Deciphering the Functions of Plasmepsins VII and VIII in *Plasmodium berghei*

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Doctor of Philosophy

By

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10LAPH06

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CERTIFICATE

This is to certify that the thesis entitled **"Deciphering the Functions of Plasmepsins VII and VIII in *Plasmodium berghei*"** submitted by **Mr. Mastanbabu Somepalli** bearing registration number 10LAPH06 in partial fulfillment of the requirements for award of Doctor of Philosophy in the School of Life Sciences is a bonafide work carried out by him under my supervision and guidance. This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other university or Institution for award of any degree or diploma.

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A. Published in the following peer reviewed journals:

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Further the student has passed the following courses towards fulfillment of coursework requirement for Ph.D.

Course Code	Course Name	Credits	Pass/Fail
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3. AS 803	Biostatistics	2	Pass
4. AS 804	Analytical Techniques	3	Pass
5. AS 805	Lab Work	4	Pass

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DECLARATION

I hereby declare that the results of the study incorporated in the thesis entitled **"Deciphering the Functions of Plasmepsins VII and VIII in *Plasmodium berghei*"** has been carried out by me under the supervision of **Dr. Kota Arun Kumar** at Department of Animal Biology, School of Life Sciences. The work presented in this thesis has not been submitted for any degree or diploma in any other University or Institute earlier.

Signature:

Date:

Name: Mastanbabu Somepalli

Registration No: 10LAPH06

Dedication

I dedicate this humble effort...

To my parents, Yogeswara Rao & Saraswati,
who have sacrificed so much for me
and encouraged me to go on every adventure,
especially this one.
&
To my brothers, Madan Kumar & Rama Naidu,
who picked me up whenever I fell
and always supported me whatever path I chose.

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To all those who encouraged and helped me to fly toward my dreams.,

~ Mastan

Abstract

Despite decades of research, malaria still remains a global health concern causing half a million deaths worldwide. Identification of novel drug targets and development of new therapeutics with novel mechanisms of action is urgently required as the parasite acquired resistance to the frontline drugs, Artemisinin-based combination therapy (ACT). Proteases are one of the most preferred enzyme families for target-based drug development due to their central role in a variety of cellular processes and human diseases and their well-characterized catalytic mechanisms and active site structures. Currently protease inhibitors are used for the treatment of AIDS, hypertension, hepatitis C and cancer. Plasmepsins (PMs), aspartic proteases of *Plasmodium,* are a group of ten proteases that perform crucial functions during the parasite development in multiple hosts and some of them are likely to be potential antimalarial targets. However, of the ten members, the roles of plasmepsin VII and VIII in the parasite biology remain elusive. Uncovering the *in vivo* functions of PMs VII and VIII in parasite biology may help in validation of these proteases as ideal antimalarial drug targets. The present study focuses on elucidating the functions of PM VII & VIII in the rodent malaria parasite *Plasmodium berghei*. Gene expression analysis by qRT-PCR in mosquito and liver stages revealed maximal expression in oocysts and midgut sporozoites implying a functional role for PM VII & VIII during parasite development in the mosquito host. To characterize the function, we generated knockout (KO) parasites using reverse genetics approach, which revealed that both PM VII & VIII were not essential for blood stage development. The systematic examination of growth kinetics, infection dynamics and development of KO parasites in mosquitoes and mice indicated that depletion of PMVII has no phenotypic effect across the life cycle. However, the genetic ablation of PM VIII causes a severe defect in gliding motility of sporozoites compromising their ability to invade salivary glands of mosquito and subsequent transmission to the rodent host. This study demonstrate that PM VIII plays a critical role in the sporozoite motility and invasion of host cells. Therefore, PM VIII is an ideal target for intervention strategies that blocks the transmission of malaria.

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Abbreviations / Acronyms

°C	Degree Celsius
µ	micro
µg	microgram
µl	microliter
µm	micrometer
µM	micro-molar
α	Alpha or Anti
aa	amino acid
ACT	Artemisinin-based Combination Therapy
AMA1	Apical membrane antigen 1
A. stephensi	*Anopheles Stephensi*
β	Beta
bp	base pairs
BSA	Bovine Serum Albumin
BSG	Basigin
cDNA	Complementary DNA
CDPK	Calcium-dependent protein kinase
CELTOS	Cell Traversal Protein for Ookinete and Sporozoite
CM	Cerebral malaria
CTRAP	Circumsporozoite and Thrombospondin Related Anonymous Protein
CTRP	Circumsporozoite and TRAP Related Protein
CITH	CAR-I/Trailer hitch
CSP	Circumsporozoite Protein
C-terminal	Carboxy terminal
DAPI	4', 6'-diamidino-2-phenylindole
DIC	Differential Interference Contrast
ddH_2O	double distilled water
dhfr/ts	dihydrofolate reductase/thymidylate synthase

DHPS	Dihydropteroate synthase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DNA	Deoxyribonuclease
dNTP	Deoxyribonucleotide
DOZI	Development Of Zygote Inhibited
E. coli	Escherichia Coli
ECP1	Egress Cysteine Protease 1
EDTA	Ethylenediamenetetraacitic acid
EEF	Exo-erythrocytic Form
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal Bovine Serum
FCM	Flow cytometry
FP	Forward Primer
GAF	Gametocyte activating factor
GAPs	Genetically attenuated parasites
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GAP45	Gliding associated protein 45
GAP50	Gliding associated protein 50
gDNA	genomic DNA
GFP	Green fluorescent protein
GLURP	Glutamate rich protein
hDHFR	Human dihydrofolate reductase
HepG2	Human hepatic carcinoma cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	High Fidelity
hpi	hours post infection
hr, hrs	hours
HSPG	Heparin sulfate proteoglycan
HSP70	Heat Shock Protein 70
HRP-II	Histidine rich protein II

HT	Host targeting signal
IFA	Immuno-Fluorescence Assay
i.p.	Intra-peritoneal
i.v.	Intra-venous
IMC	Inner Membrane Complex
iRBC	Infected Red Blood Cell
IRS	Indoor residual spraying
ITN	Insecticide treated net
IPT	Intermittent preventive treatment
IPTp	Intermittent preventive treatment in pregnancy
in vitro	in a test tube (*in vitrum*)
in vivo	in a living organism (*in vivus*)
kb	Kilo Base pairs
kDa	Kilo Daltons
KO	Knockout
L	Liter
LAMP	Loop mediated isothermal amplification
LAPs	Lectin adhesive like proteins
LB-Broth	Luria-Bertani Broth
LB-agar	Luria-Bertani Agar
LDH	Lactate dehydrogenase
LLINs	Long lasting insecticidal nets
M	Molar
MAPK	Mitogen-activated protein kinase
min	Minute
MJ	Moving Junction
ml	Milliliter
mRNA	Messenger RNA
MS	Mass spectormetry
MSP	Merozoite surface protein
MTIP	MyoA-tail interacting protein
MTOCs	Microtubule organizing centers

nm	Nano-meter
ng	Nano-gram
N-terminal	Amino Terminal
NEK4	NIMA (Never in Mitosis *Aspergillus*)-related kinase 4
OD	Optical Density
ORF	Open Reading Frame
PAGE	Poly-Acrylamide Gel Electrophoresis
P. berghei	*Plasmodium berghei*
Pb	*Plasmodium berghei*
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PEXEL	Plasmodium Export Element
Pf	*Plasmodium falciparum*
pH	potentia hydrogeni
PM	Plasmepsin
PNEPs	PEXEL negative exported proteins
PTEX	Plasmodium translocon of exported proteins
PV	Parasitophorous Vacuole
PVM	Parasitophorous Vacuolar Membrane
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RBCs	Red Blood Cells
RDT	Rapid diagnostic test
RE	Restriction Endonucleases
RESA	Ring infected erythrocyte surface antigen
RH	Relative Humidity
Rh5	Reticulocyte binding protein 5
RNA	Ribonucleic acid
RP	Reverse Prmier
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute Medium
RT	Room Temperature
RT-PCR	Reverse Transcription PCR

Σ	sigma (sum)
SDS	Sodium dodecyl sulphate
sec	Second
SERA	Serine Repeat Antigen
Spp.	species
spz	Sporozoite
Taq	Thermus aquaticus
TAE	Tris-acetate EDTA
TBS	Tris-buffer Saline
TE	Tris-EDTA
TEMED	N,N,N,N,-tetrametylethylendiamin
T. gondii	*Toxoplasma gondii*
TRAP	Thrombospondin related anonymous protein
TRSP	Thrombospondin related sporozoite protein
TSR	Thrombospondin repeat
TRX2	Thioredoxin-2
U	Unit, Biochemical unit for enzyme activity
UIS	Up regulated in infected salivary glands
UOS	Up regulated in oocyst sporozoites
UTR	Untranslated region
UV	Ultraviolet
V	Volt
v/v	Volume per volume
VTS	Vacuolar translocation signal
WB	Western Blotting
WHO	World Health Organization
WPV	Whole parasite vaccines
WT	Wild Type
w/v	Wight per volume

1. INTRODUCTION

1.1. Global burden of Malaria

Malaria is an infectious disease caused by obligatory intracellular protozoan parasites from the genus *Plasmodium* and transmitted by the bites of infected female *Anopheles* mosquitoes. Malaria has been one of the oldest and deadliest diseases in the history of mankind and it continues to be a global public health challenge. The World Health Organization (WHO) estimates that nearly half of the world's population is at risk of malaria, with ongoing malaria transmission in 91 countries and areas (World Malaria Report, 2017). Of the 91 countries, the disease burden is greatest in the least developed regions, particularly, the African region, which accounts for an estimated 90% of all malaria cases and deaths worldwide (World Malaria Report, 2017). All fifteen countries, except one, in sub-Saharan Africa carry 80% of the global malaria burden (World Malaria Report, 2017). In 2016, there were approximately 216 million clinical cases and 445 000 deaths due to malaria (World Malaria Report, 2017).

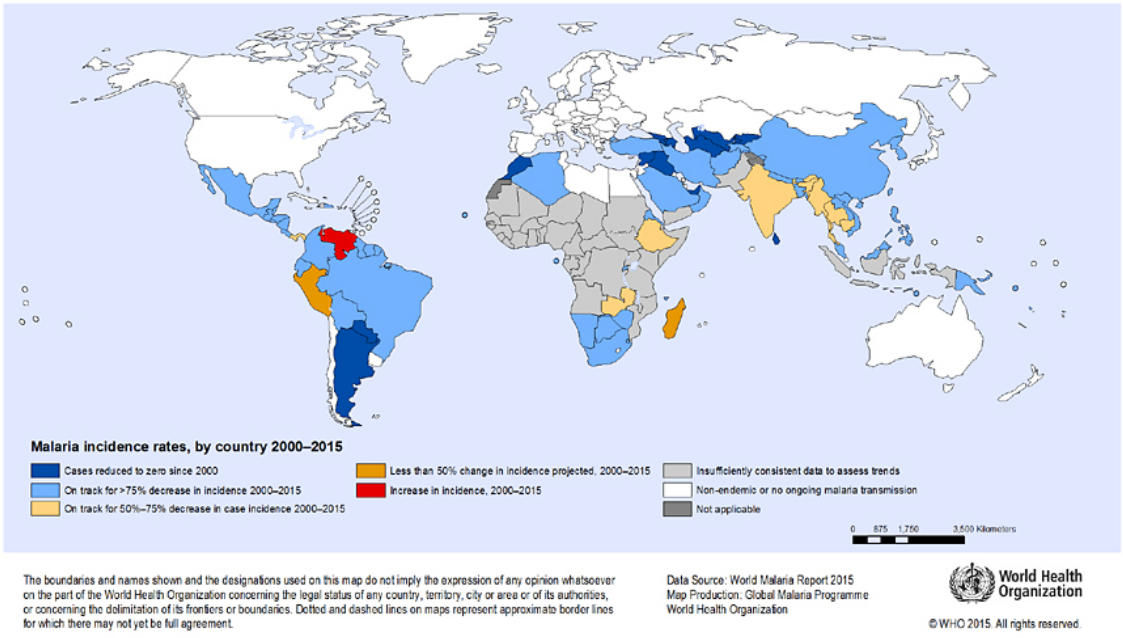


Figure 1.1. Projected changes in malaria incidence rates, by country, 2000-2015

Human malaria is caused by five species of *Plasmodium* parasites – *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae,* and *Plasmodium knowlesi*. P. knowlesi, originally known to infect monkeys, is the most recent species discovered to be infective for human beings (Cox-Singh et al., 2008). The five species have different geographical distributions. *P. falciparum* is the most predominant malaria parasite in sub-Saharan Africa, accounting for 99% of estimated malaria cases in 2016. Outside of Africa, *P. vivax* is mostly prevalent in the WHO Region of the Americas, representing 64% of malaria cases, and is above 30% in the WHO South- East Asia and 40% in the Eastern Mediterranean regions (World Malaria Report, 2017). *P. knowlesi* is particularly prevalent in Malaysia, where it caused 38% of the malaria cases reported (Ahmed and Cox-Singh, 2015). The fight against malaria has achieved significant progress in the last decade. The incidence rate of malaria is estimated to have decreased by 18% globally, from 76 to 63 cases per 1000 population at risk, between 2010 and 2016. Reduction in mortality rates were observed in all regions in 2016 compared with 2010 with the exception of WHO Eastern Mediterranean Region, where mortality rates remain unchanged (World Malaria Report, 2017). Although, globally more countries are moving towards achieving complete elimination, some countries have reported increase in number of malaria cases. This remarkable progress has been possible because of the use of preventive therapies, use of insecticide-treated nets (ITNs), availability of better diagnosis and treatment. However, malaria continues to be a major health concern and the global elimination of malaria still remains an unreality.

1.2. The Life cycle of *Plasmodium spp.*

Our efforts to eradicate or control the malaria disease necessitates a deeper understanding of the molecular and cellular mechanisms that mediate the remarkable parasite journey and the bottlenecks in the invertebrate vector and the vertebrate host. Despite remarkable progress, there are many aspects that are still needed to be comprehensively explored and this knowledge may further strengthen the efforts to control, eliminate and, perhaps, eradicate malaria in the future. Although, the ultimate aim is to understand the disease in humans, much of the existing knowledge on the malaria parasite and the pathogenesis has largely come from the animal models and the *in vitro* cultures of *P. falciparum* (Vincke and Lips, 1948; Yoeli 1964; Trager and Jensen, 1976; Haynes, 1976).

All *Plasmodium spp.* have a complex multi-stage life cycle that alternates between female *Anopheles* mosquitoes, and mammalian hosts. Once sporozoites enter the host via a mosquito bite, they infect hepatocytes and this phase is followed by asexual development in the blood. Sexual forms that arise during the blood stage development are ingested by a feeding mosquito, where the parasites undergo sexual reproduction and subsequently form sporozoites completing the cycle.

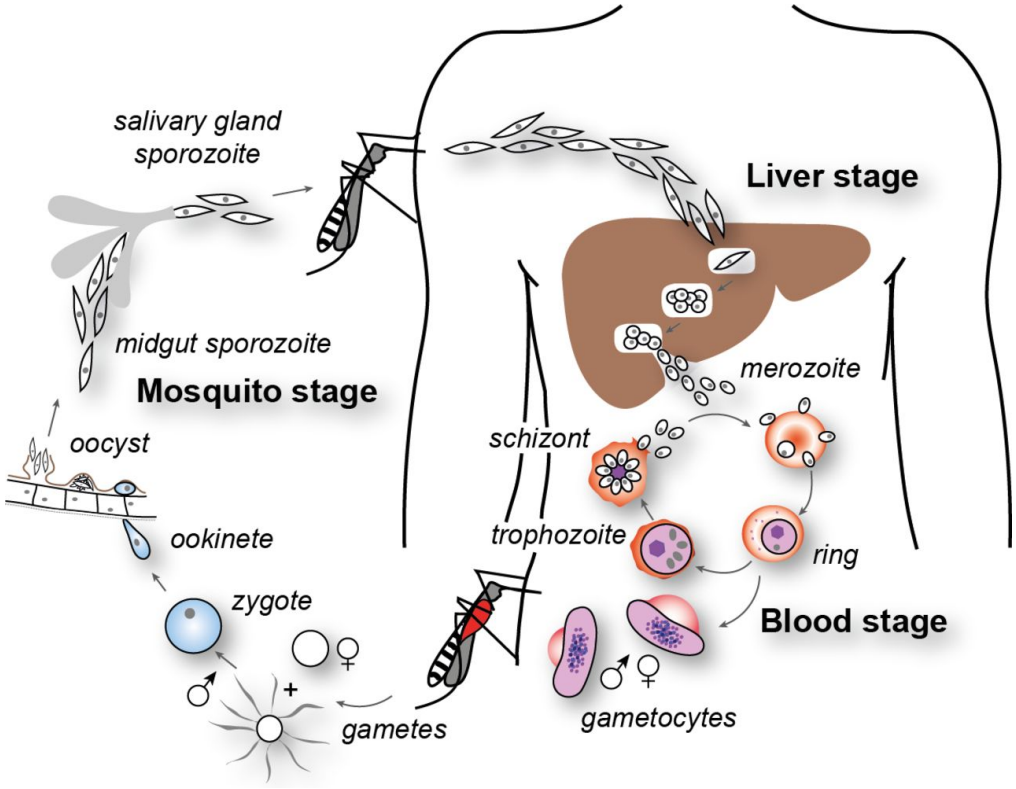


Figure 1.2. The life cycle of *Plasmodium spp.* showing different stages of the parasite in a vertebrate host and in mosquito vector. Image source: Cowman et al., 2012.

1.2.1. Development in the mammalian host

The developmental phase in the mammalian host can be divided into the skin stages (from the point of entry into the host to until they infect liver), the liver stages (infection and development in the hepatocytes up to the release of daughter parasites into the blood stream), the blood stages (development inside the erythrocytes), and the sexual stages (including sexual differentiation and gametocytogenesis).

1.2.1.1. The pre-erythrocytic phase

The life cycle is initiated with the bite of an infected female *Anopheles* mosquito, which injects crescent-shaped invasive stages known as sporozoites along with saliva into the skin of a vertebrate host during a blood meal. During the mosquito bites, sporozoites are mostly deposited in the dermis of skin tissue (Sidjanski and Vanderberg, 1997; Matsuoka et al., 2002). It has been shown in studies with rodent malaria parasites very few sporozoites are deposited in the skin by one mosquito under experimental conditions, however in natural bites less than 50 sporozoites were delivered per bite (Medica and Sinnis, 2005; Sidjanski & Vanderberg, 1997). Once injected, sporozoites follow random developmental paths, some leave the injection site by active gliding motility in one to three hours (Yamauchi et al., 2007) and eventually invade blood or lymph vessels (Amino et al., 2006). The sporozoites that remain in the inoculation site, some are destroyed in the skin, while some undergo exoerythrocytic development in the skin in case of rodent malaria parasites (Gueirard et al., 2010; Voza et al., 2012). The sporozoites that invaded the lymph vessels are taken to lymph nodes, where they are killed and processed by dendritic cells to prime the CD8(+) T lymphocytes (Amino et al., 2006; Chakravarty et al., 2007). Majority of the sporozoites that left the bite site manage to invade blood vessels and continue their onward journey to infect the liver (Amino et al., 2006). Sporozoites that successfully entered the blood stream are transported to the liver, where they migrate through few hepatocytes before settling in one to undergo differentiation into thousands of erythrocyte-infecting merozoites (Mota et al. 2001; Prudencio et al. 2006). The infection of hepatocytes in the liver involves intricate maneuvers by the sporozoites. In order to gain access to the hepatocytes, sporozoites cross the sinusoidal barrier either by traversing through kupffer cells or endothelial cells (Pradel and Frevert 2001; Amino et al., 2008; Frevert et al., 2005; Frischknecht et al., 2004). Before undergoing productive invasion and final differentiation, sporozoites migrate through multiple cells in different ways (Tavares et al., 2013). Two mechanisms have been suggested for cell traversal of the sporozoites inside the liver. First one is a wounding model, where the sporozoite enters and exits the hepatocyte by breaching the cell membrane (Mota et al. 2001). In the second one, the sporozoites pass through the cells in transient vacuoles formed by invagination of the host cell plasma membrane at the point of entry and likely a membrane fusion at the exit site without injuring the traversed cells (Risco-Castillo et al., 2015). Irrespective of the mode of transmigration, the

sporozoites finally settle in a hepatocyte for further development. The productive invasion involves formation of a parasitophorous vacuole, in which the parasite resides within the host cell. The parasitophorous vacuole is derived from the host cell membrane and the proteins released from parasite secretory organelles (Mota et al., 2001; Prudencio et al., 2006). After productive invasion, the sporozoite undergoes exponential growth and rapid multiplication (schizogony) to form merozoites. Intracellular liver stage parasites can form up to 30,000 merozoites from a single infected hepatocyte, depending on the *Plasmodium* species (Meis and Verhave 1988). After completing the development, the merozoites are released from the infected hepatocyte in membrane bound vesicles called merosomes. The merosomes are formed after the parasite mediated rupture of parasitophorous vacuole and subsequent death of the host cell (Sturm et al., 2006; Graewe et al., 2011). Eventually the merosomes are released from the liver into the neighboring sinusoids through endothelial cells. The merosomes finally rupture releasing the merozoites into the blood stream, where they invade the red blood cells to undergo blood stage development (Vaughan et al., 2008; Falae et al., 2010).

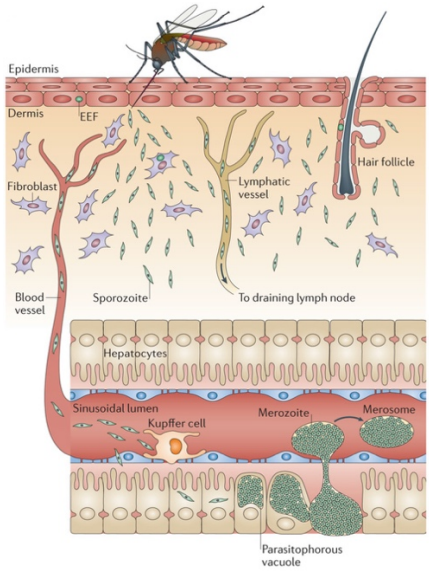


Figure 1.3. The pre-erythrocytic phase of *Plasmodium spp.* development in the mammalian host. Malaria infection is initiated when sporozoites are injected into the dermis by a feeding female *Anopheles* mosquito. Some of the sporozoites may remain in the skin while the others leave the bite site via blood or lymph. The sporozoites that enter the blood stream are transported to liver, where they exit the sinusoids through Kupffer or endothelial cells and actively transmigrate through several hepatocytes before a suitable one is found where, they form a PVM and undergo schizogony until tens of thousands of daughter merozoites are released in packets of merosomes into the vasculature. Image source: Menard et al., 2013.

1.2.1.2. The intra-erythrocytic phase

RBCs are well suited as host cells for the intracellular life style adapted by the apicomplexan parasites. Mature RBCs confer certain advantages to the parasites, as they do not display major histocompatibility complex antigens on their surfaces, they offer a perfect site to evade the host immunological attacks (Dowse and Soldati, 2004; Silvie et al., 2008; Foller et al., 2009). Also, readily available rich source of nutrients and relatively easy access to the mosquito vector might be the other factors behind Plasmodium preference to RBCs (Pasvol and Wilson, 1982). However, lack of standard biosynthetic pathways and the endocytic secretory pathways presents a challenge for the rapidly developing intracellular parasites. *Plasmodium* overcomes this hurdle by exporting a wide variety of proteins and virulence factors into the RBC cytoplasm.

Protein export into the host cell cytoplasm is facilitated by a combination of specific targeting sequence known as Plasmodium export element (PEXEL) or host targeting signal (HT) or vacuolar targeting signal (VTS) (Chang et al., 2008) and a recently discovered protein export machinery, *Plasmodium* translocon of exported proteins (PTEX) (de Koning-Ward et al., 2009). The PEXEL sequence required for protein export is a short pentameric sequence RxLxE/Q/D present in the exported proteins (Hiller et al., 2004; Marti et al., 2004). However, proteins lacking this particular sequence, termed PEXEL negative exported proteins (PNEPs), have also been found to be exported (Grüring et al., 2012; Heiber et al., 2013) suggesting redundancy in the protein export machinery. Proteins meant for export are secreted into parasitophorous vacuole lumen have shown to be associated with PTEX, which resides at the interface of parasitophorous vacuolar membrane and host cell cytoplasm. The PTEX complex consists of five components, PTEX150, PTEX88, HSP101, EXP2, and TRX2 (thioredoxin-2) (de Koning-Ward et al., 2009; Bullen et al., 2012; Matz et al., 2013). Together these five components form a complex, where the exported proteins are thought to be unfolded and translocated into the host cell cytoplasm (Haase and de Koning-Ward, 2010; Bullen et al., 2012). During intra-erythrocytic developmental cycle, malaria parasite progresses through four distinct stages, viz. the rings, the trophozoites, the schizonts, and the merozoites, about 8-24 of which are released at the end of each cycle (Lee and Fiddock, 2008). In general, in *P. falciparum*, it takes approximately 48 hours to complete one intra-erythrocytic developmental cycle and

22-24 hours for *P. berghei* (Rielly et al., 2007). Unlike *P. falciparum*, the *in vivo* blood stage development is asynchronous in *P. berghei* (Trager and Jensen, 1976). Reticulocytes are preferred host cells for *P. berghei, P. chabaudi,* and to a lesser extent for *P. falciparum*, although they can invade mature RBCs (McNally et al., 1992; Pasvol et al., 1980). The intra-erythrocytic developmental cycle begins with invasion of RBCs by liver stage merozoites. The invasion of RBCs by merozoites is an active process that includes many steps involving multiple ligand-receptor interactions (Cowman and Crabb, 2006; Silvie et al., 2008). The merozoites have specialized apical secretory organelles, such as the rhoptries, micronemes, and dense granules to aid the invasion process (Kats et al., 2006; Dowse and Soldati, 2004; Mercier et al., 2005).

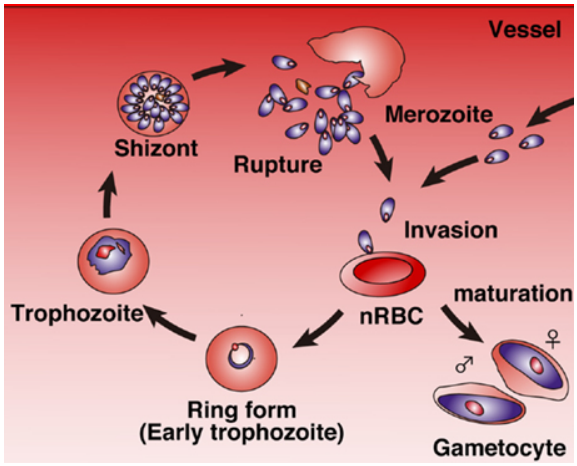


Figure 1.4. The intra-erythrocytic phase of *Plasmodium spp.* development in the mammalian host. Following intra-hepatic development, tens of thousands of merozoites are released into the blood stream, which invade red blood cells. The parasites then undergo repeated rounds of asexual multiplication, progressing through ring, trophozoite and schizont stages in each round. A small proportion (<10% of parasites) of parasites in each round develop into gametocytes, the sexual forms that are subsequently transmitted to the mosquito hosts. Image source: Inoue et al., 2013.

During invasion, the contents of secretory organelles are secreted onto the merozoite surface and subsequently into host cell enabling formation of parasitophorous vacuole, within which the parasite resides until the completion of its development (Riglar et al., 2011). Time-lapse imaging studies has revealed that invasion is a rapid process and it involves distinct phases. The initial phase includes the contact of merozoite with RBC cell surface, apical orientation of merozoite, and RBC deformation. The later phase includes the entry of merozoite into the RBC

and subsequent internalization. Also, a post invasive phase that includes alteration of RBC (Gilson and Crabb, 2009). The initial attachment of merozoites to the RBCs and subsequent reorientation is supposedly mediated by interactions between parasite cell surface proteins and host cell receptors (Mitchell et al., 2004, Sanders et al., 2005). A moving junction (MJ) is thought to be formed before invasion, which actually facilitates the entry of parasite into the host cell (Aikawa, 1978). Many surface proteins of merozoites that play role in invasion appears to be conserved between rodent and human malaria parasites (Chitnis and Blackman, 2000; Cowman et al., 2000). Recently, few host cell receptors, such as, Basigin (BSG), and reticulocyte binding protein 5 or Rh5 (*Pf*RH5) have shown to be involved in parasite invasion (Crosnier et al., 2011; van Ooij, 2011). On the other hand, initial studies have shown that invasion is an active process mediated by actin-myosin motor based machinery called glideosome, which includes a number of proteins such as actin, MyoA, MIC2 and AMA1 (Huynh and Carruthers, 2006; Matuschewski and Schuler, 2008; Soldati-Favre, 2008; Daher and Soldati-Favre, 2009). Invasion of RBCs by merozites is critical to the parasite life cycle and malaria pathogenesis thus making it crucial for the vaccine and drug development efforts. *Plasmodium* parasites progresses through distinct stages during intra-erythrocytic developmental process, which is reflected in constant changes in parasite forms inside the RBCs. These stages are distinguished based on their morphological appearance after nuclear staining (Arnot and Gull, 1998; Bannister et al., 2000). After invasion of RBC, the merozoite resides inside a parasitophorous vacuole. The shape of the parasite at this stage resembles a signet ring, hence the name ring stages. At this stage the parasite feeds on haemoglobin and synthesizes proteins required for extensive modification of RBC. The continuous export of parasite proteins leads to changes in the cyto-adherence and cellular architecture of infected RBCs. During the course of next 12 hours, parasite actively feeds and becomes more rounded as the food vacuole expands. Digestion of haemoglobin results in formation of catabolic by-products termed haemozoin crystals. The appearance of this crystals is characteristic feature of trophozoite stages (Bannister and Mitchell, 2003). After considerable growth, the parasite undergoes nuclear divisions to form about 16 daughter nuclei. These stages are called schizonts. Nuclear divisions are followed by cytoplasmic divisions, which results in formation of merozoite buds. The daughter nuclei move into the merozoite buds at the periphery of schizonts. Eventually, about 8-24 mature merozoites exit the PVM and RBC into blood circulation to invade a new RBC (Bannister and Mitchell, 1998; Lee and Fidock, 2008).

This process of repeated invasion, growth, replication and release termed asexual blood stage development. Freshly released daughter merozoites find RBCs within minutes to initiate a new intra-erythrocytic cycle. The erythrocytic developmental phase continues in a cyclical manner resulting in increased parasitemia levels in the host. The detailed molecular processes involved in infected erythrocyte rupture and subsequent release of merozoites are not yet fully understood (Lee and Fiddock, 2008). However, recent studies provided some insights into the key molecular players and their roles in merozoite egress. The whole process of parasite egress is thought to be a highly orchestrated process, which involves a variety of factors including proteases and kinases. (Miller et al., 2002; Yeoh et al., 2007; Arastu-Kapur et al., 2008; Collins et al., 2013). All the events of parasite egress are believed to be highly coordinated and controlled to ensure complete development and there is more than one model proposed for merozoite egress from the RBC (Janse and Waters, 2007; Clavijo et al., 1998; Winograd et al., 1999; Lew, 2001).

1.2.1.3. Sexual differentiation and Gameotcytogenesis

Asexual development within RBCs enables proliferation of parasites, however, transmission to mosquito hosts necessitates sexual differentiation to form gametocytes, the sexual precursor cells. During each asexual cycle, a certain but variable proportion of parasites differentiate into gametocytes. It has been observed that 5-25% of blood stage parasites undergo sexual differentiation into gametocytes (Sinden, 1983; Mons, 1986). These sexual precursor cells referred as microgametocytes (males) and macrogametocytes (females) are haploid and eventually form male and female gametes respectively. The time required for sexual differentiation and gametocyte maturation varies between rodent and human malaria parasites. *P. berghei* and *P. yoelii* require 25-33 hours post merozoite invasion for gametocyte maturation, demonstrated by the onset of exflagellation of male gametocytes (Mons et al., 1985; Garnham, 1966). Whereas, in *P. falciparum* gametocyte maturation is a much lengthier process taking 8-12 days' post RBC invasion by a merozoite (Field and Shute, 1956; Hawking et al., 1971). The sex ratios are female biased in Plasmodium, where male gametes can fertilize more than one female (Hamilton, 1967; Reece et al.,2008) and this behaviour is attribute to genetic component (Burkot et al., 1984). In *P. berghei*, gametocytes are morphologically indistinguishable from asexual stages. Morphological appearance of sexual dimorphism begins

only at 20-26 hours in *P. berghei* and around 6-10 days in *P. falciparum* (Janse and Waters, 2004; Alano and Billker, 2005). Mature gametocytes appear morphologically different from asexual parasites by their occupation of whole RBC and the characteristic presence of single large nucleus and pigmentation. However, the sexual differentiation process is believed to begin much before than the actual appearance of sexual dimorphism. Two models of sexual differentiation have been proposed for *P. falciparum*. According to the first model, the early blood stages after merozoite invasion are uncommitted and they retain the potential either to become a gametocyte or continue in the asexual cycle. The second model proposes that invading merozoites released from a single schizont are pre-committed in their previous developmental cycle either to become a gametocyte or to enter the asexual cycle (Bruce et al., 1990; Reininger et al., 2012; Silvestrini et al., 2000). The detailed molecular mechanisms behind sexual commitment and sexual differentiation remains largely unknown. However, high throughput studies in the last decade shed light on some aspects of the gametocyte development and differentiation. Transcriptome studies in *P. falciparum* suggests that 200-300 gametocyte specific transcripts are significantly upregulated in the initial stages of sexual development (Eksi et al., 2005; Silvestrini et al., 2005; Young et al., 2005). A comparative study of stage-specific proteome in *P. berghei* revealed sex-specific divergence of proteins with only 69 proteins shared among the male and female gametocytes (Khan et al., 2005). Sex-specific proteomic data shows that male gametocytes have 36% of unique proteins whereas female gametocytes have 19% of unique proteins (Khan et al., 2005). Male and female gametocytes are strikingly different with respect to cellular and molecular architecture. Female gametocytes carry transcripts necessary for zygote development post fertilization in translationally repressed state and this repression is mediated by DOZI (development of zygote inhibited) complex (Paton et al., 1993; Mair et al., 2006). Both male and female gametocytes possess osmophilic bodies, which are secretory organelles that aid the gametocyte egress and emergence (Lal et al., 2009; Ponzi et al., 2009). Reverse genetics studies in *P. falciparum* identified genes encoding proteins that play important roles during the gametocyte development and differentiation. Pfs16 and Pfg27/25 are identified as molecular markers that are expressed in the first 24 hours of gametocyte development (Silvestrinin et al., 2005; Bruce et al., 1994; Carter et al., 1989; Olivieri et al., 2009). The other proteins that are expressed during the early stages of gametocyte development include Pfpeg3 or Pfmdv1, Pfpeg4, Pfg14.744 and Pfg14.748, Pfs47, and Pfgeco (Eksi et al., 2005; Silvestrini et al., 2010).

All these genes are clustered together and indicated as *Pfge* (*P. falciparum* gametocytogenesis early) and most of them are located in the PVM to be exported (Alano, 2007; Morahan et al., 2011; Molina-cruz etal., 2013). In depth understanding of sexual commitment and sexual differentiation in *P. falciparum* appears to be difficult as it necessitates large scale purification of gametocytes, which is difficult and also long term *in vitro* cultures induces genetic variations in parasites (Alano, 2007). However, *P. berghei* is likely a suitable model for studying the gametocyte biology as sex-specific transcripts and proteins are highly conserved between two species.

1.2.2. Development in the mosquito host

1.2.2.1. Gametogenesis, Fertilization and Ookinete formation

The *Plasmodium* parasites undergo sexual reproduction in the mosquito host. The parasite journey in the mosquito begins with the ingestion of infected blood containing gametocytes. In the mosquito midgut lumen, the female and male gametocytes undergo gametogenesis, the process of gametes formation. Maturation of gametocytes into gametes is believed to be induced by exposure to environmental and mosquito-specific factors. These include a drop in temperature, rise in pH, and exposure to gametocyte activating factor (GAF), xanthurenic acid (Sinden et al., 1996; Nijhout and carter, 1978; Billker et al., 1997; Garcia et al., 1997;). Within 15-30 min of ingestion, gametocytes escape from RBCs to form gametes (Billker et al., 1998). After activation, the female gametocyte differentiates into a single, spherical female gamete (macrogamete) whereas the male gametocyte undergoes exflagellation and produces 8 haploid male gametes (microgamete). While male gametogenesis involves nuclear divisions and axoneme assembly, female gametogenesis involves switching off DOZI- mediated translational repression (Toye et al., 1977; Raabe et al., 2009; Mair et al., 2006). The exact signalling mechanisms that regulate the events of gametogenesis remains unknown. However, studies have shown that signal transduction events lead to increase in calcium levels in the cytoplasm of gametocytes, which in turn induces gametogenesis via activation of calcium-dependent protein kinase (CDPK) and mitogen-activated protein kinase (MAP) (Sinden, 2009; Tewari et al., 2010; Billker et al., 2004). In *P. berghei*, it has been shown that a MAP kinase-2 (Pbmap-2) regulates cytokinesis and axoneme motility (Tewari et al., 2005). Suppressive Subtractive Hybridization studies in *P. falciparum* identified differentially expressed genes and some of

them were implicated in signalling during gametogenesis (Ngwa et al., 2013). Soon after gametogenesis (10 min to 1 hr), fertilization takes place in the mosquito midgut lumen by fusion of haploid male and female gametes, which results in the formation of a diploid zygote (Janse et al., 1986). A number of proteins expressed by male and female gametes have been shown to play important roles in gamete fertilization and zygote formation (Thomson et al., 2001; Vandijk et al., 2001). *Plasmodium* parasites faces severe population bottlenecks and reduction in parasite numbers occurs at each developmental step during their development in the mosquito vector (Sinden et al., 1996). Therefore, successful transmission through mosquito host is critical for its survival and the outcome is determined by several factors, such as the vertebrate host, the parasite, and the mosquito. As many as 24 proteins have been discovered, which are expressed during gametogenesis and fertilization. These proteins include P48/45, P47, P230, P25, and P28 and reverse genetics studies have shown that these are essential for gametogenesis and fertilization and hence considered as potential transmission blocking vaccine candidates (Targett et al., 1990; Sinden et al., 2012). After 2-3 hrs of fertilization, the diploid zygote subsequently undergoes meiosis to become tetraploid (Sinden and Hartley, 1985; Janse et al., 1986). Meiosis is not immediately followed by karyokinesis and cell divisions. The spherical zygote with 2N or 4N DNA differentiates into a banana-shaped motile ookinetes that migrate in blood bolus to invade the mosquito midgut epithelium. It has been demonstrated that a protein kinase, NEK4 (NIMA [never in mitosis *Aspergillus*]-related kinase 4), is essential for the development of ookinetes from zygotes (Reininger et al., 2005).

1.2.2.2. Ookinete to Oocyst transformation

Similar to other motile stages of *Plasmodium*, ookinetes exhibit actin-myosin based gliding motility. The components and machinery that supports gliding locomotion is conserved among all the apicomplexan members (Matuschewshski and Schuller, 2008). The components of gliding machinery include the actin-myosin motor, an inner membrane complex (IMC), and accessory proteins; gliding associated proteins 45 (GAP 45), 50 (GAP50), and MyoA-tail interacting protein (MTIP) (Meissner et al., 2002; Morrissette and Sibley 2002; Gaskins et al., 2004; Bergman et al., 2003). Soon after transformation, the motile ookinetes which are still in the mosquito midgut lumen invade the midgut epithelium and up on reaching the basal side, begins transforming into sessile oocysts. Ookinetes like the other invasive stages of *Plasmodium*

possess an apical complex that contains secretory organelles, which facilitates invasion and traversal of cells of the midgut epithelium. Gliding motility and midgut epithelium invasion by ookinetes has been shown to be strictly regulated by cGMP and phosphodiesterase meditated signalling (Hirai et al., 2006; Moon et al., 2009). After traversing the midgut epithelium, the mature ookinetes settle between the basement cell membrane and the basal lamina of the midgut wall (Han et al., 2000; Sinden and Billingsley, 2001). The midgut epithelial cells undergo apoptosis after invasion by ookinetes (Valchou et al., 2004). The process of midgut invasion by ookinetes is a complex process and aided by a number of factor expressed on the surface of ookinetes and their interaction with mosquito midgut tissue (Gosh et al., 2011). Many of these ookinete surface proteins that interact with peritrophic matrix, midgut epithelium, and basal lamina are highly conserved between rodent and human malaria parasites. These proteins include Chitinase, Circumsporozoite and TRAP related protein (CTRP), secreted ookinete adhesive protein (SOAP), and the major GPI-anchored surface proteins, P25 and P28 (Tomas et al., 2001; Dessens et al., 2003; Yuda et al., 1999). After transmigrating through the midgut epithelium, the ookinetes come in contact with the midgut basal lamina where they initiate their subsequent development into oocysts. Transformation of ookinetes into oocysts takes place underneath the basal lamina, which is thought to be beneficial to the parasite as the developing oocyst is hidden from the mosquito immune system. The fate of the malaria parasite in the mosquito and successful transmission to the vertebrate host are largely dependent on small number of ookinetes that cross the midgut epithelium and develop into oocysts. A large fraction of the parasite losses occurs at this transition from blood bolus-born ookinete to the oocyst developing beneath the basal lamina. Ookinetes must overcome a number of challenges to successfully develop into oocysts.

1.2.2.3. Oocyst differentiation and Sporozoite formation

Oocyst represents the only extracellular developmental stage of the malaria parasite life cycle and the development of oocysts is relatively long and takes about 10-13 days. During development, growth of the oocysts is accompanied by increase in their size from 2-3 μm up to 50-60 μm in diameter, stretching the surrounding basal lamina (Meis et al., 1992; Aly et al., 2009). A remarkable feature of cell division in apicomplexan parasites is schizogony, where karyokinesis is not immediately followed by cytokinesis (Aly et al., 2009).

The signalling mechanisms that regulate coordination of these events remains unknown (Sinden and Matuschewski, 2005). During the expansion phase, the oocysts undergo several mitotic divisions resulting in the formation of several nuclei, followed by a series of complex changes, which transforms the oocyst into vacuolar structure (Terzakis et al., 1967). Throughout the growth phase, the oocyst capsule remains a bi-layered structure, where the thick outer membrane is made of laminin derived from the mosquito basal lamina and inner layer, the oocyst plasma membrane is covered with a dense coat of CSP (Circumsporozoite protein) (Hamilton et al., 1988; Posthuma et al., 1988). Eventually, the oocyst cytoplasm becomes segregated and subdivided into sporoblasts, which acts as budding centres for hundreds of sporozoites (Terzakis et al., 1967). The sporoblasts are formed by the internal invagination of oocyst plasma membrane and results in the retraction of cytoplasm into lobular structures. CSP plays an important role in oocyst development. Few days after oocyst formation, the expression of CSP begins and gradually accumulates on the oocyst plasma membrane (Thathy et al., 2002). It has been demonstrated that CSP is essential for the formation of sporoblasts, and therefore sporozoites (Menard et al., 1997). In another study, it was shown that formation of sporoblasts and cytokinesis largely depends on the GPI-anchor of CSP and interfering with the location of GPI-anchor domain within CSP resulted in developmental defects (Wang et al., 2005). Oocyst development culminates with formation of several hundreds of sporozoites by within the sporoblasts and sporozoites eventually bud out from the sporoblasts (Baton and Ranford-Cartwright, 2005). The budding process starts halfway into oocyst development with the help of microtubule organizing centres (MTOCs). The MTOCs, positioned just beneath the sporoblast membrane, facilitate the formation of apical complex and nuclei incorporation into daughter sporozoites following cytokinesis (Thathy et al., 2002). The molecular mechanisms behind the formation and organization of MTOCs remains not known. After development, the sporozoites bud off from the sporoblasts in asynchronous manner. LAPs (LCCL/lectin adhesive-like proteins), a group of proteins that are specific for female gametocytes and ookinetes shown to play an important role in sporozoite formation (Lavzec et al., 2009; Pradel et al., 2004; Trueman et al., 2004; Raine et al., 2007). The budding of sporozoites from sporoblasts appear to be asynchronous and happens in successive waves (Vanderberg and Rhodin, 1967; Sinden and Strong, 1978). The mature sporozoites develop crescent shape after completion of budding from sporoblasts. It is believed the crescent shape is formed as a result of the uneven distribution of the microtubules attached to the inner

membrance complex (IMC) of the sporozoites. Studies have shown that the sporozoite cell shape is determined by interactions of subpellicular network with microtubules (Mann and Beckers, 2001; Khater et al., 2004).

1.2.2.4. Sporozoite egress and Salivary gland invasion

After completing the sporogony, unlike the other apicomplexan parasites, thousands of sporozoites are released from the oocysts into mosquito haemolymph. Ultrastructural studies have shown that sporozoites escape to the haemolymph through small pores present in the oocyst capsule (Sinden, 1974). Initially, the sporozoite egress from oocysts was believed to be a passive process and happens as a consequence of oocyst expansion and sporozoite accumulation inside the oocysts. However, knockout studies in *P. berghei* revealed that a putative cysteine protease known as ECP1 (egress cysteine protease 1) is essential for the sporozoite egress from oocyts (Aly and Matuschewski, 2005). Targeted gene disruption of *PbECP1* resulted in mature oocysts containing viable sporozoites that fail to exit the oocysts. Losing the ability to exit the oocysts lead to continuous circular movements of sporozoites trapped within the oocysts. This intraoocyst motility suggests that sporozoite egress is an active process mediated by motility, which presides the oocyst rupture and a prerequisite for egress (Aly and Matuschewski, 2005). In another simultaneous study, mutation of positively charged residues in the thrombospondin repeat (TSR, also called region-II plus) of the CSP resulted in a phenotype that was identical to *ECP1(-)* sporozoites (Wang et al., 2005). This result implied that ECP1 is either directly or indirectly involved in CSP processing in the oocyst. Additionally, in another study, deletion of a hypothetical protein expressed in *P. berghei* oocysts resulted in an egress defective sporozoites similar to the region II plus of CSP and *ECP1(-)* mutants. These studies demonstrate that sporozoite motility and egress is a complex process regulated by multiple factors and the mechanisms that regulate sporozoite motility, sporozoite egress and the sequence of these events and molecular interactions remains to be uncovered.

After the sporozoites are released, they are carried along by haemolymph, the circulation system of the mosquito, to all tissues of the mosquito (Sinden and Matuschewski, 2005). During their passive haemolymph passage, sporozoites come in contact with the basal lamina of salivary glands and initiate a complex series of events through which the sporozoites successfully reach the ducts of salivary glands. During the brief encounter, the ligands on the

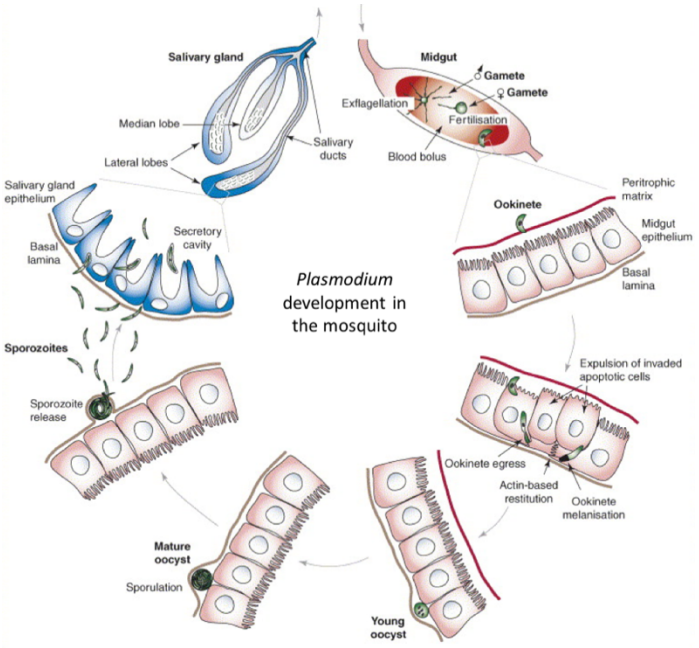


Figure 1.5. The development of *Plasmodium spp.* in the mosquito host. While feeding on an infected vertebrate host, the female *Anopheles* mosquitoes take up parasitized erythrocytes, which may include mature male and female gametocytes. In the mosquito midgut, they differentiate into micro and macro gametes, which fuse to form a zygote transforming over 24 hr into a ookinete that migrates through the mosquito midgut epithelium and encysts to become an oocyst where asexual replication occurs to form hundreds of sporozoites. These motile sporozoites are released into the hemocoel by oocyst rupture and migrate to salivary glands where they can be transmitted to the next human host. Image source: Vlachou et al., 2006.

sporozoite surface recognize specific host receptors that allow the attachment to the basal lamina of the salivary glands. The attached sporozoites eventually reach the salivary gland duct via the secretory acinar cells. Invasion and transmigration through the acinar cells is thought to occur in transient vacuoles originated from the invagination of acinar cell membrane (Pimental et al., 1994). However, the nature and molecular make up of such transient vacuoles are not yet understood. A number of proteins that facilitate sporozoite attachment to the salivary glands have been identified. Region I of CSP has been shown to specifically bind to the salivary gland lobes (Sidanjski et al., 1997). Furthermore, *P. bergehi* mutant parasites carrying *P. gallinaceum* CSP instead of endogenous copy exhibited a defect in entry of salivary glands (Tewari et al., 2005). Additionally, several studies have shown that antibodies to CSP inhibit sporozoite invasion of salivary glands

(Rosario et al., 1989; Warburg et al., 1992). Another parasite protein essential for attachment and invasion of salivary glands is the thrombospondin-related anonymous protein (TRAP). Targeted gene disruption studies resulted in viable sporozoites that lack gliding motility, fail to invade salivary glands, and remain in the haemolymph (Sultan et al., 1997). Both the extracellular region (adhesive A domain and thrombospondin repeat domain) and intracellular cytoplasmic tail domain are involved in attachment and invasion of salivary glands. These domains, which acts as a link between the transmembrane protein and the actin-myosin motor, also have a role in invasion of hepatocytes (Kappe et al., 1999; Wengelink et al., 1999; Matuschewski et al., 2002). Numerous studies have led to identifying an actin-myosin motor located between the parasite plasma membrane and the IMC powers the gliding motility and invasion of host cells by invasive stages of the parasite (Kappe et al., 2004).

A number of reverse genetics studies have identified other proteins that have important roles in gliding motility and salivary gland invasion. MAEBL, a micronemal protein expressed in oocysts, haemolymph, and salivary gland sporozoites, has a role in salivary gland invasion. Mutant *P. bergehi* sporozoites lacking MAEBL were unable to attach to the basal lamina of salivary glands (Kariu et al., 2002). Targeted deletion of cysteine repeats modular proteins (CRMPs) 1 and 2 in *P. bergehi* revealed that these sporozoite surface proteins have a role in salivary gland invasion (Thompson et al., 2007). UOS3, also named TRAP-related protein (TREP) and S6 has similar domains to that of TRAP. Genetic ablation studies in *P. bergehi* and *P. yoelii* resulted in haemolymph sporozoites that fail to invade salivary glands (Combe et al., 2009; Steinbuechel and Matuschewski, 2009; Mikolajczak 2008).

1.2.2.5. The sporozoite maturation

Plasmodium development in the mosquito vector completes when the sporozoites reach the salivary gland ducts. While awaiting transmission to the vertebrate host, sporozoites undergo maturation process, which enables them to accomplish migration and infection of a variety of cells in the mammalian host. Generation of transgenic fluorescent sporozoites facilitated the study of initial events during mammalian host infection using intravital imaging approaches (Frischknecht et al., 2004; Franke-Fayard et al., 2004; Vanderberg and Frevert, 2004; Frevert et al., 2005; Amino et al., 2006). Initial studies demonstrated that the salivary gland sporozoites

are irreversibly programmed to infect the vertebrate host (Touray et al., 1992). While the sporozoites in the salivary glands display robust gliding motility and are highly infectious *in vivo* and *in vitro*, the oocyst sporozoites are not (Vanderberg, 1974). Findings from these early studies suggest that the sporozoites in the salivary glands are different from oocyst sporozoites (Vanderberg, 1975). It is assumed that the sporozoites acquire competence for natural transmission during their haemolymph passage and subsequent salivary gland invasion. Differential gene expression profile studies using cDNA subtraction led to identification of a set of genes upregulated in infectious sporozoites (UIS) (Matuschewski et al., 2002). This study confirmed that different transcriptional repertoires are associated with different distinct sporozoite phenotypes. Functional characterization of these UIS genes using reverse genetics confirmed that UIS genes encode proteins that play important roles at different stages during the pre-erythrocytic phase of *Plasmodium* life cycle. Among them are two transmembrane proteins encoded by *UIS3* and *UIS4*, shown to be essential for liver stage development (Mueller et al., 2005). The *uis3(-)* and *ui4(-)* mutant sporozoites are shown to induce long- lasting sterile protection against natural transmission in rodent malaria model similar to radiation attenuated sporozoites (RAS) (Mueller et al., 2005). Additional UIS genes that serve functions during pre-erythrocytic phase are *UIS10*/*PL*, *SPECT*, and *PLP1/SPECT2*, which plays a role in host cell traversal by sporozoites (Ishino et al., 2004; Ishino et al., 2005; Bhanot et al., 2005). Another comprehensive study of *UIS* genes in *P. yoelii* by microarray analysis identified a set of genes that are upregulated in oocyst sporozoites (UOS) but downregulated in salivary gland sporozoites and UOS genes are shown to be essential for salivary gland invasion only (Mikolajczak et al., 2008). The ubiquitous CSP present on the sporozoite surface plays many biological functions during pre-erythrocytic development. The CSP processing is required for sporozoite gliding motility, which in turn is essential for host cell traversal and invasion (Coppi et al., 2005). The CSP cleavage triggers the signalling events that initiate host cell invasion (Mota et al., 2002). CSP even plays an important role in liver stage development (Singh et al., 2007). These findings suggest that transcriptional control plays an important role in parasite development. Another recent study demonstrated that temperature shift that occurs after sprozoite transmission to the mammalian host causes an increase in transcript abundance of a number of genes that are involved in hepatocyte infection (Siau et al., 2008). From these functional analyses it is evident that mature sporozoites are perfectly equipped to face novel host environment after their transmission.

1.3. Clinical features and Pathogenesis of malaria

In general malaria is an acute febrile illness with an incubation period of 7 days or longer. The symptoms of malaria are caused by the development of *Plasmodium* parasites in the human red blood cells. The duration of incubation period, time interval between infection and the onset of symptoms, depends on several factors such as the species of infecting parasites, the density of parasite inoculum, the mode of transmission, and the immune status of the host. Incubation period ranges from 9 to 30 days with shortest for *P. falciparum* and *P. vivax*, and the more prolonged times for *P. malariae* (Bartolini and Zammarchi, 2012). The clinical manifestations of malaria, common to all the different *Plasmodium spp.*, are non-specific and appears similar to flu-syndrome. Fever being the hallmark of malaria, other symptoms include chills, fatigue, headache, weakness, muscular aching, vomiting, diarrhea, cough and abdominal pain (Miller et al., 2002). These symptoms are followed by febrile attacks also called as the malaria paroxysms. Theses paroxysms exhibit periodicity, 48 hours for *P. falciparum*, *P. vivax*, and *P. ovale* and 72 hours for *P. malariae*. At first, these paroxysms are irregular but later become regular because of synchronous reproductive cycles of the malaria parasite within the red blood cells. The malaria paroxysm corresponds to the rupture of infected erythrocytes and release of merozoites. The severity of these paroxysms and duration of symptoms varies from species to species. *P. falciparum* causes severe malaria involving multiple organs and may lead to death if untreated, whereas the other species are rarely mortal. *P. vivax* infections rarely results in complications or death and sometimes can lead to formation of dormant forms known as hypnozoites in the liver (Shute et al., 1946). Relapses due to the activation of quiescent hepatic hypnozoites can occur in later years, which may lead to sever disease in some cases (Cogswell et al., 1992). *P. ovale* causes the most benign malaria as the paroxysms are mild and have shorter durations. *P. malariae* in general causes a mild disease, however, it is the most chronic and recrudescence have been reported several years after the infection. Pathology associated with malaria is due to the rupture of infected erythrocytes and the release of parasite material, metabolites (hemozoin), and cellular debris (Schofield et al., 2002; White et al., 2014). The most severe form of malaria, caused by *P. falciparum*, consists of two major syndromes: (i) severe anemia, caused by destruction of RBCs; and (ii) cerebral malaria (CM), caused by obstruction of small blood vessels in the brain by parasite sequestration (Good and Milon, 1992). CM is characterized by an impaired consciousness, which ranges from stupor to coma.

The clinical presentations are severe headache followed by drowsiness, convulsions and ultimately coma. These neurological symptoms are believed to be due to the sequestration of infected erythrocytes in the cerebral microvasculature (Medana and Turner, 2006; Kyes et al., 2007). Sequestration occurs due to the cytoadherence of trophozoite and schizont stages of the parasite to endothelial cells of deep vasculature in vital organs especially in brain, lungs, heart, gut and placenta (Kyes et al., 2007; Desai et al., 2007). The cytoadherence ability confers several advantages to the parasites, the major advantages include avoidance of elimination of infected erythrocytes by splenic clearance and availability of low oxygen conditions. The pathophysiology of CM is not completely understood and likely involves multiple factors and complex interactions between the host and parasite (Mackintosh et al., 2004). Moreover, in recent years, it has been recognised that severe malaria caused by *P. falciparum*, is a complex multi-system disorder with a broad range of clinical features. The key challenge is to uncover the key mechanism of disease and identifying suitable targets for adjunctive therapy.

1.4. Diagnosis, Treatment and Control of malaria

Malaria is a potential health emergency and often life threatening, hence treated accordingly. Delays in diagnosis and treatment are leading causes of death in some regions. Prompt, accurate, and effective diagnosis is crucial for the management and control of malaria. Developing novel and effective diagnostic strategies is essential especially in resource-limited endemic countries, where malaria is a substantial burden on society. Malaria diagnosis involves identification of malaria parasites or antigens/products in patient blood. Although diagnosis appears to be a simple procedure, the outcome and efficacy of the diagnosis is subject to many factors such as the infecting *Plasmodium* species, the developmental stages of infected erythrocytes, parasitemia, host immune-status, signs and symptoms, and sequestration of the parasites in the deeper tissues. Initially, the diagnosis is conducted at healthcare centers by physicians and is based on patient's symptoms, physical findings at examination and travel history. A clinical diagnosis of malaria is still challenging due to the non-specific and variable nature of malaria symptoms, which overlap with other diseases. The overlapping of symptoms

with other tropical diseases may result in misdiagnosis, non-treatment of malaria or over treatment of malaria. In general, clinical findings are always confirmed by a laboratory test for malaria (Bhandari et al., 2008). In the laboratory, malaria is diagnosed by different methods. Microscopic diagnosis using stained thin and thick peripheral blood smears, has been the gold standard for laboratory confirmation of malaria. However, it depends on the quality of the reagents, of the microscope and skills of the technician. Another improved microscopic technique is the QBC technique. This method involves staining parasite deoxyribonucleic acid (DNA) in micro-hematocrit tubes with acridine orange (Clendennen et al., 1995). Rapid diagnostic tests (RDTs) are simple, quick, cost-effective and offer a useful alternative where reliable microscopic diagnosis is not available (WHO, 1996). Various kits have been developed, currently 86 malaria RDTs are available in the market. These RDTs are relatively fast, easy to perform and do not require laboratory equipment or electricity (Bell et al., 2006). All the RDTs are based on immune-chromatographic detection of malaria antigens such as histidine rich protein II (HRP-II) and lactate dehydrogenase (LDH). Serological tests detect antibodies against malaria parasites, using either indirect immunofluorescence (IFA) or enzyme-linked immunosorbent assay (ELISA) (Sulzer et al., 1969; Doderer et al., 2007). These tests don't detect current infection but measures past exposure. Although traditional laboratory diagnostic methods are useful, they have major disadvantages. Development of novel techniques that display high sensitivity and high specificity, without subjective variation, are needed. Recent advancements in molecular biological technologies, e.g. PCR, loop-mediated isothermal amplification (LAMP), microarray, mass spectrometry (MS), and flow cytometric (FCM) assay techniques, have enabled extensive characterization of the malaria parasite and are facilitating development of new strategies for malaria diagnosis.

Malaria is an entirely preventable and treatable disease. The main objective of the treatment is rapid and complete elimination of *Plasmodium* parasites from the patient's blood. If left untreated, uncomplicated malaria may progress to severe disease or death. Timely treatment prevents chronic infection that leads to malaria-associated severe anaemia. In terms of public health perspective, the major goal of treatment is to reduce transmission of infection to other healthy individuals, reducing the infection reservoirs and to prevent emergence and spread of antimalarial resistance.

Antimalarial treatment is administered only when the patient has confirmed parasitological diagnosis either by microscopy or RDT. Treatment of a patient with malaria depends on the type (species) of infecting parasite, the geographical area where the infection was acquired and its drug resistance status, the clinical status of the patient, and any accompanying illness or condition. Most drugs used in the treatment of malaria are active against the blood stages of the *Plasmodium* parasite and they are:

Class	**Drug**	**Use**
4-Aminoquinoline	Chloroquine Amodiaquine Piperaquine	Treatment of Partner drug for ACT non-falciparum malaria ACT partner drug with dihydroartemisinin
8-Aminoquinoline	Primaquine Quinine	Radical cure and terminal prophylaxis of *P. vivax* and *P. ovale*; gametocytocidal drug for *P. falciparum* Treatment of *P. falciparum* and severe malaria
Arylamino alcohol	Mefloquine Lumefantrine	Prophylaxis and partner drug for ACT for treatment of *P. falciparum* malaria Combination with artemether as ACT
Sesquiterpene lactone endoperoxides	Artemether Artesunate Dihydroartemisinin	ACT: combination with lumefantrine ACT; treatment of severe malaria ACT: combination with piperaquine
Antifolate	Pyrimethamine/ sulfadoxine Atovaquone/ proguanil	Treatment of some chloroquine-resistant parasites; Combination with artesunate as ACT Combination for prophylaxis and treatment of *P. falciparum* (Malarone)
Antibiotics	Doxycycline Clindamycin	Chemoprophylaxis; treatment of *P. falciparum*

Table 1.1. Currently used antimalarial drugs. Source: Cui L et al., 2015

Vector control is the main way to prevent malaria incidence and transmission. If high degree of vector control is achieved in a particular geographical area, a measure of protection can be conferred among the people in that particular community. For people at risk of malaria, effective vector control measures can provide protection. WHO recommends two types of vector control: (i) usage of insecticide-treated mosquito nets (ITNs) and (ii) indoor residual

spraying (IRS). These two methods are most effective in prevention and control of malaria in high endemic regions under a wide range of circumstances. Long-lasting insecticidal nets (LLINs) are the preferred form of ITNs for public health programmes. 54% population was protected by this intervention in 2016. IRS, spraying the walls of homes with insecticides, is another effective way of prevention and controlling malaria in high endemic regions. Antimalarial medicines are also used to prevent malaria. Especially in case of pregnant women and travellers, malaria can be prevented by chemoprophylaxis, which supresses the malaria blood stage development thereby preventing malaria transmission. WHO recommends intermittent preventive treatment in pregnancy (IPTp) with sulfadoxine-pyrimethamine for pregnant women living in moderate-to-high transmission areas.

1.5. Challenges in malaria control

Remarkable progress has been achieved in malaria control, especially, the past 15 years have yielded positive results with global mortality rates decreasing by 47% between 2000 and 2013 (WHO, 2015). These achievements are largely due to unprecedented efforts in the past decade such as increased access to ITNs, the availability of a number of RDTs, expanded use of ACTs. Additionally, Increased funding along with improved function of the health systems, and the efficient use of control tools, have all contributed to reduction of disease incidence rates. However, the disease continues to be a major public health concern in Sub-Saharan Africa, where it poses the biggest challenge to global eradication initiative due to high prevalence and intensity of *P. falciparum* transmission in the region. The major challenges that global eradication efforts facing today are the lack of an effective vaccine, parasite resistance to antimalarial drugs, and vector resistance to insecticides. A combination of multiple strategies is needed to address the many challenges that the disease poses.

1.5.1. Malaria Vaccines

Currently there is no licensed vaccine against malaria and an efficacious vaccine would be an important tool in successful elimination of malaria. Despite global efforts in the past decades, research into malaria vaccines has failed to produce a commercially available and an effective vaccine that provides sterile and long lasting immunity (Crompton et al., 2010). The progress

of malaria vaccine development has been hindered by several difficulties, which may include poor understanding antimalarial immunity, lack of immune correlates of protection, and the genetic polymorphisms that cause antigenic variation in the malaria parasites (Ouattara and Laurens, 2015). Initial malaria vaccine research studies started in the 1930s with an emphasis on inactivated or killed parasites that failed to provide a protective immune response. In later studies, the addition of adjuvants enhanced the immunogenicity of malaria vaccine candidates in animal models (Freund et al., 1945). Modern malaria vaccine developmental efforts originated from immunization studies in mice with irradiated sporozoites, conducted in the 1960s (Nussenweig et al., 1967) and subsequent studies of mechanism of immunity in rodent models (Doolan et al., 1997). Pioneering immunization studies in humans demonstrated that lasting protection could be achieved in volunteers (Clyde, 1975; Nussenweig and Nussenweig, 1989). Current malaria vaccine development efforts involve a wide range of strategies focused on different parasite developmental stages: pre-erythrocytic vaccines, blood-stage vaccines, and transmission-blocking vaccines. A strong and effective immune response must act rapidly in order to kill the *Plasmodium* sporozoites during their onward journey from skin to the liver. Robust humoral and T-cell mediated responses are required to prevent liver infection, which is the main objective of pre-erythrocytic vaccines. The pre-eryhtrocytic stages (skin and liver) of *Plasmodium* are metabolically highly active but asymptomatic and silent. Attacking parasite at this stage has several advantages. This would prevent the symptomatic blood stage infection. The number of infected hepatocytes is low. The development human malaria parasites in the liver takes longer, which provides sufficient time and scope for elimination. The infected hepatocytes acts as a source of antigens to the host immune cells. However, under natural circumstances, infection with sporozoites do not confer highly effective pre-erythrocytic immunity in humans as observed in animal models.

The most effective and promising vaccine candidate to date is RTS,S a combination of hybrid protein particle and multi-component adjuvant named AS01. The RTS,S vaccine construct consists of central repeat (R) region fused to the C-terminal region of the circumsporozoite protein containing T-cell epitopes (T), which in turn is fused to the hepatitis B surface antigen (S) (Stoute et al., 1997). In a series of sporozoite challenge in studies in healthy subjects revealed that administration of RTS,S in combination with adjuvants AS01 or AS02 resulted in a of 30-50% sterile efficacy (Kester et al., 2009). Further immunological analyses have shown that

RTS,S induces a high antibody titres but modest T-cell immunogenicity (Olotu et al., 2010). Many phase I and II clinical trials of RTS,S in several African countries provided a clear evidence that RTS,S can reduce the acquisition of clinical malaria by 30-50% in different epidemiological settings (Aide et al., 2010). A recent phase III clinical trial of RTS,S/AS01 in Africa demonstrated an efficacy of 36.3% among children and 25.9% among young infants (Olotu et al., 2016). Since the efficacy of RTS,S/AS01 wanes overtime, which may lessen the usefulness of the vaccine in public health programmes. Due to limited efficacy achieved by RTS,S and other sub-unit vaccines, efforts are on to identify novel pre-erythrocytic vaccine candidates and to develop novel approaches such as whole parasite vaccines containing whole sporozoites (Hoffman et al., 2010). In parallel with efforts to develop irradiated sporozoites based whole parasite vaccines, significant progress has been made in developing genetically attenuated parasites (GAPs) that are incapable of progressing beyond liver stages due to lack of key genes (Vaughn et al., 2010).

Malaria disease is a consequence of parasite blood stage infection, hence blood-stage vaccines are aimed to prevent disease and death. The gradual acquisition of natural protection against clinical disease after repeated exposure to infection in high endemic areas prompted efforts to develop blood-stage malaria vaccines. Unlike the pre-erythrocytic vaccine development, results with blood stage developmental efforts have been disappointing and discouraging (Goodman et al., 2010). Multiple candidates have progressed to clinical trials but none of them has shown protective efficacy against clinical malaria. Several antigens expressed on the surface of infected RBCs include merozoite surface proteins 1,2, and 3; serine-repeat antigen; ring infected erythrocyte surface antigen (RESA); glutamate rich protein (GLURP); and apical membrane antigen 1 (AMA1) are considered for vaccine development. Recently 4 blood-stage vaccine candidates (AMA1, MSP1, MSP3, and GLURP) have undergone phase II clinical trials and none of the vaccines tested demonstrated significant protective efficacy (Thera et al., 2011). There are significant challenges in blood stage vaccine development. They are expression of conformationally correct antigens, low antibody titres, and extensive geneti polymorphism in many of the blood-stage vaccine candidates (Takala et al., 2009). Currently efforts are underway to identify better vaccine candidates.

Another interesting approach in malaria vaccine development efforts is mosquito stage vaccines that prevent transmission of malaria to the other people. These are called altruistic vaccines as these are not beneficial to the vaccinated people rather protect neighbour from getting infected (Carter and Chen, 1976). This type of vaccines would be deployed at community level hence can be an important tool in public health programmes. These vaccines are based on the principle that immunization with gametocyte or ookinete antigens may reduce or ablate oocyst development in the mosquito there by decreasing the scope of the transmission. To date, only one candidate has undergone clinical trials is P25 and it demonstrated significant transmission blocking activity (Wu Y et al., 2008). There has been renewed interest in development of transmission blocking vaccines candidates owing to their importance in malaria elimination and eradication efforts. The limited genetic polymorphism of the P25 encoding gene makes it an attractive target and identification of such broadly protective vaccine candidates is crucial to the success of malaria elimination efforts.

1.5.2. Antimalarial drug resistance

Antimalarial drugs are important tools for the control and elimination of malaria and remains a cornerstone in the treatment and control of malaria throughout the world. Antimalarial drugs play key roles in reducing the morbidity and mortality of malaria. First, administration of a particular drug regimen prevents severe disease and development of gametocytes, thereby blocking the transmission of malaria to mosquitoes. Second, in high endemic regions, drugs are used in various antimalarial strategies such as chemoprophylaxis, intermittent preventive therapy (IPT), and mass drug administration (Greenwood, 2010; Gosling et al., 2011). Most of the antimalarial drugs act primarily on erythrocytic stages of the malaria parasites, that are responsible for human disease. Currently, antimalarial drug resistance has become one of the greatest hurdles in controlling malaria. To date, drug resistance has been documented in three of the five malaria species known to affect humans in nature: *P. falciparum*, *P. vivax* and *P. malariae*. Antimalarial drug resistance has been defined as the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the tolerance of the subject (Bruce-Chwatt et al., 1986). Drug resistance has been implicated in the spread of malaria to new areas and re-emergence of malaria in areas where the disease had been eradicated. Drug

resistance has also played a major role in occurrence and severity of epidemics in certain parts of the world. Unfortunately, the current status appears to be bleak due to decreasing efficacies of frontline antimalarial drugs. At present, it has been reported that malaria parasites have acquired resistance to almost every antimalarial drug currently available, including the newest agents, artemisinins (Noedl et al., 2008; Dondorp et al., 2009). Drug resistance significantly increases the economic costs and complexity of achieving cure and elimination of the malaria. Further compounding the problem, the emergence and spread of drug resistance to antimalarial drugs outpaces the development of new drugs. For many drugs, the extent of resistance and the mechanisms of resistance are unknown. Drug resistance is further complicated by cross-resistance and multidrug resistance. Cross-resistance occurs when resistance to one drug confers resistance to other drugs that belong to the same chemical family or which have similar modes of action (WHO, 2010). Multidrug resistance is observed when the parasite is resistant to more than two antimalarial compounds of different chemical classes and modes of action. In case of *P. falciparum*, the two classes first affected by multidrug resistance are the 4-aminoquinolines and the antifolates. Drug resistance ultimately results in delay or failure to clear asexual parasites from the patient's blood, which subsequently allows production of gametocytes that are responsible for transmission of the disease.

In the past decade, most malaria-endemic countries have replaced failing drugs with highly effective ACTs (Artemisinin combination therapies). Despite the delayed responses to ACTs reported in the Greater Mekong sub-region, they remain the most effective treatment in Africa (WHO, 2011). Therefore, it is highly essential to contain the development and spread of Artemisinin resistance. The emergence of resistance in *Plasmodium* parasites can be attributed to several factors, including the mutation rate of the parasites, the fitness cost associated with the mutations conferring resistance, the parasite load, the strength of drug selection and the compliance to the treatment. The mutation rate of the parasite affects the frequency of resistance emergence. An increased mutation rate is advantageous to the parasite as it facilitates quick adaptation to changing environments (Sniegowski et al., 2000). Mutations associated with drug resistance often impart a fitness cost. The survival advantage attained by becoming drug-resistant is balanced by the biological cost caused by the altered function of the mutated protein (Levin et al., 2000). It has been shown that high parasitemias often can lead to faster accumulation of selective mutations (Hastings et al., 2004). Additionally,

inadequate drug exposure due to improper dosing, poor pharmacokinetic properties, poor quality of drugs and non-compliance to recommended drug regimen can all lead to parasite exposure to sub-optimal drug concentrations, which increases the likelihood of emergence of resistance.

Quinine is one of the oldest antimalarial medicines and has been in use for the treatment of malaria. The chemical synthesis of quinine was first described in 1944 and many synthetic alternatives were eventually developed (Butler et al., 2010). It is now routinely used to treat severe cases of malaria and in combination with antibiotics to treat resistance malaria. Chloroquine, a synthetic quinine, introduced in late 1940s used on a large scale for treatment and prevention of malaria. It remained a gold standard for malaria treatment due to its efficacy, affordability and safety (Alkadi, 2007). The molecular mechanism by which chloroquine acts against the *Plasmodium* is only partially understood. Similar to quinine, chloroquine has been demonstrated to accumulate in the parasite food vacuole (FV) and inhibits heme detoxification, an essential process for the survival of the parasite (Fitch, 2004). Chloroquine resistance has first emerged at Thai-Combodian border in the late 1950s (Mita et al., 2009). Chloroquine resistance is prevalent in almost all malaria endemic regions. Chloroquine is also the front-line treatment for *P. vivax* infections, however, chloroquine resistance *P. vivax* infections are rising. Recent studies demonstrated that the genetic basis for quinine and its related compounds is complex and probably multigenic with multiple genes influencing the susceptibility. Currently three transporter genes are linked to decreased sensitivity to quinine: *pfcrt* (*P. falciparum chloroquine resistance transporter*), *pfmdr1* (*P. falciparum multidrug resistance transporter 1*), and *pfnhe1* (*P. falciparum sodium/proton exchanger 1*), all of them encoding for transporter proteins (Cooper et al., 2002; Sidhu et al., 2005; Nkrumah, et al., 2009). The antifolate drugs that are used for malaria therapy are a combination of sulfa drugs (Sulfadoxine and dapsone) that inhibit the dihydropteoate synthetase (DHPS) enzyme and pyrimethamine and proguanil, which inhibits dihydrofolate reductase/thymidylate synthase (DHFR) enzyme (Srivatsava and Vaidya, 1999). This well-tolerated drug combination was introduced in 1970s and used to treat chloroquine-resistant malaria. However, resistance due to point mutations in both the target enzymes quickly emerged and currently this combination is mainly used in intermittent preventive malaria treatment of pregnant women.

Since the emergence of resistance to almost all quinolone and antifolate drugs used in treating *P. falciparum* malaria, artemisinin and its derivatives have been used as replacement therapy and currently the frontline drugs used worldwide in malaria treatment. Atremisinin is a natural product extracted from the *A. annua* (Chinese wormwood) and has been used as herbal remedy in China. Artemisinins have a complex and unique chemical structure, which contains a trioxane moiety with an endoperoxide bond (Eastman and Fiddock, 2009). Several semi-synthetic derivatives have been made for clinical use as the artemisinin has low solubility. Among the existing antimalarial drugs, artemisinins have the shortest half-lives (0.5-1.4 hrs) (Boland, 2001). Widespread use of artemisinin derivatives as monotherapy in Southeast Asia has probably contributed to delayed in parasite clearance time recently reported in Thai-Combodian border (Dondorp et al., 2009). The genes that confer this delayed parasite clearance are unknown. However, recent studies have suggested a region of chromosome 13 is involved and further studies have demonstrated strong correlation of delayed parasite clearance phenotype with four single-nucleotide polymorphisms on chromosomes 10,13, and 14 (Takala-Harrison S et al., 2013). Although, the exact mechanism that mediates the observed clinical phenotype is not known, recent genetic analysis has demonstrated that artemisinin susceptibility has a heritable component (Anderson et al., 2010). Currently to prevent the risk of resistance emergence and to prolong the life span of artemisinins, they are administered in combination with other antimalarial compounds (Muller et al., 2009).

There has been renewed interest in efforts to develop novel antimalarial medicines. Diverse strategies exist for the novel antimalarial drugs. Improving the therapeutic efficiency of existing drugs have not resulted in success. Most of the promising new classes of antimalarial compounds have come from high-throughput screens. In this strategy, a library of compounds is screened by phenotypic based or whole-cell based assays to identify compounds that are active against the parasite. Alternatively, the same libraries can be screened for activity against targets, parasite proteins that are essential for parasite survival. With the advent of new technologies, ongoing efforts are focused on identification of compounds that are active against all stages of the parasite life cycle (Peatey et al., 2011; Derbyshire et al., 2011). Target-based drug discovery is another popular approach for lead identification. Target are typically proteins with essential cellular functions, the inhibition of which results in cellular death. In this approach, targets can be identified based on the essentiality for parasite viability, which is

determined by genetic validation. After identification of the target, recombinant protein activity based screens are performed to identify potential chemical inhibitors. Identification of the key targets is crucial in target-based drug discovery approaches. Apart from the discovery and development of novel antimalarial drugs, improved understanding of the role of genetic polymorphisms in the parasite and the influence of drug resistance on the parasite fitness may aid in the identification of optimal dosing strategies.

1.6. The role of proteases in *Plasmodium* biology

Proteases are a group of enzymes that degrade proteins by peptide bond hydrolysis. Based on the mechanism of catalysis, proteases are classified into five distinct groups, aspartic, cysteine, metallo, serine, and threonine proteases. Proteases regulate the fate, localization, and activity of many proteins, modulate protein-protein interactions, create new bioactive molecules, contribute to the processing of cellular information, and generate, transduce, and amplify molecular signals. As a consequence of these multiple activities, proteases influence diverse biological processes. Likewise, many infectious organisms use proteases for their survival and replication. The availability of detailed information on active site structures and mechanism of action, make proteases attractive targets for drug development. Several protease inhibitors are already in clinical use for treatment of human diseases (Drag 2010).

Malaria parasites have a complex multistage life cycle that involves progression through different host environments and distinct parasite stages. Analysis of the existing *Plasmodium* genome sequences revealed that *Plasmodium* has evolved with sophisticated proteolytic machinery consisting of ~115-137 putative proteases, representing all five groups (Kuang 2009). The proteolytic repertoire of malaria parasites is likely to be involved in a large number of crucial processes during parasite development. However, to date, only few of these proteases have been studied and characterized. A series of studies based on chemical inhibition of proteases and functional genetics identified *Plasmodium* proteases that play regulatory as well as effector functions in a variety of essential cellular processes. Most of the well characterized proteases belong to *Plasmodium falciparum*, as it is the most virulent and extensively studied *Plasmodium* species. However, it is of utmost significance to identify and validate which among the predicted proteases are likely to be ideal therapeutic targets. *Plasmodium* parasites while progressing through a complicated series of sexual and asexual developmental phases,

the motile, non-replicating extracellular forms of the parasites known as zoites first invade host cells, grow and replicate within these cells, and rupture in the end to establish further infections (Black and Boothroyd, 2000; Striepen et al., 2007). These molecular events necessitate the expression of many specialized proteins to coordinate these diverse cellular activities.

1.6.1. Invasion of host cells

The developmental cycle of malaria parasites involves multiple invasion events, with invasion of salivary gland and hepatocytes by sporozoites, erythrocytes by merozoites, and the mosquito midgut by ookinetes. Being obligate intracellular parasites, it is essential for the *Plasmodium* extracellular forms, called zoites, to rapidly invade host cells. Invasion of host cells takes place in a very short time as the zoites cannot survive outside host cells for longer periods of time. Merozoites and sporozoites share conserved mecahnisms of invasion. Invasion begins with initial contact of the host cell by the polar zoite, which reorients itself in a way, the apical end contacts the host cell membrane (Soldati et al., 2004). A moving junction is formed between the between the parasite and host cell as a result of the multiple interactions between parasite proteins host membrane receptors. During progress of the invasion, this junction moves using an actin/myosin motor from apical to the posterior end of the zoite. The continuous trafficking of proteins on the parasite surface, helps creating an invagination within the host cell and the parasite drags the host cell membrane resulting in formation of parasitophorous vacuole (PV), and sealing of the PV and host cell membranes (Kim K, 2004; Blackman, 2004). When invasion in progress, the zoite must shed the surface proteins initially required for attachment and penetration. Specialized secretory organelles such as rhoptries, micronemes, located at the apical end, secrete surface proteins that facilitate invasion (Cowman and Crabb, 2006). However, the proteases mediating these events are still unknown. During the invasion of merozoites, *Plasmodium* rhomboids and subtilisin-like protease 2 (SUB2) process the surface adhesins (Baker et al., 2006). Two rhomboids *in P. falciparum* have been shown to shown to cleave the transmembrane domain of adhesin proteins. *Pf*ROM4 localized at the parasite surface, has been shown cleave EBA175 (O'Donnell et al., 2006). The other rhomboid *Pf*ROM1 localised to the apical end of merozoites (Baker et al., 2006), and is shown to cleave AMA1 (apical membrane antigen 1) *in vitro* (Srinivasan et al., 2009). The

other important protein for RBC invasion is SUB2, a transmembrane calcium dependent protease, secreted from micronemes into the merozoite surface (Harris et al., 2003). SUB2 has been shown to process the MSP1/6/7 complex as well as AMA1 (Howell et al., 2005). and PTRAMP (Green et al., 2006). Gene knockout attempts for SUB2 has been unsuccessful, inidcating an essential role. Interestingly, a recent study reported that SUB2 was detected in the secretory osmiophilic bodies of gametocytes, and also secreted from ookinetes during invasion of midgut epithelial cells, suggesting a role in sexual stages and in midgut wall transversal, respectively (Suárez-Cortés et al., 2016). Knockout studies in *P. berghei* demonstrate that *Pb*ROM1 is dispensable druing erythrocytic cycle but probably it plays an important role in sporozoite invasion of hepatocytes (Srinivasan et al., 2009). One of the dominant sporozoite surface protein TRAP could be potential substrate for the rhomboid. As mutation of rhomboid cleavage sites resulted in non-motile sporozoites (Ejigiri et al., 2012). A recent reverse genetics screen reported only half of the eight *P. berghei* rhomboids seem to be essential in blood stages (*Pb*ROM4, 6, 7 and 8), and *Pb*ROM3 is required to produce sporozoites (Lin et al., 2013). The potential functions of ROM6–8, but ROM6 and ROM7 are predicted to be localised to the mitochondria and apicoplast, respectively (Lin et al., 2013).

1.6.2. Egress from host cells

After completing the development, the daughter parasites egress out of host cells to progress to the next life-cycle phase. Parasite egress therefore is a tightly controlled process, wherein proteolytic events playing major regulatory and effector roles. Proteases have been shown to play important role in these pathways as the disruption of the parasitophorous vacuole and host cell membranes are essential for successful parasite egress. Before egress, signalling events trigger release of proteins into the parasitophorous vacuole (PV) and onto the merozoite surface (Collins et al., 2013). One such secreted protein is the exonemal subtilisin-like protease 1 (SUB1), which processes several substrates that are crucial for egress and invasion (Yeoh et al., 2007). Among these are SERA (serine repeat antigen) family proteins. *P. falciparum* contains 9 and *P. berghei* contains 5 SERA proteins. All the SERA proteins have a conserved papain-like protease domain. Gene-knockout studies in *P. falciparum* demonstrated that SERA 2, 3, 7, and 8 are dispensable and SERA 4, 5, and 6 are essential for development of erythrocytic stage (Miller 2002). Knockouts of SERA 1 and 2 in *P. berghei* and *P. yoelii* were successful (Putrianti et al., 2010; Huang et al., 2013). However, knockout of *Pb*SERA5 (also

known as ECP1) led to arrest of sporozoite egress from oocysts (Aly 2005). Expression and localization studies have shown that SERA 3-6 and SERA 9 are expressed in erythrocytic stages (Aoki et al., 2002; Miller et al., 2002), and SERA 3–6 are localized to the parasitophorous vacuole (Aoki 2002; Knapp 1991; Delplace 1988). A recent study showed that SERA6 is necessary for disruption of the RBC membrane (Thomas et al., 2016). SUB1 is also expressed in liver stages where it has also been shown to play a role in egress using a conditional knockout (cKO) approach in *P. berghei* (Tawk et al., 2013). Moreover, an independent study of *Pb*SUB1 cKO have shown a defect in schizont development and merozoite maturation within hepatocytes, suggesting additional functions for SUB 1 in liver stages (Suarez et al., 2013). During egress, proteases such as human calpain 1 is activated at the RBC membrane and thought to degrade components of the host cytoskeleton (Das et al., 2009). DPAP3 (dipeptidyl aminopeptidase 3) was also reported to participate in egress as DPAP3 inhibitors block egress upstream of SUB1 activation (Arastu-Kapur et al., 2008). Interestingly, a recent study showed that DPAP2, which is exclusively expressed in gametocytes (Suárez-Cortés et al., 2016), resides in osmophilic bodies, and knockout of DPAP2 decreases gamete egress (Tanaka et al., 2013).

1.6.3. Manipulation of host cells

In order to grow and replicate within the RBCs, the Plasmodium parasites extensively modify their host cell. This thorough renovation of host cells facilitates nutrient acquisition, cyto-adherence, and immune evasion. The parasite exports ~10% of its proteome into the RBC across the PVM through a sophisticated protein export machinery, the PTEX complex (Plasmodium translocon for exported proteins) (Gilson et al., 2017). Most of the exported proteins possess a N-terminal PEXEL (protein export element) motif downstream of the secretory signal peptide. Plasmepsin V (PM V), an endoplasmic reticulum (ER) resident protease recognizes and cleaves the PEXEL motif in proteins destined for export (Klemba and Goldberg, 2005; Russo et al., 2010; Boddey et al., 2010). The PEXEL cleavage exposes an N-terminal sequence that is in turn recognised by PTEX. Since the protein export is central to virulence and pathology of malaria parasites, PM-V is likely a very promising target.

1.6.4. Nutrient acquisition

For growth and development within RBCs, the parasites need a variety of nutrients. To achieve this, parasites import proteins from the host cytosol into the PV and subsequently into food vacuole, a specialized lysosome-like organelle, where the imported proteins are degraded into single amino acids. Haemoglobin, the major protein of RBCs, is processed by several different proteases to provide amino acids and also to create space within the RBC for the parasite to grow. This haemoglobin degradation pathway has been extensively studied for its important role in parasite development. However, various biochemical and knockout studies have shown that a high level of functional redundancy exists in this pathway. The proteolytic machinery regulating this pathway consists of four aspartic proteases, plasmepsins I-IV (PM I, PM II, PM III and PM IV) (Liu et al., 2005), three papain-like proteases, falcipains 2A, 2B and 3 (FP2A, FP2B & FP3) (Shenai et al., 2000; Sijwali et al., 2001; Singh et al., 2006) and the metalloprotease falcilysin (Eggleson et al., 1999).

The concerted action of these proteases breaks down haemoglobin into smaller oligopeptides. At the downstream of the haemoglobin degradation pathway acts a group of aminopeptidases. Dipeptidyl aminopeptidases (DPAPs), homologues of human cathepsin C, acts on oligopeptides and sequentially cleaves dipeptides from N-terminus. *P. falciparum* contains three dipeptidyl aminopeptidases (DPAP1–3). The DPAP1 and DPAP3 were shown to be expressed during intra-erythrocytic stage development (Klemba et al., 2004; Wang et al., 2011). Furthermore, several food vacuole and cytosolic aminopeptidases (*Pf*A-M1, *Pf*A-M17, *Pf*-APP, *Pf*A-M18) cleave dipeptides and oligopeptides into single amino acids (Dalal et al., 2007; Teuscher et al., 2011). Among the aminopeptdiases, only *Pf*A-M18 KO parasites are viable and knockout attempts for DPAP1, *Pf*A-M1, *Pf*A-M17 or *Pf*-APP have been unsuccessful, suggesting that these proteases are important for parasite development. In addition, potent inhibitors against aminopeptidases and DPAP1 have been shown to have antiparasitic activity both *in vitro* and *in vivo* (Flipo et al., 2003; Harbut etal., 2011; Mistry et al., 2014; Drinkwater et al., 2016).

1.6.5. Protein trafficking and Homeostasis

In the secretory pathway, soluble proteins require co-translational insertion of a hydrophobic N-terminal signal peptide into the ER membrane, translocation of the polypeptide chain within the ER, and cleavage of the signal peptide. In *Plasmodium* this is mediated by the signal peptidase complex, which recognises and cleaves the signal peptide in the lumen side of the membrane, and by a transmembrane aspartic SPP, which cleaves it within the ER membrane. In *P. falciparum*, the two serine protease subunits of the signal peptidase complex (SP18 and SP21) have been reported to have proteolytic activity, and the latter localises in the ER (Sharma et al., 2005; Tuteja et al., 2008). In addition, *Pf*SPP has been chemically validated as important for parasite development, and knockout attempts of this gene have been unsuccessful (Marapana et al., 2012).

Protein homeostasis plays a significant role in *Plasmodium* species given its complex life cycle and the distinct morphological stages, where each stage requires a special set of proteins to develop and replicate in different host environments. Especially, most of the genes expressed in erythrocytic phase are regulated in cyclical manner in sync with the parasite life style (Bozdech et al., 2003). Therefore, proteases that regulate protein quality control, temporal and spatial degradation of damaged and unwanted proteins are likely to be essential for proper parasite development, hence potential drug targets. Methionine aminopeptidases (MetAPs) are metalloproteases that catalyse the removal of the N-terminal initiator methionine during protein synthesis. *Plasmodium* Spp., encode five MetAPs, and *in vitro* and *in vivo* chemical inhibition studies revealed that indeed MetAP1b and MetAP2 could be targeted for drug development (Chen et al., 2003, 2006; Arico-Muendel et al., 2009). The proteasome system is highly conserved machinery involved in degradation of damaged misfolded proteins and also plays a very crucial role in signalling cascades that regulate diverse biological processes. Recent structural studies of *P. falciparum* proteasome have shown that there are considerable differences in specificity between the human and malaria parasite proteasomes. These differences can be explored in depth to design safer *P. falciparum* proteasome inhibitors (Li H et al., 2014).

The Ubiquitination proteasome pathway (UPP) is the principle mechanism for protein catabolism. This pathway is highly conserved from yeast to mammals. In *Plasmodium* spp., ubiquitination has been shown to regulate vital biological process such as the ER-associated protein degradation (ERAD) pathway, response to oxidative stress, protein trafficking and drug resistance. Bioinformatics analysis has revealed existence of ~30 deubiquitinating enzymes (DUBs) in *P. falciparum*. Recent studies indicated that some of these proteases are likely to be essential for parasite development (Artavanis et al., 2006; Frickel et al., 2007; Borrmann et al., 2013). However, further investigation is needed to understand their functional roles in parasite biology. Moreover, exploration of the ubiquitination and deubiquitination might provide new drug targets. Site-2 proteases (S2P) that belong to M50 family of metalloproteases are intra membrane proteases, which perform essential roles by mediating activation of membrane-bound transcription factors through regulated intramembrane proteolysis (RIP). A recent study has shown that PbS2P is expressed throughout the parasite life cycle and is localized to the periphery of the nucleus. Gene-knockout of PbS2Pstudies revealed reduced growth rates during liver and blood infection, although the knockout phenotype is non-lethal (Koussis et al., 2017).

1.6.6. Organelle biogenesis and maintenance

Plasmodium spp. have evolved with two special organelles, the mitochondrion and the apicoplast as a result of endosymbiotic events with ancestral prokaryotes. Not only proteases are required to deliver the nuclear encoded proteins into these organelles, but also for their biogenesis, growth and division of these organelles. If significant differences exist between human and *Plasmodium* homologs, mitochondrial and apicoplast proteases could be novel antimalarial drug targets. Interestingly, a homolog of bacterial proteasome system component (*Pf*HslV or ClpQ) is identified in *Plasmodium* spp., and shown be to be localized to the mitochondria (Tschan et al., 2010). Conditional overexpression of a dominant negative ClpQ mutant in *P. falciparum* resulted in abnormal mitochondrial morphology, blocks organelle growth and division, and disrupts transcription of mitochondria-encoded genes (Jain et al., 2013). Additionally, two other ATP-dependent proteases were shown to be important for the function of these organelles: the mitochondrial metalloprotease FtsH (Tanveer et al., 2013) and the ATP-dependent apicoplast Ser protease system ClpAP (Rathore et al., 2010).

1.6.7. Programmed cell death

Programmed cell death (PCD) forms an integral physiological part of multicellular organisms, where it plays an essential role in normal development and maintenance of integrity and homeostasis (Engelbrecht et al., 2012). This phenomenon has also been demonstrated in unicellular organisms, including parasitic protozoa (Zandbergen et al., 2010; Lüder et al., 2010). Thus far, no true caspases have been identified in *Plasmodium* (Deponte and Becker, 2004) although plant-like metacaspases have been found (Nedelcu 2009). Interestingly, *Plasmodium* metacaspase-1 (PfMCA-1) has been shown to be active and able to complement the function of yeast metacaspase (Meslin et al., 2011). However, knockout of metacaspase in *P. berghei* has no effect in parasite development (Le chat et al., 2007).

1.7. Plasmepsins, the aspartic proteases of *Plasmodium*

1.7.1 Overview of plasmepsins

Plasmepsins (PMs), the aspartic proteases of malaria parasites, are the most extensively studied amongst the proteolytic repertoire of *Plasmodium*. Genomic analysis of sequence information led to the identification of ten PMs in *P. falciparum*, namely Pf PMs I, II, IV-X, and III also known as HAP (Histo-Aspartic Protease) (Coombs et al., 2001). The genes encoding PMs are located in five different chromosomes.

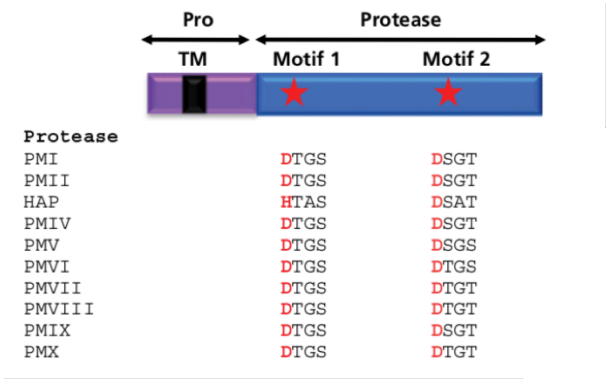


Figure 1.6. Schematic representation of domain architecture of plasmepsins. General architecture of plasmepsins is shown on the top. All the plasmepsins possess a prodomain (Pro), which contains a transmembrane region (TM) and a mature protease domain (Protease), which contains two active site motifs, motif1 and motif2. The amino acid sequences of active site motifs of each plasmepsin are indicated below the schematic. Image source: Advances in malaria research, 2016.

Most of the PMs are produced as proenzymes, with an amino-terminus (N) containing a proregion and a carboxy-terminus (C) containing aspartic protease domain (Figure 1.6). The N-terminus of the proregion also contains a transmembrane domain, which likely facilitates localization these proteases as integral membrane proteins in some cases (Klemba and Goldberg, 2005). The aspartic protease domain of the ten PMs has two catalytic motifs containing the aspartate residues. Except that of HAP, which is unique in having a histidine residue instead of the aspartate residue (Figure 1.6) (Banerjee et al., 2002; Xiao et al., 2006). Although, the domain organization is broadly conserved, PMs are distinct from each other in their structural, biochemical and functional properties. The first four PMs; *Pf* PM I-IV are clustered together in a 20-kb-long region of chromosome 14, and share a high amino acid sequence identity among themselves. These four PM paralogs were initially identified in the food vacuole (FV), an acidic organelle unique to the genus *Plasmodium* where degradation of hemoglobin of red blood cells (RBCs) occurs, hence they are called as the FV *Pf*PMs (Francis et al., 1994; Gluzman et al., 1994; Banerjee et al., 2002). *Pf*PMs V to X share a low amino acid sequence identity with the FV PMs, and their sequence structures are distinct from each other and from those of the FV PMs, suggesting diversity among the PM family members with respect to biological functions and enzymatic features.

1.7.2. Expression and *In vivo* synthesis of plasmepsins

The four FV *Pf*PMs exhibit distinct temporal expression patterns during the intra-erythrocytic development. While *Pf*PM I and *Pf*PM II are expressed as early as the ring stage and *Pf*PM IV expression starts in the early trophozoites, *Pf*HAP expression begins only in the mid stage trophozoites. All the four FV PMs continue to be expressed until the schizont stages (Banerjee et al., 2002). From the expression data, it is believed that haemoglobin degradation is a highly ordered process regulated by FV PMs. Interestingly, recent mass spectrometry (MS)-based studies have identified presence of FV PMs in gametocytes, merozoites, oocysts and sporozoites (Florens et al., 2002; Lasonder et al., 2008; Lindner et al., 2013). FV PMs are synthesized as type II integral membrane proteins, with the putative trans- membrane motif residing in the N-terminal pro-segment. Immuno-electron microscopy studies have shown that *Pf*PM I and *Pf*PM II were localized in the lumens of transport vesicles and FVs, in the parasite plasma membrane (PPM), in small vesicular structures near PPM and in the cytostome (Francis et al., 1994; Klemba et al., 2004). Similar to other aspartic proteases, FV

PMs are cleaved in their pro-segments to achieve catalytic activity and the cleavage site is conserved (Banerjee et al., 2003). Furthermore, studies showed that the cleavage of pro-segment occurs in an acidic environment and is completed within half an hour in cultured *P. falciparum* at the trophozoite stages (Banerjee et al., 2003). The enzymes likely responsible for maturation of FV PMs are shown to be cysteine proteinases, falcipain (FP) 2 and 3 (Drew et al., 2008). The maturation of FV PMs occurs in the acidic milieu of FV and transport vesicles. The best studied among the non-FV PMs (PM V to X) is PM V. Expression of *Pf*PM V is detected throughout the life cycle of the parasite (Banerjee et al., 2002; Lopez-Barragan et al., 2011; Singh et al., 2007). *Pf*PM V synthesized in its pro-form contains a type I integral membrane protein comprising an N-terminal pro-segment, a catalytic domain, a C-terminal transmembrane domain and a cytoplasmic tail. *Pf*PM5 is shown to be localized in the endoplasmic reticulum and at the nuclear envelope (Klemba and Goldberg, 2005). The C-terminal transmembrane domain is essential for the localization of *Pf*PM5 (Russo et al., 2010). Recent studies have provided information on expression of PMs VI to X. Genes encoding *Pf* PM IX and *Pf*PM X, are transcribed in the intra-erythrocytic phase (Le Roch etal., 2003) and they exhibited a diffuse localization pattern throughout the cytoplasm (Banerjee et al., 2002). Interestingly, a recent MS-based analysis indicated the presence of *Pf*PM IX in sporozoites and the presence of both *Pf*PM VI and *Pf*PM X in merozoites and sporozoites (Florens et al., 2002; Lasonder et al., 2008; Lindner et al., 2013). Further, expression of *Pf*PM VII and PfPM X is detected in zygotes and ookinetes (Zhous et al., 2008; Li et al., 2016).

1.7.3. Biological functions of plasmepsins

Based on their cellular location, plasmepsins can be categorized into two groups. Food vacuole plasmespins (FV PMs) and non-food vacuole plasmepsins. The primary cellular function of FV PMs is haemoglobin digestion and degradation. Haemoglobin catabolism takes place mainly between the ring and the early schizont stages during intra-erythrocytic development (Asawamahasakda et al., 1994; Orjih et al., 1993). The processing of haemoglobin occurs in FVs and to small extent in transport vesicles (Rudzinska et al., 1965). Early investigations indicated that aspartic and cysteine proteases are the enzymes involved in haemoglobin digestion (Gyang et al., 1982; Rosenthal et al., 1988; Vander Jagt et al., 1986). The isolation of

FVs from *in vitro* cultured trophozoites allowed the identification and characterization of enzymes responsible for haemoglobin digestion and degradation (Goldberg et al., 1990). The first enzyme isolated and purified from FVs is *Pf*PM I, an aspartic protease (Goldberg et al., 1991). *Pf*PM II, a second aspartic protease isolated from FVs, shares a 73% amino acid sequence identity with *Pf*PM I and found to be involved in haemoglobin processing, although less efficient than *Pf*PM II (Francis et al., 1994). Now it is known that both PM I and PM II digests haemoglobin into smaller peptides, although PM1 initiates the catalytic process (Gluzman et al., 1994). *Pf*PM III or HAP, a third PM isolated from FVs, acts on native haemoglobin less efficiently than *Pf*PM II but cleaves more efficiently the denatured haemoglobin (Banerjee et al., 2002). *Pf*PM IV, another FV PM prefers digestion of denatured than the native haemoglobin, similar to *Pf*HAP (Banerjee et al., 2002). Other proteolytic enzymes, such as the falcipains, falcilysin and aminopeptidases, are actively involved in further degradation of haemoglobin fragments to oligopeptides and amino acids (Coombs et al., 2001; Chugh et al., 2013). The participation of several enzymes in haemoglobin digestion and degradation in *P. falciparum* indicates that it is a highly ordered and dynamic process, in which *Pf*PM I and *Pf*PM II initiate the cleavage and various other proteases catalyse additional processing.

The reason behind extensive haemoglobin catabolism is still debatable. Initially it is assumed that malaria haemoglobin is a major source of nutrients for malaria parasites, which is supported by their limited capacity for *de novo* biosynthesis (Sherman et al., 1970; Sherman et al., 1977; Naughton et al., 2010). A second hypothesis states that the parasite degrade haemoglobin extensively to make space for their growth and development (Krugliak et al., 2002). A third hypothesis based on an experimental modelling study assumes that haemoglobin catabolism is essential for the parasites to maintain the osmotic stability of infected erythrocytes (Lew et al., 2003). *Pf*PM II, apart from haemoglobin digestion, catalyses hydrolysis of host cytoskeleton proteins such as spectrin, actin and protein 4.1 at near neutral pH conditions (Le Bonniec et al., 1999). Therefore, it is likely that FV PMs play a role in host cell remodelling in the intra-erythrocytic phase of malaria parasites development.

During the intra-erythrocytic development, malaria parasites express and export hundreds of proteins to the infected erythrocytes for acquisition of nutrients, host cell-remodelling, evasion of host immune system and to promote virulence (Marti et al., 2004; Hiller et al., 2004). Some of the exported proteins contains a pentameric sequence motif RxLxE/Q/D (x represents any natural amino acid) at the N-terminus. This motif is known as the *Plasmodium* export element (PEXEL) (Marti et al., 2004) or the vacuolar transport signal (VTS) (Hiller et al., 2004). A cleavage within the PEXEL motif at the C-terminus of leucine allows the PEXEL-containing proteins to be exported into the host cell cytosol (Boddey et al., 2009). PM V, an ER resident aspartic protease catalyses this cleavage reaction following the translation of PEXEL-containing proteins (Russo et al., 2010; Boddey et al., 2010). Recent studies revealed that host-targeted protein export is not limited to the intra-erythrocytic phase but also occurs throughout the course of the parasite life-cycle (Singh et al., 2007). Additionally, the spatio-temporal expression pattern of PM V supports the assumption that PM V could also play a role in protein export at other stages of the parasite life cycle (Silverstrini et al., 2010). The PM IX and X were shown to be expressed in blood stages and knock out attempts to disrupt the PMs IX and X were not successful thus indicating an essential role during intra-erythrocytic development. However, recent studies demonstrated that PM IX plays an important role in erythrocyte invasion, while the PM X plays key roles in merozoite egress and invasion (Pino et al., 2017; Nasamu et al., 2017). PM VI, VII, and VIII are not expressed in blood stages implying a functional role for these PMs in mosquito and liver stages. Indeed, a recent reverse genetics screen uncovered the function of PM VI in mosquito stages. PMVI knock out parasites were shown to have defective and morphologically abnormal oocysts which failed to undergo sporulation thus precluding infection to the mammalian host. However, the functions of PM VII and VIII remain unknown.

1.8. Plasmepsins as antimalarial drug targets

Given the established role of FV PMs in haemoglobin digestion and degradation, they can be potential targets for novel antimalarial drugs. Early studies have shown that peptidomimetic compounds strongly inhibited the growth of cultured parasites, indicating that targeting FV PMs could be a promising antimalarial strategy (Francis et al., 1994; Bailly et al., 1992; Andrews et al., 2006). Knockout studies of individual (*Δpfpmiv, Δpfpmi, Δpfpmii* and *Δpfhap*)

or multiple FV PMs (*Δpfpmiv/i/ii/hap*) were performed to determine their essentiality and druggability. During the intra-erythrocytic development, genetic ablation of any individual FV PM does not affect either the mRNA expression or protein expression of other three paralogs (Bonilla et al., 2006). The formation of hemozoin, catabolic by-product of haemoglobin digestion, is not affected in the knockout lines except in the *Δpfpmiv* line (Omara-Opayene et al.,2004; Bonilla et al., 2007). However, the knockout lines have slower replication rates than the wild type, all the knockout lines *Δpfpmiv, Δpfpmi, Δpfpm2, Δpfpmiv/i* and *Δpfpmiv/i/ii/hap* exhibited reduced growth rates in amino-acid rich media (Bonilla et al., 2007; Liu et al., 2006). Together, gene-knockout studies revealed that FV PMs are dispensable for parasite survival despite, slower growth rates. *In vitro* studies with known PM inhibitors indicated that these inhibitors exhibit anti-parasitic activity via off-target effects. Despite the discouraging results, further investigation is warranted to expand our limited understanding of the functions of FV PMs. A series of recent studies have shown that PM V can be considered as an ideal target for the development of novel antimalarial drugs. Gene-knockout studies indicated that PM V is essential in blood stages and a dominant negative mutation of *Pf*PM V also found to be lethal (Boddey et al., 2010; Russo et al., 2010). Apart from being essential, PM V is conserved among all the *Plasmodium* spp. with no functional redundancy (Silvestrini et al., 2010). Two more features that makes PM V a better candidate are, one PM V is expressed throughout the life cycle of *Plasmodium* and shares a low amino acid sequence identity with human aspartic proteases (Lopez-Barragan et al., 2011; Singh et al., 2007). WEHI-916, a statine-based compound mimicking the non-prime- side RVL motif of the PEXEL, shows a strong inhibition of *Pf*PM V and *Pv*PM V. *In vitro* studies with *P. falciparum* have shown that WEHI-916 blocked the PEXEL cleavage in a dose-dependent manner, and impairs protein export to host erythrocytes (Sleebs et al., 2014). The precise cellular functions and mechanistic details of PMs VII and VIII yet to be determined, in order to evaluate these enzymes as potential antimalarial drug targets. Therefore, further studies are warranted to understand their functions and characteristics.

1.9. *P. berghei* as model malaria parasite

The study of malaria parasites and their complex interactions with the human and mosquito hosts has been challenging. However, malaria research has immensely benefitted with the development of laboratory culture of malaria parasites (Trager & Jensen, 1976; Haynes, 1976). It has enabled studies on different aspects of parasite biology, which led to major discoveries that have uncovered the intricacies of cellular and molecular aspects of the malaria parasite. The study of malaria parasites under laboratory conditions have its own limitations such as lack of access to human tissue samples, the inability to study the entire life cycle or examine the host-pathogen interactions, and the gradual accumulation of adaptive changes within laboratory parasite strains (Wu et al., 1995). This challenge been overcome by the use of model malaria parasites which provide unique insights into the developmental aspects of parasite biology and its interaction with mammalian and mosquito hosts. The model malaria parasites grown in controlled environments allows detailed investigation *in vitro* and also permits *in vivo* investigation across the various stages of the parasite life cycle. *Plasmodium berghei*, originally isolated from the African thicket rats, is one of the most popular model among rodent malaria parasite species. *P. berghei* permits the *in vivo* investigation of synchronized blood stage development, gametocyte formation, gamete fertilization, zygote development and the other mosquito stages (Carlton et al., 2005). Comparative genomic studies have revealed a high degree of conservation (almost 80%) between *P. falciparum* and *P. berghei* (Janse et al., 1994; Rich and Ayala, 2003; Kooij et al., 2005). Despite many similarities, some aspects of malaria disease are not well conserved, such as progression of cerebral malaria and sequestration of infected erythrocytes in the host brain (Langhorne et al., 2011). The increased availability of a wide variety of well-established reverse genetics technologies for *P. berghei*, coupled with the enhanced transfection efficiency and the ease of analysing the development of transgenic parasites throughout the life cycle, both *in vitro* and *in vivo*, have made *P. berghei* the most preferred model for gene function analysis (de Koning-Ward et al., 2000; Carvalho and Menard, 2005; Janse et al., 2006). Therefore, rodent malaria parasites, especially, *P. berghei* have been an invaluable tool for understanding several aspects of parasite biology and host-pathogen interactions.

1.10. Aims and objectives

Plasmodium parasites have a complex life cycle with multiple stages alternating between mammalian and mosquito hosts. Their obligatory intracellular lifestyle necessitates precise coordination of many cellular processes during their highly complex life cycle inside multiple host microenvironments. *Plasmodium* genome encodes several distinct classes of proteases that play important roles in crucial biological processes in all stages of the parasite life cycle. Amongst the parasite proteolytic repertoire of *Plasmodium*, recent studies have demonstrated that the aspartic proteases, known as plasmepsins, play diverse roles in parasite biology, ranging from haemoglobin degradation, host-targeted protein export, egress of merozoites and host cell invasion. Given their critical roles in parasite biology, plasmepsins have been considered as potential antimalarial drug targets. Of the ten plasmepsins in *Plasmodium*, the functional roles of PM VII and VIII are not yet known. This study is aimed at characterizing the cellular functions of PM VII and VIII in *Plasmodium berghei* using the genetic, molecular and cell biological approaches. Determining their stage specific expression and *in vivo* functions during the parasite life cycle is crucial for validating the PMs VII and VIII as antimalarial targets.

2. MATERIALS & METHODS

2.1. Materials

2.1.1. Technical and mechanical devices

Device	Type / Model	Manufacturer
Autoclave	#SA35	Ketan
Table centrifuge	#R-8 C	Remi
Refrigerated centrifuges	#5415R and #5810R	Eppendorf
Mini centrifuge	MiniSpin®	Eppendorf
Fluorescent microscope	#Eclipse Ni-E	Nikon Instruments
Light optical microscope	#LM-52-1704	Lawrence & Mayo
Inverted microscope	#MEIJI TC5300	Lawrence & Mayo
Stereo zoom microscope	#080774	Lawrence & Mayo
Digital camera	Neo 5.5 sCMOS	Andor
Incubator for HepG2 cells	#51030302	Thermo Scientific
Incubator for *P. berghei*	Orbitek	Scigenics Biotek
Incubator for bacteria	Orbitek	Scigenics Biotek
Incubator for Mosquitoes	Walk-in Stability Chambers	Cassia Siamia
Freezer -20°C	Top chest (#CHF150C) Vertical (#VTS254)	Blue Star Vestfrost Solutions
Freezer -80°C	ES Series (#232F-AEV-TS)	Thermo Scientific
Fridge	Eon series	Godrej
Heating block	#RS232	Major Science
Water bath	Stirnew #8428	Lequitron
Magnetic stirrer	#1MLH	Remi
Vortex	#CM 101	Remi
Rocker/Shaker	#Li-G-R-M-100	GeNei
pH meter	#380734	Eutech Instruments
Microwave oven	#GMG 27A	Godrej

Device	Type / Model	Manufacturer
Agarose gel apparatus	Mini-Sub Cell GT system	Bio-Rad
SDS-PAGE gel apparatus	Mini-Protean	Bio-Rad
Western blotting apparatus	Mini Trans-Blot	Bio-Rad
Power supply unit	Broviga	Balaji Scientific Services
Liquid nitrogen container	Locator™ Plus (#CY509108)	Thermo Scientific
Mosquito cages	20 cm x 15 cm x 15 cm	Advanced Technocracy Inc
Larvae trays	31 cm x 26 cm x 7 cm (#PCHTNNO89)	Chetan Plastic Industries
Electroporator	Nucleofector™ II/2b (#AAB-1001)	Lonza
Spectrophotometer	Nanodrop 2000	Thermo Scientific
Spectrophotometer	UV-1800	Shimadzu
Pipettes	P10/P200/P1000 µl	Thermo Scientific
Pipette aid	Easypet 3 (#4430000018)	Eppendorf
PCR machine	Mastercycler (#Z316091)	Eppendorf
Real time machine	Realplex 2	Eppendorf
Sterile laminar hood	Grade III, Advance	Laminar Systems
Mouse restrainer	Tail veiner (#TV-150)	AgnTho's
Analytical balances	GE 212 and ENTRIS64-1S	Sartorius
Vacuum pump	#13158	Pall
UV Transilluminator	MUV Series (#MUV21)	Major Science
Gel imaging system	Molecular Imager Gel Doc XR+ System (#1708195)	Bio-Rad
Infrared Lamp	#ML0040	Murphy
Dryer	#KM8893	Kemei
Ice maker	Scotsman (#WW-55100-05)	Cole-Parmer
Water purification system	Milli Q	Millipore

2.1.2. Laboratory materials and disposables

Labware & disposables	Type / Specifications	Manufacturer
Pipette tips	0.2-10 µl (Cat No: #521000) 2-200 µl (Cat No: #521010) 200-1000 µl (Cat No: #521020)	Tarsons
Filter tips	2-200 µl (Cat No: #527104)	Tarsons
Micro centrifuge tubes	0.5 ml (Cat No: #500000) 1.5 ml (Cat No: #500010) 2.0 ml (Cat No: #500020)	Tarsons
PCR tubes	0.2 ml (Cat No: #510051)	Tarsons
Falcon tubes	15 ml (Cat No: #546021) 50 ml (Cat No: #546041) 50 ml (Cat No: #430829)	Tarsons Tarsons Corning
Petri dishes	35 mm (Cat No: #460035) 90 mm (Cat No: #460090)	Tarsons
Cryogenic vials	2.0 ml (Cat No: #CLS430488)	Corning
24 well plates	Nunclon (Cat No: #142475)	Thermo Scientific
Microscope cover slips	Circular, f 12mm (Cat No: #CG134-10X100NO)	Himedia
Cell culture flasks	T25 cm^2 (Cat No: #430641) T75 cm^2 (Cat No: #430720) T75 cm^2 (Cat No: #430641)	Corning
Microscope slides	Frosted micro slides (1.35 mm)	Polar Industries
Immersion oil (for microscopy)	Low viscosity (Cat No: #GRM225)	Himedia
Pasteur pipettes	1 ml (Cat No: #940080)	Tarsons
Serological pipettes	10 ml (Cat No: #4488)	Corning
Neubauer counting chamber	Bright-line Hemacytometer (Cat No: #Z359629)	Sigma-Aldrich
Nitrocellulose membrane	0.2 µm (Cat No: #1620112)	Bio-Rad
Nylon blotting membrane	Hybond-N+ (Cat No: #RPN119B)	GE Healthcare

Labware & disposables	Type / Specifications	Manufacturer
Filter paper	No-3 (Cat No: #1004110)	Whatman Ltd
Sterile filters	Acrodisc 0.2µm (Cat No: #PN4612)	Pall Corporation
Sterile filtration units	500 ml (Cat No: #431097)	Corning
Syringes	Insulin (Cat No: #328418) 1 ml DISPO VAN	BD HMD
Parafilm	4" x 125' (Cat No: #PM996)	Bemis
Aluminium foil	Superwrap 11 micron	Hindalco
Saran wrap	Cling plus	Saran
Gloves	LA885	Himedia
Scalpels	LA769-1X100NO	Himedia

2.1.3. Chemicals

Chemical / Reagent	Manufacturer
Acrylamide/Bis-acrylamide (Cat No: #124263)	SRL Pvt. Ltd.
Agarose (Cat No: #16500500)	Invitrogen
Alsever's solution (Cat No: #A3551)	Sigma-Aldrich
Ampicillin (Cat No: #A1593)	Sigma-Aldrich
Ammonium persulfate (Cat No: #28575)	SRL Pvt. Ltd.
Antibiotic-Antimycotic (Cat No: #15240062)	Gibco, Invitrogen
Bovine serum albumin (Cat No: #85171)	SRL Pvt. Ltd.
Bromophenol Blue (Cat No: #11458)	SRL Pvt. Ltd.
Chloramphenicol (Cat No: #C3175)	Sigma-Aldrich
Calcium chloride (Cat No: #31944)	SRL Pvt. Ltd.
Coomasie brilliant blue (Cat No: #64222)	SRL Pvt. Ltd.

Chemical / Reagent	Manufacturer
DMSO (Dimethyl Sulfoxide) (Cat No: #D4540)	Sigma-Aldrich
DAPI (4',6-diamidino-2-phenylindole) (Cat No: #D9542)	Sigma-Aldrich
Deoxynucleotide Triphosphates (dNTPs) (Cat No: # 18427088)	Invitrogen
DMEM (Dulbecco's Modified Eagle Medium) (Cat No: # 11995065)	Gibco, Invitrogen
Ethanol (Cat No: #792780)	Sigma-Aldrich
Ethylenediamine Tetraacetic Acid (EDTA) (Cat No: #45247)	SRL Pvt. Ltd.
Ethidium bromide (EtBr) (Cat No: #17220)	SRL Pvt. Ltd.
Foetal Bovine Serum (FBS) (Cat No: #16000044)	Gibco, Invitrogen
Gentamycin (Cat No: #G1272)	Sigma-Aldrich
Giemsa Stain (Cat No: #48900)	Sigma-Aldrich
Glycerol (Cat No: #15514029)	Invitrogen
Heparin (Cat No: #H3393)	Sigma-Aldrich
Hydrochloric acid (Cat No: #AC366520025)	Thermo Scientific
Isopropanol (Cat No: #38445)	SRL Pvt. Ltd.
Ketamine (Cat No: Ketamax* 50)	Troikaa Pharmaceuticals Ltd.
Luria Bertani Agar, Miller (Cat No: #M1151)	Himedia
Luria Bertani Broth, Miller (Cat No: #M1245)	Himedia
Magnesium chloride (Cat No: #31196)	SRL Pvt. Ltd.
Milk Powder (Cat No: #GRM1254)	Himedia
Methanol (Cat No: #65524)	SRL Pvt. Ltd.
Nuclease-free water (Cat No: #LSKNF0500)	Sigma-Aldrich
Nycodenz / Histodenz (Cat No: #D2158)	Sigma-Aldrich
Para-formaldehyde (Cat No: #47608)	Sigma-Aldrich
Phosphate Buffer Saline (PBS) 10x (Cat No: #10010049)	Invitrogen
PCR Master Mix EmeraldAmp (Cat No: #RR310A)	Takara, Clonetech

Chemical / Reagent	Manufacturer
RPMI 1640 Medium (Cat No: #CC3014) (Roswell Park Memorial Institute) with 25mM HEPES and L-Glutamine	Genetix Biotech Asia Pvt. Ltd.
RNAse-Free Water (Cat No: #750024)	Thermo Scientific
Saponin (Cat No: #47036)	Sigma-Aldrich
Sodium bicarbonate (Cat No: #89399)	SRL Pvt. Ltd.
Sodium chloride (Cat No: #33205)	SRL Pvt. Ltd.
Sodium Dodecyl Sulphate (SDS) (Cat No: #54468)	SRL Pvt. Ltd.
Sodium hydroxide (Cat No: #68151)	SRL Pvt. Ltd.
Sucrose (Cat No: # PCT0607)	Himedia
TEMED (Cat No: #84666) (N,N,N,N-Tetramethylethylenediamin)	SRL Pvt. Ltd.
Tetracycline (Cat No: #87128)	Sigma-Aldrich
TRIS (Cat No: #34969)	SRL Pvt. Ltd.
Trypsin-EDTA (Cat No: #25300054)	Gibco, Invitrogen
Triton X-100 (Cat No: #93443)	Sigma-Aldrich
Tween 20 (Cat No: #MB067)	SRL Pvt. Ltd.
Xylazine (Xylaxin)	Indian Immunologicals Ltd.
Xylene Cyanol FF (Cat No: #A1593)	SRL Pvt. Ltd.

2.1.4. Enzymes

Enzyme	Catalogue No	Manufacturer
Taq DNA polymerase	#10342020	Thermo Scientific
Phusion DNA polymerase	#F530S	Thermo Scientific
T4 DNA ligase	#EL0014	Thermo Scientific
Restriction endonucleases		Thermo Scientific
DNAse I	#18047019	Thermo Scientific
SuperScript III reverse transcriptase	#18080093	Thermo Scientific

2.1.5. Kits

Kit	Catalogue No	Manufacturer
PureLink RNA Mini kit	#12183018A	Thermo Scientific
GeneJET PCR Purification and Gel Extraction kit	#K0701	Thermo Scientific
GeneJET Plasmid Miniprep kit	#K0503	Thermo Scientific
Sureprep Plasmid Maxi kit	#NP-15161	Genetix Biotech
Nucleospin Tissue kit	#740952.10	Macherey-Nagel
InsTAclone PCR Cloning kit	#K1214	Thermo Scientific
Rapid DNA ligation kit	#K1422	Thermo Scientific
Amaxa Mouse T Cell Nucleofector® kit	#VPA-1006	Lonza
Western blot-ECL detection kit	#RPN2232	GE Healthcare
DNA Labeling and Detection Starter Kit	#11745832910	Roche

2.1.6. Molecular size markers

Marker	Catalogue No	Manufacturer
GeneRuler™ DNA Ladder Mix	#SM0333	Thermo Scientific
PageRuler™ Prestained Protein Ladder	#26616	Thermo Scientific

2.1.7. Antibodies

Antibody	Dilution	Source / Manufacturer
Anti- *Pb*CSP (mouse)	1:1000 (WB) 1:500 (IFA)	Yoshida et al., 1981
Anti-*Pb*HSP70 (mouse)	1:500 (IFA)	Tsuji M, NYU
Anti-UIS4 (rabbit)	1:500 (IFA)	Sinnis P, JHU
Anti-Mouse Alexa Fluor 488 (Goat)	1:500 (IFA)	Molecular Probes
Anti-Rabbit Alexa Fluor 594 (Goat)	1:500 (IFA)	Molecular Probes
Anti-Mouse-IgG-HRP (Rabbit)	1:2000 (WB)	Molecular Probes

2.1.8. Organisms

Organism	Species/Strain	Source/Supplier
Mice	Swiss Webster, BALB/C and C57BL/6	Thermo Scientific
Mosquitoes	*Anopheles stephensi* (SD-500)	Thermo Scientific
Parasites	*Plasmodium berghei* ANKA, *Plasmodium berghei* ANKA-GFP	Thermo Scientific
Bacteria	*Escherichia coli* XL-1 Blue (Cat No: 200130)	Genetix Biotech
Mammalian cells	HepG2 (human liver carcinoma cells)	Macherey-Nagel

2.1.9. Media, Buffers and Solutions

Media / Buffer / Solution	Composition
LB Broth	1% (w/v) Tryptone 0.5% (w/v) Yeast extract 0.5% (w/v) NaCl add to ddH2O, adjust the pH to 7.5 Autoclave
LB Agar	1% (w/v) Tryptone 0.5% (w/v) Yeast extract 0.5% (w/v) NaCl 1.5% (w/v) Agar Autoclave and pour into petri dishes (~25 ml/100 mm plate)
Transfection media for *P. berghei*	160 ml of RPMI 1640 with 25 mM HEPES 2.05 mM L-Glutamine 40 ml of heat-inactivated FCS 50 µl gentamycin (50 mg/ml)
Mosquito dissection media	10 ml RPMI with antibiotic-antimycotic 3% Bovine albumin serum in 10 ml RPMI (for gliding motility experiments)
Hepatocyte cell culture media (HepG2)	450 ml DMEM 50 ml FBS 5 ml Penicillin/Streptomycin

Media / Buffer / Solution	Composition
50x TAE	2 M Tris base 0.5 mM EDTA 250 mM sodium acetate adjust the pH to 7.8
5x DNA Loading dye	40% Gycerol (v/v) 2.5% Xylene cyanol (w/v) 2.5% Bromophenol Blue (w/v) in ddH_2O
Tris-EDTA (TE) buffer	10 mM Tris-HCl 1 mM EDTA pH 8.0
6x SDS sample buffer (Laemmli Buffer)	375 mM Tris HCl pH 6.8 12% SDS (w/v) 60% Glycerol (v/v) 0.6 M DTT 0.06% Bromophenol blue (w/v)
SDS-PAGE stacking buffer (5%) For two gels	0.75 ml 1 M Tris-HCl, pH 6.8 4.35 ml ddH_2O 750 µl Acryl amide (40%) 60 µl SDS (10%) 60 µl APS (10%) 6 µl TEMED
SDS-PAGE resolving buffer (12%) For two gels	2.5 ml 1.5 M Tris-HCl, pH 8.8 4.2 ml ddH2O 3 ml Acryl amide (40%) 100 µl SDS (10%) 100 µl APS (10% (w/v) in ddH_2O) 4 µl TEMED
10x SDS-PAGE Running buffer	250 mM Tris base 1.92 M Glycine 1 % SDS (w/v) in ddH2O
10x Western transfer buffer	250 mM Tris base 1.92 M Glycerol 0.1 % SDS (w/v) in ddH_2O
Blocking solution	5% skimmed (w/v) milk in 1x TBS

Media / Buffer / Solution	Composition
1x Western-Transfer buffer	10% 10x Western transfer buffer 20% Methanol in ddH2O
20x TBS / TBS-T	20 mM Tris 137mM NaCl dissolve in H_2O adjust pH to 7.6 and make up to 1L add 0.05 or 0.1 % Tween 20 for TBS-T
Antibiotics (1000x)	Ampicillin Chloramphenicol Tetracycline
Anaesthetic solution	0.8 ml Ketamine 0.3 ml Xylazine 3.9 ml sterile PBS
Freezing solution	For *P. berghei* blood stages Glycerol + Alsever's solution (1:9 v/v) Freezing solution + Infected blood (1:2 v/v) For HepG2 cells 10 % DMSO + 90 % FCS (v/v) For *E. coli* XL-1 Blue 50 % (v/v) glycerol + 1 ml LB culture
Pyrimethamine stock (100x)	7 mg pyrimethamine dissolve in 1 ml of DMSO adjust the pH to 3.5-5.5 make up the volume to 100 ml with ddH_2O
Giemsa-stain solution	20 % Giemsa Solution in ddH_2O
Nycodenz-stock solution	5 µM Tris pH 7.5 (1 M stock) 3 mM KCL (250 mM stock) 0.3 mM EDTA (0.5 M stock) 110.4 g Nycodenz (27.6 g/100 ml) dissolve in 400 ml ddH_2O autoclave the solution and store at 4°C
Mosquito breeding	For larvae and pupae 60% Kellogg's + 40% Wheat germ For adult mosquitoes 10% sucrose in ddH_2O
Erythrocyte lysis solution	0.02% Saponin (w/v) in 1x PBS

2.1.10. Oligonucleotides

Custom DNA Oligonucleotides were ordered and purchased from Sigma-Aldrich and Integrated DNA Technologies. All the oligonucleotides used in this study are described below, restriction sites are indicated in underlined bold case and overhangs in lower case.

Oligonucleotide	Sequence (5'-3')
Plasmepsin VII	
For qRT-PCR	
pm vii FP	AAGAGATGATGAAGTGATTGTA
pm vii RP	CAAGAATTTATTGTCAGATCATTT
18s rRNA FP	AAGCATTAAATAAAGCGAATACATCCTTC
18s rRNA RP	GGAGATTGGTTTTGACGTTTATGT
For Targeting Construct	
5' UTR FP (75F)	cgc**CTCGAG**GGAGCAATTATGTTACTATATC
5' UTR RP (75R)	att**ATCGAT**GGTTTATACACTTGTACGACA
3' UTR FP (73F)	att**GCGGCCGC**CCTGAATGGAAAAGAATACATA
3' UTR RP (73R)	att**GGCGCGCC**CCACTATTTAACCACACGATT
For Diagnostic PCR	
5' Integration FP (P1)	GATAAAATGAGAGGATATATTA
5' Integration RP (P2)	TTCCGCAATTTGTTGTACATA
3' Integration FP (P3)	GTTGTCTCTTCAATGATTCATAAATAG
3' Integration RP (P4)	CATACTCCATATTTATAAAAGA
WT FP (P5)	AGAAGAGATGATGAAGTGATTGTAA
WT RP (P6)	TTATAGTGGCCCATTATAACTCTTT
Plasmepsin VIII	
For qRT-PCR	
pm viii FP	TGATGGGCTATTTGGATTAGGCA
pm viii RP	GTCATATCCACCAAATGTAATTGC
18s rRNA FP	AAGCATTAAATAAAGCGAATACATCCTTC
18s rRNA RP	GGAGATTGGTTTTGACGTTTATGT

Oligonucleotide	Sequence (5'-3')
For Targeting Construct	
5' UTR FP (85F)	ccc**CTCGAG**TGCATAGAGAGATTATACCTA
5' UTR RP (85R)	att**ATCGAT**TGCATGTGATTTCCTATTTAAA
3' UTR FP (83F)	att**GCGGCCGC**ATTCATTCTTCCCGATAACTT
3' UTR RP (83R)	att**GGCGCGCC**TTTGAACGACGACTATGAATA
For Diagnostic PCR	
5' Integration FP (P1)	GAGATATAGTGTGAATTTGG
5' Integration RP (P2)	TTCCGCAATTTGTTGTACATA
3' Integration FP (P3)	GTTGTCTCTTCAATGATTCATAAATAG
3' Integration RP (P4)	CTCATGAACTCAAAATTCTG
WT FP (P5)	CGGATGAAAATGGTATTTATAATA
WT RP (P6)	TTATATCGATTTAGCTAAGCCTA

*Restriction sites are indicated in underlined sequences

2.1.11. Computer software

Various software used in the course of this study are mentioned below:

- NIS-Elements Advanced Research
- EndNote
- GraphPad Prism
- Microsoft Office
- Adobe Photoshop
- Adobe Illustrator
- Sanger sequencing software

2.1.12. Databases and online bioinformatics tools

- BLAST http://blast.ncbi.nlm.nih.gov/Blast.cgi
- ClustalW http://www.ebi.ac.uk/Tools/msa/clustalw2/
- MotifScan http://myhits.isb-sib.ch/cgi-bin/motif_scan
- PlasmoDB http://plasmodb.org/plasmo/
- GeneDB http://www.genedb.org/Homepage
- RMgmDB http://www.pberghei.eu/index.php
- PubMed http://www.ncbi.nlm.nih.gov/pubmed

- Google scholar https://scholar.google.co.in/
- Search engine http://www.google.co.in/
- DNA sequence analyser
    - https://pga.mgh.harvard.edu/web_apps/web_map/start
- Primer designing tools
    - OligoCalc - Oligonucleotide Properties Calculator
      http://biotools.nubic.northwestern.edu/OligoCalc.html
    - OligoAnalyzer Tool - Integrated DNA Technologies
      https://eu.idtdna.com/calc/analyzer
- Multiple alignment tool
    - http://multalin.toulouse.inra.fr/multalin/

2.2. Methods

2.2.1. Methods for molecular biology

2.2.1.1. Isolation of RNA from *P. berghei*

To study the gene expression analysis, RNA was isolated from different *P. berghei* life cycle stages. For isolating the mRNA from mosquito stages of the parasite, the midguts and salivary glands were obtained at different time points after infection by dissection of 30 female Anopheles stephensi mosquitoes infected with *P. berghei* wild type GFP. The infected midguts and salivary glands were pelleted by centrifugation. Similarly, EEFs or infected HepG2 cells were harvested at different time points by trypsinization. The trypsinized cells were washed three times with sterile RNAse free PBS and pelleted by centrifugation. RNA was extracted from different parasite stages using PureLink RNA isolation kit (Invitrogen) following manufacturer's instructions. Finally, the RNA was eluted in 50 µl of nuclease-free water and quantified using Nanodrop® ND-2000 spectrophotometer. Afterwards, RNA was either subsequently processed to cDNA or stored at -80°C until further use.

2.2.1.2. Complementary DNA (cDNA) synthesis

Prior to CDNA synthesis, total RNA was subjected to DNAse I (Invitrogen) treatment to ensure purity of RNA samples by getting rid of genomic DNA contamination. Purified total RNA ~2 µg was added to DEPC treated water containing 10x DNAse I reaction buffer and 2 U of DNAse I in microcentrifuge tubes. The reaction mixture was incubated room temperature for 15 min. Afterwards, DNAse I was neutralized by mixing with 1 µl of 25mM EDTA and heat inactivation at 65°C for 10 min. The DNAse I treated RNA was subsequently used for cDNA preparation. The cDNA synthesis was done using M-MLV reverse transcriptase (Thermo Scientific). The reaction mix consisted of the following made in a final volume of 30 µl - M-MuLV Reverse Transcriptase, dNTPs, MgCl2, RNase inhibitor, random hexamer primers (for short length cDNA fragments) or Oligo $(dT)_{18}$ primers (for full length cDNA), and 1x reaction buffer. cDNA was generated using following thermal cycling conditions: 25°C for 10 min, 42°C for 20 min, 98°C for 5 min. Finally, the cDNA generated was stored in aliquots at -20°C.

2.2.1.3. Quantitative real time PCR (qRT-PCR)

This technique was used for gene expression profiling of plasmepsins VII and VIII through mosquito and liver stages of *P. berghei*. It is a PCR based technique, which allows simultaneous amplification and quantification of the targeted DNA molecule. During double strand DNA synthesis, the fluorescent nucleic acid marker SYBR green is incorporated and fluorescence is measured after each cycle. A 150 bp fragment was amplified from each of the plasmepsins VII and VIII using target specific primers and was cloned in pTZ57R/T vector. After confirming the clones, a series of log dilutions of plasmids were made in a dynamic range of 10^2 copies / µl to 10^8 copies / µl and used as gene specific standards. Similarly, 18S rRNA standards were made to be used as internal control. The qRT-PCR reaction was performed in triplicates using Eppendorf Mastercycler® RealPlex real time PCR system and iQ SYBR Green Supermix (BioRad), according to the manufacturer's instructions.

The reaction mixture consisted of the following in a final volume of 20 µl –

- cDNA or the standards as a template
- 0.25µM each of forward and reverse primers
- SYBR Green mix
- nuclease-free water

The target genes and internal control samples were run together with the following standard PCR program and an additional step for melting curve.

Standard qRT-PCR program:

- Step 1: 95°C for 5 min
- Step 2: 95°C for 15 sec
- Step 3: 60°C for 1 min
- Step 4: 40 cycles of Step 2 and Step 3

Melting curve:

- Step 5: 95°C for 15 sec
- Step 6: 60°C for 15 sec
- Step 7: 95°C for 15 sec

Gene expression levels were determined by a ratio of absolute copy numbers of *Plasmepsin vii* and *viii* versus absolute copy number of *18S rRNA* and the normalized values were plotted in a graph.

2.2.1.4. Isolation of genomic DNA (gDNA) from *P. berghei*

For genotyping the transfected or clonal lines of recombinant parasites and other molecular biology applications such as gene/insert amplification, restriction digestion, molecular cloning, etc., genomic DNA was isolated from blood stage parasites. The parasites were isolated from infected blood as described in later sections (2.2.4.7.). The parasite pellet was further processed according to the manufacturer's user manual of the Nucleospin Tissue Kit (MACHEREY-NAGEL). Finally, the gDNA was eluted either in 50 µl of elution buffer or nuclease-free water and stored at -20°C.

2.2.1.5. Polymerase chain reaction

PCR was performed to obtain specific DNA sequences from *P. berghei* wild type genomic DNA and other sources having the template DNA for gene expression analysis, molecular cloning, parasite genotyping, and sequencing. This technique is based on the ability of thermo-stable DNA polymerases to synthesize short pieces of single-stranded DNA complementary to target sequences using primers and deoxynucleotide triphosphates (dNTPs). Sequence specific primers were designed with 5' extensions containing restriction endonuclease sites to facilitate molecular cloning. *Taq* DNA polymerase was used for analytical PCR and *Pfu* DNA polymerase was used for preparative PCR.

Standard PCR reaction

1. 1x reaction buffer
2. 1.5 mM of Mgcl2
3. 200 µM of dNTPs
4. 0.2 µM of each primers
5. 10-50 ng of template DNA
6. 1 unit of DNA polymerase
7. Nuclease-free water

Standard PCR program

1. Initial denaturation	94°C, 5 min
2. Denaturation	94°C, 30 sec
3. Primer annealing	54 to 60°C, 30 sec
4. Extension	72°C, 30 sec to 3 min
5. Repetition of steps 2-4	30-35 cycles
6. Final extension	72°C, 10 min
7. Hold	4°C

2.2.1.6. Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA molecules according to their charge, size, and conformation. Due to the presence of phosphate backbone the DNA molecule is negatively charged, hence when placed in an electric field, DNA fragments migrate to the positively charged anode. The rate of migration depends on length and conformation of the DNA fragments. 0.8-1% gels were prepared by dissolving agarose in 1x TAE buffer. The solution was boiled in a microwave oven until the agarose was dissolved and allowed to cool down to 50-60°C before the addition of Ethidium bromide (EtBr) (0.5 µg/ml). The DNA samples were loaded in 1x loading dye along with 10 kb ladder and separated by 100 V for 1 hr. Later, the separated DNA fragments were visualized under Ultraviolet light (UV) using a gel documentation system.

2.2.1.7. DNA isolation by PCR purification

PCR Purification procedure removes primers, nucleotides, enzymes, salts, and other residual impurities from DNA samples. This method was preferentially used to purify DNA fragments after PCR amplification and restriction endonuclease digestion. Using this method DNA fragments of sizes up to 10 kb can be purified. The DNA samples were processed according to manufacturer's instructions using the the GeneJET PCR Purification Kit (Thermo Scientific).

2.2.1.8. DNA isolation by gel extraction

PCR fragments as well as plasmids digested with restriction endonucleases were purified by gel extraction. This method was especially used for ligation purpose. DNA fragments were separated by agarose gel electrophoresis. The gel block containing the band of interest was carefully excised using a sharp scalpel under UV illumination. Precautions must be taken to minimize the exposure of UV light to the DNA while excising the gel block. The DNA fragments of interest were then purified according to manufacturer's instructions using the the GeneJET Gel Extraction Kit (Thermo Scientific).

2.2.1.9. Determination of DNA concentration

Measuring the absorbance of UV light is the most comprehensive method to determine DNA yield and purity. DNA absorbance is highest at 260nm, therefore the absorbance value (A_{260}) at this wavelength can be used to estimate the DNA concentration using the following equation.

The concentration of pure double-stranded DNA with an A260 of 1.0 is 50 mg/ml.

Unknown (mg/ml)/ Measured A260 = 50 (mg/ml)/ 1.0 A260

Since absorbance is directly proportional to concentration,

Unknown mg/ml = 50 mg/ml x Measured A260 x dilution factor

However, DNA preparations will have contaminants such as RNA, protein, and salts. Purity of DNA is assessed by ratio of the absorbance at 260nm divided by the reading at 280nm. Good-quality DNA should have an A260/A280 ratio of 1.7–2.0. Alternatively, DNA concentration can be quantified using a Nanodrop Spectrophotometer.

2.2.1.10. Insert and vector preparation

One of the most critical steps in molecular cloning is the preparation of gene of interest and the vector. Initially, the PCR amplified gene product and plasmid were digested with appropriate restriction endonucleases (REs). One unit of restriction enzyme digests 1 μg of DNA under ideal conditions, such as the appropriate buffer and the optimal temperature. Directional cloning can be achieved by cutting with two unique REs that flank the gene of interest and are also present in multiple cloning sites (MCS) in the vector. Using two different REs for digestion allows the generation of two non-compatible ends. This minimizes the chances of self-ligation of the vector alone and facilitates directional cloning. DNA can be digested with two different REs simultaneously with buffers compatible for both the enzymes. Usually, in the cloning procedure, the required restriction sites are introduced in the primer sequences of the gene of interest prior to PCR amplification and restriction digestion.

Standard restriction digestion reaction

1. 1x Enzyme buffer
2. 1-2 μg of DNA
3. 1-2 units of restriction enzymes
4. Nuclease-free water
5. Incubation at appropriate temperature

After incubation, the digested DNA samples were further processed using either the GeneJET PCR Purification Kit or the GeneJET Gel Extraction Kit (Thermo Scientific).

2.2.1.11. Ligation of DNA fragments

This technique is based on the ability of DNA ligase enzyme, which covalently joins the phosphate backbone of DNA with blunt or compatible cohesive ends. In molecular cloning it is routinely used for the insertion of restriction enzyme-generated DNA fragments into vector backbones. Following digestion and purification the insert was ligated with the vector using the DNA Ligation Kit (Thermo Scientific) at 1:5 molar ratio of vector to insert. According to manufacturer's instructions the ligation reaction was carried out either at 22°C for 1 hour or at 4°C overnight. After ligation, the reaction mixture was immediately transferred into competent bacterial cells.

Standard Ligation reaction

1. 1x T4 DNA ligase buffer
2. 20-100 ng of linear vector DNA
3. 1:5 molar ratios of insert DNA
4. 1 unit of T4 DNA ligase
5. Nuclease-free water
6. Incubation at appropriate temperature

2.2.1.12. Isolation of plasmid DNA from *E. coli*

Bacterial colonies were inoculated in 3-5 ml of liquid LB medium with appropriate antibiotics. After overnight incubation, the cultures were spun down and the recombinant plasmids were extracted using the GeneJET Plasmid Miniprep Kit (Thermo Scientific) as per the manufacturer's instructions. Samples of the obtained plasmids were subjected to restriction digestion by appropriate restriction enzymes to confirm the presence of the insert. The large-scale plasmid preparations of final targeting vector used for electroporation were obtained using the Sureprep Plasmid Maxi Kit (Genetix). A detailed description of the isolation procedure is described in the user manual. Briefly, bacterial over-night cultures (500 ml of LB Broth medium) were centrifuged at 4°C for 15 min at 10000 rpm. The pellet was resuspended in resuspension buffer and lysed with the lysis buffer for 5 min at room temperature. Next, the samples were incubated for 5 min on ice with neutralization buffer, the bacterial lysate was clarified with a filter provided in the kit. The clarified filtrate was transferred to a column previously equilibrated with equilibration buffer. After washing twice with wash buffer, the DNA was eluted in 15 ml of elution buffer, precipitated with isopropanol and centrifuged at at 10000 rpm for 40 min at 4°C. The pellet was washed with 5 ml of 70% ethanol and centrifuged at 4°C for 20 min at 10000 rpm. After discarding the supernatant, the DNA pellet was air-dried, resuspended in 150 µl of either 1x TE or nuclease-free water. The concentration of the plasmid was estimated using nanodrop and stored at -20°C for further use.

2.2.1.13. Preparation of DNA for transfection

The final targeting plasmids containing the introduced homology arms were linearized with suitable restriction enzymes overnight at 25 µg per reaction with 10U/reaction of each of the

restriction enzymes in the appropriate buffer, at the appropriate temperature to ensure complete digestion. For the double homologous recombination, the plasmid was linearized using restriction sites at each end of the target construct to separate the plasmid backbone from the desired linear recombination construct. Four reactions were setup and all the reactions were pooled and the digested plasmid was fractionated on an 0.7% agarose gel containing a minimal amount of EtBr (Himedia) along with the undigested plasmid as a control to distinguish uncut and the linearized plasmid DNA. The band corresponding to the linearized targeting construct was cut out of the gel with standard precautions to minimize exposure to UV rays. DNA was extracted from the gel using GeneJET Gel Extraction Kit (Thermo Scientific) and eluted in nuclease-free water (Sigma). All the elutions were pooled and resuspended in nuclease-free water to make a final concentration of about 1 µg/µl. For transfection of P. berghei with the Amaxa® Mouse T-cell Nucleofector™ solution (Lonza), 5-7 µg of DNA was used for each transfection.

2.2.1.14. Southern blot analysis

Approximately 5 µg of *P. berghei* wild type and *pm viii(-)* gDNA was digested with EcoRV at 37°C for 8 hrs. After the digestion, the samples were loaded on a 0.8% agarose gel in 1x TAE buffer and fractionated at 20 Volts / cm overnight. When the gel has run more than 3/4th of its length, the gel was depurinated by incubating with 0.25 N HCl on a slow shaker for 15 min at room temperature (RT). Afterwards, the gel was rinsed with double distilled water (ddH_2O) and subjected to denaturation in 1.5 M NaCl / 0.5 M NaOH followed by neutralization in 1.5 M NaCl / 0.5 M Tris, pH 7.0 by incubating on a shaker for 30 min at RT. Finally, the DNA was transferred from gel to Nylon membrane (Amersham Hybond-N+) using Vacuum Blotter (BioRad) in 20x SSC buffer. After transfer, the membrane was washed in 2xSSC. Probe was amplified using primers PM85F - PM85R using PCR DIG Labelling Mix (Roche). 15 µl of probe was added in hybridization buffer and membrane was incubated overnight at 65°C. Membrane was washed two times with low and four times with high stringent buffer. Further the membrane was washed two times with washing buffer (see materials for the composition of buffers). The signal was revealed using DIG Nucleic acid detection kit (Roche), according to the manufacturer's instructions. Briefly, the membrane was blocked for 3 hrs in blocking solution followed by incubation with polyclonal sheep anti-digoxigenin Fab-fragments,

conjugated to alkaline phosphatase for 2 hrs, followed by three washes in washing buffer. The alkaline phosphatase substrate BCIP/NBT was added to the membrane, developed for 2 hrs and the reaction was terminated using TE buffer.

2.2.1.15. Sequencing of DNA

Sequencing is an important step to confirm the correct DNA sequence of the transgenes and inserts after molecular cloning to ensure functionality for subsequent downstream applications and also to reduce the number of mice for transfection and cloning. The sequencing of DNA fragments used in this study for molecular cloning and transfection were performed by Sandor Lifesciences Pvt. Ltd (Hyderabad, India). The samples were prepared as follows:

Plasmids: 20 µl, 50-100 ng/µl

PCR products: 20 µl, 10-50 ng/µl

2.2.2. Methods for microbiology

2.2.2.1. Culturing of *Escherichia coli (E. coli)*

The study of microorganisms requires techniques for isolating cells from natural sources and growing them in the laboratory on synthetic media. Free-living microorganisms like the bacterium *E. coli* can be grown in synthetic media. Under suitable conditions, a single cell of bacterium grows into a colony of cells on agar medium. The resulting population of genetically identical cells derived from a single parent cell is called a clone. This simple technique facilitates isolation of genetically distinct clones of cells. Luria-Bertani (LB) broth is the most widely used medium for the growth of bacteria (Bertani, et al., 1951). It contains peptides, peptones, yeast extract, and trace elements that are important for bacteria growth and proliferation. Often, culture media is prepared in solid form such as in the slants and the petri dishes. Culture media is solidified with the addition of Agar, a purified carbohydrate obtained from a marine seaweed. Culturing of *E. coli* on semi-solid surface of the agar plates facilitates production of discrete colonies. Generally, transformed bacteria is grown on LB agar plates with antibiotics, allowing selective growth of bacteria. Bacterial cultures for plasmid preparation or recombinant protein production are grown in liquid medium in order to

produce large quantities of either DNA or protein. Appropriate antibiotics were added to the growth medium to allow only the strain of bacteria with the required DNA can grow and the liquid cultures were incubated overnight at 37°C with continuous shaking.

2.2.2.2. Preparation of chemo-competent *E. coli* cells

The ability to transform or take up the exogenous DNA is called competence. Natural competence exists in some bacterial species. Most of the laboratory strains do not possess natural competence. To enable the bacterial cells to accept the exogenous DNA, they have to be made competent and competence can be artificially induced with chemical treatment. Chemically competent cells are regularly used in cloning procedures for the production of recombinant DNA or proteins. Briefly, a frozen glycerol stock of *E. coli* XL-1 Blue was streaked onto a LB agar plate containing tetracycline and incubated overnight 37°C. A single colony from the plate was inoculated in 3 ml LB broth containing tetracycline and incubated overnight 37°C with continuous shaking at 200 rpm. Next morning, 100 ml of fresh LB broth with tetracycline was inoculated with 1% of overnight culture and incubated overnight 3at7°C with continuous shaking at 200 rpm. The growth of bacterial culture was monitored by measuring the optical density (OD_{650}) in a spectrophotometer. When the culture reached logarithmic growth phase (OD_{650} 0.4–0.5), the bacterial culture was transferred to two 50-mL Falcon tubes and placed on ice for 30 min. The bacterial cells were pelleted by centrifugation at 5000 g for 10 min. The pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and placed on ice for 30 min. Following incubation, the cells were again spun down and pelleted at 5000 g for 10 min. Finally, the pellet was resuspended in 4 ml of ice-cold 0.1 M $CaCl_2$/15% glycerol and stored in 100 µl aliquots at -80°C for long term usage.

2.2.2.3. Transformation of chemo-competent *E. coli* cells

This is a routinely used molecular biology technique to introduce foreign DNA, either in a linear or circular conformation (plasmid), into bacterial cells *(E. coli)*. The introduction of plasmids or ligation products into *E. coli* cells is an essential step in the molecular cloning procedures. After transformation, the bacterial cells will replicate the foreign DNA along with its own to produce large DNA quantities, which can be isolated for further use in downstream applications. Using plasmids containing antibiotic resistance marker facilitates the selection

of transformed cells. The chemically competent *E. coli* strain XL-1 Blue was used for cloning procedures and also for final target vector amplification. For transformation, a frozen vial of competent *E. coli* XL-1 blue cells 100 µl was thawed on ice and 10 µl of the ligation mixture or 50- 100 ng of the plasmid was added to the competent cells, mixed gently by stirring with a pipette tip. The mixture was immediately incubated on ice for 20min and given heat shock at 42°C for 60 seconds for uptake of the DNA, followed by incubation on ice for 5min. One ml of fresh LB broth was added to the transformation mixture and incubated at 37°C for 1 hr in a shaker incubator. After incubation, the cells were spun down at 3000 rpm for 5min. The pellet was resuspended in 100µl fresh LB broth and plated on to the LB agar plates containing appropriate antibiotics. The plates were incubated overnight at 37°C to obtain transformed bacteria in single colonies.

2.2.3. Methods for microscopy

2.2.3.1. Live cell imaging

Mixed blood stage parasites were obtained by tail vein puncture of infected mouse. The blood was diluted in pre-warmed enriched RPMI containing DAPI (1µg/ml) and incubated at 37°C for 15 min. The cells were washed and pelleted in RPMI. Finally, the cells were mounted in 40 % glycerin/PBS on a standard microscopic slide under sealed cover slips. Afterwards, the imaging was performed using a fluorescent microscope with 100x immersion oil objective lens. All mosquito stages were dissected in RPMI. The infected midguts and salivary glands were mounted in dissection media under sealed coverslips on standard microscopic slides. The samples were imaged using a fluorescent microscope with 20x objective lens. Similarly, the oocysts and sporozoites were dissected in 3% BSA/RPMI media and mounted in dissection media under sealed coverslips on standard microscopic slides. For visualizing oocyst derived sporozoites, the infected midguts were dissected in RPMI and mounted under sealed coverslips on microscopic slide. The sporozites were mechanically liberated out of oocysts by applying force on the coverslip using forceps. The samples were imaged using a fluorescent microscope under 100x immersion oil objective lens. For long-term imaging of oocysts, the infected midguts at days 14-21 post infection were dissected in 3% BSA/RPMI media and mounted on a microscopic slide. The samples were sealed under cover slips and observed for sporozoite movement within oocysts and egress events using a fluorescent microscope with

100x immersion oil objective lens. 10-20 midguts were imaged for each parasite line (knockout and wild type). Movies of 1-2 min duration were recorded in DIC mode with 100x magnification. To perform the live motility assays, the hemocoel sporozoites were isolated as described in earlier section. The sporozoites were pelleted down by centrifugation at 7000 rpm for 5 min. The sporozoites were resuspended in 200 µl of 3% BSA/RPMI and incubated at 37°C for 30 min. 5-10 µl of sporozoite suspension was placed on microscopic slides and sealed under coverslips. The movements of sporozoites were observed in DIC mode with 100x magnification. Movies of 1-2 min duration at a rate of 30 frames per sec were recorded. Only sporozoites glidng in a circular manner for at least one complete circle over the time of two min were considered as motile. The other patterns, such as floating, bending, patch gliding, waving, twitching and flexing (Hegge et al., 2009), were considered non-motile.

2.2.3.2. Immunofluorescence assays (IFA)

2.2.3.2.1. IFA of sporozoites

For *in vitro* gliding motility assays, after incubation at 37°C, medium was carefully removed and sporozoites were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature. After fixation, the sporozoites were washed in PBS once and then permeabilized with ice-cold methanol for 10 min at room temperature. Following permeabilization, sporozoites were blocked in 3% BSA/PBS for 1 hr at room temperature. Sporozoites were incubated with the primary antibody solution, mouse anti-PbCSP mAb 3D11 in 3% BSA/PBS (Yoshida et al., 1980), for 1 hr at room temperature in the dark and subsequently washed three times with PBS. After the final wash, the sporozoites were incubated in secondary antibody solution, Alexa Fluor 488 goat anti-mouse 3% BSA/PBS, for 1 hr at room temperature in the dark. Afterwards, sporozoites were washed again three times with PBS. The coverslips were allowed to air dry before mounting in 5-10 µl of ProLong Gold Antifade solution (Thermo Scientific) on standard microscopic slide. The coverslips were sealed in nail polish and the slides were stored at 4°C or directly examined using fluorescent microscope with 100x magnification in immersion oil. For cell traversal assays, after incubation at 37°C, medium was carefully removed and cells were fixed in 4% PFA) for 30 min at room temperature. After fixing, the cells were washed in PBS.

The nuclei were visualized with Diamidino-phenylindole (DAPI, 10 µg/ml) staining by incubation in DAPI/PBS for 15 min at room temperature. The samples were washed with PBS twice after DAPI staining. The coverslips were mounted in antifade solution and sealed with nail polish. The number of traversed host cells were counted in a minimum of 50 fields by fluorescence microscopy. For invasion assays, after incubation at 37°C, the medium was removed and the cells were fixed in 4% PFA for 30 min at room temperature. After fixation, the cells were washed two times with PBS. After the last wash, the cells were blocked with 3% BSA/PBS for 1 hr at 37°C. Following blocking, the extracellular non-invading sporozoites were detected without permeabilization by incubating with primary antibody solution rabbit polyclonal anti-GFP IgG in 3% BSA/PBS for 1 hr at 37°C in the dark. The cells were washed three times with PBS followed by incubation with secondary antibody solution goat anti-rabbit IgG-Alexa Flour 488 in 3% BSA/PBS for 1 hr at 37°C in th dark. Cells were washed with PBS three times after the incubation. Afterwards, cells were permeabilized with ice-cold methanol and total sporozoites were detected by incubating with mouse anti-PbCSP mAb 3D11 in 3% BSA/PBS for 1 hr at 37°C in the dark followed by goat anti-mouse IgG-Alexa Flour 594 in 3% BSA/PBS for 1 hr at 37°C in the dark. The nuclei were stained with DAPI added along with the secondary antibody solution. The cells were washed two times with PBS. The coverslips were air-dried and mounted in antifade solution on microscopic slides. The coverslips were sealed with nail polish on the periphery before the examination using a fluorescence microscope. A minimum of 50 fields were counted, each filed was counted using both green and red filters.

2.2.3.2.2. IFA of EEFs / infected HepG2 cells

HepG2 cells were cultured and infected as described in earlier section. After the indicated time periods, medium was carefully removed from the wells. The cells were washed with PBS once and fixed with 4% PFA for 20 min at room temperature. After fixation, cells were washed with PBS and permeabilized with ice-cold methanol for 10 min at room temperature. Afterwards, cells were blocked with 3% BSA/PBS for 1 hr at 37°C. EEFs were detected by incubating cells in 3% BSA/PBS for 1 hr at 37°C with primary antibodies mouse monoclonal anti-HSP70 that stains parasite cytoplasm (Tsuji et al., 1994) and rabbit polyclonal anti-UIS4 that stains parasitophorous vacuole membrane (Mueller et al., 2005). Afterwards, cells were washed three

times with PBS and incubated with secondary antibodies; goat anti-rabbit IgG conjugated with Alexa Fluor 594 and rabbit anti-mouse IgG conjugated with Alexa Fluor 488 for 1 hr at 37°C in the dark. DAPI was added along with the secondary antibodies to visualize the nuclei. Following incubation, the cells were washed three times with PBS. The coverslips were allowed to air dry and mounted in antifade solution on microscopic slides. The coverslips were sealed with nail polish on the periphery. The slides were either stored at 4°C in the dark or directly examined using fluorescent microscope. For all the IFA experiments, after processing, the samples were analyzed using Nikon Eclipse Ni-U fluorescence microscope equipped with appropriate filters at different magnifications based on the experimental needs. The images were captured and processed using NIS-Elements Advance Research microscope imaging software.

2.2.4. Methods for parasitology

2.2.4.1. Generation of gene knockout constructs

DNA constructs for knockout vectors were generated using standard molecular biology techniques. The plasmid pBC-GFP-Hdhfr was used to generate the gene knockout constructs. It contains hDHFR cassette which would favour selection of recombinant parasites and GFP cassette, under the control of HSP70 regulatory elements, which would facilitate visualization of recombinant parasites. It also contains chloramphenicol resistance cassette which allows selection in *E. coli*. Double crossover constructs were made by PCR amplification of approximately 500 to 700 bp of upstream and downstream DNA homology sequences (inserts) flanking the open reading frame of the gene to be knocked out, using wild type *P. berghei* genomic DNA. The desired restriction sites, which facilitates cloning, were incorporated in the oligonucleotides designed for amplifying the inserts. After the PCR amplification, the inserts were sequentially cloned into the the vector by digesting the inserts and the vector with compatible restriction enzymes to generate the final targeting vector consisting of homology sequences flanking the GFP cassette and the drug selectable marker cassette. The targeting plasmid pBC-GFP-hDHFR was a kind gift from Robert Menard, Pasteur Institute, France.

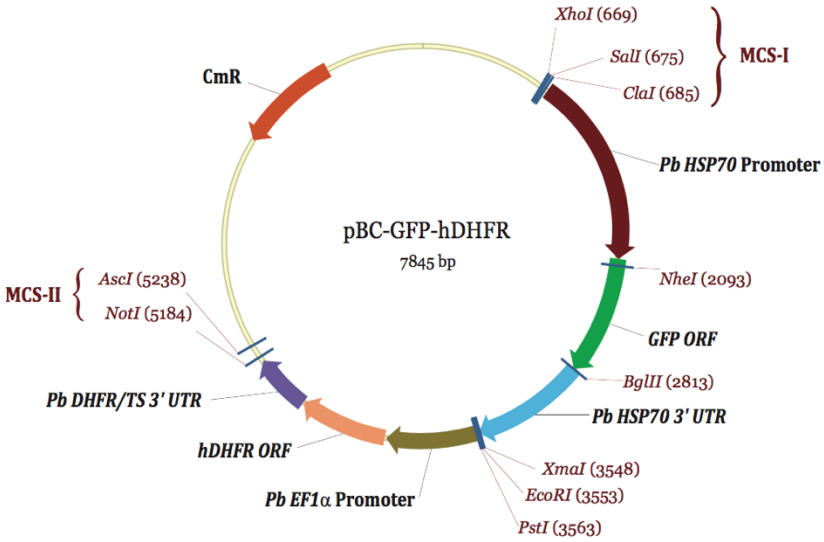


Figure 2.1. Pictorial representation of the pBC-GFP-hDHFR plasmid. This plasmid (size 7.8 kb) was used to generate the targeting constructs for gene knockout studies in *P. berghei*. Note that it has two selectable markers, one for bacteria (CmR) and the other for *P. berghei* (hDHFR). Two multiple cloning sites for introducing the homologous sequences and a GFP cassette for visualizing the recombinant parasites.

2.2.4.2. *In vitro* culture of *P. berghei* blood stage parasites

Short term culture of *P. berghei* blood stage parasites can be carried out to obtain mature schizonts, which were used to transfect the linearized targeting DNA construct. Transfection was performed as described earlier (Janse et al., 2006). Briefly, to obtain mature schizonts, a Swiss-Webster mouse was infected with cryopreserved *P. berghei* ANKA parasites by intra-peritoneal injection. When the parasitemia reached about 1%, blood was collected by retro-orbital bleeding and passaged to another four mice intravenously (i.v.). Mice having 2-3% parasitemia and low gametocyte density were used for the schizont culture. The blood was harvested by cardiac puncture from ketamine (50 mg/ml) / xylazine (20 mg/ml) anesthetized animals using heparin-treated syringes. The blood was collected into a 50 ml Falcon tube containing 250 µl heparin/PBS (200 U/ml). After collection, the blood was washed once in 10 ml of pre-warmed schizont media and erythrocytes were pelleted down by centrifugation at 800 g for 8 min in a swing bucket rotor without brake at room temperature. The blood pellet was carefully resuspended in 5 ml of pre-warmed schizont media and was pipetted equally into five unvented T75 cell culture flasks each containing 20 ml of schizont media. The flasks were gassed for 3 min with a mixture of 5%O2, 5% CO2, and 90% N2 before incubated at 37°C for

15-16 hours with gentle shaking at 50-60 rpm in a shaker incubator. During the overnight culture, most of the blood stages transform into mature schizonts. The quantity and quality of schizonts were determined by making a giemsa-stained blood smear from the culture prior to transfection.

2.2.4.3. Isolation and purification of schizonts

Before electroporation, the mature schizonts were separated from uninfected erythrocytes by density gradient centrifugation using 60% nycodenz solution. The overnight culture was carefully transferred to four 50 ml falcon tubes, 25 ml in each tube and 10 ml of the nycodenz solution was gently added to the culture suspension using a 10 ml pipette. The culture suspension was then centrifuged at 380g for 20 min in a swing bucket rotor without the brake at room temperature. Mature schizonts were collected with a pasteur pipette from the brownish interphase ring between the two suspensions. The schizonts were then pelleted and washed in two falcon tubes with schizont media by centrifugation at 1500 rpm for 15 min without the brake. After the washes the schizont pellet was subsequently suspended in 5 ml of schizont media.

2.2.4.4. Transfection of schizonts by electroporation

For each transfection, 1ml of schizonts was pelleted by centrifuging at 13000 rpm for 1 min at room temperature. The schizont pellet was resuspended in 90 µl of Mouse T-cell nucleofector solution mixed with 20 µl of supplement 2 solution and 5-10 µl of linearized targeting DNA construct. The whole mixture was transferred to a transfection cuvette and electroporation was performed with Amaxa Nucleofector II device using the U33 program. Immediately after the electroporation, 100 µl of schizont media was added and the transfected parasites were immediately injected into the tail vein of pre-warmed naïve Swiss-Webster mice.

2.2.4.4. Positive selection of recombinant parasites

24 hours after transfection the parasitemia was checked by observing Giemsa-stained blood smears made from the mice i.v. infected with transfected parasites. The selection of recombinant parasites was achieved by administering the antifolate drug pyrimethamine (7 mg/100 ml; pH 3.6- 5.0) to the mice in drinking water. While the mice were under

pyrimethamine, parasitemia was monitored daily for a period of 5-10 days by Giemsa-stained blood smears. Initially, the parasitemia decreased gradually, followed by a complete absence of parasites in the blood. The drug resistant parasites started to appear on day 7 and the parasitemia levels increased gradually. The pyrimethamine resistant parasites were also observed under fluorescence microscope for GFP expression. The blood from positive mice was collected by retro-orbital bleeding and cryopreserved. Some of the blood was used for genomic DNA preparation to perform genotyping of recombinant parasites or was transferred to naïve mice for parasite cloning. Two independent transfections were performed for each targeting construct.

2.2.4.5. Giemsa staining and determination of parasitemia

Giemsa staining is used to monitor infection of red blood cells, differentiate the developmental stages, and determination of parasitemia, the percentage of *Plasmodium* infected erythrocytes. Thin blood smears were made on standard microscopic slides by venipuncture of mice tail. The smears were air dried and fixed in methanol for 30 sec. Afterwards, the smears were stained with 20% Giemsa solution (v/v in water) for 10 min. The smears were then rinsed gently with tap water and allowed to air dry. The stained blood smears were examined in immersion oil under 100x objective lens using a standard light microscope. Parasites were identified by the unique morphological features of their developmental stage. A minimum of 10-20 fields, each containing uniformly spread 300-500 red blood cells were counted and the final parasitemia was calculated using the following formula.

Parasitemia % = ∑ of infected erythrocytes / (∑ of erythrocytes per field x ∑ of counted fields)

2.2.4.6. Genotyping of transfectants

After transfection, the mice intravenously injected with transfected parasites were subjected to pyrimethamine in drinking water. The parasitemia was monitored by Giemsa smears. When the parasitemia reached 5% blood was collected and cryopreserved. Some of the collected blood was lysed and used for genomic DNA preparation. To check if the drug resistant population obtained after transfection contained recombinant parasites and to determine the relative proportion of wild type parasites that survived the drug selection, diagnostic PCR was performed using specific primers. Integration events at 5' and 3' ends were

determined using upstream and downstream primers, outside of the homology sequences used for integration and also corresponding reverse or forward primer on either GFP cassette or drug selection cassette respectively. These primers amplified 5' and 3' integration products only if the correct integration of the homology sequences and selection cassettes were present in the parasite population. Also, to ascertain whether the target gene was knocked out, PCRs were performed using primers which amplify a portion of the open reading frame (ORF) of the target gene. The same combination of primers was used to confirm the clonality of isogenic parasite lines obtained after cloning by limiting dilution. Further, the targeted deletion and the clonality of isogenic parasite lines was also confirmed by southern blotting analysis.

2.2.4.7. Cryopreservation of *P. berghei* blood stage parasites

The blood stages of recombinant parasites were cryopreserved in liquid nitrogen for long term usage or at -80°C for short term storage. The infected blood was collected from the mice by cardiac puncture using heparin treated syringes. Two parts of freezing solution was added to one part of infected blood (800 µl of freezing solution + 400 µl of infected blood) in a 1.5 ml microcentrifuge tube. After proper mixing, the parasites were transferred to 1.2 ml cryovials as 300 µl aliquots. The cryovials were immediately stored at -80°C in a cryocooler for few days and subsequently transferred to liquid nitrogen container.

2.2.4.8. Isolation of *P. berghei* from infected blood

Approximately 250 µl of blood was collected from *P. berghei* infected mouse in a heparinized 1.5 ml microcentrifuge tube. Erythrocytes were spun down and pelleted at 1500 rpm for 8 min at room temperature. The erythrocyte pellet was washed twice in 1x PBS by centrifugation at 1500 rpm for 8 min at room temperature. Parasites were harvested by resuspension of the erythrocyte pellet in 500 µl of 0.02% saponin in 1x PBS. After saponin lysis, parasites were pelleted by centrifugation at 4000 rpm for 8 min at room temperature. The parasite pellet was washed twice in 1x PBS by resuspension and centrifugation at 6000 rpm for 5 min at 4°C. The parasite pellet was subsequently processed to obtain either genomic DNA or protein lysates for western blotting.

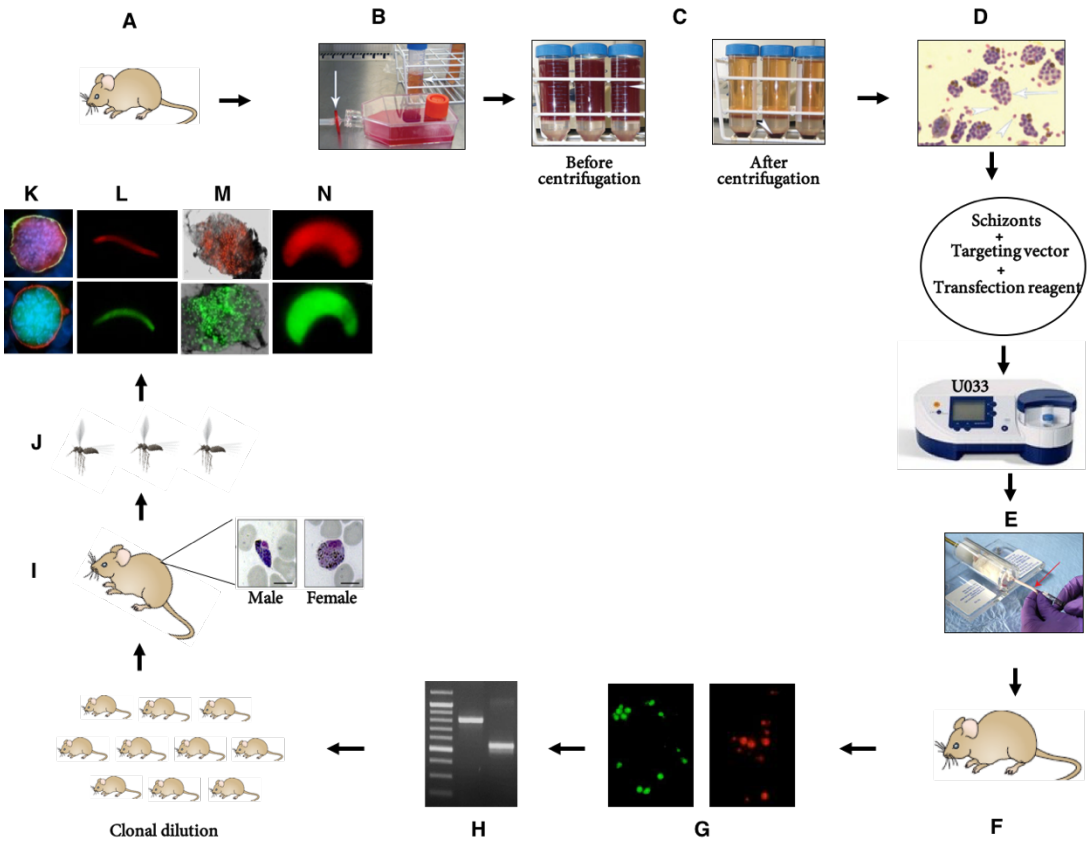


Figure 2.2. Schematic representation of *P. berghei* transfection, drug selection, confirmation of site specific integration and phenotypic characterization of genetically modified parasites. P. berghei infected blood was collected from the mouse having 2-5% parasitemia **(A)** and an overnight in vitro culture was set up **(B)**. Next day, schizonts were enriched on a 60% nycodenz density gradient by centrifugation **(C)**. The purified schizonts **(D)** were collected and electroporated with the targeting construct using Amexa nucleofector device and immediately injected intravenously into mouse **(E)**. Pyrimethamine, an antimalarial drug was provided orally in drinking water to mouse harbouring transfected parasites that facilitate selection **(F)**. The transfected parasites were observed for either GFP or mCherry fluorescence **(G)** depending on the reporter cassette used in the targeting construct. Site specific integration was confirmed by PCR **(H)**. Clonal population of successful transfectants was obtained by limiting dilution and the mice were infected with clonal lines **(I)** and subsequently passed through mosquitoes **(J)** to analyze the phenotype in stages like ookinete **(K)**, oocyst **(L)**, sporozoite **(M)** in mosquito and liver stages **(N)** in the vertebrates. Images adapted and modified from Janse et al, 2006.

2.2.4.9. Generation of isogenic parasite lines by limiting dilution

Drug-resistant parasite population obtained after transfection usually consists of both the recombinant as well as the wild type parasites that survived the drug selection. A homogenous recombinant parasite population derived from a single parent is necessary for characterizing the phenotype of the knockout (KO) parasites. Isogenic clones of recombinant parasite populations were generated *in vivo* by intravenous injection of single parasite-infected erythrocytes obtained by limiting dilutions into naïve BABL/C mice. Briefly, a donor mouse was infected with 200 µl of cryopreserved transfected parasites of the line to be cloned by intraperitoneal (IP) injection. When the parasitemia was <1%, blood was collected from the infected mouse by cardiac puncture. The blood was further diluted 10^5 times in incomplete RPMI and the number of uninfected and infected erythrocytes per µl of cell suspension was calculated. The blood was further diluted to a final concentration of 0.5 parasite / 200 µl in incomplete RPMI. A group of ten mice was infected intravenously with 0.5 parasites/mouse to obtain isogenic parasite clones. At day 8 after infection, mice were checked for infection by Giemsa-stained blood smears, usually, 20 - 40% of mice become infected. When the parasitemia reached 5-10% in the positive mice, blood was collected for cryopreservation and simultaneously gDNA was isolated from the blood as described in earlier sections, for genotyping the isogenic clonal parasite populations.

2.2.4.10. *In vivo* analysis of *P. berghei* blood stage development

The isogenic recombinant parasite clones obtained were subsequently used for phenotypic characterization. To assess the growth rates and development of blood stages of KO and wild type parasites *in vivo*, two groups of 6-week-old female BALB/C mice (5/group) were intravenously injected with $1x10^3$ mixed blood stage parasites. A *Plasmodium berghei* ANKA line constitutively expressing GFP was used as wild type control for all the subsequent experiments throughout this study (Al-Nihmi et al., 2017). After infection, parasitemia was measured from day 3 onwards by daily microscopic observation of Giemsa-stained thin blood smears. Growth rates were analyzed, by plotting % of parasitemia against time of infection.

2.2.4.11. Rearing and maintenance of *A. stephensi* mosquitoes

The maintenance of laboratory colonies of *Anopheles stephensi* mosquitoes, vectors for human and rodent malaria, is essential to study the development and infection dynamics of *P. berghei* in mosquito vector and vertebrate host (mice). All stages of *A. stephensi* mosquitoes were reared and maintained in the insectary in a 14 hrs. light and 10 hrs. dark cycle under favourable growth conditions of 26-28°C temperature and 70-80% relative humidity. Adult mosquitoes were bred and maintained in netted cloth cages attached to metal frames. Each cage can hold 1000 – 2000 mosquitoes. Adult mosquitoes were fed with an uninterrupted supply of 10% sucrose solution by means of soaked cotton pads placed on top of the cages.

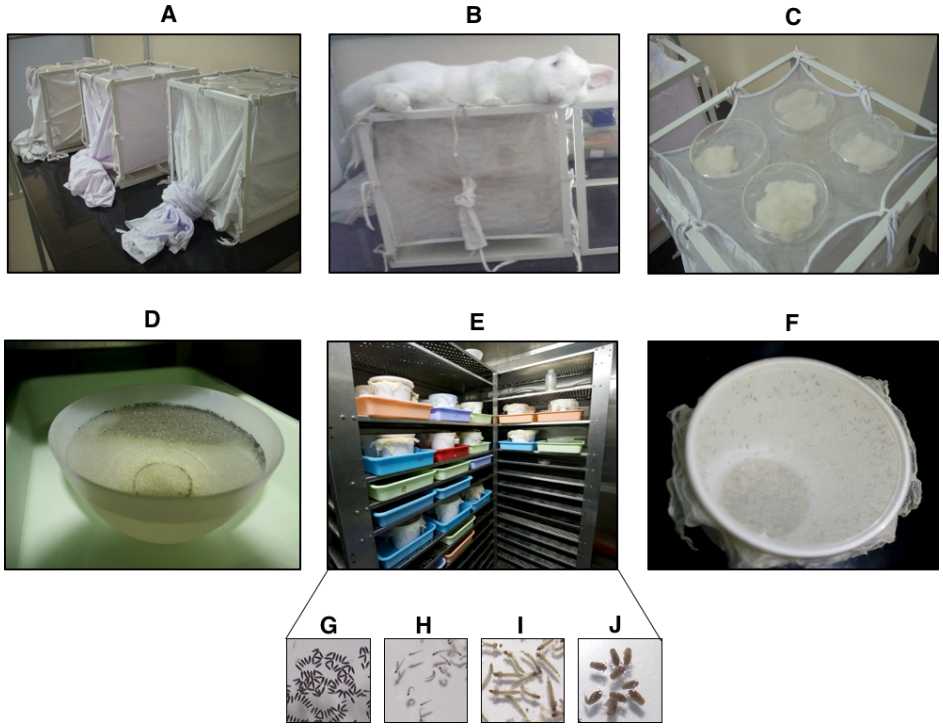


Figure 2.3. Rearing and maintenance of *A. stephensi* mosquitoes. (**A**) Breeding cages were prepared from freshly emerged mosquitoes. Adult mosquitoes typically mate within 24-36 hrs post emergence. **(B)** For blood feeding of female mosquitoes, anaesthetized rabbit was placed on the breeding cage. **(C)** After the blood meal, both male and female mosquitoes were fed with sugar (sucrose) pads. **(D)** After the blood meal, eggs were collected on daily basis for the next 3-4 days by placing a water bowl inside the breeding cage. **(E)** The eggs were then transferred to stability chamber maintained at 26-28°C temperature and 70-80% relative humidity, where **(G)** eggs undergo transformation into **(H)** and **(I)** larvae, **(J)** pupae and finally into adults. **(F)** When pupae start to emerge, they were collected and transferred to plastic cages, where they transform into adult mosquitoes in 24-48 hrs. Pictures courtesy: Ravi Jillapalli.

Once in two weeks the adult mosquitoes were blood fed for 20 – 30 min on 3-month old New Zealand White male rabbit. Prior to the blood feeding the mosquitoes were starved for 8 hours by removing the sucrose pads and the rabbit was anaesthetized with a mix of 0.4 ml of Xylazine (20mg/ml) and 0.8 ml of Ketamine (50mg/ml) injected intramuscularly. Two days after feeding, eggs from adult female mosquitoes were collected in an egg bowl containing a moist filter paper. The eggs were allowed to hatch in another bowl containing low levels of water for two days. Upon hatching, the young hatchlings were transferred to plastic trays and fed with larval food made with a combination of cereal (Kellogs) and wheat germ extract (Avees). After 7-10 days, the larvae were transformed into pupae. The pupae were manually collected into

2.2.4.12. Infection of female *A. stephensi* mosquitoes with *P. berghei*

For *P. berhei* infection of mosquitoes, first a donor mouse was infected with the parasite line to be studied by i.p injection of cryopreserved blood stages. When the parasitemia reached 3-5%, blood was collected and five 6-8 week old female naïve Swiss-Webster mice were intravenously infected. Similarly, another group of mice were infected with wild type parasites to be used a control. Three days after infection, mice were monitored for the emergence of gametocytes by daily observation of the Giemsa-stained blood smears. Meanwhile female mosquitoes were heat selected by placing the hand around netted plastic cages containing freshly emerged adult mosquitoes. Prior to hand selection, the mosquitoes were starved for 4-6 hours to facilitate the isolation of female mosquitoes and to enhance the quality and the amount of blood meal. The female mosquitoes are collected into freshly made netted plastic cages using a vacuum pump. Usually infection cages were made just before the blood meal. Infection rates of mosquitoes are dependent on the number of gametocytes present in the blood. Therefore, when appropriate gametocytemia prevailed, the mice were anesthetized by intraperitoneal injections of Ketamine/Xylazine. Once, the mice were sedated, they were placed on top of the netted plastic infection cages such that the ventral surface (primarily abdomen) of each mouse was accessible for the mosquitoes, which were allowed to feed for 20 minutes. To obtain uniform infection rates among the mosquitoes, the sleeping positions of mice were rotated during the blood meal.

Following the blood meal, the infected mosquitoes were maintained in stability chambers under appropriate conditions (20°C temperature and 75% relative humidity) for optimal parasite development in the mosquito vector. The infection cages were fed with 10% sucrose solution on daily basis.

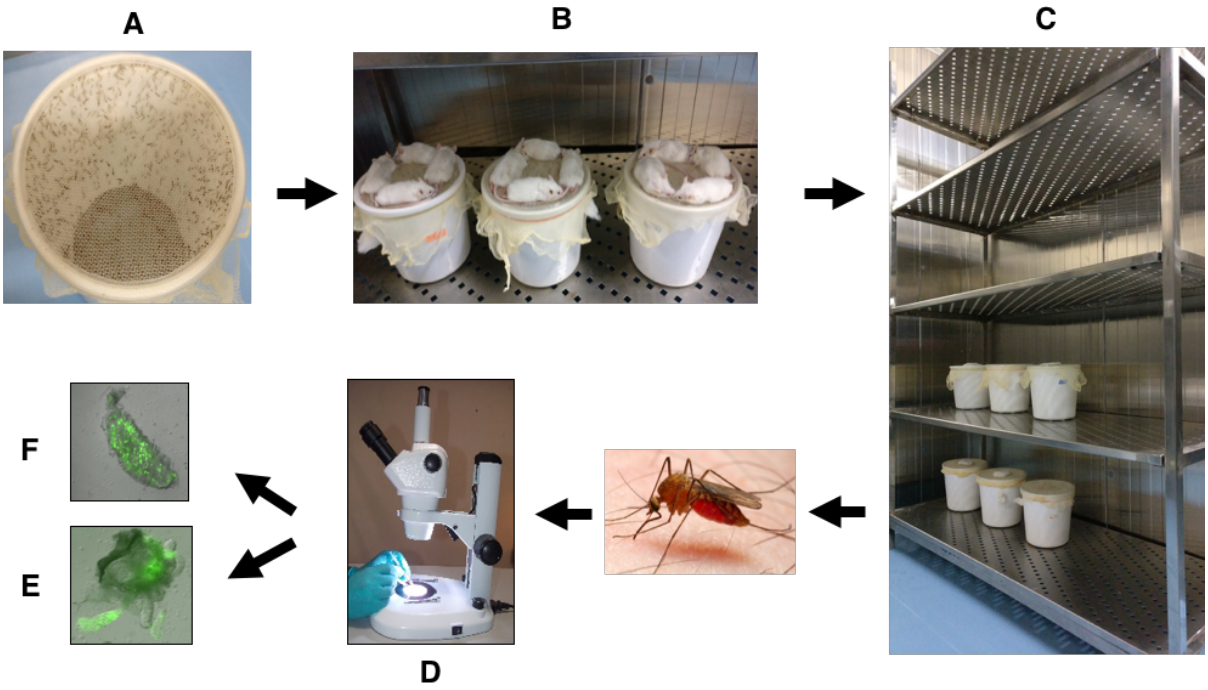


Figure 2.4. Infection of female *A. stephensi* mosquitoes with *P. berghei*. (**A**) Infection cages were prepared using freshly emerged female mosquitoes, typically few hours before infectious blood meal. **(B)** Infected mice carrying gametocytes in peripheral blood were anaesthetized and placed on the mosquito cage. **(C)** After the blood meal, mosquitoes were maintained at 20°C temperature and 80% relative humidity and fed with sugar (sucrose) pads. **(D)** At appropriate times post infection, mosquitoes were collected and dissected to examine the **(E)** salivary gland and **(F)** midgut infectivity.

2.2.4.13. Mosquito dissection and determination of midgut infectivity

At day after infection, 20-30 infected mosquitoes were collected into a 50 ml falcon tube using vacuum pump. The mosquitoes were sedated by keeping the tube on ice for 20 min. Mosquito dissection was performed in PBS or incomplete RPMI using a binocular dissection microscope. Initially, the mosquitoes were washed twice in 70% ethanol followed by a single wash in 1x PBS. Dissections were done in RPMI containing the Antibiotic-Antimycotic solution. Using insulin syringes, the mosquito was held in place at anterior end with one syringe tip and from the other end the midgut was gently pulled out with the help of another

syringe tip after chopping off a piece of posterior end of abdomen region. Mosquito midguts were stored either in PBS or RPMI based on the experimental requirements. For determining the midgut infectivity, the dissected midguts were spread uniformly in RPMI on a standard microscopic slide and was observed under a fluorescent microscope with 20x objective lens. Infectivity can be easily estimated by observing the GFP fluorescence of the oocysts at day 10 after infection.

The midgut infectivity was calculated as follows:

$$\text{Infectivity \%} = \sum \text{of infected mosquitoes} / \sum \text{of total mosquitoes' X } 100$$

2.2.4.14. Determination of oocyst numbers and sporulation

At day 14 after infection, oocyst formation was examined following mosquito midgut dissection as described earlier. 20-30 infected midguts were placed on a standard microscopic slide and mounted in RPMI under sealed rectangular cover slips. Images were captured using a fluorescent microscope under 10x objective lens. The number of oocysts were determined by manual counting of GFP expressing oocysts on captured images of infected midguts. The same mosquito midguts were used to observe the sporulation. To check sporulation of oocysts, the same midguts were also imaged under 100x oil immersion objective lens using a fluorescent microscope as well as in differential interference contrast (DIC) mode with 100x objective lens.

2.2.4.15. Isolation of midgut and salivary gland sporozoites

Sporozoites were isolated from midguts and salivary glands of infected mosquitoes at days 14 and 21 post infection respectively. For each time point at least 20-30 infected mosquitoes were dissected. Midguts were dissected and collected as described earlier. To isolate salivary gland sporozoites, mosquitoes were washed briefly in 70% ethanol twice followed by a single wash with sterile PBS. First, the head was severed carefully using insulin syringes. The salivary glands are very tiny and located in pairs at the base of the head. The salivary glands were gently dislodged from the base of the head and collected into a 1.5 ml microcentrifuge tube in RPMI containing the Antibiotic-Antimycotic solution. The pooled midgut and salivary gland tissues were crushed and ground extensively using a loosely fitted plastic micro pestle. The samples were centrifuged at 800 rpm for 3 min at 4°C. The supernatants were collected in a fresh 1.5 ml microcentrifuge tube and the numbers were quantified by counting in a Neubauer

chamber. Prior to enumeration, the stock samples were diluted in RPMI and 10 µl of the diluted sample was used for counting. The entire procedure was done on ice. Similarly, matching number of mosquitoes infected with wild type parasites were analyzed as controls. The total number of sporozoites was calculated as follows:

$$\sum \text{ of Sporozoites} = \text{Average no. of sporozoites from 4 quadrants X 10 (dilution factor)} \text{ X } 10^4 \text{ (Haemocytometer correction)/ml}$$

2.2.4.16. Isolation of haemolymph associated sporozoites

Haemolymph sporozoites were dissected between days 15-21 after mosquito infection. After collecting in a 50 ml falcon tube, the infected mosquitoes were sedated on ice. The dry mosquitoes were placed under the dissection microscope. Using the insulin syringes, the last segments of the abdomen were cut off. The haemolymph of the mosquitoes was flushed with RPMI containing the Antibiotic-Antimycotic solution using a finely stretched glass capillary tube inserted into the lateral side of the thorax. The haemolymph thus drained from the abdomen, collected in a plastic 1.5 ml microcentrifuge tube. The haemolymph sporozoites were quantified as previously described for midgut and salivary gland sporozoites.

2.2.4.17. Analysis of sporozoite infectivity by mosquito bites and intravenous injections

To determine the capacity of sporozoites of knockout parasite lines to undergo successful transmission from vector to host, transmission experiments were performed with infected mosquito bites and sporozoite injections. Once injected the sporozoites travel to the liver, via blood stream, where the invade hepatocytes and develop into exoerythrocytic forms (EEFs). After 68-72 hr of liver stage development, the daughter merozoites are released into blood where they infect red blood cells. With *P. berghei* it takes usually 3 to 4 days to detect the appearance of blood stage infection. Between 18-21 days of infection, the infected mosquitoes were transferred to medium sized netted paper cups, 10-15 mosquitoes per each cup and starved for 4-6 hr. Three female C57BL/6 mice were used for each experiment and anaesthetized using 80-100 µl of ketamine/xylazine mixture in 1X PBS. One mouse was placed on each cup and mosquitoes were allowed to bite on the ventral side for 30 min.

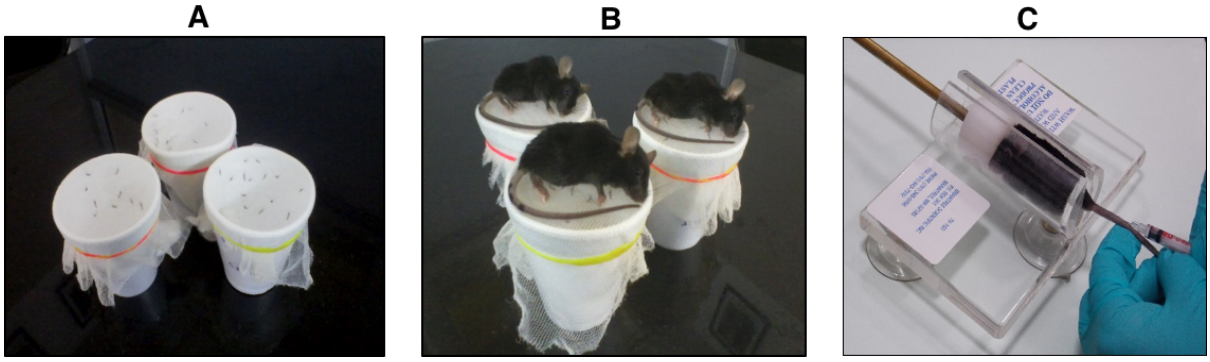


Figure 2.5. Malaria transmission experiments. **(A)** Approximately 15 infected mosquitoes harbouring either WT or KO parasites were placed in captivity in a small cage. **(B)** Anesthetized C57BL/6 mouse were placed over the cage net to facilitate malaria transmission by mosquito bites while obtaining a blood meal. **(C)** C57BL/6 mouse was kept still inside a mouse restrainer and sporozoites were delivered into tail vein by intravenous injections.

After the feeding, the blood fed mosquitoes were dissected to examine the parasite burden in midguts and salivary glands. Salivary glands were not dissected for mosquitoes infected with *pm viii(-)* as they never contained any salivary gland sporozoites. For sporozoite injections, the salivary gland sporozoites and hemocoel sporozoites were isolated as described earlier. Sporozoites were counted in a Neubauer chamber and later diluted in RPMI to the desired numbers in 200 µl aliquots. For each experiment three to five female C57BL/6 mice were injected intravenously. Bitten and injected mice were monitored for blood stage infection from day 3 to day 15 after exposure to infected mosquitoes or i.v. injections. The prepatency was checked by daily observation of Giemsa-stained blood smears using a light microscope. The time taken between infection to first appearance of the parasites in blood was calculated as prepatent period.

2.2.4.18. *In vitro* culturing and maintenance of HepG2 cells

Human liver carcinoma HepG2 cell line was obtained from National centre for cell sciences (NCCS, Pune, India). Upon arrival, the frozen cells were removed from dry ice, thawed and the culture was initiated immediately. Alternatively, the frozen cells can be stored in liquid nitrogen until the culture is initiated. The frozen vial was thawed by placing the vial in a 37°C water bath for 3-5 min. The vial was removed from the water bath as soon as the contents were thawed, and decontaminated by spraying with 70% ethanol.

The remaining procedure was carried out under strict aseptic conditions i.e. in Laminar air flow chamber (LAFC). The vial contents were transferred to a 15 ml falcon tube containing 5 ml pre-warmed complete culture medium and spun at 1500 rpm for 5 min. After discarding the supernatant, the cell pellet was resuspended in culture medium and dispensed into 75 cm^2 culture flasks. The flasks were transferred to incubator and maintained at 37°C temperature and 5% CO_2. After a day, the morphology and overall health of the cells was checked by observing under inverted phase-contrast microscope. The medium was changed twice in a week. The actively growing cells were subcultured when they reached 70-80% confluence. The medium was removed and the monolayer of cells was briefly rinsed with Trypsin-EDTA. Afterwards, 3 ml of Trypsin-EDTA solution was added and incubated for 5-10 min, until cells were detached and dispersed. The cells were gently aspirated into 15 ml falcon tube containing 7 ml of pre-warmed culture medium. The cells were spun down and pelleted at 1500 rpm for 5 min. The cell pellet was resuspended in fresh culture medium and appropriate aliquots were dispensed into new culture flasks, which were maintained in the incubator. Simultaneously, frozen stocks were prepared by dissolving the cell pellet in freezing medium, which contains DMSO apart from culture medium.

2.2.4.19. *In vitro* infection of HepG2 cells / EEF development assay

The human liver carcinoma cell line HepG2 was used to study *P. berghei* liver stage development *in vitro*. Wild type, *pm vii(-)* salivary gland sporozoites were isolated on day 21 post infection as described in earlier section. *pm viii(-)* hemocoel sporozoites were isolated on day 21 post infection as described in earlier section. A day before infection, $1x10^5$ HepG2 cells were seeded in collagen pre-treated 24 well plates containing coverslips. Collagen treatment enhances the attachment of cells on to the coverslips. 10,000 sporozoites of each line were added to the wells in 250 µl of complete DMEM. After centrifugation at 3000 rpm for 5 min, the plates were incubated for 2 hrs at 37°C. Afterwards, wells were washed thrice with complete DMEM to get rid of unattached sporozoites and finally incubated in 500 µl of complete DMEM at 37°C for 65-72 hrs. Medium was changed every 6 hrs to prevent contamination. The cultures were stopped at different stages of development (12, 24, 36, 48, 60, and 72 hrs) washed once with PBS.

The samples were stored at 4°C for further processing by immunofluorescence assay. Alternatively, the infected cultures for used for RNA isolation to study the gene expression profile at different time points of the parasite development in hepatocytes.

2.2.4.20. *In vitro* sporozoite gliding motility assays

One striking feature of *Plasmodium* sporozoites is their ability to move by gliding locomotion (Vanderberg, 1974). Gliding is a substrate-dependent form of motility and can be visualized by staining the circumsporozoite protein (CSP) trails left by *Plasmodium* sporozoites on the substrate using anti-CSP antibodies (Stewart and Vanderberg, 1988). Briefly, salivary gland and hemocoel sporozoites of wild type and knockout parasite lines were dissected and isolated on days 17-21 post infection. Glass coverslips were coated with 3% BSA/RPMI in 24 well plates at 37°C for 1 hr, in a humid chamber. Afterwards, 10,000-20,000 sporozoites in 3% BSA /RPMI were added to each well and incubated at 37°C for 1 hr in a humid chamber. After the incubation, the sporozoites were further processed by immunofluorescence assay. Finally, the percentage of gliding motility was determined by quantifying the CSP trails.

2.2.4.21. *In vitro* sporozoite cell traversal assays

To test the cell traversal ability, hemocoel sporozoites of wild type and *pm viii(-)* were isolated on days 17-21 post infection. 5000-10,000 hemocoel sporozoites of each line were added, in the presence of 0.5 mg/ml of Dextran conjugated with Texas Red to the sub-confluence monolayers of $1x10^5$ HepG2 cells seeded before 24 hrs in 24 well plates containing glass coverslips. After the addition of sporozoites, the plates were incubated for 2 hrs at 37°C, 5% CO_2. After the incubation, cells were further processed and the number of traversed host cells was determined by immunofluorescence assay. The percentage of traversed cells was calculated as follows: 100 X (Dextran positive cells / DAPI positive cells).

2.2.4.22. *In vitro* sporozoite invasion assays

To test the ability of hemocoel sporzoites to adhere to and invade HepG2 cells *in vitro*, the invasion assay was performed using a slightly modified version of earlier described protocol (Renia et al., 1988). Hemocoel sporozoites of wild type and *pm viii(-)* were isolated on days 17-

21 post infection. 5000-10,000 hemocoel sporozoites of each line were added to the sub-confluence monolayers $1x10^5$ HepG2 cells seeded before 24 hrs in 24 well plates containing coverslips. After the addition of sporozoites, the plates were incubated for 2 hrs at 37°C, 5% CO_2. After the incubation, cells were further processed and analyzed by dual color immunofluorescence assay to distinguish between invading and non-invading sporozoites. Finally, the percent invasion was calculated as follows:

[(Total sporozoites – Extracellular sporozoites) / Total sporozoites] × 100

2.2.4.23. Infecting the mice with parasites

Mice were infected with parasites through one of the following methods based on the experimental requirements.

(i) Intraperitoneal injections (i.p.) were performed either on lower left quadrant or lower right quadrant of un-anaesthetized mice. The mice were held by the nape of the neck and extended by pressing the tail to the palm of the hand (all with the left hand). The abdominal wall was penetrated at 45-degree angle with a sterile needle held in the right hand. This route of infection was preferred when mice were infected either with cryopreserved blood stages or when mechanically passaging the parasites from an infected mouse to a naïve mouse. Briefly, a cryogenic vial containing infected blood was taken out of the liquid nitrogen storage container and thawed at room temperature. Once thawed, 0.2-0.3 ml of the suspension was then i.p. injected into lower abdomen of the mice with a sterile hypodermic 1 ml syringe. For mouse to mouse mechanical passage, a drop of tail blood was collected from an infected mouse (parasitemia 5% to 10%) into incomplete RPMI (without FBS). The infected blood was then diluted and used for i.p. injection.

(ii) Intravenous injections (i.v.) were performed in the tail vein of un-anaesthetized mice. This route was preferred when infecting mice with sprozoites or the blood stage parasites for clonal dilution or for synchronization of infection or for transfection. Prior to the injections, the mice were put in a warm box at 37°C for 10 to 15 min to enhance the dilation of tail vein. Once properly dilated, immediately 0.2 ml of the infected blood suspension was injected into one of the tail vein with a sterile hypodermic insulin syringe. During the procedure mice were held steady with the help of a restrainer.

2.2.4.24. Animal handling and maintenance

Animals were procured from National Institute of Nutrition (Hyderabad) and maintained in animal house for experiments. During the course of the study, animals were provided with clean water and food *ad libitum*.

2.2.4.25. Ethics statement

All animal experimentations in this study were performed in strict accordance with the CPCSEA guidelines for care and use of animals in scientific research, India. The animal experimentation protocols were reviewed and approved by Institute Animal Ethics Committee at University of Hyderabad (Permit Number: UH/SLS/IAEC/2014-1/9b & 9c). A combination of ketamine and xylazine was used for anaesthesia and minimal suffering was ensured during the course of all experiments.

2.2.5. Methods for protein biochemistry

2.2.5.1. Preparation of sporozoite lysates

Between 17 to 21 days after infection with either wild type or knockout parasites, infected female mosquitoes were dissected and hemocoel associated sporozoites were isolated and enumerated as described in earlier section. To obtain hemocoel associated sporozoite lysate, the isolated sporozoites were pelleted by centrifugation at 5000 g for 3 min at 4°C. The pellet was resuspended in 2x Laemmli SDS sample buffer buffer and boiled for 5 min at 95°C. The lysates were either immediately used for separation of proteins by SDS-PAGE or stored in aliquots at -20°C for future use.

2.2.5.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analysed by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions (Laemmli, 1970). The proteins are separated based on the molecular weight. The natural protein conformation is altered by SDS, and the surface of the denatured polypeptides becomes negatively charged, hence migrates inside an electric field. The stacking gel allows the proteins to settle at the same level before chromatographic

resolution is started. Proteins are separated inside the resolving gel where the pore size determines the migration speed and distance. Resolving gels were prepared at a concentration of 12 % acrylamide, cast between two glass plates in a casting gel stand (Bio-Rad) and polymerized for 30 min. Stacking gels were prepared at a concentration of 5% acrylamide and layered over the polymerized resolving gel. The protein samples were separated in 1x running buffer at room temperature for 2 hrs at 100 V or until the dye front reached the end of the gel. The gels were either stained with Coomassie Brilliant Blue or further analysed by Western Blot.

2.2.5.3. Western blot (WB) analysis

Proteins resolved in polyacrylamide gels can be transferred in exact fashion of the original gel pattern on to nitrocellulose membranes and the immobilized proteins can be further identified with specific antibodies (Towbin et al., 1979). A nitrocellulose membrane (Bio-Rad) was thoroughly soaked in 1x transfer buffer together with two blotting foams and six whatman filter paper pieces. The SDS gel was removed carefully from the glasses and laid over the membrane, and further covered with Whatman filter papers and a fibre blotting pad. The gel and the membrane were sandwiched with three filter papers and a fibre blotting pad on either side in the blotting cassette (Bio-Rad). Proteins were transferred in a wet blotting system (Bio-Rad) filled with pre-cooled transfer buffer at 15 V for overnight at 4°C. After the membrane transfer, the protein of interest was detected by a specific primary monoclonal or polyclonal antibody, which was then recognised by a secondary anti-IgG antibody conjugated to horseradish peroxidise (HRP). After the transfer, the membrane was first transferred to a plastic container and blocked with 5% skimmed milk in 1x TBS for at least 1 hr at RT on a shaker mixer. After wards, the primary antibody diluted in blocking solution was added to the membrane and incubated on a shaker mixer for at least 2 hrs at RT or overnight at 4 °C. The membrane was washed three times in succession once with TBS, followed by TBST and again TBS. Each wash lasted at least 20 min. Thereafter, the membrane was incubated with the HRP-conjugated secondary antibody for 2 hours at RT. After the incubation, the membrane was thoroughly washed three times with TBS, TBST, and TBS. The washed membrane was incubated in dark for 2 minutes at RT with a freshly prepared mixture of solutions A and B in a ratio of 1:1 provided with an ECL kit (Amersham, GE Healthcare) and

the chemi-luminescence was detected. Briefly, the washed membrane was placed on transparent plastic sheet, the detection solution was pipetted on to the membrane. After incubation, the excess detection solution was drained and the membrane was covered with another transparent plastic sheet in such a way that bubble formation was prevented. Then the membrane was exposed to different time intervals to record light emission and to optimize the better signal. Signals intensities were recorded with a Chemi Doc XRS imaging system (Bio-Rad) and densitometric analysis was performed with Image Lab Software 5.2 (Bio-Rad).

3. RESULTS

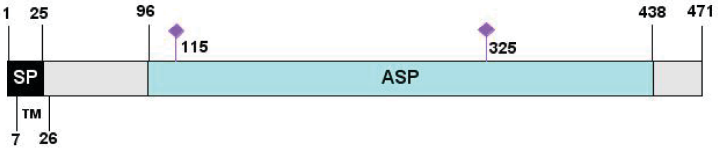
3.1. Functional characterization of *Plasmodium berghei* plasmepsin VII (PbPM VII)

3.1.1. *Pbpm vii* encodes a conserved aspartic protease

In order to characterize *Pb*PM VII, a detailed bioinformatic analysis was performed initially. The *Pb*PM VII (PBANKA_0517600) has orthologues in other *Plasmodium* species including *P. berghei*, *P. falciparum, P. vivax*, *P. yoelii*, *P. chabaudi*, and *P. knowlesi*. The gene structure and genomic organization is conserved among the orthologues. It has an 8 exon gene structure and encodes an aspartic protease (Figure 3.1A). A conserved domain search revealed that the predicted protein sequence encompasses a signal peptide region, a transmembrane region and a highly conserved aspartic domain with two aspartic acid residues essential for catalytic activity (Figure 3.1B). The overall amino acid sequence identity of PM VII proteins indicated differential conservation among the orthologues (Figure 3.1C).

A

B



C

Proteins	PbPM VII	PfPM VII	PvPM VII	PyPM VII	PcPM VII	PkPM VII
PbPM VII	100	56.8	54.7	96.3	95.1	53.9
PfPM VII		100	53.11	56.6	56.6	53.5
PvPM VII			100	54	54.5	82.8
PyPM VII				100	95.3	53.1
PcPM VII					100	53.4
PkPM VII						100

Figure 3.1. PbPM VII gene structure, protein structure, and conservation among *Plasmodium* species. **(A)** Schematic representation of genomic organization of PbPM VII gene (not to scale). The PbPM VII gene (PBANKA_ 0517600, 2380 bp) is composed of eight exons (shaded bars) and seven introns (lines). **(B)** Schematic representation of protein domain architecture of PbPM VII (not to scale). PbPM VII protein (PBANKA_ 0517600, 471 amino acids) contains a signal peptide (SP), a transmembrane domain (TM) and an aspartic domain (ASP) typically found in eukaryotic aspartic proteases. The positions of two catalytic aspartic acid residues are indicated in purple colour. **(C)** The table shows amino acid sequence conservation of PM VII orthologues among six *Plasmodium* species.

3.1.2. Gene expression profiling of *Pbpm vii*

In order to determine the importance of PbPM VII in *P. berghei* life cycle, the temporal expression of *PM VII* (PBANKA_0517600.1) gene across different stages of *P. berghei* was quantified using standard quantitative real-time PCR (qRT-PCR). cDNA samples were prepared from mosquito stage parasites (zygotes, ookinetes, young oocysts, mature oocysts, midgut sporozites, and salivary gland sporozoites) and from the *in vitro* grown liver stages corresponding to time points (16, 24, 42, 50, 65 hours) obtained following addition of sporozoites to HepG2 cells. Expression values were normalized against the *P. berghei 18s rRNA*.

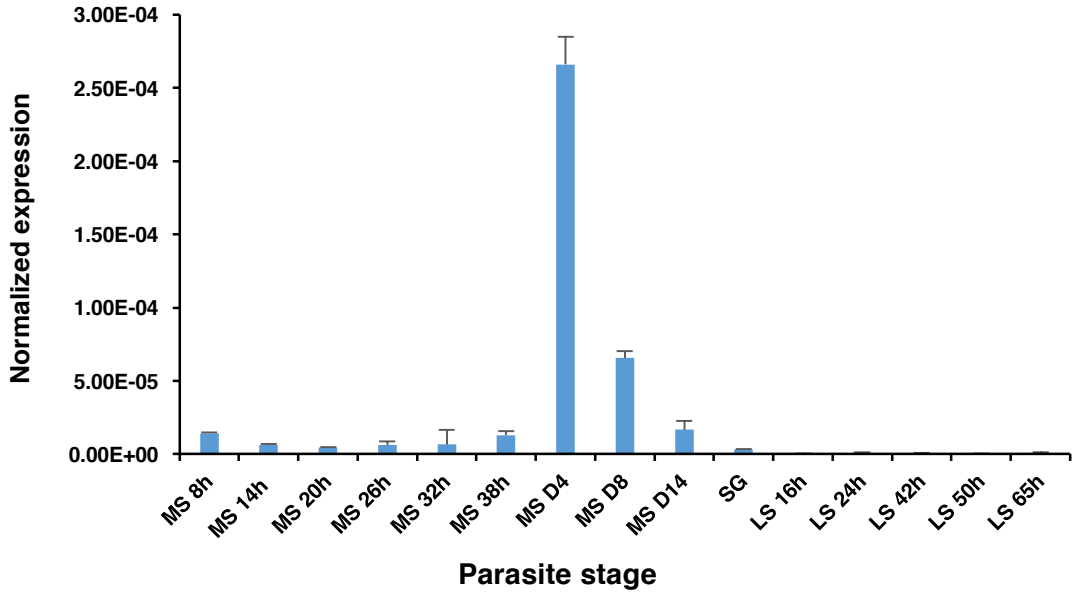


Figure 3.2. Expression profile of *Pbpm vii* in *P. berghei* mosquito and liver stages. Stage-specific gene expression analysis of *Pbpm vii* was performed by quantitative real-time PCR (qRT-PCR) using cDNA samples prepared from *P. berghei* mosquito stages and liver stages. Values were normalized to *P. berghei 18s rRNA* control. The abbreviations used to describe parasite stages are: mosquito stages (MS) in hours (h) at 8, 14, 20, 26, 32, 38 post infection and at days (D) 4 and 8 post infection (MS D), midgut sporozoites (MG), salivary gland sporozoites (SG), and liver stage parasites (LS) in hours (h) at 16, 24, 42, 50, 65 post infection.

Gene expression analysis revealed that *Pbpm vii* expression was most abundant in day 4 young oocysts followed by moderate expression in day 8 oocysts (Figure 3.2). The *Pbpm vii* expression was lowest in early mosquito stage parasites (MS 8h to 38h) and mature oocysts (D14). Surprisingly, *Pbpm vii* expression was not detected during liver stage development (LS

16h to 65h) (Figure 3.2). Taken together, the prominent expression of *Pbpm vii* gene in day 4 and day 8 oocysts likely suggested a functional role for PM VII during parasite development in mosquito stages.

3.1.3. Targeted gene deletion of *Pbpm vii*

To determine the cellular functions of PbPM VII in parasite life cycle, loss-of-function mutant (knockout) parasites were generated using classical reverse genetics. The *Pbpm vii* gene was targeted with a gene replacement construct that disrupts the target gene locus. This construct, containing 5' and 3' untranslated regions of the *Pbpm vii* ORF as homology sequences, inactivates the gene by double crossover homologous recombination and replaces the target gene with the GFP and hDHFR positive selection cassettes (Figure 3.3A). The 5' and 3' homology sequences were PCR amplified from wild type *P. berghei* genomic DNA and cloned into the targeting plasmid pBC-GFP-DHFR. The targeting plasmid was linearized and transfected into purified wild type *P. berghei* ANKA schizonts by electroporation. The transfected parasite population was immediately i.v. injected into a naïve mouse. Drug resistant population was obtained after selection with pyrimethamine. The parental drug resistant population contained the mixture of recombinant and wild type parasites. These parasites were subsequently cloned by limiting dilution to obtain two independent clonal lines lacking *Pbpm vii* and clone 7 (cl7) was used for further analysis and here after referred as *Pbpm vii(-)*. Genotyping by diagnostic PCR using specific primer combinations confirmed the expected integration and target gene replacement after homologous recombination in *Pbpm vii(-)* parasites (Figure 3.3B). Furthermore, taking advantage of constitutive GFP expression in recombinant parasites, live cell imaging of *Pbpm vii(-)* blood stage parasites was performed to confirm the disruption of *Pbpm vii* gene locus (Figure 3.3C). The successful generation of *Pbpm vii* knockout parasites (referred as *pm vii(-)*) in *P. berghei* blood stages demonstrates that this gene is dispensable during blood stage multiplication of the parasite.

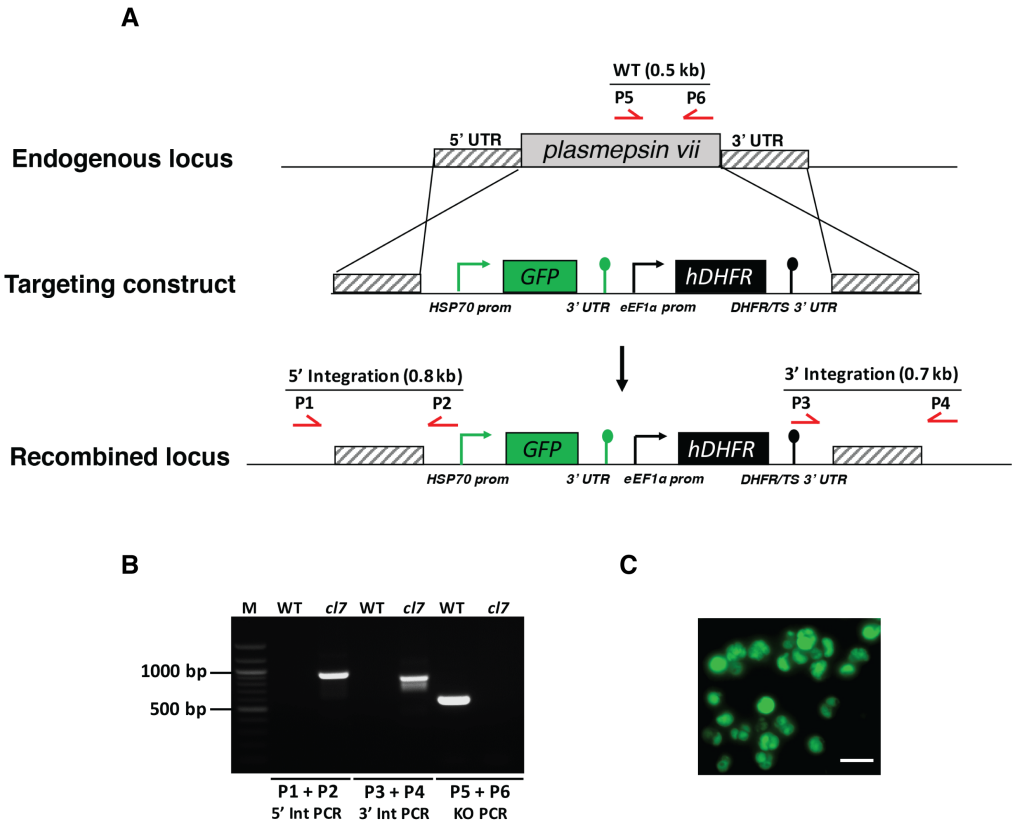


Figure 3.3. Generation of *Pbpm vii* knockout parasites in *P. berghei*. (**A**) Schematic representation of gene replacement strategy for the generation of *Pbpm vii(-)* knockout parasites (not to scale). The endogenous locus was replaced with a targeting plasmid pBC-GFP-DHFR/*Pbpmvii* containing the 5' and 3' untranslated regions (UTRs) of *Pbpm vii* flanking *GFP* and the selection marker human dihydrofolate reductase (*hDHFR*). After double crossover homologous recombination, the *Pbpm vii* open reading frame was replaced with *GFP* and the *hDHFR* selection cassettes. HSP70 prom, Heat shock protein 70 gene promoter; eEF1α prom, elongation factor 1 alpha gene promoter; DHFR/TS, dihydrofolate reductase - thymidylate synthase. **(B)** Genotypic analysis of *Pbpm vii(-)* parasites by diagnostic PCR with specific primers as indicated in schematic. The integration events at 5' and 3' ends were confirmed by primer combinations P1+P2 and P3+P4 respectively. The absence of wild type (WT) locus was confirmed by knockout (KO) PCR with primers P5+P6. WT gDNA was used as control. **(C)** Live cell imaging of *Pbpm vii(-)* parasites expressing GFP. Scale bars, 20 µm.

3.1.4. PbPM VII is dispensable for growth of asexual blood stages and sexual differentiation *in vivo*

To further corroborate the earlier findings, whether Pb*PM VII* has any supplementary role during blood stage development, *in vivo* growth assays were performed by intravenous

injection of 1x10^3 either WT or *Pbpm vii(-)* infected asexual parasites. Growth was monitored by parasitemia counts every 24 hours post infection (Figure 3.4A). Notably, proliferation of *Pbpm vii(-)* blood stage parasites was indistinguishable from WT parasites. Further, to investigate, if the loss of *Pbpm vii* affects sexual differentiation into male and female gametocytes, which is a prerequisite for transmission of malaria to mosquitoes, was determined. Daily microscopic observation of Giemsa stained thin blood smears revealed that *Pbpm vii(-)* blood stage parasites were morphologically indistinguishable from WT and undergo normal sexual differentiation into gametocytes (Figure 3.4B). Thus these data indicate that genetic ablation of PbPM VII affects neither multiplication of asexual stages nor sexual differentiation.

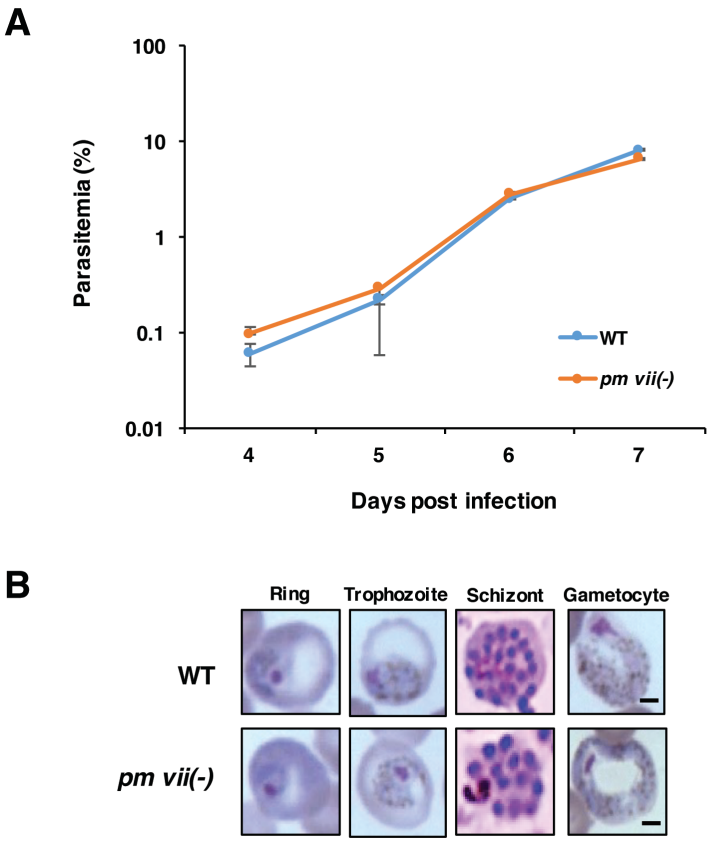


Figure 3.4. PbPM VII is dispensable for blood stage development *in vivo*. (A) *Pbpm vii(-)* blood stage parasites develop normally in the mammalian host. Asexual blood stage development was determined by injecting female BALB/C mice (n = 5) intravenously with 1x10^3 infected erythrocytes. Parasitemia and the gametocyte formation in the recipient mice was monitored by daily microscopic observation of Giemsa-stained blood smears. Shown are the values of mean (± SD). Asexual blood stage propagation was performed three times and a representative experiment is shown. **(B)** Shown are representative micrographs of blood stage parasites, at different stages of development, stained with Giemsa. Scale bars, 10 µm.

3.1.5. *Pbpm vii(-)* parasites develop normally in the mosquito vector

The predominant expression of PbPM VII in day 4 and day 8 oocysts prompted an in-depth characterization of *Pbpm vii(-)* parasites during their development in the *Anopheles* mosquitoes. A *Plasmodium berghei* ANKA line constitutively expressing GFP under the *HSP70* promoter was used as a wild type (WT) control throughout the course of this study for the phenotypic characterization of knockout parasites (Al-Nihmi et al., 2017). To test the effect of lack of the PbPM VII on the mosquito phase of the life cycle, the knockout parasite clone cl7 was fed to female *A. stephensi* mosquitoes on mice infected with mature gametocytes. In parallel, mosquitoes were allowed to feed on mice infected with WT mature gametocytes. Between days 10 and 12 after blood feeding on infected mice, infected mosquito midguts were dissected and oocyst numbers were determined based on intrinsic GFP expression using fluorescence microscopy. High oocyst burdens were observed in most of the infected mosquitoes irrespective of the parasite line. Quantification of oocyst numbers revealed similar mean numbers in mosquitoes infected with WT as well as *Pbpm vii(-)* parasites (Figure 3.5A and B). These data show that disruption of *Pbpm vii* does not affect oocyst formation and the preceding events including gametogenesis, fertilization, ookinete formation, and traversal of epithelium in the mosquito midgut. Next sporozoite development within oocysts, sporozoite egress, and subsequent invasion of salivary glands by WT and *Pbpm vii(-)* parasites were analysed by fluorescence microscopy. Initially, at day 14 post infection WT and *Pbpm vii(-)* oocysts were imaged to determine whether the oocysts contained mature or budding sporozoites (sporulated). Fluorescence microscopic analysis revealed that no morphological difference was observed between WT and *Pbpm vii(-)* sporulating oocysts and the quantification of midgut sporozoites revealed no differences between the two parasite lines (Figure 3.5C and D).

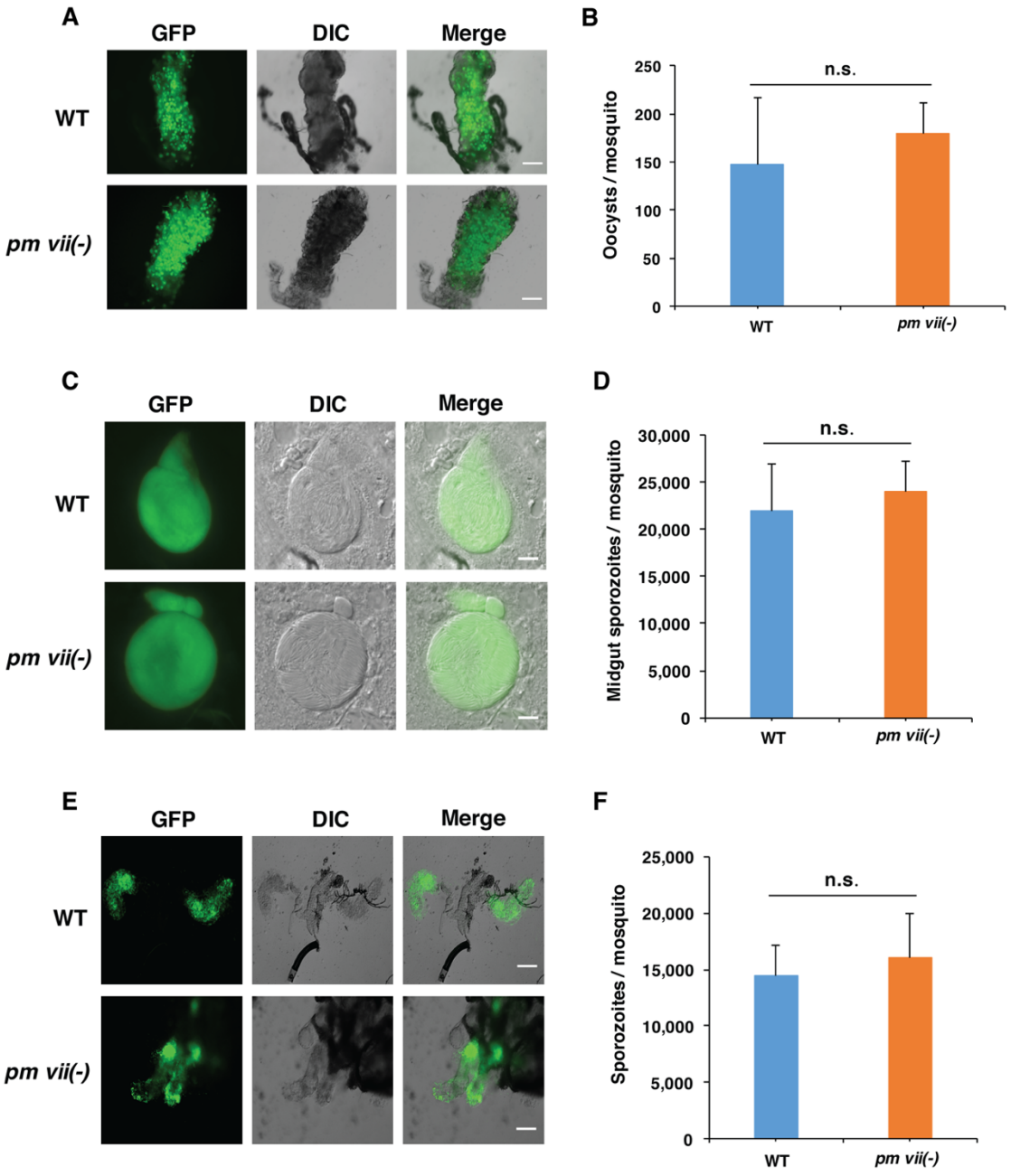


Figure 3.5. Depletion of PbPM VII does not affect development in mosquitoes. **(A)** Shown are representative images of mosquito midguts infected with WT and *Pbpm vii(-)* line. Scale bars, 200 µm. **(B)** Quantification of oocyst numbers per infected mosquito for WT and *Pbpm vii(-)* line. **(C)** Shown are representative images of sporulating WT and *Pbpm vii(-)* oocysts. Scale bars, 20 µm. **(D)** Quantification of midgut sporozoite numbers per infected mosquito for WT and *Pbpm vii(-)* line. **(E)** Shown are representative images of salivary glands infected with WT and *Pbpm vii(-)* sporozoites . Scale bars, 200 µm. **(F)** Quantification of salivary gland sporozoite numbers per infected mosquito for WT and *Pbpm vii(-)* line. Shown are mean (± SD) values from at least three independent feeding experiments. Differences were not significant (n.s.).

Further, at day 21 post infection salivary glands were isolated from mosquitoes infected with WT and *Pbpm vii(-)* parasites and the numbers of salivary gland sporozoites were determined. Quantification of salivary gland sporozoites revealed similar numbers in WT and *Pbpm vii(-)* parasite lines (Figure 3.5E and F). These results suggest that lack of PbPM VII did not compromise sporogony, sporozoite egress or the ability of hemolymph sporozoites to invade salivary glands. Collectively, these data demonstrate that PbPM VII is dispensable for the part of *P. berghei* life cycle that occurs in the mosquito vector, despite predominant gene expression in the mosquito stages.

3.1.6. *Pbpm vii(-)* parasites progress normally in the mammalian host

To investigate a potential role of PbPM VII during the parasite development in the mammalian host, characteristics of pre-erythrocytic stages (sporozoites and liver stages) were analysed. *Plasmodium* sporozoites rely on actin based gliding motility to invade host cells and cross cellular barriers. Gliding motility is crucial for productive invasion and successful establishment of infection. Motility of *Pbpm vii(-)* sporozoites was investigated with gliding assays on glass slides and subsequent analysis by indirect immunofluorescence against the CSP protein to detect sporozoite trails. Both WT and *Pbpm vii(-)* sporozoites showed no difference in numbers and patterns of CSP trails and displayed normal gliding motility (Figure 3.6).

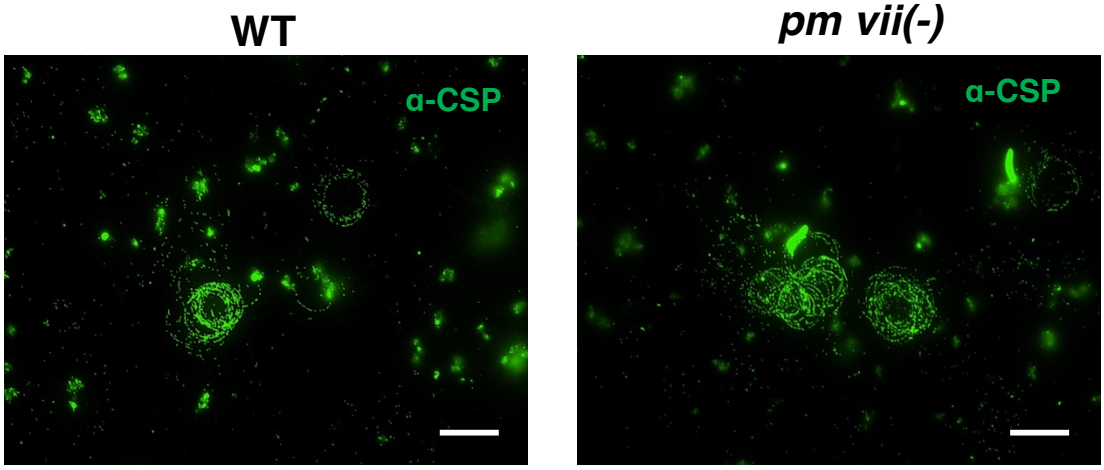
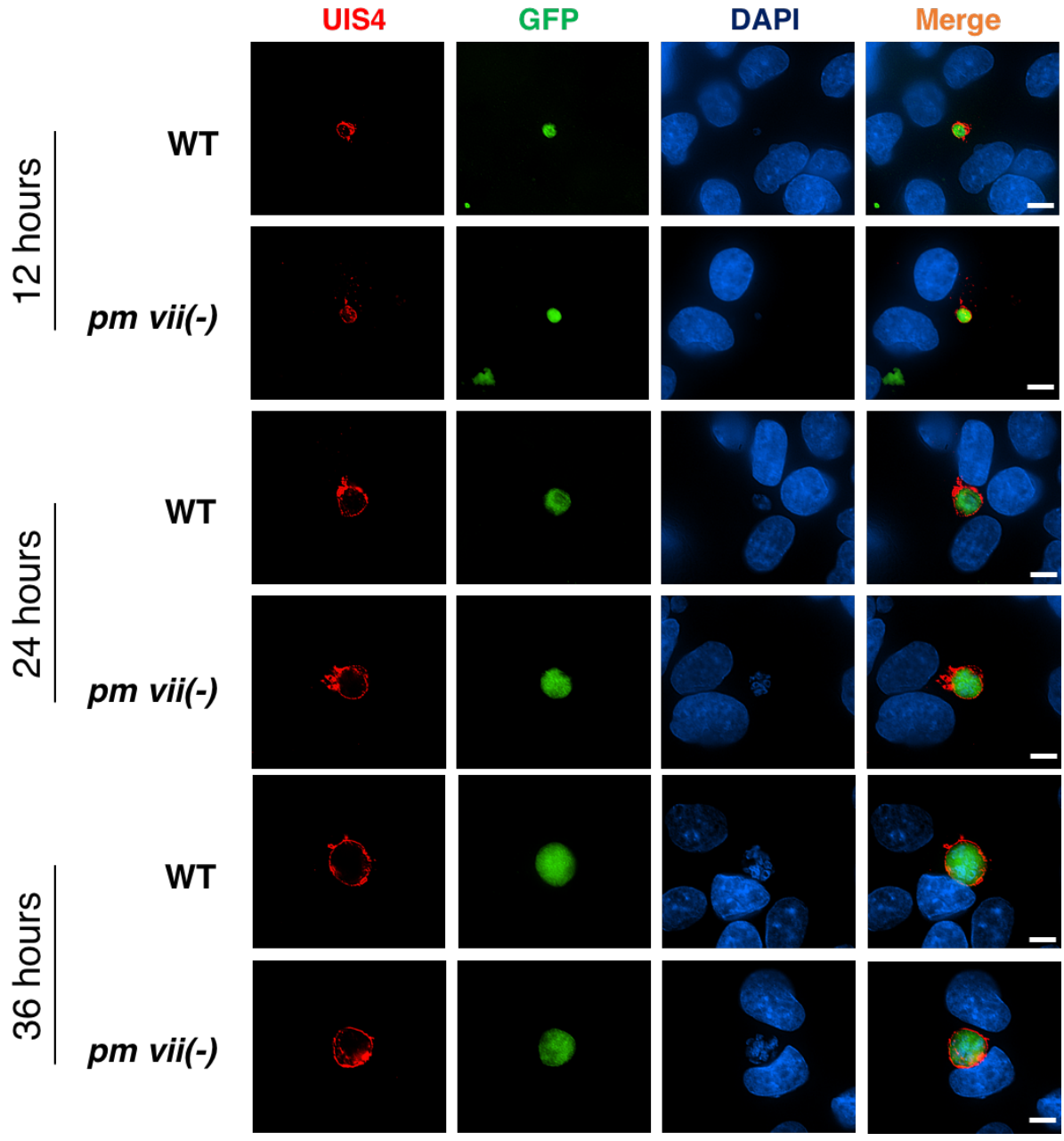


Figure 3.6. *Pbpm vii(-)* sporozoites perform continuous gliding locomotion. Shown are the representative immunofluorescence images of CSP gliding trails by WT and *pm vii(-)* sporozoites. Both the parasite lines displayed the typical continuous and circular gliding motility pattern. Scale bars = 10 µm.

Next, *Pbpm vii(-)* sporozoites were evaluated for their ability to successfully complete the liver stage development and initiate blood stage infection. First, the infectivity and development of knockout parasites was determined in cultured hepatoma (HepG2) cells *in vitro*.



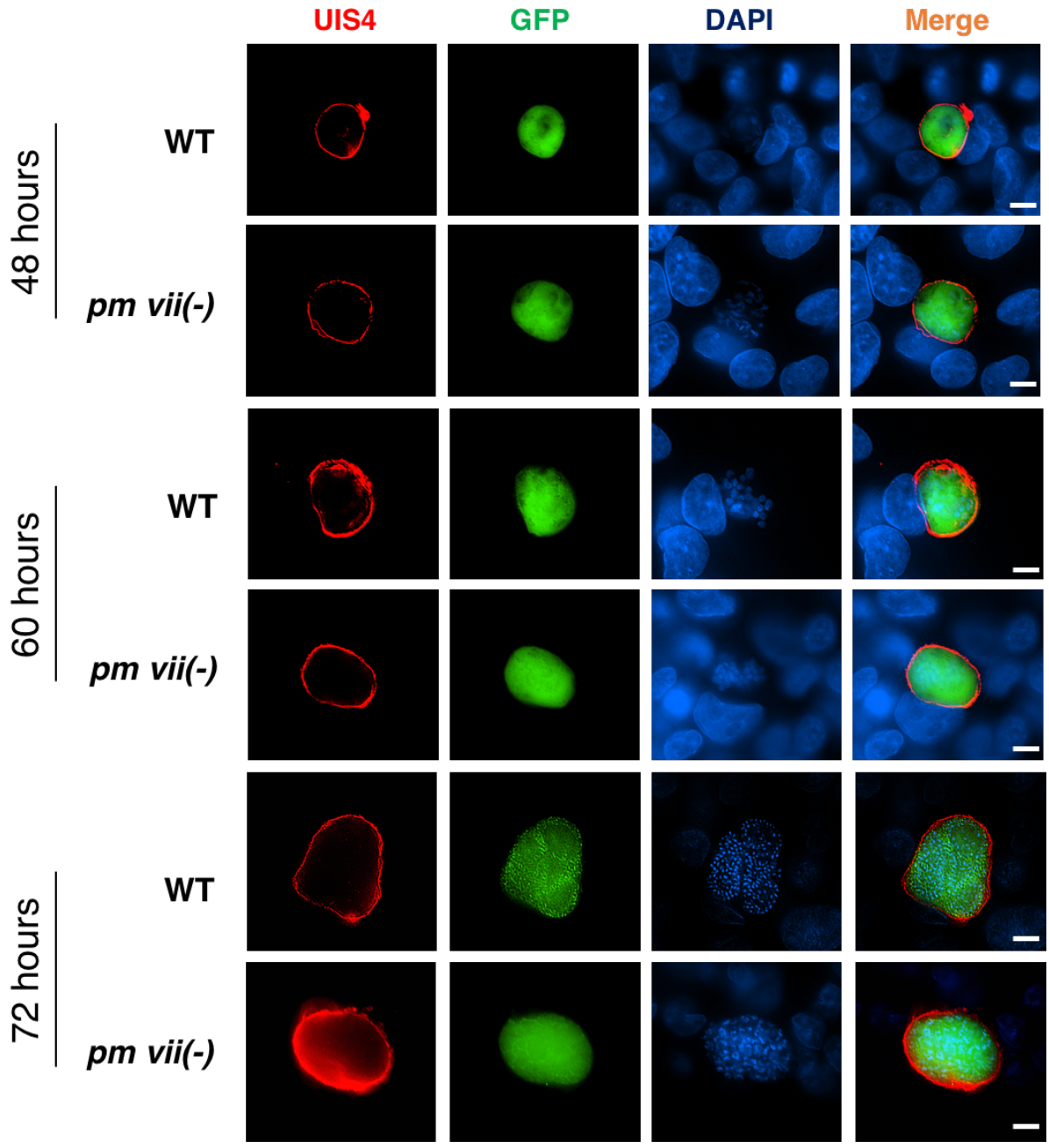


Figure 3.7. *Pbpm vii(-)* parasites develop normally in HepG2 cells *in vitro*. HepG2 cells were infected with WT and *Pbpm vii(-)* sporozoites and incubated for 72 hours to allow development of liver stages. WT (top panel) and *Pbpm vii(-)* (bottom panel) liver stages show similar morphology and size. Cells were stained at indicated time points with anti-UIS4 (up-regulated in infective sporozoites gene 4) that stains the parasitophorous vacuole membrane (PVM, red). Nuclei were visualized with DAPI (blue). Scale bars, 10 µm.

HepG2 cells were infected with WT *and Pbpm vii(-)* sporozoites and development was monitored for 72 hours. WT *and Pbpm vii(-)* liver stages showed a similar development as judged by endogenous GFP fluorescence. In addition, staining with anti-UIS4 antibodies, a resident protein of the parasitophorous vacuole membrane (PVM) revealed no discernible differences in the PVM structure between WT and *Pbpm vii(-)* EEFs (Figure 3.7). Thus, the *Pbpm vii(-)* parasites appear to develop normally inside HepG2 cells. Next to test whether *Pbpm vii(-)* sporozoites could successfully initiate blood stage infection *in vivo*, malaria transmission experiments by natural mosquito bites were performed. Mosquitoes harbouring either WT or *Pbpm vii(-)* sporozoites in their salivary glands were allowed to feed on anaesthetized C57BL/6 mice. Additionally, 5,000 of either WT or *Pbpm vii(-)* salivary gland sporozoites were intravenously injected into groups of C57BL/6 mice. The mice were monitored for prepatency, i.e. the time to detection of blood stage parasites in peripheral blood. Analysis of prepatency revealed the appearance of blood stage infection at day 4 in mice infected with *Pbpm vii(-)* line similar to WT (Table 3.1). Blood stage infection was detectable after an average of 4 days in all mice. This finding is in good agreement with *in vitro* intrahepatic development data (Fig. 2B) and supports the earlier observation that the *Pbpm vii(-)* salivary gland sprozoites are infectious and virulent. Collectively, these results establish that in the absence of PbPM VII, the parasites progress normally through their life cycle. In particular, genetic ablation of PbPM VII, neither affects intrahepatic development *in vitro* nor the development in mice *in vivo*. Therefore, PbPM VII is not essential for completion of the *Plasmodium* life cycle.

Parasite line	Route of inoculation	Mice positive / Mice infected	Pre-patency in days
WT	mosquito bite	9/9	4
Pbpm vii(-)	mosquito bite	12/12	4
WT	5000 sporozoites (i.v.)	9/9	3
Pbpm vii(-)	5000 sporozoites (i.v.)	11/11	3

Table 3.1. Infectivity of *Pbpm vii(-)* sporozoites to C57BL/6 mice. Data are shown for the *PbPM VII* gene knockout line in comparison to WT (wild type *P. berghei* strain *ANKA*). Salivary gland sporozoites were either injected intravenously (i.v.) into the tail vein at the doses indicated or delivered by natural mosquito bite via exposure of anesthetized mice to infected *A. stephensi* mosquitoes. Pre-patency is the time until the first detection of blood stage parasites in Giemsa-stained blood smears after sporozoite infection.

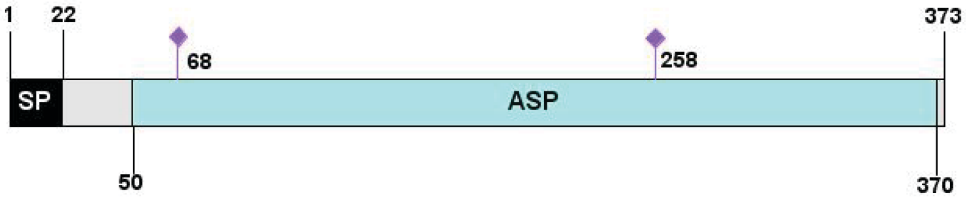
3.2. Functional characterization of *Plasmodium berghei* plasmepsin VIII (PbPM VIII)

3.2.1. PbPM VIII encodes a conserved aspartic protease

Ten aspartic proteases had been identified in *Plasmodium* based on the presence of an aspartic domain and all the proteins contain a signal sequence. This study was initiated with *in silico* analysis of orthologous proteins in *Plasmodium* species (Figure 3.8A). The PM VIII gene structure and genomic organization is conserved among *Plasmodium* species, including *P. berghei*, *P. yoelii*, *P. chabaudi*, *P. falciparum*, *P. vivax* and *P. knowlesi*. All the PM VIII orthologs possess a 13 exon gene structure and encode an aspartic protease with 373-385 amino acids. Sequence analysis revealed that PbPM VIII has a signal peptide and an aspartic domain consisting of two active site regions providing two catalytic aspartate residues (Asp68 and Asp258) (Figure 3.8B). Multiple sequence alignment of 6 orthologous proteins revealed overall conservation of amino acid identities (Figure 3.8C).

A

B



C

Proteins	PbPM VIII	PfPM VIII	PvPM VIII	PyPM VIII	PcPM VIII	PkPM VIII
PbPM VIII	100	71	75	98.1	95.1	73.1
PfPM VIII		100	75	71.3	71.5	73.1
PvPM VIII			100	74.7	74.7	91.4
PyPM VIII				100	95.9	73.1
PcPM VIII					100	73.7
PkPM VIII						100

Figure 3.8. PbPM VIII gene structure, protein structure, and conservation among *Plasmodium* species. **(A)** Schematic representation of genomic organization of PM VIII gene (not to scale). The *Plasmodium berghei* PM VIII gene (PBANKA_ 1329100, 2380 bp) is composed of eight exons (shaded bars) and seven introns (lines). **(B)** Schematic representation of protein domain architecture of PbPM VIII (not to scale). PbPM VIII protein (PBANKA_ 0517600, 471 amino acids) contains a signal peptide (SP), a transmembrane domain (TM) and an aspartic domain (ASP) typically found in eukaryotic aspartic proteases. The positions of two catalytic aspartic acid residues are indicated in purple colour. **(C)** The table shows amino acid sequence conservation of PM VIII orthologues among six *Plasmodium* species.

3.2.2. Gene expression profiling of *Pbpm viii*

To determine the gene expression profile of *Pbpm viii* (PBANKA_1329100.1) across different stages of parasite life cycle, standard quantitative real time PCR (qRT-PCR) was performed. To obtain mosquito stages (MS) of parasite, samples were prepared at samples were prepared at 8 h, 14 h, 20 h, 26 h, 32 h and 38 h post blood meal, on days (D) 4 and day 8 post infection, and from midgut (MG) and salivary gland (SG) associated sporozoites. The liver stage (LS) samples were obtained from *in vitro* cultured sporozoite-infected HepG2 cells at different time points (16, 24, 42, 50, 65 hours) post infection. The parasite *18s rRNA* was used for normalization of *Pbpm viii* gene expression data.

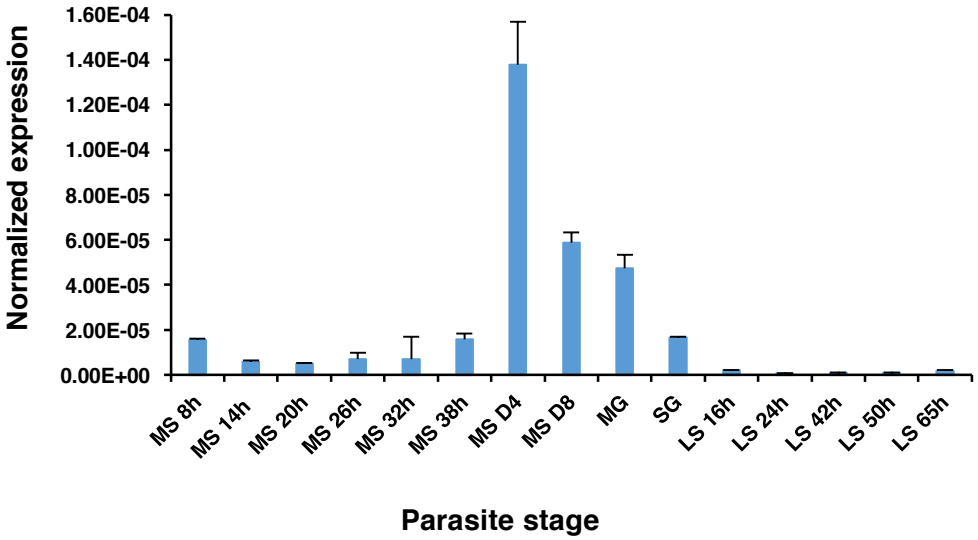


Figure 3.9. Expression profile of *Pbpm viii* in mosquito and liver stages. Stage-specific gene expression analysis was performed by quantitative real time PCR (qRT-PCR) using cDNA samples prepared from the mosquito stage parasites and liver stage parasites of *P. berghei*. Values were normalized to *P. berghei 18s rRNA* control. The abbreviations used to describe parasite stages are: mosquito stages (MS h) at 8, 14, 20, 26, 32, 38 hours post infection

and on days 4, 8 post infection (MS D), midgut sporozoites (MG), salivary gland sporozoites (SG), and liver stage parasites (LS) at 16, 24, 42, 50, 65 hours post infection.

Gene expression analysis of *Pbpm viii* by qRT-PCR revealed highest expression was highest in young oocysts at day 4 after infection followed by moderate expression in oocysts at day 8 after infection and midgut sporozoites (Figure 3.9). The *Pbpm viii* expression was lowest in early mosquito stage parasites (MS 8h to 38h) and salivary gland sporozoites (SG). Interestingly, *Pbpm viii* expression was reduced to background levels during liver stage development (LS 16h to 65h) (Figure 3.9). Collectively, *Pbpm viii* gene is differentially expressed in mosquito stage parasites with predominant expression in day 4 young oocysts and moderate expression in day 8 oocysts and midgut sporozoites. Thus, the *PM VIII* gene expression data suggests a possible role for *Pbpm viii* during parasite development within the mosquito vector.

3.2.3. Targeted gene deletion of *Pbpm viii*

To systematically study the *in vivo* function of PM VIII during the *Plasmodium* life cycle, knockout parasites were generated in *P. berghei* using a gene replacement strategy (Menard and Janse, 1997). The endogenous *Pbpm viii* gene was deleted with a targeting plasmid (pBC-GFP-DHFR/*Pbpm8*) consisting of 5' and 3' homologous untranslated regions (UTRs) of the *Pbpm viii* flanking the *GFP* and pyrimethamine resistance conferring *hDHFR* cassettes. The 5' and 3' UTRs facilitate integration into the *Pbpm viii* genomic locus by double crossover homologous recombination and simultaneous deletion of the endogenous *PM VIII* gene (Figure 3.10A). The homologous DNA fragments were PCR amplified from wild type *P. berghei* gDNA with specific primers for 5' UTR and 3' UTR and ligated into the pBC-GFP-hDHFR using XhoI-ClaI for the 5' UTR and NotI-AscI for the 3' UTR. Approximately, 10 µg of the final targeting plasmid linearized with XhoI-AscI was transfected into purified schizonts of *P. berghei ANKA* strain by electroporation (Janse et al., 2006). After transfection, the parental blood stage population was subjected to pyrimethamine selection. Two independent clonal populations of *Pbpm viii(-)* knockout parasites were obtained by limiting dilution and named clone 5 (cl5) and clone 8 (cl8). Genotyping of two independent *Pbpm viii(-)* lines by diagnostic PCR confirmed successful integration of *GFP* and *hDHFR* cassettes flanked by 5' UTR and 3' UTR into the wild type locus.

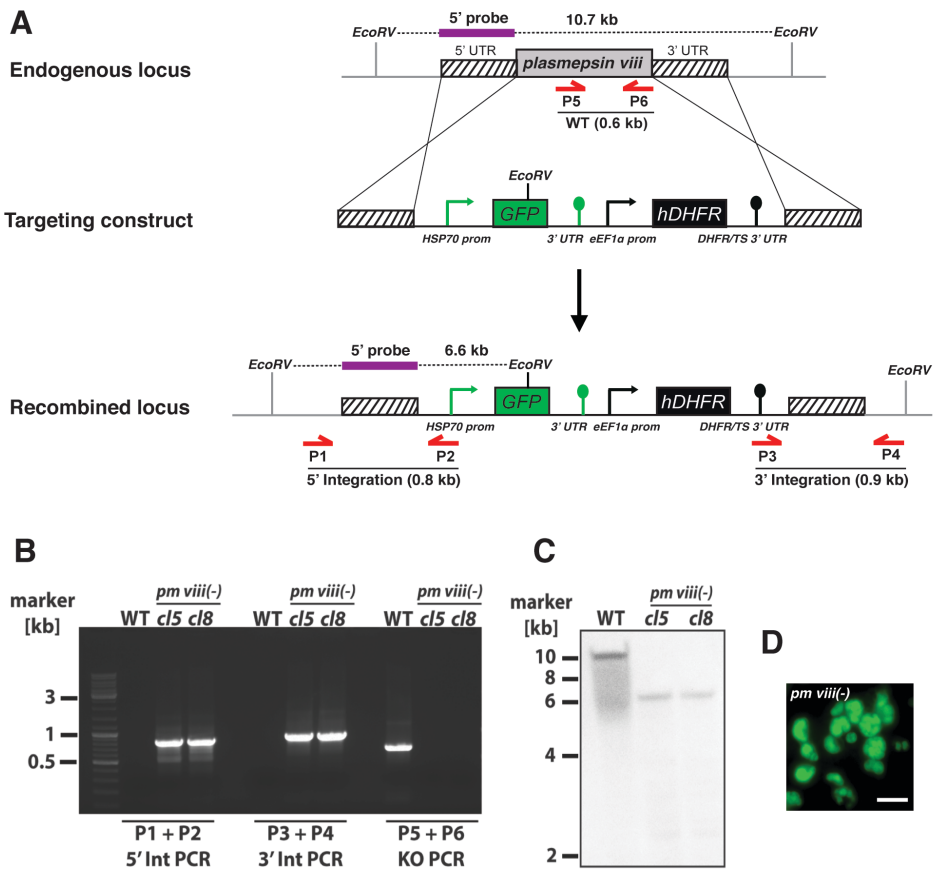


Figure 3.10. Generation of *Pbpm viii* knockout parasites. (**A**) Schematic representation of gene replacement strategy for the generation of *P. berghei pm viii(-)* knockout parasites (not to scale). The endogenous locus was replaced with a targeting plasmid pBC-GFP-DHFR/*Pbpm8* containing the 5' and 3' untranslated regions (UTRs) of *PM VIII* flanking *GFP* and the selection marker human dihydrofolate reductase (*hDHFR*). After double crossover homologous recombination, the *Pbpm viii* open reading frame was replaced with *GFP* and the *hDHFR* selection cassettes. HSP70 prom, Heat shock protein 70 gene promoter; eEF1α prom, elongation factor 1 alpha gene promoter; DHFR/TS, dihydrofolate reductase - thymidylate synthase. **(B)** Genotypic analysis of *Pbpm viii(-)* parasite clones 5 and clone 8 by diagnostic PCR with specific primers as indicated in schematic. The integration events at 5' and 3' ends were confirmed by primer combinations P1+P2 and P3+P4 respectively. The absence of a wild type (WT) locus was confirmed by knockout (KO) PCR with primers P5+P6. WT gDNA was used as control. **(C)**Genotypic analysis by southern blot. gDNA of WT parasites and *Pbpm viii(-)* parasite clones 5 and clone 8 was analyzed by southern blotting after digestion with EcoRV. A probe, indicated as a purple line in schematic, homologous to the *PM VIII* 5′ UTR recognizes a 10.7 kb fragment in the endogenous locus and 6.6 kb fragment in the recombined locus. **(D)**Live cell imaging of *Pbpm viii(-)* parasites expressing GFP. Scale bars, 20 μm.

PCR with primers specific for recombination events at 5' (P1+P2) and 3' (P3+P4) ends resulted in amplification of ~0.8 kb (5' integration) and ~0.9 kb (3' integration) long fragments in *Pbpm viii(-)* parasites but not in wild type parasites, confirming the integration into the *Pbpm viii* genomic locus (Figure 3.10B). The wild type *Pbpm viii* gene locus was detected as a 0.6 kb long fragment (WT) in parental parasites, but not in *Pbpm viii(-)* clonal lines (Figure 3.10B). Furthermore, successful integration of the targeting construct and deletion of the *Pbpm viii* was also confirmed by Southern blot hybridization using a PCR DIG labelling and detection kit (Roche). The hybridization of the probe with EcoRV digested gDNA from wild type (WT) and *Pbpm viii(-)* parasites resulted in bands of 10.8 and 6.7 kb, respectively (Figure 3.10C), which revealed the expected size shift and homogenous presence of *Pbpm viii(-)* parasites only. Since the targeting construct contains GFP cassette, the GFP expression in knockout parasites was confirmed by live cell imaging of *Pbpm viii(-)* blood stage parasites (Figure 3.10D). Taken together, the targeted gene deletion of *Pbpm viii* is achievable in blood stages and resulted in viable *pm viii(-)* parasites. Therefore, PbPM VIII is not essential in blood stages of parasite.

3.2.4. PM VIII is dispensable for blood stage development *in vivo*

To ascertain the importance of PM VIII during *P. berghei* life cycle progression, the proliferation of *Pbpm viii(-)* blood stage parasites was analysed *in vivo*. To check, if the *Pbpm viii(-)* parasites exhibit any defect during asexual growth and replication, two groups of mice (n=5) were intravenously injected with $1x10^3$ RBCs infected with mixed blood stages of both clones of *Pbpm viii(-)* and wild type parasites. The parasitemia was monitored daily by microscopic observation of Giemsa-stained blood smears. The *in vivo* multiplication rates of the *Pbpm viii(-)* parasites were similar to wild type parasites and revealed no significant difference during exponential phase of asexual development in the blood (Figure 3.11A). Furthermore, the *pm viii(-)* parasites were able to undergo gametocytogenesis, the process of formation of gametocytes, similar to wild type parasites (Figure 3.11B). Together, these results indicate *Pbpm viii(-)* parasites does not have any defect either in growth and development of asexual blood stages or in gametocyte formation. Thus implying a non-essential role for PM VIII during intra-erythrocytic development and sexual differentiation.

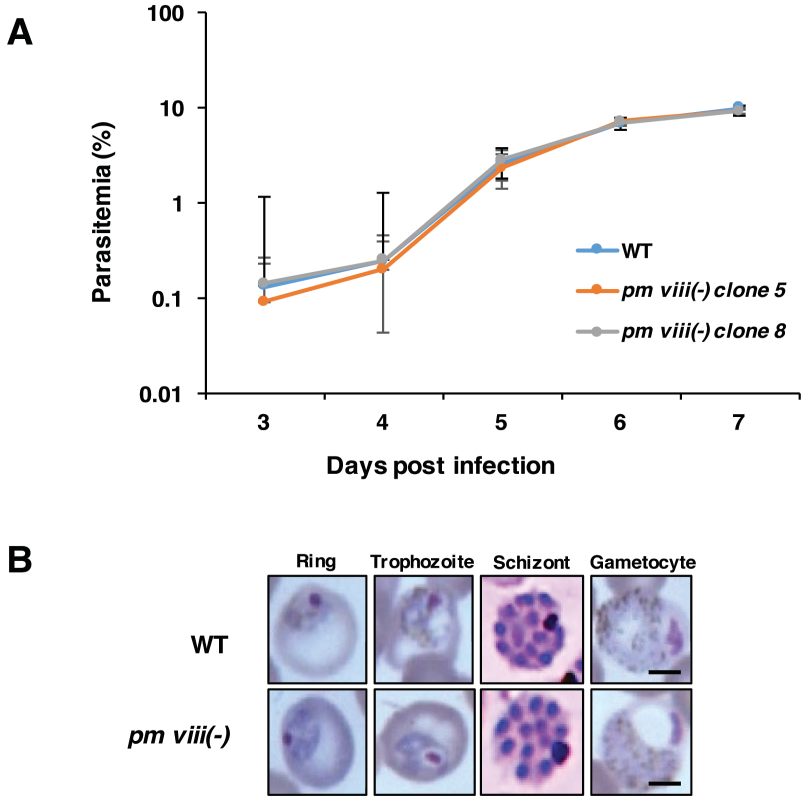


Figure 3.11. PbPM VIII is dispensable for blood stage development *in vivo*. (**A**) *Pbpm viii(-)* blood stage parasites develop normally in the mammalian host. Asexual blood stage development was determined by injecting female BALB/C mice (n = 5) intravenously with 1x10^3 infected erythrocytes. Parasitemia and the gametocyte formation in the recipient mice was monitored by daily microscopic observation of Giemsa-stained blood smears. Shown are the values of mean (± SD). Asexual blood stage propagation was performed three times and a representative experiment is shown. **(B)** Shown are representative micrographs of blood stage parasites, at different stages of development, stained with Giemsa. Scale bars, 10 µm.

3.2.5. PM VIII is not essential for oocyst development

Considering the predominant expression *Pbpm viii* in the mosquito stages, the phenotype of *Pbpm viii(-)* parasites was characterized in detail during life cycle progression in the invertebrate host, the *A. stephensi* mosquitoes. A *Plasmodium berghei* ANKA line constitutively expressing GFP under the *HSP70* promoter was used as a wild type (WT) control throughout this study for the phenotypic characterization of knockout parasites (Al-Nihmi et al., 2017). For mosquito transmission, female *A. stephensi* mosquitoes were allowed to feed on

anaesthetized Swiss-Webster mice infected with either clonal *Pbpm viii(-)* parasites or wild type parasites. After infection, the parasite development was monitored over a period of 25 days and three independent feedings of *A. stephensi* were done for each phenotypic characterization experiment. Infectivity and oocyst development of *Pbpm viii(-)* parasites were determined by live microscopic analysis of infected midguts between days 10 to 12 post infection and compared to WT. No significant differences were observed between *pm viii(-)* and WT parasites in terms of midgut infectivity and oocyst numbers (Fig. 3.12A and B). This suggests that ablation of *PM VIII* did not affect the initial events of parasite development in mosquito midgut such as gametogenesis, fertilization, ookinete formation, followed by transmigration of ookinetes and subsequent transformation into oocysts (Aly et al., 2009).

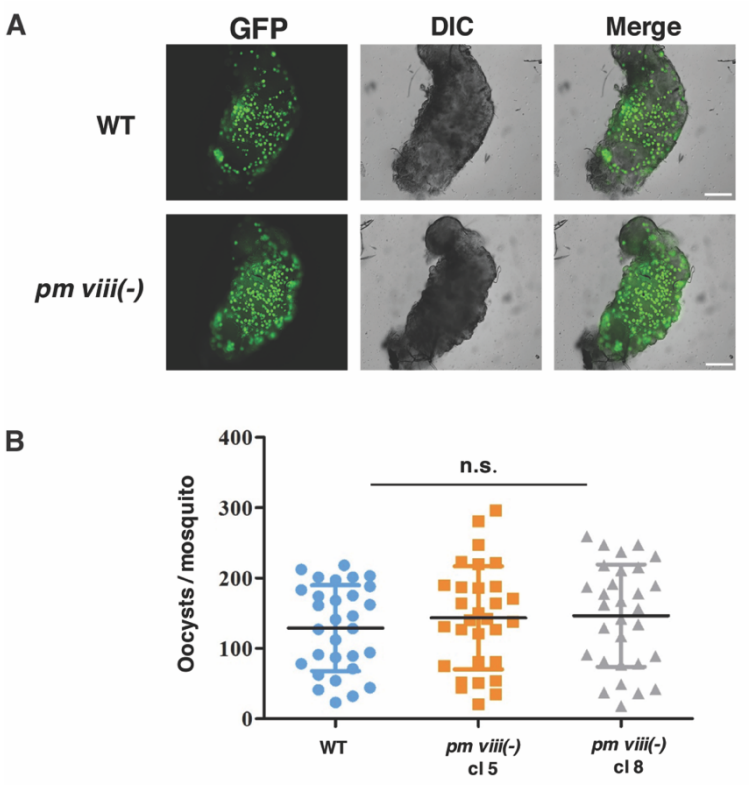


Figure 3.12. Lack of *Pbpm viii* did not affect midgut infectivity and oocyst formation. **(A)** Live microscopy images of mosquito midguts colonized with WT or *Pbpm viii(-)* oocysts at day 10 post feeding. Note that parasites were visualized by endogenously expressed GFP. **(B)** Quantification of oocyst numbers in the infected mosquito midguts. Note that WT and *Pbpm viii(-)* parasites produced similar number of oocysts. Oocysts from 15 infected mosquitoes were observed per mosquito cycle, shown are the data from two cycles at day 10 post infection. Horizontal lines indicate the median number of oocysts. Scale bars, 200µm.

3.2.6. *Pbpm viii(-)* sporozoites fail to colonize mosquito salivary glands

After complete development and maturation, the sporozoites exit out of the oocyst and migrate to salivary glands (SG) via the hemolymph, the circulatory system of the mosquito (Pimenta et al., 1994; Vlachou et al., 2006). Live microscopic examination of salivary glands of infected mosquitoes was perforemd between days 17 to 25 for determining the successful invasion of salivary glands by *Pbpm viii(-)* sporozoites. Intriguingly, compared to WT, no SG associated sporozoites were detected in the *Pbpm viii(-)* clonal lines (Fig. 3.13A and B) despite the robust midgut infectivity and high number of oocysts. Even until day 25 post infection, *Pbpm viii(-)* salivary gland sporozoites could not be detected.

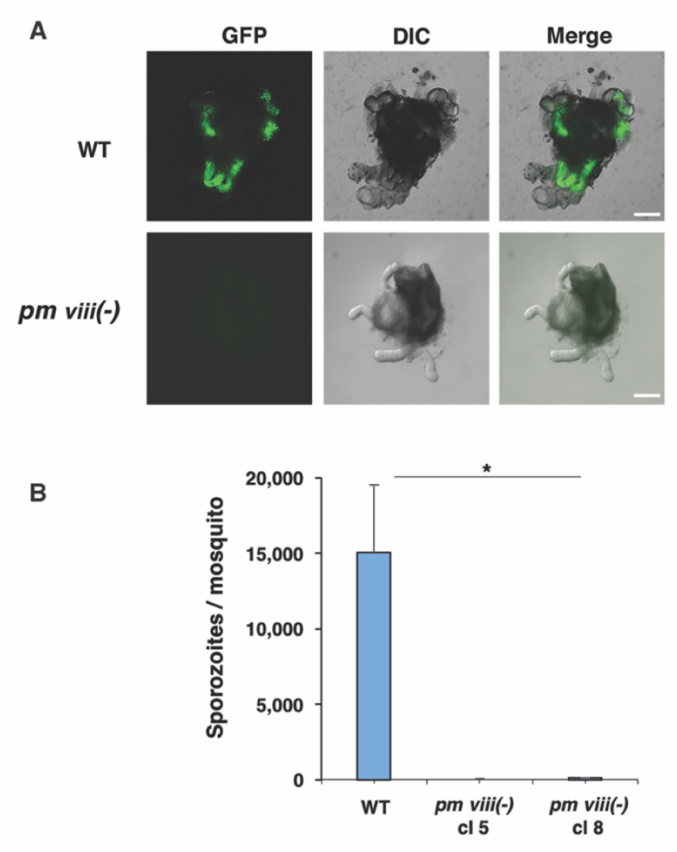


Figure 3.13. PbPM VIII is essential for successful colonization of salivary glands. **(A)** Live microscopy images of salivary glands of mosquitoes infected with WT or *Pbpm viii(-)* parasites at day 18 post infection. Note that in contrast to WT, no *Pbpm viii(-)* sporozoites could be detected in salivary glands of infected mosquitoes, while WT sporozoites were abundant. Scale bars, 200µm. **(B)** Quantification of sporozoite numbers in salivary glands of WT and *Pbpm viii(-)* infected mosquitoes. Shown are the mean ± S.E.M. of three independent experiments (n = 20 mosquitoes/experiment, *P = 0.029).

3.2.7. Oocysts lacking PM VIII develop normally and forms mature sporozoites

The failure to colonize salivary glands could be due to either failure to form mature sporozoites or a sporozoite specific defect. Initially, to address whether the sporulation occurred in *pbpm viii(-)* oocysts, infected midguts were examined in detail. Oocyst development and differentiation is relatively a long and complex process that culminates with the release of thousands of sporozoites into the hemocoel (Ghosh et al., 2000). The oocyst development is accompanied by extensive growth and multiple rounds of asynchronous mitotic divisions, termed sporogony, followed by cytokinesis resulting in formation of hundreds of sporozoites in specialized structures called sporoblasts (Terzakis et al., 1967; Sinden and Garnham, 1973; Sinden, 1974). To further characterize oocyst development and sporulation in the *Pbpm viii(-)* clonal lines, live microscopic studies were performed on infected midguts at day 14 post infection. Similar to WT, the *Pbpm viii(-)* oocysts contained the typical radially aligned individual sporozoites that mark the final step of sporulation (Fig. 3.14A). Further, the individual oocysts containing mature sporozoites were examined and midgut sporozoites of the WT as well as *Pbpm viii(-)* clonal lines were quantified (Fig. 3.14B and C). *Pbpm viii(-)* parasites showed no significant difference either in oocyst sporulation or midgut sporozoite numbers compared to WT. Taken together, these data indicate PM VIII is not required for the oocyst formation, sporulation and sporozoite maturation.

3.2.8. *Pbpm viii(-)* parasites are severely impaired in oocyst egress

Given the normal sporulation and comparable midgut sporozoite numbers in *Pbpm viii(-)* oocysts, the number of hemolymph sporozoites were examined in the mosquitoes infected with *Pbpm viii(-)* parasites. In mosquitoes infected with wild type parasites, midgut sporozoites begin to egress out of the oocysts on days 12-14 post infection. As a consequence of gradual sporoozite egress from the oocysts, the midgut sporozoite numbers decrease, while the hemolymph and salivary gland sporozoite numbers increase over time. To address whether the *Pbpm viii(-)* parasites have any defect in oocyst egress, hemolymph sporozoites were isolated from infected mosquitoes between days 15 to 21 post infection and quantified. Surprisingly, the numbers of *Pbpm viii(-)* haemolymph sporozoites were drastically reduced when compared to WT (Fig. 3.15A).

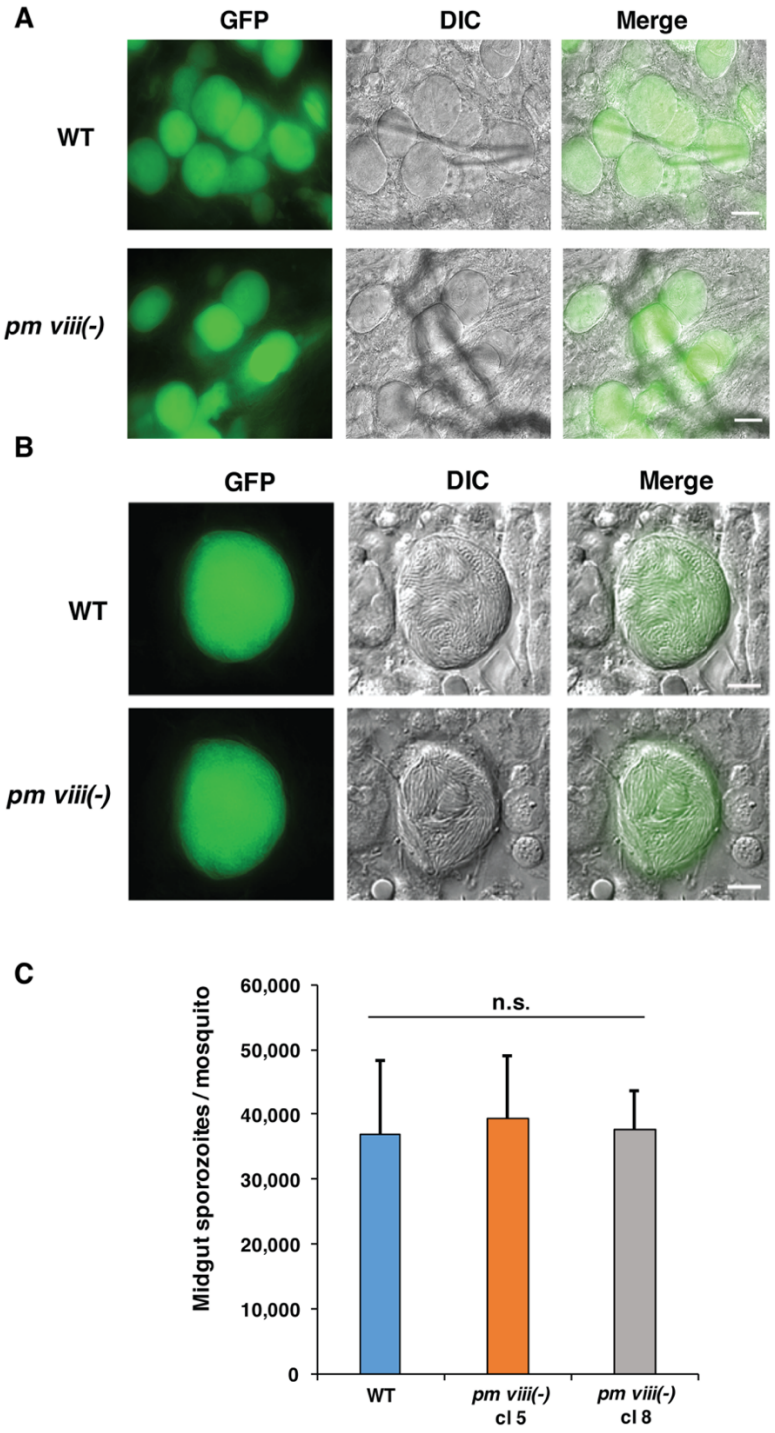


Figure 3.14. PbPM VIII is not essential for oocyst development and sporulaiton. **(A)** Live microscopy images of sporulating oocysts in the mosquito midguts infected with WT and *Pbpm viii(-)* parasites at day 14 post infection. Scale bars = 20 µm. **(B)** Live microscopy images of WT and *Pbpm viii(-)* oocysts harbouring mature sporozoites at day 16 post infection. Scale bars = 20 µm. **(C)** WT and parasites produced similar numbers of midgut sporozoites at day 14 post infection. n.s: not siginificant. Shown are the mean ± S.E.M. of three independent experiments (n = 20 mosquitoes/experiment).

Alternatively, sporozoites were mechanically liberated from *Pbpm viii(-)* and WT oocysts and examined with fluorescence microscopy. Interestingly, no morphological differences were observed between WT and *Pbpm viii(-)* oocyst-derived sporozoites (Fig. 3.15B). Very few hemolymph sporozoites in mosquitoes infected with *Pbpm viii(-)* parasites indicate a defect in oocyst egress. Altogether, these data demonstrate that PM VIII plays a critical role in oocyst egress and subsequent colonization of salivary glands.

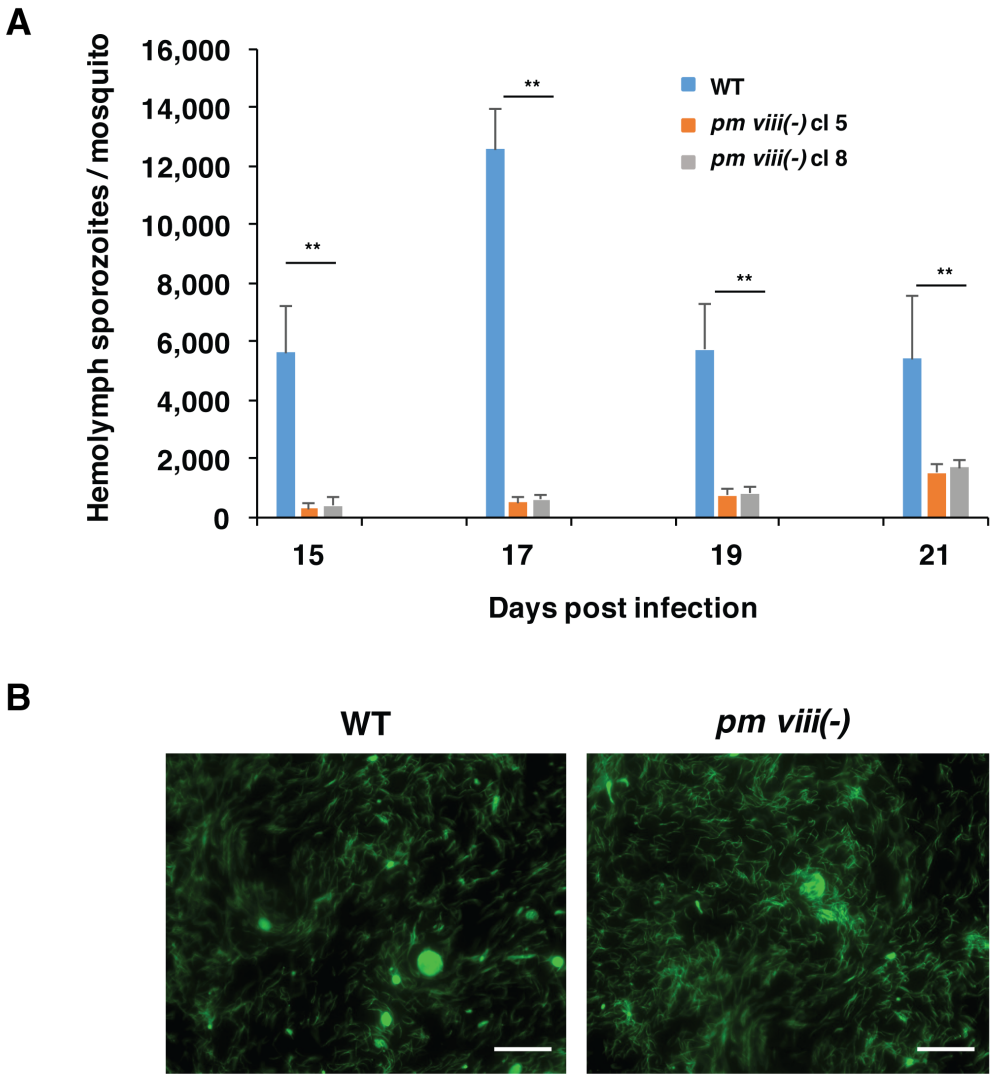


Figure 3.15. *Pbpm viii(-)* sporozoites are impaired in oocyst egress. (A) Quantification of hemolymph sporozoites in mosquitoes infected with WT and *Pbpm viii(-)* parasites. Shown are the mean ± S.D. from one experiment (n = 15 mosquitoes/experiment, **P<0.0001). This experiment was performed five times with similar results. **(B)** Fluorescence microscopy images of mechanically ruptured WT and *Pbpm viii(-)* oocyst-derived sporozoites at day 20 post infection. Scale bars = 10 µm.

3.2.9. *Pbpm viii(-)* sporozoites show defect in gliding motility

The failure to colonize salivary glands clearly established a role for PM VIII in invasion and traversal of host cell barriers, but no role in development of sporozoites. The severe impairment of sporozoite egress from oocysts could be either due to an intrinsic defect in gliding motility of sporozoites or a defect in oocyst rupture or a combination of both. In order to delineate the events, *Pbpm viii(-)* oocysts were checked for intra-oocyst motility of sporozoites as reported for *ecp1(-)* parasites, between days 15 to 21 post infection (Aly and Matuschewski, 2005). Intra-oocyst motility of sporozoites indicate the failure to egress out of oocysts despite the sporozoites being motile. No intra-oocyst motility was observed in *Pbpm viii(-)* oocysts, hinting a defect in sporozoite gliding motility (Videos M1 and M2). The sporozoite egress is a complex process regulated by several proteins especially, The circumsporozoite protein (CSP) (Menard et al., 1997). Initially, the CSP resides in the inner membrane of the oocysts and after the sporogony, CSP will become the major surface protein of sporozoites and is important for motility and invasion of both the salivary glands of mosquito and hepatocytes of the mammalian host (Coppi et al., 2011). The processing of CSP is a prerequisite for sporozoites exit from oocysts (Wang et al., 2005). CSP is synthesized as an ~54-kDa precursor form, which is proteolytically processed to a mature ~44-kDa protein during the sporozoite maturation. To determine, whether PM VIII plays a role in CSP processing during the sporozoite egress, oocyst-derived sporozoites were analysed by western blotting. Interestingly, no difference in CSP processing was observed between WT and *Pbpm viii(-)* lines, suggesting that lack of PM VIII did not affect CSP processing in oocyst-derived sporozoites (Fig. 3.16A). Impaired sporozoite egrees, very less numbers of hemolymph sporozoites and especially the absence of salivary gland sporozoites in *pm viii(-)* lines indicated that PM VIII could be playing a critical role in gliding motility of the sporozoites. Gliding motility of *Plasmodium* and related apicomplexan parasites is characterized by continuous production and processing of cell surface proteins, which serves as anchors for parasite-host cell contact points, are processed by multiple parasite proteases (Munter et al., 2009; Ejigiri et al., 2012). The circumsporozoite protein (CSP), the most predominant surface protein, is processed and released as a trail from the posterior end of the gliding sporozoites and these trails can be recognized using anti-CSP antibodies (Stewart and Vanderberg, 1988).

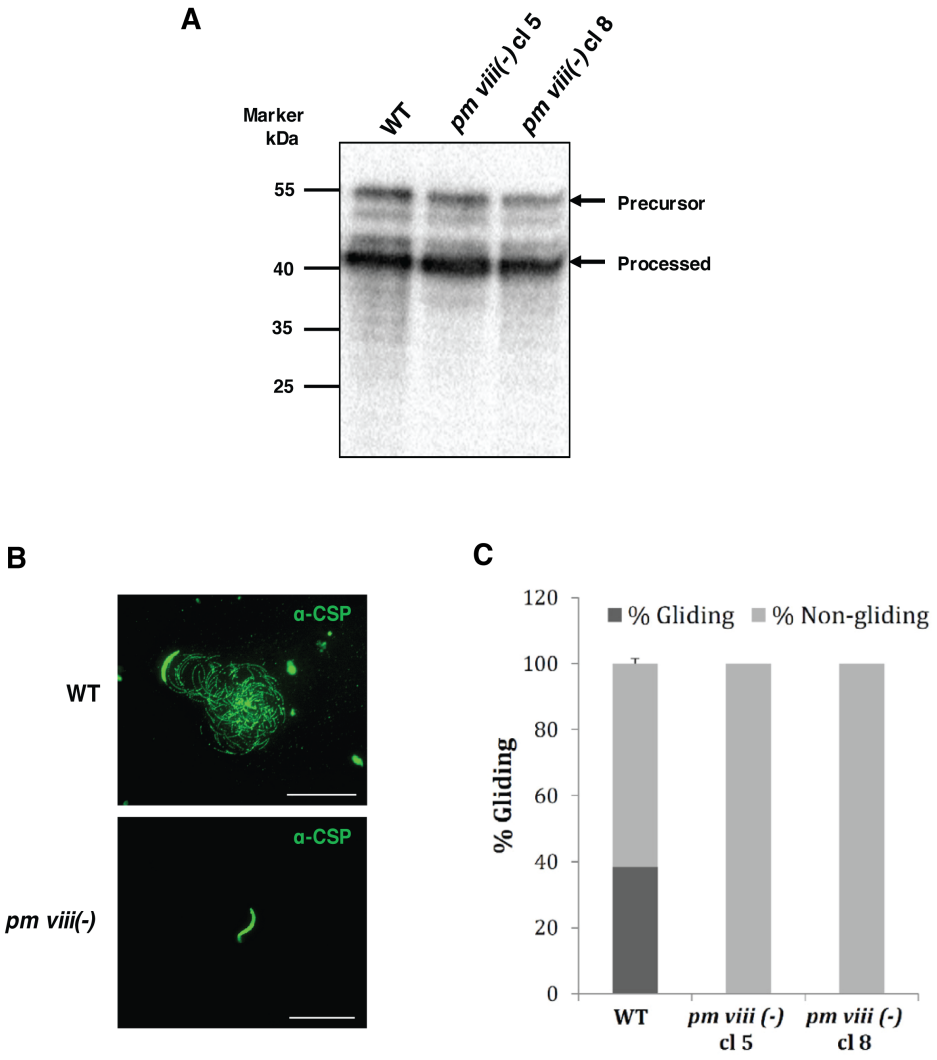


Figure 3.16. Gliding motility is abolished in *Pbpm viii(-)* sporozoites. (**A**) Western blot analysis of circumsporozoite protein (CSP) processing in wild type (WT) and *Pbpm viii(-)* midgut sporozoites from clones 5 and 8, showing no difference in precursor and proteolytically processed forms. **(B)** Gliding motility was severely impaired in *Pbpm viii(-)* hemolymph sporozoites. Immunofluorescence images of CSP gliding trails by WT and *Pbpm viii(-)* hemolymph sporozoites. WT displayed the typical continuous and circular gliding motility pattern while *Pbpm viii(-)* sporozoites were non-motile. Scale bars = 25 μm. **(C)** Quantification of hemolymph sporzoites gliding motility. A total of 252, 192 and 266 immunofluorescent-stained sporozoites from WT, *Pbpm viii(-)* cl 5 and cl 8 were evaluated, respectively.

Therefore, the motility behaviour of *Pbpm viii(-)* hemolymph sporozoites was further examined by *in vitro* gliding motility assays as well as live microscopic studies. The hemolymph sporozoites were isolated and allowed to glide on glass slides. The CSP trails were visualized and quantified by immunofluorescence using anti-CSP antibodies. As expected, circular trails of CSP were found in a substantial fraction (38%) of WT sporozoites but *Pbpm viii (-)* sporozoites could not produce CSP trails at all (Fig. 3.16B and C). To further confirm this finding, live gliding motility assays were performed and the motility of individual sporozoites was recorded in differential interference contrast (DIC) mode by fluorescence microscopy. WT sporozoites derived from hemolymph, on day 15 post infection, displayed different forms of motility such as gliding, attached waving, flexing and bending (Video M3), by contrast *Pbpm viii (-)* hemolymph sporozoites were never observed gliding or exhibited any form of motility (Video M4). Together, these results show that the PM VIII plays a vital role in gliding motility and is thus essential for sporozoite egress from oocysts and subsequent invasion of salivary glands.

3.2.10. *Pbpm viii(-)* sporozoites fail to transmit malaria to the mammalian host

Although, no *Pbpm viii(-)* sporozoites could be detected in salivary glands of infected mosquitoes, few of the hemolymph sporozoites that were able to egress out of oocysts could passively reach salivary glands. To further address the importance of PM VIII role in malaria transmission to the mammalian host, transmission experiments were performed using either mosquito bites on day 21 post infection or intravenous injections of hemolymph sporozoites. Initially, mosquitoes infected with either *Pbpm viii(-)* or wild type were allowed to feed on anaesthetized C57BL/6 mice and parasitemia was checked by microscopic examination of Giemsa-stained blood smears on daily basis until day 15 post bite to detect the blood stage infection. None of the mice exposed to bite of the mosquitoes infected with *Pbpm viii(-)* parasites developed blood stage infection as opposed to mosquitoes infected with WT parasites, where all the mice turned positive for blood stage infection (Table 3.2). Additionally, to test the infectivity of haemolymph sporozoites, C57BL/6 mice were intravenously injected with $5x10^3$ of either wild type or *Pbpm viii(-)* haemolymph sporozoites. The intravenous injections revealed that mice infected with *Pbpm viii(-)* parasites remained negative for blood stage infection, whereas mice injected with wild type parasites become positive (Table 3.2).

These transmission experiments showed that the clonal lines of *Pbpm viii(-)* parasites could not transmit malaria to mice, the mammalian host, either by mosquito bites or intravenous injections. The lack of *Pb*PM VIII renders the sporozoites non-infectious.

Parasite line	Route of inoculation	Mice positive / Mice infected	Prepatency in days
WT	mosquito bite	7/7	4
Pbpm viii(-) clone 5	mosquito bite	0/5	NA
Pbpm viii(-) clone 8	mosquito bite	0/9	NA
WT	5000 HL sporozoites (i.v.)	3/3	3
Pbpm viii(-) clone 5	5000 HL sporozoites (i.v.)	0/3	NA
Pbpm viii(-) clone 8	5000 HL sporozoites (i.v.)	0/4	NA

Table 3.2. Infectivity of *Pbpm viii(-)* sporozoites to C57BL/6 mice. Data are shown for the *Pbpm viii* gene knockout lines *pm viii(-)* clone 5 and clone 8 in comparison to WT (wild type *P. berghei* strain *ANKA*). Haemolymph (HL) sporozoites were either injected intravenously (i.v.) into the tail vein at the doses indicated or delivered by natural mosquito bite via exposure of anesthetized mice to infected *A. stephensi* mosquitoes. Prepatency is the time until the first detection of blood stage parasites in Giemsa-stained blood smears after sporozoite infection. NA- not applicable.

3.2.11. PM VIII is essential for EEF development *in vitro*

The haemolymph derived *Pbpm viii(-)* sporozoites failed to initiate blood stage infection in the rodent hosts. To further corroborate this finding and to ascertain whether lack of *PM VIII* affects the liver stage development, *in vitro* EEF development assays were performed. Haemolymph derived WT or *Pbpm viii(-)* sporozoites were added to the *in vitro* cultures of HepG2 cells. 40 hours after infection (hpi), the cultures were stopped and the infected cells were examined and quantified by immunofluorescence assay using the endogenous GFP based fluorescence and antibodies that stains the parasitophorous vacuole membrane (UIS4). At 40 hpi, EEFs were consistently detected in HepG2 cultures infected with WT sporozoites, in contrast the *Pbpm viii(-)* sporozoites did not produce EEFs at all (Figure 3.17A and B). Thus reiterating the earlier observation that the *Pbpm viii(-)* haemolymph sporozoites are non-infectious *in vitro* and *in vivo*.

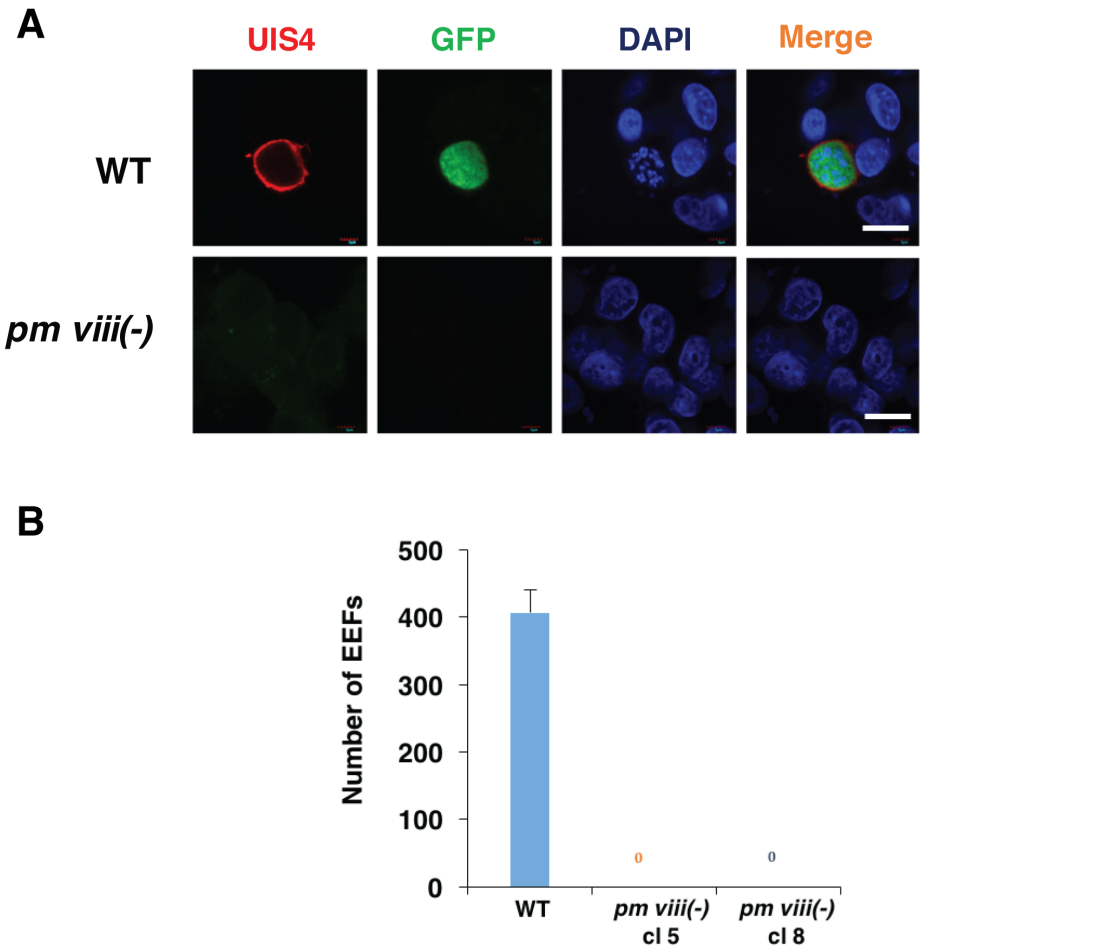


Figure 3.17. *Pbpm viii(-)* sporozoites are non-infectious *in vitro*. (A) HepG2 cells infected in vitro with WT or *pm viii(-)* hemolymph sporozoites were fixed at 40 hours post infection, and examined by IFA, after probing with antibodies against the PVM protein UIS4 (red), and anti-HSP70 antibodies (green). Nuclei were counterstained with 4,6-diamidino-2-phenylindol (DAPI, blue). Note that *pm viii(-)* sporozoites failed to undergo EEF development in contrast to the WT. Scale bars = 10 µm. (B) Quantitation of development of exoerythrocytic forms (EEFs) in cultured HepG2 cells. Shown are the mean (±SD) values of three independent experiments.

3.2.12. PbPM VIII is essential for sporozoite invasion and transmigration of hepatocytes

The *Pbpm viii(-)* sporozoites failed to transmit malaria to the mice due to a severe defect in gliding motility and, therefore, it was not surprising that *Pbpm viii(-)* sporozoites were not able to undergo successful intrahepatic development. To investigate whether the defect in gliding motility compromises the ability of *Pbpm viii(-)* sporozoites to invade and traverse

through hepatocytes, *in vitro* invasion and transmigration assays were performed.To determine the invasion capacity, hemolymph sporozoites were incubated with monolayers of HepG2 cells for 2 hrs at 37°C. Sporozoites that successfully invaded the host cells (inside) and the sporozoites that remained extracellular (outside) were quantified by immunofluorescence assay (Renia et al., 1988; Sinnis et al., 2013). 53% of WT sporozoites invaded the HepG2 cells, but remarkably none of the *Pbpm viii(-)* sporozoites were able to invade HepG2 cells (Figure 3.18A). Similarly, quantification of transmigration by immunofluorescence assay revealed complete failure of *Pbpm viii(-)* sporozoites to traverse hepatocytes compared to WT (Figure 3.18B). Together, these results demonstrate that *Pbpm viii(-)* sporozoites are severely impaired in their cell invasion and traversal capabilities. Therefore, it was not surprising that *Pbpm viii(-)* sporozoites were not able to invade and transmigrate through host cells as those two important functions depend, to certain extent, on gliding motility. Thus, the lack of *Pbpm viii* has severely affected many aspects of sporozoite biology including gliding motility, invasion and transmigration.

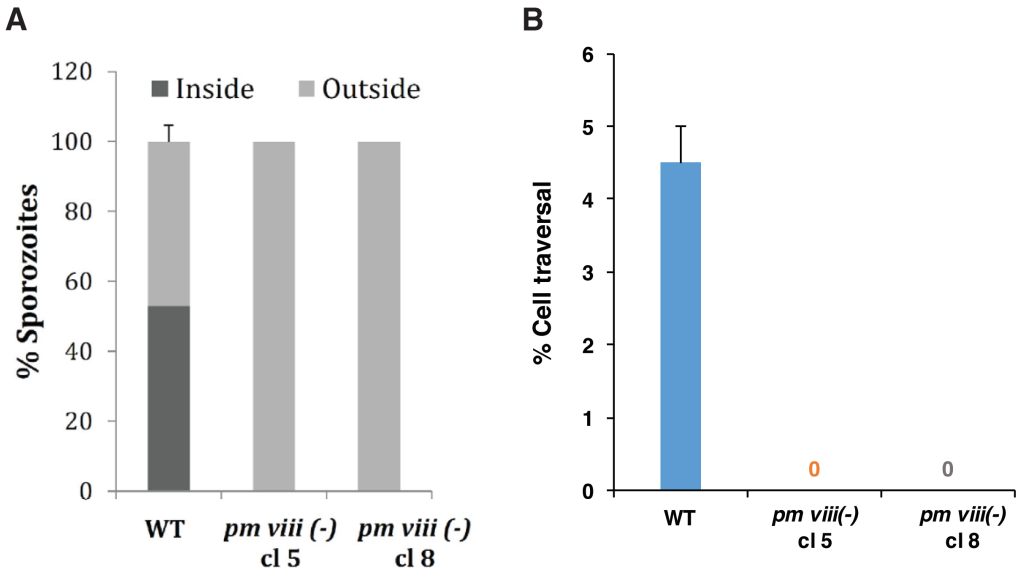


Figure 3.18. *Pbpm viii(-)* sporozoites are severely impaired in cell invasion and transmigration capabilities. **(A)** Sporozoite hepatocyte invasion assay. WT and *Pbpm viii(-)* haemolymph sporozoites were added to HepG2 cells incubated for 1 h, fixed, and differentially stained with anti-CSP antibody. **(B)** Sporozoite hepatocyte traversal assay. HepG2 cells were incubated with WT and *Pbpm viii(-)* hemolymph sporozoites for 1 hour in the presence of dextran-Texas Red. Cells were fixed and analyzed by IFA to enumerate the percentage of dextran-positive cells. Shown are the mean (±SD) values from three independent experiments.

4. DISCUSSION

4. Discussion

Despite humongous efforts in the past decades to control malaria, an entirely preventable and treatable disease, it still remains one of the most formidable global health challenges. Although there are several potential vaccine candidates in the pipeline, the most promising and advanced subunit candidate is the RTS,S/AS01 that targets the parasite pre-erythrocytic stages (Casares et al., 2010). Considering the unsatisfactory outcome of recent Phase 3 clinical trials of RTS,S/AS01 and the limitations of developing highly effective and efficient vaccine candidates, judicious use of antimalarial drugs will remain a prudent strategy to keep the malaria deaths and disease burden under control (Agnandji et al., 2012; Moorthy et al., 2013). In recent years the Artemisinin combinatorial therapies (ACTs) has been the backbone of global malaria control. Unfortunately, there have also been recent reports describing parasite populations resistant to Artemisinins on the Thailand-Cambodia border (Dondorp et al., 2009). The emergence of resistance to the Artemisinins and the impending risk of spreading of drug resistance to malaria endemic regions in Africa or elsewhere is a major concern. If unchecked, it will likely increase the malaria mortality rates and deaths in coming years undermining the progress achieved lately. Therefore, discovery and development of new antimalarial compounds with novel mechanisms of action is the need of the hour.

As an obligate intracellular parasite, *Plasmodium* progress through a highly complex and multistage life cycle during which, the parasites invade, grow and replicate within host cells and eventually egress to ensure successful transmission. The precise coordination of these molecular events require specialized proteins in different host microenvironments. Research in recent years revealed proteases regulate a variety of key physiological functions in parasite biology. Specifically, plasmepsins, the aspartic proteases of *Plasmodium* have been shown to regulate a variety of functions in the parasite life cycle. Thus, they present attractive targets for developing novel antimalarial therapeutics. This thesis was aimed at elucidating and understanding the hitherto unknown functions of plasmepsins VII and VIII in *Plasmodium berghei*, the rodent malaria parasite. This study was carried out to expand the existing knowledge on aspartic proteases of *Plasmodium* and to validate these enzymes as antimalarial drug targets using advanced experimental genetics.

4.1. PM VII has a non-essential role in completion of the *Plasmodium* life cycle

Gene expression profiling of *PM VII* in mosquito and liver stages of *P. berghei* has shown differential expression of *PM VII* compared with other plasmepsins (PM I, II, III or HAP, IV, V, IX and X), which are exclusively expressed in asexual blood stages (Banerjee et al., 2002). Young and developing oocysts showed highest expression of *PM VII* suggesting a possible functional role in oocyst development. Surprisingly, a recent study reported expression of *PM VII* and X in *P. falciparum* zygotes and ookinetes, where they have been suggested to play a role in midgut traversal (Li et al., 2016). The *in vivo* function of PM VII was characterized using experimental genetics. The *PM VII* endogenous copy was disrupted by double crossover homologous recombination. The resulting knockout blood stage parasites were viable, indicating a dispensable role of PM VII in asexual blood stages of the parasite life cycle. Additionally, *in vivo* growth rates in susceptible mice exhibited no noticeable differences between wild type and *pm vii(-)* parasites. These results demonstrate that the depletion of *pm vii* has no apparent quantifiable impact on invasion, development, and replication of blood stages. *pm vii(-)* parasites were similar to wild type parasites in terms of gametocyte formation, which is an obligatory step for transmitting malaria to mosquitoes. Since a discernible function of PM VII could be excluded in blood stages, phenotypic analysis was extended to the rest of the *Plasmodium* life cycle. Sexual differentiation into gametes, ookinete formation, and subsequent development of oocysts was not altered in *pm vii(-)* parasites although high *PM VII* expression was observed in these stages. Dissection of infected mosquitoes and subsequent quantification revealed similar midgut infectivity (oocyst numbers), numbers of midgut sporozoites as well as salivary gland sporozoites in wild type and *pm vii(-)* parasites. Consequently, PM VII is also not essential for either sporogony or maturation of sporozoites. Analysis of salivary gland sporozoite infectivity to host hepatocytes *in vivo* and *in vitro* revealed no striking phenotype in the knockout parasites. In good agreement with the *in vivo* data, the gliding locomotion of *pm vii(-)* salivary gland sporozoites was not affected. The *in vitro* transformation of *pm vii(-)* sporozoites into EEFs within HepG2 cells was indistinguishable from that of wild type EEFs. Similarly, when the *in vivo* infectivity was ascertained by intravenous injections or natural transmission experiments by mosquito bites, the susceptible mice exhibited similar pre-patency compared to inoculation of wild type

sporozoites. Altogether, these findings indicate that genetic ablation of *pm vii* did not affect the life cycle progression of malaria parasites.

The major conclusion from this study is the non-essential role for PM VII in the life cycle of *P. berghei,* the rodent malaria parasite. The objective of this study is to assess the potential of plasmepsin VII, an aspartic protease of *Plasmodium*, as a suitable target for drug development. By employing experimental genetics, the current studies establish that PM VII is not essential for *Plasmodium* growth and developmental progress within the mosquito and mammalian hosts. These findings show that PM VII is not an ideal candidate for target based drug discovery approaches. Successful generation of *pm vii(-)* parasites facilitated an in depth investigation of the *in vivo* function of PM VII during life cycle progression in *P. berghei*. No critical role in any stage of the parasite development inside the host cells was observed. Thus, targeting of plasmepsin VII is not a suitable transmission-blocking candidate or ideal for developing chemo-therapeutic intervention strategies.

A plausible explanation for the non-essential role of PM VII in malaria parasites could be due to the existence of functional redundancy among the mosquito stage expressed plasmepsins (PM VI, VII, and VIII). Hemoglobin catabolism is the chief source of nutrients for blood stage parasites. Four plasmepsins (PM I, II, IV and HAP or PM III) present in the food vacuole (FV) catalyse the semi-ordered degradation of hemoglobin. Experiments with knockout parasites demonstrated high degree of functional overlap among the FV plasmepsins as the knockout parasites were viable and morphologically normal thus compensating for each other (Bonilla et al., 2007; Liu et al., 2005; Omara et al., 2004). Interestingly, in a recent study it is found that triple knockouts and quadruple knockouts of plasmepsins are still killed by inhibitors assumed to be specific for PM I or II (Moura et al., 2009). Thus, highlighting the functional redundancy extends beyond four plasmepsins. The role of mosquito stage expressed plasmepsins has only started to be explored recently. The first study in this direction was the ablation of PM VI in *P. berghei* that revealed its role in mosquito stages where oocyst developed normally but no salivary gland sporozoites were detected (Ecker et al., 2008). Based on the presence of transmembrane domain and signal peptide, it is assumed that PM VI might be a membrane protease and likely to be cleaved and activated by romboid-3, a member of intra membrane serine protease family (Lin et al., 2008). Such speculation is based on the finding that mutants

lacking ROM-3 also manifest identical phenotype as *PM VI* knockouts where oocyst were produced but no functional sporozoites were observed. While such hypothesis needs validation, these findings suggest a possibility that both PM VI and ROM-3 may play a role in pathway that regulates sporogony in oocysts. Subcellular localization data along with the *in vitro* pull down assays may provide information regarding the target substrates and precise molecular function of plasmepsin VII. Nevertheless, compensatory mechanisms operating within the parasite might confer survival advantage despite the lack of a specific protease.

4.2. PM VIII is essential for gliding motility and host cell invasion

In this objective we carried out a detailed functional analysis of PM VIII in *P. berghei* using experimental genetics. The initial gene expression analysis revealed that *PM VIII* is predominantly expressed in oocysts and midgut sporozoites similar to the other PMs expressed in mosquito stages (*PM VI* and *PM VII*). A recent study reported expression of *P. falciparum* PM VI and VIII in gametocytes and are shown to be localized in osmophilic bodies (Weißbach et al., 2017). However, their function in gametocytes remain unknown. The gene expression data suggested that the PM VIII could play an important role during parasite development within the mosquito vector. The *in vivo* role of PM VIII was addressed using reverse genetics approach. The endogenous copy of *PM VIII* gene was replaced with a targeting construct by double homologous recombination. The targeted gene deletion experiments resulted in viable knockout parasites indicating the dispensable role of PM VIII in blood stages of the parasite life cycle. Furthermore, *in vivo* growth assays to determine the multiplication rates and sexual differentiation into gametocytes revealed no differences between wild type and *pm viii(-)* parasites. These results suggest that the genetic ablation of PM VIII has no effect on growth, development and sexual differentiation of blood stage parasites.

Given the abundant expression of *PM VIII* in oocysts and migut sporozoites, the developmental fate of knockout parasites was further examined in *A. stephensi* mosquitoes. There was no defect observed in the initial developmental events in the mosquito and oocyst formation. This was a surprising observation given the predominant expression of *PM VIII* in young and developing oocysts. Apparently, the lack of *PM VIII* has no effect on midgut infectivity and formation of oocysts. The next phase in *P. berghei* life cycle is the egress of

mature sporozoites from oocysts and subsequent invasion of salivary glands. When salivary glands of infected mosquitoes were visualized by live microscopy, surprisingly no sporozoites were associated with the salivary glands of mosquitoes infected with *pm viii(-)* parasites in contrast to wild type. Additionally, quantification of salivary gland sporozoites confirmed that *pm viii(-)* sporozoites failed to colonize salivary glands. These results demonstrate that the absence of PM VIII compromised the ability of sporozoites to invade salivary glands.

The oocysts in the mosquito midgut undergo sporulation or sporogony to form sporozoites. Next, the knockout parasites were analysed for their ability to undergo sporulation by live microscopy and the number of midgut sporozoites were quantified. No significant differences were observed between wild type and *pm viii(-)* parasites with respect to sporulation and number of midgut sporozoites. This implies that PM VIII is not essential for sporozoite formation and maturation within the oocysts. Further the observed phenotype was closely examined to determine whether the defect is associated with oocyst egress or sporozoite gliding motility. Quantification of haemolymph sporozoites showed significant reduction in mosquitoes infected with *pm viii(-)* parasites. In addition to that no morphological differences were observed between wild type and *pm viii(-)* oocyst-derived sporozoites. Thus, lack of *PM VIII* apparently caused a defect in oocyst egress and slaviary gland invasion. The matured sporozoites within the oocysts perform gliding motility in order to egress out of the oocysts and to invade salivary glands. The experimental results so far strongly suggested a defect in gliding motility. Therefore, if PM VIII plays a critical role in gliding motility of sporozoites, then the ablation of *PM VIII* should affect the oocyst egress and salivary gland invasion. The processing of circumsporozoite protein (CSP), one of the important cell surface proteins required for gliding locomotion of the sporozoites, was not affected in *pm viii(-)* oocyst-derived sporozoites. This implied that PM VIII could be regulating the motility of the sporozoites in CSP independent manner. The results of *in vitro* gliding motility experiments and live cell imaging of haemolymph sporozoites behaviour were in good agreement with our prediction and showed that the *pm viii(-)* haemolymph sporozoites were severely impaired in gliding locomotion.

Further progression of the parasite life cycle takes place in the mammalian host after successful transmission. Assuming that very few sporozoites managed to reach salivary glands, the infectivity of *pm viii(-)* sporozoites was checked by transmission experiments *in vivo* either using mosquito bites or intravenous injections of haemolymph sporozoites. Both the experiments revealed that *pm viii(-)* sporozoites failed to transmit malaria to the mammalian host. Furthermore, the *in vitro* infectivity was analysed in HepG2 cells following addition of *pm viii(-)* sporozoites. This experiment revealed that *pm viii(-)* haemolymph sporozoites failed to invade and undergo development within liver cells. Therefore, it is evident that PM VIII is necessary for efficient malaria transmission as the *pm viii(-)* parasites could not transmit malaria either *in vivo* or *in vitro*. Once inside the mammalian host, gliding motility is crucial for the sporozoites as it facilitates their journey from inoculation site to the liver. When the sporzoites reach liver, they transmigrate and invade several hepatocytes before establishing productive infection. *In vitro* invasion and transmigration experiments indicated that the *pm viii(-)* haemolymph sporozoites were heavily affected in their invasion and transmigration abilities as a consequence of defective gliding motility.

Plasmodium and other related Apicomplexan parasites are a group of obligatory intracellular protozoan parasites that are uniquely adapted to grow within the boundaries of host cells. This intracellular life style provides considerable protection from host immune responses and also provides plenty of nutrients required for the growth and development. During infection, these parasites employ sophisticated strategies to minimize their exposure to the host immune system and also to egress out of the host cells when the development and reproduction is completed, after which they continue their life cycle.

A remarkable feature of *Plasmodium* parasites is the repeated cycles of host cell invasion, intracellular multiplication and egress of parasites (Black and Boothroyd, 2000). *Plasmodium* and *Toxoplasma* share broadly similar mechanisms of invasion and egress. The critical components of this conserved machinery are specialized apical secretory organelles, cell surface molecules, transmembrane proteins and an actin-myosin motor complex, coordinating all these components (Cowman and Crabb, 2006; Carruthers and Boothroyd, 2007). Malaria parasites progress through different developmental stages in multiple host during their life cycle. These developmental stages encounter hostile conditions in different

tissue microenvironments, such as skin, liver, blood and mosquito midgut and salivary glands. Malaria parasites rely on gliding motility for navigating through various physiological barriers and invasion of a variety of host cells in mosquito and mammalian hosts. The extracellular motile forms, sporozoites, merozoites and ookinetes, are morphologically different yet share conserved components of gliding motility apparatus. The glideosome complex consists of an intracellular actin-myosin motor situated in the inner membrane complex (IMC) of plasma membrane. The IMC is linked to the substrate through cell surface adhesins (Bargieri et al., 2014; Baum et al., 2008; Harding and Meissner, 2014; Kono et al., 2013). Upon contact with the host cells, the adhesins are discharged from secretory organelles (Carruthers and Sibley, 1997). Some of the adhesins, such as the ookinete SOAP and WARP are secreted into the extracellular milieu (Dessens et al., 2003; Yuda et al., 2001; Li et al., 2004) and some are retained within the plasma membrane to engage with receptors of the host cells. During gliding, these adhesins move backwards along the cell surface and are continuously shed and deposited onto the substrates as detectable gliding trails (Stewart and Vanderberg, 1992; Ejigiri et al., 2012; Kappe et al., 1999; Kariu et al., 2006), as they are processed by intramembrane proteases like rhomboid and serine proteases like subtilisin (Baum et al., 2008; Brossier et al., 2005; Baker et al., 2006; Ejigiri et al., 2012).

In *Plasmodium* several factors involved in adhesion, invasion and motility are expressed in stage-specific manner throughout the life cycle. So far the known key molecules include CTRP (Yuda et al., 1999; Dessens et al., 1999), WARP (Yuda et al., 2001; Li et al., 2004; Ecker et al., 2008), SOAP (Dessens et al., 2003), CHT1 (Li et al., 2004; Dessens et al., 2001) and MAOP (Kadota et al., 2004), which are exclusive to the ookinetes. TRAP (Kappe et al., 1999; Sultan et al., 1997; Matuschewski et al., 2002), AMA1 (Giovannini et al., 2011; Bargieri et al., 2013; Silvie et al., 2004), MAEBL (Kariu et al., 2002; Saenz et al., 2008), SPECT (Ishino et al., 2004), SPECT2 (Ishino et al., 2005a), GEST (Talman et al., 2011) and P52 (Ishino et al., 2005b; Annoura et al., 2014; Labaied et al., 2007a; van Dijk et al., 2005), which are exclusive to sporozoites. CelTOS is expressed in both the stages (Jimah et al., 2016). In addition to them, the other surface proteins shown to be required for parasite motility and host cell invasion include S6/TREP/UOS3 (Mikolajczak et al., 2008; Steinbuechel and Matuschewski, 2009; Combe et al., 2009), TLP (Hellmann et al., 2011; Moreira et al., 2008; Heiss et al., 2008; Lacroix and Ménard, 2008), PCRMP1 and 2 (Thompson et al., 2007), the rhoptry-resident proteins

TRSP (Kaiser et al., 2004; Labaied et al., 2007b) and RON4 (Giovannini et al., 2011), the GPI-anchored circumsporozoite protein (CSP) of the sporozoite (Coppi et al., 2011) and the recently reported small solute transporter PAT (Kehrer et al., 2016a). How all these molecules interact with each other and with the host cell receptors and how their concerted action efficiently coordinates gliding motility and host cell invasion, which ultimately leads to productive infection remains poorly understood.

It is assumed that genes encoding proteins that regulate gliding motility and host cell invasion are transcribed and translated instantly according to the parasite needs. However, recent studies revealed that some of the mRNAs are transcribed but do not undergo translation (Mair et al., 2006, 2010; Guerreiro et al., 2014). These untranslated mRNAs are repressed in female gametocyte and therefore maternally inherited to the subsequent developmental stages (ookinetes and oocysts) (Ecker et al., 2008; Guerreiro et al., 2014; Santos et al., 2016; Saeed et al., 2013; Rao et al., 2016; Sebastian et al., 2012; Raine et al., 2007). PM VIII transcript is found to be one of the many translationally repressed mRNAs associated with DOZI and CITH complex and the protein is shown to be localized to osmophilic bodies in female gametocytes (Guerreiro et al., 2014; Weißbach et al., 2017). The protein localization studies indicate a role for PM VIII in the female gametocytes. Nonetheless, there was no defect observed with respect to gametocyte formation and subsequent developmental stages. The lack of PM VIII, however, resulted in sporozoites with severely impaired egress as a result of defective gliding motility. The exit of sporozoites from oocysts and their further journey towards salivary glands appears to be a highly complex and regulated process and involves an array of specialized parasite molecules. The factors that are identified to date are the ECP1, the GPI-anchored circumsporozoite protein, CSP (Wang et al., 2005; Aly and Matuschewski, 2005), the *Plasmodium* cysteine repeat modular proteins PCRMP3 and PCRMP4 (Douradinha et al., 2011) and the LCCL-domain-containing proteins PfCCp2 and PfCCp3 (Pradel et al., 2004). Mutant sporozoites that lack proteins involved in gliding motility do not enter into salivary glands (Sultan et al., 1997; Ganter et al., 2009) and impaired gliding motility (S6/TREP/UOS3, Coronin, CSP and PAT) often results in decreased salivary gland invasion (Coppi et al., 2011; Kehrer et al., 2016b; Steinbuechel and Matuschewski, 2009; Combe et al., 2009; Bane et al., 2016; Tewari et al., 2002; Mikolajczak et al., 2008). Thus, the mutant parasites lacking proteins that regulate gliding motility are also impaired in host cell invasion in the mammalian host

and therefore they are essential for life cycle progression at two subsequent steps (salivary gland invasion and hepatocyte invasion). In conclusion, in the present study, we characterized a *Plasmodium* aspartic protease, PM VIII, which plays a critical role in sporozoite gliding motility and infection of host cells in the rodent malaria parasite *P. berghei*. Although the exact mechanism of action remains elusive, it is likely that the PM VIII participates in the proteolytic cascade essential for gliding motility of sporozoites. However, in the absence of information on sub-cellular localization within in the sporozoites and its proteolytic substrates, it is difficult to ascertain whether *Pb*PM VIII directly regulates gliding motility by participating in the processing of cell surface adhesins other than CSP or is involved in upstream signalling events that govern the motility of sporozoites. Further investigation is needed to determine the mechanistic details of *Pb*PM VIII role in sporozoite gliding motility. Uncovering the *in vivo* functions of proteases involved in sporozoite motility could help in identification of new targets for the development of novel antimalarial drugs.

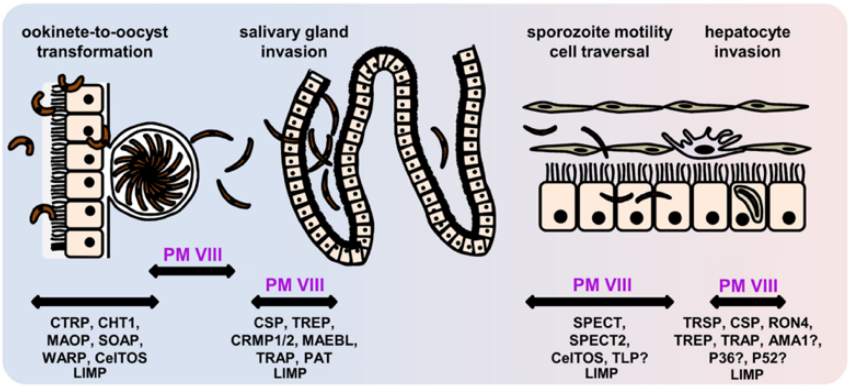


Figure 4.1. Pictorial representation of various factors involved in motility, invasion and egress of *Plasmodium* parasites during mosquito stage development and malaria transmission. While, previously identified malaria proteins take part in just one or few steps of motility and invasion stage-specific manner, PM VIII plays a key role in sporozoite egress from oocysts, salivary gland invasion, sporozoite gliding motility, and traversal and invasion of hepatocytes. (Image adapted & modified from Santos et al., 2017).

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Short communication

Gene disruption reveals a dispensable role for Plasmepsin VII in the *Plasmodium berghei* life cycle

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ABSTRACT

Plasmepsins (PM), aspartic proteases of *Plasmodium*, comprises a family of ten proteins that perform critical functions in *Plasmodium* life cycle. Except VII and VIII, functions of the remaining plasmepsin members have been well characterized. Here, we have generated a mutant parasite lacking PM VII in *Plasmodium berghei* using reverse genetics approach. Systematic comparison of growth kinetics and infection in both mosquito and vertebrate host revealed that PM VII depleted mutants exhibited no defects in development and progressed normally throughout the parasite life cycle. These studies suggest a dispensable role for PM VII in *Plasmodium berghei* life cycle.

Plasmepsins are aspartic proteases of *Plasmodium falciparum* (*P. falciparum*) that have been extensively studied in blood stages for their role in hemoglobin (Hb) degradation and hence as potential drug targets. *P. falciparum* encodes for ten plasmepsins [1], out of which four paralogues *viz.*, PM I, II, III (HAP) and IV have been shown to reside in the acidic food vacuole of *P. falciparum* infected red blood cells. These plasmepsins orchestrate an ordered process of Hb degradation where PM I and PM II likely catalyze the initial cleavage. Further catabolism of Hb to free amino acids is facilitated by combined action of histoaspartic protease (HAP, PM III), PM IV, cysteine proteases and metalloproteases [2]. Though PM V, IX and X are expressed in the blood stages, they do not have a function in the food vacuole [3]. PM V is a parasite endoplasmic reticulum resident protease [4] that cleaves the export cargo containing PEXEL motif to facilitate their translocation into cytosol to promote virulence and erythrocyte take over [5,6]. Both PM IX and X were shown to localize in trophozoite stage [3]. While PM IX locus is recalcitrant to gene disruption reiterating its essential role in blood stages [7], the function of PM X is not known.

PM VI, VII and VIII are not expressed in the blood stages [3,8] implying a possible role in other stages. An evidence corroborating for an extra erythrocytic function of PM VI was recently reported in mosquitoes stages of *Plasmodium berghei* (*P. berghei*), where depletion of *pm vi* led to absence of salivary gland sporozoites, though functional oocyst were observed [7]. While gene expression data for PM VII in mosquito transmission stages have been reported earlier [9,10] its functional role has not been investigated. A better understanding of its role in other *Plasmodium* stages may provide novel insights into their biological roles unique to these stages.

Towards this end, we have undertaken a genetic approach to investigate the role of *P. berghei* PM VII (PBANKA_051760) in the parasite life cycle. We first analyzed the gene expression of PM VII both in the mosquito and liver stages by quantitative real time PCR. The cDNA samples were prepared at different time points from both mosquito and liver stages as described in supplementary material. Normalized data obtained as a ratio of *P. berghei PM VII*/*P. berghei 18S rRNA* revealed highest level of transcript abundance on day 4 (MSD4) post blood meal. While other time points of mosquito stages showed modest expression, no expression was detected in the liver stages (Fig. 1A). In order to reveal the function of the PM VII, we have generated a loss of function mutant using gene replacement strategy (Fig. 1B). To achieve this, we amplified 650 bp of 5′ and 550 bp of 3′ sequence flanking the target (PBANKA_051760) by PCR. The primer pair CTCGAGGGAGCAATTATGTTACTATATC and ATCGATGGTTTATACACTTGTACGACA were used to amplify the 5′ end and the primer pair GCGGCCGCCCTGAATGGAAAAGAATACATA and GGCGCGCCCCACTATTTAACCACACGATT were used to amplify the 3′ end. The restriction sites in the sequence are underlined.

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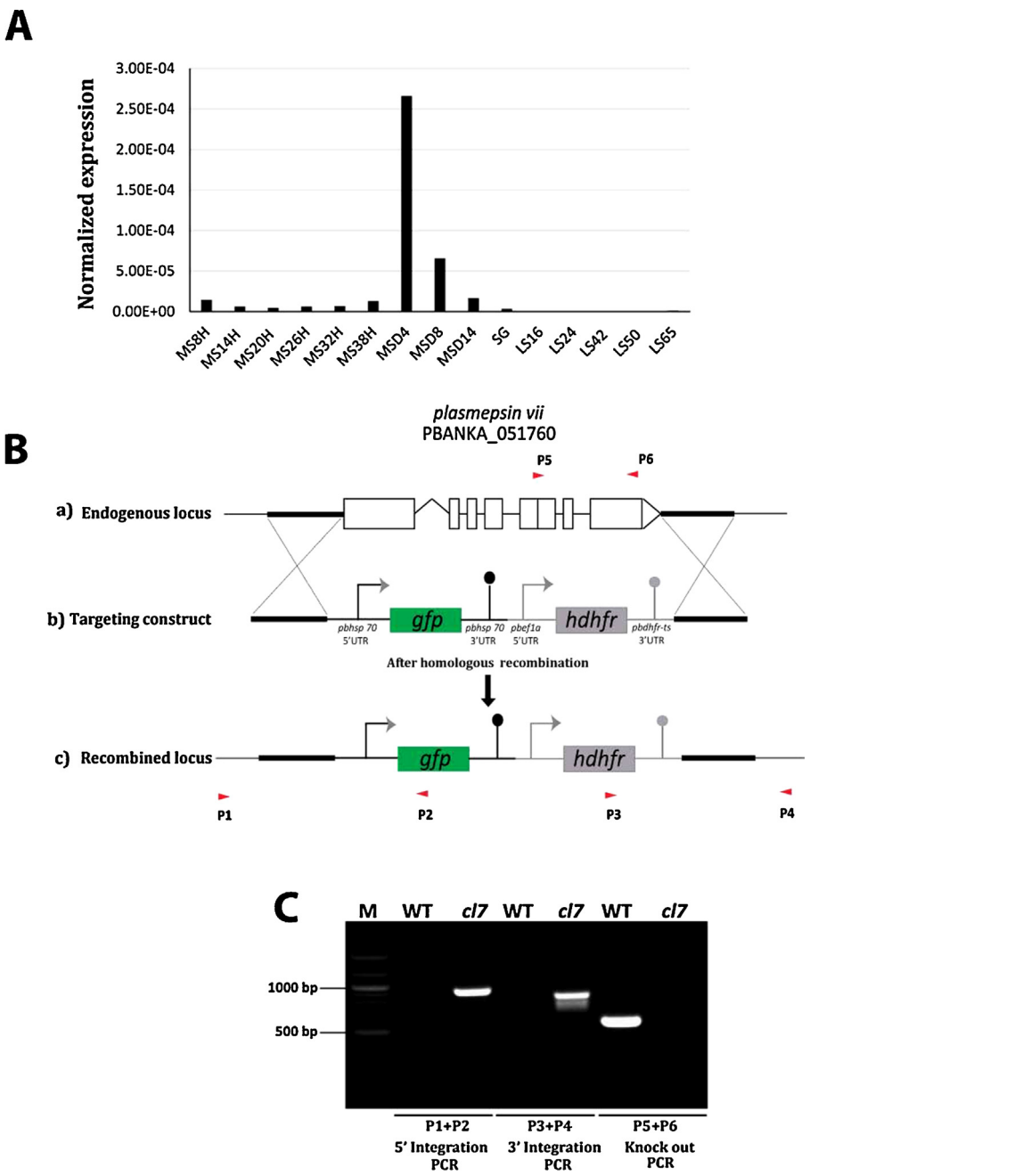

Fig. 1. (A) Quantitative determination of the transcript levels of PM VII in mosquito and liver stages of *P. berghei*. Expression levels were measured in mosquito stages (MS) post infection at hours (H) 8, 14, 20, 26, 32, 38 and on day (MSD) 4, 8, 14, salivary gland sporozoites (SG), and liver stages (LS) at hours 16, 24, 42, 50, 65. Normalization of target gene expression was performed by obtaining a ratio of absolute number of *pm vii*/*Pb 18S rRNA*. (B) Genomic organization of *P. berghei pm vii* and strategy used to replace the target by double cross over recombination (DCO). (a) *P. berghei pm vii* locus. (b) Targeting construct having 650 pb of 5′ and 550 bp of 3′ part of *P. berghei pm vii* cloned on either side of GFP and hDHFR cassette. (c) Recombined locus after DCO. (C) Diagnostic PCR to confirm the site specific integration: Genomic DNA obtained from either WT or *pm vii* KO were subjected to PCR using indicated sets of primers. A PCR product corresponding to 850 bp using P1 + P2 and 750 bp using P3 + P4 in *pm vii* KO confirmed respectively the 5′ and 3′ end specific integration. A wild type specific PCR product corresponding to 550 bp using P5 + P6 confirmed the absence of *pm vii* in the KO.

Following sequence confirmation, the 5′ and 3′ fragments were cloned into pBC-GFP-DHFR vector using restriction sites XhoI/ClaI and NotI/AscI respectively. The targeting vector was linearized using restriction sites XhoI and AscI. Ten micro gram of targeting construct was electroporated into wild type (WT) synchronized blood stage schizonts as described earlier [11] and the transfected parasites were immediately injected intravenously into mouse followed by selection on pyrimethamine. Successful integration of the targeting construct by double cross over recombination was confirmed by diagnostic PCR (Fig. 1C) and also by GFP expression, observed under fluorescent microscope (Nikon Eclipse NiE AR). The *pm vii* KOs derived from two independent transfections were subjected to limiting dilution and two clones were selected for further phenotypic characterization. While identical results were obtained in all experiments using two independent clones (data not shown), we report the results of one clone (cl7) in this study.

Successful generation of loss-of-function mutant indicated that PM VII was dispensable for blood stages. However to determine whether absence of PM VII has any effect on growth of blood stages, 10^3 infected RBC of either WT or *pm vii* KO were intravenously

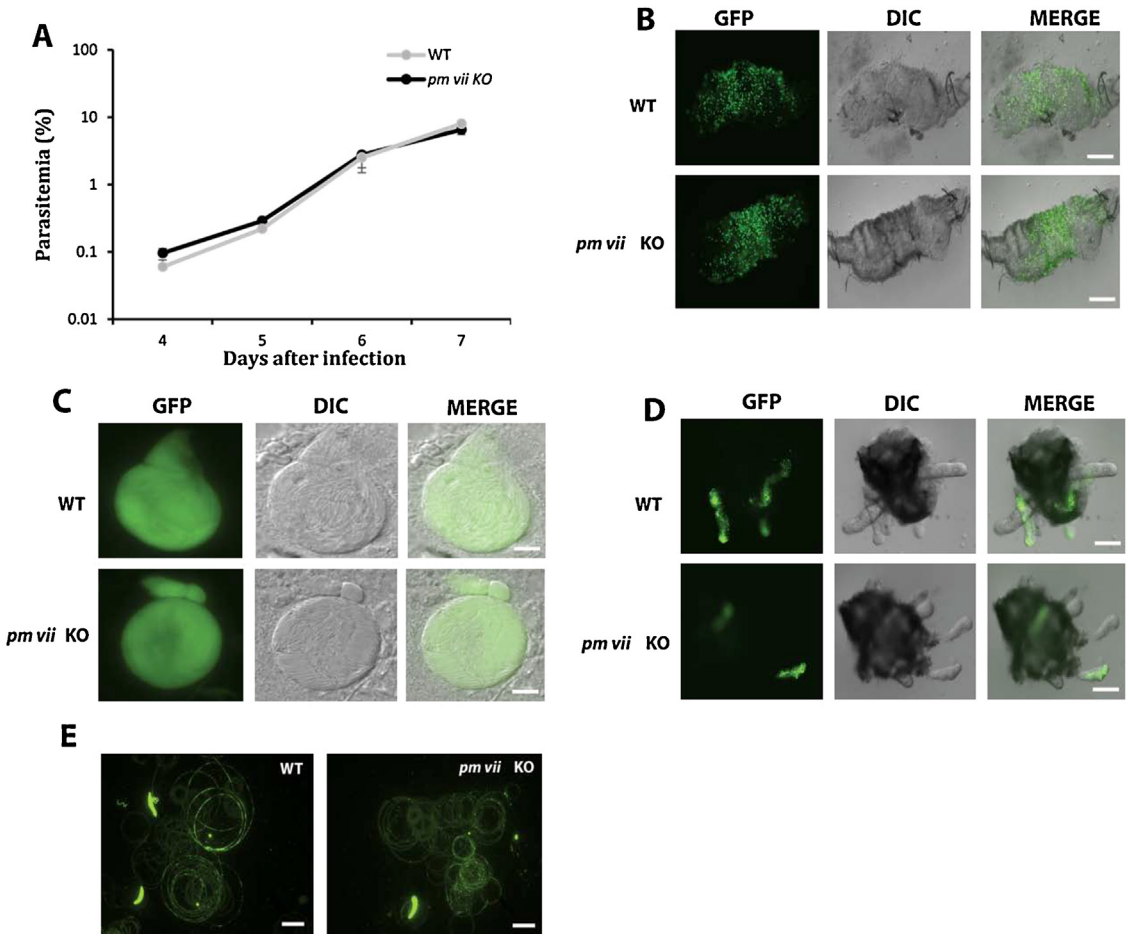

Fig. 2. Phenotypic characterization of *pm vii* KO parasites. (A) Genetic ablation of pm vii does not affect blood stage development. *In vivo* growth curves of wild type (gray) and KO (black) parasites. Female BALB/c mice ($n = 3$) were intravenously injected with 1000 mixed blood stage parasites and parasitaemia was determined by microscopic examination of Giemsa-stained blood smears. Shown values are mean ± standard deviation. (B) Dissected midguts. (C) Magnified oocyst showing sporulation. (D) Dissected salivary glands. (E) Trails released by WT and *pm vii* KO sporozoites during gliding motility on glass slides (scale bars, 200 μm for midguts, 20 μm for oocyst, 200 μm for salivary glands and 10 μm for sporozoites showing gliding motility).

injected into a group of 3 female BALB/c mice and parasitaemia was daily monitored by microscopic examination of Giemsa stained blood smears for a period of 7 days. Both WT and *pm vii* KO blood stages showed similar multiplication rates (Fig. 2A). Further to investigate the role of PM VII in the mosquito stages, we propagated cloned *pm vii* KO parasites into 4–5 female BALB/c mice. GFP expressing WT *P. berghei ANKA* parasites were used as control. All gametocyte positive mice were selected for feeding female *Anopheles stephensi* mosquitoes. The infected mosquitoes were maintained at 20 °C and 75–80% relative humidity (RH). On D14 post blood meal, the mosquito midguts were dissected and the oocyst numbers were determined under fluorescent microscope (Fig. 2B and Table 1A). No apparent differences in morphology were observed in *pm vii* KO oocysts as compared to WT (Fig. 2C). Dissected salivary gland of *pm vii* KO infected mosquitoes contained viable sporozoites whose numbers were comparable to that of the WT sporozoites (Fig. 2D and Table 1A). These results suggest that lack of PM VII did not compromise development of oocysts or ability of haemocoel sporozoites to invade salivary glands.

Table 1A
Oocyst and salivary gland sporozoite numbers was determined at D12 and D21 respectively, post infectious blood meal. Three independent feeding experiments were performed.

Parasites	Number of oocyst/mosquito Mean (±SD)	Number of salivary gland sporozoites/mosquito Mean (±SD)
WT	147 (±70)	14,500 (±3700)
pm vii KO	180 (±80)	16,000 (±3900)

To analyze the characteristics of pre-erythrocytic stages, we studied sporozoite gliding motility by allowing both *pm vii* KO and WT parasites to glide on glass slides coated with 3D11 mAb (anti-PbCS). To visualize the trails of circumsporozoite protein (CSP) released by sporozoites, the slides were fixed with 4% paraformaldehyde, probed with biotinylated 3D11 antibody and revealed by FITC-streptavidin [12]. No differences were observed in the numbers and pattern of trails produced by *pm vii* KO sporozoites as compared to WT (Fig. 2E). To determine if the *pm vii* KO sporozoites successfully initiate blood stage infection *in vivo*, female C57BL/6 mice were infected through mosquito bite. Two groups of three mice were kept on individual cages, each containing 11–16 infected mosquitoes of either WT or *pm vii* KO (D21 post blood meal) to initiate infection. The number of mosquitoes that took blood meal ranged from 54% to 90% in both groups. The number of blood fed mosquitoes that were positive for salivary gland infection ranged from 58% to 100% (Supplementary Table 1), suggesting a successful infection in mice through bite. Analysis of the prepatent period in *pm vii* KO revealed the appearance of blood stage infection on D4 that was similar to wild type parasites (Table 1B). From these studies, we conclude that depletion of PM VII neither alters *in vivo* cell traversal activity or hepatocyte tropism and these sporozoites behave identical to the WT sporozoites with

Table 1B
Monitoring of pre-patent period following mosquito bite. Pre-patent period: after sporozoite inoculation, number of days until the detection of blood stage parasites by microscopy.

Wild type		*pm vii* KO	
Infected/total	Pre-patent period	Infected/total	Pre-patent period
3/3	Day 4	3/3	Day 4

respect to commitment to hepatocyte infection and completion of exo-erythrocytic development.

The role of extra erythrocytically expressed plasmepsins has only started to be explored recently. The first study in this direction was the ablation of PM VI in *P. berghei* that revealed its role in mosquito stages where oocyst developed normally but no salivary gland sporozoites were detected [7]. Based on the presence of transmembrane domain and signal peptide that may facilitate its attachment to plasma membrane, it is predicted that PM VI may be a likely substrate for the romboid-3, a member of intra membrane serine protease family [13]. Such speculation is based on the finding that mutants lacking ROM-3 also manifest identical phenotype as *pm vi* KO where oocyst were produced but no functional sporozoites were observed. While such hypothesis needs validation, these findings suggest a possibility that both PM VI and ROM-3 may play a role in pathway that regulates sporogony in oocyst. Based on the evidence of gene expression data that PM VII is expressed in the transmission stages [9,10] we took a reverse genetics approach to dissect the role of PM VII by generating a KO. By systematic comparison of developmental kinetics of *pm vii* KO and WT parasites in mosquito and vertebrate host, we report that ablation of PM VII had no apparent fitness loss with respect to progression through the life cycle. Our preliminary results indicate a similar pattern of gene expression for PM VII and VIII in the mosquito stages (Babu S. Mastan and Kumar KA, unpublished data). While our studies reveal a dispensable role of PM VII in *Plasmodium* life cycle, further investigation is required to prove if any functional redundancy exists among mosquito stage expressed plasmepsins that may compensate for loss of function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2014.05.004.

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Succinctus

Plasmodium berghei plasmepsin VIII is essential for sporozoite gliding motility

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ABSTRACT

Plasmodium aspartic proteases, termed plasmepsins (PMs) play many critical roles such as haemoglobin degradation, cleavage of PEXEL proteins and sporozoite development in the parasite life cycle. Most of the plasmepsins are well characterized, however the role of PM VIII in *Plasmodium* remains unknown. Here, we elucidate the functions of PM VIII (PBANKA_132910) in the rodent malaria parasite *Plasmodium berghei* (*Pb*). By targeted gene deletion, we show that *Pb*PM VIII is critical for sporozoite egress from an oocyst and gliding motility, which is a prerequisite for the invasion of salivary glands and subsequent transmission to the vertebrate host.

Malaria is a life threating disease caused by an obligate intracellular parasite that belongs to the genus *Plasmodium*. Despite significant advances in malaria research, it remains a global health concern accounting for an estimated 214 million cases and 438,000 deaths worldwide (WHO, 2015). Identification of novel drug targets and development of new antimalarial treatments are urgently needed as the parasite has acquired resistance to the existing frontline drug, artemisinin (Dondorp et al., 2009). A comprehensive understanding of parasite biology can aid in the development of novel chemotherapeutic agents and effective vaccines. The *Plasmodium* parasites have a complex life cycle with multiple stages switching between the vector and the vertebrate host, which involves many events of invasion, development and dissemination (Aly et al., 2009). Parasite-derived proteases located in distinct cellular compartments mediate many functions that are essential for successful development and propagation of the *Plasmodium* parasites (Klemba and Goldberg, 2002).

Among different classes of proteases, aspartic proteases were shown to be excellent drug targets owing to their critical roles in a variety of physiological and pathological processes (Eder et al., 2007). *Plasmodium falciparum* encodes for 10 aspartic proteases referred to as plasmepsins (PMs). Most of those were shown to govern several biological processes of the parasite (Coombs et al., 2001). Seven PMs (I, II, III, VI, V, IX, X) are expressed in blood stages. The first four paralogues, PMs I, II, IV, and Histo-aspartic protease or PM III, were shown to be located in the food vacuole and have been extensively characterized for their role in haemoglobin catabolism, a vital function for the survival of intraerythrocytic parasites (Banerjee et al., 2002; Liu et al., 2005). Together with other cysteine proteases and metalloproteases, these four PMs catalyse the ordered degradation of haemoglobin into amino acids (Eggleson et al., 1999; Sijwali and Rosenthal, 2004). PM V is an endoplasmic reticulum resident protease that recognizes and cleaves *Plasmodium* Export Element sequence (PEXEL) containing cargo proteins destined for export into erythrocytes, which is crucial for parasite virulence and survival (Klemba and Goldberg, 2005; Boddey et al., 2010; Russo et al., 2010). Despite being shown to be expressed in blood stages, the precise functions of PMs IX and X are not yet known. Knockout attempts to disrupt both of these PMs were not successful, thus indicating an essential role during intraerythrocytic development (Banerjee et al., 2002; Ecker et al., 2008; Hu et al., 2010). Interestingly PMs VI, VII and VIII are not expressed in blood stages, hinting at a probable role for these PMs either in mosquito stage (MS) or liver stage (LS) parasites. Indeed, a recent reverse genetics screen uncovered the function of PM VI in the MS with the mutants displaying a defect in oocyst sporulation (Ecker et al., 2008). An earlier study from our group revealed a non-essential role of *Plasmodium berghei* (*Pb*) PM VII

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during the *Plasmodium* life cycle, although maximal transcription was observed at day 4 of the MS (MS D4; Mastan et al., 2014). However, the function of *Pb*PM VIII in *Plasmodium* biology is currently unknown.

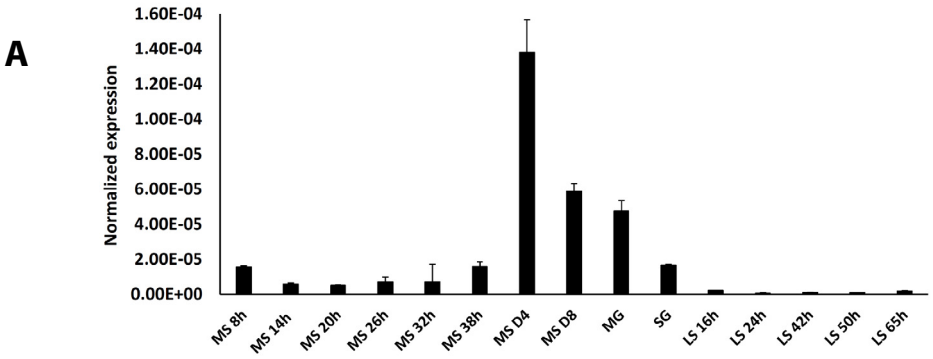
Here, we investigated the in vivo role of aspartic protease *Pb*PM VIII during the malaria parasite life cycle using the rodent malaria model *P. berghei*. We demonstrate that *Pb*PM VIII is dispensable for blood stage development in the mammalian host, whereas in the mosquito vector it is necessary for the sporozoite egress from the oocyst and gliding motility of the sporozoite.

We initiated our study with gene expression analysis of *pbpm viii* by quantitative reverse transcription (qRT)-PCR using cDNA prepared from MS and LS parasites at different time points (in h and days). For the MS, whole midguts were used whereas for midgut (MG) and salivary gland (SG) sporozoites, dissected midguts and salivary glands were disrupted by mechanical crushing and sporozoites were purified by centrifugation. Normalization of gene expression data with parasite *Pb18s rRNA* control revealed that *pbpm viii* expression was highest for MS D4 followed by modest expression in MS D8 and MG sporozoites (Fig. 1A). While very low expression was detected in early MS (8–38 h) and SG sporozoites, meager or no expression was observed in LS (16–65 h). Collectively, gene expression data suggested a possible role for *Pb*PM VIII during parasite development within the mosquito vector.

In order to gain insight into the function of *Pb*PM VIII during the *Plasmodium* life cycle, we generated knockout parasites in *P. berghei* using a gene replacement strategy (Fig. 1B). To delete the endogenous *pbpm viii* gene, we prepared a targeting construct comprising constitutively expressing GFP and human dihydrofolate reductase (hDHFR) cassettes flanked by 5′ and 3′ untranslated regions (UTRs) of *pbpm viii*. The 5′ and 3′ UTRs of *pbpm viii* were amplified from *P. berghei* wild type (WT) genomic DNA (gDNA) using primers 85F-85R and 83F-83R, respectively (primer sequences are provided in Supplementary Table S1). Both fragments were cloned into a pBC-GFP-hDHFR vector using *Xho*I-*Cla*I for the 5′ UTR and *Not*I-*Asc*I for the 3′ UTR. The final plasmid was linearized with *Xho*I-*Asc*I and electroporated into purified schizonts (Janse et al., 2006). The transfected schizonts were injected i.v. into Swiss Albino mice and subjected to pyrimethamine selection. Since knockout parasites constitutively express GFP, we performed fluorescence microscopy of asexual blood stages and observed a strong GFP expression. Diagnostic PCR was carried out to confirm the site-specific integration of the selection cassette and deletion of the target gene (Fig. 1C). Successful deletion of *pbpm viii* was also confirmed by Southern blot hybridization using a PCR DIG labelling and detection kit (Roche, Switzerland). The hybridization of the probe with *Eco*RV digested gDNA from wild type (WT) and *pbpm viii (-)* parasites resulted in bands of 10.8 and 6.7 kb, respectively (Fig. 1D), which revealed the expected size shift and homogenous presence of *pbpm viii (-)* parasites only. Two independent clonal populations of knockout parasites were obtained by limiting dilution and named clone 5 (cl 5) and clone 8 (cl 8). We next examined the phenotype of *pbpm viii (-)* parasites during the *Plasmodium* life cycle progression. To determine whether the deletion of *pbpm viii* affected the development of blood stages, 10^3 red blood cells (RBCs) infected with either WT or *pbpm viii (-)* parasites were i.v injected into a group of five mice and their parasitemias were monitored daily by Giemsa staining. This analysis revealed that *pbpm viii (-)* parasites were indistinguishable from WT parasites with respect to propagation of asexual stages (Fig. 1E). Taken together these data indicate that lack of *Pb*PM VIII did not affect growth and development of asexual blood stages. All animal experiments performed in this study were approved by the Institutional Animal Ethics Committee at the University of Hyderabad, India (approval nos: UH/SLS/IAEC/2014-1/9b and UH/SLS/IAEC/2014-1/9c) and CSIR-Central Drug Research Institute, India (approval no: IAEC/2013/83).

We further sought to investigate the developmental fate of *pbpm viii (-)* parasites in the mosquito vector. A *P. berghei* ANKA line constitutively expressing GFP under the HSP70 promoter (Abdul Al-Nihmi FM and Kumar KA, personal communication) was used as a WT control throughout this study for the phenotypic characterization of knockout parasites. For mosquito transmission, we allowed female *Anopheles stephensi* mosquitoes to feed on anesthetized Swiss Albino mice carrying either WT or *pbpm viii (-)* mature gametocytes. Post blood meal, mosquitoes were maintained at 20 °C and 80% relative humidity. Infected mosquitoes were dissected to perform a detailed analysis of parasite development in the MS. Live microscopic studies of infected midguts ($n = 30$) on day 10 revealed no differences in midgut infectivity and oocyst formation (Fig. 2A and B), which suggests that ablation of *pbpm viii* did not affect the formation of ookinetes and subsequent transformation into oocysts. Moreover, we observed the sporulation patterns inside the oocysts and quantified the numbers of MG sporozoites at day 14 after the blood meal and found no difference between *pbpm viii (-)* and WT (Fig. 2C and D). In contrast, at day 18 after the blood meal, no SG associated sporozoites were detected in the *pbpm viii (-)* line ($n = 60$) (Fig. 2E and F) despite robust midgut infectivity and a high number of oocyst sporozoites. Although we continued to look for SG sporozoites until day 25 p.i., we failed to detect *pbpm viii (-)* salivary gland sporozoites (data not shown). As the failure to reach salivary glands could be a consequence of impaired sporozoite egress from oocysts or a defect associated with sporozoite motility, we next quantified the hemolymph-associated sporozoites and examined the presence of sporozoites in the midgut of infected mosquitoes. While we observed no difference in the sporozoite load in the mosquito midgut (Fig. 2G) released from a mechanically ruptured oocyst, we found that the number of *pbpm viii (-)* sporozoites in hemolymph was severely reduced compared with WT (Fig. 2H). Altogether, these data demonstrate that *pbpm viii (-)* sporozoites have a defect in egress from oocysts. The sporozoite

Fig. 1. Gene expression analysis of *Plasmodium berghei* plasmepsin VIII (*Pb*PM VIII) and generation of *pbpm viii (-)* parasites. (A) Stage-specific gene expression analysis was performed by quantitative reverse transcription (qRT)-PCR using cDNA prepared from the mosquito (MS) and liver stage (LS) parasites of *P. berghei*. Values were normalized to a *Pb*18s rRNA control. The abbreviations used to describe parasite stages are: MS at 8, 14, 20, 26, 32, 38 h p.i. and on days 4, 8 p.i. (MS D), midgut sporozoites (MG), salivary gland sporozoites (SG), and LS at 16, 24, 42, 50, 65 h p.i. (B) Schematic representation of gene replacement strategy used to generate *pbpm viii (-)* parasites. The endogenous locus was targeted with a replacement plasmid pBC-GFP-DHFR/*pbpm viii* containing the 5′ and 3′ untranslated regions (UTRs) of *Pb*PM VIII flanking GFP and the selection marker human dihydrofolate reductase (hDHFR). After double crossover homologous recombination, the *pbpm viii* open reading frame was replaced with GFP and the selection cassette. HSP70 prom, Heat shock protein 70 gene promotor; eEF1α prom, elongation factor 1 alpha gene promotor; DHFR/TS, dihydrofolate reductase - thymidylate synthase. (C) Confirmation of gene deletion (knockout, KO) by diagnostic PCR analysis. The integration (Int) at 5′ and 3′ UTR ends were confirmed by primer combinations that amplified the recombinant locus, P1 + P2 for 5′ and P3 + P4 for 3′ end. The absence of a wild type (WT) locus was confirmed with primers P5 + P6 (shown in B). (D) Southern blot hybridization of WT, *pbpm viii (-)* clones (cl) 5 and cl 8 is shown to confirm integration of targeting construct into the *pbpm viii* locus. The probe represented by a purple line in B was used for hybridization with *Eco*RV-digested genomic DNA. Integration of the cassette was confirmed by the presence of a 6.7 kb band. (E) Asexual blood stage development was unaffected in *pbpm viii (-)* parasites. Female BALB/C mice ($n = 5$) were injected i.v. with 10^3 infected red blood cells and the parasitemias of the recipient mice were monitored daily by microscopic observation of Giemsa-stained blood smears. Shown are the values of mean ± S.D. Asexual blood stage propagation was repeated three times, and a representative experiment is shown.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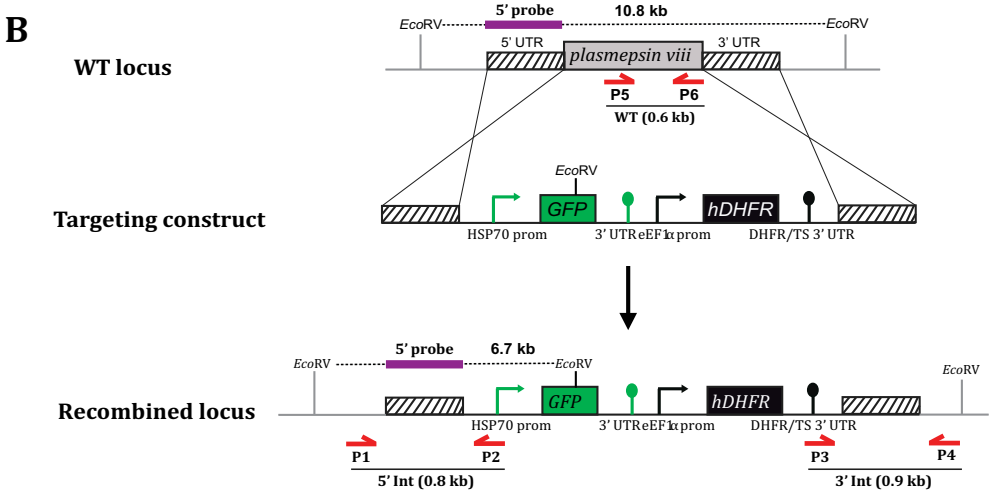
1.40E-04
1.20E-04
1.00E-04
8.00E-05
6.00E-05
4.00E-05
2.00E-05
0.00E+00
MS 8h
MS 14h
MS 20h
MS 26h
MS 32h
MS 38h
MS D4
MS D8
MG
SG
LS 16h
LS 24h
LS 42h
LS 50h
LS 65h


B
WT locus
EcoRV
5' probe
10.8 kb
EcoRV
5' UTR
plasmepsin viii
3' UTR
P5
P6
WT (0.6 kb)
Targeting construct
EcoRV
GFP
hDHFR
HSP70 prom
3' UTR
eEF1α prom
DHFR/TS 3' UTR
Recombined locus
5' probe
6.7 kb
EcoRV
EcoRV
EcoRV
GFP
hDHFR
HSP70 prom
3' UTR
eEF1α prom
DHFR/TS 3' UTR
P1
P2
P3
P4
5' Int (0.8 kb)
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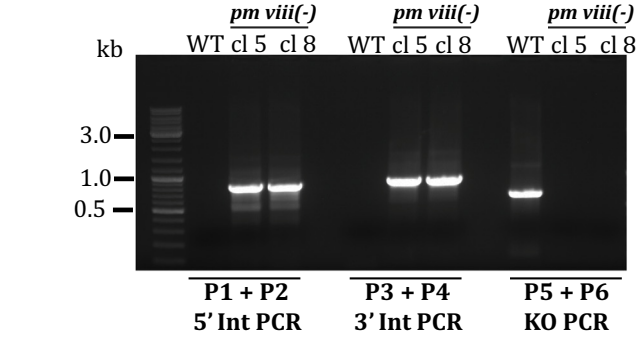
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WT cl 5 cl 8
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P1 + P2
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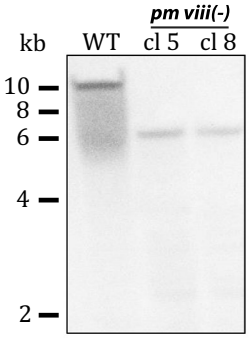
P3 + P4
3' Int PCR
P5 + P6
KO PCR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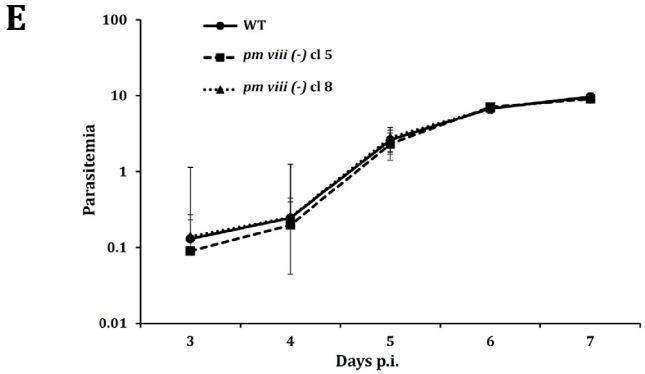
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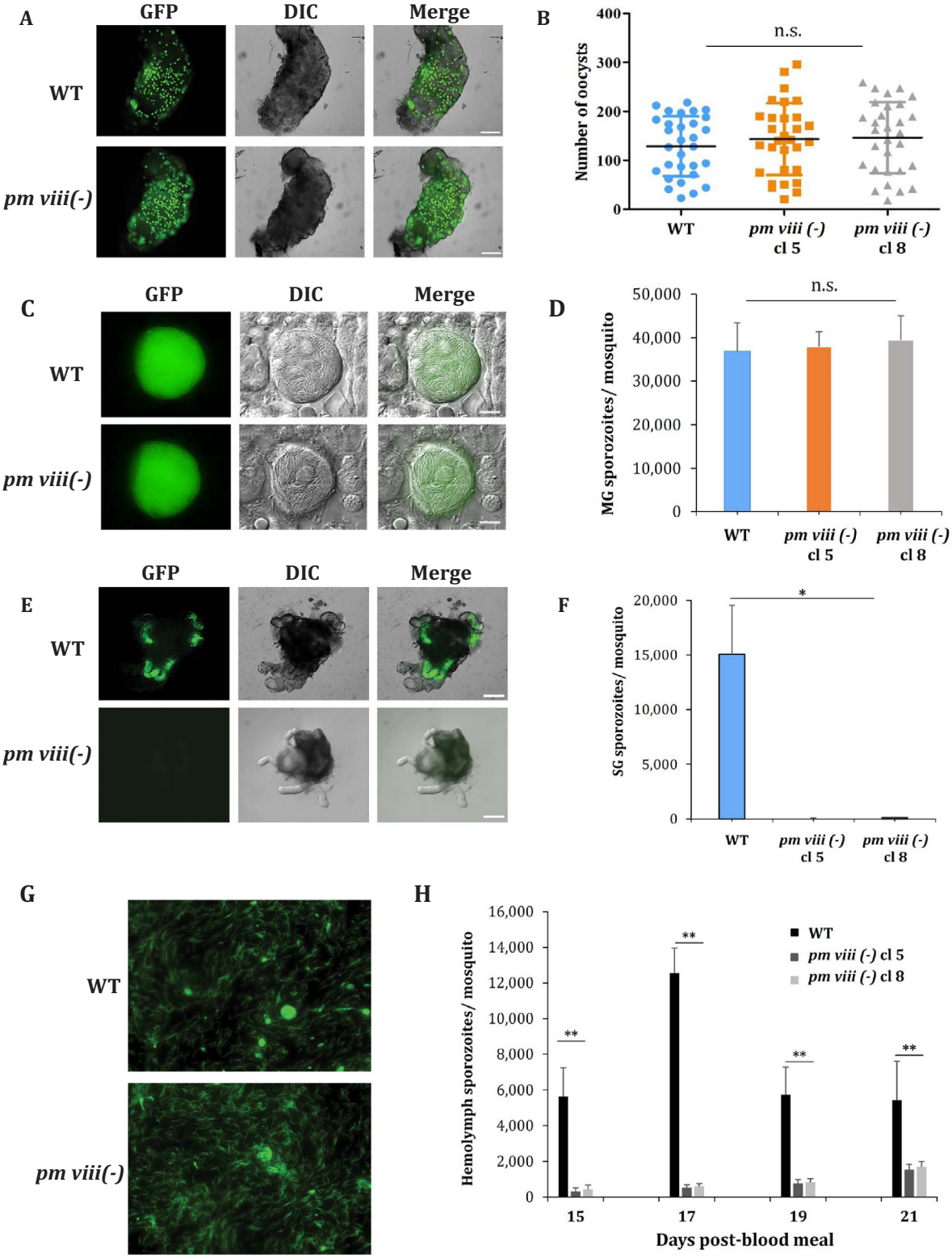

Fig. 2. Phenotypic characterization of *Plasmodium berghei* plasmepsin VIII gene-deficient (*pbpm viii* (-)) parasites. Female *Anopheles stephensi* mosquitoes were fed on anesthetized Swiss Albino mice carrying either wild type (WT) or *pbpm viii (-)* mature gametocytes. Post blood meal, mosquitoes were dissected at certain time points to analyse parasite development. (A) Live microscopy images of mosquito midguts infected with wild type (WT) or *pbpm viii (-)* oocysts at day 10 post feed. Scale bars = 200 μm. (B) WT and *pbpm viii (-)* parasite clones (cl) 5 and 8 produce similar numbers of oocysts. Oocysts from 15 mosquitoes were observed per mosquito cycle; shown are the data from two cycles at day 10 post feed. n.s., not significant. (C) Live microscopy images of sporulating oocysts of WT and *pbpm viii (-)* at day 14 post feed. Scale bars = 20 μm. (D) WT and *pbpm viii (-)* parasites produced similar numbers of midgut-derived (MG) sporozoites at day 14 post feed. Shown are the mean ± S.E.M. of three independent experiments (n = 20 mosquitoes/experiment). (E) Dissected salivary glands at day 18 post feed. Scale bars = 200 μm. (F) In contrast to WT, no *pbpm viii (-)* sporozoites could be detected in salivary glands (SG) of infected mosquitoes at day 18 post feed. Shown are the mean ± S.E.M. of three independent experiments (n = 20 mosquitoes/experiment, $^{*}P = 0.029$). (G) Fluorescence microscopy images of WT and *pbpm viii (-)* oocyst-derived sporozoites on day 20 post feed. (H) Quantification of hemolymph sporozoites in mosquitoes infected with WT and *pbpm viii (-)* parasites. Shown are the mean ± S.D. from one experiment (n = 15 mosquitoes/experiment, $^{**}P < 0.0001$). This experiment was performed five times with similar results.

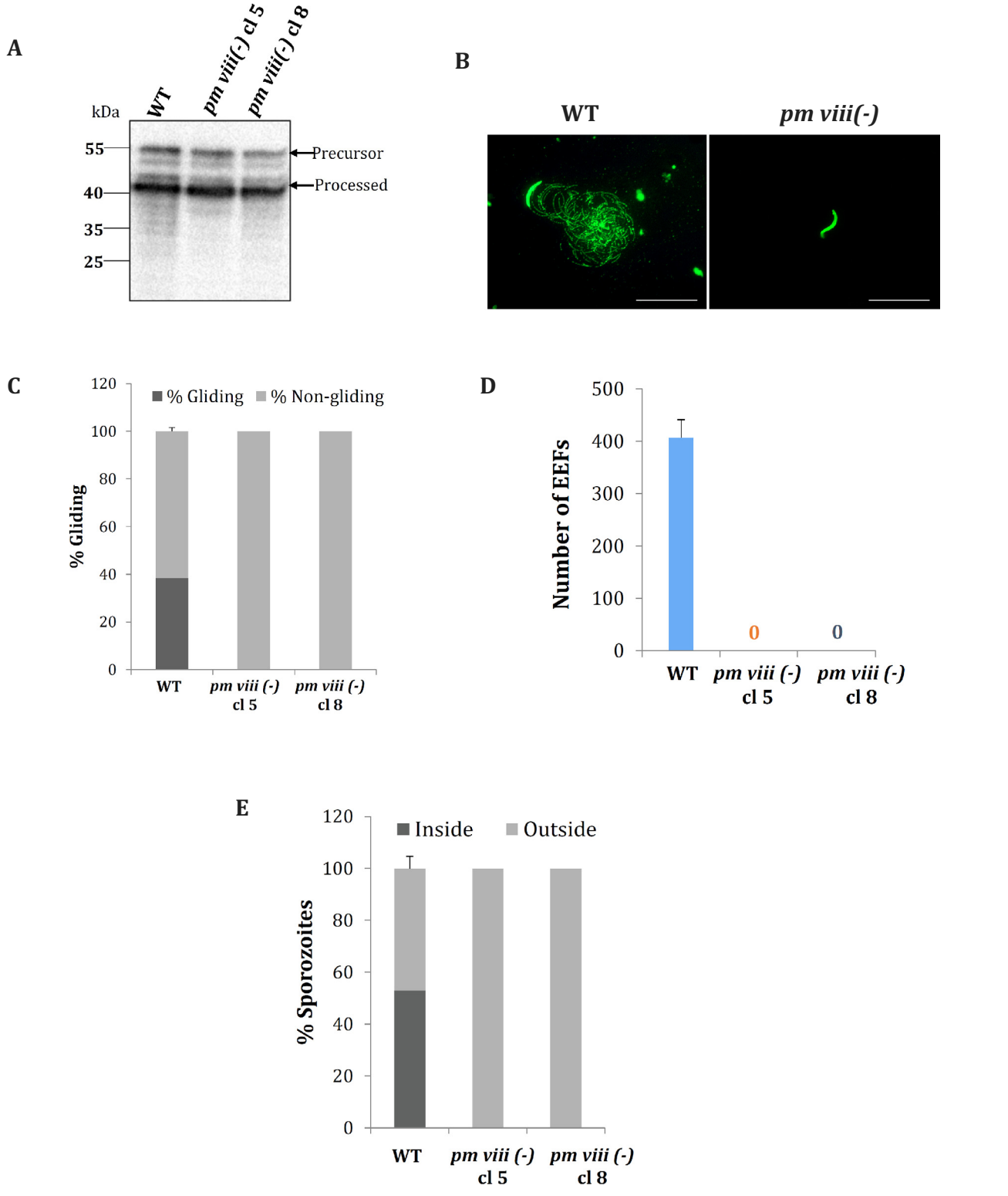

Fig. 3. *Plasmodium berghei* plasmepsin VIII gene-deficient (*pbpm viii (-)*)sporozoites from hemolymph are severely impaired in gliding motility and are non-infective in vitro and in vivo. (A) Western blot analysis of circumsporozoite protein (CSP) processing in wild type (WT) and *pbpm viii (-)* midgut sporozoites (from clones (cl) 5 and 8), showing no difference in precursor and proteolytically processed forms. (B) Gliding motility was abolished in *pbpm viii (-)* sporozoites from hemolymph. Using immunofluorescent staining with an anti-CSP antibody (Stewart and Vanderberg, 1988), WT sporozoites displayed the typical continuous and circular gliding motility pattern while *pbpm viii (-)* sporozoites were non-motile. Scale bars = 25 μm. (C) Quantification of hemolymph-derived sporzoite gliding motility. A total of 252, 192 and 266 immunofluorescent-stained sporozoites from WT, *pbpm viii (-)* cl 5 and cl 8 were evaluated, respectively. (D) Quantitation of in vitro exoerythrocytic form (EEF) development of WT, *pbpm viii (-)* cl 5 and cl 8, 40 h p.i. Infected human liver HepG2 cells were fixed and EEFs were counted. (E) Inside/outside assay to assess the invasion capacity of hemolymph-derived sporozoites. Hemolymph-derived sporozoites were added to HepG2 cells and incubated for 1 h, fixed and stained with anti-CSP antibody. Fifty-three percent of WT hemolymph-derived sporozoites were found to be intracellular, whereas all of the visualised *pbpm viii (-)* sporozoites remained extracellular. Experiments C, D and E were performed three times with similar results.

Table 1
Infectivity by natural transmission or with haemolymph *Plasmodium berghei* sporozoites injected into C57BL/6 mice. Sporozoites, inoculated by mosquito bite or injected i.v. (5×10^3), as either wild type (WT) parasites or plasmepsin VIII gene-deficient (*pm viii (-)*) parasite clones (cl) 5 and 8, were monitored for the development of blood stage infection. The pre-patent period was determined by daily microscopic observation of Giemsa-stained blood smears. The pre-patent period was the number of days until the detection of blood stage infection.

Experiments	Parasites	Route	Sporozoites	Mice positive/mice inoculated	Pre-patent period (Day)
1	WT	Mosquito bite	From 10 mosquitoes	7/7	4
	pm viii (-) cl 5	Mosquito bite	From 10 mosquitoes	0/5	NA
	pm viii (-) cl 8	Mosquito bite	From 10 mosquitoes	0/9	NA
2	WT	Intravenous[a]	5000	3/3	3
	pm viii (-) cl 5	Intravenous[a]	5000	0/3	NA
	pm viii (-) cl 8	Intravenous[a]	5000	0/4	NA

NA, Not applicable.
[a] Hemolymph sporozoites were inoculated.

egress from an oocyst is a proteolytic process that involves the processing of circumsporozoite protein (CSP) present on the inner surface of the oocyst capsule (Wang et al., 2005). To determine whether *Pb*PM VIII plays a role in CSP cleavage during the sporozoite egress, we next analysed the processing of CSP on oocyst-derived sporozoites by immunoblotting. Surprisingly, we observed no difference in CSP processing between WT and *pbpm viii (-)* oocyst- derived sporozoites (Fig. 3A), suggesting that deletion of *Pb*PM VIII did not affect CSP processing in oocyst-derived sporozoites. These findings prompted us to gain a deeper insight into the phenotype, and we examined the intraoocyst motility of sporozoites as reported for *ecp1(-)* parasites (Aly and Matuschewski, 2005) and observed no intraoocyst motility in *pbpm viii (-)* parasites (data not shown). Further, we assessed the gliding motility of the *pbpm viii (-)* hemolymph sporozoites. For this, hemolymph sporozoites were allowed to glide on glass slides and the shedding of CSP trails was revealed by immunofluorescence using an anti-CSP antibody (Stewart and Vanderberg, 1988). As expected, we observed circular trails of CSP in a substantial fraction (38%) of WT sporozoites but failed to detect CSP trails in any of the *pbpm viii (-)* sporozoites (Fig. 3B and C). To further confirm this finding, we performed live microscopy on sporozoites. We observed that sporozoites from WT hemolymph displayed different forms of motility such as gliding, attached waving, flexing and bending (Supplementary Movie S1), while sporozoites from *pbpm viii (-)* hemolymph were never observed gliding or motile in any form (Supplementary Movies S2, S3). Together, these results show that the *Pb*PM VIII plays a vital role in gliding motility and is thus essential for sporozoite egress from oocysts and subsequent invasion of salivary glands.

To test whether the *pbpm viii (-)* sporozoites produce exoerythrocytic forms (EEFs), we added sporozoites from *pbpm viii (-)* hemolymph to cultures of human liver hepatocellular carcinoma (HepG2) cells. After 40 h cells were fixed and EEFs were counted. We consistently detected WT EEFs in HepG2 cells but failed to find *pbpm viii (-)* EEFs (Fig. 3D). To further corroborate this finding, first we tested natural transmission to mice by mosquito bites despite the absence of sporozoites in salivary glands of *pbpm viii (-)* infected mosquitoes. None of the mice bitten by mosquitoes carrying *pbpm viii (-)* parasites became positive for blood stage infection. Second, we injected 5×10^3 sporozoites from *pbpm viii (-)* hemolymph i.v into highly susceptible C57BL/6 mice and in good agreement with the in vitro EEF development assay, they did not develop a blood stage infection (Table 1), while infected WT mice became patent on day 3.

Finally, we investigated whether the defect in gliding motility compromises the ability of *pbpm viii (-)* sporozoites to invade hepatocytes. To determine the invasion capacity, sporozoites from hemolymph were incubated with monolayers of HepG2 cells for 1 h. Sporozoites inside and outside the HepG2 cells were quantified using a two color immunofluorescence assay by staining before and after permeabilization (Renia et al., 1988). We found 53% of WT sporozoites invaded the HepG2 cells, but none of the *pbpm viii (-)* sporozoites were found inside (Fig. 3E), indicating that *Pb*PM VIII plays an exclusive role in gliding motility and invasion. In summary, our results demonstrate that *Pb*PM VIII is critical to sporozoite motility and efficient malaria transmission.

As obligate intracellular parasite, *Plasmodium* progress through a highly complex and multistage life cycle where extracellular forms of the parasite invade, grow and replicate within host cells and eventually egress to ensure successful transmission. The precise coordination of these molecular events requires specialized proteins in different host micro environments. *Plasmodium* and other apicomplexan parasites share a broadly similar mechanism of host cell invasion and egress (Bargieri et al., 2014). The critical components of this conserved machinery are cell surface molecules, transmembrane proteins, and an actin-myosin motor complex. Several distinct classes of proteases have been implicated in these vital processes (Dowse et al., 2008). In the present study, we characterized the function of *Pb*PM VIII, a conserved aspartyl protease, specifically expressed in MS parasites. Targeted gene deletion revealed that it is dispensable in the development of asexual blood stages but plays an essential role in sporozoite transmission to the mammalian host. *pbpm viii* loss-of-function mutants displayed a dramatic defect in the egress of sporozoites from oocysts and abolished gliding motility of sporozoites in hemolymph and hence were unable to reach salivary glands of the mosquitoes and transmit malaria to susceptible mice. The phenotype reported in this study closely resembles the mutants of sporozoite invasion-associated protein 1 (SIAP-1) (Engelmann et al., 2009), which was shown to be partially affect sporozoite egress from oocysts and to abolish gliding motility and invasion of salivary glands. *pbpm viii (-)* sporozoites also resemble the mutants of inhibitor of cysteine proteases (ICP), that exhibit normal egress from oocysts but fail to glide and invade salivary glands and hepatocytes (Boysen and Matuschewski, 2013). However, in the absence of subcellular localization data and its proteolytic substrates, it is difficult to ascertain whether *Pb*PM VIII directly modulates gliding motility by participating in the processing of cell surface molecules other than CSP or is involved in upstream signaling events that govern the egress and motility of oocyst sporozoites. Further investigation is needed to determine the precise role of *Pb*PM VIII in sporozoite egress from oocysts and migration to targeted tissues. Uncovering the in vivo functions of proteases involved in sporozoite egress could help in identification of new targets for the development of novel antimalarial drugs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2016.11.009.

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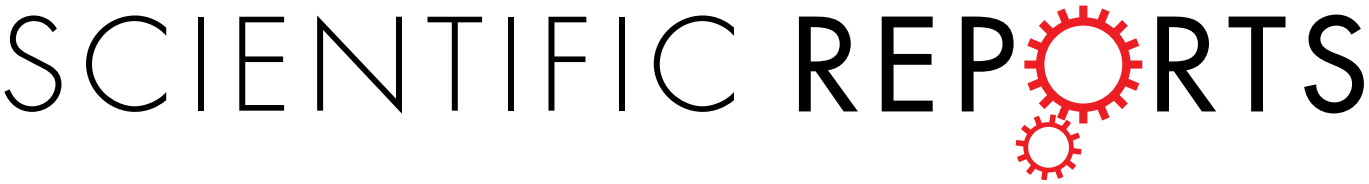

OPEN

A Novel and Conserved *Plasmodium* Sporozoite Membrane Protein SPELD is Required for Maturation of Exo-erythrocytic Forms

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Plasmodium **sporozoites are the infective forms of malaria parasite to vertebrate host and undergo dramatic changes in their transcriptional repertoire during maturation in mosquito salivary glands. We report here the role of a novel and conserved *Plasmodium berghei* protein encoded by *PBANKA_091090* in maturation of Exo-erythrocytic Forms (EEFs) and designate it as Sporozoite surface Protein Essential for Liver stage Development (PbSPELD). *PBANKA_091090* was previously annotated as *PB402615.00.0* and its transcript was recovered at maximal frequency in the Serial Analysis of the Gene Expression (SAGE) of *Plasmodium berghei* salivary gland sporozoites. An orthologue of this transcript was independently identified in *Plasmodium vivax* sporozoite microarrays and was designated as Sporozoite Conserved Orthologous Transcript-2 (*scot-2*). Functional characterization through reverse genetics revealed that PbSPELD is essential for *Plasmodium* liver stage maturation. mCherry transgenic of PbSPELD localized the protein to plasma membrane of sporozoites and early EEFs. Global microarray analysis of *pbspeld* ko revealed EEF attenuation being associated with down regulation of genes central to general transcription, cell cycle, proteosome and cadherin signaling. *pbspeld* mutant EEFs induced pre-erythrocytic immunity with 50% protective efficacy. Our studies have implications for attenuating the human *Plasmodium* liver stages by targeting SPELD locus.**

Malaria is an infectious disease caused by a protozoan parasite that belongs to the genus *Plasmodium*. In 2013 alone, the reported mortality associated with malaria was about 854,586 cases[1]. Malaria is transmitted to humans by the bite of a female *Anopheles* mosquito that injects sporozoites into the skin of the host[2]. The sporozoites make their way to the liver where they transform into EEFs or liver stages. Following asexual exo-erythrocytic schizogony, the hepatic merozoites are released into the blood stream to initiate an erythrocytic cycle. During this phase, a proportion of parasites undergo differentiation to sexual forms called as gametocytes. When a female *Anopheles* mosquito ingests these gametocytes during the process of obtaining a blood meal, the male and female gametes fuse and result in the formation of a zygote. The zygote transforms into a motile ookinete that breaches the mosquito midgut epithelium and settles on hemocoel side of gut. The end product of sexual reproduction are the oocysts that undergo sporulation and upon rupture, release sporozoites into hemocoel[3]. The sporozoites migrate to the salivary glands and wait for transmission to humans when the mosquito probes for a blood meal.

High throughput methods of gene expression analysis have offered an insight in understanding the malaria parasite biology and allowed the appreciation of stage specifically regulated gene expression in modulating the infectivity or virulence of parasites[4–7]. Significant changes occur in the transcriptional repertoire of salivary gland sporozoites rendering them highly infective for hepatocytes[8]. The first comprehensive transcriptomic analysis of sporozoites[9] opened the possibility of understanding the regulation of *Plasmodium* gene expression in mosquito stages that further led to investigating the differential gene expression between salivary gland sporozoite stages

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WILEY

RESEARCH ARTICLE

Modulation of host cell SUMOylation facilitates efficient development of *Plasmodium berghei* and *Toxoplasma gondii*

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Summary

SUMOylation is a reversible post translational modification of proteins that regulates protein stabilization, nucleocytoplasmic transport, and protein–protein interactions. Several viruses and bacteria modulate host SUMOylation machinery for efficient infection. *Plasmodium* sporozoites are infective forms of malaria parasite that invade mammalian hepatocytes and transforms into exoerythrocytic forms (EEFs). Here, we show that during EEF development, the distribution of SUMOylated proteins in host cell nuclei was significantly reduced and expression of the SUMOylation enzymes was downregulated. *Plasmodium* EEFs destabilized the host cytoplasmic protein SMAD4 by inhibiting its SUMOylation. SUMO1 overexpression was detrimental to EEF growth, and insufficiency of the only conjugating enzyme Ubc9/E2 promoted EEF growth. The expression of genes involved in suppression of host cell defense pathways during infection was reversed during SUMO1 overexpression, as revealed by transcriptomic analysis. The inhibition of host cell SUMOylation was also observed during *Toxoplasma* infection. We provide a hitherto unknown mechanism of regulating host gene expression by Apicomplexan parasites through altering host SUMOylation.

KEYWORDS

conjugation enzyme E2, host SUMOylation, *Plasmodium* EEFs, SMAD4, SUMO1, *Toxoplasma*

1 | INTRODUCTION

Malaria infection is initiated when sporozoites are introduced in the mammalian host by the bite of a female *Anopheles* mosquito. The sporozoites selectively invade hepatocytes and develop into exoerythrocytic forms (EEFs) inside a parasitophorous vacuole (Prudencio, Rodriguez, & Mota, 2006). The intrahepatic development of *Plasmodium* is an obligatory step before the onset of disease. Very little is known about the parasite liver stage and their interactions with host cell. Apicomplexan parasites subvert their host cell functions to access essential nutrients and escape from host defense mechanisms. The host processes that are targeted include modulation of gene expression, protein synthesis, membrane trafficking, antigen presentation, and apoptosis (Plattner & Soldati-Favre, 2008). In addition to the orchestration of the above host cellular mechanisms, parasites have evolved to manipulate specialized host cellular processes—the posttranslational modifications (PTMs). The first study providing evidence for a pathogen-derived toxin in catalyzing PTM of host protein was reported 5 decades ago (Collier & Cole, 1969). Since then, significant numbers of host PTMs induced, mediated, or counteracted by different pathogens have been reported (Randow & Lehner, 2009; Ribet & Cossart, 2010).

In addition to ubiquitin, a family of substrates called ubiquitin-like proteins (UBLs) is covalently linked to the target protein and influences diverse biological processes. One such member of UBLs is small ubiquitin-like modifier (SUMO), a 12-kDa polypeptide found ubiquitously in the eukaryotic kingdom. SUMOylation, the covalent linkage of SUMO moiety on Lys residue of the target protein, is mediated by the sequential action of three enzymes: E1-SAE1/SAE2 heterodimer (activating enzyme), E2/Ubc9 (conjugating enzyme), and E3 enzymes (ligating enzymes). SUMOylation is essential for many cellular functions such as transcription regulation, intracellular transport, maintenance of genome integrity, protein stability, stress responses, and many other biological functions (Zhao, 2007).

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Genetic ablation of plasmoDJ1, a multi-activity enzyme, attenuates parasite virulence and reduces oocyst production

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Malaria parasites must respond to stresses and environmental signals to perpetuate efficiently during their multistage development in diverse environments. To gain insights into the parasite's stress response mechanisms, we investigated a conserved *Plasmodium* protein, which we have named plasmoDJ1 on the basis of the presence of a putative cysteine protease motif of the DJ-1/PfpI superfamily, for its activities, potential to respond to stresses and role in parasite development. PlasmoDJ1 is expressed in all intraerythrocytic stages and ookinetes. Its expression was increased 7–9-fold upon heat shock and oxidative stress due to H_2O_2 and artemisinin; its expression in a stress-sensitive *Escherichia coli* mutant conferred tolerance against oxidative stress, indicating that plasmoDJ1 has the potential to sense and/or protect from stresses. Recombinant plasmoDJ1 efficiently neutralized H_2O_2, facilitated renaturation of denatured citrate synthase and showed protease activity, indicating that plasmoDJ1 is a multi-activity protein. Mutation of the catalytic cysteine residue, but not other residues, reduced H_2O_2-neutralization activity by ~90 % and significantly decreased chaperone and protease activities, indicating that these activities are intrinsic to plasmoDJ1. The plasmoDJ1 gene knockout in *Plasmodium berghei* ANKA attenuated virulence and reduced oocyst production, suggesting a major role for plasmoDJ1 in parasite development, which probably depends on its multiple activities.

Key words: chaperone, DJ-1/PfpI, malaria, peroxiredoxin, *Plasmodium*, reactive oxygen species (ROS).

INTRODUCTION

Malaria, a disease caused by protozoan parasites of the genus *Plasmodium* and transmitted by female anopheline mosquitoes, is one of the most important infectious diseases of humans. The 2012 WHO (World Health Organization) report estimated 219 million cases and 0.66 million deaths from malaria (http://www.who.int/malaria/publications/world_malaria_report_2012/report/en/index.html), and the disease is becoming uncontrollable because of the parasite's resistance to most of the existing antimalarials [1]. Hence new medicines are needed to control the disease. Malaria parasite is challenged with oxidative bursts and change in temperature and pH during its multistage development in different environments [2–4], and it must have competent systems to sense physiologically relevant stress signals and neutralize harmful stresses. Unusual sensitivity of malaria parasites to pro-oxidants, including potent antimalarials such as artemisinins and primaquine, suggests that parasite antioxidant defence system is an attractive drug target [5–14]. Protective effects of G6PD (glucose-6-phosphate dehydrogenase) deficiency and certain haemoglobinopathies, which have been proposed to be due to increased oxidative stress in parasite-infected erythrocytes [15], underscore drug target potential of the parasite antioxidant defence system. Thus investigation of parasite proteins with key roles in protective and/or regulatory stress responses is required, which may lead to targets of therapeutic importance. The *Plasmodium* antioxidant system consists of two SODs (superoxide dismutases), thioredoxin-dependent peroxidase or peroxiredoxin, and glutathione and thioredoxin redox systems [16–20]. Additionally, malaria parasites import the host peroxiredoxin-2 from infected erythrocytes [21], which contributes to almost half of the total peroxidase activity of the parasite. Moreover, all malaria parasites encode a conserved protein belonging to the DJ-1/PfpI superfamily, whose members have been shown to protect from a variety of stresses, including oxidative, thermal and pH stress [22,23]. However, the *Plasmodium* DJ-1/PfpI superfamily protein has not yet been investigated.

The Parkinson's disease protein PARK7 or human DJ-1 (hDJ1), the *Escherichia coli* proteins YajL and Yhbo, the intracellular protease I of *Pyrococcus furiosus* (PfpI) and *Pyrococcus horikoshii* (PhpI), heat-shock protein 31 of *E. coli* (EcHsp31), protein Ydr533c from *Saccharomyces cerevisiae* (ScYdr533c) and catalases [22,23] are some of the most studied DJ-1/PfpI superfamily proteins. All DJ-1/PfpI members share a region designated GATase1-DJ-1 (type 1 glutamine amidotransferase-DJ-1) on the NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov) or DJ-1_PfpI on the Pfam database (http://pfam.sanger.ac.uk). This region contains a typical nucleophile elbow with a positionally conserved cysteine residue in structures of several DJ-1/PfpI proteins [24–31]. Hence a large number of these proteins are predicted to be proteases and have been put in the peptidase family C56 (http://merops.sanger.ac.uk). However, protease activity is reported for PfpI, PhpI, hDJ1 and EcHsp31 only [24,32–35]. hDJ1, the most studied member of this superfamily, was first

Abbreviations: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); EcHsp31, heat-shock protein 31 of *Escherichia coli*; gDNA, genomic DNA; hDHFR, human dihydrofolate reductase; hDJ1, human DJ-1; HRP, horseradish peroxidase; IFA, immunofluorescence assay; Ni-NTA, Ni^{2+}-nitrilotriacetate; PbDJ1, *Plasmodium berghei* plasmoDJ1; PfDJ1, plasmoDJ1 of *Plasmodium falciparum*; PfpI, intracellular protease I of *Pyrococcus furiosus*; PhpI, intracellular protease I of *Pyrococcus horikoshii*; PvAc, *Plasmodium vivax* actin; PyαTb, *Plasmodium yoelii* α-tubulin; PyCRT, *Plasmodium yoelii* chloroquine-resistance transporter; ROS, reactive oxygen species; ScYdr533c, protein Ydr533c from *Saccharomyces cerevisiae*; SOD, superoxide dismutase; TgDHFR-TS, *Toxoplasma gondii* dihydrofolate reductase–thymidylate synthase; TNB, 5-thio-2-nitrobenzoic acid.

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Dominant negative mutant of *Plasmodium* Rad51 causes reduced parasite burden in host by abrogating DNA double-strand break repair

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Summary

Malaria parasites survive through repairing a plethora of DNA double-stranded breaks (DSBs) experienced during their asexual growth. In *Plasmodium* Rad51 mediated homologous recombination (HR) mechanism and homology-independent alternative end-joining mechanism have been identified. Here we address whether loss of HR activity can be compensated by other DSB repair mechanisms. Creating a transgenic *Plasmodium* line defective in HR function, we demonstrate that HR is the most important DSB repair pathway in malarial parasite. Using mouse malaria model we have characterized the dominant negative effect of PfRad51^{K143R} mutant on *Plasmodium* DSB repair and host–parasite interaction. Our work illustrates that *Plasmodium berghei* harbouring the mutant protein (PfRad51^{K143R}) failed to repair DSBs as evidenced by hypersensitivity to DNA-damaging agent. Mice infected with mutant parasites lived significantly longer with markedly reduced parasite burden. To better understand the effect of mutant PfRad51^{K143R} on HR, we used yeast as a surrogate model and established that the presence of PfRad51^{K143R} completely inhibited DNA repair, gene conversion and gene targeting. Biochemical experiment confirmed that very low level of mutant protein was sufficient for complete disruption of wild-type PfRad51 activity. Hence our work provides evidence that HR pathway of *Plasmodium* could be efficiently targeted to curb malaria.

Accepted 19 August, 2014. *For correspondence. E-mail mkbsl@uohyd.ernet.in; Tel. (+91) 40 23134528; Fax (+91) 40 23010120.

Introduction

Malaria is one of the biggest killers in today's world, especially in developing countries. Of the five species of *Plasmodium* that cause human malaria, *P. falciparum*, causative agent of malignant malaria, is the deadliest one resulting into several million deaths per year. Due to the rapid emergence of parasites resistant to all the available drugs (Wongsrichanalai and Sibley, 2013) and due to the lack of an effective vaccine, it is of utmost importance to find new drug targets.

Targeting DNA double-strand break (DSB) repair pathways could be an excellent choice because a single unrepaired DSB leads to death of a unicellular organism (Frankenberg-Schwager and Frankenberg, 1990). The parasite navigates through its life while facing extensive DNA damage from various sources, such as free haem, innate immune system of the host, errors during DNA replication etc. Parasites must repair such DNA damages especially DNA double-strand breaks in order to survive. Eukaryotes use non-homologous end joining (NHEJ) and homologous recombination (HR) to repair DNA DSBs. In *P. falciparum* homologous recombination pathway has been identified and characterized (Bhattacharyya and Kumar, 2003) and it seems to be the predominant DSB repair mechanism (Kirkman *et al.*, 2014). Canonical NHEJ is apparently absent in this parasite and alternative end joining pathway is utilized at a very low frequency to repair DSB (Kirkman *et al.*, 2014). Thus, targeting HR mechanism, the main DSB repair mechanism of *Plasmodium* appears to be a very lucrative option in curbing the disease.

Rad51 is the RecA recombinase in eukaryotes and it plays a central role during HR mediated DSB repair which occurs due to exposure to some genotoxins. Additionally, Rad51 is essential for restarting a replication fork that has been stalled upon encountering DNA damage or reduced nucleotide pools. During meiosis, Rad51 is involved in pairing between the two parental homologous chromosomes. To promote all these activities, Rad51 forms a

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