, A. Krueger, J. M. Pollok, R. Menard & V. T. Heussler, (2006) Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science* **313**: 1287-1290.

Takemoto, K., E. Kuranaga, A. Tonoki, T. Nagai, A. Miyawaki & M. Miura, (2007) Local initiation of caspase activation in *Drosophila* salivary gland programmed cell death *in vivo*. *Proc Natl Acad Sci U S A* **104**: 13367-13372.

Takemoto, K., T. Nagai, A. Miyawaki & M. Miura, (2003) Spatio-temporal activation of caspase revealed by indicator that is insensitive to environmental effects. *J Cell Biol* **160**: 235-243.

Taylor, H. M., L. McRobert, M. Grainger, A. Sicard, A. R. Dlugowski, C. S. Hopp, A. A. Holder & D. A. Baker, (2009) The malaria parasite cGMP-dependent protein kinase plays a central role in blood stage schizogony. *Eukaryotic cell*.

The All-Party Parliamentary Group on Malaria and Neglected Tropical Diseases, (2010) The Control of Malaria 2005-15: progress and priorities towards eradication. In. London: House of Commons,, pp.

Tine, J. A., D. E. Lanar, D. M. Smith, B. T. Wellde, P. Schultheiss, L. A. Ware, E. B. Kauffman, R. A. Wirtz, C. De Taisne, G. S. Hui, S. P. Chang, P. Church, M. R. Hollingdale, D. C. Kaslow, S. Hoffman, K. P. Guito, W. R. Ballou, J. C. Sadoff & E. Paoletti, (1996) NYVAC-Pf7: a poxvirus-vectored, multiantigen, multistage vaccine candidate for *Plasmodium falciparum* malaria. *Infect Immun* **64**: 3833-3844.

Tonkin, C. J., G. G. van Dooren, T. P. Spurck, N. S. Struck, R. T. Good, E. Handman, A. F. Cowman & G. I. McFadden, (2004) Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method. *Mol Biochem Parasitol* **137**: 13-21.

Topolska, A. E., A. Lidgett, D. Truman, H. Fujioka & R. L. Coppel, (2004) Characterization of a membrane-associated rhoptry protein of *Plasmodium falciparum*. *J Biol Chem* **279**: 4648-4656.

Torii, M., J. H. Adams, L. H. Miller & M. Aikawa, (1989) Release of merozoite dense granules during erythrocyte invasion by *Plasmodium knowlesi*. *Infect Immun* **57**: 3230-3233.

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

Trager, W., C. Rozario, H. Shio, J. Williams & M. E. Perkins, (1992) Transfer of a dense granule protein of *Plasmodium falciparum* to the membrane of ring stages and isolation of dense granules. *Infect Immun* **60**: 4656-4661.

Treeck, M., S. Zacherl, S. Herrmann, A. Cabrera, M. Kono, N. S. Struck, K. Engelberg, S.

Haase, F. Frischknecht, K. Miura, T. Spielmann & T. W. Gilberger, (2009) Functional analysis of the leading malaria vaccine candidate AMA-1 reveals an essential role for the cytoplasmic domain in the invasion process. *PLoS Pathog* **5**: e1000322.

Triglia, T., J. Healer, S. R. Caruana, A. N. Hodder, R. F. Anders, B. S. Crabb & A. F. Cowman, (2000) Apical membrane antigen 1 plays a central role in erythrocyte invasion by *Plasmodium* species. *Molecular microbiology* **38**: 706-718.

Triglia, T., W. H. Tham, A. Hodder & A. F. Cowman, (2009) Reticulocyte binding protein homologues are key adhesins during erythrocyte invasion by *Plasmodium falciparum*. *Cell Microbiol* **11**: 1671-1687.

Trucco, C., D. Fernandez-Reyes, S. Howell, W. H. Stafford, T. J. Scott-Finnigan, M. Grainger, S. A. Ogun, W. R. Taylor & A. A. Holder, (2001) The merozoite surface protein 6 gene codes for a 36 kDa protein associated with the *Plasmodium falciparum* merozoite surface protein-1 complex. *Mol Biochem Parasitol* **112**: 91-101.

Tyndall, J. D., T. Nall & D. P. Fairlie, (2005) Proteases universally recognize beta strands in their active sites. *Chem Rev* **105**: 973-999.

Uellner, R., M. J. Zvelebil, J. Hopkins, J. Jones, L. K. MacDougall, B. P. Morgan, E. Podack, M. D. Waterfield & G. M. Griffiths, (1997) Perforin is activated by a proteolytic cleavage during biosynthesis which reveals a phospholipid-binding C2 domain. *The EMBO journal* **16**: 7287-7296.

Ungureanu, E., R. Killick-Kendrick, P. C. Garnham, P. Branzei, C. Romanescu & P. G. Shute, (1976) Prepatent periods of a tropical strain of *Plasmodium vivax* after inoculations of tenfold dilutions of sporozoites. *Trans R Soc Trop Med Hyg* **70**: 482-483.

Urban, S., J. R. Lee & M. Freeman, (2001) Drosophila rhomboid-1 defines a family of putative intramembrane serine proteases. *Cell* **107**: 173-182.

Uzureau, P., J. C. Barale, C. J. Janse, A. P. Waters & C. B. Breton, (2004) Gene targeting demonstrates that the *Plasmodium berghei* subtilisin PbSUB2 is essential for red cell invasion and reveals spontaneous genetic recombination events. *Cell Microbiol* **6**: 65-78.

Valero, M. V., R. Amador, J. J. Aponte, A. Narvaez, C. Galindo, Y. Silva, J. Rosas, F. Guzman & M. E. Patarroyo, (1996) Evaluation of SPf66 malaria vaccine during a 22-month follow-up field trial in the Pacific coast of Colombia. *Vaccine* **14**: 1466-1470.

van der Wel, A. M., A. M. Tomas, C. H. Kocken, P. Malhotra, C. J. Janse, A. P. Waters & A. W. Thomas, (1997) Transfection of the primate malaria parasite *Plasmodium knowlesi* using entirely heterologous constructs. *J Exp Med* **185**: 1499-1503.

van Dooren, G. G., S. B. Reiff, C. Tomova, M. Meissner, B. M. Humbel & B. Striepen, (2009) A novel dynamin-related protein has been recruited for apicoplast fission in *Toxoplasma gondii*. *Curr Biol* **19**: 267-276.

van Ooij, C., P. Tamez, S. Bhattacharjee, N. L. Hiller, T. Harrison, K. Liolios, T. Kooij, J. Ramesar, B. Balu, J. Adams, A. P. Waters, C. J. Janse & K. Haldar, (2008) The malaria secretome: from algorithms to essential function in blood stage infection. *PLoS Pathog* **4**: e1000084.

VanBuskirk, K. M., M. T. O'Neill, P. De La Vega, A. G. Maier, U. Krzych, J. Williams, M. G. Dowler, J. B. Sacci, Jr., N. Kangwanransan, T. Tsuboi, N. M. Kneteman, D. G. Heppner, Jr., B. A. Murdock, S. A. Mikolajczak, A. S. Aly, A. F. Cowman & S. H. Kappe, (2009) Preerythrocytic, live-attenuated *Plasmodium falciparum* vaccine candidates by design. *Proc Natl Acad Sci U S A* **106**: 13004-13009.

Venter, J. C., M. D. Adams, E. W. Myers, P. W. Li, R. J. Mural, G. G. Sutton, H. O. Smith, M. Yandell, C. A. Evans, R. A. Holt, J. D. Gocayne, P. Amanatides, R. M. Ballew, D. H. Huson, J. R. Wortman, Q. Zhang, C. D. Kodira, X. H. Zheng, L. Chen, M. Skupski, G. Subramanian, P. D. Thomas, J. Zhang, G. L. Gabor Miklos, C. Nelson, S. Broder, A. G. Clark, J. Nadeau, V. A. McKusick, N. Zinder, A. J. Levine, R. J. Roberts, M. Simon, C. Slayman, M. Hunkapiller, R. Bolanos, A. Delcher, I. Dew, D. Fasulo, M. Flanigan, L. Florea, A. Halpern, S. Hannenhalli, S. Kravitz, S. Levy, C. Mobarry, K. Reinert, K. Remington, J. Abu-Threideh, E. Beasley, K. Biddick, V. Bonazzi, R. Brandon, M. Cargill, I. Chandramouliswaran, R. Charlab, K. Chaturvedi, Z. Deng, V. Di Francesco, P. Dunn, K. Eilbeck, C. Evangelista, A. E. Gabrielian, W. Gan, W. Ge, F. Gong, Z. Gu, P. Guan, T. J. Heiman, M. E. Higgins, R. R. Ji, Z. Ke, K. A. Ketchum, Z. Lai, Y. Lei, Z. Li, J. Li, Y. Liang, X. Lin, F. Lu, G. V. Merkulov, N.

Milshina, H. M. Moore, A. K. Naik, V. A. Narayan, B. Neelam, D. Nusskern, D. B. Rusch, S. Salzberg, W. Shao, B. Shue, J. Sun, Z. Wang, A. Wang, X. Wang, J. Wang, M. Wei, R. Wides, C. Xiao, C. Yan, et al., (2001) The sequence of the human genome. *Science* **291**: 1304-1351.

Voskoboinik, I., M. C. Thia, J. Fletcher, A. Ciccone, K. Browne, M. J. Smyth & J. A. Trapani, (2005) Calcium-dependent plasma membrane binding and cell lysis by perforin are mediated through its C2 domain: A critical role for aspartate residues 429, 435, 483, and 485 but not 491. *J Biol Chem* **280**: 8426-8434.

Wandless, T. J., (2000) A confederacy of bunches: fundamentals and applications of a self-associating protein. *Proc Natl Acad Sci U S A* **97**: 6921-6923.

Wanyiri, J. W., P. Techasintana, R. M. O'Connor, M. J. Blackman, K. Kim & H. D. Ward, (2009) Role of CpSUB1, a Subtilisin-Like Protease in *Cryptosporidium parvum* Infection in Vitro. *Eukaryotic cell*.

Ward, G. E., L. H. Miller & J. A. Dvorak, (1993) The origin of parasitophorous vacuole membrane lipids in malaria-infected erythrocytes. *J Cell Sci* **106 (Pt 1)**: 237-248.

Waters, A. P., A. W. Thomas, J. A. Deans, G. H. Mitchell, D. E. Hudson, L. H. Miller, T. F. McCutchan & S. Cohen, (1990) A merozoite receptor protein from *Plasmodium knowlesi* is highly conserved and distributed throughout *Plasmodium*. *J Biol Chem* **265**: 17974-17979.

Wee, L. J., T. W. Tan & S. Ranganathan, (2006) SVM-based prediction of caspase substrate cleavage sites. *BMC Bioinformatics* **7 Suppl 5**: S14.

Wel, A., C. H. Kocken, T. C. Pronk, B. Franke-Fayard & A. W. Thomas, (2004) New selectable markers and single crossover integration for the highly versatile *Plasmodium knowlesi* transfection system. *Mol Biochem Parasitol* **134**: 97-104.

Wertheimer, S. P. & J. W. Barnwell, (1989) *Plasmodium vivax* interaction with the human Duffy blood group glycoprotein: identification of a parasite receptor-like protein. *Exp Parasitol* **69**: 340-350.

White, N. J., (1997) Assessment of the pharmacodynamic properties of antimalarial drugs *in vivo*. *Antimicrob Agents Chemother* **41**: 1413-1422.

Wickham, M. E., J. G. Culvenor & A. F. Cowman, (2003) Selective inhibition of a two-step egress of malaria parasites from the host erythrocyte. *J Biol Chem* **278**: 37658-37663.

Wickramarachchi, T., A. L. Cabrera, D. Sinha, S. Dhawan, T. Chandran, Y. S. Devi, M. Kono, T. Spielmann, T. W. Gilberger, V. S. Chauhan & A. Mohammed, (2009) A novel *Plasmodium falciparum* erythrocyte binding protein associated with the merozoite surface, PfDBLMSp. *International journal for parasitology*.

Winograd, E., C. A. Clavijo, L. Y. Bustamante & M. Jaramillo, (1999) Release of merozoites from *Plasmodium falciparum*-infected erythrocytes could be mediated by a non-explosive event. *Parasitol Res* **85**: 621-624.

Withers-Martinez, C., E. P. Carpenter, F. Hackett, B. Ely, M. Sajid, M. Grainger & M. J. Blackman, (1999) PCR-based gene synthesis as an efficient approach for expression of the A+T-rich malaria genome. *Protein Eng* **12**: 1113-1120.

Withers-Martinez, C., L. Jean & M. J. Blackman, (2004) Subtilisin-like proteases of the malaria parasite. *Molecular microbiology* **53**: 55-63.

Withers-Martinez, C., J. W. Saldanha, B. Ely, F. Hackett, T. O'Connor & M. J. Blackman, (2002) Expression of recombinant *Plasmodium falciparum* subtilisin-like protease-1 in insect cells. Characterization, comparison with the parasite protease, and homology modeling. *J Biol Chem* **277**: 29698-29709.

Woehlbier, U., C. Epp, F. Hackett, M. J. Blackman & H. Bujard, (2010) Antibodies against multiple merozoite surface antigens of the human malaria parasite *Plasmodium falciparum* inhibit parasite maturation and red blood cell invasion. *Malar J* **9**: 77.

World Health Organisation, (1973) Chemotherapy of malaria and resistance to antimalarials. Report of a WHO Scientific Group. In: WHO Technical Report Series. pp.

World Health Organisation, (2009) WHO World Malaria Report 2009. In.: World Health Organisation, pp.

Wu, Y., L. Kirkman & T. Welles, (1996) Transformation of *Plasmodium falciparum* malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. *PNAS* **93**: 1130-1134.

Wu, Y., C. D. Sifri, H. H. Lei, X. Z. Su & T. E. Wellems, (1995) Transfection of *Plasmodium falciparum* within human red blood cells. *Proc Natl Acad Sci U S A* **92**: 973-977.

Wu, Y., X. Wang, X. Liu & Y. Wang, (2003) Data-mining approaches reveal hidden families of proteases in the genome of malaria parasite. *Genome Res* **13**: 601-616.

Yamauchi, L. M., A. Coppi, G. Snounou & P. Sinnis, (2007) *Plasmodium* sporozoites trickle out of the injection site. *Cell Microbiol* **9**: 1215-1222.

Yang, Z. R., (2005) Prediction of caspase cleavage sites using Bayesian bio-basis function neural networks. *Bioinformatics* **21**: 1831-1837.

Yeoh, S., R. A. O'Donnell, K. Koussis, A. R. Dluzewski, K. H. Ansell, S. A. Osborne, F. Hackett, C. Withers-Martinez, G. H. Mitchell, L. H. Bannister, J. S. Bryans, C. A. Kettleborough & M. J. Blackman, (2007) Subcellular discharge of a serine protease mediates release of invasive malaria parasites from host erythrocytes. *Cell* **131**: 1072-1083.

Young, M. D. & D. V. Moore, (1961) Chloroquine resistance in *Plasmodium falciparum*. *Am J Trop Med Hyg* **10**: 317-320.