"Role of *Plasmodium* PhIL-1 Interacting Protein (PhIP) in malaria transmission"

Thesis submitted to University of Hyderabad for the award of
Doctor of Philosophy
In Department of Animal Biology

By
Sandeep Dey
14LAPH04

Department of Animal Biology
School of Life Sciences
University of Hyderabad
Hyderabad - 500 046
India

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University of Hyderabad
(A Central university by an act of Parliament)
Department of Animal Biology
School of Life Sciences
P.O. Central University, Gachibowli, Hyderabad-500046

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2	AS 802	Research Ethics Data Analysis and Biostatistics	3	PASS
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KOTA ARUN KUMAR
Professor
Department of Animal Biology
School of Life Sciences
University of Hyderabad
P.O. Central University, Gachibowli,
Hyderabad-500 046. Telangana. INDIA

Supervisor

16/1/23

Head of the Department
HEAD / HEAD
जंतु जैविकी विभाग
Department of Animal Biology

16/1/23

DEAN
Dean of the School
School of Life Sciences
University of Hyderabad
Hyderabad-500 046.

UNIVERSITY OF HYDERABAD
Central University (P.O.), Gachibowli, Hyderabad-500046

DECLARATION

I hereby declare that the results of the study incorporated in the thesis entitled **"Role of *Plasmodium* PhIL-1 Interacting Protein (PhIP) in malaria transmission"** has been carried out by me under the supervision of **Prof. Kota Arun Kumar** and this work has not been submitted for any degree or diploma of any other university earlier.

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Date

Sandeep Dey

Sandeep Dey
(Ph.D. Research Scholar)

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"The Lord is my Shepherd; I shall not want: Psalm 23.1"

Sandeep…

Dedicated to
my
Family & Friends

Table of Contents

Abbreviations

ACT	Artemisinin combination therapy
AMA	Apical membrane antigen
Bp	Base pairs
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CDK	Cyclin dependent kinase
cDNA	Complementary DNA
CDPK	Calcium dependent protein kinase
CELTOS	Cell traversal protein for ookinetes and sporozoites
CSP	Circumsporozoite Protein
DAPI	4', 6' diamidino-2 phenyl indole
DIC	Differential interference contrast
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
DNA	Deoxy ribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DOZI	Development of zygote inhibited
EBL	Erythrocyte binding like
ECP	Egress cysteine protease
EDTA	Ethylene diamine tetra acetic acid
EEF	Exo-erythrocytic form
EMP	Erythrocyte Membrane Protein
ETRAM	Early transcribed membrane protein
EXP	Exported protein
FBS	Fetal bovine serum
FLP	Flippase
FP	Forward primer
FRT	Flippase recognition target site
GAP	Genetically attenuated parasite
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
HBV	Hepatitis B virus
hDHFR	Human Dihydrofolate reductase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
HSPG	Heparin sulfate proteoglycan
i.p.	intraperitoneal
i.v.	intravenous
IFN	Interferon
IMC	Inner membrane complex
iRBC	Infected red blood cell
ITN	Insecticide treated net
Kb	Kilobase pairs
KO	Knockout
LB broth	Luria-Bertani broth
LISP	Liver specific protein
MAP/MAPK	Mitogen activated protein kinase
mRNA	Messenger RNA
MSP	Merozoite surface protein
MTIP	Myosin tail interacting protein
Ng	nanogram
NIMA	Never in mitosis Aspergillus
^{0}C	Degrees Celsius
OD	Optical density
ORF	Open reading frame
P. falciparum	*Plasmodium falciparum*
P. knowlesi	*Plasmodium knowlesi*
P. malariae	*Plasmodium malariae*
P. ovale	*Plasmodium ovale*
P. vivax	*Plasmodium vivax*
Pb or P. berghei	*Plasmodium berghei*
PBS	Phosphate buffer saline

PEXEL	*Plasmodium* export element
PK	Protein kinase
PKG	cGMP dependent protein kinase/ protein kinase G
PL	Phospholipase
PTEX	*Plasmodium* translocon of exported proteins
PUF	Pumilio and fem3 transcription binding factor
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuolar membrane
RBC	Red blood cell
RH	Relative humidity
RNA	Ribonucleic acid
RON	Rhoptry neck protein
RP	Reverse primer
RPM	Revolutions per minute
RPMI	Roswel Park Memorial Institute medium
SAP	Sporozoite asparagine rich protein
SAP	Sporozoite asparagine rich protein
SBP	Skeletal binding protein
SERA	Serine repeat antigen
SIAP	Sporozoite invasion associated protein
SIMP	Structural Integrity Maintenance Protein
SPECT	Sporozoite protein essential for cell traversal
SRPK	Serine arginine rich protein kinase
SSH	Suppression subtractive hybridization
SSR	Site specific recombination
SUB	Subtilisin like protease
TAE	Tris acetate EDTA
TBS	Tris buffer saline
TBV	Transmission blocking vaccines
TE	Tris EDTA
*Tg*DHFR	*Toxoplasma gondii* dihydrofolate reductase
TRAP	Thrombospondin related anonymous protein
TRSP	Thrombospondin related sporozoite protein
TVN	Tubulovesicular network

UIS	Up-regulated in infected salivary glands
UOS	Upregulated in oocyst sporozoites
UTR	Untranslated region
UV	Ultraviolet
v/v	Volume per volume
VTS	Vacuolar translocation signal
w/v	Weight per volume
WHO	World health organization
WT	Wild type
XA	Xanthurinic acid
µg	Microgram(s)
µL	Microliter(s)
µm	Micrometer(s)
µM	Micromolar(s)

Chapter I:

Review of Literature

1.1 Malaria through History

In the annals of history, malaria always holds a special place. Victims of this deadly disease include neolithic inhabitants, ancient residents from China and Greece, the kings and the paupers. The malaria epidemics go back to the early stages of civilization (1). Considering the current situation, the residents of Sub-Saharan Africa, the basin of Amazon and regions having tropical weather are the principal victims today. Forty percent of the population dwells in these affected areas where this lethal disease is highly transmissible. Numerous ancient artifacts and works of literature have established malaria's long supremacy. Mesopotamian clay tablets from 2000BC and Egyptian papyri from 1570BC provide evidence regarding deadly malarial fever. Even a few vedic period of Indian writing, *The oldest Chinese medical canon, Nei Ching*, has shown testimonies of malaria stating tertian and quatrain fevers with an enlarged spleen, headaches, chill, and fevers. The early Greek poet like Homer (circa 850BC), Plato (428-347BC), Sophocles (496-406BC), Aristophanes (445-385BC), and Aristotle (384-322BC) have mentioned about malaria (2). The word malaria has been extracted from an Italian word "mal'aria" which means spoiled air or bad air. For the first time in the year 1880, Charles Louis Alphonse Laveran, a French army doctor, observed d bodies nearly translucent bodies having crescent-shape, except for a tiny point of a pigment known as hemozoin. Camillo Golgi showed asexual cycle of the parasite, getting ruptured and released from blood schizonts on third and fourth day of fever caused by *P. vivax* and *P. malariae*. Both of them were awarded with Nobel prize because of the discovery. Sir Ronald Ross dissected few infected *Anopheles* mosquitos and observed larger pigment containing bodies and published his observation in 1897 that confirmed that malaria grows inside mosquitos. The discovery of mosquito stages of malaria led Sir Ross to receive Nobel prize in 1902. Confirmation of developmental stages in human and avian malaria was done by a group of Italian scientists between 1898 and 1900.

1.2 Malaria epidemiology

Malaria, an acute febrile disease, mainly found in poor tropical and subtropical areas of the world, caused because of an obligatory intracellular protozoan parasite from the genus *Plasmodium*. Areas with a high infection transmission rate include

young boys and pregnant women as the most vulnerable group (3). There are five *Plasmodium* parasite species responsible for human malaria: *Plasmodium falciparum (P. falciparum), Plasmodium ovale (P. ovale)*, *Plasmodium knowlesii (P. knowlesii), Plasmodium vivax (P.vivax)* and *Plasmodium malariae (P. malariae)*. Out of these, *P. falciparum* and *P. vivax* claim the most number of lives. Moderate to high transmission countries like Sub-Saharan Africa accounts for the most global malaria burden. Globally, 241 million malaria cases were registered in 2020 post COVID-19, which spiked compared to 2019 because of the pandemic (**Fig. 1**). Children under the age of five, pregnant women, HIV AIDs patients, and people with low immunity are considered to be at high risk of exposure to the disease (4). Half of the world's population is at risk of getting infected, as they live in areas of risk.

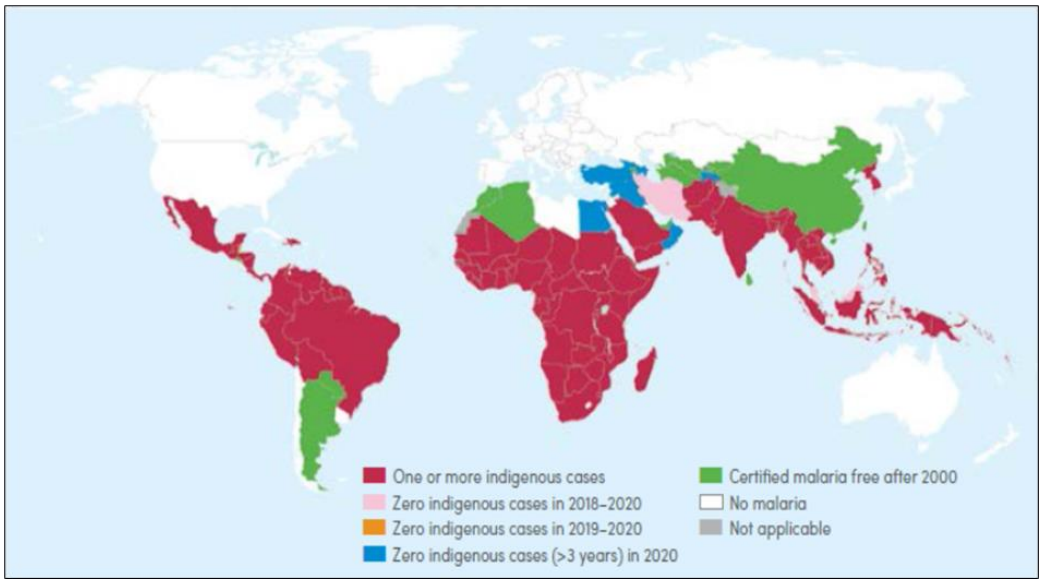


Fig. 1: **Indigenous malaria cases in 2000 and their status by 2020**. Tropical and subtropical countries have shown a higher tendency in malaria transmission. Due to COVID-19 pandemic a spike of cases can be seen in regions of south America, Africa and India (Red). Parts of South America, China and Algeria has been certified as malaria free (Green). Countries with absolutely no malaria cases are indicated in white. Source: World Malaria Report, 2021.

Due to disruptions in health services due to pandemic in 2020, number of deaths also increased, which was registered to be almost 627,000, of which 95% were from the WHO African region.

The frequency of malaria cases has reduced from early 2000 to 2019. Cases between 2000 to 2015 have decreased by 27% compared to only 2% between 2015 to 2019. The number of deaths was 736000 in 2000, also reduced to 409000 in 2019. (WHO world malaria report 2020). Reason behind a fall in malaria cases in the last

decade is the overall intervention strategies. Involvement of world powers, introduction of advanced techniques to control the disease, an augment in funding for malaria control, and immediate procurement of tools for prevention and handling of the disease plays a significant role in quick fall of the malarial burden in areas like southern Africa. Apart from African countries, other countries have replaced the ineffective drug chloroquine with pyrimethamine, sulfadiazine, or artemisinin, resulting in low case occurrence. Similarly, the use of insecticidal bed nets and indoor insecticidal spray has significantly helped control vectors from spreading the disease (7).

1.3 Life cycle of *Plasmodium*

Plasmodium life cycle is quite fascinating and one of the most complicated among organisms. In poor and developing countries like sub-Saharan Africa and South Asian areas, it has wreaked havoc on their public health and economy (5). The intricate and exciting biology of the parasite has always drawn attention of several immunologists, vaccinologists, and parasite biologists to address the challenges and towards possible discovery of an ideal drug or a vaccine (6). The complicated life cycle of the parasite occurs in two hosts, where one is a vertebrate intermediate host and the second one is the invertebrate definitive insect host. Consecutive rounds of asexual replication occur in several stages and tissues in vertebrate host, followed by sexual stages in the *Anopheles* mosquito upon transmission (7). Beginning of life cycle is marked by inoculation and deposition of sporozoites in to the vertebrate host skin by an infected female mosquito, while probing for blood (**Fig. 2**). Sporozoites are motile cells that breach several cellular barriers prior to reaching blood stream. They get selectively arrested in the liver and upon crossing liver sinusoids, invade hepatocytes with by a process of productive invasion (8). Productive invasion involves invagination of hepatocyte plasma membrane during internalisation of the sporozoite, that eventually subsumes resulting in a vacuolar structure called as Parasitophorous vacuolar membrane (PVM). Within this intracellular niche, the spindle shaped sporozoites transforms into a round form called as exo-erythrocytic form.

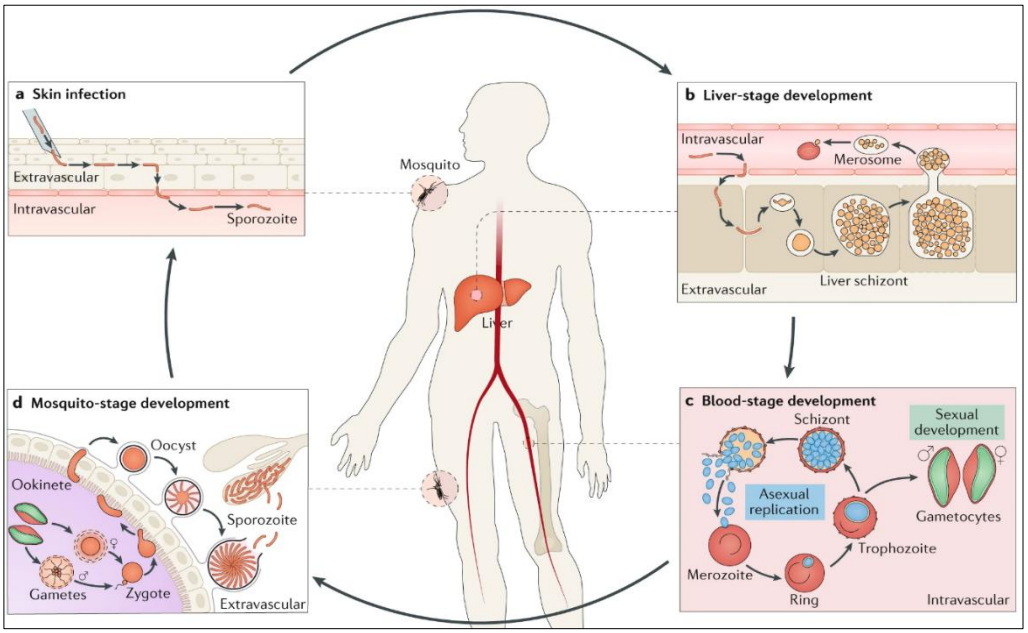


Fig. 2: Life cycle of *Plasmodium* in humans and mosquitoes. (a) Beginning of life cycle starts with injection of sporozoites (orange) into the skin while mosquito taking a blood meal where the infective forms after a short time period in skin migrate and enter in the blood stream. **(b)** Next is liver sinusoids after leaving the blood stream to invade hepatocytes after several traversal activities through cells. Asexual replication undergoes inside parasitophorous vacuole (PV) to produce thousands of merozoites (yellow). Upon releasing from the vacuole merozoites enter blood stream to initiate intra-erythrocytic cycle by infecting Red Blood Cells (red). **(c)** Blood stage is marked with several forms where the parasite transforms from ring to trophozoite and then schizonts. Mature schizonts start another replication cycle which generates several more merozoites to initiate the cycle again. A subset of this population commits towards sexual development producing male and female gametocytes (green). **(d)** A healthy female *Anopheles* mosquito while taking blood meal ingests these gametocytes from an infected human. Both the gametocytes undergo gametogenesis in mosquito midgut followed by fertilization of male and female gametes to form a zygote (green). Zygote slowly transforms into motile ookinete. Ookinetes cross through the midgut epithelium to form an oocyst beneath the mosquito midgut basal lamina. Sporulation in oocyst lead to form thousands of sporozoites which upon bursting the oocyst wall glide through the hemolymph. Sporozoites finally invade the salivary gland and transmit to the next human during the subsequent mosquito bite. Image has been adapted from (8).

As the EEF grows in size, asexual replication is initiated resulting in the formation of first generation hepatic merozoites. Following complete EEF maturation, the merozoites are bundled together in a membrane bound vesicle called merosomes (9), a structure that is derived both from hepatocyte plasma membrane and parasite membrane (10). The merosomes are delivered into the sinusoidal lumen and its rupture within the blood capillaries leads to release of hepatic merozoites into blood. These merozoites invade RBCs where they transform into various developmental stages like rings, trophozoites and schizonts over a time course of 24-72 hours. The schizonts typically contain 32 merozoites with RBC and its rupture initiates multiple iterative cycles of parasite replication. A small proportion of schizonts are committed towards

sexual cycle producing male and female gametocytes. These are terminally mature stages of the parasite that does not undergo any further development, until it is ingested during a blood meal by a female *Anopheles* mosquito.

After ingestion of infected blood by a female *Anopheline* mosquito, gametocytes get activated to produce gametes. The male gametocyte produces 8 microgametes by a process of exflagellation, while the female gametocyte produces only one female macro gamete (11). Fusion of the gametes produces a zygote which transforms into motile ookinete within 18-24hrs. Motile ookinetes traverse through the epithelial layer of midgut and form oocyst which settles in between midgut epithelia and basal lamina, on the hemocoel side. Within oocyst, a third round of asexual replication takes place to yield thousands of sporozoites by a process of sporogony. This occurs over a course of 10-15 days. Rupture of sporulated oocyst results in egress of oocyst sporozoites into the haemocoel that make their way to salivary glands. Their movement is aided by both passive action of hemocoel fluid and by an inherent ability of hemocoel sporozoites to perform gliding motility. After reaching the salivary glands, they reside in this compartment until they are released along with mosquito saliva into a vertebrate host when a mosquito is probing for a blood meal (10,12).

Parasite endures huge loss because of continuous changes in environment during switching of host together with the stringent defense responses triggered in both human and the mosquito host. However the parasites strategically surpass host cellular immune response and asexual replication in three life cycle stages likely accounts for successful continuity of parasite life cycle that compensates for large losses in their number (11).

1.4 Detailed insight into development of parasite in mammalian host.

Parasite development in the mammalian host is divided in two main categories.

(a) The pre-erythrocytic stage **(b)** The erythrocytic stage

1.4.1 The pre-erythrocytic stage

The pre-erythrocytic stages include extracellular sporozoite stage and the liver stages that occurs within the hepatocyte. The sporozoites are injected into the skin during

a mosquito bite and few sporozoites that fail to leave the injection site, can transform into skin stages, that manifest schizogony. However, these forms are non-infective and do not lead to initiation of blood stage infection (12). It is currently speculative if these skin stages are able to prime the immune system to generate protective immune responses (13) . The remaining sporozoites that successfully leave the site of injection get into the blood capillaries and selectively get arrested in the liver.

1.4.1.1 Sporozoite transmission and migration- the dermis stage

Female infected mosquitoes while probing for a blood meal, inject their proboscis and stylets into the skin, in search for dermal blood capillaries (14). During this process, the mosquitoes inject saliva at the site of bite. The saliva contains vasodilators, anticoagulants along sporozoites that are deposited in dermis. Approximately 20-200 sporozoites are injected in a single bite and it was believed that sporozoites remain motile for 30 mins at the bite site (15). A successful inoculum does not always ensure an infection as skin resident fibroblasts, leukocytes and capillary endothelial cells serve as physical and cellular barriers and limit the successful transit of sporozoites into blood stream, and hence to the liver (16). To move away from the site of inoculation, the sporozoite use a unique kind of substrate dependent movement (17). Although the majority of invasive stages do not possess any obvious specialised structures such as cilia or flagella, yet they are highly motile and show rapid movement of solid substrate. This mode of locomotion is referred as gliding motility (18,19). One of the sporozoite proteins- TRAP (thrombospondin related anonymous protein) connects the molecular motor with sporozoite membrane and likely aids in the gliding motility and *Plasmodium* mutants lacking TRAP showed diminished motility and failed to establish infection (20,21). Using substrate dependent motility the sporozoites depart from the site of injection within 30- 60mins and enter in the blood vessel (22). There are multiple fates for sporozoites that have not successfully entered the systemic circulation. Few sporozoites are phagocytosed, while others enter in the lymphatic circulation, and end up in the lymph nodes and get degraded. Alternatively, some sporozoites that manifest constitutive exocytosis activate a first cohort of antigens specific T cells in lymph nodes. These T cells are likely to get reactivated when they encounter processed antigens on surface of infected hepatocytes leading to elimination of parasite-infected cells (15,23). Interestingly, sporozoite's cell traversal activity has been linked to several important

proteins like sporozoite microneme protein essential for cell traversal (SPECT) and perforin-like protein 1 (PLP1), cell traversal protein for ookinetes and sporozoites (CelTOS), TRAP-like protein (TLP) and *P. berghei* phospholipase (*Pb*Pl). Parasites lacking SPECT 1 or 2 were not able to perform traversal yet were capable to hepatocyte invasion and formation of mature exo-erythrocytic forms (EEFs). But deleting both the genes manifested an impaired liver stage development and delayed pre patency in mice (24-26). Targeted disruption of CelTOS and TLP in *P. berghei* resulted a severe reduction in cell passage activity and decreased sporozoite infectivity (27,28). Similarly, parasites lacking *Pb*Pl previously known as UIS10, showed a drastic decrease in sporozoite infectivity followed by a significant reduction in liver infection with a one-day delay in the prepatency period (29).

1.4.1.2 The liver stages

Sporozoites penetrate through the blood vessel endothelium to enter in to the blood and migrate via hepatic artery to liver sinusoids. Prior to successful hepatocyte invasion, parasite has to pass through the sinusoidal cell layer. The sinusoid is made up of interspersed liver resident macrophages known as Kupffer cells and fenestrated endothelial cells. Sporozoites passage through both the cell barriers to extravasate from circulation into the liver parenchyma for successful hepatocyte infection. (30).

Ability of migration through the host cells is an intriguing characteristic of sporozoites before establishing infection. It directly penetrates through host cell plasma membrane to traverse through several cells before engaging with the final hepatocyte where they develop and replicate to establish infection (31). During the course of migration, activation of *Plasmodium* sporozoites is triggered by encountering host cell factors that make them infection competent. Specific molecules of host cell trigger sporozoite Ca^{2+} regulated signaling pathway which induces the apical organelle mediated exocytosis. Exocytosis provides essential molecules for vacuole formation and hepatocyte infection. Hence, migration is a crucial step during liver infection as it is involved in regulated exocytosis followed by activation of sporozoites and establishment of intra-hepatic infection (32).

Random breaching of the sporozoites through host cell also causes secretion of several host cell factors that very likely sensitise the neighbouring cells to infect. One of

the important factors is hepatocyte growth factor (HGF), which activates MET (receptor of HGF) and is required during early stages of parasite development (33). HGF/MET signaling includes activation of Rho GTPases for a brief period time which causes a dramatic remodeling of cell actin cytoskeleton, helping the parasite to create a specialised niche for its own survival in the hepatocyte (34). In addition to that, to sustain the rate of infection and uninterrupted proliferation, HGF/MET signaling also inhibits host cell apoptosis during sporozoite infection. It has been reported that, inhibiting the HGF/MET signaling pathway led towards increase in apoptosis of infected cells resulting a decline in parasite load (**Fig. 3**) (35).

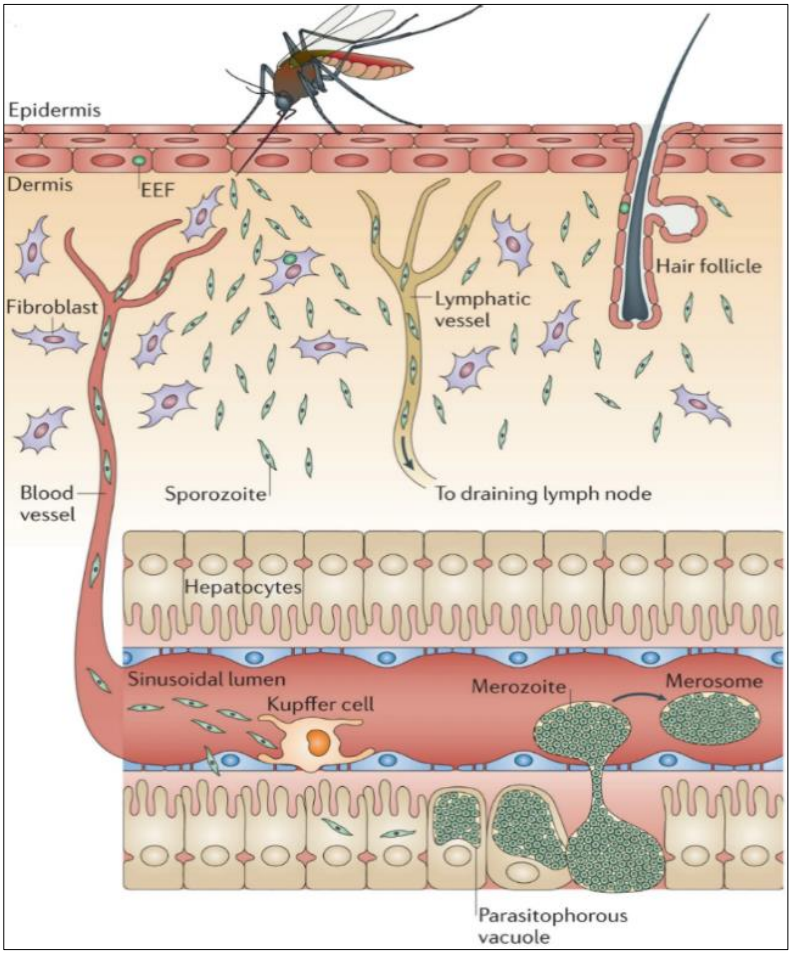


Fig. 3: The pre-erythrocytic phase of *Plasmodium* in mammalian host. Motile sporozoites inoculated into the skin may harbor at the bite site or leave via blood or lymph vessel. Sporozoites staying in skin invade dermis associated with hair follicle or those getting drained by lymphatic vessels can invade host cells in proximal lymph node. Sporozoites passing through blood stream halt at liver cells, glide into the sinusoids, wound Kupffer cells and cross the barrier to traverse through hepatocytes and finally settle down. They form parasitophorous vacuole (PV) to undergo schizogony. This produces tens of thousands of daughters merozoites which are finally released in packets of merosomes into the vasculature. Image adapted from (36).

After getting selectively arrested in liver, sporozoite retention is highly dependent on two important secretory antigens known as circumsporozoite protein (CSP) and TRAP (thrombospondin related anonymous protein) (36,37). Infection of sporozoites in liver is based upon binding of CSP to the heparin sulfate proteoglycan (HSPGs) produced by hepatocytes and stellate cells in the space of Disse.

CSP forms one of the major parasite surface proteins and is a well characterised protein in *Plasmodium*. Structure of CSP is almost similar in all the *Plasmodium* species, having two conserved regions: a region I which contains five amino acids and a C terminal highly adhesive region similar to type I thrombospondin repeat (TSR) region II. Apart from this there is central tandem repeat region containing amino acids which are highly species specific (38). Productive invasion by the parasite is associated with a proteolytic cleavage of CSP at region I upon contact with hepatocytes (39). Genetically engineered sporozoites lacking the region I had poor CSP cleavage followed by low efficiency in hepatocyte invasion (38). To decipher the exact enzyme, E-64 inhibitor was used to inhibit the protease that cleaves CSP which suggested that it is a Clan CA papain family cysteine protease and N terminal end of CSP is targeted during this process. Further when E-64 was treated in *in vivo* model infected with *Plasmodium*, it was found that the mice were completely protected from the infection (40).

The second important protein, known as TRAP. It is a type 1 transmembrane protein. It has two adhesive domains on its extracellular portion: a type I thrombospondin motif (TSR) and integrin like A type domain. During hepatocyte invasion both of them interact with proteoglycans (37). TRAP is associated with actin-myosin motor complex through its carboxy-terminal cytoplasmic domain and is secreted in large quantity onto the zoite surface, which shows its significant role during the process of invasion (41). Sporozoites lacking either A domain or TSR of TRAP likely to have reduced infectivity in hepatocytes because of diminished adhesive interaction of the domains (42,43). Recent study has shown that AMA-1 which is originally related to *Plasmodium* merozoite invasion during blood stages is reported to be expressed on surface of the sporozoite like TRAP. Prior to secretion, it also gets proteolytically processed in sporozoites, losing its N terminal fragment and gets cleaved in the C terminal. A large concentration of AMA-1 antibody inhibits sporozoite invasion of hepatocytes (44).

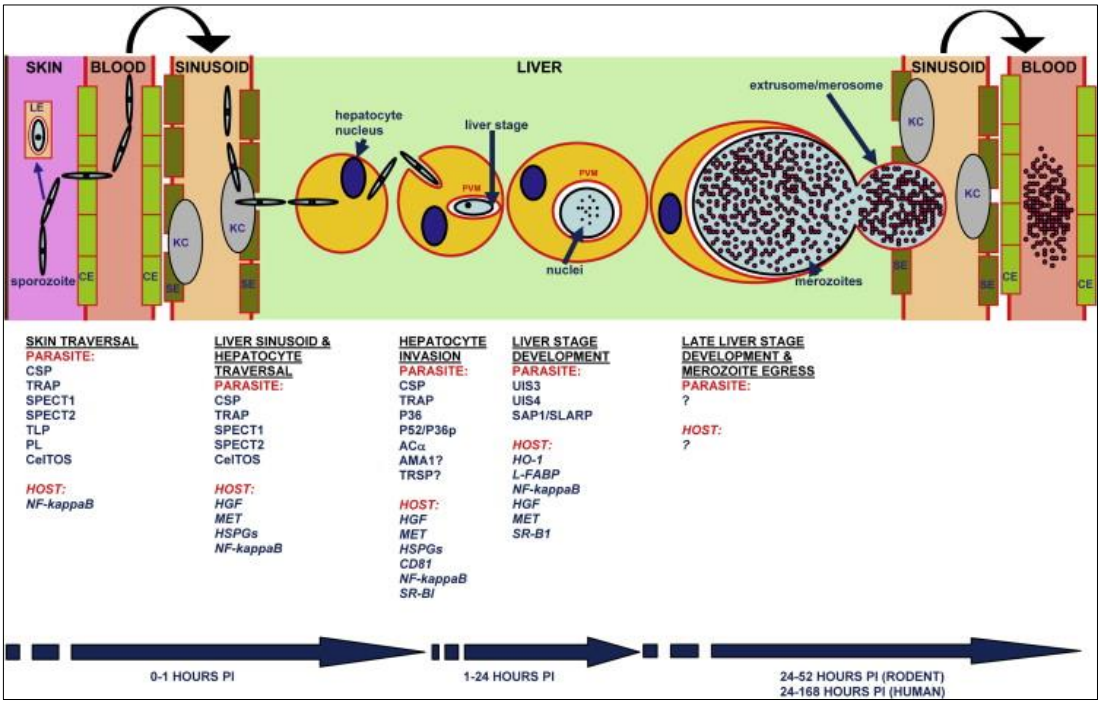


Fig. 4: Proteins involved during sporozoite injection to liver stage development. Several proteins are involved during the journey of sporozoite from skin to traversal through hepatocytes and establishing infection in liver cells. Massive replication of parasite inside parasitophorous vacuole demands a high intake of proteins and nutrients from host which in turn helps for production of thousands of merozoites to carry forward the cycle. Image adapted from (45).

During the process of invasion, host cell receptors also pay an equally important role. The tetraspanin CD81 receptor has been identified as one of the essential molecules and is needed for an effective sporozoite invasion. Interestingly it is essential for both rodent malaria parasite *P. yoelii* and human malaria parasite *P. falciparum*. Whereas *P. berghei, a*nother rodent malaria parasite, can use both CD81 dependent and independent pathways during hepatocyte infection. Mouse lacking CD81 inhibits infection of *P. berghei* and *P. yoelii* sporozoites (46). Similarly, scavenger receptor BI(SR-BI) facilitates selective uptake of cholesteryl during infection. Deficiency of it causes decrease in CD81 expression on hepatocyte surface leading towards decline in hepatocyte infection (47). Host cell receptors like CD68 and Ephrin A2 have evident roles in liver infection. Expression of CD68 is found exclusively in liver sinusoid and Kupffer cells and silencing their expression with the help of short interfering RNA (siRNA), resulted in a decline in entry of sporozoites through Kupffer cells (48). Similarly, another host cell receptor Ephrin A2(EPHA2) is involved during sporozoite invasion in *P. yoelii* and *P. falciparum* and *P. berghei*. *EPHA2* KO mice showed a decreased liver parasite load (**Fig. 4**) (49).

1.4.1.3 Parasitophorous vacuole membrane (PVM)

Upon invasion, sporozoite actinmyosin motor powers a dynamic access into the host cell which leads towards invagination of host cell membrane and concealing the intracellular parasite within an intracellular structure called the parasitophorous vacuole membrane (PVM) (50). PVM acts as barrier within the host cell membrane. The PVM is a dynamic interface for communication between the host and parasite and aids in acquisition of nutrients and also helps parasite to escape from host immunity. Within PVM, parasite undergoes active asexual replication by a process of schizogony. The parasite at this stage is known as liver stage or exo-erythrocytic form (EEF), when extensive remodeling occurs. This involves disassembly of invasive organelles and inner membrane complex (IMC), fragmentation of IMC, expansion of globular plasma membrane and contraction of distal ends (51). Development of parasite inside host occurs in juxtanuclear region and associated closely with host endoplasmic reticulum (ER). Two members of 6 cys-domain proteins P52 and P36 are found to be crucial for PVM formation in *P. yoelii* parasites. Deletion of P52 and P36 in parasite did not form PVM both *in vivo* and *in vitro* thus blocking the development of parasite in liver stages (52). Liver stage development of parasite starts with schizogony which matures to form invasive exoerythrocytic merozoites (56). It is a rapid process involving biogenesis of mitochondria, apicoplast and DNA replication leading towards transformation of one sporozoite to nearly 29000 exo-erythrocytic merozoites (57,58).

The parasitophorous vacuole resident proteins like Up-regulated in infective sporozoites (UIS3) and UIS4 are exclusively transcribed in sporozoites. These are important for early stages of EEF development. Targeted deletion of *UIS3* and *UIS4* showed essential role in liver stage development of the parasite (53). UIS3 helps in parasite survival by promoting uptake of nutrients. It has been shown that it directly interacts with L-FABP1 (with liver fatty acid binding protein) and downregulation of expression of L-FABP showed a drastic reduction in parasite liver growth (54). Similarly, UIS4 is localised in a vesicular protrusion known as liver stage tubovesicular network (TVN) that extends from periphery of PVM into the hepatocyte cytoplasm. *UIS4* depleted parasites had a normal early-stage growth, likely to have no function during sporozoite transformation into liver trophozoites but with the onset of replication phase, deficiency of UIS4 dramatically impaired the production the merozoites (55). In late liver stage

development two PVM associated protein are expressed, known as liver specific proteins (LISP1) and LISP2 (66). LISP1 helps in release of daughter merozoites and LISP2 is present in PV which gets exported to cytoplasm and plasma membrane of infected hepatocytes. Depletion of *LISP 1* and *2* seizes maturation of parasites during late liver stage development.

During liver stage development a repertoire of proteins are exported by *Plasmodium* in to the host cytosol. These export proteins have a canonical sequence known as PEXEL/HTS (*Plasmodium* export element/host targeting signal) that is a pentameric amino acid sequence of RxLxE/Q/D (56,57). PEXEL motif is present in membrane bound and in soluble proteins. Plasmepsin V, an aspartyl protease cleaves the PEXEL on C-terminal side of conserved leucine (RxL ↓ xE/D/Q) (58,59). Following cleavage the protein gets N-acetylated (60). PTEX complex present in PVM constitutes of Exp2, HSP10, PTEX150, thioredoxin and PTEX 88 which helps in transportation of these proteins (61). The proteins after being cleaved by plasmepsin V, unfolds inside PV and get transported via PTEX complex to host cytosol where heat shock proteins help in refolding of these exported proteins. Parasite also exports proteins that do not contain PEXEL, revealing that the motif is not indispensable for export. (62). The first protein to be detected in an infected liver is CSP, which has two PEXEL motifs (63) and other proteins include IBIS1, Sporozoite and liver stage expressed tryptophan rich protein (SLTRiP) and LISP2 (64,65).

After successful invasion in hepatocytes, sporozoites undergo differentiation to attain an early active replication stage known as liver trophozoite (51). Only essential organelles are retained during this process viz. mitochondria, endoplasmic reticulum, apicoplast and there is a disassembly of cellular and molecular components that was needed for motility (66). During the process of schizogony, genome of the parasite gets multiplied by 10^4-10^5 times along with the cell organelles. This is followed by cytokinesis which produces thousands of daughter merozoites (9). To form individual exoerythrocytic merozoite, parasite plasma membrane expands and invaginates to form cytomere. A coordinated organellar and nuclear fission provides equal segmentation of nuclei and organelles to nascent merozoites (56). The whole process is dependent upon liver stage specific RNA binding protein, PlasMei2 which has major role in division of nuclear DNA. Plasmei2 mutants in *P.yoelii* had a normal liver stage growth but failed in

formation of exo-erythrocytic merozoite (57). Members of serine repeat antigen (SERA) family are observed to be conserved across *Plasmodium* species and is important during egress of merozoites and oocyst sporozoites. *Pb*SERA1 and 2 are targeted to PVM showing abundant expression during formation of merozoite (67,68). A conserved serine protease known as subtilisin like protease (SUB1), was reported to be essential during egress of merozoites in both liver and blood stages (69). SERA proteases are cleaved and gets activated by SUB1 to initiate an array of protease events essential during the process of egress (70,71).

Breakdown of PVM releases packed merozoites in to a membranous structure called merosomes which gets extruded to liver sinusoid from infected liver cell (9,72). Merosomes are host cell derived structures and provide protection from liver sinusoid resident Kupffer cells. Merozoites are released out of merosomes by membrane disruption into hepatic circulation for initiating erythrocytic cycle (9).

1.4.2 The erythrocytic stage

Exo-erythrocytic stages are dynamic and metabolically active but are asymptomatic. Disease symptoms of malaria are associated with proliferation and invasion of merozoites during erythrocytic cycle. Disruption of merosome releases 10,000 to 30,000 merozoites into the blood stream to initiate infection by invading RBCs (73). Parasite maintains a balance between rapid rate of reproduction and also maximaisation of host cell survival to attain the sexual stage transmission (74). Merozoites are elliptical in shape and relatively small with a length and breadth of approximately 1–2 μm (75). The apical end has a canonical protuberance which contains polar rings and essential secretory organelles viz. rhoptries, micronemes, and dense granules. Merozoite surface coat is composed of glycosylphosphatidylinositol (GPI) anchored proteins (86). The initial contact between merozoite and erythrocyte is a sequential and a well-organized process (76).

Initial attachment between merozoite and erythrocyte is mediated by a dominant surface antigen protein called merozoite surface proteins (MSPs) with band 3, serving as the host cell receptor (77). Parasite lacking MSP1 expression can still invade RBCs but is essential during the process of egress out of erythrocytes (87,88). Second step is reorientation which is mediated by a major merozoite surface protein known as apical

membrane antigen (AMA1), considered to be a potential vaccine candidate. Major role of AMA1 is, juxtaposition of the apical end of merozoite with erythrocyte membrane for a closer association (78,79). For successful completion of invasion, interaction between merozoite surface proteins with actin-myosin motor residing in inner membrane complex (IMC) is considered to be a crucial step (80). Drug targeting the actin-myosin motor blocks the process of invasion by parasite (81). Following reorientation, merozoites bind to the erythrocyte surface to initiate invasion. In order to do that merozoites are equipped with adheshins, secreted during the process of invasion and daughter cell formation (75). Tight junction is formed between host membrane and the parasite which extends from anterior apical end to the posterior end of parasite with a series of complex events supported by actin-myosin motor (82). It is regulated by two major families of adhesins, released from microneme. First one is duffy binding like protein (DBL or also known as erythrocyte binding like protein EBL), initially identified in *P. knowlesi* and shown to bind the duffy antigen (DARC) (83).

This family includes EBA175 which binds to host cell receptor glycophorin A, and leads to discharge of the contents of rhoptries. Additionally, EBL1 and EBA140 binds to glycophorin B and glycophorin C respectively. These important processes, not only facilitate host and parasite binding but also activate a cascade of downstream signaling associated with successful parasite invasion (84-86). The second family of proteins is reticulocyte binding-like homologues (RBL) or Rh which was first identified in *P. vivax* and *P. falciparum* (94). In case of Rh, erythrocyte binding regions are well defined where complement receptor 1 (CR1) and basigin serve as a receptor of *Pf*RH4 and *Pf*RH5 respectively (87,88). Rh sensing and attachment with an appropriate host erythrocyte triggers the merozoites for a successful invasion (89). After the attachment of merozoite-erythrocyte, a parasite protein called calcineurin initiates Ca^{2+} signaling to strengthen host parasite adhesion by EBL stabilization and dimerization of *Pf*Rh (98) (**Fig. 5**).

The interaction of merozoites with erythrocytes leads not only in deformation and wrapping of cell membrane but also reorganization of the underlying cytoskeleton to extend support during the process of invasion. A complex is formed between *Pf*RH5, *Pf*Rh5-interacting protein (*Pf*Ripr) and the cysteine-rich protective antigen (CyRPA) (90-92). The interaction between *Pf*Rh5-basigin is important for invasion which is related to increase in Ca^{2+} concentration in erythrocyte, likely suggesting formation of a pore

between the host and parasite (93). Conditional deletion of *PfRipr* and *CyRPA* blocked the invasion process and found out to be essential for release of Ca^{2+} from merozoite to erythrocyte during invasion (94).

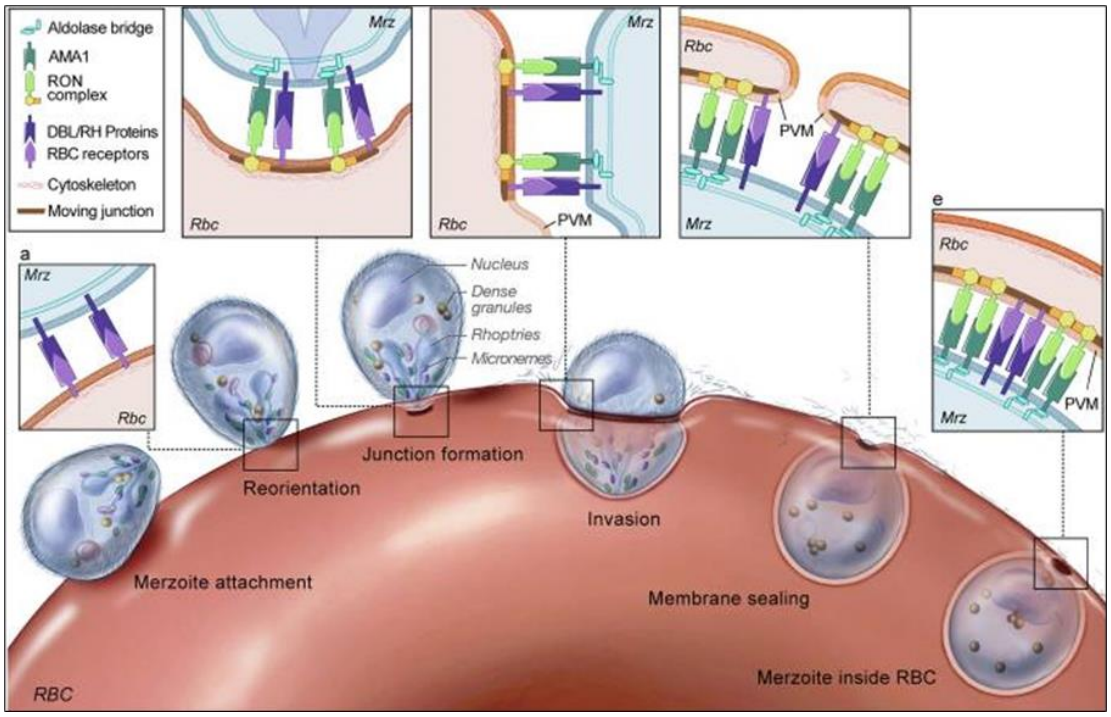


Fig. 5: Steps involved during merozoite invasion. Merozoite attaches to target RBC and then reorients. Two parasite ligand families DBL and RH brings the parasite to close apposition with RBC membrane. Neck of the parasite releases parasite proteins called RONs, to be inserted into and across the RBC membrane. An electron dense structure forms a tight junction between parasite and RBC membrane. Rhoptry bulb proteins induces parasitophorous vacuole as the parasite moves inside RBC. Interaction between AMA1 and RON2 play a major role in invasion. Image adapted from (95)

Tight junction is comprised of AMA1 and RON2. AMA1 is located on the surface of merozoite and binds to RON2 (a part of larger RON complex) to get inserted into the erythrocyte (76,102-105). During each step of invasion, subsets of secretory organelles secrete specific ligands. One of such subsets is called exoneme which releases a protease known as subtilin (SUB1) which is essential for merozoite egress. It is released into the parasitophorous vacuole where it cleaves several parasite proteins (96). As the surface of merozoite comes first in contact with erythrocyte, the apical organelles have a major role during this process of invasion (97).

After successful invasion, the merozoites form parasitophorous vacuole inside the host cell. Erythrocyte are devoid of biosynthetic activities as they lack intracellular

organelles. A tubulovesicular network (TVN), extending from PVM to erythrocyte membrane facilitates acquisition of nutrients from blood plasma like purines, proteins and lipids (98,99). Similar to liver stages, *Plasmodium* exports several proteins to host cytosol with the help of export motif known as *Plasmodium* export element (PEXEL) or vacuolar translocation signal (VTS) which participates in protein trafficking, likely suggesting to have role in maintenance of parasite virulence and survivability (100). Several proteins that lack a PEXEL motif (PEXEL negative exported proteins, PNEPs) such as skeletal binding protein (SBP1), *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1), Ring Exported proteins 1 and 2 (REX1 and 2) are also exported to RBC cytoplasm (101-103). It has been reported that a shift in expression of *var* genes during cycles helps *Plasmodium* to successfully escape host immunity. *Pf*EMP1 family encodes almost 60 *var* genes. STEVOR and RIFIN are *var* gene family members which show antigenic variation in the parasite (104). Apart from this *Pf*EMP1 also regulates cytoadherence in *P. falciparum* iRBCs and mediates iRBCs sequestration in brain capillaries and placenta (105).

Inside erythrocyte, *Plasmodium* undergoes dramatic structural transitions. It transforms into ring which is a vacuole surrounded by cytoplasm with nucleus and internal organelles. In next phase they transforms into a metabolically active trophozoite stage, that exhibits maximal biosynthetic activity (106). During this stage it uses hemoglobin as a source of nutrition and produces a byproduct called heme a toxic substance to the survival of parasite. The parasite converts heme to nontoxic hemozoin crystals that appear in the food vacuole (107). A wide range of antimalarial drugs viz. mefloquine and chloroquine target the heme biosynthetic pathway, by inhibiting the conversion of heme to hemozoin (108). After a successful round of schizogony trophozoites transform into schizont stage having 16-32 merozoites. As described earlier SERA family proteins- SERA 5 and 6 play a vital role during the egress of merozoites and hence are important during asexual propagation (109,110). Prior to egress, release of SUB1 activates SERA 4,5 and 6. Further these SERA proteins mediate merozoite egress out of iRBCs by damaging the PVM. Loss of function of *Pf*SUB1 abolished processing of SERA proteins. Merozoites released into blood infect fresh erythrocyte to continue with the life cycle whereas few of them commit to form sexual form and differentiate into male and female gametocytes (111,112).

1.4.2.1 Sexual stages of *Plasmodium*

Plasmodium life cycle has a sexual phase that also occurs within the erythrocytes which is a pre-requisite for disease transmission into mosquito vector. In case of *Plasmodium*, sexual commitment does not depend on sex chromosomes, but rather dependent on differential gene expression. However male and female gametocyte formation occurs from a single haploid parasite asexually replicating in RBC (113,114). GFP reporter expression during sexual commitment is driven by male and female specific markers viz α-tubulin II and *Pf*g377 respectively (115,116).

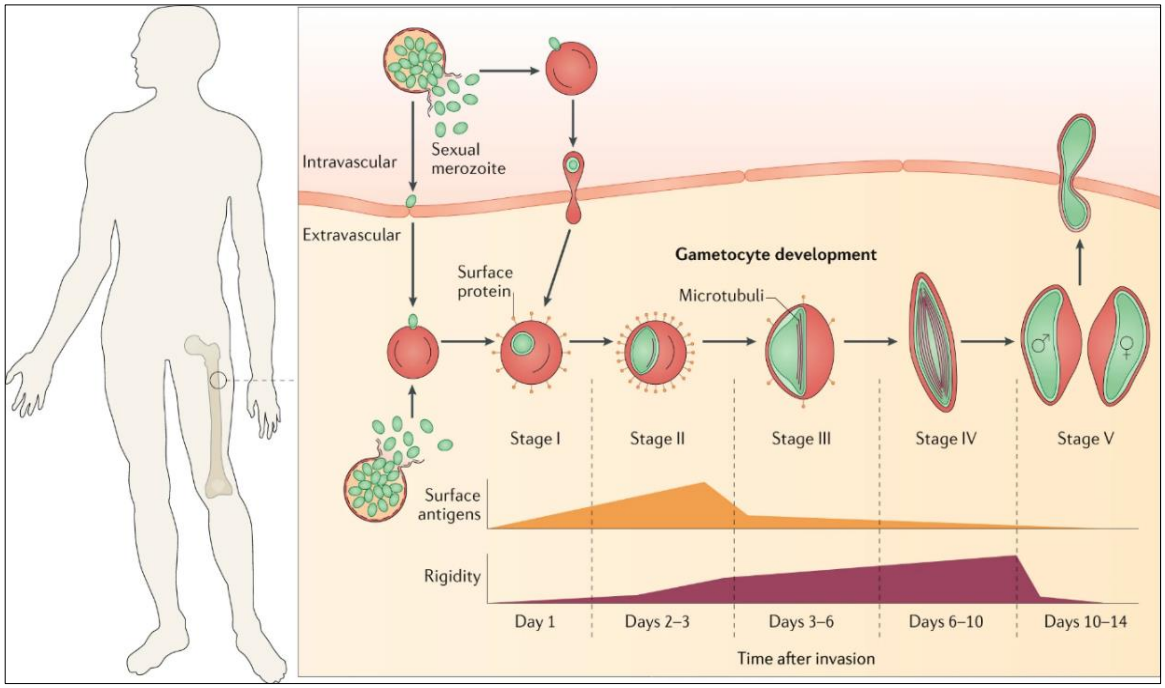


Fig 6: Sexual development of *Plasmodium* in hematopoietic niche of host. Sexual merozoites and young gametocytes (Green) resides in bone marrow. In bone marrow parenchyma, gametocytes develop into stage I to Stage IV. Remodeling of membrane of host RBC result in deposition of surface antigen and a reversible increase in cellular rigidity. Upon maturation to stage V, gametocytes released into the bloodstream where they will be taken by mosquito bite. Image adapted from (9)

Commitment towards sexual stage in *P. falciparum* and *P. berghei* is regulated by a transcription factor known as AP2-g gene (117,118). Additionally, histone deacetylase2 (HDAC2) and heterochromatin protein 1 (HP1) are the epigenetic regulators of sexual commitment that control expression of AP2-g (119,120). A recent study has revealed that a *P. falciparum* perinuclear protein known as gametocyte development 1 (GDV1) mediates enhancement of gametocyte formation (121). Interaction of GDV1 with HP1

helps in activation of AP2-GP2 locus, facilitating transcription of AP2-g which results in sexual commitment in small set of schizonts (122). Concomitant with the above information, deletion mutants of *Pf*GDV1 and Ap2-g resulted in loss of gametocyte production there by downregulating gametocytogenesis (**Fig. 6**) (118,121).

Stage I form of gametocyte is not morphologically well differentiated when compared to asexual trophozoites. In stage II a minor change in appearance can apparently be visible with a pointed end. Stage III converts itself into a D shape by elongating and flattening. Eventually parasite out spaces the residing RBC and occupies most of the cytoplasm except a small membrane fold called Laveran's bib. Stage IV and V are marked by elongation, deformation and finally attaining a crescent shape respectively (113). One of the major complexes called inner membrane complex (IMC) plays vital role in gametocyte metamorphosis (113,114). IMC has proteins like GAP40 and GAP45 which interact with RBC spectrin-ankyrin network (115). Multigene STEVOR family members are highly expressed during this process and stabilises the membrane during gametocyte elongation. Similarly, dephosphorylation of STEVOR helps in shape shifting during different stages (116). Cyclic AMP dependent kinesin regulates deformability in mature gametocytes and drugs that target cAMP expression confers stiffness to the gametocytes preventing their maturation (117). In vertebrate host, gametocytes are the cells which are differentiated terminally and arrested in G0 phase of cell cycle. These avoid DNA synthesis during circulation (96-98). Stage I to IV of gametocytes sequesters in bone marrow and only the stage V gets released into circulation (123). The mature stage V gametocytes are infectious for mosquitos and collected near microvasculature of the dermis of skin to enable easy ingestion while taking a blood meal by mosquito (124,125).

Translational regulators which are present in repressed state during sexual stages are required during fertilization in mosquito vector. Such as DOZI (development of zygote inhibited) and CITH (CAR-I and fly trailer hitch) present in *P. berghei* are well characterised (126). Translational control is governed by an array of RNA binding proteins (RBPs) that monitors mRNA stability, localisation, and translatability (127). One of the notable examples is the Puf (Pumilio and fem-3 binding factor) family RBPs, which are pervasively prevalent across all eukaryotic branches (128). RNA binding domain is conserved in it and is known as the Pumilio homology domain (129). As *Plasmodium* is a digenetic parasite, host transitions events include, translational activation of dormant

mRNAs, required in stages responsible for transmission viz. gametocytes and sporozoites. The translationally repressed (TR) version of the important ookinete surface proteins *Pbs*25 and *Pbs*28 in *Plasmodium berghei* is deposited in gametocytes (130,131). A multi protein complex comprising the DEAD-box RNA helicase DOZI appears to be responsible for maintaining the steadiness of these "maternal" mRNAs in the gametocytes (126). The maternal transcripts, including the mRNAs for *Pbs*25 and *Pbs*28, were significantly lost upon disruption of either DOZI or CITH from the complex (132).

There are two conserved *Plasmodium* Puf proteins which are known to be expressed in gametocyte and sporozoite viz. Puf1 and Puf2 (133,134). Studies reveal that Puf1 is dispensable in *P. berghei* (135,136). Puf2 plays a noticeable role in both gametocytes and sporozoites. Deletion of *Puf2* gene increases the male to female sex ratio by promoting gametocyte proliferation in both *P. berghei* and *P. falciparum* (137). It has also been noticed that deletion of *Puf2* in *P. berghei* sporozoites causes early expression of a group of genes, which causes the sporozoites to prematurely transform into forms that resembles early intra-hepatic stages inside the mosquito salivary glands (135,136). Comparison of gametocyte transcriptome between *P. falciparum* wildtype and *Puf2* deletion mutants with the help of microarray analysis identifies putative transcriptionally repressed transcripts including *Pfs*25 and *Pfs*28. The data reveals that both the transcripts encoding these proteins are upregulated in *Puf2* deleted mutants. *Pf*Puf2 binds to PBEs (Puf-binding element) in the UTRs of these genes to mediate translational repression. Earlier it was identified that PBEs are localised to be in the 3'UTR region but later found out to be also present on 5'UTR (138).

1.5 *Plasmodium* life cycle in mosquito:

1.5.1 Gametogenesis

Gametocytes are taken up by healthy female *Anopheles* mosquitos at the time of blood meal. Except the stage V gametocytes, all other blood stages are digested. Female gametocyte attains a rounded structure to egress out of RBC to form female macrogametes whereas the male gametocyte undergoes a rapid process called exflagellation to produce 8 microgametes. This process takes place within 10-15 mins of post blood meal. Induction of gamete formation is accelerated by drop in temperature (by 5^0C), mosquito derived factors like xanthurenic acid (XA) and change of extracellular pH

from 7.2 to 8 (139,140). Process of exflagellation is preceded by a cascade of events. This includes a rise in intracellular calcium level, phospholipase C and guanylyl cyclase activation which raises intracellular cGMP level (141). Disruption of *P. berghei* guanylyl cyclase (*Pb*GCβ) revealed normal events of exflagellation but motility of ookinetes were impaired (142). Similarly, calcium dependent protein kinase (CDPK4) plays an significant role during sexual stage signaling in *P. berghei*. Disruption of CDPK4 manifested defective male gametogenesis. Activation of CDPK4 is triggered by rise in calcium levels which in turn activates XA. This results in replication of microgametocyte genome (143). Members of conserved 6-cys protein family have been reported to be essential during gamete stages. Such as P48/45 are specific for male gamete and loss of function of P48/45 manifested a defective fertilization capability of male gamete (141). Similarly female gamete expresses exclusively P47 a paralog of P48/45. Targeted deletion of P47 resulted defective female gamete production (142). Another member of 6 cysteine repeats protein family P230 is expressed on both the gametes and genetic disruption in *P. falciparum* showed a block in fertilization (143,144). *P. berghei* male development 1 (MDV1) has an important role in activation of female gametocyte and development of macrogamete whereas *P. falciparum* MDV1 reported to have a role in male gamete activation (**Fig. 7**) (146,147). In *Plasmodium berghei* mitogen activated protein kinnase2(MAPK2) is required during maturation of male gamete (144).

1.5.2 Fertilisation and formation of ookinetes

Plasma membrane of both the gametes fuse together with the involvement of a plant sterility factor known as HAP-2. HAP-2 mutants showed normal male and female gamete production but failed in zygote formation (151). Knock out of RNA helicase- DOZI (development of zygote inhibited) leads to the degradation of almost 370 translationally repressed transcripts which manifested an unsuccessful fertilisaton followed by arrest in sexual development (109). Another protein known as high mobility group protein 2 (HMGP2) is essential for expression of 35 transcripts and essential during formation of zygote, ookinete and oocyst development (152). In the entire *Plasmodium* life cycle, zygote is the only diploid stage. Within 3 hours of formation, it undergoes meiosis to become tetraploid (145). NIMA (never in mitosis Aspergillus) related protein kinase (Nek 2 and 4) are essential during development of zygote and also help in conversion from zygote to ookinete (146,147)

Within 18-22 hours of post gamete fusion the round shaped zygote transforms into a banana shaped motile ookinete. An array of morphological changes makes the elongated protruded mature ookinetes. ISP I and III, two important acetylated IMC proteins helps in linking subpellicular microtubule (SPM) and IMC together to maintain the overall pellicular structure. Lack of both of these proteins showed a reduced rate of differentiation from zygote to ookinete (148,149). IMC anchors the glideosome which confers a stable structure and exerts a forward motion. Parasite secreted chitinase which mediates the degradation of mosquito midgut peritrophic matrix helps ookinete to traverse through mosquito midgut epithelium (150,151). P25 and P28 contains EGF domain and GPI anchor which confers protection to the ookinete against mosquito gut lumen proteolytic activity (152).

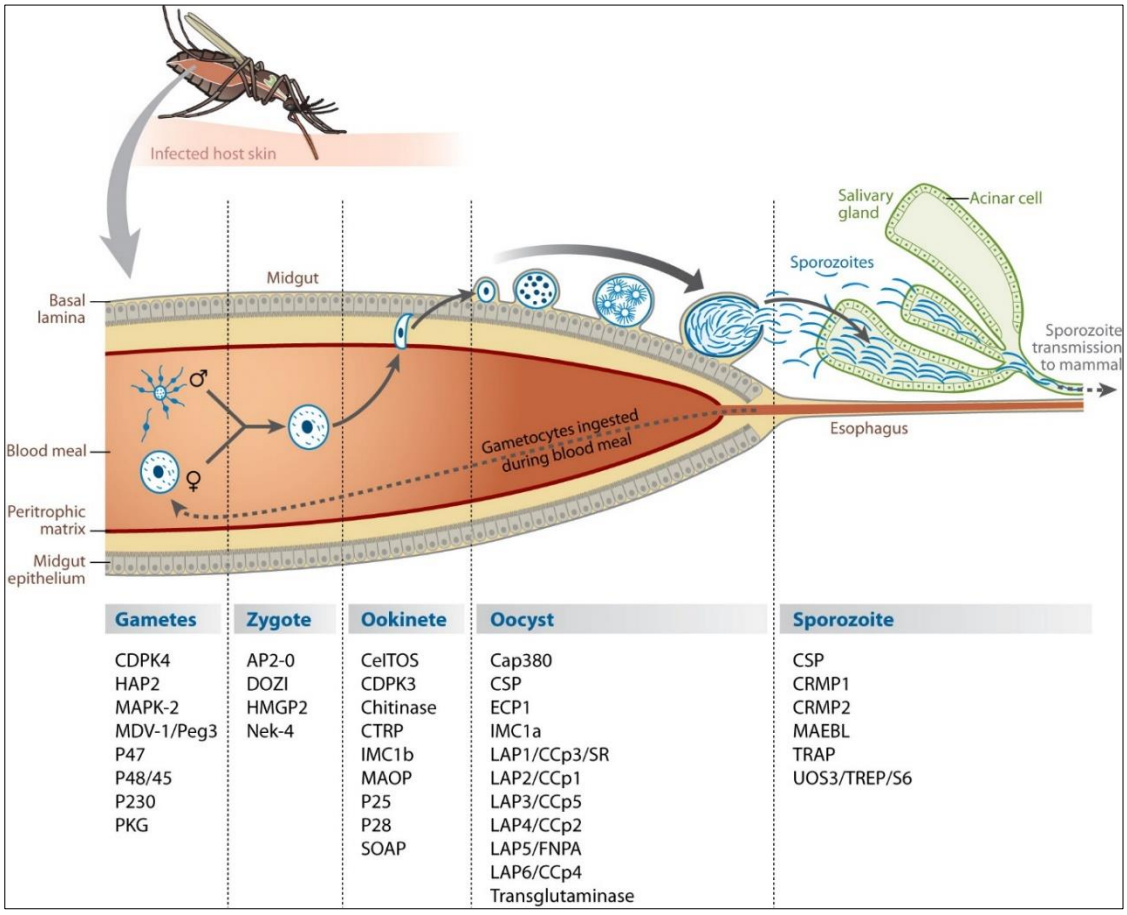


Fig. 7: Development of *Plasmodium* parasite inside a mosquito vector. Gametocytes ingested by *Anopheles* mosquito during blood meal, transforms to male and female gametes. Both the gametes fuse to form zygote which undergoes meiosis and transforms into ookinete. Ookinete breach through the peritrophic matrix to enter into the apical end of midgut epithelium and then traverse through several midgut epithelium. Further they transform into oocyst within 10-14 days after exiting epithelium. Sporulation inside oocyst produces numerous sporozoites, burst upon to swim through the hemolymph before attaching to the basal side of salivary gland acinar cells. Finally, they enter into the duct and wait to be transmitted to a mammalian host during blood meal. Image adapted from (153)

Disruption of circumsporozoite and TRAP related protein (CTRAP) resulted loss of motility in ookinetes and failed in oocyst colonization (154). Similarly, CDPK3 is also important in ookinete development and deletion mutants of CDPK3 produces immotile ookinetes which failed to invade midgut epithelium (155).

Perforin like membrane attack complex domains known as membrane attack ookinete protein (MAOP) is essential for parasite to breach peritrophic matrix and penetrates apical end of mosquito midgut epithelium (162). Disruption of IMC1b and ALV5, IMC proteins reduced ookinete motility followed by reducing the number of oocyst colonization in midgut. Ookinete changeover to oocyst also happens with loss of IMC (156-158).

1.5.3 Oocyst formation

The ookinetes transmigrate through the cells of epithelium to finally settle below the basal lamina of midgut (148,159). Oocyst is the longest stage of development in *Plasmodium* which takes around 10-14 days (160). Basal lamina of mosquito consists of laminin and collagen. Laminin persuades transformation of a moving ookinete to sessile oocyst with association of host factors. Important parasite ligands, glycosylphosphatidylinositol (GPI)-anchored ookinete surface proteins- P25 and P28, interacts with laminin to induce laminin mediated transformation of oocyst. Double gene deletion mutants of P25 and P28 completely failed in oocyst transformation and midgut epithelium invasion (163,164). Another important *P. berghei* protein known as secreted ookinete adhesive protein (SOAP) and CRTP interreacts with *Anopheles* laminin for successful production of midgut oocysts. Disruption of both these genes impaired invasion ability and transformation capability of ookinetes (165,166).

Oocyst development is the sole form of developmental stage which happens extracellularly in malaria parasite. LCCL/lectin adhesive like proteins (LAPs) are expressed in gametocytes (female), ookinetes and oocyst. The parasite surrounds the entire oocyst surface with a secreted amorphous capsule which consists of transglutaminase (161). The oocyst wall is bipartite with a thicker external layer formed of laminin and an inner plasma membrane of the host mosquito (162). Immune system of mosquito eliminates the parasite during the transition towards basal lamina but the encapsulation confers protection from mosquito defense molecules. Mitotic division take

place after oocyst formation where cytokinesis followed by karyokinesis happens (163). Circumsporozoite protein (CSP) has an important role during oocyst development, sporoblast formation and sporulation. It has a N-terminal signal peptide and it is embedded in the plasma membrane with the help of a GPI anchor at its C-terminus (164). The plasma membrane of oocyst gets retraced with an internal invagination including a nucleus to form a cytoplasm lobe, called as sporoblast to undergo final mitotic division (165). CSP is present in the endoplasmic reticulum, cytoplasm and on the plasma membrane of sporoblast (166,167). Mutants lacking CSP showed defect in sporozoite formation (168). Beneath the plasma membrane of sporoblast, MTOCs (microtubule organizing centers) are present which helps sporozoites to incorporate apical complex and nuclei. Probably formation and organization of MTOCs are facilitated by CSP (169). Assembly of apical rings, formation of subpellicular microtubules, polymerization and attachment of IMC helps sporozoites to bud off from sporoblast (170). IMC places approximately 30nm below the plasma membrane with a presence of a minor gapped highly organized flattened homogenous vesicles (136). IMC1 decides the shape of sporozoites and lack of IMC1 resulted in deformed and immotile sporozoites. This reveals the role of IMC1 in crescent shape of mature sporozoites (171).

A small fenestration in the wall of oocyst leads the path for sporozoite to escape into the hemocoel. An egress cysteine protease 1- referred to as ECP1, is expressed in oocyst and *P. berghei* deletion mutants of ECP1 produces sporozoites which were not capable to egress (172). Disruption of another hypothetical protein in oocyst displayed the similar degree of defect like ECP1 mutants, which infers that there are several proteins engaged in the process of egress (176).

1.5.4 Salivary gland invasion

Sporozoites egressed out of the oocyst are released into mosquito circulatory system, known as hemolymph. Parasite ligands recognises and binds to the receptors present on the basal lamina of salivary gland. Upon attachment sporozoites invade the salivary gland lobes (distal, medial and lateral) (173). Parasite proteins like CSP play an essential role during attachment of sporozoites to the salivary gland (174). It is reported that anti-CSP antibodies when injected into the infected mosquito hemocoel blocks salivary gland invasion. Disruption of N terminal region of CSP, arrested the attachment and invasion of sporozoites (178,179). Similarly, sporozoites lacking region II of CSP

protein were unable to invade salivary gland and infect the vertebrate host as well (175). A recent study has shown that a hypothetical gene encoding for conserved *Plasmodium* structural integrity maintenance protein (SIMP) is localised to the membrane of the sporozoite. Deletion mutants showed deformed sporozoites, lacking a regular shape with an extensive membrane blebbing and detachment. Mutant sporozoites lost their capability of gliding motility and failed to infect hepatocytes *in vivo*, as seen by complete absence of blood stage infection (176).

As described earlier TRAP plays a vital role during sporozoite gliding motility and is associated with parasite actin-myosin motor. It has a functional A-domain of von Willebrand factor and thrombospondin type I repeat (TSR) domain (177). Disruption of TRAP results in accumulation of immotile sporozoites in mosquito hemocoel lacking the capability to glide. Mutagenesis study of *Pf*TRAP has reported that both the above-mentioned domains of TRAP has absolutely no role in sporozoite gliding motility but contribute for infectivity of sporozoite in both insect and mammalian host (42). Another important sporozoite micronemal protein known as MAEBL is expressed prior to invasion of salivary gland. Parasite lacking MAEBL produced sporozoites having a major defect in attachment to the basal lamina of mosquito salivary gland (178). But interestingly these MAEBL deficient parasites were having normal gliding motility and were able to infect mammalian host (178). Gene regulation of MAEBL involves alternative splicing and post-translational processing. Inhibitory studies of MAEBL reveals its important role in hepatocyte invasion and development, infected erythrocyte resetting and recombinant surface expression. Above mentioned functions are exerted by variants of MAEBL (179).

Sporozoites residing in salivary gland have a significant difference in virulence as compared to the sporozoites egressing out of the oocysts. The enhanced infectivity in salivary gland sporozoites is achieved by altering its transcriptional repertoire. During the course of maturation of *Plasmodium* sporozoites within salivary glands, they undergo a developmental program, that enhances the sporozoite infectivity for mammalian host. As suggested by transcriptomic studies this includes upregulation of nearly about 30 genes in salivary gland sporozoites, known as upregulated in infectious sporozoites or UIS genes (180). UIS genes have shown to have important functions during establishment and growth of EEFs in hepatocytes. For example, the sporozoite transformation in liver

is dependent on the phosphorylation status of *Plasmodium* eIF2α mediated by a kinase-IK2 (UIS1) and this modification can be reversed by a phosphatase. IK2 inhibits translation of stalled mRNAs while in the salivary glands however, once the sporozoites are inoculated into vertebrate host, dephosphorylation of eIF2, by phosphatase alleviates the translational repression. This facilitates the liver stage transformation of sporozoites. Lack of UIS1 in sporozoites resulted in early transformation into EEFs in mosquito salivary gland itself (181). UIS3 and UIS4 are found to be essential in liver stages development of parasite and elimination of either of the genes arrested the development (53,55). SAP1 (sporozoite asparagine-rich protein 1) is important for UIS gene expression and perturbing the SAP1, abolished the factors responsible for infectivity for during liver stage infection (182). Mice exposed to *uis3* and *uis4* KO sporozoites, that failed to cause a break through infection resulted in generation of $CD8^+$ T cell immunity (183).

1.6 Clinical manifestations of malaria

Symptoms of malaria become clinically apparent when threshold parasitemia in erythrocytic cycle crosses around 100 parasites per μL. Incubation period could be different in different parasite species ranging from 10-14 days in case of *P. falciparum, P. knowlesi, P. ovale, P. malariae* or as long as to 3-6 months in case of some strains of *P. vivax*. A periodic fever can be observed at intervals relating to blood stage cycle length of the infected species which can be from 48 hrs. to 72 hrs. Pregnant women may get affected with placental malaria due to sequestration in the intervillous space causing low birth weight, anemic patient, pre term labor and risk of abortion. Common features of malaria disease are anemia, intravascular hemolysis, bone marrow suppression and dyserythropoiesis. Symptoms may include vomiting, mild diarrhea, pain in abdomen, myalgia, dizziness, tiredness and nausea. Along with that other physical signs like jaundice, hypotension, hepatomegaly, cough, tachycardia, orthostatic, pallor and splenomegaly can also be seen (184). Cerebral malaria and acute lung injury are the most common indicators of severe malaria, causing acute respiratory distress syndrome.

1.7 Diagnosis

An appropriate and rapid diagnosis of malaria is one of the prime strategies of disease management especially for vulnerable and non-immune population. There are

two methods, widely used for diagnosis which is done by the help of light microscopy and rapid diagnostic tests (RDTs). RDTs help in detection of parasite specific antigen which helps in recognising different *Plasmodium* species. The microscopic way of detection is much simpler and more economic than RDTs. It can be applied where the parasite load of the patient is high. But practicing the microscopic way of detection needs skilled staff with an extensive training to identify parasitic stages. Moreover, maintenance of the instruments and a constant source of power is also a prerequisite.

Though RDTs are expensive but it is more sensitive and specific towards confirmatory detection yet has constraints. Blood films prepared for the patients observed under microscope to be negative but can be diagnosed correctly using RDTs as it detects *Pf*HRP2. Apart from that parasite lactate dehydrogenase (pLDH) is produced by asexual and sexual stages of parasite and detected by RDTs (185-187).

1.8 Treatment

Plasmodium resistance towards drugs is one of the major challenges to control the disease. In order to reduce the risk factors, prophylactic vaccines have been proposed to be the effective way to prevent the spread as compared to drugs. Due to the complex life cycle of *Plasmodium*, polymorphic antigens are expressed in different stages. A successful vaccine, therefore is expected to be comprising of antigens expressed in different life cycle stages.

Sporozoite and liver stage targeting vaccines are known as pre-erythrocytic vaccines, and are aimed at neutralizing the infectivity of extracellular sporozoites or eliciting cell mediated immunity against liver stage antigens. (188). RTS, S is considered to be a highly effective sporozoite vaccine which targets a major surface protein of sporozoites known as CSP (189). Radiation-attenuated *Plasmodium* sporozoites were observed to be arrested in liver stage development. The degree of attenuation of genetically attenuated parasites (GAP) varies from early liver stages to late liver stages. As described earlier *uis3* KO and uis*4* KO parasites revealed a block in early liver stage development and triggered $CD8^+$ T cell immunity (183).

Erythrocytic vaccines primarily targets multiplication and invasion of merozoites by inducing high antibody titers to protect the host. Few important candidates, having highest expression on surface of merozoites are as follows: glutamate-rich Protein

(GLURP), merozoite surface protein 1 (MSP1/2/3), erythrocyte-binding antigen-175 (EBA-175), apical membrane antigen 1, (AMA1), serine-repeat antigen 5 (SERA5). These candidates can be used as blood stage vaccines (190).

To reduce the transmission of the disease within a community, transmission blocking vaccines (TBV) are being used to block the development of sexual stages of *Plasmodium.* Important proteins like *Pf*s25 and 28 (zygote/ookinete surface proteins) or *Pf*s48/45 and *Pf*s230 (gamete and gametocyte surface proteins) are considered to be effective vaccine candidates. Phase I trial of *Pv*s25 showed decreased immunogenicity (191).

Chemotherapy is also one of the mainstays for treatment of malaria. Quinine is a derivative of chloroquine. It is extracted from cinchona plant and is considered to be the first choice for treatment of malaria (192). Similarly, primaquine targets hypnozoites of *P. vivax* and used with other antimalarial drugs as a combination therapy (193). Artemisinin is isolated from *Artemisia annua* plant and used in Chinese traditional medicines as a natural inhibitor of malaria. It has become one of initial drugs used for the treatment in a combination with other classes of antimalarial drugs, called as artemisinin combination therapy (ACT). Other synthetic derivatives of artemisinin are artesunate, dihydroartemisinin, showing higher antimalarial activity (194). WHO is also recommending ACTs for better treatment of malaria due to widespread resistance towards other existing drugs (195).

Several antibiotics have shown to be affecting the *Plasmodium* metabolic pathways of mitochondria or apicoplast due to its bacterial origin. Like doxycycline and tetracycline block translation of protein in mitochondrion (196). Clindamycin is reported to be potent inhibitor of protein translation in apicoplast (197).

1.9 Control measures

Prevention of transmission is one of the major ways to regulate outbreak of disease and also considered to be a new world strategy. Efforts are more concentrated towards vector control where use of insecticidal nets, repellants, larvicides and insecticides are employed as a protective mechanism to eliminate the spread of the disease in high-risk groups like pregnant women and young infants or children (198). Insecticide treated nets (ITNs), keep the insects away up to 3 years almost (199). Global malaria eradication campaign has introduced indoor residual spraying (IRS) in regions of

low or seasonal transmission. Dichloro-diphenyl-trichloroethane (DDT) is one of the most popular insecticides to be used as IRS in several countries (200). To target the aquatic habitats, a strategy called larval source management (LSM) is implemented which includes habitat modification and manipulation by using biological control with the help of predatory fish like *Gambusia affins* and using larvicidal drugs (201-203). Other vector control strategies include use of attractive toxic sugar bait (ATBS), mass drug administration (MDA) with a combination of sulphadoxine-pyrimethamine or a combination of primaquine with chloroquine. One of the novel approaches, that can be tested on an experimental basis is to modify the genome of mosquitoes by CRISPR/Cas9 gene editing technique. Infertile mosquitoes produced by this approach can be released in the environment, that may have a potential to inhibit growth of the mosquito colony (201,204,205).

1.10 Current challenges to malaria

In the recent years, considerable efforts are being put in the direction to eradicate malaria and many countries has almost achieved the goal. However increased resistance towards artemisinin and chloroquine in *P. falciparum*, and *P. vivax* is leading to an alarming situation.

Histidine rich protein 2 and 3 (*Pf*hrp2 and *Pf*hrp3) are used in rapid diagnostic tests (RDTs) for patients suffering from *P. falciparum* and *P. vivax* parasites. Malaria endemic regions have started showing natural deletion of *Pfhrp2/3* in *P. falciparum* which has become a major threat in diagnosis and treatment (206). Resistance towards chloroquine was first reported in Thailand in 1957. Similarly, SVMNT haplotype of *P. falciparum* chloroquine resistance transporter (*Pf*CRT) was first reported in 2004 in India (207). Primaquine is mainly used for the *P. vivax* hypnozoites but is restricted in case of pregnant women, infants and also G6PD deficient individuals (4).

Point mutations in *Pfdhps* (dihydropteroate synthase) and *Pfdhfr* (dihydrofolate reductase) genes are associated with antifolate resistance where the parasites develop resistance to pyrimethamine (208). In the current conditions artemisinin-based combination therapy (ACT) is the only working drug used for treatment. Mutation in *Pfkelch13* propeller gene(K13) is the major reason for artemisinin resistance (209).

Anopheles is a primary vector for *P. falciparum* and *P. vivax*. Insecticidal resistance is a biological process which has hindered elimination strategies. Surveillance of disease is an important aspect of malaria eradication strategy. Poor surveillance has become a major problem in Asian, African and Southern American countries where multiple gaps in disease observation and reporting have been observed (210-212).

Another major challenge that has come upon recently is outbreak of coronavirus 2 (SARS-CoV-2, COVID-19). Towards the end of 2019 it was initially reported in patients with severe pneumonia in Wuhan, China (213). Within a short period of time sars-CoV2 transmitted all over the world and WHO officially declared it as pandemic thus hampering all other major health systems as the focus of government was completely shifted to fight against the virus. This might affect control efforts taken for malaria as the symptoms for both the diseases are nearly overlapping (214,215). In the past decade malaria was controlled in an effective way and few countries were declared with zero reports of malaria cases. But after the pandemic, there is a sudden spike in new malaria cases and deaths which shows the lack of knowledge related to treatment of both the disease together and a major gap in healthcare system (216).

1.11 *Plasmodium berghei* as model malaria parasite

Laboratory culture of malaria has immensely helped several research groups to uncover particulars of cellular and molecular aspects of the parasite. Laboratory research on human malaria species of *Plasmodium* is restricted mostly to asexual blood stages and investigation of other life cycle stages have ethical and logistic concerns (217,218). Keeping this in view, the rodent malaria parasite model offers several advantages with reference to ease of handling and a close orthology with human malaria parasite. *Plasmodium berghei* infects specifically rodents. It was first discovered by Vincke and Lips in 1948. *P. berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei* are the rodent malaria parasites. These are transmitted in laboratory rats and the erythrocytic cycle is 22-24 hrs. It is a popular model organism also used for studying *Plasmodium* genetics (219,220). Particularly *P. berghei* is one of the most extensively used rodent malaria species that mimics several symptoms of human malaria like sequestration and cytoadherence (218). Malaria can be readily transmitted to mosquito by using *P. berghei* parasite, that allows investigation of *Plasmodium* stages occurring in mosquito gut and salivary gland. The

genome of parasite is highly conserved across different *Plasmodium* species and around 80% of the genes are orthologous that roughly corresponds to 5300 genes (221). The feasibility of genetic manipulations like promoter swap, single or double homologous cross over and conditional silencing is possible with *P. berghei*. The genome is 18-20mb in size with 14 chromosomes. It has 3890 orthologous genes, showing 63% protein sequence identity and near about 70% nucleotide sequence similarity when compared with *P. falciparum* (222). With the use of variety of genetic engineering tools, genome of *P. berghei* can be manipulated proficiently to decipher the function of a gene (223,224). Use of endogenously tagged GFP, mCherry parasites or obtaining parasite proteins as translational fusions with certain reporters, enables the localisation and investigation of protein expression in a stage specific manner (220).

1.12 Research Objectives

Malaria eradication presents several challenges, including a complex *Plasmodium* life cycle, asexual blood stage parasites showing antigenic variation, development of antimalarial drug resistance by parasites, and *Anopheline* strains getting resistant to insecticides. To unravel roles of different essential genes performing important functions to successfully complete the parasite life cycle, an effective system of gene manipulation is required. *P. berghei* is an excellent model organism for genetic manipulation and generating knock out (KO) mutants with the help of reverse genetics approach. It is a very efficient way of understanding the functions of certain genes by cycling the KO mutants through both the insect and mammalian host. The targeted gene is being replaced by double cross over recombination technique with a fluorescent reporter marker along with a drug resistance cassette which facilitates selection of recombinant parasites. An arrest in any parasitic stage is considered to be a probable role of the deleted gene, likely providing information regarding the function of that gene in a stage specific manner. If the targeted gene is found out to be important during selection stages like blood stages in turn preventing the investigation of the gene in further life cycle stages, a yeast FLP (flippase, recombinase)/FRT (recognition sequence) conditional mutagenesis system is employed. The Flp recombinase is expressed particularly in mosquito stages of *Plasmodium* leading towards excision of FRTed gene sequence which subsequently helps to characterise the phenotype of the target gene in further life cycle stages. With the aforementioned approaches, we have

(a) localised PhIL-1 interacting protein (PhIP) protein in *Plasmodium* life cycle stages followed by complete genotypic and phenotypic characterisation (Chapter 2).

(b) generated mutant parasites by conditionally silencing Heme detoxification protein (HDP) and investigated its role in mosquito stages (Chapter 3).

Chapter II:

Decoding the role of PhIL1 Interacting Protein (PhIP) (Pb ANKA_1409200) in Plasmodium berghei

2.1 Introduction

Malaria is one of the most lethal tropical parasitic diseases claiming maximum lives worldwide yearly. *Plasmodium* species are the causative organism for the disease (8). These digenetic apicomplexan parasites have a multifaceted life cycle that cycles within a mosquito vector and a vertebrate host (225). The ability of parasites to deceive the host immune system by antigenic variations confers survival fitness to the parasite, that in turn induces virulence in the mammalian host (226). Thus, there is an urgent need to validate newer targets, that can prevent malaria. Current anti-malarial drugs mostly target the asexual blood stages and gametocytes. However, due to the ability of *Plasmodium* to develop resistance against several antimalarials, malaria biologists are forced to identify novel targets (227).

To understand the pathogenesis of the disease a detailed investigations on multiple aspects of parasite biology is needed that includes transmission dynamics, molecular basis of invasion and host defense mechanisms (228). Blocking malaria transmission could be one of the major breakthroughs in preventing the disease spread among population, but yet is an underrated approach owing to practical challenges in demonstrating its proof of principle. Developing transmission blocking drugs would have immense public benefit, especially for the poor countries (229). To strengthen such approaches a detailed understanding of all the events associated with sexual reproduction is highly imperative. Gametocytes are the sexually dimorphic forms of *Plasmodium* that arise from a subset of merozoites, seem to be committed while inside a schizont. Infact all the daughter merozoites of one schizont are committed to either form male or female gametocytes only. Transition from asexual to sexual stages is an irreversible process that leads to terminal differentiation of gametocytes which can only undergo further development when it is taken up by next vector. In the past few years many recent advancements has boomed towards transmission blocking strategies reduces the risk of overall disease burden in population, specially belonging to tropical region (230).

Initial developmental transformation of gametocytes is almost morphologically indistinguishable from early asexual stages. Unlike the primary stages of gametocytes, final stage known as stage V is visible in peripheral blood circulation. The D shaped stage

I gametocytes lose their rigidity to form the characteristic crescent shape forms that mark the beginning of stage IV and finally transforms into a more rounded structure- referred to as the stage V gametocytes. Both the onset of gametocytogenesis and the entire metamorphosis of gametocytes is highly dependent on a complex structure called the Inner membrane complex (IMC) which occurs below the parasite plasmalemma structurally reinforcing it (231). Ultrastructural analysis reveals IMC as a double layer membrane organelle consisting of several flattened membranal sacs called alveoli and a cytoplasmic face connected to a highly organised network of intermediate filament like proteins present in apicomplexan parasites (158,232). Apicomplexan life cycle is highly dependent on motility and migration through insect host to vertebrate host for invasion and internalisation (232). Therefore in unicellular pathogens like *Plasmodium*, *Toxoplasma* and *Cryptosporidium* , the IMC plays a vital role where it not only helps in motility and invasion but also confers shape and stability to the cell by acting as scaffolding compartment daughter cell during formation (233). Due to the indispensable role of IMC in parasite life cycle, targeting unknown proteins that make up the complex would offer novel intervention options to prevent malaria infection.

The *de novo* biogenesis of IMC occurs during the process called schizogony, when new daughter cells are formed to become merozoites and each merozoite will be having their own IMC on the apical end prior to nuclear division (233). The growing IMC gradually develops from apical end further surrounding the newly formed daughter cells in the form of a ring. The heading edge of increasing IMC migrates from apical pole to basal pole of daughter cell till the completion of schizogony (232). During maturation of parasite, the IMC gets flattened and appears to arise from Golgi- associated vesicle to form a large membranous sheet, enveloping the parasite (234).

Plasmodium completes its complex life cycle through several stages of which three stages are endowed with the capability of motility and invasion. These stages are: the merozoites, the ookinetes and the sporozoites- that invade respectively the RBC, the midgut epithelium and salivary glands of female *Anopheles* vector. These stages are together referred to as zoites (235,236). Motile zoites are having a unique cortical structure called pellicle. Additionally, all zoites are highly dependent on substrate-dependent locomotion called as gliding motility. Gliding motility is one of the most important aspects in the parasitic life cycle. It helps for rapid penetration through cell

membrane for invading intracellular niche or to cross non-permissive biological barriers by an actinomyosin based motor system. It is a part of bigger invasion machinery present beneath the plasma membrane, called glideosome (235,237). Glideosome is positioned within the pellicle that is composed of parasite plasma membrane, IMC and sub pellicular network beneath the IMC (238).

Targeting proteins which are responsible for the synthesis and organisation of pellicle and apical complex could be an interesting strategy of controlling infection caused due to apicomplexan parasites (239). Till date there are 45 IMC proteins, identified in *Plasmodium* (232). Phylogenetic investigation of existing IMC proteins has shown that ancestral eukaryotic proteins like coronin and alveolate specific proteins like alveolins, have together contributed to the evolution of IMC. The IMC manifest diverse functionalities in apicomplexan ranging from structural integrity, invasion and division inside a specific host cell, which is also attributable for the phenomenal evolutionary diversity in the parasite (233).

Alveolins are an interesting group of peripheral membrane proteins constituting a subpellicular network towards the cytoplasmic face of IMC. Proteins of this family have been identified to be involved in stage specific expression and also in parasite morphogenesis, gliding motility in ookinete and sporozoites. This multi-protein family consists of 13 proteins in total and are reported to be expressed across several life cycle stages (e.g., IMC1a/ALV1, IMC1c/ALV5) of *Plasmodium* spp. While few are restricted only to asexual blood stages or gametocyte stages (e.g. IMC1/ALV6) (240-242). Proteins which are directly associated with IMC are IMC1 a, b and h and those with gliding motility include glideosome associated proteins 40, 45, 50 and 70 (GAP40, GAP45, GAP50 and GAP70) and glideosome associated proteins with multiple membrane spans (GAPMs), together with Myosin A (MyoA) and the myosin A tail-interacting protein (MTIP) in *Plasmodium* species (237). Apart from IMC proteins there are IMC associated peripheral membrane proteins like the IMC sub-compartment-proteins (ISPs) which are also characterised in *Plasmodium* (**Fig. 8**). Mostly they are attached to IMC with the help of lipid anchor (157). Two of these ISPs (ISP1 and ISP3) are important for defining apical polarity (239).

Keeping in the view the diverse roles of proteins in IMC for regulating *Plasmodium* biology and maintaining stability during various motile life cycle stages, yet certain proteins are under explored. One of the reasons of this could be the abundance of GPI anchored family proteins dominated on the surface of the parasite which gives less accessibility to identify and investigate non-GPI linked proteins. To overcome this problem non-GPI anchored proteins were labelled with photoactivatable compound 5-[^{125}I] iodonaphthalene-1-azide (INA) which can be activated by photosensitizing fluorochromes. Due to this approach a protein called photosensitized labeling with 5-[^{125}I] iodonapthaline-1-azide (INA Labeled protein 1(PhIL1) could be recognised for the very first time in *T. gondii*. This protein is conserved among apicomplexan parasites and localised towards periphery in IMC (243). Disruption of *TgPhIL1* showed a shorter and wider morphology of parasite as compared to wild-type, concomitant with the mutants being outcompeted in growth when compared to control parasites (244). The C-terminal part of *Plasmodium* PhIL1 shares 56% similarity with *TgPhIL1* whereas the N-terminal region is highly divergent. *Plasmodium* PhIL1 was shown to have a spine like structure, appeared as longitudinal orientation with an array of microtubule surrounding them with a ribbon like arrangement (245). Further *Plasmodium falciparum* PhIL1 was found to be localised to IMC and present in all motile cum invasive stages. The expression of PhIL1 has also been detected in male gametogenesis during sexual stages (225).

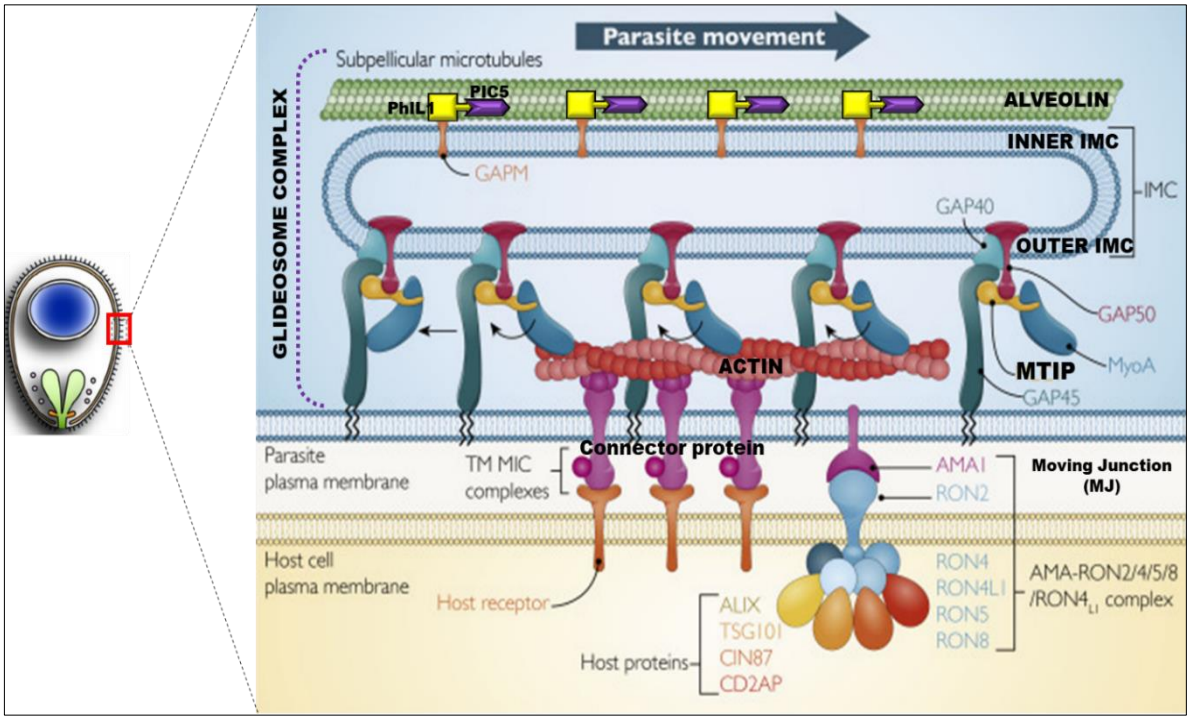


Fig. 8: Model IMC gliding machinery partially overlaying protein complex. Photosensitized 5-[125I] Iodonaphthalene-1-azide Labelled Protein-1 (PhIL1); PhIL1 Interacting Protein (PhIP). Glideosome-Associated Protein 40, 45 and 50 (GAP40, 45 and 50); Glideosome-associated protein with multiple membrane spans (GAPM); myosin-A (MyoA); Myosin-A Tail domain Interacting Protein (MTIP). Connector Protein between actin and protein of the merozoite surface (CP). C-terminal cytosolic domain (CD) of transmembrane (TM); microneme proteins (MICs); (Apical membrane antigen) AMA1-(Rhoptry Neck Protein) RON2/4/4L1/5/8 complex, which is proposed to form the MJ during host parasite interaction. Picture adapted and modified from (246)

The *Plasmodium falciparum* PhIL1 consists of 224 amino acids (247,248). It was found to be expressed in sexual, sporogonic, liver stages, including blood stages like ring, trophozoite, schizonts and male gametocytes. There seems to be a likely transcriptional repression of *PhIL1* in female gametocytes as the localisation studies shows its presence in zygote. When compared to its IMC-associated counter parts, PhIL1 has both apical and basal polarity. Further, knock out of *PhIL1* in *Plasmodium berghei* was also not successful which implies an important role in blood stage development of parasite. Knock down of *PhIL1* was not completely successful in gametocytes and ookinete stages as a fractional expression of PhIL1 was sufficient for the parasite to survive (225).

PfPhIL1 was used as a bait for identifying several interacting candidates and novel IMC proteins, known as PhIL1 interaction candidates (PIC). These included the plant-like vacuolar-type H+-pyrophosphatase (PF3D7_1235200, *Pf*VP2), that showed localisation both in parasite plasma membrane and as punctate intracellular inclusion (247,249). Other interacting partners included KIC8 (PF3D7_1014900) and MCA2 (PF3D7_1438400) proteins that are linked to Kelch13. Kelch13 mediates host cell haemoglobin uptake by endocytosis pathway and that is crucial for artemisinin resistance. Kelch13 and its interacting proteins mediate resistance upstream of both drug activation and action. Point mutation in *Kelch13* is mainly associated with artemisinin resistance and conditional inactivation of Kelch13 causes a developmental block in ring stage (250). Finally, the interactome also detected *Plasmodium* myosin (PF3D7_0613900, MyoE) which showed differential gene expression and function in invasive and proliferative stages (251).

Apart from the above-mentioned, a total of 6 putative IMC proteins were identified which are as follows: PF3D7_1229300(PIC1), PF3D7_0822900(PIC2), PF3D7_0415800(PIC3), PF3D7_0308300(PIC4), PF3D7_1310700(PIC5) and PF3D7_0530300(PIC6).

Functional inactivation of PIC1, PIC3 or PIC4 revealed no growth phenotype. PIC2 has previously been studied to be involved in maturing schizonts and segmented merozoites till the egress and invasion to the fresh host cells (252). PIC5 was localised to be in IMC of schizonts and daughter merozoites. It is also having a RNA binding domain (RBD) (253). 3'UTR of PIC5 is refractory to any modification or tagging due to which it was expressed under blood stage promoter AMA1 to detect the localisation. Owing to the presence of RBD, and protein being recruited to IMC in schizonts and daughter merozoites, functional investigation of the role of PIC5 across other life cycle stages is worth exploring (247).

In *Plasmodium falciparum* PIC5 or PhIL1 interacting Protein (PhIP) is localised to IMC along with two well established proteins- ALV5 and GAPM2 as expected also colocalises with PhIL1 protein in asexual blood stages. Knock down of *PfPhIP* impacted the reinvasion of the newly released daughter merozoites that additionally manifested a disoriented structure though few of the PhIP depleted merozoites behaved normally and invaded new host cells. This suggests that PhIP might be important during cell division for both maintenance of cellular integrity of daughter cells along with its role in reorientation of merozoites during the overall process of invasion (237). IMC integrated PhIL1 associated protein complex has a different role than glideosome complex. This helps in parasite survival and invasion in erythrocytic stages which was impaired when two or more proteins from this complex were targeted. Further this also gives an insight for new investigation regarding different candidates residing in IMC (237).

In the current study we investigated the role of PhIP in sexual stages of *Plasmodium berghei*, owing to high sequence similarity of almost 64% between the rodent and human *Plasmodium* parasite. In addition to that we did not find any other homolog of *PbPhIP* while probing for its evolutionary relatedness using nucleotide BLAST. Further conserved domain analysis revealed that the protein sequence has two essential domains, having major function in exporting and stabilizing RNA. Our approaches to endogenous tag either HA-mCherry or HA alone yielded non-viable parasites in mosquito stages reiterating the previous reports of 3' regulatory element of *PbPhIP* being refractory to genetic modification. However, *PbPhIP* antisera localised the protein to parasite periphery/membrane in multiple life cycle stages, suggesting its IMC association. To inspect the role *PbPhIP* in mosquito stages and liver stages of *Plasmodium*,

we generated a yeast based Flp/FRT conditional mutant where an engineered FRTed *PbPhIP* locus experiences excision or editing when the conditional mutants produce Flp recombinase system. The mutant lone was validated for presence of FRT sites in the genomic locus. The characterisation of *PbPhIP* conditional mutants in mosquito and liver stages are currently underway.

2.2 Materials and methods

2.2.1 Animals used for experiment

Swiss Albino female mice were used for propagation of *Pb*PhIP~3XHA/HA-mCherry, GFP expressing WT or *Pb*PhIP KO (C1 and C2) and WT mCherry transgenic parasites. Female *Anopheles stephensii* mosquitos were allowed to obtain a blood meal from gametocyte positive mice to initiate sexual cycle in mosquito. The mice were around 6-8 weeks old and weighed approximately 20-25 grams. For maintenance of *Anopheles stephensii* colony, female New Zealand rabbit of 3-4 weeks old and weighing around 1.5 kilograms was used. All the animals were obtained from National Institute of Nutrition (NIN), Tarnaka Hyderabad and Hylasco Biotechnology (India) Pvt. Ltd. Use of animals in all the experiments was done in accordance with Institutional Animal Ethical Committee (IAEC) protocols.

2.2.2 Parasite lines

For localisation of *Pb*PhIP, *Pb*PhIP~3XHA/HA-mCherry tagged parasites were used along with *Pb*SPELD-mCherry as a positive control line. For phenotypic characterisation, *PbPhIP* KO was used along with *Pb*ANKA WT as control. For cross experiments *Pb*ANKA WT mCherry line, *Pb*ANKA WT GFP and both GFP reporter clones of *PbPhIP* KO were used. For generation of conditional KO of *Pb*PhIP, transgenic parasite line expressing FlpL recombinase driven by TRAP promotor (active in oocyst stages of *Plasmodium*) were used as parental line.

2.2.3 Bacteria

For the purpose of cloning and propagation of targeting constructs in *E. coli* cells DH5α (F$^-$ Φ80*lac*ZΔM15Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^- ,$_k^+$) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ$^-$) and DH10β™(F– *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ (*ara-leu*)7697 *galU galK* λ– *rpsL*(Str^R) *nupG*) were used and made chemically competent.

2.2.4 Retrieval of *Pb*PhIP gene and protein sequences from PlasmoDB and GeneDB databases

Retrieval of DNA and protein sequence of *Pb*PhIP, was done by using *Plasmodium* database Plasmodb (www.plasmodb.org) and GeneDB database (http://www.genedb.org/Homepage/P*berghei*) hosted by Sanger Institute. Gene identity and orthologues of other *Plasmodium* species were also retrieved from the same database.

2.2.5 Bioinformatics analysis

Sequences of rodent *Plasmodium* strains *P. berghei (PB, PBANKA_1409200)*, *P. yoelli (PY, PY02798) and human Plasmodium strains P. vivax (PV, PVX_122365)*, *P. falciparum (PF, PF3D7_1310700)* were retrieved from PlasmoDB. Sequences were aligned using multiple sequences alignment tool MUSCLE (MUltiple Sequence Comparison by Log Expectation) (http://www.ebi.ac.uk/Tools/msa/muscle/) to check the homology across different *Plasmodium* species. Pairwise identity and similarity among amino acid sequences of *Plasmodium* PhIP was calculated using SIAS (Sequence Identity And Similarity) tool (http://imed.med.ucm.es/Tools/sias.html). To identify any known functional motifs/domains within *Pb*PhIP, the amino acid sequence was used as a query in NCBI Conserved domain database (CDD) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Conservation of the domain across *Plasmodium* species was investigated by using Motif search tool (https://www.genome.jp/tools/motif/).

2.2.6 Primer information

S. No.	Name of the primer	Primer sequence (5'- 3')
1.	*Pb PhIP* RT FP	GAAATATGGTGCTTTACAAGTTTAC
2.	*Pb PhIP* RT RP	TCGTTATTTTCTGAGGATTCTTT
3.	*Pb*18S rRNA TA FP	AAGCATTAAATAAAGCGAATACATCCTTC
4.	*Pb*18S rRNA TA RP	GGAGATTGGTTTTGACGTTTATGT
5.	*Pb PhIP* KO 5' FP1	CAG**CTCGAG**TTTGGCTTGTTTTTACAACATCA
6.	*Pb PhIP* KO 5' RP1	ACG**ATCGAT**AGTTGCACGATAACTAAATAGG
7.	*Pb PhIP* KO 3' FP2	TAG**CGGCCGCG**TTCTAATTCATAGCATTTATATTGTG
8.	*Pb PhIP* KO 3' RP2	GAT**GGCGCGCC**TTTAATGTGCTTATGTCTTGTC
9.	*Pb PhIP* KO 5' confirmation FP3	TTAATAGTGTGCATTTTTTGG
10.	HSP70 5' UTR RP3	TTCCGCAATTTGTTGTACATA
11.	*Pb PhIP* KO 3' confirmation RP4	GATAATTTGGAAATATAAACAC
12.	hDHFR FP4	GTTGTCTCTTCAATGATTCATAAATAG
13.	*Pb PhIP* LOC 3'CDS FP5	GTA**CTCGAG**TCGTGCAACTTTTCCATTATTAA
14.	*Pb PhIP* LOC 3'CDS RP5	TAC**AGATCT**ATCTTCTTCTTCATCGTTATCAT
15.	*Pb PhIP* LOC 3' UTR FP6	TGC**GCGGCCGC**ACTGGTTCTAATTCATAGCATTTAT
16.	*Pb PhIP* LOC 3' UTR RP6	GAG**GGCGCGCC**TATTTCTCAATAATTAAAAACGGAA
17.	*Pb PhIP* LOC confirmation 3' CDS FP7	TTAGTAAAATTAATTCTATAATTTG
18.	mCherry RP7	TCGCCCTCGCCCTCGATCT
19.	3XHA RP9	GCGTAATCTGGAACATCGTATG
20.	*Pb DHFR/TS 3'UTR FP4*	AAAATGTGTATGTTGTGTGCATA
21.	*Pb PhIP* LOC 3' confirmation RP8	GATAATTTGGAAATATAAACAC
22.	WT *Pb* mCherry FP1	AA**CTCGAG**TCATGGATCATATCCACTAACAAT
23.	WT *Pb* mCherry RP1	AAT**ATCGAT**TGTGTTTTATTTGGATGTGCAAT

24.	WT *Pb* mCherry FP2	AT**GCGGCCGC**TTCTCTTGAGCCCGTTAATGAA
25.	WT *Pb* mCherry RP2	AT**GGCGCGCC**TAGGAAATTTGTTTATTTTTAT
26.	*Pb PhIP* cKO 5'FP1	GGC**GGCGCGCC**TGTGTGTGGTGTAAAATATATGT
27.	*Pb PhIP* cKO 5'RP1	AGG**GTCGAC**TGTGCTGTGAGAGAAAAATATTT
28.	*Pb PhIP* cKO ORF FP2	GTG**CTCGAG**ATGGAAAACAATCATAAACTTTAAC
29.	*Pb PhIP* cKO ORF RP2	TTG**GCGGCCGC**CCGAAACATTTTCATTTATTTACT
30.	*Pb PhIP* cKO 3'FP3	GGA**CTGCAG**ATTTGGTGAGGGCATACAATTTT
31.	*Pb PhIP* cKO 3'RP3	CCT**GGTAAC**TTATATACTAATTTCGAATGTGAAT
32.	*PHIP* CKO confirmation 5'UTR FP4	TACAATTTAAGTAATATAGAATAA
33.	*PhIP* CKO confirmation 5'UTR RP 4	TACTTTCTAGAGAATAGGAACT
34.	*PhIP* CKO confirmation 3'UTR FP 5	TACACTTTATGCTTCCGGCT
35.	*PhIP* CKO confirmation 3'UTR RP 5	ATAAATTTACAAATCGGTTAGTT

Table 1: List of primers and their sequences used for the real time PCR analysis, construction of transfection plasmids, confirmation of site-specific integrations.

2.2.7 Phylogenetic tree

For analysis of evolutionary relatedness of *PbPhIP*, nucleotide sequence was used as a query to retrieve homologs using NCBI blast. With the help of ClustalW, the sequences were subjected to multiple sequence alignment. Evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-2252.01) was generated. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 34 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. Evolutionary analyses were conducted in MEGA11 software.

2.2.8 Total RNA isolation and cDNA synthesis

Total parasite RNA was isolated from different developmental stages of *P. berghei*. Swiss Albino mice were infected with WT *P. berghei* line. When the parasitemia reached around 10-12%, mixed blood stages were collected in heparinised RPMI. Infected

erythrocytes were lysed twice using 0.5% saponin and centrifuged at 8000 rpm for 5 mins. The pellet was further washed two times with RNAse free PBS prepared in DEPC treated distilled water. The parasite pellet was stored in -80^{0}C until RNA extraction. For isolating RNA from mosquito stages of *Plasmodium*, 100 *Anopheles stephensii* mosquitos were infected with wildtype *P. berghei* ANKA parasites. Midguts and salivary glands were collected by dissecting the mosquitoes on day 14th and 18th respectively. The glands were later disrupted mechanically with the help of sterile plastic pestle and subjected to centrifugation at 800 RPM for 3mins at 4^{0}C. The supernatant containing sporozoites was gently collected, pelleted and stored in -80^{0}C until further use. Different developmental stages of EEFs derived from sporozoites added to HepG2 cultures were harvested at 13 hr, 24 hr, 36 hr, 47 hr and 65 hr. The cells were washed, pelleted and stored in -80^{0}C. From all the frozen pellets obtained from different life cycle stages, total RNA was extracted using Ambion pure link RNA mini kit (Cat no# 12183020). The procedure included lysing the sample in 500µl of lysis buffer containing 1% β-mercaptoethanol (78.13 g/mol) was added and vortexed vigorously. The mixture was passed through 25-gauge insulin syringe (28 G x 1/2 in. U-100 BD Micro-Fine™, Cat no# 329410) for 10-15 times to ensure effective lysis. The contents of the lysates were mixed with equal volume of 70% ethanol was vortexed for 30 sec. The mixture was then applied on to a filter cartridge assembled in a collection tube and centrifuged at 13000 rpm for 15 secs at room temperature. Flow through was discarded followed by addition of 700µl wash buffer I to the cartridge and centrifuged at 13000 rpm for 15 secs at room temperature. Washing step was continued by discarding the flow through. 500µl of wash solution II was added and centrifuged at 13000 rpm for 15 secs at room temperature. Previous wash step was repeated one more time and then the spin column was dried by centrifuging at 13000 rpm for 2 mins at room temperature. RNA was eluted using 50µl of preheated (70^{0}C) RNAse free water at 13000 rpm for 2 mins at room temperature. The concentration of eluate was estimated using nanodrop 2000 (Eppendorf, Cat no# ND-2000) at wavelength of 260 nm using RNase free water as a blank. Further to avoid DNA contamination, DNAse I treatment was performed in 10µl reaction mixture that contained 1X DNAse I buffer, 2µg RNA sample and 1 µl (1U) of DNase I. The mixture was then incubated at room temperature for 20 mins followed by addition of 1µL of 25mM EDTA and heating the tube at 65^{0}C for 10 mins to inactivate DNAse I activity. The sample was subsequently used for cDNA synthesis using the components as tabulated below (**Table no. 2**). The thermal

cycling conditions used for cDNA synthesis was 25^0C for 10 min, 42°C for 20 min, 98°C for 5 min. Following completion of reverse transcription, the cDNA samples were stored in -20°C.

1.	RNA - 2.0µgm
2.	10X PCR buffer - 3.0µl
3.	dNTPs (2.5mM each) - 1.5µl
4.	Random hexamers - 1.5µl
5.	RNase inhibitor - 1.5µl
6.	MulV reverse transcriptase (Applied biosystems, Cat no# N8080018) - 1.5µl
7.	DEPC treated water- volume made up to 30µl

Table 2: List of components used for cDNA synthesis

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.

2.2.9 Quantitative real time (qRT-PCR) expression analysis of *PbPhIP* expression across all the life cycle stages of *P. berghei.*

Expression of *PbPhIP* was measured in different life cycle stages by absolute quantification method using real time PCR. cDNA from all life cycle stages (as mentioned above) was used as template for gene expression analysis. As quantification was through absolute quantification method, gene specific standards were generated by cloning 165 bp of fragment of *PbPhIP* ORF in pTZ57R/T vector. To generate an internal control, standards for *Pb*18SrRNA were similarly generated by cloning 180bp fragment in TZ57R/T vector (Cat no# K1213). Both the clones were confirmed by restriction digestion using vector specific enzymes. For both gene specific standards, the plasmid concentration was adjusted in a way such that each microliter contained target gene copies in the range of 10^8 copies/µl to 10^2 copies/µl. qRT PCR was performed using SYBR Green PCR master mix (TAKARA TB Green® Premix Ex Taq™ II Tli RNase H Plus, Cat no.# RR82WR) that contained 0.25µM gene specific primers and template (either cDNA or varying log dilutions of plasmid standard) in a 10µl reaction mixture. EFFENDORF REALPLEX 2 qPCR machine (Cat no.#2894) was used for real time PCR. During qRT-PCR,

amplification and simultaneous detection of copy numbers of target gene and internal control will occur. The quantified target gene and *Pb18SrRNA* levels were expressed as plasmid equivalents. The data normalisation was done by obtaining the ratio of absolute copy numbers of *PbPhIP* and *Pb18S rRNA* for each sample and graph was plotted.

2.2.10 Agarose gel electrophoresis

Agarose gel electrophoresis was used for resolving DNA fragments released from plasmid or for analysis of the PCR product size following amplification of target DNA sequence. 1% agarose gel was prepared in 1X TAE buffer (Tris base, Acetic acid and EDTA). The solution was heated in microwave for 3 mins to solubilised the agarose (Lonza, Cat no# 50010). 2-4 µl of ethidium bromide (0.4 µg/ml, Sigma, Cat no# 1239-45-8) was added to the agarose solution and poured on the gel tray fixed priorly with an appropriate comb. After solidification, DNA samples were mixed with 6XDNA loading dye (ThermoScientific, Cat no# R0611) and the samples were loaded into the wells. To confirm the correct size of resolved DNA fragments, a DNA ladder was used as a marker in the range of 100 bp-1 kb (ThermoScientific, Cat no# SM0333). The gel was subjected to 100- 150 volts current for 40 mins to 1 hour. The agarose electrophoretic unit contained 1X TAE as tank buffer. After completion of run, the agarose gel was visualised using UV illuminator and documented using gel documentation system – E-BOX CX5 Edge (Vilber).

2.2.11 Generation of *Pb*PhIP (*Pb*ANKA_1409200) localisation construct

For localisation of *Pb*PhIP, pBC~3XHA-mCherry-hDHFR and pBC~3XHA-hDHFR vectors were used. The following elements were integrated into plasmids viz., MCS-1, 3XHA-mCherry or 3XHA, 3' UTR of *Plasmodium* HSP 70, hDHFR drug selection marker and MCS-II. The region between MCS-I and MCS-II carried the chloramphenicol resistance cassette. To generate translational fusions of both 3XHA-mCherry and 3XHA with *Pb*PhIP, ORF sequence preceding the stop codon was cloned in MCS-1 using XhoI and BglII enzyme. In the MCS-2, 647 bp of 3' region of *Pb*PhIP *was* cloned using NotI and AscI restriction enzymes. The primers used in the amplification of both fragments are listed in **table no. 1.** Both localisation constructs were confirmed for correct sequence. Both constructs were linearised using terminal restriction enzymes- Xho1 and Asc1. The targeting construct was electroporated in *P. berghei ANKA* line and recombinant

transfectants were enriched by pyrimethamine (Sigma, Cat no# 46706) selection. Successful double cross over recombination following transfection facilitated an in tandem placement of either 3XHA-mCherry or 3XHA to the *Pb*PhIP ORF. DNA was isolated from both reporter lines and sequencing through the recombined locus confirmed that both reporters were in frame to *Pb*PhIP ORF. Further to confirm correct integration of the reporter construct, integration products were amplified using primers that flanked beyond sites of integration and within the elements of targeting construct at both 5' CDS region and 3' region. The 5' CDS (FP7/RP7, FP7/RP9) and 3' regions (FP4/RP8) were amplified using primer sets as mentioned in the above **table no. 1**. Expression of endogenous reporter signals were investigated using Nikon AR fluorescent microscope either directly by or indirectly (by anti-HA staining) in both asexual and mosquito stages.

2.2.12 Generation of rabbit and mouse anti- *Pb*PhIP antibody

Two epitopes within *Pb*PhIP protein corresponding to position 88-107aa (EKAIEDAEKALENKESSEN, peptide 1 or P1) and 129-146 (EENDESVENLENEINEDG, peptide 2 or P2) were commercially synthesised from K.R. instruments & chemicals, Kolkata. The selection of P1 and P2 was based on their antigenicity and hydrophobicity index. Both peptides were conjugated to keyhole limpet hemocyanin (KLH) to enhance their immunogenicity. Female New Zealand rabbit, weighting 1.5 kg and aged 12-14 weeks was used for immunisation. Prior to immunisation, pre-immune bleed was collected to be used as a negative control in western blot and immuno fluorescence analysis. The rabbit was primed with a mixture of both synthetic peptides- P1& P2 (0.5mg/ml each) along with 0.5ml (0.1mg/ml) Freund's complete adjuvant (CFA) (Sigma, CFA, Cat no# F5506-10ml). The mixture was delivered through an intramuscular route. Two weeks following priming, the first booster was given with 0.5mg/ml of both peptides, mixed in 0.5ml of Freud's incomplete adjuvant (Sigma, IFA, Cat no# F5881-10ml). Subsequently, 3 boosters were given in an interval of 10-12 days. Ten days after final booster, the blood collected and was stored in 4^0C overnight. Next day, the sample was centrifuged at 9000 rpm for 10 mins to collect the serum. Serum was used to perform ELISA to determine the antibody titer. Rabbit was euthanised after collection of blood. To generate anti-mouse *Pb*PhIP antibody eight female Swiss Albino mice of 4-6 weeks were used and injected subcutaneously with both the synthetic peptides- P1&P2 (12.5 µg/ml each). After collecting pre-immune bleed, all the mice were primed with mixture of

peptides and CFA, followed by 4 boosters with IFA. The final collection was done as described above and the sera was preserved at -20^{0}C for future use.

2.2.13 ELISA

The enzyme-linked immunosorbent assay (ELISA) was used for measuring PhIP antibody titers. 50mM coating buffer was prepared with Na_2CO_3/$NaHCo_3$ (MERK, Cat no# 497-19-8) and 100 µg of both PI and P2 were dissolved. The pH was adjusted to 9.6. 100µl of coating solution was added in all wells of a 96 well plate and incubated overnight at room temperature. After removing the coating solution, the wells were washed once with sterile 1X PBS (ThermoScientific, Cat no# AM9624). Nonspecific blocking was performed with 3% BSA (Merk, Cat no# 1076192) prepared in 1X PBS at 37^{0}C for 1 hour. Antisera was initially diluted in 1% BSA in a ratio of 1:50 and incubated in the first well and in all subsequent steps, a, two-fold dilution was made. The dilutions were done in a total of 24 wells. The plate was incubated at 37^{0}C for 1 hour. All wells were washed with 1XPBS, PBST and PBS for 20 mins each. Anti-rabbit antibody conjugated to HRP (ThermoScientific, Cat no# 31460) was diluted at 1:5000 ratio in 1% BSA and 100µl of this solution was added to each well and incubated in 37^{0}C. After incubation, all the wells were washed with 1XPBS, PBST and PBS again. 50µl of ABTS chromophore (Sigma, Cat no# 194434) was added to each well for developing the chromogenic reaction. Following visualisation of optimal intensity of the reaction, 1M H_2So_4 was added to stop the reaction. O.D. was recorded in ELISA plate reader (AGILENT BioTek 800 TS Absorbance Reader) and titer value was calculated.

2.2.14 Immunofluorescence assay

To perform localisation studies, cells were spotted on a 10 well spotted slide (ThermoScientific, Cat no#1 014326412 white). The sample was air dried and fixed in 10% paraformaldehyde (Sigma, Cat no# HT5011) for 20 mins. and the sample was washed once with sterile 1XPBS (pH 7.2) for 5 mins. The cells were permeabilised for 15 mins using ice cold acetone and methanol in the ratio of 1:3, followed by washing with 1X PBS for 5 mins. Nonspecific blocking was done for 1.5h in 3% BSA prepared in 1X PBS. Primary antibody was prepared in 3% BSA and the samples were incubated with this solution for 1 h at 37^{0}C followed by 3 washes with PBS, PBST (0.1% Tween20, Merk, Cat no# 9005-64-5 in 1XPBS) and PBS for 20 mins each. Immunoreactivity was revealed by

using appropriate secondary antibody either anti-rabbit or anti-mouse conjugated respectively to Alexa flour 594 (ThermoScientific, Cat no# A-11062/A21442) or Alexa flour 488 (ThermoScientific, Cat no# A-11001/08), diluted in 3% BSA along with DAPI (Sigma, Cat no# 9564). The secondary antibody dilutions were made at 1:250 dilution in 3% BSA and incubated at 37^0C for 45 mins. Following incubation, the cells were washed 3 times with 1X PBS, PBST and PBS, 20 mins each. After the final wash the slide was air dried and mounted with ProLong gold antifade mountant (ThermoScientific, Cat no# P36930). Mounted slides were visualised under an upright fluorescent microscope (Nikon Eclipse AR). The images were captured, processed and deconvoluted by NIS elements software.

2.2.15 Generation of *PbPhIP* (*Pb*ANKA_1409200) knockout construct

2.2.15.1 PCR amplification

For disruption of *PbPhIP* gene, a double homologous recombination strategy was followed. To generate *PbPhIP* KO construct, pBC-GFP-hDHFR was used as targeting vector. *Pb* WT genomic DNA was used as template to amplify respectively 637bp and 679bp of 5' and 3'UTR regions of *PbPhIP* using primers pair FP1/RP1 and FP2/RP2 (**Table no. 1**). The PCR reaction mixture contained 1x NEB PCR Buffer, 1mM dNTPs each (ThermoScientific, Cat no# R72501), 2.5 mM $MgCl_2$(BIORAD, Cat no# 1708872), 0.25 µM each of forward and reverse primers, 50ng of gnomic DNA, 1.25U of Taq polymerase (NEB, Cat no# M0273). PCR thermal cycling was performed using Eppendorf master cycler using following conditions: initial denaturation at 95^0C for 3 mins followed by 95^0C for 30s, annealing at 60^0C for 45s and extension at 68^0C for 1 min. This thermal cycling was repeated for 30 times except the initial denaturation and final extension was at 68^0C for 10 mins. 5µl of the amplified product was run on 1% agarose gel and visualized in UV transilluminator. Further PCR amplified products were purified using DNA clean up and extraction kit (ThermoScientific, Cat no# K0832) and measured using nanodrop 2000.

2.2.15.2 Competent cell preparation by calcium chloride treatment method

DH10β *E. coli* strain was inoculated in 5ml of LB broth to initiate a primary culture (without antibiotic). The culture was maintained overnight in an orbital shaker incubator (Scigenics Orbitek LT-Orbital Laboratory Incubator Shaker) at 37^0C with 180 rpm.

Following day, a secondary culture was initiated by adding 1% overnight inoculum to 25ml antibiotic free LB broth and was incubated further for 2 to 3 hours kept at 37^0C with agitation at 180 rpm. The secondary culture was grown until the OD reached 0.35-0.4. Subsequently, the cultures were kept on ice for 30mins. Further the culture was centrifuged at 6000 rpm for 10 mins and supernatant was discarded. 3 ml of 0.1 mM $CaCl_2$ was added to the pellet and the cells were uniformly resuspended. The mixture was then incubated on ice for 1 hour and centrifuged at 6000 rpm for 10 mins to collect the pellet. The pellet was resuspended with 13% glycerol (Sigma, Cat no# G5516) prepared in 0.1mM $CaCl_2$ (Sigma, Cat no# G5516) for long term storage. Approximately 75-100µl aliquots were dispensed in 1.5 ml Eppendorf tube. The tubes were snap frozen in liquid nitrogen and preserved in -80^0C for future storage. The cells were used for bacterial transformation.

Prior to transformation, the cells were thawed on ice. 10-20µl of ligation mixture was added to the cells and incubated for 20 mins on ice. Later heat shock was given at 42^0C for 90 secs on a preheated block followed by a cold shock on ice for 5 mins. Further 1 ml of LB broth (HiMedia, Cat no# M1245) was added to the cells and incubated for 1 hour at 37^0C with 180 rpm. Later the cells were centrifuged at 3000 rpm for 5 mins and the supernatant was discarded. The pellet was resuspended in 100µl of LB broth and added to the LB agar (HiMedia, Cat no# M1151) plate containing the suitable antibiotic for selection of transformed bacteria. The plate was kept in 37^0C overnight in upside condition to allow the growth of recombinant/transformed colonies.

2.2.15.3 Cloning of 5' homologous region of *PbPhIP*

5' homologous region of *PbPhIP* was amplified by PCR and the product was purified. Concentration of the purified amplicon was measured using nanodrop 2000. Amplified product was cloned into the targeting vector- pBC-GFP-hDHFR which was a generous gift by Dr. Robert Menard from Pasteur Institute, France. The pBC-GFP-hDHFR vector contained two multiple cloning sites (MCS) sites- MCS-I and II located respectively upstream and downstream to the pBC-GFP-hDHFR cassette. To produce compatible restriction sites on the PCR product, 1- 2 µg of the amplified product was subjected to digestion in a total volume of 30µl. The cocktail contained 1U of XhoI (ThermoScientific, Cat no# FD0694), 1U of ClaI (ThermoScientific, Cat no# FD0143) and 3µl fast digest buffer and the volume was made up with nuclease free water (Ambion, Cat

no# AM9932). Similar restriction digestion was performed with 2-2.5µg pBC-GFP-hDHFR vector using same set of enzymes and fast digest buffer. Both the digested vector and insert were purified using DNA clean up and gel extraction kit (Fermentas, Cat no# K0831) and concentration was measured using nanodrop 2000. Both the digested and purified vector and insert were kept for ligation at 1:3 ratio in a reaction mixture of 20µl containing 1X ligation buffer (ThermoScientific, Cat no# EL0014), 1U of T4 DNA ligase and nuclease free water. The ligation mixture was incubated at 22^0C for 2-4 hours. 10µl of ligation mixture was added to the DH10β competent cell for transformation and after 1 hour of incubation, the cells were plated on a LB agar plate with chloramphenicol (HiMedia, Cat no# CMS218) antibiotic. The plates were incubated overnight for 37^0 C to allow growth of transformed bacteria. Following day, 10-15 colonies were inoculated in 200µl of LB broth and following 6-8 hours of bacterial growth at 37^0C, colony PCR was performed to select the positive clone. The positive clone was inoculated again in 5-10ml of culture to isolate plasmid using GeneJET Plasmid Miniprep Kit (ThermoScientific, Cat No# K0502). The clones were confirmed via double digestion to check the presence of the 5' insert using XhoI and ClaI. The vector was named as recombinant 5' *PbPhIP* + pBC-GFP-hDHFR.

2.2.15.4 Cloning of 3' region of *PbPhIP*

Similarly, 3' region of *PbPhIP* was PCR amplified and purified. The recombinant pBC-GFP-hDHFR vector carrying 5' *PbPhIP* was considered for ligating the 3' region of *PbPhIP* in the MCS-II. The restriction sites used for ligation was NotI (ThermoScientific, Cat no# FD0593) and AscI (ThermoScientific, Cat no# FD1894). As described in previous section, both the vector and insert were purified following restriction digestion with NotI and AscI enzymes for 1 hour. After measuring the concentration in nanodrop, ligation was kept with 1:3 ratio at 22^0C for 2-6 hours. For transformation, 10µl of the ligation mixture was mixed with DH10β cells and plated on LB agar plate after containing chloramphenicol. Positive clones were screened by colony PCR and the insert was confirmed by double digestion using NotI and AscI restriction enzymes. The pBC-GFP-hDHFR vector containing both the 5' and 3' regions of *PbPhIP* were sequenced and following confirmation of a mutation free sequence, the *PbPhIP* KO vector was purified in a large scale using PureLink™ HiPure plasmid filter maxiprep kit, (Invitrogen, Cat no# K210016).

2.2.15.5 Release of *PbPhIP* targeting cassette from knockout vector by restriction digestion.

The *PbPhIP* KO vector was digested with the terminal enzymes- Xho1 and Asc1 to release the GFP-hDHFR cassette containing the 5' and 3' flanking regions of *PbPhIP*. To ensure digestion of the back bone, an additional enzyme Kpn2I (BspEI) (ThermoScientific, Cat no# FD0534) was added to digest the remaining vector back bone in two segments. The digested product was run on 1% agarose gel for confirmation and purified using DNA clean up and gel extraction kit (Fermentas, Cat No# K0831). The concentration was measured using nanodrop 2000. The purified targeting cassette was used during electroporation of WT *P. berghei* schizonts to target the *PbPhIP* endogenous locus.

2.2.15.6 Propagation of *P. berghei* ANKA WT line in SA mice and giemsa staining of blood stage parasites.

A *P. berghei* ANKA WT stabilate maintained in -80^{0}C was thawed on ice and the blood was injected intraperitoneally into Swiss Albino mice. From third day post infection, parasitemia was monitored by making blood smears from the caudal vein puncture. The smear was air dried and the cells were fixed in 100% methanol for 5-8 secs. Fixation was followed by staining the smears with Giemsa (Sigma, Cat no# GS1L), prepared by diluting the stock solution at 1:5 ratio and further incubated for 10-12mins. The slide was then washed under tap water to remove any extra stain and air dried. Smear was observed under a 100x objective lens magnification using immersion oil under a light microscope (Lynx Lawrence & Mayo Binocular Microscope, Cat no# LM521710). Several parasitic stages were identified in infected RBC by the distinctive morphological features of each stage. To estimate parasitemia, a minimum of 25 uniform fields having approximately 280-300 RBCs each, were examined and parasitemia was determined.

2.2.15.7 *In vitro* culture of *P. berghei* ANKA asexual stages

The *in vitro* culture of *P. berghei* ANKA asexual blood stages, parasite transfection and drug selection of recombinant parasites were performed according the protocol described in the Nature article (254).

To initiate *in vivo* infection, Swiss Albino mice was injected intraperitoneally with *Pb ANKA* WT parasites. Parasitemia was monitored until it reaches around 2-3% and then the infection was passage into three recipient mice (around 10^7 parasites per mouse). When the parasitemia reached to 3-5%, the recipient mice were anesthetized by injecting 200µl premade anesthesia (300µl of xylazine, 800µl of ketamine and volume made up to 5ml with 1X PBS) into each mouse. Further the mice were dissected and blood was collected using heparinised pasture pipettes into 50ml falcon containing 250U of 1X heparin (Sigma, Cat no# H3393). The blood was cultured overnight in a media containing RPMI (Gibco™ RPMI 1640 Medium Green features, Cat no# 11875085), 20% Fetal Bovine Serum (Gibco™ Fetal Bovine Serum, Premium Plus Green features, Cat no # A4766801) and gentamycin (3.5 ml for 500 ml of media) (Gibco™ Gentamicin (10 mg/ml) Cat no #15710064). The media was filtered using a sterilized filtration unit (Corning®, Cat no #431097). This media is referred to as schizont culture media and the parasites were grown overnight to allow one additional cycle. This culture was used for schizont enrichment. 10ml of schizont culture media was added to the blood collected from animal and centrifuged at 200 g in a swing bucket rotor for 8min with an acceleration of 5 and deceleration of 0 (Eppendorf, Cat no #5180R). After spin the supernatant was discarded and remaining 1ml of pellet was added to two T75 cell culture flasks (T75 closed caps flasks, Nunc, Cat no# 156472) containing 20-25ml of prewarmed schizont culture media. Further the cultures were gassed with mixture composed of 5% O_2, 5% CO_2 and 90% N_2 for 3mins. Then the flasks were kept in an incubator at 36.5^0C for 16-18 hours with a gentle agitation at 37 rpm. Next day, after 12-14 hours of incubation 100µl of culture was taken and centrifuged for maximum speed for 10 sec at room temperature. The supernatant was discarded and a smear was made with the pellet and stained with giemsa to observe the frequency maturation of schizonts per field. Outline of the transfection protocol has been shown in **Fig. 9.**

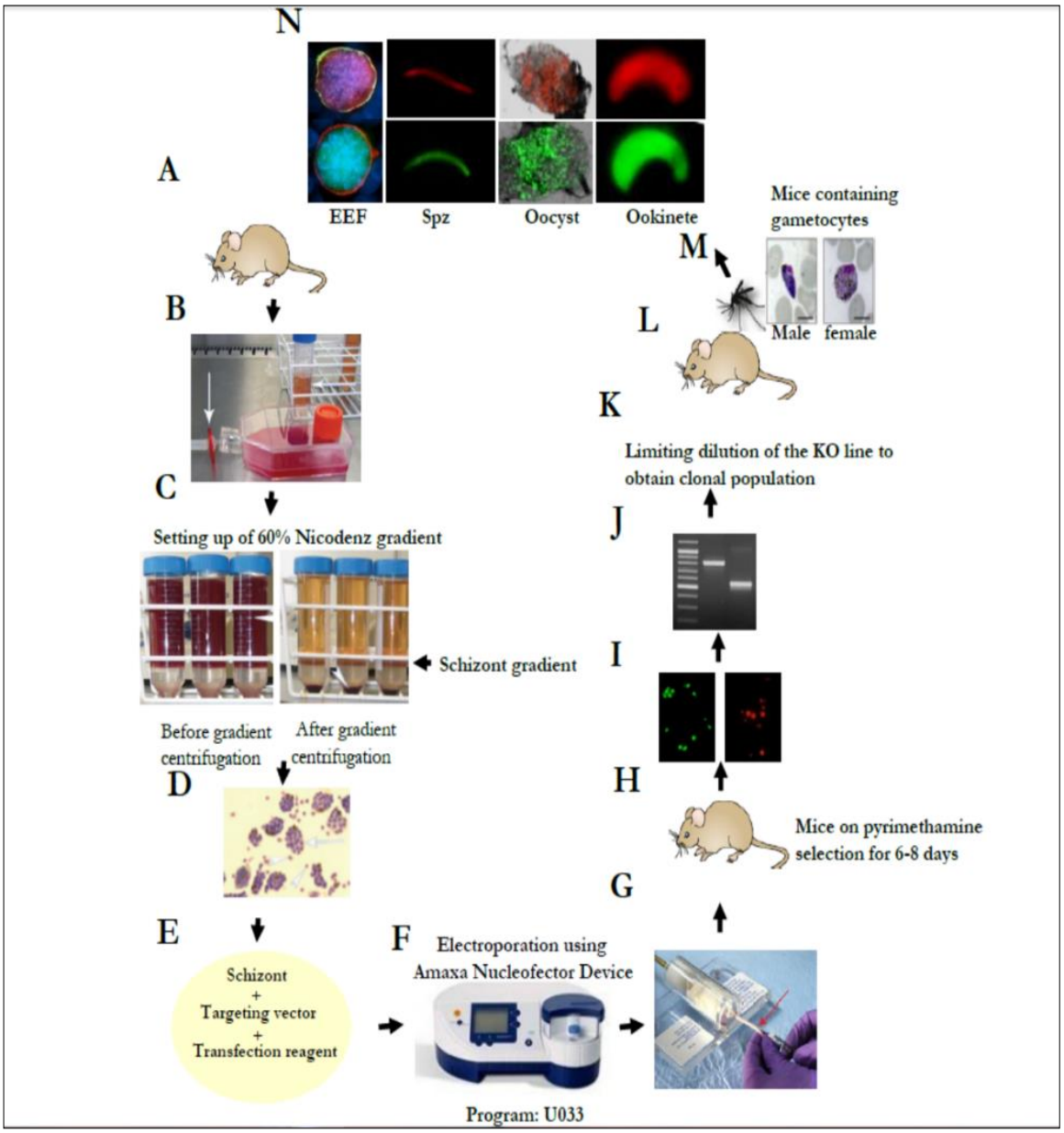


Fig. 9: **Schematic representation of transfection in *P. berghei*, drug selection, confirmation of target construct site specific integration and phenotypic characterisation of transgenic parasites**. A) *P. berghei* parasites were injected into mice. When parasitemia reached to 3-5%, blood was collected and B) Schizont culture was kept overnight. C) Following day, schizonts were enriched by 60% Nycodenz density gradient centrifugation. D) Purified schizonts confirmed by Giemsa staining. E) To the purified schizonts, targeting construct and transfection reagent were mixed, F) Electroporation was done using Amaxa electroporator and G) medium was added to the electroporate contents and immediately injected by intravenous route to Swiss Albino mouse. H) The mice were subjected to pyrimethamine drug administered orally in drinking water. I) Following 7 days of stringent drug selection, the parasites were analysed for GFP/mCherry fluorescence. J) Genomic DNA was isolated from drug resistant parasites and correct integration was confirmed by integration PCR. K) Limiting dilution was done to isolate single clonal population and to eliminate drug resistant wild type and non-transfectants. L) The clones were used for phenotypic characterisation; M) asexual blood stage propagation in the mouse, N) ookinete, oocyst and salivary gland sporozoite stages in the mosquito and liver stages the vertebrate host. (Images adapted and modified from the thesis of Dr. Segireddy Rameswara Reddy)

2.2.15.8 Purification of schizonts

Presence of mature schizonts were confirmed by Giemsa staining. Further the culture was subjected to schizont enrichment by Nycodenz density gradient method. A 100% Nycodenz solution was made by mixing 27.6 g Histodenz (Sigma, Cat no# D2158), 3 mM KCl, 5 mM Tris-HCl, 0.3 mM EDTA in a flask where volume was made up to 100ml using sterile double distilled water. Then it was autoclaved and stored in 4^0C. Prior to use, 60 % Nycodenz was prepared with 1X PBS and kept for warming at 37^0C for 15-20 mins. 25ml of schizont culture was taken along with 60% of Nycodenz and added to the culture in a 50ml falcon. Nycodenz was added in the culture directly to the bottom slowly without any disturbance to make an even layer. Culture was layered over the Nycodenz where a clear difference between both the media were observed. Further the culture was centrifuged at 380 g for 20mins with an acceleration of 5 and deceleration of 0 in a swing bucket rotor. After the spin a clear layer of schizonts forming a brown ring was seen between both the layers. The ring was collected with capillary tube and transferred to a new falcon. The enriched schizonts were washed with 5ml of prewarmed schizont culture medium twice by spinning at 1500 rpm for 7mins with an acceleration of 5 and 0 deceleration. Collected schizonts were used for electroporation.

2.2.15.9 Electroporation of *PbPhIP* knockout construct into schizonts

For electroporation of *PbPhIP* KO construct, Amaxa nucleofector kit (Lonza, Cat no# VPA-1006) was used. As instructed in the kit, 90µl of mouse T cell nucleofector solution was mixed with 10µl of supplement to make the nucleofector solution. Next 10µg of digested and purified *PbPhIP* KO construct was mixed along with the transfection mixture. Purified schizonts were pelleted down before electroporation and mixed with solution and slowly transferred to the nucleofector cuvette and placed in Amaxa nucleofector machine. Program U033 was used for electroporation, followed by addition of 100µl of prewarmed schizont culture media to the mixture. Following electroporetic pulse, the contents in the cuvette was immediately injected to Swiss Albino mice intravenously.

2.2.15.10 Drug selection of transfected parasites

After 48 hours of transfection, tail vein blood was collected and a smear was prepared to check the parasitemia. Once the parasitemia was confirmed to be 0.5-1%, the mice were orally administered with antifolate drug pyrimethamine to select the recombinant parasites. Drug was prepared by weighing 7mg of pyrimethamine (Sigma, Cat no# 46706) dissolved in 1 ml of dimethyl sulphoxide (DMSO) (Sigma, Cat no# D2650), mixed in drinking water. As the drug does not dissolve readily in water due to neutral pH, white precipitate can be observed after addition. To dissolve the drug completely in water, the pH was adjusted to 3.5-4.0 using 1N HCl (Sigma, Cat no# 258148). After third day of post drug treatment, a blood smear was prepared to confirm clearance of non-transfected parasites. On day 6^{th} or 7^{th} of post drug, a smear was prepared to observe the emergence of drug resistant parasites and on subsequent days blood smears were prepared to monitor the steady growth of recombinant parasitemia under drug cover.

2.2.15.11 Steady emergence of GFP expressing parasites during selection under pyrimethamine cover

Once the drug resistant or recombinant parasitemia reaches up to 3-5 %, GFP fluorescence was observed owing to the stable integration of the reporter cassette in the target gene locus. 5 µl of blood was collected by mouse caudal vein puncture and mixed with 2U of heparin. Blood was pelleted down and washed twice with 1X PBS and incubated with DAPI (4', 6' diamidino-2 phenyl indole, Sigma, Cat no# 9542- prepared by adding 1 mg of DAPI in 1ml of 1XPBS) at 37^0c for an hour. Cells were pelleted down after incubation and resuspended with 10µl of 1XPBS. 3µl of the mixture was placed on a slide and cover slip (Fisherbrand™, Cat no# 12-544-18) was placed on it. The sides of the cover slip were covered with transparent nail polish. The GFP fluorescence was observed under an upright Nikon Eclipse microscope. The images were processed with NIS software.

2.2.15.12 Cryopreservation of the *P. berghei* blood stage parasites

Parasites expressing GFP confirmed the presence of recombinant population or transfectants. To preserve the recombinant parasites, infected blood was collected in freezing medium with 250U heparin. The freezing medium contained nine parts of Alsever's solution (Sigma, Cat no# A3551) and one part sterile glycerol (Invitrogen, Cat

no# 15514011). To cryopreserve the parasites, one part of blood was collected by retro orbital puncture and mixed with 2 parts of freezing medium. 250µl of the mixture was aliquoted to each cryovials (Corning, Cat no # CLS430658) and kept in -20^{0}C for four hours and then transferred to -80^{0}C for longer storage. Further the vials were shifted to liquid nitrogen for a long-term storage.

2.2.15.13 Confirming the stable integration of *PbPhIP* knockout construct in endogenous locus

Infected blood was collected in an eppendorf tube and centrifuged at 8000 rpm for 5 mins at room temperature to pellet RBCs. The cells were lysed in 0.5% saponin (Sigma, Cat no# 84510) twice. The pellet was resuspended in sterile 1X PBS once and centrifuged at 8000 rpm for 5 mins. Genomic DNA was isolated from the parasite pellet using NucleoSpin tissue, mini kit for DNA from cells and tissue (Macherey-Nagel, Cat no #740952.250).

Manufacturer's protocol was followed to isolate genomic DNA. In brief, parasite pellet was resuspended in 200 µl of T1 buffer. To this, 25 µl of proteinase K (20mg/ml) and 200µl buffer B3 was added. The mixture was vortexed vigorously and incubated at 70^{0}C for 15 mins. Following incubation, 100% ethanol was added and vortexed to precipitate DNA. The solution was added to nucleospin column and subjected to centrifugation at 11000g for 1 min at room temperature. The column was washed first with 500µl BW buffer followed by 600µl of B5 buffer and centrifuged at 11000 g for 1 min at room temperature. The column was air dried and placed in a separate tube. For elution preheated 50µl buffer BE was added to the center of the column and incubated for 1 min at room temperature. The column was centrifuged for 2mins at 13000 rpm and the concertation was measured using nanodrop 2000. To confirm the integration of *PbPhIP* knock out construct in the endogenous locus, integration PCR was performed using specific primers designed beyond the site of integration. For confirmation both 5' UTR (FP3/RP3) and 3' UTR (FP4/RP4) integration primer pairs were used as mentioned in **table no. 1**.

2.2.15.14 Clonal dilution of *PbPhIP* knockout parasites

To enrich the integrants, transfected parasites were selected using pyrimethamine. WT parasites that survive and become drug resistant has to be eliminated to obtain a homogeneous parasite population from a single parent. To achieve this, limiting dilution was performed. After confirming correct integration of the knockout cassette into the endogenous locus, blood was collected from the infected mice when the parasitemia reaches around 2-4%. The blood was diluted to 10,000 fold in incomplete RPMI (Gibco, Cat no# 11875093) and the total number of RBCs were calculated using a hemocytometer. Further the number of iRBC/µl was calculated and the sample was diluted to obtain 1or 0.5 parasites/µl. A group of 10 mice were intravenously injected with one parasite each suspended in 200 µl of incomplete RPMI and other 10 were injected with 0.5 parasite per mouse (one parasite per two mice). After 7 days of post injection, blood smears were prepared to check infection. Out of 20 injected mice, 2-4 mice were found to be positive for infection. To confirm that the rise in parasitemia was of clonal nature and was devoid of WT parasites, a diagnostic PCR was performed using genomic DNA as template using primers as shown in **table no. 1**. The products of correct integration were analysed on 1% agarose gel along with a 1Kb DNA ladder.

2.2.15.15 Phenotypic analysis of *PbPhIP* knockout (KO) parasites in blood stages

Since *PbPhIP* KO line was obtained as GFP expressing line, a suitable control of WT *P. berghei* ANKA GFP line was generated by targeting non-essential *p230P* locus with GFP-hDHFR cassette and the line was named as *Pb* WT GFP line. To check the asexual growth of both control *Pb* WT GFP and *PbPhIP* KO mutant line, around $1x10^3$ parasites were intravenously injected to 6 weeks old Swiss Albino mice. From day 5 post injection, the parasitemia was monitored till 8^{th} day by preparing giemsa stained blood smears on a daily. The kinetics of asexual growth of KO in comparison with WT control was investigated by observing 25 random fields daily under light microscope. The mean percentage of parasitemia was calculated and plotted as a graph.

2.2.15.16 Gametocyte enrichment and investigation of gametocytemia

Pb WT GFP and *PbPhIP* KO mutant parasites were injected intraperitoneally to two different mice. Once the parasitemia reached up to 3-4%, two separate group of mice (4 each) were treated with phenylhydrazine (Sigma, Cat no# P26252) to enrich

reticulocytes. 250 mg of phenylhydrazine was weighed and dissolved in 0.9% NaCl prepared in sterile 1XPBS. The mixture was vortexed vigorously and each mouse was injected with 100 µl of phenylhydrazine intraperitoneally. After 24 hours of injection the mice were passaged with either *Pb* WT GFP or *PbPhIP* KO mutant parasites (10^7 parasites per mouse). Giemsa-stained blood smear was prepared after 3rd days post infection to monitor the parasitemia. Once the parasitemia reached to 10-12% with having 3-5 gametocytes per field, the infected mice were treated with sulphadiazine drug (Sigma, Cat no# S8626-30mg/1L) prepared by adding 7.5 mg of sulphadiazine in 1ml of DMSO. The mixture was vortexed thoroughly and dissolved in 250ml of drinking water and administrated orally to the infected group of mice. The drug cleared all asexual stages and selectively enriched the gametocytes. After 24 hours post drug treatment, a confirmatory slide was prepared and stained to check the clearance of asexual stages and enrichment of gametocytes per field. After 48 hours of drug treatment, a giemsa stained slide was made using a drop of blood taken from tail vein to confirm complete enrichment of gametocytes. Gametocytes were calculated from 7th day of post infection till 10th day. After calculating percentage of gametocytemia in both WT and mutant, a bar diagram was plotted.

2.2.15.17 Flow cytometric analysis of asexual growth and enriched gametocytes in WT vs Kos

Blood vials of WT GFP, WT mCherry and *PbPhIP* KO cloned lines were thawed. Swiss Albino mice were injected with respective lines intraperitoneally. After 4 days of post infection 25 giemsa stained fields from each infected mouse were counted to determine percentage of parasitemia. For positive control, a mixture of WT GFP and WT mCherry ($1X10^3$ parasites of each reporter line) were injected intravenously to a group of two mice. Similarly, for experiment group, KO clone1 and clone2 (each) were injected two mice respectively along with WT mCherry parasites in both ($1X10^3$ parasites of each line). Giemsa-stained smears were prepared from day 4th of post injection to observe the growth of parasites. On day 6th of post injection 2µl of blood was collected from each group and diluted up to 200 times with sterile 1XPBS for FACS analysis. The flow cytometric analysis of GFP and mCherry positive parasites were performed using a BD LSR fortessa II. Samples were acquired using BD FACS Diva software at a sorting speed of 1,00,000 events per second. Excitation of GFP and mCherry was done at a wavelength

of 488nm and 594nm respectively. All the gating was performed according to the control parasites and applied to other experimental samples. Doublets gate strategy was performed to exclude the doublets and to select the single population (FSC-H vs FSC-W). Subsequently, forward and sideward scatter gating was used to gate the erythrocyte population and to eliminate small particles viz. blood platelets and predominantly leukocytes. The erythrocyte population were sorted by measuring the fluorescent intensity of GFP vs mCherry. Flow Jo software was used for all flow cytometry analyses. Above mentioned strategy was followed on 8th day of post infection too. Further the same set of mice were subjected to gametocyte enrichment. The groups of mice were kept under observation to confirm 3-4 gametocytes per field as judged by giemsa staining. Gametocyte enrichment was done according to the protocol discussed above using sulphadiazine drug. After calculating the mean for the GFP +ve, mCherry +ve and double +ve infected RBCs for both asexual growth and enriched gametocytes, graph was plotted using GraphPad prism software to analyze the results.

2.2.15.18 Breeding of *A. stephensii* mosquitoes

After investigating asexual blood stage growth of the mutant parasites, they were further transmitted to female *Anopheles* mosquitoes to initiate a sexual cycle. To maintain the mosquito colony, freshly emerged male and female mosquitoes held in captivity were allowed to mate for 2 days. The mosquitoes were starved for 6 h and then allowed to obtain a blood meal from a rabbit (female New Zealand rabbit) anesthesised intramuscularly with a mixture composed of 0.8 ml of ketamine (50 mg/ml) and 0.4 ml of xylazine (20 mg/ml). The sedated rabbit was placed on the breeding cage to allow the mosquitos to take blood meal for 30-35 mins on two successive days. 48 hours after the blood meal, a water bowl was kept inside the mosquito cage to collect eggs layed by the female mosquitos. Eggs were collected up to 4 days, and the bowls were shifted on a daily basis to mosquito breeding chamber maintained at 27^{0}C and 75-80% relative humidity (RH). Once the eggs hatched into larvae, they were transferred to large plastic trays with 2 liters of autoclaved single distilled water. For providing food to the growing larvae a mixture of powered Kellogg's special K low fat cereal with wheat germ (Avees) in the composition of 60:40 ratio was prepared and added to the trays. Trays were filtered on alternative days and a pinch of food was added to each tray on a daily basis. After 6-7 days post hatching, the larvae transformed to pupae. The pupae were collected manually and

kept in a petri plate within a plastic cage for the mosquitoes to emerge as shown in **Fig. 10**

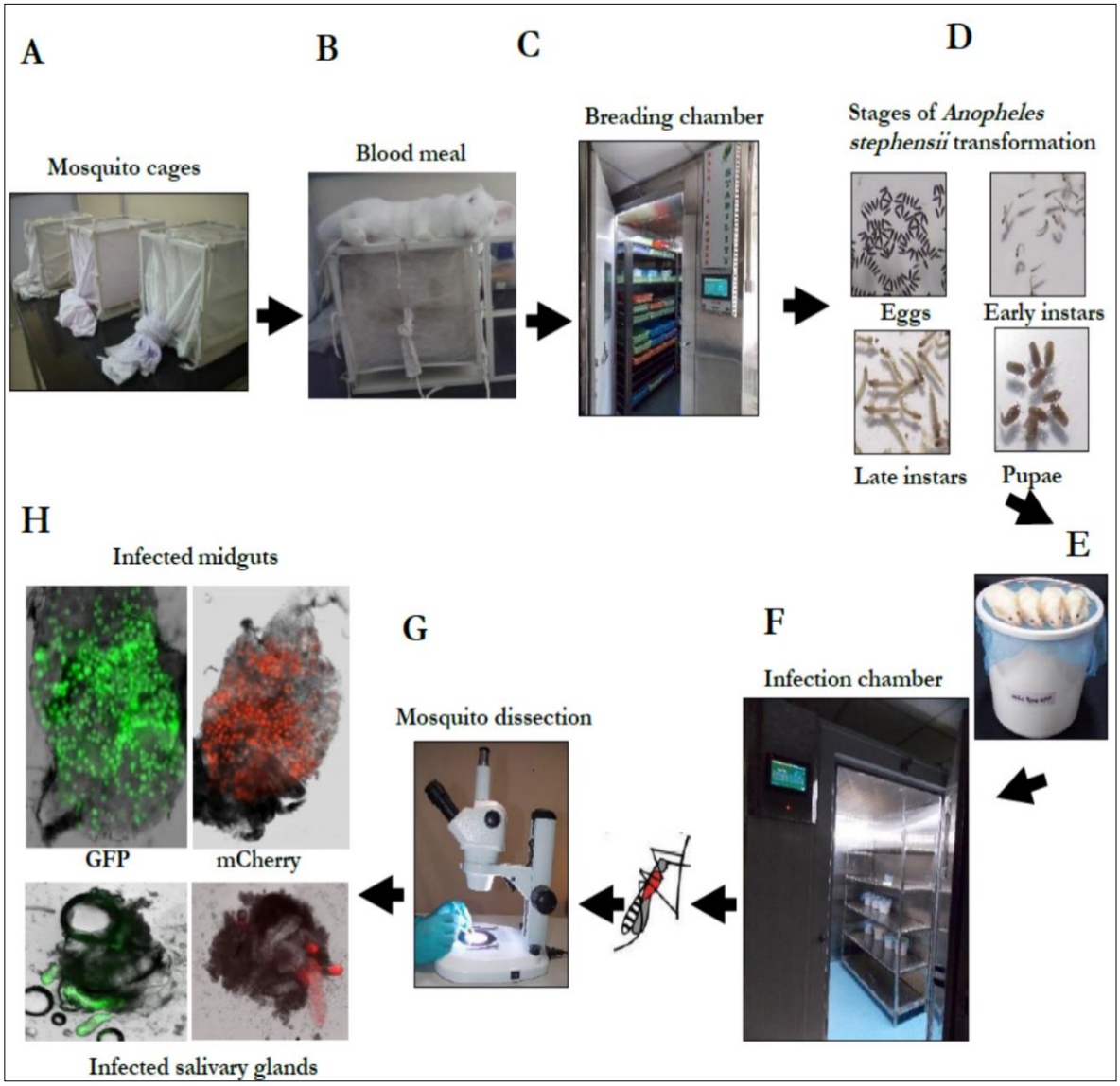


Fig. 10: **Breeding of *Anopheles stephensii* mosquitoes and transmission of *P. berghei* parasites to the mosquitoes to study the ookinete, oocyst and salivary gland sporozoite stages.** A) Emerged male and female mosquitoes were kept in breeding cage. B) Anesthetized rabbit blood meal was given to mosquitoes for 20 min with 24 h interval for 2 successive days. C) Following day, water bowl was kept in the cage to facilitate egg laying by the mosquitoes. Next day, eggs were shifted to mosquito transformation chamber maintained at 28^0C with 80% relative humidity. D) Eggs hatch into larvae, that further develop into pupae. E) Pupae were manually collected, kept in a mosquito cage and within 24 h, pupae emerged into mosquitoes. Female mosquitoes were collected with the help of vacuum pump and allowed to feed on mice harboring gametocytes in the blood circulation. F) The blood fed mosquitoes were shifted to infected mosquito chamber maintained at 20^0C with 80% relative humidity for 20 days. G) Mosquitoes were dissected at day 14 for midgut oocysts and on day 18 for salivary gland sporozoites. H) Dissected midgut oocysts and salivary glands were visualised under fluorescence microscope for oocyst numbers, sporulation pattern and salivary gland sporozoite load respectively. (Images adapted and modified from the thesis of Dr. Surendra Kumar Kolli).

2.2.15.19 Transmission of *PbPhIP* KO mutant to *A. stephensii* to study mosquito stages of the parasite

For transmitting *PbPhIP* KO line to *Anopheles* mosquitos, approximately 10^7 iRBCs of both *Pb* WT GFP and KO parasites were injected intravenously to 6 weeks old Swiss Albino mice. For preparing an infection cage, female mosquitos were separated by body temperature cue. In this technique, the left hand (with open palm) is placed on the outer side of the plastic cage harbouring the mosquitoes and is held tightly. The female mosquitoes contained within the cage gradually get attracted to region where the palm is placed. In few minutes all female mosquitoes segregate on the inner side of the cage sensing the body temperature. A collection tube attached to vacuum pump was used for collecting all the female mosquitos in the cage. Around 300- 350 female mosquitos were separated and kept in a clean cage. After three days of post injection, gametocyte positive mice were confirmed on a blood smear. The infected mice were anaesthetized and kept on the mosquito cages for blood meal. Mosquitos were allowed to take blood for 15 mins with a change of position of each mouse every 3 mins. This allowed mosquitos to take up infected blood from more than one gametocyte positive mouse. Similarly, second successive blood meal was given for 20 mins after 24h. The mosquito cages were maintained in infection chamber at 20^0C with 80% RH to complete the sexual development. The colonisation of oocysts and formation of salivary gland sporozoites were observed on day 14 and 18-20 respectively. During this time, the mosquitoes were maintained on 10% sugar-soaked cotton balls kept on the cages. To check for the development of ookinetes, blood was collected at 20-22h post meal by dissecting female midguts. The samples were stained with giemsa and observed under light microscope using 100X objective lens (Lynx Lawrence & Mayo Binocular Microscope, Cat no# LM521710). After day 14 post infection, mosquito midguts were dissected to check midgut oocyst infectivity of WT and mutant lines under fluorescence microscope which provided an idea of parasite burden in the mosquito. Similarly on day 18^{th}, 30-40 mosquitos were dissected and investigated for salivary gland sporozoite load. Once the midguts and salivary glands were collected, it was mechanically disrupted with a plastic pestle and centrifuged at 4^0C at 800 rpm for 3 mins. Supernatant was collected that contained partially purified sporozoites. The sporozoite numbers were estimated using a hemocytometer. All images of midgut oocysts and salivary gland sporozoites were

captured by using 100X objective lens with the help of Nikon Eclipse upright microscope and processed with NIS Software.

2.2.15.20 Exflagellation assay

Both WT and *PbPhIP* KO infected Swiss Albino mice were monitored for the parasitemia to reach up to 10-15%. Later, fresh mice were injected with 100µl of phenylhydrazine (Prepared in 0.9% NaCl in 1XPBS) prior to infection. WT and KO parasites were intravenously injected in the phenylhydrazine treated mice after 24 hours. Giemsa-stained smears were prepared to observe gametocytes. With a confirmation of 3-4 gametocytes per field, sulphadiazine drug was prepared and administered orally for the clearance of all other asexual stages. After 48 hours giemsa stained smears were prepared from both the infected mice to confirm the presence of gametocytes. A drop of blood from tail vein was collected on slide and incubated at room temperature for 10-15mins and observed under microscope with 100x magnification to check for the exflagellated male gametes both in WT and KO. The evidence of exflagellation was recorded using AVI acquisition with the help of NIS element software in upright fluorescent microscope (Nikon Eclipse).

2.2.15.21 Generation of *P. berghei* ANKA mCherry wild type parasite by targeting non-essential P230p locus

For successful replacement of PBANKA_030600 (P230p) locus, the 5' and 3' fragments within the target gene were amplified and cloned into the targeting vector pSKC-mCherry-hDHFR. To achieve this, 537 bp of 5' fragment was amplified using the primers WT *Pb* mCherry FP1 (containing XhoI) and WT *Pb* mCherry RP1 (containing ClaI) and 918 bp of 3' fragment was amplified using the primers WT *Pb* mCherry FP2 (containing NotI) and WT *Pb* mCherry RP2 (containing AscI), and cloned into pTZ57R/T vector after adenylation. Both the clones were confirmed by double digestion and sequencing. The 5' and 3' fragments were released following restriction digestion with XhoI/ClaI and NotI/AscI enzymes respectively and subcloned in the targeting vector pSKC-mCherry-hDHFR using same restriction sites to obtain mCherry@P230p targeting plasmid. The targeting cassette was released from the vector backbone by using XhoI and AscI enzymes and purified using Purelink Gel extraction kit (Life Technologies) following manufacturer's instructions. The targeting construct was electroporated into WT P.

berghei schizonts and the mCherry expressing lines were selected and a clonal population was established. The mCherry line was used in cross experiments.

2.2.15.22 Genetic crosses to investigate the effect of *PbPhIP* depletion on gamete formation

Transmission of gametocytes into the female *Anopheles* mosquitos induces gametogenesis in mosquito midgut, forming male and female gametes. This eventually fuses to form zygote and further differentiates to form motile ookinetes. Investigation of defect in sexual stage development of the parasite was done with the help genetic cross experiment. To examine whether *PbPhIP* mutants are forming functional gametes, *Pb* WT GFP and *P. berghei* ANKA mCherry parasites (control) were mixed and injected in equal proportion (1x 10^3 parasites each) to Swiss Albino mice. Similarly asexual stage parasites of *PbPhIP* KO were mixed with *P. berghei* ANKA mCherry (WT mCherry) parasites with equal proportion (1x 10^3 parasites) and injected. If either of the male or female gametes of *PbPhIP* mutant parasites are functional then it should be fertilizing with WT mCherry female or male gametes respectively, producing oocysts which expresses both mCherry and GFP simultaneously. WT GFP and WT mCherry lines were crossed and considered as an appropriate control.

2.2.15.23 Preparation of RNA samples for *Plasmodium berghei* whole genome microarray

To generate RNA for micro array studies gametocyte stage was enriched *in vivo* as described previously in both WT and KOs. Further the mice were sacrificed and gametocytes were harvested by density gradient centrifugation. They were subjected to two washes using 1X PBS. Further the samples were shipped to Genotypic Technology Private Limited, Bangalore, India where RNA isolation was done using TRIzol based precipitation method.

2.2.15 24.1 Microarray analysis of WT and KO gametocytes

For microarray analysis, an Agilent custom *Plasmodium berghei*_4X44k format (AMADID: 067226) was used. The hybridisation, scanning, data extraction and data analysis was performed under project number SO_9155 at Genotypic Technology Private Limited, Bangalore, India.

2.2.15.24.2 RNA Quality Control

The concentration and purity of the RNA extracted were evaluated using the Nanodrop Spectrophotometer (Thermo Scientific, Cat no# ND 1000). An aliquot of the samples was run on an Agilent RNA TapeStation to check for integrity. The integrity of the extracted RNA was analysed on the bioanalyzer (Agilent, Cat no# 2100). The RNA was considered to be of good quality based on the 260/280 values (Nanodrop), rRNA 28S/18S ratios and RNA integrity number (RIN) (Bioanalyzer).

2.2.15.24.3 Labeling and microarray hybridisation

The samples for gene expression were labeled using Agilent Quick-Amp labeling kit (Cat no# p/n5190-0442). Five hundred nanogram each of total RNA was reverse transcribed at 40°C using OligodT primer with T7 promoter based-linear amplification to generate labeled complementary RNA (One-Color Microarray-Based Gene Expression Analysis). Synthesised double stranded cDNA were used as template for cRNA sample generation. cRNA was generated by *in vitro* transcription and the dye Cy3 CTP(Agilent) was incorporated during this critical step. The cDNA synthesis and *in vitro* transcription steps were carried out at 40°C. Labeled cRNA was purified using Qiagen RNeasy columns (Qiagen, Cat no# 74106) and quality assessed for yields and specific activity using the Nanodrop ND-1000.

2.2.15.24.4 Hybridisation and scanning

600 ng of labeled cRNA sample were fragmented at 60°C and hybridized on to a *Plasmodium berghei*_4X44k format (AMADID: 067226) arrays. Fragmentation of labeled cRNA and hybridisation were performed using the gene expression hybridisation kit from Agilent Technologies (In situ Hybridisation kit, Part Number 5190-6420). Hybridisation was carried out in Agilent's surehyb chambers at 65° C for 16 hours. The hybridized slides were washed using the Agilent gene expression wash buffers (Agilent Technologies, Part Number 5188-5327) and slides were scanned using the Agilent microarray scanner (Agilent Technologies, Part Number G2600D).

2.2.15.24.5 Image Quality Control

The images were manually verified and found to be devoid of uneven hybridisation, streaks, blobs and other artifacts. Hybridisation across the slide was good

based on number of features that were "g is PosAndSignif" which indicates feature is positive and significantly above background.

2.2.15.24.6 Microarray data analysis

Feature extracted raw data was analyzed using Agilent GeneSpring GX software. Normalisation of the data was done with GeneSpring GX using the 75th percentile shift method. Percentile shift normalisation is considered as a global normalisation, where the locations of all spot intensities in the array are adjusted. This normalisation takes each column in an experiment independently, and calculates the *nth* percentile of the expression values for this array, across all the spots (where n has a range from 0-100 and n=75 is the median). It subtracts this value from the expression value of each entity and fold change values were obtained by comparing test samples with respect to specific control samples. Significant genes up regulated fold> 0.8 (logbase2) and down regulated <-0.8 (logbase2) in the test samples with respect to the control sample were identified. Statistical student T-test p-value among the replicates was calculated based on volcano Plot Algorithm. GO-mean values were compared between control and test samples and a heat map was generated. Clusters of functionally related proteins were separated on the basis of gene ontology and plotted on different pie charts with separate boxes. Differentially regulated genes were clustered using hierarchical clustering based on the Pearson coefficient correlation algorithm to identify significant gene expression patterns. Genes were classified based on functional category and pathways using Biological Analysis tool DAVID (http://david.abcc.ncifcrf.gov/).

2.2.15.25 Generation of *PbPhIP* conditional KO parasites

Defect in sexual stage development in *PbPhIP* KO parasites yielded lack of oocyst formation in mosquito midgut. The phenotype did not allow the investigation of the mutants in the mammalian host. In order to explore whether *PbPhIP* has additional role in parasite biology in sporozoite and liver stages, a conditional mutagenesis approach was employed. In this approach, the endogenous *PbPhIP* locus is replaced with a FRTed locus in a line expressing Flp recombinase, driven through TRAP promotor. As TRAP is active in oocyst stages, the recombinase is expected to be editing the FRTed locus, thus conditionally depleting the gene. The sporozoites bearing the edited locus can further continue the life cycle, if the product of FRTed gene is not essential in salivary gland stage.

Likewise, the conditional sporozoite mutants can also be evaluated for their ability to invade hepatocytes and cause blood stage infection. Therefore, the conditional system offers a chance to investigate the function of indispensable genes in mosquito and liver stages of *Plasmodium*. The schematic of the conditional gene silencing is shown in the figure below (**Fig. 11**).

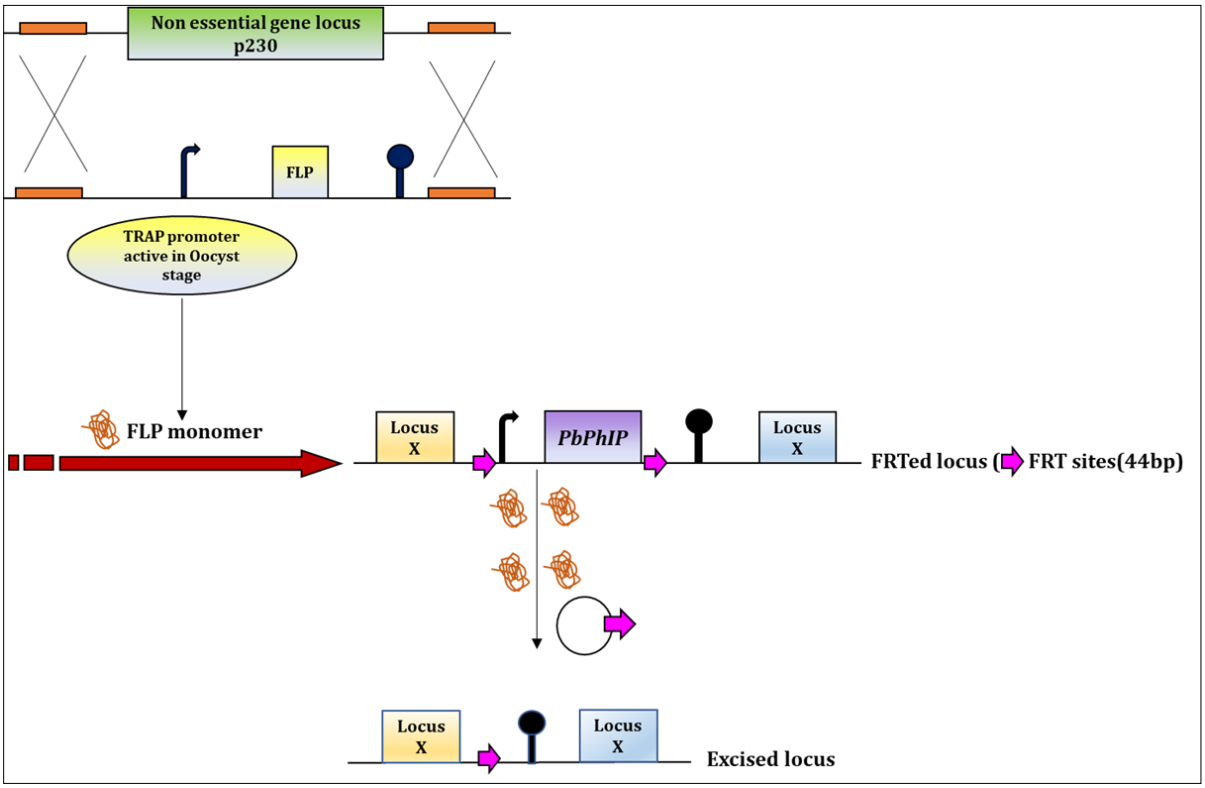


Fig. 11: **Schematic showing conditional silencing of *PbPhIP* using yeast FLpL/FRT system**. TRAP/FLpL transgenic line was generated by integrating the ORF of FLpL recombinase under oocyst stage specific promoter TRAP and targeting this construct stably into a non-essential gene locus. For conditional silencing of *PbPhIP*, PCR amplified 527bp of 5' homologous region, 623bp of 3' homologous region and 1984bp of Promoter+ORF+3'UTR region was cloned using respective restriction enzymes. Finally, the targeting vector was linearised using restriction enzymes AscI and KpnI and Kpn2I(BspEI) and PCR purified prior to transfection. FLpL (a thermolabile variant of FLP) expression is driven by TRAP promoter that is active in the oocyst stages. After successful double homologous recombination, the endogenous locus will be replaced by a FRTed locus and will be excised by FLpL recombinase. Tjis system allows tracking of the conditional mutant in mosquito and on liver stages.

For generating *PbPhIP* conditional knock out construct, a 527bp of 5' homologous region (cKOFP1/RP1), 623bp of 3' homologous region(cKOFP3/RP3), was amplified and cloned using AscI/SalI and PstI/KpnI restriction enzymes respectively into the targeting vector. For excision a combination of 180bp of promoter, 465bp of ORF and 359bp of 3'UTR (cKOFP2/RP2) was amplified, digested with XhoI/NotI and cloned into the

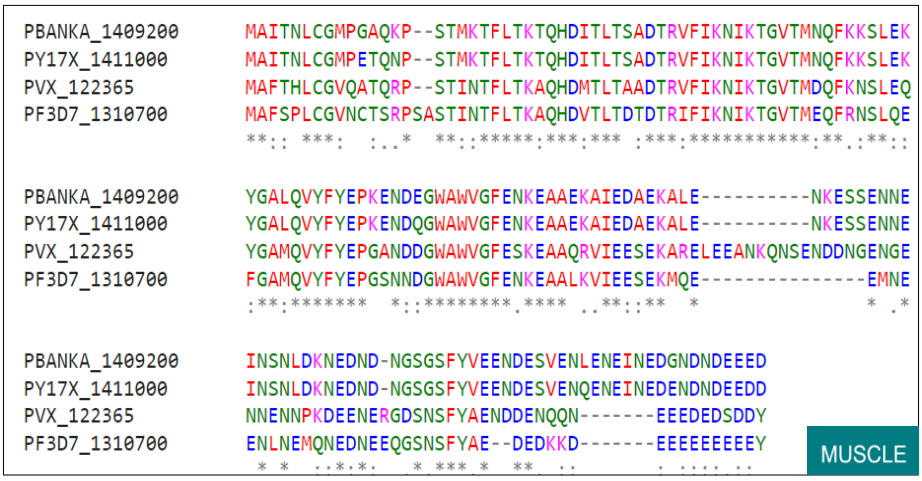
targeting vector pSKc-2FRT flanked by 2 FRT sites. Finally, the targeting vector was linearised using restriction enzymes AscI and KpnI (ThermoScientific, Cat no# FD0524) and the backbone was further digested into two parts with Kpn2I (BspEI). The linearised cassette contained the following genetic elements; 5'homologous region of the *PbPhIP*, first FRT, promoter/ORF/3'UTR of *PbPhIP*, hDHFR, second FRT, 3' homologous region of *PbPhIP*. After the final digestion, the cassette was PCR purified and the concertation was measured by nanodrop 2000. Further the cassette was electroporated in to a TRAP/FLpL parental line where the expression of FLP recombinase is driven by TRAP promoter during oocyst stage. Further after transfection the parasites were kept on drug and after 7-9 days of drug treatment genomic DNA was isolated from the drug resistant parasite to check the stable integration of the cassette with the help of integration PCR. Integration of both 5' and 3' region was confirmed using primers cKOFP4/RP4 and cKOFP5/RP5 respectively as shown in **table no 1**.

2.3 Results

2.3.1 Bioinformatics search reveals *Pb*PhIP is conserved across different *Plasmodium* species

*Pb*PhIP has been annotated as PBANKA_1409200 in PlasmoDB. Amino acid sequence of PBANKA_1409200 was aligned with other orthologues of *Plasmodium* using MUSCLE (MUltiple Sequence Comparison by Log Expectation), a tool that offers better average accuracy than ClustalW2 or T-Coffee (**Fig. 12 A**). The sequence alignment results revealed a high degree of conservation. By using SIAS (Sequence Identity And Similarity) tool, pairwise identity and similarity were calculated (**Fig. 12 B)**. This analysis revealed 96% similarity with rodent *Plasmodium* species- *PyPhIP* (PY17X_1411000) and up to 62% and 65% similarity with human parasites-*PfPhIP* (PF3D7_1310700) and *PvxPhIP* (PVX_122365) respectively.

A



B

SIMILARITY RESULTS				
PBANKA_1409200	100%			
PY17X_1411000	96.1%	100%		
PF3D7_1310700	64.33%	64.33%	100%	
PVX_122365	61.68%	61.68%	74.82%	100%
	PBANKA_1409200	PY17X_1411000	PF3D7_1310700	PVX_122365

PbPhIP: Plasmodium berghei (rodent) PBANKA_1409200

PyPhIP: Plasmodium yoelii (rodent) PY02798

Pf PhIP: Plasmodium falciparum (human) PF3D7_1310700

PvPhIP: Plasmodium vivax (human) PVX_122365

Fig. 12: A) **Multiple amino acid sequence alignment of *Plasmodium* PhIP orthologues**. *P. berghei (PB, PBANKA_1409200), P. yoelli (PY, PY02798), P. vivax (PV, PVX_122365), P. falciparum (PF, PF3D7_1310700).* B) Degree of amino acid residue conservation in *Plasmodium* PhIP orthologs indicated in percentage.

Analysing the evolutionary relatedness of *Pb*PhIP showed no homologs in species other than *Plasmodium*. The retrieved sequences were aligned and a phylogenetic tree was generated with the help of MEGA 11 which informed PhIP to be unique only to *Plasmodium* (**Fig. 13**)

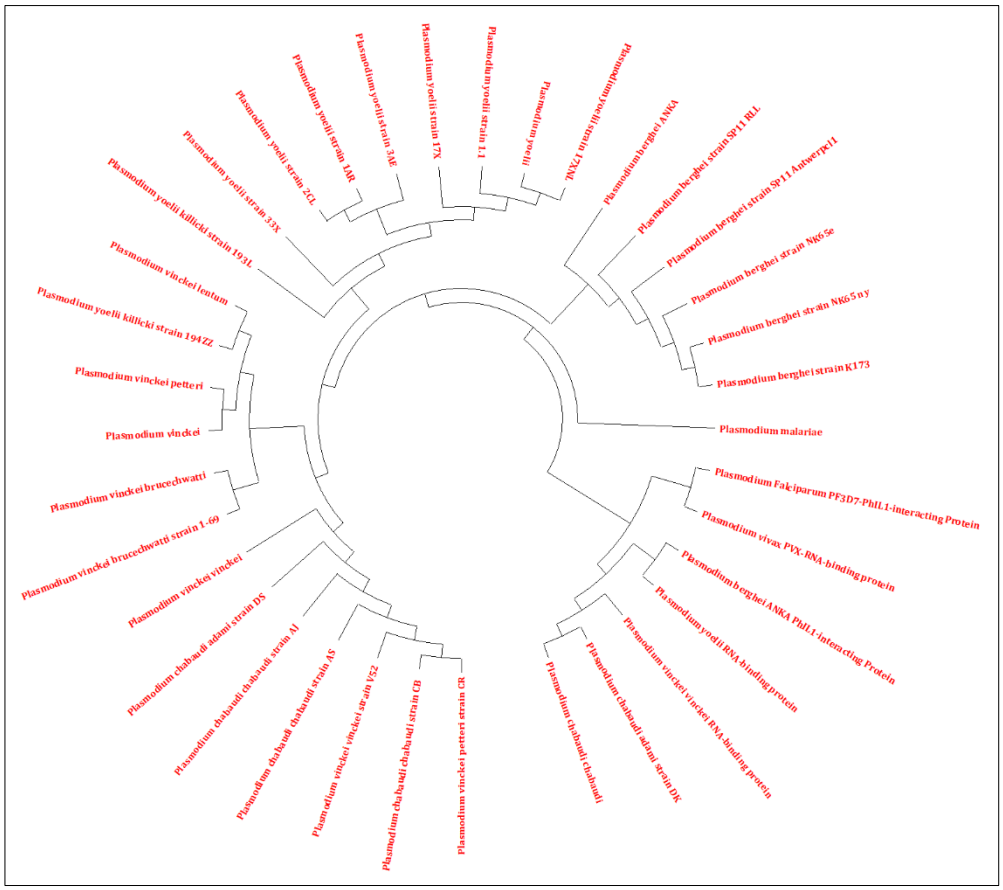


Fig. 13: **Phylogenetic analysis of *PbPhIP* with other *Plasmodium* orthologues**. The nucleotide sequences of the *PbPhIP* and their orthologs were derived and their ancestral states were inferred using the Maximum Likelihood method. All the *Plasmodium* species infecting the rodents formed a separate clade from those infecting primates. Evolutionary analyses were conducted using MEGA11 software.

Conserved domain analysis revealed that PhIP belongs to RRM (RNA recognition motif) superfamily. The RRM motif is also called as RBD (RNA binding domain) or RNP (ribonucleoprotein domain) (**Fig. 14 A**). This domain is present in eukaryotic proteins and regulates post transcriptional gene expression like processing of mRNA and rRNA, RNA export and RNA stability (255,256). The RRM domain consists of a 4 stranded beta-sheet packed against two alpha helices and is typically ninety amino acids in length and. RRM can interact with a variety of targets like ssRNA, ssDNA as well as proteins. RRM binds to variable number of nucleotides, typically 2-8, residues. The active site of RRM includes three aromatic side-chains located within the conserved RNP1 (spanning from

aa 38-aa 94) and RNP2 (spanning from aa 38-aa 110). Interestingly, the RRM motifs were also conserved in other *Plasmodium* species as revealed by motif finder (**Fig. 14 B**). The RRM is also found in other proteins like heterogeneous nuclear ribonucleoproteins (hnRNPs), proteins implicated in regulation of alternative splicing, and protein components of small nuclear ribonucleoproteins (snRNPs) (257-259).

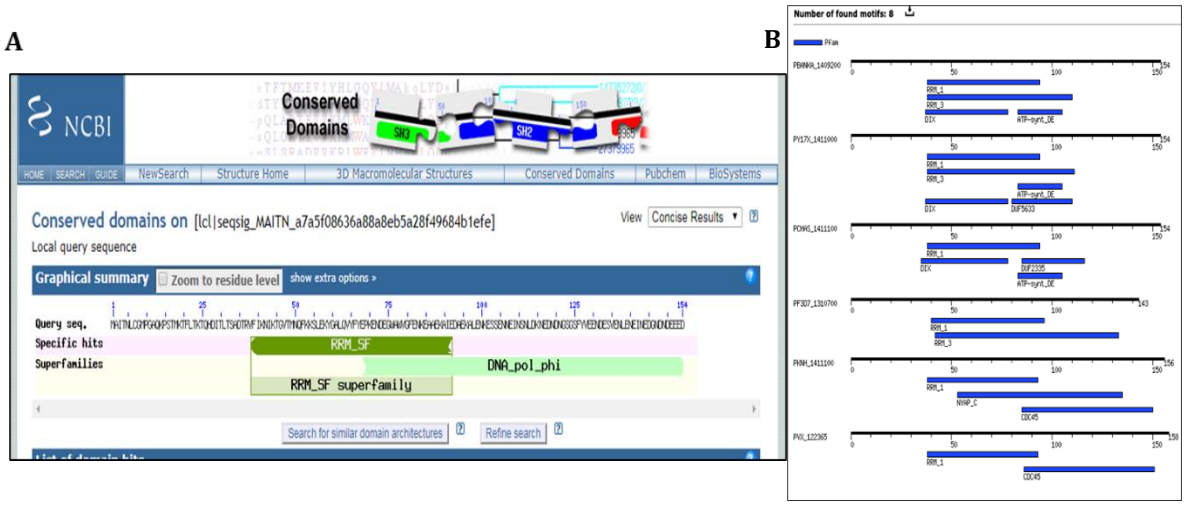
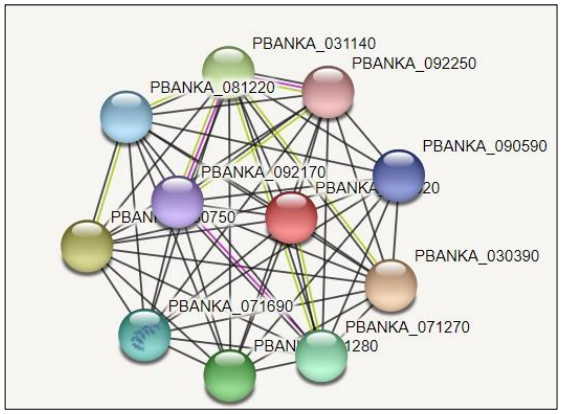


Fig. 14: **Conserved domain analysis of *Pb*PhIP** A) NCBI conserved domain analysis reveals *Pb*PhIP contains RNA recognition motif (RRM). B) Motif finder showing the presence of conserved RRM motifs among other *Plasmodium* orthologues.

To predict the likely interacting partners of *Pb*PhIP, we next resorted to STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database. The STRING database aims to collect, score and integrate all publicly available sources of protein–protein interaction information, and complements this information with computational predictions. Its goal is to achieve a comprehensive and objective global network, including direct (physical) as well as indirect (functional) interactions. The association evidence in the STRING database is divided into one of 7 independent 'channels' viz., neighbourhood, gene fusion, cooccurrence, coexpression, experiments, databases and text-mining (**Fig. 15**).

A



B

Your Input:

PBANKA_140920 *Uncharacterized protein (154 aa)*

Predicted Functional Partners:

	Neighborhood	Gene Fusion	Cooccurence	Coexpression	Experiments	Databases	Textmining	[Homology]	Score
PBANKA_030390 *annotation not available*				•					0.999
PBANKA_030750 *annotation not available*				•					0.999
PBANKA_031140 *annotation not available*				•					0.999
PBANKA_051280 *annotation not available*				•					0.999
PBANKA_071270 *annotation not available*				•			•		0.999
PBANKA_071690 *annotation not available*				•					0.999
PBANKA_081220 *annotation not available*				•					0.999
PBANKA_090590 *annotation not available*				•					0.999
PBANKA_092170 *annotation not available*				•					0.999
PBANKA_092250 *annotation not available*				•					0.999

Fig. 15: **Prediction of possible interacting partners of *Pb*PhIP by STRING analysis.** STRING analysis showing the possible interaction of *Pb*PhIP with ten interacting partners. As STRING database did not annotate any of these interacting partners, we used PlasmoDB for probable annotation. Given within brackets following the gene ID are the probable PlasmoDB annotations. PBANKA_030390 (pantothenate transporter), PBANKA_030750 (a protein CERL1, putative), (PBANKA_031140 (serine/threonine protein kinase, STK2), PBANKA_051280 (merozoite TRAP like protein), PBANKA_071270 (zinc finger protein, putative), PBANKA_071690 (Armadillo-domain containing rhoptry protein, putative), PBANKA_081220 (START domain containing protein, putative), PBANKA_090590 (AP2 domain transcription factor AP2-SP), PBANKA_092170 (Conserved *Plasmodium* protein, unknown function), PBANKA_092250 (kelch domain-containing protein putative). The association evidence was categorised under coexpression category for all candidates and additionally under text mining category for PBANKA_071270.

2.3.2 Gene expression analysis revealed highest *PbPhIP* expression in salivary gland sporozoite stage

Analysis of stage specific expression of *PbPhIP* was performed using cDNA obtained from all life cycle stages. Gene expression was quantified by qPCR, using absolute quantification method. This revealed highest expression of *PbPhIP* in salivary gland sporozoite followed by a mild expression in midgut sporozoite stages (D14 oocyst) (**Fig. 16**) We noted no detectable expression of *PbPhIP* in other life cycle stages. Considering that most genes expressed at certain stage of life cycle translate their gene products immediately or in successive stages, it may likely be possible that *PbPhIP* may have a role in sporozoite, development in oocyst, maintenance of salivary gland sporozoite infectivity or EEF development.

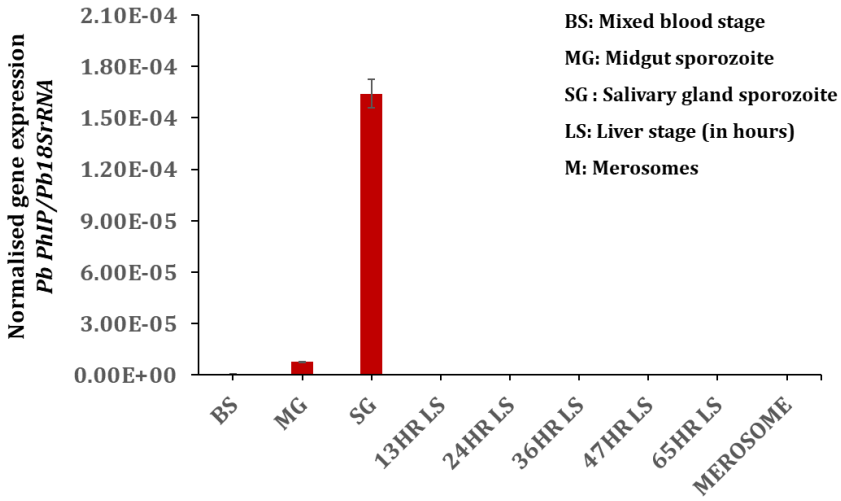


Fig. 16: **Gene expression analysis. Determination of *PbPhIP* transcript levels across *Plasmodium berghei* life cycle stages by quantitative real time PCR**. cDNA samples were generated from different stages as indicated. MBS: mixed blood stages, Mg spz: midgut sporozoites, Sg spz: salivary gland sporozoite stage, liver stages (LS) in hours (h): 13h LS, 24h LS, 36h LS, 47h LS, 65h LS and M: merosomes. The expression values were normalized with *Pb*18SrRNA (internal control gene).

2.3.3 Generation of *PbPhIP*~HA-mCherry & *PbPhIP*~HA tagged parasites for subcellular localisation of PhIP across life cycle stages

We next investigated the subcellular localisation of *Pb*PhIP by generating a transgenic parasite where PhIP was expressed as translational fusion protein along with either 3XHA-mCherry or 3XHA (hemagglutinin). This was achieved by fusing the two tags in frame with *PbPhIP* ORF, excluding the stop codon (**Fig. 17 A-E, Fig. 18 A-D**) Following stringent selection, genotyping and clonal selection, both recombinant transgenic parasites were passed through mosquito. We used *Pb*SPELD-mCherry expressing line as a positive control, that expresses mCherry both in midgut sporozoite and salivary gland sporozoite stage. On day 14 post infection, we dissected infected mosquito midguts to observe the reporter expression of *Pb*PhIP in oocyst (**Fig. 17 F-G, Fig 18 E-F**).

Surprisingly we noted no oocyst formation in several batches of mosquitoes dissected. However, we noted reporter expression in the *Pb*SPELD -mCherry line, as expected. To rule out the possibilities of any frameshift mutation contributing to the observed phenotype, the recombined genomic locus of transfectants were amplified and sequenced. Sequence analysis revealed correct integration of both the tags in frame with *PhIP* ORF (**Fig. 18 G**). The lack of oocyst formation following correct integration suggested that C-terminal tagging of either HA or HA-mCherry rendered *Pb*PhIP protein non-functional yielding a block in parasite development in mosquito stages.

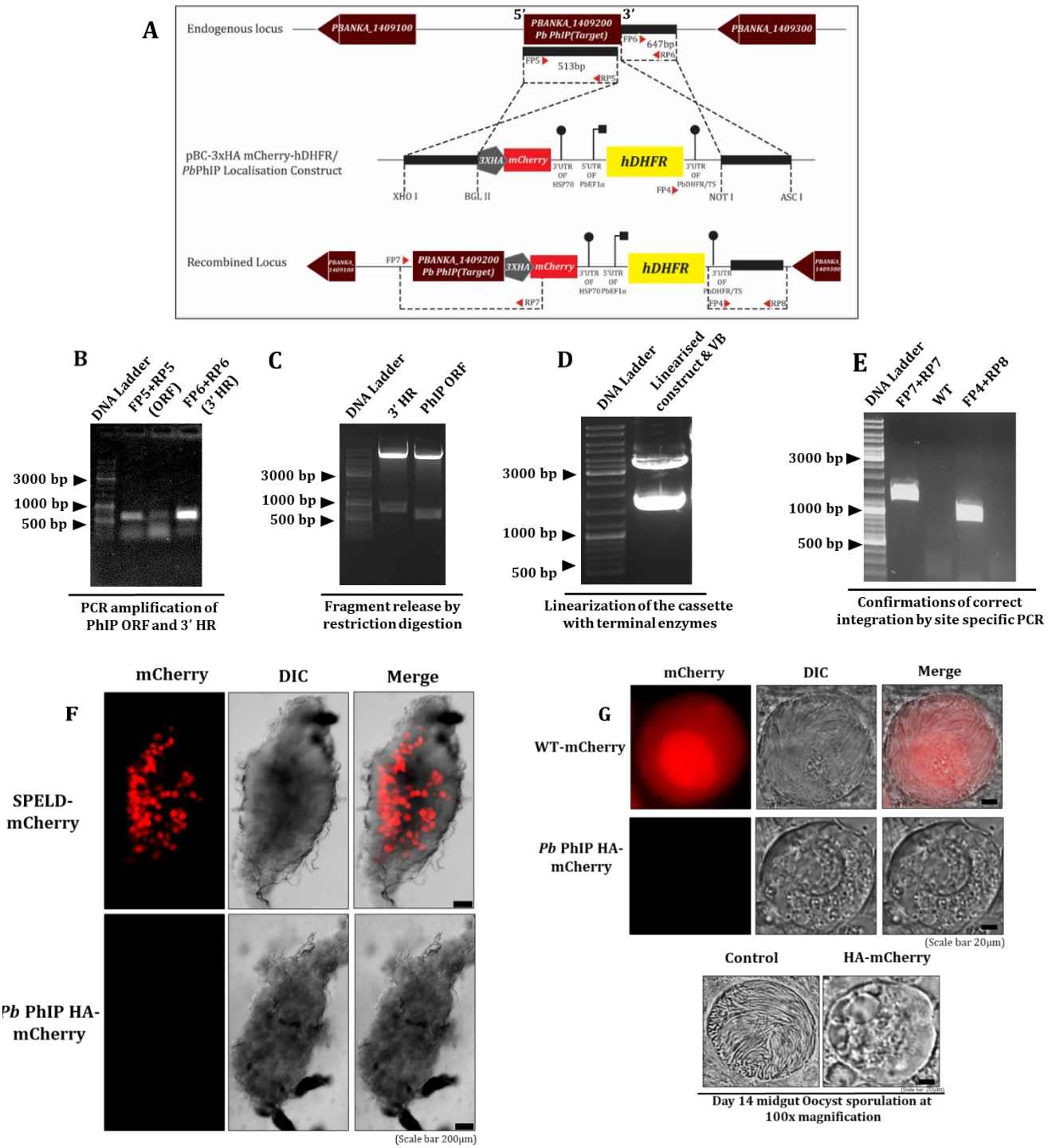
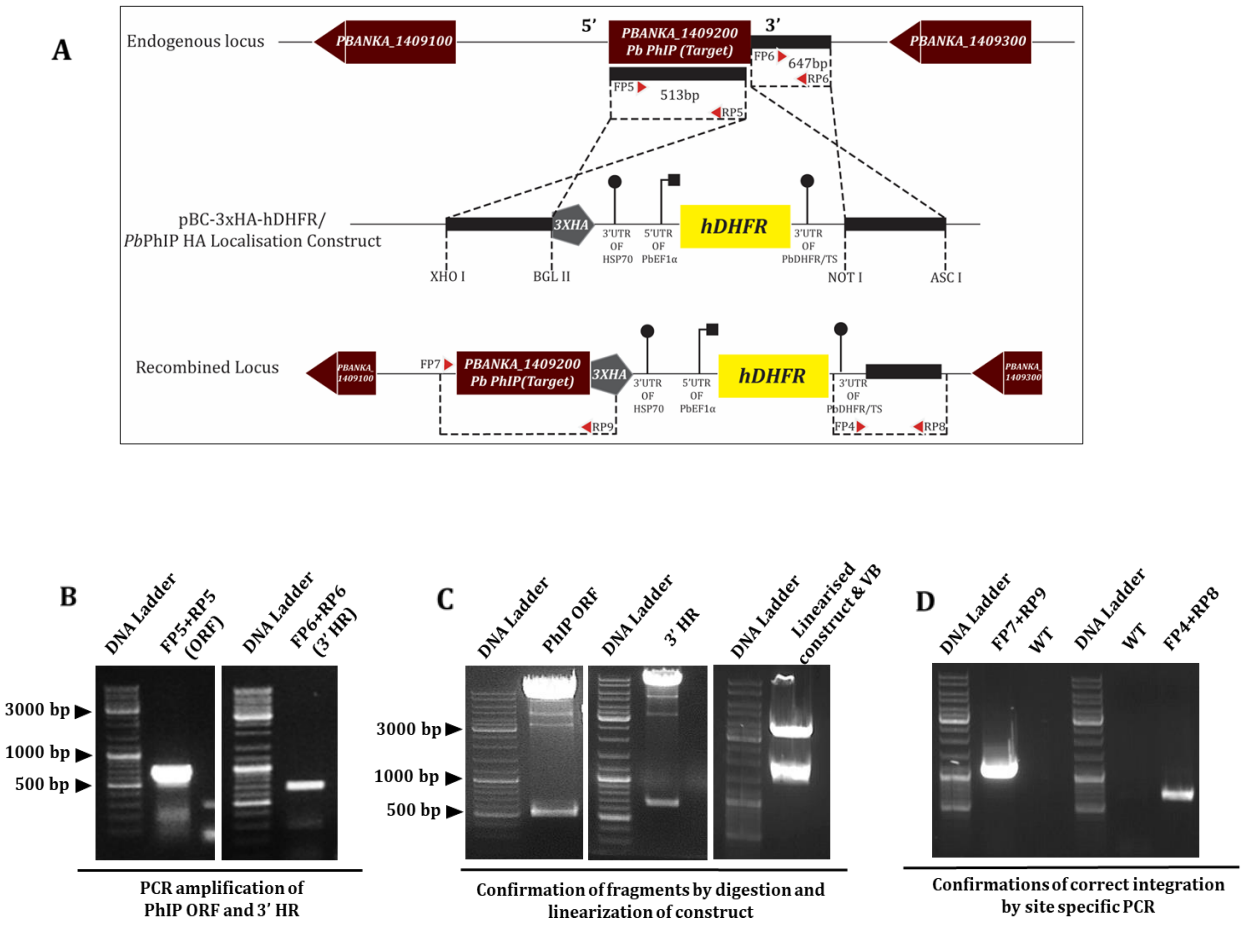
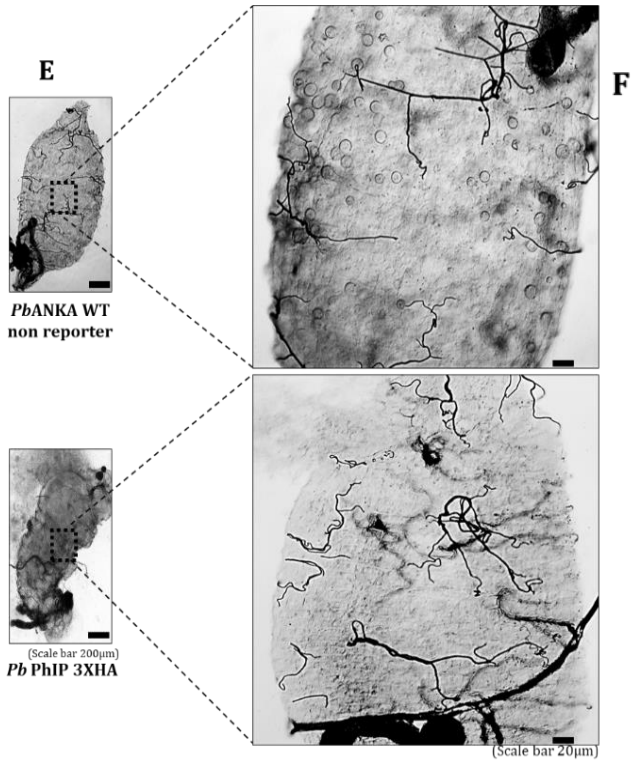


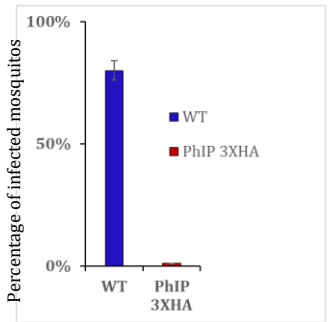
Fig. 17: Generation of *PbPhIP*~3XHA-mCherry transgenic parasites for expression and localisation of sporozoites. A) Strategy for generation of *PbPhIP*~3XHA-mCherry transgenic line. Coding sequence and 3' UTR were amplified and cloned in pBC~3XHA-mCherry-hDHFR to generate the targeting construct. After successful homologous recombination, a 3XHA-mCherry was introduced in frame to *PbPhIP* ORF. B) Amplification of coding sequence (CDS) and 3′ UTR regions for generating the *PbPhIP*~3XHA-mCherry targeting construct. C) Digestion products of 647bp of coding region and 513bp 3' of UTR of *PbPhIP* following release with XhoI/BglII and Not1/Asc1respectively. D) Vector was linearised with XhoI/AscI and targeting cassette was gel purified. E) diagnostic PCRs for confirming correct integration of 3XHA-mCherry epitope tagged to *PbPhIP* CDS. Primers in red triangles and PCR product sizes are indicated. F) Infection was transmitted to *Anopheles* mosquitoes from mice infected with *PbPhIP*~3XHA-mCherry and SPELD mCherry parasites as WT control to initiate sexual cycle. Dissected mosquito midguts on day 14 lack oocyst in tagged parasites as compared to WT parasites.





F

Parasite	Experimental group	Number of mosquitoes /group	Number of mosquitoes positive for midgut infection	Percentage of infected mosquitoes *
Wild Type	Group 1	50	40	80%
	Group 2	50	42	84%
	Group 3	50	38	76%
Pb PhIP 3XHA	Group 1	50	0	0%
	Group 2	50	0	0%
	Group 3	50	0	0%



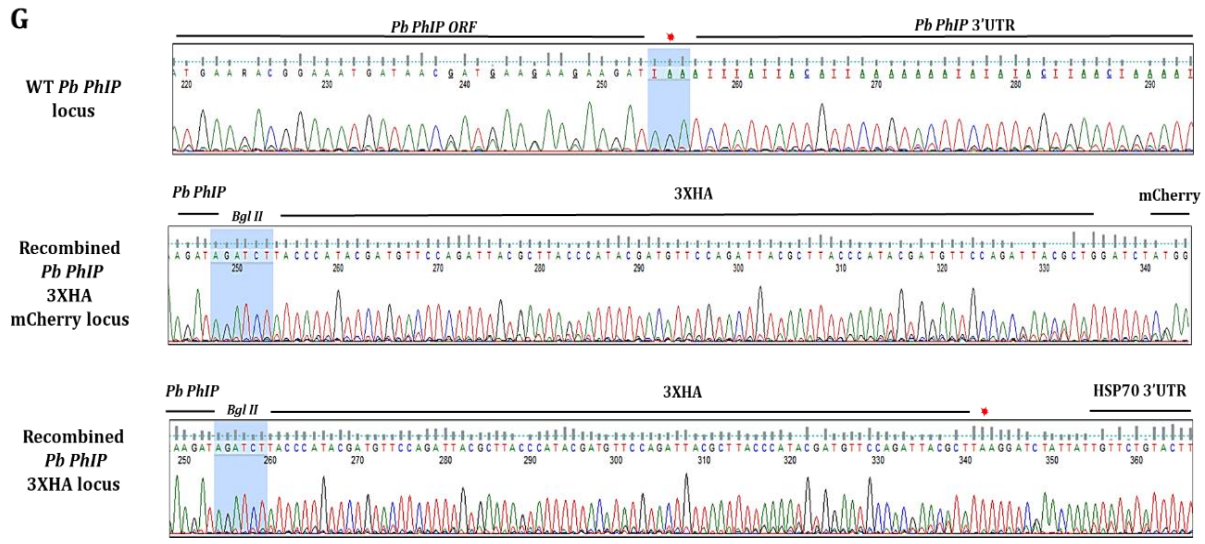


Fig. 18: Generation of *PbPhIP*~3XHA transgenic parasites for expression and localisation of sporozoites A) Strategy for generation of *Pb*PhIP~3XHA transgenic line. Coding sequence and 3' UTR were amplified and cloned in pBC~3XHA-mCherry-hDHFR to generate the targeting construct. After successful homologous recombination, 3XHA was introduced in frame to *PbPhIP* orf B) Amplification of coding sequence (CDS) and 3′ UTR regions for generating the *Pb*PhIP-3XHA targeting construct C) Digestion products of 647bp of coding region and 513bp 3'UTR of *PbPhIP* following release with XhoI/BglII and Not1/Asc1respectively. Transfection vector was linearised with XhoI/AscI and targeting cassette was gel purified. D) Diagnostic PCRs for confirming correct integration of 3XHA epitope tagged to Pb*PhIP* CDS. Primers in red triangles and PCR product sizes are indicated. E) *PbPhIP*~3XHA and *Pb*ANKA WT non reporter parasites were transmitted to mosquitoes to initiate sexual cycle. Dissected mosquito midguts on day 14 lack oocyst in tagged parasites as compared to WT. F) Bar graph showing quantification of infected midguts out of total number of mosquitos. G) Recombined locus was sequenced and confirmed that *PbPhIP* was in frame with 3XHA-mCherry and 3XHA.

The interesting phenotype observed with the tagged transgenic lines, that in a way mimicked protein destabilisation approach, likely hinting to the role of *Pb*PhIP in completion of sexual reproduction, though this does not rule out the possibility of *Pb*PhIP being required in other stages as well.

2.3.4 PhIP localisation studies using *Pb*PhIP antisera

As our attempts to obtain tagged transgenic lines was not successful, we next resorted to generate anti- *Pb*PhIP antisera by immunising rabbits with 2 synthetic (immunodominant) peptides within *Pb*PhIP protein (**Fig. 19**).

Following complete immunisation strategy, antisera was collected from rabbit and its titers was determined to be in the range of $16X10^5$.

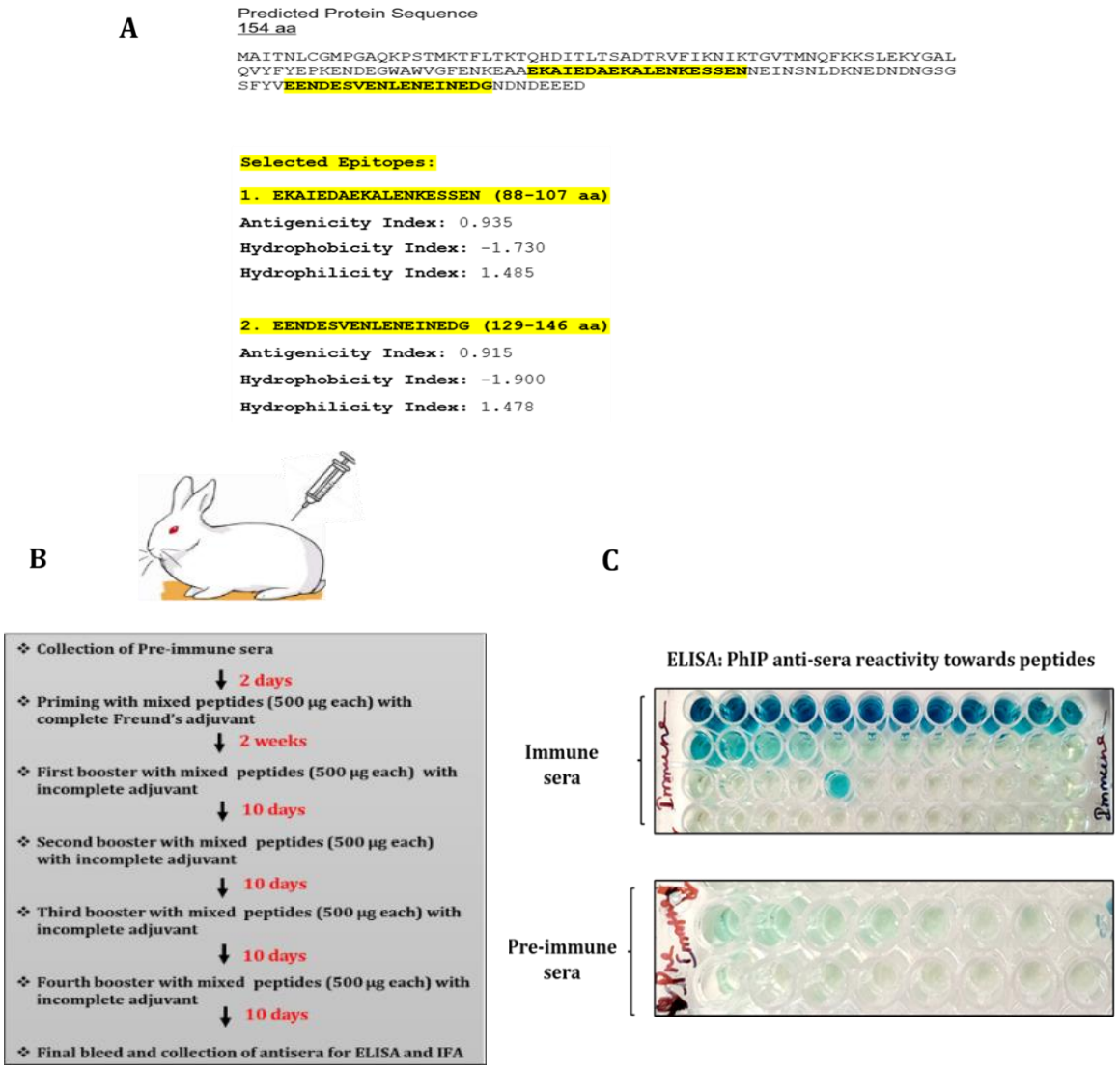


Fig. 19: **Immunogenic epitope selection and immunisation strategy to generate *Pb*PhIP antisera** A) Complete protein sequence of *Pb*PhIP and prediction of two immunodominant sequences showing antigenicity, hydrophobicity and hydrophilicity index. B) Prime boost regimen strategy followed for rabbit immunisation C) ELISA showing immunoreactivity of antisera as compared to pre-immune sera.

The antisera was used for localisation of *Pb*PhIP across all life cycle stages. We noted expression of *Pb*PhIP in schizonts, free merozoites, gametocytes, ookinetes, midgut and salivary gland sporozoites (**Fig. 20**). Anti-*Pb*spt4 (mouse) antibody was used as marker for schizont stage and free merozoite stage. The immunoreactivity of anti-spt4 and anti-PhIP were revealed respectively with anti-mouse Alexa 488 and anti-rabbit Alexa 594. For other stages like gametocytes, ookinetes, midgut and salivary gland sporozoite stage, anti-SIMP (rabbit) antibody was used as marker (176) and PhIP was detected using mouse specific antibody. The immunoreactivity of *Pb*SIMP and PhIP was

revealed using anti-rabbit secondary conjugated to Alexa 594 and anti-mouse secondary conjugated to Alexa 488. DAPI was used to stain parasite nuclei.

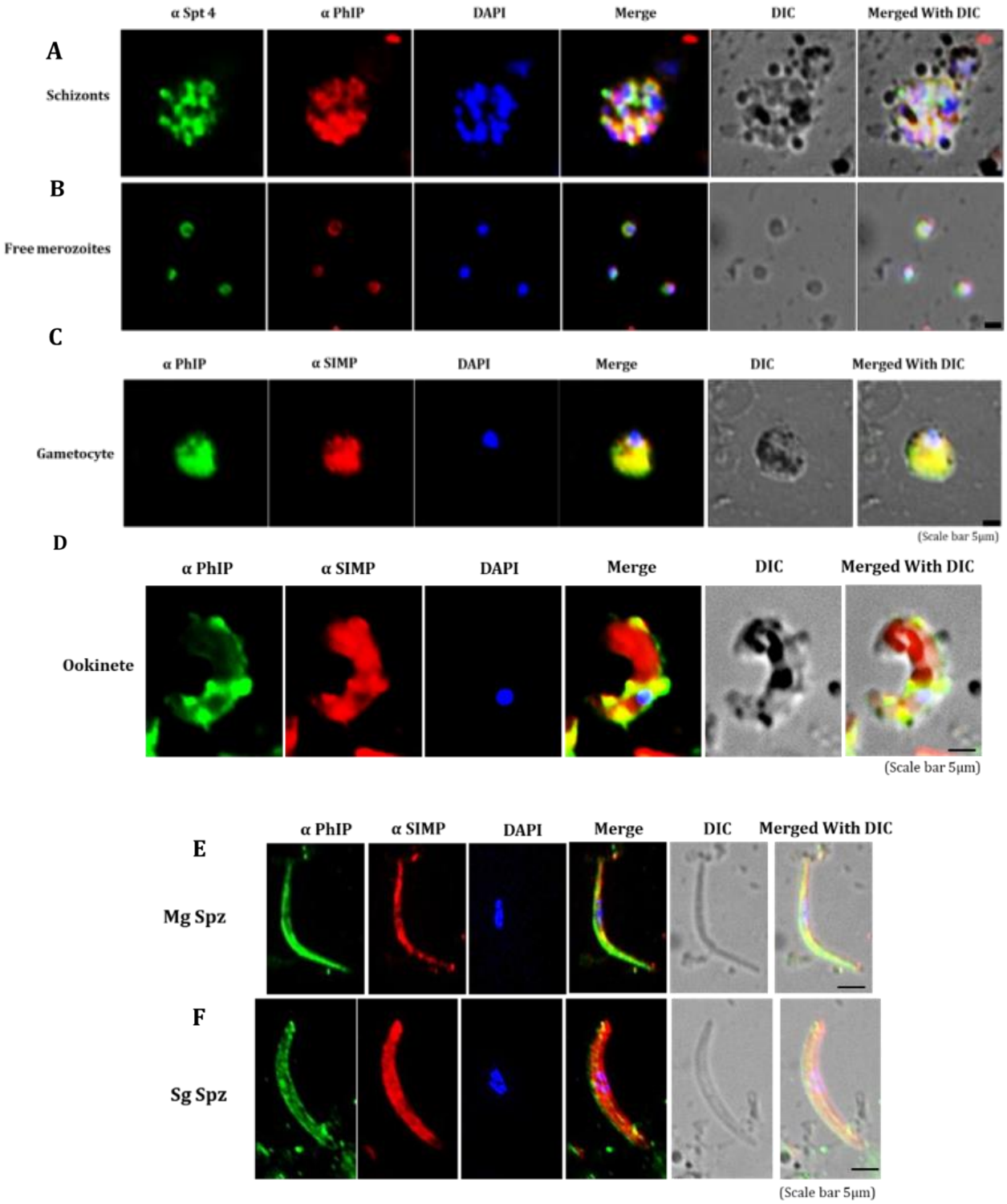


Fig. 20: Investigation of PhIP expression across all *Plasmodium* life cycle stages using *Pb*PhIP antisera. Analysis of PhIP expression in A) Schizonts B) Free merozoites C) Gametocytes D) Ookinetes E) Midgut sporozoites, and F) Salivary gland sporozoites. The PhIP expression was detected by staining with anti-PhIP (rabbit/mouse) anti-spt4 and anti-*Pb*SIMP were used as stage specific marker. Parasite nucleus was stained with DAPI. Scale bar 5 μm.

2.3.5 Generation of *Pb*ANKA_1409200 (*PbPhIP*) KO parasite line

Having shown that *Pb*PhIP is expressed in multiple stages of the life cycle, we next resorted to functionally characterise its role by generating a knock out (KO) mutant. A targeting construct was generated to replace the endogenous *PbPhIP* locus with a GFP fluorescent reporter and a drug resistance marker-hDHFR that confers resistance against pyrimethamine, an anti-malarial drug (**Fig. 21 A-E**).

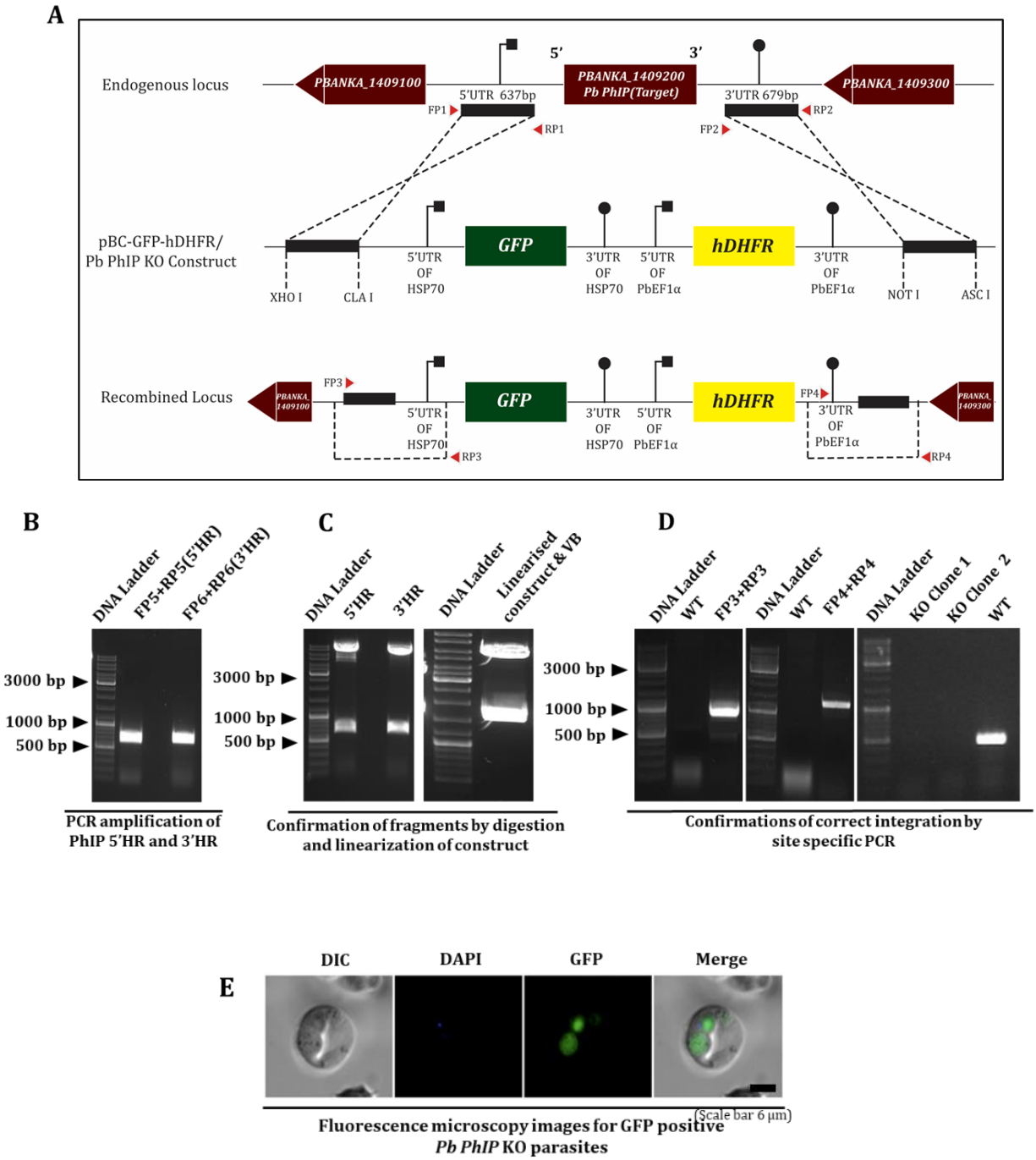


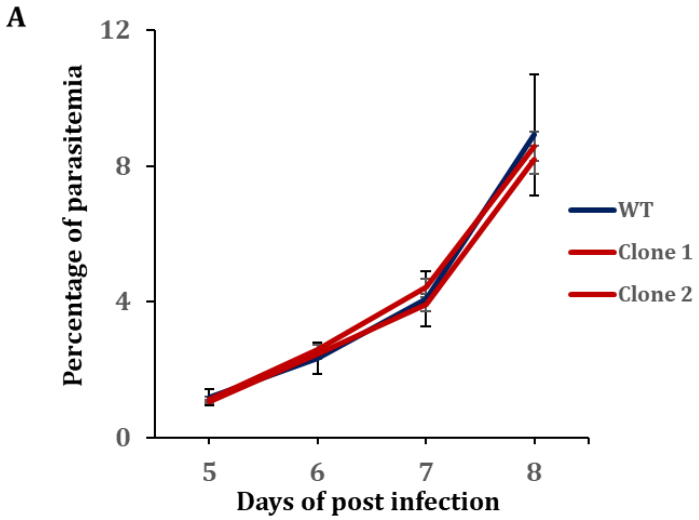
Fig. 21: Generation of *PbPhIP* knockout parasites. A) Strategy for generating *PbPhIP* KO. *PbPhIP* 5' and 3' UTR regions used for generating the targeting construct for homologous recombination are indicated as solid lines. Recombined locus shows the replacement of *PbPhIP* orf with GFP and hDHFR cassettes. B)

1%agarose gel showing PCR amplified products of 5' and 3' UTR regions for generating the *PbPhIP* KO targeting construct. C) Digestion products of 637 bp of 5' UTR and 679 bp 3' of UTR of *PbPhIP* following release with XhoI/ClaI and NotI/AscI respectively. Vector was linearised with XhoI/AscI and targeting cassette was gel purified. D) Diagnostic PCRs for confirming correct integration of KO cassette at *PbPhIP* locus. Primers used for diagnostic PCRs were designed flanking the homology arms used for recombination. Genotyping both KO lines by PCR showed absence of *PbPhIP* orf in KO clones from two independent transfections (C1 and C2). F) Merged DIC image showing *PbPhIP* KO expressing GFP reporter (Scale 5µm).

To facilitate the replacement of endogenous *PbPhIP*, the 5' and 3' regions of *PbPhIP* were amplified and cloned into the pBC-GFP-hDHFR transfection vector. The 5' and 3' regions of *PbPhIP* were cloned in the MCS flanking the GFP reporter cassette and drug resistance marker. The final transfection vector was sequenced and the gene targeting region was released from the transfection vector. Following transfection with KO construct and selection, correct integration was confirmed by diagnostic PCR, using a set of primers designed beyond the site of integration. The drug resistant parasites showed the constitutive expression of GFP. Two independent clones were selected for further phenotypic characterisation after clonal dilution.

2.3.6 Analysing the asexual blood stage propagation and gametocyte transformation efficiencies in *PbPhIP* KO clones

The asexual propagation of *PbPhIP KO* mutants were analysed by intravenously injecting 1X10^3 iRBCs of either *PbPhIP* KO mutants or WT *Pb* GFP parasites in a group of 5 mice and their growth was monitored for 8 days by making giemsa stained smears on a daily basis (**Fig. 22**).



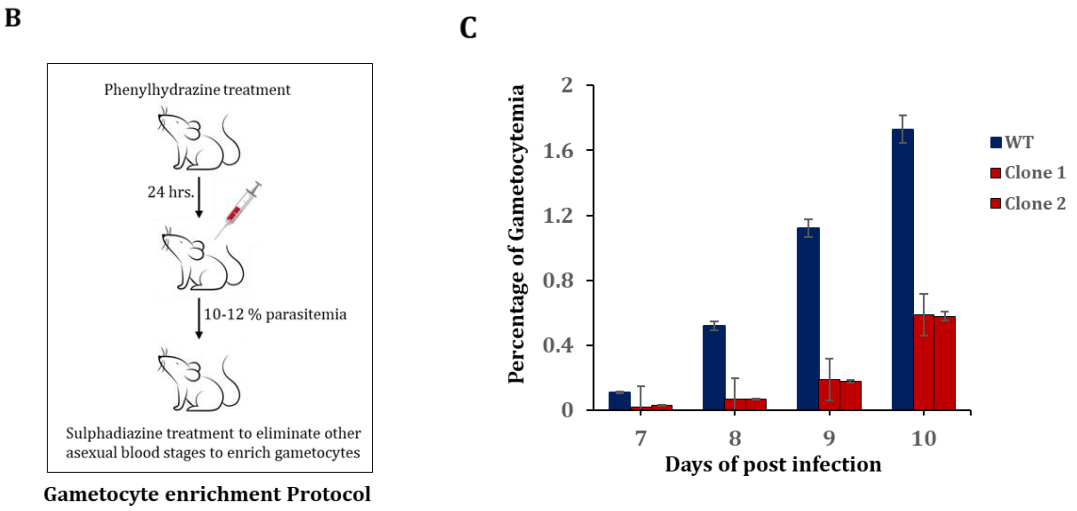


Fig. 22: Asexual blood stage propagation. A) 1X10^3 infected RBCs of WT GFP and *PbPhIP* KO parasites (C1 and C2) were injected intravenously into mice and parasitaemia was monitored daily for 7 days by microscopic observation of Giemsa-stained blood smears. B) Protocol for enrichment of gametocytes C) Quantification of gametocytes monitored for 10 days post infection.

We noted no difference in the propagation of *PbPhIP* KO parasites as compared to WT *Pb* GFP (**Fig. 22 A**). In a different group of mice, gametocytes were enriched following phenyl hydrazine and sulphadiazine treatment (**Fig. 22 B**). Quantification of enriched gametocytes revealed a dramatic decrease in their production in *PbPhIP* KO mutants when compared with WT *Pb* GFP as judged by Giemsa staining (**Fig. 22 C**). To reiterate that this phenotype was indeed true, we designed another elegant experiment that allowed us to monitor gametocyte formation competitively in the same host mice, that harboured two reporter lines expressing GFP and mCherry. The control group included mice that received equal dose of WT GFP and WT mCherry parasites while two experimental groups included mice that received clone 1 *PbPhIP* KO (GFP line) along with WT mCherry or clone 2 *PbPhIP* KO (GFP line) along with WT mCherry (**Fig. 23**).

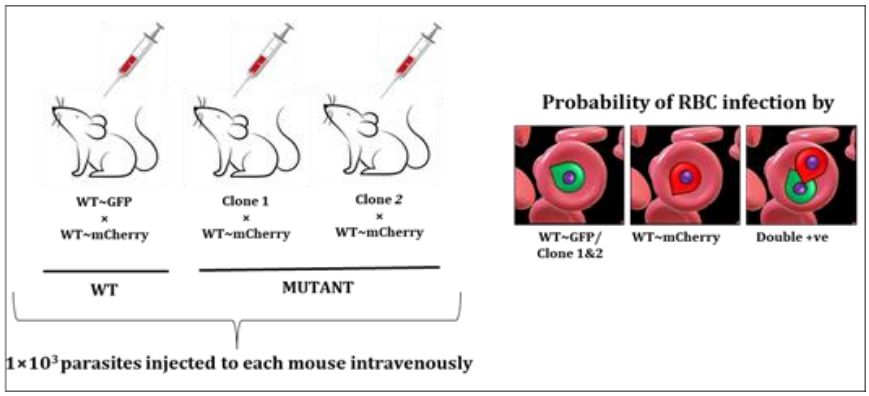


Fig. 23: Strategy to monitor the competitive growth of both GFP and mCherry parasites. All the groups of mice received equal number of GFP and WT mCherry parasites. First group was considered to be control and other two groups received *PbPhIP* KO C1 and C2 GFP parasites. Each erythrocyte may have

three probabilities of getting infected by either GFP or mCherry parasites or by both parasites called as double positive RBC.

Following delivery of 1X10^3 iRBCs of GFP expressing WT or *PbPhIP* KO (C1 and C2) and WT mCherry in different groups of mice, we monitored their asexual propagation by FACS. Gating strategy used for analysis of control parasites (both GFP and mCherry) was applied to experimental samples. To consider singlets, doublets gate strategy was performed which removed all the possible doublets. Erythrocyte population was gated exclusively, using forward/sideward scatter and sorted according to their fluorescent intensity (GFP vs mCherry). This eliminated all small and overly large cells. Later flow cytometry analyses were done using FlowJo software (detailed description in M&M). Analysis of parasitemia on D6 and D8 by FACS revealed similar patterns in the asexul growth of all (GFP expressing WT or *PbPhIP* KO (C1 and C2) and WT mCherry) reporter lines. However, following gametocyte enrichment, we noted nearly 9-10 fold decrease in gametocyte production in the mutants while no difference in the rates of propagation was noted in the WT mCherry line. Interestingly however, the rate at which the gametocytemia progressed for WT mCherry line was comparably less (nearly 6 fold) than WT GFP (**Fig. 24**). This could be inherent to the mCherry line and does not affect the interpretation of our results. Taken together, we conclude that depletion of *PbPhIP* impacted significantly the gametocyte production.

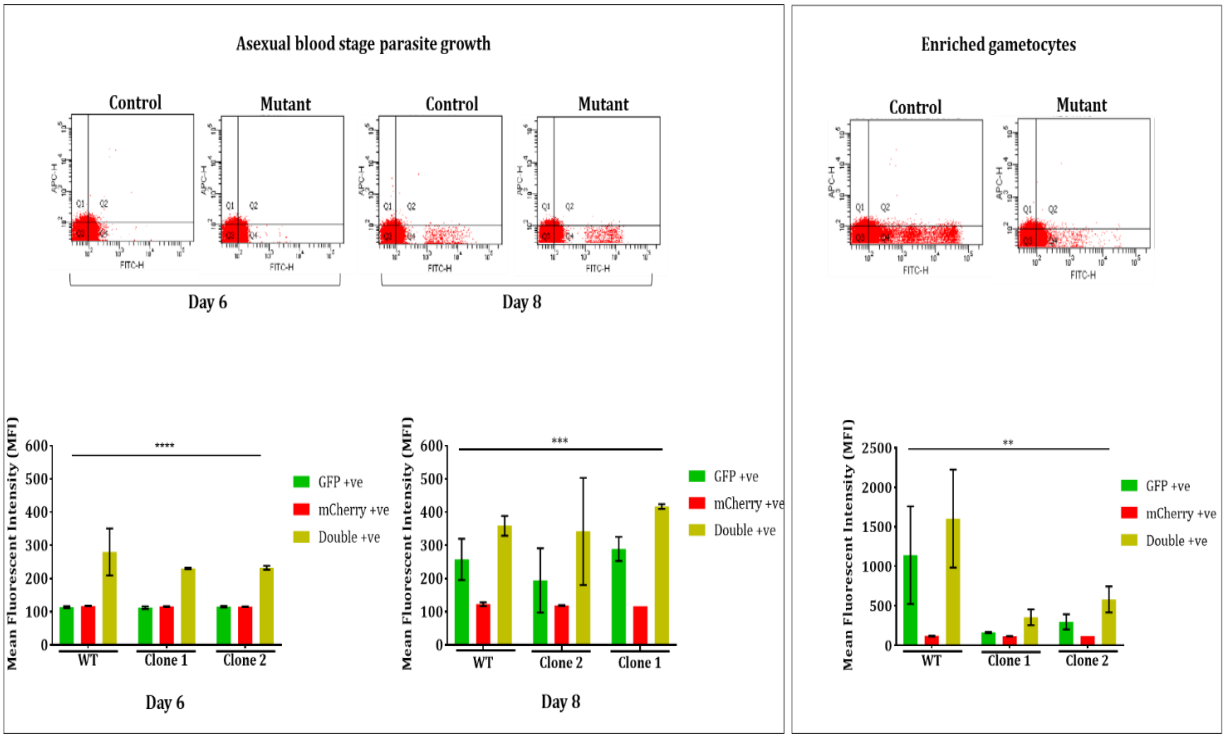
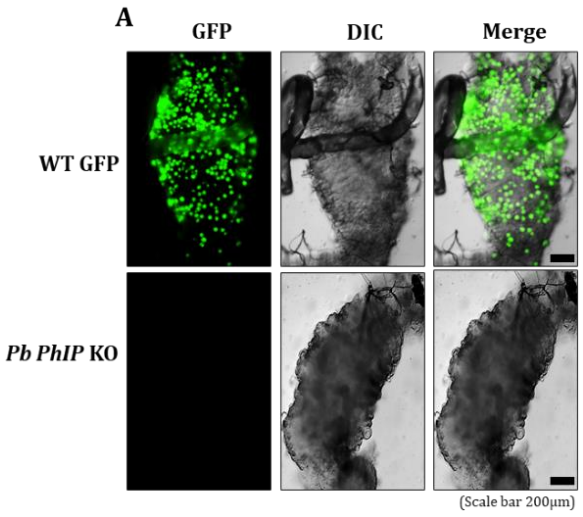


Fig. 24: Quantification of asexual propagation and gametocytemia by FACS. A) Quantification of GFP expressing WT or *PbPhIP* KO (C1 and C2) and WT mCherry on day 6 and 8 post infection. B) Enrichment and quantification of gametocytes in GFP expressing WT or *PbPhIP* KO (C1 and C2) and WT mCherry.

2.3.7 *PbPhIP* KO failed to produce oocyst in mosquito

The mutants were next analysed for their ability to form oocyst, the end products of sexual reproduction. The WT GFP line and both clones of *PbPhIP* KO were transmitted to female *Anopheles* mosquitoes for initiating a sexual cycle. We dissected the infected mosquitoes on D14 to analyse oocyst burden. Interestingly, we noted that the mutants failed to produce oocyst (**Fig 25 A**). We dissected mosquitoes from 3 feedings (n=70) each for clone 1 and clone 2 and noted that none of the mosquitoes that received blood meal harbouring mutant parasites produced oocyst. In WT GFP parasites, we noted mosquito infectivity in the range of 73-86% from 3 independent mosquito feedings (n=30) mosquito (**Fig 25 B-C**).



Kinetics of transmission dynamics of Wild Type and *Pb PhIP* KO parasite in *Anopheles stephensii* mosquitoes

B

Parasite	Experimental group	Number of mosquitoes /group	Number of mosquitoes positive for midgut infection	Percentage of infected mosquitoes*
Wild Type	Group 1	30	25	83%
	Group 2	30	22	73%
	Group 3	30	26	86%
KO Clone1	Group 1	70	0	0%
	Group 2	70	0	0%
	Group 3	70	0	0%
KO Clone2	Group 1	70	0	0%
	Group 2	70	0	0%
	Group 3	70	0	0%

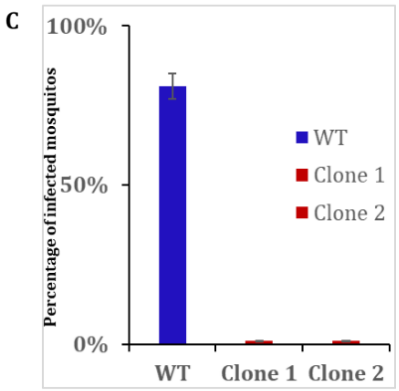


Fig. 25: *PbPhIP* KO does not produce oocysts. A) Dissected mosquito midguts on day 14 showing lack of oocysts. Scale bar 200µm B) Percentage of infected midguts out of total number of mosquitos analysed in

WT vs KO C1 and C2. C) Bar diagram showing the percentage of infectivity in WT vs KO C1 and C2. Infectivity was scored based on the presence of oocyst and not on absolute numbers.

Taken together we conclude that *P. berghei* lacking *PbPhIP* fail to produce oocyst.

2.3.8 Analysis of gametogenesis in *PbPhIP* Kos

We next wanted to investigate if drop in gametocytemia also affected gametogenesis. Male gametes are formed by a process of exflagellation, an event that occurs spontaneously within 5-10 minutes in mosquito midgut after ingestion of gametocytes. Exflagellation can also be visualised *in vitro*, in gametocyte enriched blood within 8-10 minutes of exposure to ambient temperature. Live monitoring of exflagellation though video recording revealed that the mutants failed to manifest this activity or exhibited very rarely (**Fig. 26**). This indicated that *PbPhIP* depletion affected male gamete formation.

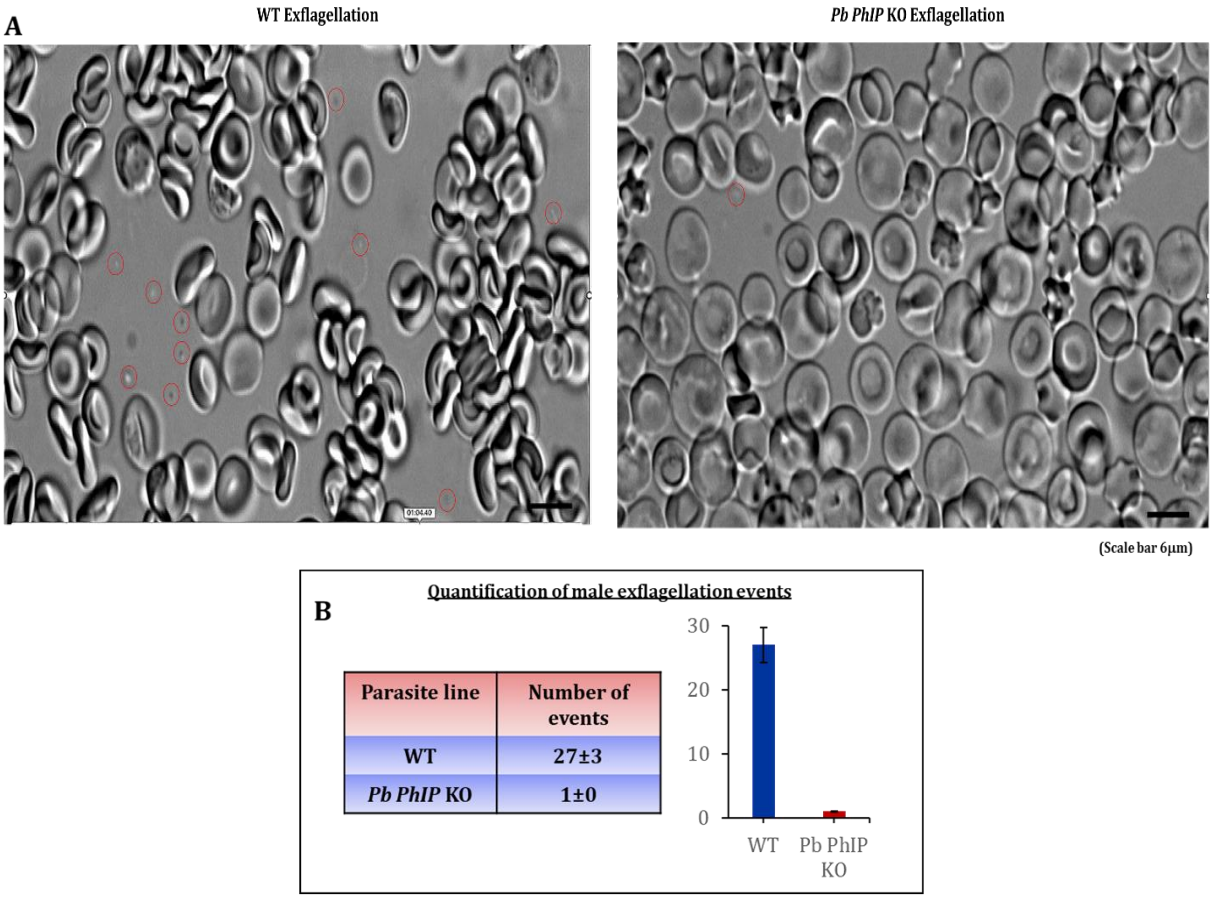


Fig. 26: Quantification of male gamete exflagellation events A) Total number of exaflagelleting male gametes captured in WT vs KO. Scale bar 6µm B) Bar diagram showing the number of events captured in a certain field.

2.3.9 Generation of *P. berghei* ANKA mCherry Wild Type parasite by targeting non-essential *P230p* locus

PbPhIP depletion clearly affected oocyst formation. Our investigations also revealed that mutants had a dramatic drop in gametocytemia and exflagellation. While absence of any one functional gamete (male or female) would render a block in sexual reproduction, we also wanted to confirm additionally the fate of female gametes under conditions of *PbPhIP* depletion. Unlike easy scoring of functionality for male gametes, there are no methods to assay the functionality of female gametes. Therefore, we resorted to perform cross experiments to unravel if *PbPhIP* mutants produced functional female gametes. To this end,

we generated WT *Pb* mCherry transgenic parasites by replacing a partial region with a dispensable p230P locus with mCherry cassette (**Fig. 27 A-G**). The mCherry line was validated for its fitness by comparing its infectivity with WT line at all life cycle stages. These investigations revealed that mCherry were comparable to WT parasites in terms of infectivity, across all life cycle stages (**Fig. 28 A-E**).

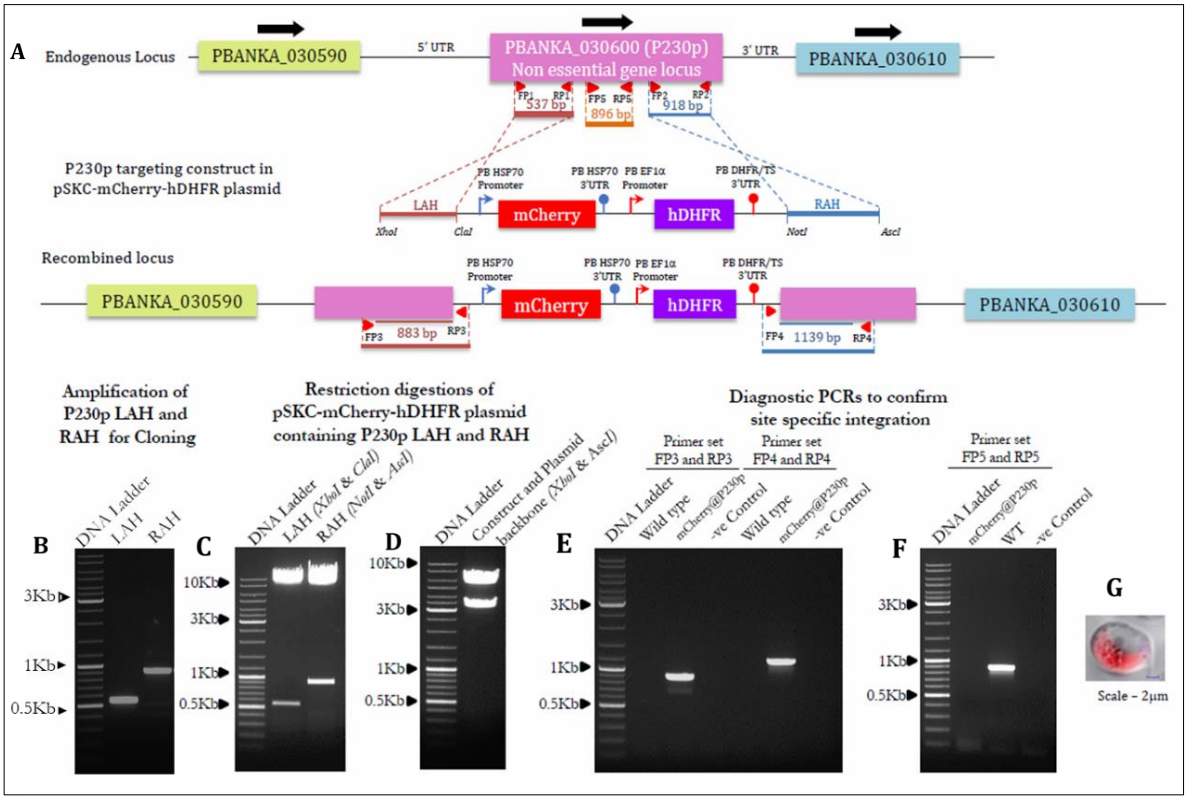
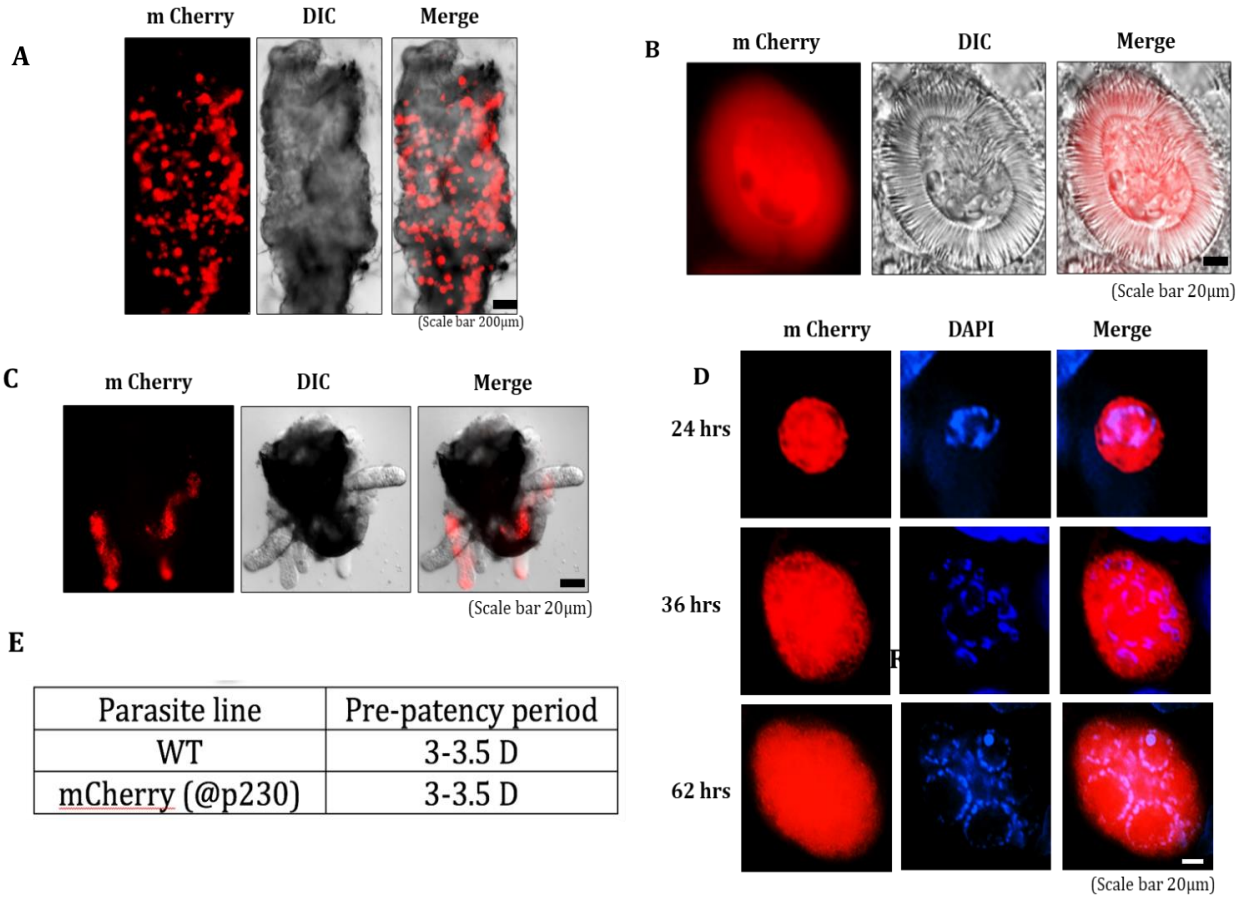


Fig. 27: Generation of *P. berghei* WT mCherry line targeting the non-essential gene locus P230p. A) Genomic locus of *Pb P230p* (*PBANKA_030600*) showing *5'* and *3' UTRs*. Elements of the targeting vector showing regions selected for homology, *mCherry* and *hDHFR* cassettes. A 537 bp 5' fragment and 918 bp 3'

fragment of *Pb P230p ORF* were cloned at *XhoIClaI* and *NotI/AscI* sites of the targeting vector respectively. Recombined locus following successful double crossover recombination resulting in disruption of target gene, *Pb P230p* by *mCherry-hDHFR* cassettes. B) A 1% agarose gel showing the PCR products of 5' and 3' fragments. The 5' and 3' fragment were amplified with primer sets N FP1/N RP1 and N FP2/NRP2 respectively. C) Release of 5' and 3' fragments from transfection vector using restriction enzymes XhoI/ClaI and NotI/AscI respectively. D) Release of targeting cassette (*5'*fragment+*mCherry-hDHFR* cassettes+*3'* fragment) from the vector using restriction enzymes XhoI/AscI. E) Diagnostic PCR using primers within the targeting cassette and beyond sites of recombination revealing the correct site-specific integration. PCR products of 883 bp and 1139 bp with primer sets N FP3/H RP and D FP/N RP3 indicated a correct *5'* and *3'* integrations respectively F) A PCR product of 896 bp was amplified using primer set N FP4/N RP4 from the genomic DNA of WT parasites but not from cloned WT mCherry line. G) A merged DIC image showing an mCherry expressing WT mCherry parasite inside RBC.



E

Parasite line	Pre-patency period
WT	3-3.5 D
mCherry (@p230)	3-3.5 D

Fig. 28: Mosquito stages of *P. berghei* WT mCherry do not show any defect in sexual development, oocyst formation, sporulation and migration of sporozoites to salivary glands. Malaria was transmitted to female *Anopheles* mosquitoes from the mice harboring gametocyte stages of WT mCherry. A) Microscopic images of day 14 midguts oocysts post feeding derived from WT mCherry. B) Microscopic images of oocyst sporulation on day 14 post feeding in WT mCherry. C) Salivary glands showing sporozoites on day 18 post feeding from WT mCherry and D) The EEFs derived from WT mCherry sporozoites were comparable to that of WT at all indicated time points. E) Kinetics of malaria transmission by mosquito bite and i.v. routes showing the prepatent period is same for both WT mCherry parasites vs WT.

2.3.10 Genetic crosses to investigate if *PbPhIP* depletion affected female gamete formation

To reveal if *PbPhIP* mutants produced functional female gametes, we resorted to perform genetic cross experiments. These involved transmission of malaria from mice harbouring two reporter strains of *P. berghei*- GFP and mCherry as describe earlier (**Fig. 29**).

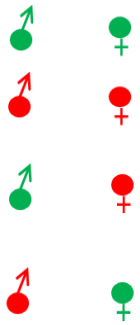
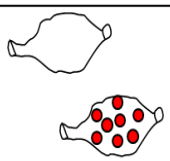
S.No	Nature of sample	Cross between	Possible combinations of gametes formed	Phenotype of oocyst in midguts	Inference
1.	+ve control	WT~GFP X WT~mCherry			**Both male and female gametes are functional**
	Test	*Pb PhIP* KO (GFP) X WT~mCherry			*Pb PhIP* KO has no male & female gamete

Fig. 29: Strategy of genetic cross experiments. Schematic showing the genetic cross of WT GFP with WT mCherry and cross of *PbPhIP* KO (GFP) with WT mCherry. The possible gametes derived from each line and oocysts formed are indicated. Genetic cross reveals that *PbPhIP* KO do not form functional female gametes.

We propagated GFP expressing clone 1 or clone 2 KO mutants with WT mCherry in one group of mice. The control group included mice that harboured GFP and WT mCherry expressing parasites. Both groups of mice were allowed to transmit malaria to mosquito. On day 14 post blood meal, the mosquito guts were dissected and observed for presence of oocyst. As expected, we noted both GFP and mCherry expressing oocyst in mosquito guts that received blood from mice harbouring mixed WT lines (GFP and mCherry) (**Fig. 30 A**).

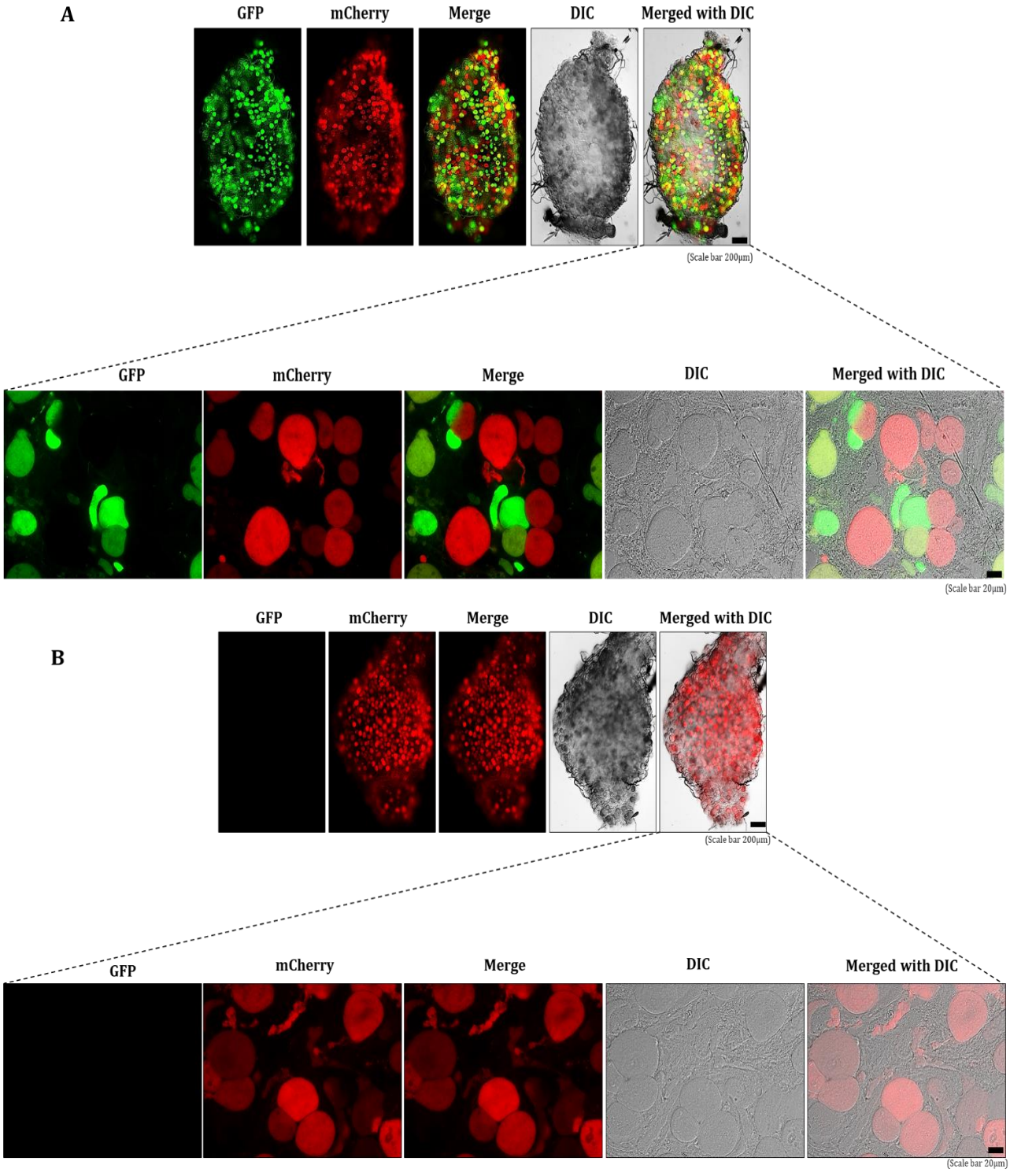


Fig. 30: Genetic cross experiment: A) Top panel shows a cross of WT GFP and WT mCherry, having 3 kinds of oocysts include, oocyst that were positive only for GFP, oocyst that were positive only for mCherry and oocyst that were positive for both mCherry and GFP expression, and B) shows a cross of *PbPhIP* KO with WT mCherry having oocysts expressing only mCherry.

However, in case of mosquitoes that were fed with mix of mutant parasites viz., GFP expressing clone 1 or clone 2 KO mutants and WT mCherry, we only noted mCherry expressing oocyst (**Fig. 30 B)**. Having shown that male gamete formation is impaired in

PbPhIP mutants, the absence of GFP expressing oocyst clearly reiterated that even female gametes were defective.

2.3.11 Microarray analysis (RNA Seq) reveal significant global gene expression changes in *PbPhIP* mutants

As the *PbPhIP* KO parasites manifested a dramatic decline in gametocyte production, we next resorted to investigate the transcriptome at gametocyte stage. Microarray analysis was performed to investigate the differential gene expression under condition of *PbPhIP* depletion. An intra-array normalisation showed no difference in the RNA prep for the duplicate samples of WT and *PbPhIP* KO clone 1 and clone 2 (**Fig. 31**).

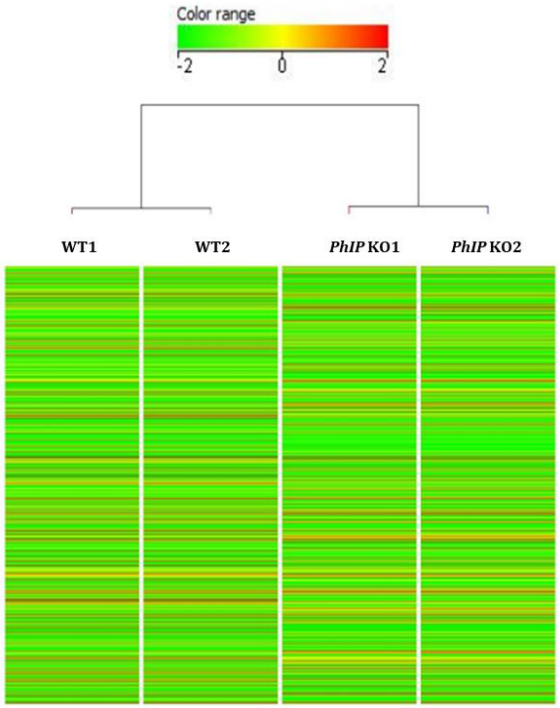


Fig. 31: Quality control report showing intra-array normalisation: no difference in the RNA prep for the duplicate samples provided for both WT and *PbPhIP* KO C1 and C2.

The analysis revealed a total number of 1208 genes being downregulated and 1590 genes upregulated after excluding the non-significantly expressed transcripts. Further a heat map was generated by considering the GO mean values of duplicates in both WT and *PbPhIP* KO clones to see the similarity in expression among the duplicates and appreciate the significance of differentially expressed transcripts (**Fig. 32**).

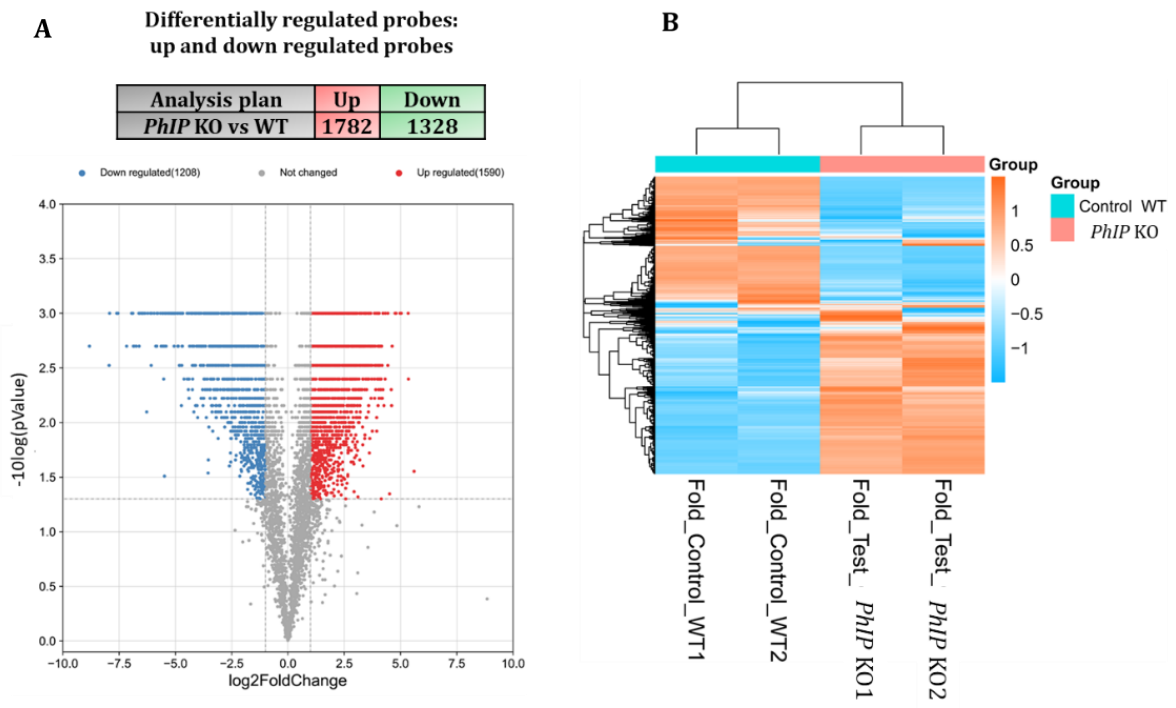


Fig. 32: Volcano plot and heat map reveals significant differential expression of transcripts. A) Total number of upregulated and downregulated probes. B) Volcano plot with log2fold change cutoff value to eliminate non-significant transcripts. This yielded 1208 downregulated and 1590 upregulated transcripts. C) GO mean of both WT and KOs reveal similar expression patterns. Noteworthy, *PbPhIP* KO C1 and C2 showing a dramatic change of expression as compared to WT.

Clustering of functionally related proteins were made on the basis of gene ontology (**Fig. 33 A-B**).

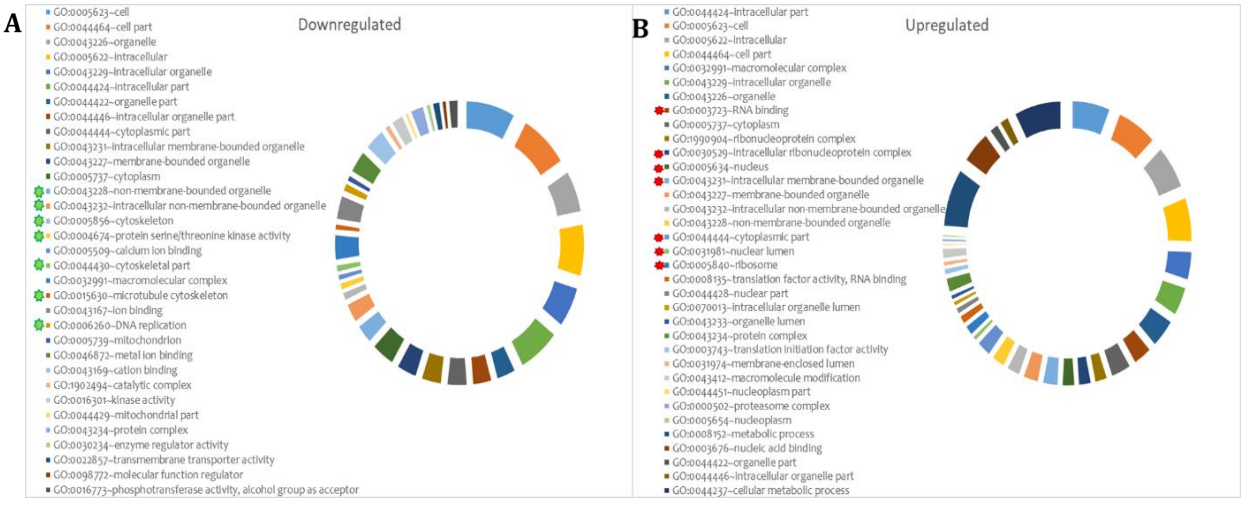
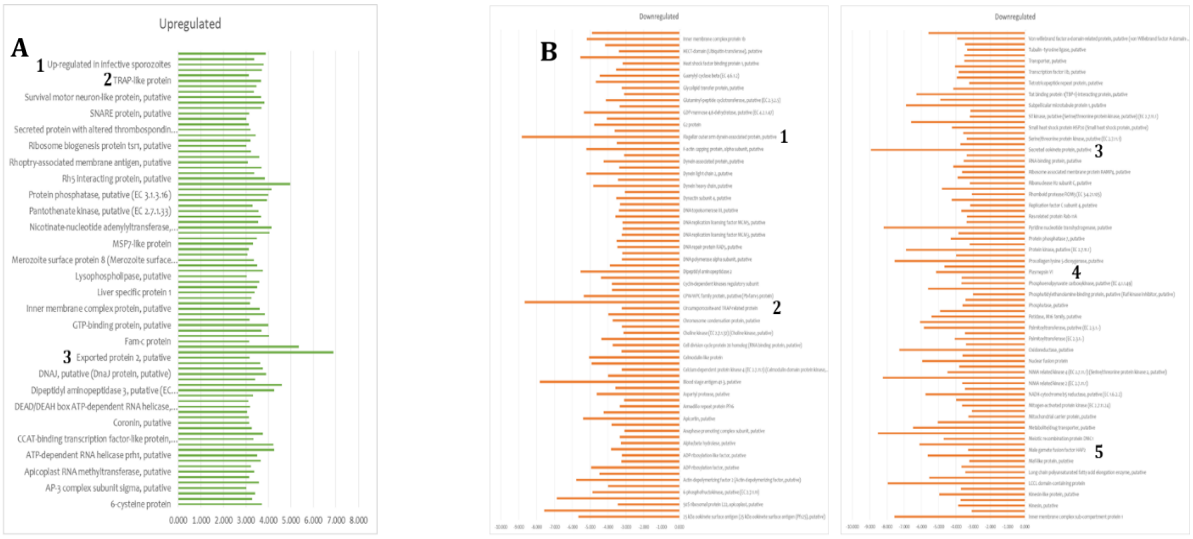


Fig. 33: Pie chart of functionally related proteins on the basis of gene ontology. Proteins having similar functions are grouped together and represented in a box with different colours. Thickness of the box shows the number of proteins accommodated in the group. We clustered the differentially expressed proteins into either down or upregulated categories. The most significantly affected groups included A) GO terms related to non- bounded organelle, cytoskeleton, microtubule and DNA replication were downregulated. B) GO terms related to RNA binding, ribonucleoproteins, membrane bound organelle, nuclear lumen and ribosome were upregulated.

To identify transcripts showing major fold changes, we fixed a cut off fold change value between 3 to 8 (8 being the maximum fold change observed). We noted transcripts that encode for proteins involved in sporozoite maturation, invasion and infection establishment and host cell remodeling were upregulated. On contrary, transcripts that encode for flagellar outer arm dynein protein, male gamete fusion factor, secreted ookinete protein and plasmepsins showed downregulation (**Fig. 34 A-B**).



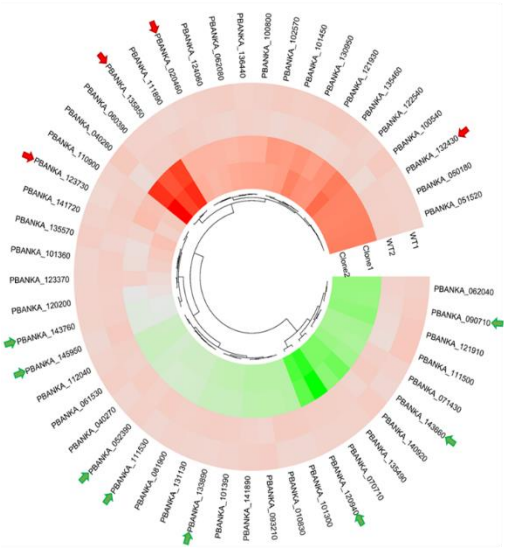
Imp. Upregulated transcripts	Probable function
Up-regulated in infective sporozoites	Sporozoite maturation
Trap-like protein	Establishment of infection
Export protein 2	Host cell remodeling

Fold change 3-8	
Up	Down
118	196

Imp. Downregulated transcripts	Probable function
Flagellar outer arm dynein-associated protein	Motility
Circumsporozoite-trap like protein	Infection and motility
Male gamete fusion factor	Gametogenesis
Secreted ookinete protein	Sexual reproduction
Plasmepsin	Survival in mosquito stages

Fig. 34: Complete data set of total upregulated and downregulated transcripts in the range of 3-8 fold. A) Transcripts were tabulated further into upregulated and B) downregulated category.

Considering that *PbPhIP* is a PhiL-1 interacting protein that localises to IMC, we also analysed expression of transcripts that encode for IMC resident proteins. From the complete data set, a total number of 51 proteins were identified and a pie chart was created according to their expression profile. Our analysis reported that 29 and 22 IMC specific transcripts were down and upregulated respectively, of which a subset were members of PhiL-1 interactome. Few of the important reported proteins are tabulated below with their probable function (**Fig. 35**).



Inner membrane complex residing proteins	
Down	Up
29	22

Up/Down	*Pb*ANKA gene id	Name of the proteins
Downregulated genes	PBANKA_052390	Glideosome associated protein with multiple membrane spans 2
	PBANKA_090710	Inner membrane complex protein 1b
	PBANKA_111530	Glideosome-associated protein 40
	PBANKA_120940	Inner membrane complex sub-compartment protein 1
	PBANKA_133890	Glideosome associated protein with multiple membrane spans 1, putative
	PBANKA_135570	Myosin A (Myosin a, putative)
	PBANKA_143660	Inner membrane complex protein 1h
	PBANKA_143760	Glideosome-associated protein 45
	PBANKA_145950	Myosin A tail domain interacting protein
Upregulated genes	PBANKA_020460	Photosensitized INA-labeled protein 1
	PBANKA_123730	Inner membrane complex protein
	PBANKA_132430	Inner membrane complex sub-compartment protein 3
	PBANKA_135850	Inner membrane complex protein

Fig. 35: Fate of IMC residing proteins upon depletion of PhIP**.** Pie chart showing IMC residing proteins with significant fold changes tabulated for upregulated and downregulated category.

Currently we cannot reason for the altered expression of IMC specific genes following depletion of *PbPhIP.*

2.3.12 Successful generation of *PbPhIP* conditional KO parasite line

As *PbPhIP* KO mutant experience a block in the sexual stages, we were not able to investigate the role of *PbPhIP* in oocyst stages, salivary gland sporozoites stages and liver stages. To overcome this limitation, we resorted to generate a conditional mutant by using a yeast FLP-FRT based system. In this approach, we employ a *P. berghei* line that expresses Flp recombinase through the TRAP promotor, active in late oocyst stages. This recombinase expressing line was further engineered by replacing the endogenous *PbPhIP* locus with a FRTed copy of *PbPhIP* (that carries FRT sites flanking *PbPhIP* locus). This manipulation renders the FRTed site for editing or excision when the recombinase is expressed in oocyst stages. Upon successful excision, the parasites can be tracked in salivary glands by analyzing the *PbPhIP* locus integrity by PCR. Considering the utility, application and the ease of determining excision by simple diagnostic PCR, we used this system to generate conditional mutants of *PbPhIP* (**Fig. 36 A-F**).

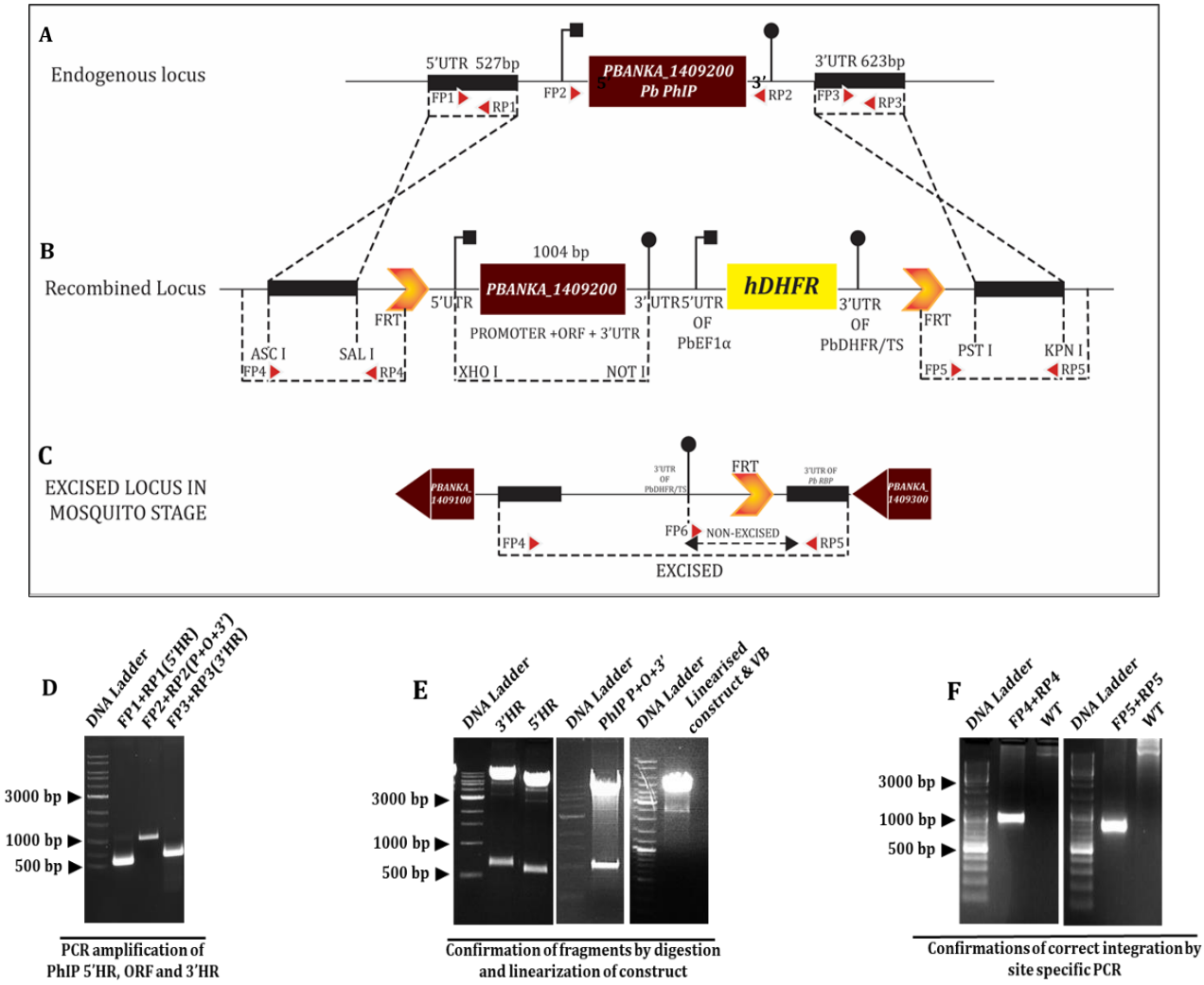


Fig. 36: Generation of *PbPhIP* conditional knockout parasites. A) Representation of *PbPhIP* endogenous locus showing *PbPhIP* ORF, 5' and 3'UTRs. B) Recombined locus showing elements of the targeting plasmid showing, 527 bp of *PbPhIP* 5' homology region followed by a 1004 bp of *PbPhIP* promotor+ORF+3'UTR, hDHFR cassette, GFP cassette and 623 bp of *PbPhIP* 3' flanking region. The first FRT site is present in between *PbPhIP* 5' homology region and promoter+ORF+3'UTR, and the second FRT is after the hDHFR cassette. C) excised locus after transmitting in to *Anopheles* mosquitos D) 1% agarose gels showing PCR amplified products of 527 bp of 5' HR, 1004 bp of promoter+ORF+3' UTR, and 623 bp 3' HR of *PbPhIP* using primer set FP1/RP1, FP2/RP2 and FP3/RP3 respectively. E) digestion products of 527 bp of 5' HR, 1004 bp of promoter+ORF+3'UTR, and 627 bp 3' HR of *PbPhIP* following release with AscI/SalI and XhoI/NotI and PstI/KpnI respectively F) diagnostic PCRs showing the confirmation of site-specific integration of targeting cassette using a primer pair, one at beyond the site of integration in endogenous locus and the other within the targeting cassette.

By PCR and sequential ligations, we engineered a DNA fragment that encompassed the promotor, ORF and 3' regulatory elements and selection marker, flanked by FRT sites. The construct was directed to *PbPhIP* locus by using sequences that flank the endogenous locus. Following transfection, we successfully obtained the transfectant parasites as

judged by sequencing the recombined locus, that retained the engineered FRT sites (**Fig. 37**).

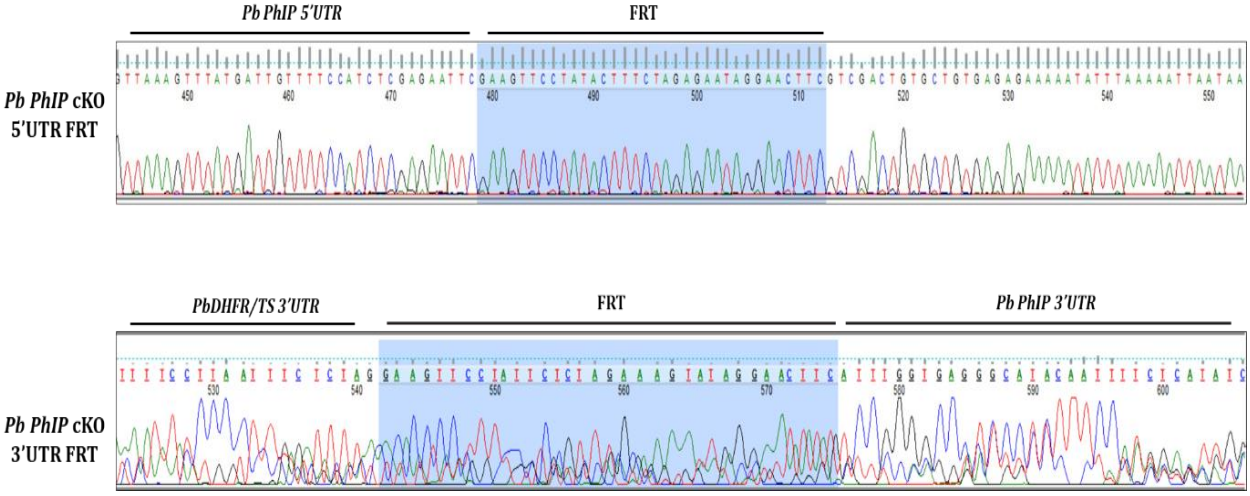


Fig. 37: Sequencing of recombined locus to show the intact FRT sites on both 5' and 3' end.

2.4 Discussion

In the current study, we investigated the functional role of PBANKA_1409200, referred to as PhiL-1 interacting protein (PhIP) by a reverse genetics approach. We were intrigued to investigate the functional role of PhIP owing to its high expression in sexual and asexual stages (260,261), detection of the protein in salivary gland sporozoite stages (262), being a conserved *Plasmodium* gene and having no orthologues in other species (263).

Our studies demonstrate the role of *PbPhIP* in the regulation of sexual reproduction. PhIP was identified as one of the interacting partners of PhIL1-identified in an approach that involved labeling of non-GPI anchored proteins with photoactivatable compound 5-[^{125}I] iodonaphthalene-1-azide (INA) followed by the activation by photosensitizing fluorochromes. As this method utilised photosensitized labelling with 5-[^{125}I] iodonapthaline-1-azide and resulted in identifying an INA (iodonapthaline-1-azide) labelled protein 1, the newly identified protein was assigned the name of PhIL1 in *T. gondii* and is conserved among apicomplexan parasites and is localised to be in the periphery of inner membrane complex (243). Using PhIL1 as a bait, a proximity-dependent biotin identification (BioID)- based proteomics approach has further identified several new IMC candidates collectively referred to as PIC (PhIL1 interacting candidates, PIC 1-6, PIC 5 being PhIP, the candidate investigated in the present study) as well as three putative apical annular proteins- *Pf*AAP 2, 4 and 6) (247). Surprisingly, though the putative annular proteins resided in apical region, they however, did not colocalise with apical organelles. Interestingly, the PhIL-BioID interactome also identified kelch13- a candidate that is linked to artemisinin resistance (247,250). Further systematic analysis of PIC 1-6 and *Pf*AAP 2,4,6 were performed to get an insight into their biological functions. Fluorescent microscopy confirmed the IMC localisation of PIC 1-4 and 6 by endogenous GFP tagging approach that was further reiterated by using IMC markers-ALV5 (Alveolin 5) (247). Interestingly, the 3' region of PIC5 was refractory to genetic modification thus precluding its translational fusion with GFP. Nonetheless, episomal expression of PIC5 also confirmed its IMC localisation (247). Functional inactivation of PIC 1, 3 and 4 did not affect the viability of the parasites likely suggesting redundancy in their function (247). Analysis of the functional motifs in the PIC proteins shed some light on the nature of their function. For example, PIC 1 and 6 contain an N-

terminal signal sequence (247), while PIC 1, 3 and 6 contains at least one predicted transmembrane domain (264). PIC5 contains putative RNA binding domain (253), as also predicted from our studies. PIC5 was also detected in pull down using *Pb*PhIL1 (225) and *Pf*CINCH (265). PIC2 was detected in IP experiments using MyoA (252) as well as PhIL1 in *P. falciparum* and *P. berghei*. Taken together, the PhIL1 interactome likely acts a subcompartment of IMC whose functions are only started to be deciphered gradually and may include but not b limited to motility, endocytosis and very likely in regulation of gene expression.

Functional studies on PhIP/PIC5 have also been reported in *P. falciparum* (237). It has demonstrated the presence of a PhIL1-associated complex and outlined the significance of novel proteins associated with PhIL1, like IMC1c or ALV5, PF3D7_1310700 (PhIL1 Interacting Protein (PhIP) and a well-known member of the glideosomal complex known as GAPM2 in merozoites etc. Genome wide screening of *P. falciparum* with the help of piggyBac transposon mutagenesis shows *Pf*PhIP as an essential candidate, whereas ALV5 was scored as dispensable. However conditional mutation of *Pf*ALV5 slightly affected the growth of late asexual stages yet the parasites successfully complete the cycle and produced new rings. This possibly points out to the redundancy in members of alveolin family proteins (266). In contrast to ALV5 results, knock down of PhIP exhibited a noticeable defect in parasite invasion. With the help of light, immunofluorescence, and electron microscopy, it was observed that PhIP knockdown manifested a distorted segmentation of merozoites causing a firm attachment with each other whereas a prominent division of merozoites encased in schizonts was evident in the wild type. The reason behind this phenotype may likely be due to failure of IMC biogenesis and impaired cytoskeletal stabilization. However, few PhIP-depleted merozoites were fully segmented and egressed normally from the schizonts, but they were not able to breach host RBC. These findings imply that despite early attachment of merozoites, its inability to penetrate RBC probably is related to diminished capability of merozoites during the process of reorientation. The above results hypothesized that PhIP might serve a double role during cell division where it renders cellular integrity of the daughter cells and also in the process invasion by establishing the capability of reorientation in released merozoites.

Our studies clearly point to the effect of PhIP depletion on gametogenesis, that likely stabilizes IMC for production of viable gametes, consistent with a dramatic decrease in gametocytemia in the absence of PhIP. Extensively remodeling of IMC occurs during the transition of *Pf* gametocytes from stage I-V. No detectable IMC is noted in stage I, however its biogenesis begins at stage II, with appearance of IMC markers like PhIL1 (245), GAP45 (231) and GAPM2, ISP3 and PF3D7_1345600 (233). A distinct association of PhIL1 with microtubules, that may serve as a scaffold for assembly of Golgi derived vesicles leading to formation of IMC. In stage III, IMC extends progressively and appear dense at the growing edge and in stage IV, the IMC completely surrounds the parasite (245). In stage V, there is disassembly of microtubule network, that makes the parasite more flexible, a requisite for reverse migration from bone marrow to peripheral blood. Upon ingestion by mosquito, the gametocytes are activated, round up and egress from RBC to form male and female gametes very spontaneously (2 mins) (267) and during this step the disassembly of IMC is critical for rounding up. Taken together, IMC plays largely a structural role in gametogenesis enabling changes in shape and size (233,245,268), that contrasts to the function in motility and invasion in zoites stages of *Plasmodium*. The inability of mutants to exflagellate as well as form functional female gametes in PhIP mutants corelates well with the aforementioned functions of IMC. The PIC interactome being a sub-compartment of IMC, it is understandable that disruption of PhIP/PIC5 seems to have more dramatic effect on gamete production, than other PIC counterparts. A decline in gametocyte production prompted us to investigate the transcriptional snapshot at this stage. As the *PbPhIP* KO parasites manifested a dramatic decline in gametocyte production, we next resorted to investigate the transcriptome at gametocyte stage. Microarray analysis was executed to understand the differential gene expression under condition of *Pb*PhIP depletion, the rationale being that PhIP had 2 RNA binding motifs.

Though the regulatory functions of these motifs are not validated, nonetheless it was intriguing to analyse if PhIP depletion led to any dramatic changes in global gene expression. The transcriptome analysis revealed a total number of 1208 genes being downregulated and 1590 genes upregulated after excluding the non-significantly expressed transcripts. Clusters of functionally different proteins were made on the basis of gene ontology. We noted transcripts that encode for proteins involved in sporozoite maturation, invasion, infection establishment and host cell remodeling, were

upregulated. On contrary, transcripts that encode for flagellar outer arm dynein protein, male gamete fusion factor, secreted ookinete protein and plasmepsins showed downregulation. The molecular basis alteration in gene expression needs further investigation.

Proteomic analysis also detected the presence of PhIP in salivary gland sporozoite stages. However, given that *PbPhIP* KO mutants manifested a block in sexual reproduction, we could not identify the role of PhIP in mosquito and liver stages. Towards addressing this problem, a conditional mutant was generated by replacing the PhIP ORF with an engineered ORF flanked with FRT sites. This genetic manipulation was done in a line expressing Flp recombinase that recognises the FRT sites. This conditional mutant was generated and confirmed for the presence of FRT integration. The line is currently under investigation for analyzing the role of PhIP in mosquito and liver stages of *Plasmodium*.

Our investigations on PhIP contrasts the findings of (237,247). (247) reported that the 3' region of *PfPhIP* was refractory to genetic modification. However, in our genome tagging approach, we stably integrated the reporter tags HA and HA-mCherry in frame with *Pb*PhIP and obtained the recombined parasites that however failed to produce oocyst. It is tempting to speculate if translational fusions inactive the protein, thus recapitulating the phenotype of a KO, as noted in our study. *PbPhIP* mutants also propagated at rates comparable to WT, and did not have any defect in correct attachment and reorientation as reported in *P. falciparum* (237). The synchronous rounds of nuclear division during mitosis tightly coordinates with IMC biogenesis (269). This may likely explain the basis for the observed phenotype in *Pf*. However, PhIP depletion did not negatively affect the asexual propagation in our study. Asexual propagation, egress and reinvasion of newly formed merozoites is by far a conserved theme irrespective of the *Plasmodium* species. Why PhIP depletion behaves differently in *Pf* and *Pb* is indeed an interesting question that needs to be further addressed. One likely explanation may be that given the dynamic nature of the IMC and the diversity of the sub-compartments it forms with varying protein composition; one cannot rule out the possibility of other proteins substituting for the function of PhIP/PIC5. Such assumptions are justifiable by the observation that depletion of PIC 1,3 and 4 does not lead to a lethal phenotype and likely suggests redundancy in function. Given the notion that IMC may exist as

subcompartment with other protein complexes like glideosome, basal complex or apical annuli, the stability of IMC may be largely determined in a spatio-temporal context (269). More concrete evidence for such assumptions can only come from high resolution imaging techniques, given that the IMC lies in great proximity to the parasite plasma membrane (20-30nm) and the IMC bilayers are themselves separated by approximately 10nm distance. While cryo immuno- EM may provide with the needed spatial resolution to distinguish the PM and IMC, the information captured is not dynamic in nature but rather a snapshot in time. Advent of more promising techniques like lattice light sheet microscopy into the field of *Plasmodium* may provide high resolution temporal insights to address the dynamics of IMC.

Taken together we show for the first time that an IMC resident protein- PhIP is critical for successful completion of sexual reproduction. Functional genomics offers the potential to address questions about some fundamental mechanisms governing diversity of IMC composition, engagement of microtubule for achieving cytoskeletal stability in a spatio-temporal manner, unraveling membrane complex interactome and assess the kinetics of cellular deformity to achieve successful transmission. An insight into all these aspects can aid in identification of new transmission-blocking targets. In line wit this approach, we show for the first time that an IMC resident protein- PhIP is critical for successful completion of sexual reproduction. Given that the IMC protein complex interactions are highly intricate and orderly process, developing inhibitors for PhIP to block its clientship with IMC may have a pivotal significance in the prevention of malaria transmission.

Chapter III:

Elucidating the role of Heme Detoxification Protein (HDP) PbANKA_1310600 in Plasmodium berghei

3.1 Introduction

Blood contains 60% of plasma and 40% of cellular constituents comprising WBCs and RBCs. RBCs lack nucleus which allows them to have more space to store haemoglobin- a substance that can bind to oxygen with high affinity and permit its transport through RBC during systemic circulation (270). Nearly 95% of the cytosolic protein of RBC is composed of haemoglobin (Hb). Intraerythrocytic cycle of the parasite degrades around 60-80% of the haemoglobin (271), that provides essential nutrients in the form of amino acids required for the growth of the parasite (272). Hb taken up by *Plasmodium,* starts a catabolic process where it gets proteolyzed into constituent amino acids in an acidic vacuole called as food vacuole (FV) (273). However even haemoglobin degradation alone seems insufficient for the parasites to meet its broad metabolic needs as it completely lacks isoleucine and is a poor source of other amino acids like cysteine, glutamine, glutamate and methionine (274). Owing to this reason, the parasite is dependent upon exogenous amino acid in addition to haemoglobin proteolysis for its growth (275). Previous studies have pointed to the role of both cysteine and aspartic proteinase in proteolysis of haemoglobin. Specific inhibitors which block the hydrolysis of globin, results in accumulation of undegraded globin in the food vacuole resulting in developmental arrest of the parasite (276). Infact, an appropriate equilibrium exists inside parasite between degradation of haemoglobin extracellular amino acid influx and incorporation of proteins in the parasite (277).

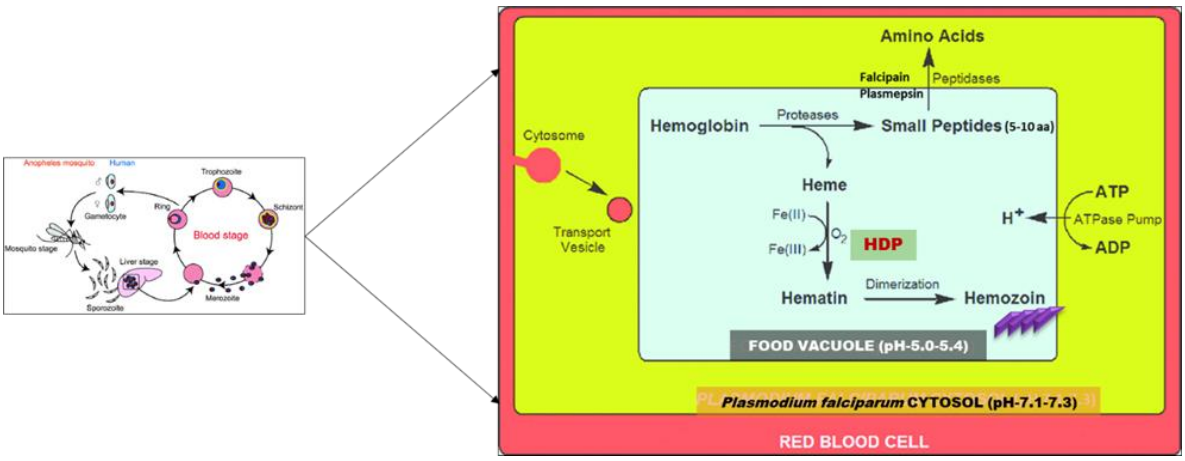


Fig. 38: Schematic depicting haemoglobin degradation and hemozoin synthesis in *Plasmodium falciparum*: Haemoglobin is taken up into the parasite via cytostome, passing through parasite membrane and parasitophorous vacuole membrane (PVM). Double-membrane vesicle containing RBC delivers Hb into the food vacuole. A series of proteases in the food vacuole like plasmepsin (PM), falcipain (FP) and falcilysin

(FLN) family digest Hb into either free amino acid or short peptide. The detoxification of heme begins with the self-oxidation of the Fe (II) in heme group into Fe (III) to form potentially toxic hydroxyferriprotoporphyrin IX (hematin, HO-Fe (III)PPIX. This detoxification ends with the formation of highly insoluble brown crystals known as hemozoin also called as β-hematin. Heme from digested Hb is packed into polymerized crystal called hemozoin with help from heme detoxification protein (HDP) and histidine-rich protein (HRP). Picture adapted and modified from (226).

Proteolysis of haemoglobin releases free heme. Within the erythrocyte, heme iron resides in ferrous (+2 state) but subsequently upon degradation in digestive vacuole it oxidises to ferric (+3 state). This ferric form of free heme is extremely toxic to the parasite. Unlike *Plasmodium,* the vertebrates produce homeostatically hemopexin and heme oxygenase enzymes that can tackle the degradation of heme moiety. However, *Plasmodium* spp. lack such biochemical regulation, but nonetheless possess an alternate pathway that facilitates their detoxification. This process involves conversion of the toxic free heme (α-hematin) into an inert biocrystal called hemozoin (Hz) or β-hematin, a dark brown malarial pigment (278). Interestingly, nearly 75% of the free heme is processed to hemozoin (279). Considering the indispensable need for the parasite to convert inert heme into Hz, this pathway is central to developing therapeutic inhibitors that can act against Hz formation (**Fig. 38**).

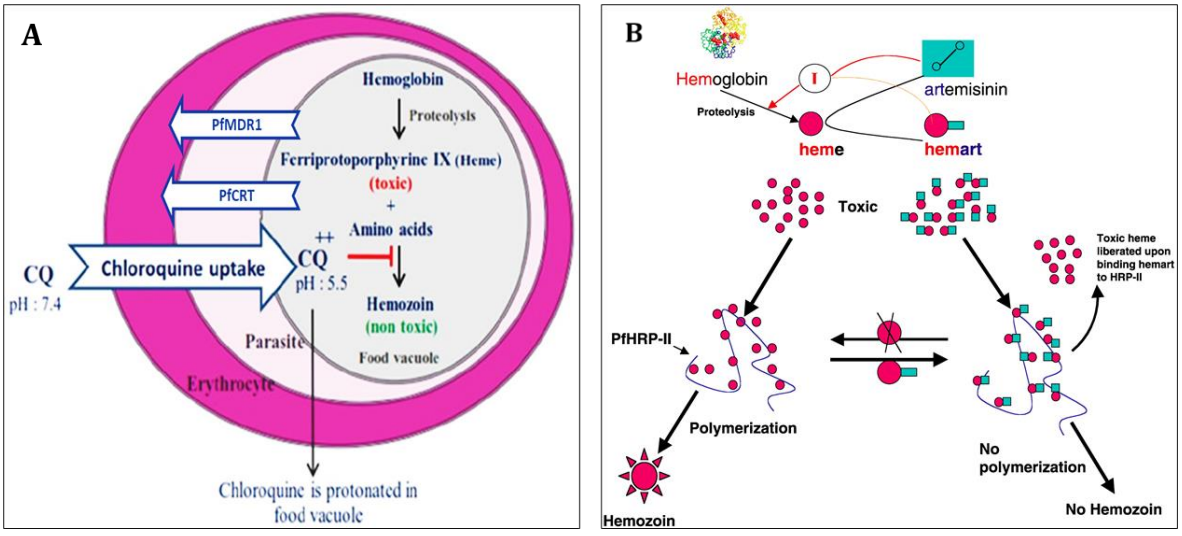


Fig. 39: Schematic model showing acquisition of anti-malarial Chloroquine and the likely mechanism of resistance and in *Plasmodium falciparum*. A. CQ (Chloroquine) is transferred to the food vacuole (FV) of the parasite and trapped in its acidic form. Mutations in transporter proteins (*Pf*CRT and *Pf*MDR1) decrease CQ accumulation in the parasite's food vacuole thus leading to anti-malarial activity; B. A model of *Pf*HRP II (histidine-rich protein II)-mediated heme polymerization and inhibition of the same by artemisinin; Proteolysis of haemoglobin liberates the toxic heme. In the presence of artemisinin, heme undergoes a reaction with the drug to form "hemart" which is toxic to parasite. Compared to heme, hemart exhibits a higher affinity for binding to *Pf*HRP II. As a result, the process of heme polymerization is expected to halt, and the excessive hemart would manifest their toxicity to the parasite. Picture adapted and modified from (280,281).

Several antimalarial quinolines like chloroquine, quinine, quinidine and mefloquine primarily have their effect during polymerization of free heme. These drugs bind with free heme in a 1:2 stoichiometry, that leads to preventing their detoxification, culminating the death of parasite. After binding to free heme in the digestive vacuole, it forms a drug-heme complex which results in accumulation of toxic free heme and starts an irreversible process of parasite demise (282). Similarly, another anti-malarial drug known as artemisinin has proven to be effective against multi drug resistant malaria (283). Artemisinin aggregates with hemozoin and causes damage to digestive vacuole. Unlike chloroquine, artemisinin's mode of action is distinct where free iron and heme accelerate the chemical modification of artemisinin leading to formation of free radicals which alkylates parasite proteins and forms adducts with heme (**Fig. 39**)(284) (285,286).

The precise mechanism for formation of Hz in parasite is not known and the topic is a subject of extensive debate. Within the parasite the likely possibilities of Hz formation may include preformed Hz facilitating an autocatalytic conversion of free heme into Hz (287) or lipids playing an important role in this process (288,289). Such assumptions point to no definitive role of parasite factors in Hz production (290-292). In contrast, in *in vitro,* Hz synthesis have been proposed to be aided by a protein (293) and lipid dependent processes (294,295).

An unexpected role of heme detoxification protein (HDP) in Hz formation was for the first time deciphered by Jani et al. (296) while working towards functional characterization of *Plasmodium falciparum* genes that contributed to malarial pathogenesis. Bioinformatics approaches were primarily employed to search for candidates that contained signal peptide, transmembrane or extracellular domains. Such approaches lead to identification of *Pf*HDP encoded by *PF*14_0446. Over expression studies of HDP in *Pf* revealed its localisation to parasite FV and also to cytosol of RBC.

The immuno EM observations of co-occurrence of HDP with in the vesicles containing Hb, likely pointed to the possibility of its binding with heme, though the amino acid sequence did not specify for any canonical heme binding domains (18). Infact elegant biophysical and biochemical studies revealed several interesting aspects of HDP that account to its heme binding and conversion into Hz. Isothermal titration calorimetry informed that pure recombinant HDP binds to heme with a K_d of 80nM and further possesses 2.7 heme binding sites. To infer, if this binding resulted in Hz production, *in*

vitro assays were set up mimicking typical enzyme and substrate like reactions with minimal HDP concentration (0.5uM) as compared to several hundred-fold excess heme (300 and 600μM). The assay revealed HDP's ability to convert nearly 50% heme to Hz. Further, the Hz formed from HDP resembled that of β-hematin, as evident by similar diffraction pattern and profile of X-Ray diffraction to that of β-hematin (297). Further SEM enabled measurements of the Hz crystals revealed dimension in the range of 0.1-0.2μm long and 0.05μm wide, that were consistent with the Hz crystals formed in FV (282).

In order to further identify the precise domain of HDP that is associated with the Hz formation activity, two truncated versions of the proteins were recombinantly expressed (18). One retaining the C- terminus fasciclin domain (spanning aa 88-205) referred to as HDP3 and other- the HDP2 region including aa 1-87. Fasciclin is an evolutionarily conserved adhesive motif present both in the prokaryotes and eukaryotes and are present in secreted and membrane anchored proteins. Both recombinants though manifested heme binding activity, however failed to produce Hz. This implicated the insufficiency of fasciclin domain alone with an indispensable full-length HDP in Hz formation. Further in order to estimate the efficiency of Hz formation by HDP, it was compared with other known Hz producers like *Pf* histidine rich protein (293) and neutral lipids (288,289,294). Multiple reactions set up with heme as a substrate (with fixed concentration of 300μM), and increasing concentrations of either HDP, HRP-2 or various neutral lipids like monoplamitic glycerol (MPG), mono-oleoyl glycerol (MOG) and oleic acid (OA) revealed HDP mediated Hz formation to be most robust. Such studies pointed to a 1500-2000-fold efficient conversion of HDP to Hz as compared to MPG and MOG (296).

Measuring precise levels of HDP in parasite is not feasible and it may be linked to the cellular levels of Hz at a given point of time. This is due to the fact that Hb acquisition and other enzymes that process Hb may be highly variable owing to parasites ability to adjust or modulate this machinery through a "just in time" concept. Thus, Hz levels are likely to be influenced by the rate at which Hb is endocytosed, the proportion of Hb proteolyzed, the quantum of HDP, lipids that concomitantly reach the FV through cytostome mediated pathways and finally how rapidly the HDP and lipids act on newly formed heme. Nonetheless, cellular levels HDP levels have been approximated. Since

HDP is secreted into the cytosol of infected cells, the total cellular content of HDP was measured by dot blot analysis (296), by plotting unknown intensity against intensity values obtained from defined amounts of HDP. Through a semiquantitative method of estimation, the approach revealed that RBC harbouring trophozoites harboured nearly 40 zmol of HDP/RBC. Considering that approximately 75% of Hb is acquired by the intracellular parasite and a nearly similar amount of HDP arrives to the FV through cytostome mediated pathways, these presumptions may account for nearly 30 zmol of protein may be present in FV for catalysing the Hz formation. Given that the heme content of trophozoite is approximately 0.55-0.6 fmol in the form of Hz (298) and the ability of HDP to convert 1566 molecules of heme into Hz/hour (296). This kinetics reveal HDP as a major player in Hz formation, *in vivo*. Taken together the rapid *in vitro* conversion rates mediated HDP and their arbitrary cellular levels likely support HDP as a major play in Hz formation, *in vivo* (296).

Attempt to analyse the cellular trafficking route taken by HDP, that accounted for its food vacuole localisation, uncovered an unusual secretion pathway. HDP does not possess any canonical secretory signals/motifs that can explain for its FV localization. Nonetheless, immuno EM revealed the accumulation of HDP in host cytosol, that however was not secreted outside the infected RBC. With maturation of parasite, the HDP was trafficked along with Hb to FV through the cytostome mediated pathway. Independent localisation snap shots associated with cytostome, transport vesicles and finally FV all suggested an unusually long route of secretion and eventual uptake by parasite. Two independent observations lead to the possible presence of a non-canonical export motif in HDP viz., an unaltered secretion of HDP in the presence of brefeldin A, a classical inhibitor of constitutive exocytosis and lack of any discernible effect on the secretion of both N-term or C-term tagged myc HDB. The later observation reiterated that translational fusion of HDP with myc tag did not affect any possible secretion mediated by N or C terminal signal sequences that may act as trafficking motifs. Taken together these findings indeed account for a yet to be identified export motif in HDP that accounts for its secretion into RBC cytosol (296).

Analysis of the constituents of the FV revealed the presence of a mixture of saturated and unsaturated fatty acids and also neutral lipids (288) and it has been demonstrated that within FV, the Hz crystals are delimited by capsules that contain a mixture of mono

and diacyl glycerols (288). As discussed earlier, the role of neutral lipids like MPG and MOG in Hz production, *in vitro*, the possibility of HDP forming Hz, chaperoning their delivery of lipid nanosphere to form Hz crystals cannot be ruled out (107) and their *in vivo* interactions need to be further investigated.

In order to characterise the heme binding residues of *Pf*HDP, the crystal structure of *Pf*HDP was modelled. This approach revealed two well characterised histidine rich heme binding domains viz., HHAHHAADA and HHAAD that were referred as HeD1 and HeD2 in *PfHDP* (299). To decipher the functional role of these motifs, two deletion mutants -$PfHDP_{HeD1}$ & $PfHDP_{HeD2}$ were generated. These mutants showed a significant reduction in heme to Hz formation as compared to WT, that however did not account for a total loss of Hz formation. Another independent study also arrived at a similar conclusion where complete deletion of all the histidine residues still retained 49% of Hz formation activity as compared to the WT (300). These observations likely pointed to the possibility of having other heme binding domains in HDP. Interestingly, an additional role of *Pf*HDP was also documented in vesicles that transports haemoglobin. Hb binding region of *Pf*HDP was aligned against the known sequence of falcipain-2 which revealed a partially homologous sequence of 14 unique amino acids. *PfHDP* mutant (*Pf*HDP_{HbD}) lacking this region had reduced capacity to bind haemoglobin as compared to wild type (301).

Heme which is an important by-product of Hb hydrolysis, is also synthesized by the malaria parasite. Infact, the *de novo* synthesis of heme can also additionally serve as a therapeutic target for control of malaria (302). Apart from intraerythrocytic stages, heme is also required for development of mosquito stage and liver stages of *Plasmodium*. Depletion of the first enzyme- δ-aminolevulinate synthase (ALAS) and the last enzyme-ferrochelatase (FC), in the heme-biosynthetic pathway in *Plasmodium berghei* manifested dramatic reduction in oocyst colonization and absence of sporozoites in salivary gland. Treatment of infected mosquitoes with aminolevulinic-acid (ALA) supplement restored the infectivity of sporozoites delivered naturally or via intravenous injection yielding a break through infection with pre-patency similar to WT. Overall this indicates that the parasite heme synthesis is also essential for both mosquito and liver stages (303) in niches that are not naturally abundant in heme. While heme biosynthesis occurs in multiple life cycle stages, high abundance from host in blood stages may call for an

indispensable role of HDP to achieve detoxification. This likely hints to the essential role of HDP in erythrocytic stages. Infact, *PfHDP* locus has been shown to be recalcitrant to genetic modification as evident by two independent observations (296). Firstly, a single cross over approach failed to replace the endogenous locus, as the transfectants still expressed HDP as revealed by immunofluorescence assay. Secondly, attempts to replace the endogenous locus with tagged chimeric version of HDP though yielded integrants, as confirmed through southern blotting, revealed that the HDP locus was intact and the targeting construct was integrated in a non-specific manner in the genome. This and other studies reiterated the indispensable nature of *Pf*HDP (304,305).

As HDP is essential for *Pf* blood stages, its functional analysis in other life cycle stages of *Plasmodium* is not feasible. HDP is highly conserved with orthologues in seven other *Plasmodium* species (306) with homologs in other protozoans like *Theileria* (296), *Babesia* and *Toxoplasma* (296). Within *Plasmodium* species, HDP shows 60% sequence identity, while exhibiting less than 15% sequence identity outside the *Plasmodium* genus. Hz formation assays performed with purified recombinant protein of *Py*HDP, yielded kinetics on par with *Pf*HDP, likely suggesting the functional equivalence of HDPs across species. These findings may also mean that the biological function of HDP is also similar in *in vivo* settings. Therefore, we resorted to investigate the functional role of HDP in rodent model of *Plasmodium berghei*. As rodent models offer easy accessibility to study mosquito and liver stages of *Plasmodium*, functional investigation of HDP mutants could be done with relative ease. However, considering the indispensable nature of HDP in *Plasmodium*, we resorted to use a yeast based Flp/FRT conditional mutagenesis system. In this approach the endogenous HDP locus is replaced with an engineered copy of HDP, flanked with FRT site in a line that expresses Flp recombinase through the TRAP promotor, active in oocyst stage. As FRT sites are recognition sequences for Flp recombinase, the FRTed locus undergoes excision in oocyst stage yielding conditionally silenced HDP midgut sporozoites. These conditional mutants can further be analysed for their ability to infect salivary glands, liver cells and initiation of blood stage infection, by genotyping and confirming presence of excised/edited HDP locus in multiple life cycle stages. This approach will help us to infer the role of *PbHDP* in mosquito stages liver stages of the parasite.

3.2 Materials and methods

Since most of the research methodologies are common for the study and have already been discussed in great detail in the previous chapter, only a brief mention is made in this chapter, to avoid redundancy.

3.2.1 Primer Information

SL NO.	NAME OF THE PRIMER	SEQUENCE (5' TO 3')
1.	*Pb* HDP RT FP	TCATCGCTAATTCACGAACTAT
2.	*Pb* HDP RT RP	TATACATCCACAGACCTTTAG
3.	*Pb* HDP CKO 5'HR FP1	ACG**CCGCGG**TATCTGCTCCATCATGCCA
4.	*Pb* HDP CKO 5'HR RP1	CTA**GTCGAC**ATTGAAAATATGAAAATGAGAAAA
5.	*Pb* HDP CKO ORF FP2	GTA**GAATTC**GTGAAAACATGCACTTTAAATT
6.	*Pb* HDP CKO ORF RP2	CAG**GCGGCCGC**GGGAAATATGAAAATTGTTTATG
7.	*Pb* HDP CKO 3'HR FP3-	GGA**CTGCAG**TACTATTTAAATGAAGATTTTCC
8.	*Pb* HDP CKO 3'HR RP3	CCT**GGTAAC**GGGAAATATGAAAATTGTTTATG
9.	*Pb* HDP CKO confirmation 5UTR FP4	AAATCTTCATCAACTTCATCTTC
10.	*Pb* HDP CKO confirmation 5'UTR reverse primer RP4	TACTTTCTAGAGAATAGGAACT
11.	*Pb* HDP CKO Confirmation 3'UTR forward primer FP5	TACACTTTATGCTTCCGGCT
12.	*Pb* HDP CKO confirmation 3'UTR reverse primer RP5	TTTATTTCATAGCAAAACATGG

Table 3: List of primers and their sequences used for the real time PCR analysis, construction of transfection plasmids, confirmation of site-specific integrations.

3.2.2 Retrieval of *Plasmodium berghei HDP* (*Pb*HDP) gene and protein sequences for alignment and similarity analysis

PlasmoDB (www.plasmodb.org) was searched to retrieve *Pb*HDP gene and amino acid sequences. The gene ID of *Pb*HDP is PBANKA_1310600. Amino acid sequence of rodent and human *Plasmodium* orthologues were obtained from the database and aligned using online alignment tool Clustal Omega (ClustalW) (http://www.ebi.ac.uk/Tools/msa/clustalo/). The gene ID for rodent orthologue- *P. yoelli* (*Py*HDP) is PY17X_1314400. The gene orthologues of human malaria species- *P. falciparum* (*Pf*HDP), *P. vivax* (*Pvx*HDP) and *P. knowlesi* (*Pknh*HDP) were respectively PF3D7_1446800, PVX_118155 and PKNH_1235400. NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) was used to find the similarity of *Pb*HDP amino acid across other *Plasmodium* species. The sequence information was also used to design primers (**Table no. 3**) for generation of *Pb*HDP conditional knock out and for *PbHDP* quantitative gene expression analysis using qRTPCR.

3.2.3 Quantitative real time PCR analysis of *PbHDP* expression across all the life cycle Stages

For quantification of stage specific gene expression of *PbHDP,* absolute quantification method was used. RNA was extracted from all life cycle stages viz., mixed blood stages, mosquito stages (D14 midgut and salivary gland sporozoites), developing liver stages (13h, 24h, 36h, 47hr, 65hr EEF and merosomes). From all the above stages, 2µg RNA was reverse transcribed to generate cDNA. To generate gene specific standard for *PbHDP*, a 169bp was amplified from 2nd exon by PCR and the product was cloned in to pTZ57R/T vector. The insert was confirmed with specific restriction enzymes.

Following estimation of the DNA of *PbHDP*, it was diluted in a logarithmic manner to establish a dynamic range from 10^8 to 10^2 copies of plasmid per µl. Identical volumes of stage specific cDNA samples were used for analysing the expression status of *PbHDP*. Real time PCR was set up in a total reaction mixture of 10µl containing SYBR green PCR master mix (TAKARA TB Green® Premix Ex Taq™ II Tli RNase H Plus Catalogue No.#RR82WR), 0.25µM of each primer, template and nuclease free water to make up the volume. The reaction mixture contained as template either known concentrations of the *PbHDP* standards or cDNA samples. At the end of qRT-PCR run, the target gene was expressed as equivalents of gene specific standards. A ratio of absolute copy numbers of

PbHDP and *Pb 18S rRNA* was obtained to normalize the gene expression data and graph was plotted.

3.2.4 Generation of *PbHDP* (*PB*ANKA_1310600) conditional KO (cKO) construct

Due to indispensable role of *PbHDP* in asexual blood stages, a regular knock out to disrupt the gene was not possible. This precluded the investigation of HDP in other life cycle stages. Therefore, we employed a conditional mutagenesis approach, that involved replacing the endogenous *PbHDP* locus with an engineered construct that carried the *PbHDP* ORF and its regulatory elements (5' and 3') flanked by Flp recombinase recognition sequence-FRT (*PbHDP* cKO cassette). Double homologous recombination strategy was followed for replacing the FRTed version of *PbHDP* into the endogenous locus. Towards the construction of *PbHDP* cKO cassette, we used pSKC-TRAP-GFP-2FRT vector. To achieve correct locus specific integration of the FRTed version of *PbHDP*, the 5' and 3' homology arms flanking *PbHDP*, together with *PbHDP* ORF and its regulatory sequence were cloned into the pSKC-TRAP-GFP-2FRT. A 765bp of 5'UTR, a 629bp of 3'UTR and 1478bp DNA sequence (spanning the promotor, ORF and 3' UTR regions of *PbHDP*) were amplified from genomic DNA using primers (FP1/RP1, FP2/RP2, FP3/RP3 respectively) as mention in **table no. 3**. PCR was set up in a reaction mixture containing 1x NEB PCR buffer, 1mM dNTPs each (Thermo Scientific, cat no.# R72501), 2.5 mM $MgCl_2$ (Biorad, cat no.#1708872), 0.25 µM forward and reverse primers, 50ng of *P. berghei* genomic DNA and 1.25U of Taq DNA Polymerase (NEB cat no.# M0273). Thermal cycling was performed at using Eppendorf master cycler (cat no.# Z316091). The thermal cycling conditions were: 95^0C for 3 min and 95^0C for 30 sec, annealing at 60^0C for 45 sec followed by synthesis at 68^0C for 1 min. The final extension was kept for 10 mins at 68^0C and the PCR cycles was repeated for 30 times except the initial denaturation and final extension part. The amplified product was analysed on 1% agarose gel (w/v) and later visualizing it under UV. Amplified DNA was subjected to purification using DNA clean up and gel extraction kit (Thermo scientific cat no.#KO832).

3.2.5 Transfection of *Pb*HDP cKO construct into a *Plasmodium berghei* parental line expressing FlpL recombinase and selection of transfected parasites.

Transfection was performed using Amaxa nucleofector kit (Lonza cat no.#VPA-1006) as described earlier. Propagation of FlpL parental line was done in Swiss Albino

mice. After attaining a parasitemia of 3-5%, blood was collected from mice and was cultured for schizont enrichment at 37^0C for 16h. Next day, the enriched schizonts were purified and electroporated with *PbHDP c*KO construct along with a mixture of mouse T cell nucleofector solution (90µl) and supplement (10µl) using Amaxa nucleofector II device following previously optimized program U-033. Transfected parasites were selected under pyrimethamine drug, administered orally to the mice. After successful selection, drug resistant recombinant parasites were cryopreserved with a composition of 1 part blood and 2 parts cryopreservative. 250µl of this mixture was aliquoted in each cryovial and transferred to -80^0C in a freezing container (Nalgene, cat no.#5100) that allows cooling of the samples at the rate of 10^0C per/min. Subsequently the samples were stored in liquid nitrogen.

3.2.6 Confirming the stable integration of cKO construct in PbHDP locus endogenous locus

Integration of cassette in the endogenous locus was confirmed by PCR using primers in **table no. 3** that amplified products from endogenous (non- recombined region) and recombined locus. For genotyping the transfected population, infected blood containing recombinant parasites was lysed in 0.5% saponin followed by centrifugation at 8000 rpm for 5 mins at room temperature. Above process was repeated twice, followed by a wash with sterile 1X PBS and centrifuged at 8000 rpm for 5 mins at room temperature. Then genomic DNA was isolated using DNAsure tissue mini kit (Genetix, cat no.# NP-61305). The 5' and 3' integrations were confirmed using primers pairs (FP4/RP4 and FP5/RP5) as indicated in **table no. 3**. After confirming integration, the recombinant parasite line was transmitted to mosquito.

3.2.7 Growth analysis of *PbHDP* cKO parasites in blood stages

Approximately $1x10^3$ infected parasites of both *Pb* WT GFP and *PbHDP* cKO parasites were intravenously injected in Swiss Albino female mice (4-6weeks old). Parasitemia was monitored daily for 8 days to compare and assess the asexual growth kinetics of *PbHDP* cKO parasites with WT line. Giemsa-stained blood smears were prepared on a daily basis and a minimum of 25 fields were analysed randomly. The mean percentage of parasitemia was calculated and plotted as a graph. To monitor gametocyte propagation in *PbHDP* cKO line, gametocytes were enriched using previously described

methodology. Following enrichment, gametocytemia was estimated from day 7 to day 10 post infection (I.V.) with asexual stages and the results were plotted and represented as bar diagram.

3.2.8 Transmission of *PbHDP c*KO parasites to *A. stephensii* to study mosquito stages of the parasite

For transmission of *PbHDP c*KO parasites to *Anopheles* mosquitos, around 10^7 iRBCs of both *Pb* WT GFP and *PbHDP* cKO were passaged to 4-6 weeks old Swiss Albino female mice. Smears were made from infected mice and stained with giemsa. The mice that were positive for gametocytes were selected for mosquito feeding. Two blood meals were given to mosquitoes for a duration of 15-20 min with a gap of 20-24h. The infected mosquitos were maintained at 20^0C at 80% RH for 16 days. Subsequently, the mosquito cage was shifted to 25^0C with 80% RH, to induce maximal activity of FlpL recombinase in the parasite. The maximal expression of FlpL is expected in oocyst (D14 post blood meal), as the parasite specific promotor TRAP is active at this stage and the recombinase is driven through TRAP promotor element. We noted the presence of GFP expressing oocyst under an upright fluorescent microscope (Nikon Eclipse). The images were captured, processed and deconvoluted by NIS software. Currently we are analysing the fate of these conditionally silenced sporozoites for their ability to colonize the salivary gland and infect hepatocytes.

3.3 Results

3.3.1 Stage specific gene expression *PbHDP* shows maximum expression in salivary gland sporozoite

*Pb*HDP has been annotated as PBANKA_131600. Towards understanding the functional role of *PbHDP* (PBANKA_131600) stage specific gene expression analysis was performed by qPCR using absolute quantification method. Highest expression was observed in salivary gland sporozoite stage followed by a mild expression in mixed blood stages and midgut sporozoite stages (**Fig. 40**).

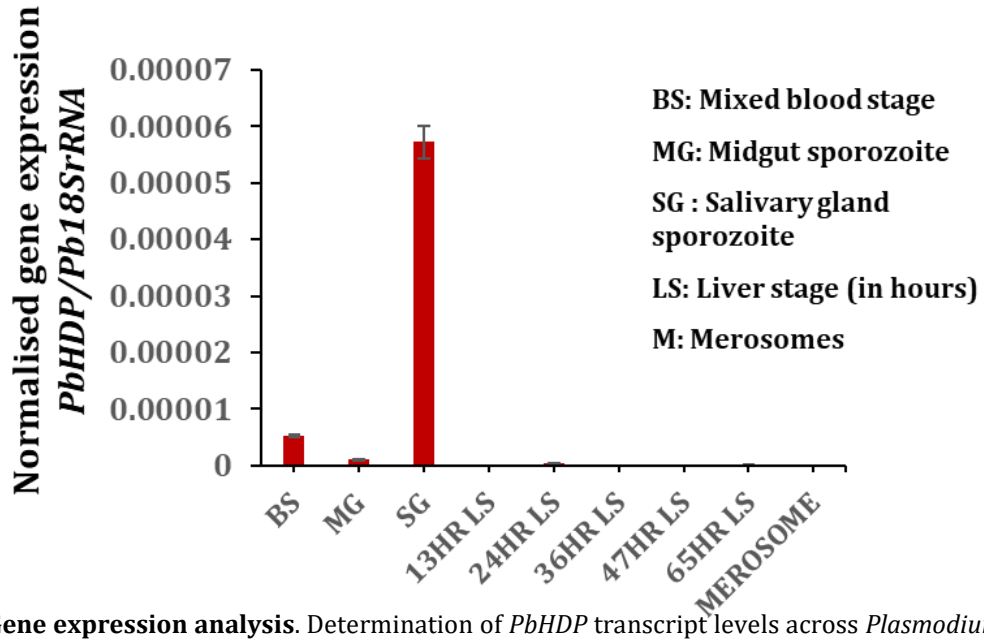
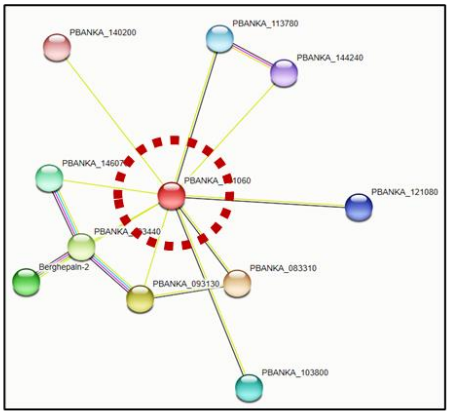


Fig. 40: Gene expression analysis. Determination of *PbHDP* transcript levels across *Plasmodium berghei* life cycle stages by quantitative real time PCR. cDNA samples were generated from different stages as indicated. MBS: mixed blood stages, Mg spz: midgut sporozoites, Sg spz: salivary gland sporozoite stage, liver stages (LS) in hours (h): 13h LS, 24h LS, 36h LS, 47h LS, 65h LS and M: merosomes. The expression values were normalized with *Pb*18SrRNA (internal control gene). Gene expression analysis of *PbHDP* by real-time PCR reveals maximal expression in the salivary gland sporozoite stage.

3.3.2 STRING database showing possible interacting *Plasmodium* proteins with *PbHDP*

STRING database, predicted several proteases as interacting partners like M18 aspartyl aminopeptidase, dipeptidyl aminopeptidase, plasmepsin, falcipin (**Fig. 41**).



Your Input:		Neighborhood	Gene Fusion	Cooccurence	Coexpression	Experiments	Databases	Textmining	[Homology]	Score
PBANKA_131060	annotation not available (204 aa)									
Predicted Functional Partners:										
PBANKA_083310	M18 aspartyl aminopeptidase, putative; Aminopeptidase, putative ; Belongs to the peptidase M18 family							•		0.658
PBANKA_093130	Dipeptidyl aminopeptidase 1, putative; Uncharacterized protein; Belongs to the peptidase C1 family							•		0.652
PBANKA_103440	Plasmepsin, putative; Plasmepsin ; Belongs to the peptidase A1 family							•		0.643
Berghepain-2	Falcipain 2, putative ; Belongs to the peptidase C1 family							•		0.630
PBANKA_146070	Dipeptidyl aminopeptidase 2; Preprocathepsin c, putative ; Belongs to the peptidase C1 family							•		0.562
PBANKA_103800	Cytochrome c, putative; Electron carrier protein. The oxidized form of the cytochrome c heme group can accept an ele...							•		0.428
PBANKA_113780	annotation not available							•		0.427
PBANKA_121080	Hypoxanthine-guanine phosphoribosyltransferase, putative; Hypoxanthine-guanine phosphoribosyl transferse (HGPR...							•		0.427
PBANKA_144240	annotation not available							•		0.420
PBANKA_140200	annotation not available							•		0.416

Fig. 41: Prediction of possible interacting partners of *PbHDP* by STRING analysis. STRING analysis showing the possible interaction of *PbHDP* with ten interacting partners. The query sequence yielded 10 interacting partners. Following are the gene IDs (in bracket) of the associating partners. M18 aspartyl aminopeptidase (PBANKA_083310), dipeptidyl aminopeptidase 1 (PBANKA_093130), plasmepsin (PBANKA_103440), berghepain-2 (Flacipain-2), dipeptidyl aminopeptidase 2 (PBANKA_146070), cytochrome C (PBANKA_103800), glideosome-associated connector (PBANKA_1137800), hypoxanthine-guanine phosphoribosyltransferase (PBANKA_121080), protein KIC2, putative (PBANKA_1442400) and sodium/hydrogen exchanger, putative (PBANKA_1402000) The association evidence was mentioned under the category of text mining for all the candidates.

While STRING predicted interactions need validation from *in vivo* pull-down experiments, it is only speculative if HDP has any role in stabilizing or facilitating the functions of these interacting clients. Since majority of the interacting partners are proteases, it is likely possible that constant digestion of haemoglobin and detoxification of heme may be also aided by these proteases and tightly regulated by *PbHDP*. This function of HDP could be in addition to its role as a protease mediating the progressive degradation of haemoglobin and convert toxic heme to hemozoin.

This explain why *PbHDP* is indispensable in blood stages. Further, *PbHDP* showed the highest expression in salivary gland stage, which may imply to some important moonlighting functions of this protein in mosquito stages of *Plasmodium*, that needs to be further investigated.

3.3.3 Retrieval of *Plasmodium berghei* (*Pb*) target gene, protein sequences, alignment and similarity analysis of *Pb*HDP

Multiple sequence alignment of amino acid sequence of *Pb*HDP was performed using ClustalW. The sequence alignment revealed that *Pb*HDP is conserved in other *Plasmodium* orthologues (**Fig. 42**).

```
Clustal Omega 1.2.3 Multiple Sequence Alignments

Click here to view a phylogenetic tree of the alignment.

PBANKA_1310600.1-p1   1 .......... .....MKKKL YNLVLKRNYT RSGGLRKPQK VTNDPESINR KVYWCFEHKP VRRTVINLIF SHNELKNFST
 PY17X_1314400.1-p1   1 .......... MFPKNMKKKL YNLVLKRSYT RSGGLRKPQK VTNDPESINR KVYWCFEHKP VRRTVINLIF SHNELKNFST
 PF3D7_1446800.1-p1   1 .......... ....MKNRFY YNLIIKRLYT RSGGLRKPQK VTNDPESINR KVYWCFEHKP VKRTIINLIY SHNELKIFSN
  PKNH_1235400.1-p1   1 MRTHLPKIRP KYKRMKKSHP PFLIIKRLYT RSGGLRKPQK VTNDPESINR KTYWCFEHKP IKRTMVNLIY SHNELKLFSR
     PVX_118155.1-p1   1 .......... ....MKKSRP PFLVIKRLYT RSGGLRKPQK VTNDPESINR KTYWCFEHKP IKRTLVNLIY SHNELKLFSR

PBANKA_1310600.1-p1  66 LLKNTNASSS LIHELSLEGP YTGFLPSDEA LNLLSTNSLN KLYKDDNKMS EFVLNHFTKG LWMYRDLYGS SYQPWLMYNE
 PY17X_1314400.1-p1  71 LLRNTNASSS LIHELSLEGP YTGFLPSDEA LNLLSTNSLN KLYKDDNKMS EFVLNHFTKG LWMYRDLYGS SYQPWLMYNE
 PF3D7_1446800.1-p1  67 LLNHPTVGSS LIHELSLDGP YTAFFPSNEA MQLINIESFN KLYNDENKLS EFVLNHVTKE YWLYRDLYGS SYQPWLMYNE
  PKNH_1235400.1-p1  81 FLSHPNVGTS LIHELSLEGP YTGFLPSNEA LKLISPESLA KLYEQGDKLM EFVLGHFTKD FWLYRDLYGS SYQPWLVFNE
     PVX_118155.1-p1  67 FLNHPNVGTS LVHELSLEGP YTGFLPSNEA LKLISPESLA KLYEEGDKLM EFVLGHFAKD FWLYRDLYGS SYQPWLVFNE

PBANKA_1310600.1-p1 146 KREAPEKIPT LVNNDIIVKI EGEFKNCDHS IYLNEAKIIR PNMKCHNGII HIIDKPIIF.
 PY17X_1314400.1-p1 151 KREAPEKIQT LVNNDIIVKI EGEFKNCDHS IYLNEAKIIR PNMKCHNGII HIIDKPIIF.
 PF3D7_1446800.1-p1 147 KREAPEKLRN LLNNDLIVKI EGEFKHCNHS IYLNGSKIIR PNMKCHNGVV HIVDKPIIF.
  PKNH_1235400.1-p1 161 KREAPEKITN LVNKDLLVKI TGEFKNCDHS IFLNGAKIIR PNMKCHNGVV HIVDRPIIQR
     PVX_118155.1-p1 147 RRDAPEKITN LVNRDLLVEI TGEFKNCDHS ISLNGAKIIR PNMKCHNGVV HIVDRPIIQR
```

Fig. 42: Multiple amino acid sequence alignment of *Plasmodium Pb*HDP orthologues.
P. berghei (Pb), P. yoelli (PY), P. falciparum (PF), P. knowleshi (PKNH), and P. vivax (PV).

RMgmDB- Rodent Malaria genetically modified Parasites (https://www.pberghei.eu/index.php) database also pointed out the unsuccessful attempt to disrupt the gene which rendered the parasites nonviable in blood stage of the parasites thus failing to generate a KO mutant (**Fig. 43**).

A

Rodent Malaria genetic modifications (RMgmDB) Download Data sets

Search this table... Showing 2 rows

RMgmDB	PubMed	Successful Modification	Modification Type
RMgm-3455	28708996	NO	disrupted
Phenotype:			
RMgm-807	25941254	NO	disrupted

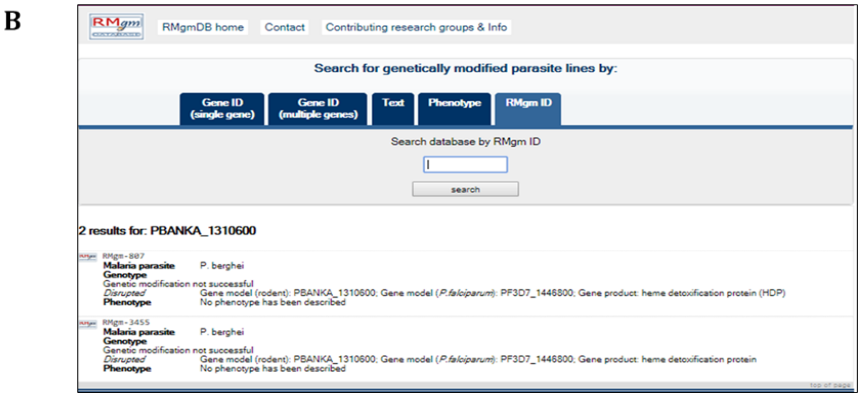


Fig. 43: Information retrieved from RMGM database related to *PbHDP*. A) Unsuccessful attempts to disrupt the gene which rendered the nonviable parasites in blood stage. B) Information regarding attempts of gene modification by individual research groups.

3.3.4 Generation of conditionally silenced *PbHDP* parasites

Based on the previous reports that *PbHDP* locus is recalcitrant to gene disruption, we resorted to a conditional mutagenesis approach as described in earlier section (**Fig. 44 A-G**).

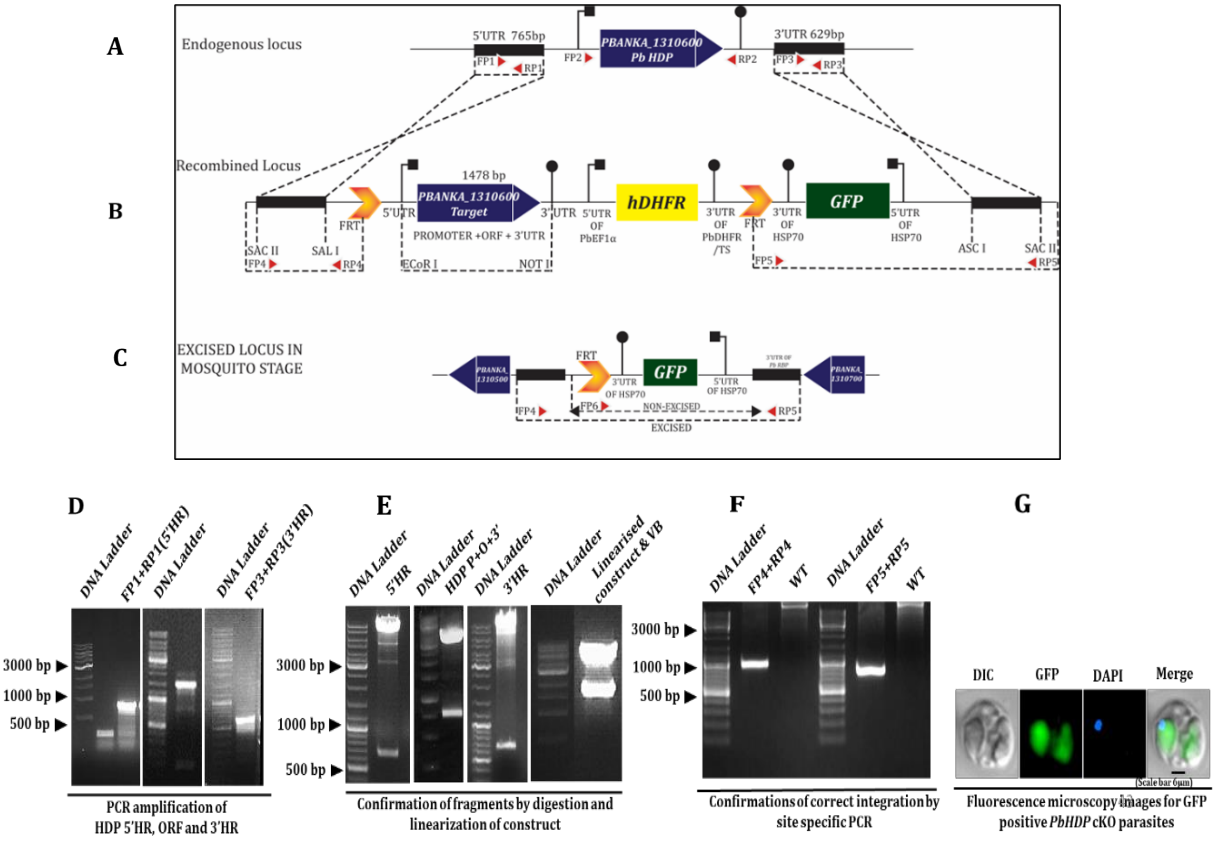


Fig. 44: Generation of *PbHDP* conditional knockout parasites. A) Representation of *PbHDP* endogenous locus showing *PbHDP* ORF, 5' and 3'UTRs. B) Recombined locus showing elements of the targeting plasmid

showing, 765 bp of *PbHDP* 5' homology region followed by a 1478 bp of *PbHDP* promotor+ORF+3'UTR, hDHFR cassette, GFP cassette and 629 bp of *PbHDP* 3' flanking region. The first FRT site is present in between *PbHDP* 5' homology region and promoter+ORF+3' UTR, and the second FRT is after the hDHFR cassette. C) Excised locus after transmitting in to *Anopheles* mosquitos D) 1% agarose gels showing PCR amplified products of 765 bp of 5' HR, 1478 bp of promoter+ORF+3' UTR, and 629 bp 3' HR of *PbHDP* using primer set FP1/RP1, FP2/RP2 and FP3/RP3 respectively. E) Confirmation of cloned products in conditional transfection vector. Agarose gel showing digestion products of: 5' HR (765 bp, obtained using restriction enzymes SacII/SalI), promoter+ORF+3'UTR (1478 bp, obtained using restriction enzymes EcoRI/NotI), and 3' HR of *PbHDP* (629 bp, obtained using restriction enzymes AscI/SacII). F) Diagnostic PCRs showing the confirmation of site-specific integration of targeting cassette using diagnostic primers (FP4/RP4, FP5/RP5). Forward primer (FP16) was designed upstream to the site of integration in endogenous locus and the reverse primer (RP 16) was designed within the targeting cassette. Expected products size with these diagnostic primers that confirmed correct integration. G) Merged DIC image of *PbHDP* conditional knockout parasites expressing GFP reporter.

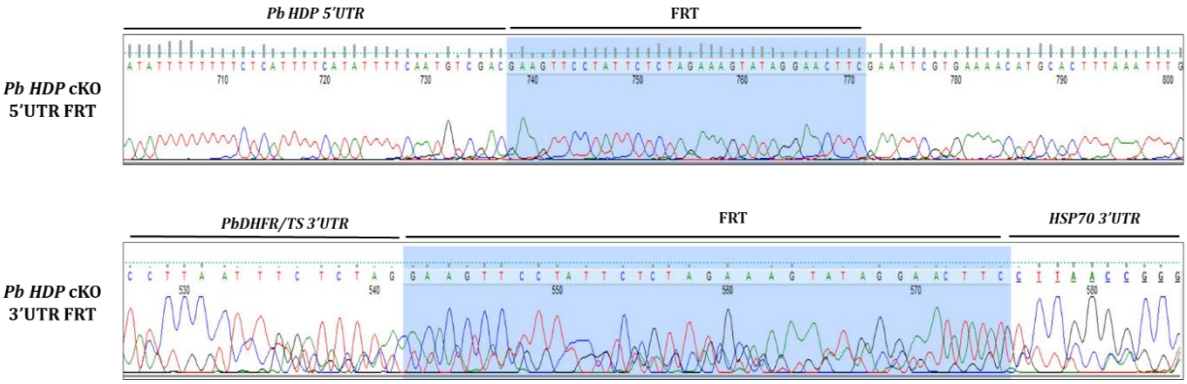


Fig. 45: Sequencing of recombined locus to show the intact FRT sites on both 5' and 3' sides.

We successfully obtained conditional mutants for HDP as confirmed by sequencing the recombined locus that revealed stable integration of FRT sites, flanking the *PbHDP* locus (**Fig. 45**).

3.3.5 *PbHDP* conditional KO parasites were comparable to WT in asexual; propagation and gametocyte formation.

The conditional KO (cKO) recombinant parasites were analysed for asexual blood stage propagation and gametocyte formation as compared to *Pb* WT GFP parasites. In this regard, approximately $1X10^3$ infected RBCs of either *PbHDP* cKO or WT parasites were injected via intravenous injections in a group of 4 mice and parasitemia was monitored for 8 days, daily by observing giemsa stained blood smears (**Fig. 46**). Further enrichment and quantification of gametocytes was also done in different group of mice and the gametocytemia was monitored for 10 days.

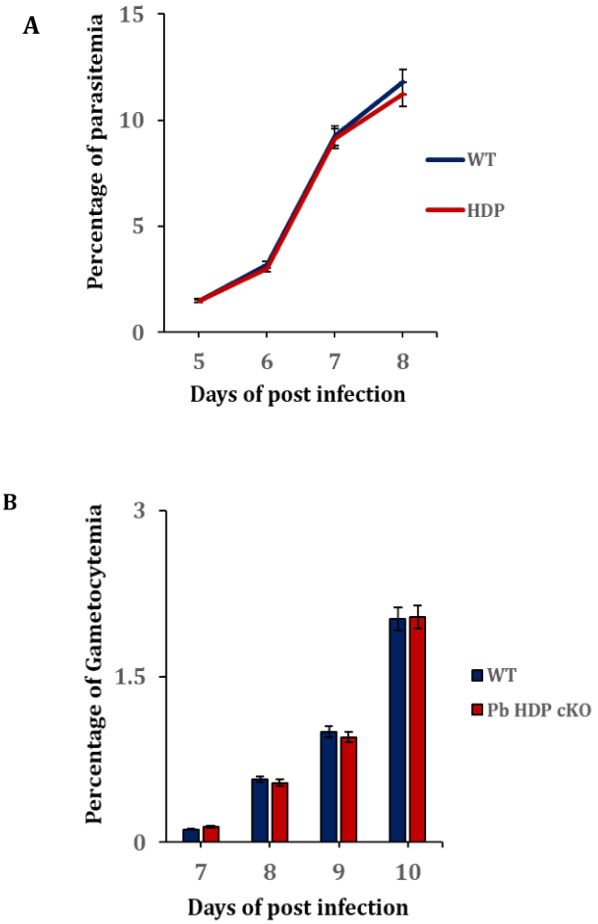


Fig. 46: Asexual blood stage propagation. A) 1X10^3 infected RBCs of *Pb* WT GFP and *PbHDP* cKO parasites were injected intravenously into mice and parasitaemia was monitored daily for 8 days by microscopic observation of Giemsa-stained blood smears. B) Bar diagram showing quantification of enriched gametocytes monitored for 10 days post infection.

PbHDP conditional KO parasites showed no defect in both asexual blood stage propagation and gametocyte production in comparison with WT (**Fig. 46 A-B**). This concludes that replacement of endogenous *PbHDP* locus with a FRTed locus of *HDP,* did not affect parasite viability. Interestingly, while generation of KO was not possible, replace of the HDP locus with an engineered version harbouring flanking FRT sites was readily integrated into the HDP locus and such genetic modification did not late the viability of parasites. The retention of the FRT sites at both 5' and 3' regions of *PbHDP* was confirmed by sequencing the locus (**Fig. 45**.)

3.3.6 Transmission of *PbHDP* conditional KO parasites to *A. stephensii*

To study the role of HDP beyond sexual stages, *PbHDP* cKO parasites were transmitted to *Anopheles* mosquitos. The mosquito midguts were dissected on day 14th to analyse the oocyst burden (**Fig. 47**).

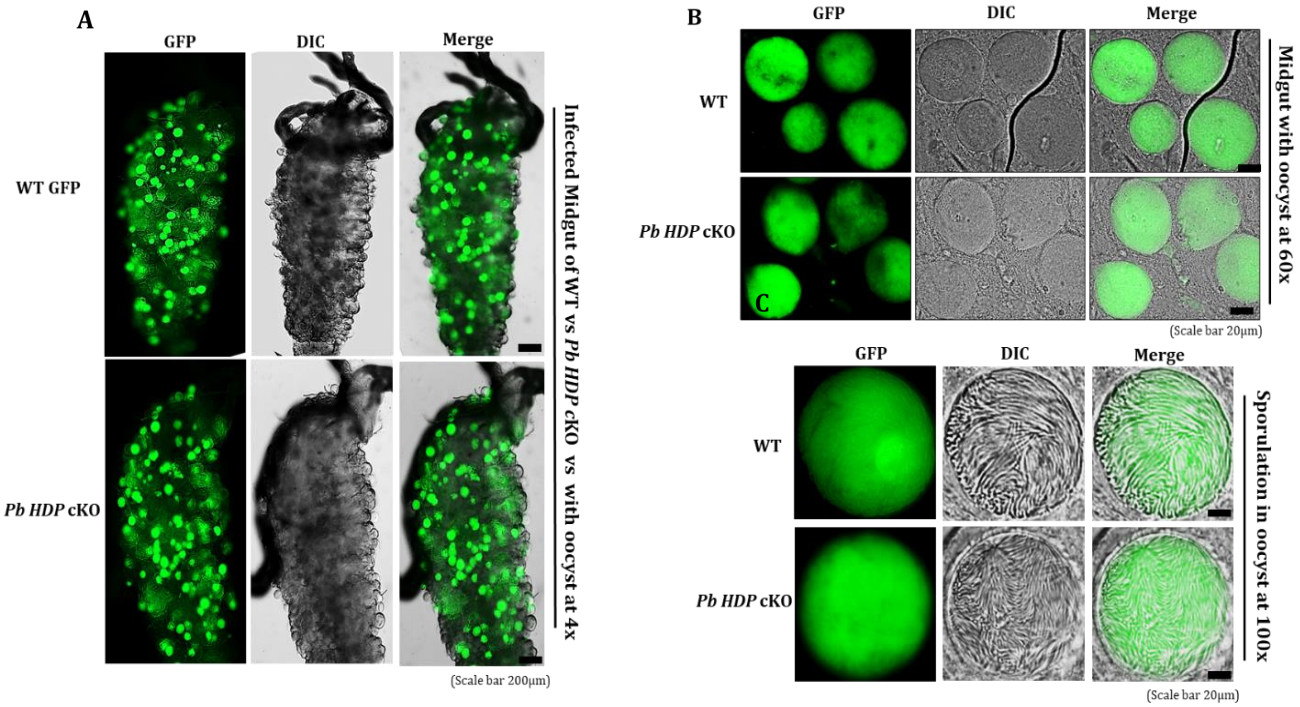


Fig. 47: ***PbHDP* cKO parasites showed similar oocyst infectivity to that of *Pb* WT GFP parasites**. Infection was transmitted to *Anopheles* mosquito from mice harboring gametocyte stages of either *Pb* WT GFP or *PbHDP* cKO parasites to initiate sexual cycle in the mosquito. A) Dissected mosquito midguts on day 14 post-feeding showing oocysts for WT GFP and *PbHDP* cKO parasites at similar rates. B) Infected mosquito midgut showing oocysts at 60X. C) Sporulation pattern in a single oocyst from both WT GFP and *PbHDP* cKO parasites.

We noted that *PbHDP* conditional KO parasites were able to successfully colonise in mosquito midgut and form oocysts similar to WT parasites. Further, the sporulation pattern inside the oocyst was also comparable to WT. Currently the line is under investigation to understand the role of *PbHDP* in salivary gland stages and liver stages.

3.4 Discussion

In the current study, we have assessed the functional role of PBANKA_1310600, referred to as Heme detoxification protein (HDP) by conditional mutagenesis approach. We were interested to investigate the functional role of HDP owing to its high expression in asexual blood stages (296,307) and detection of the protein in salivary gland sporozoite stages (303), being a conserved *Plasmodium* gene.

Our study demonstrates the expression of *PbHDP* in blood stage parasites and a significantly robust expression in salivary gland sporozoites. We have also shown that conditionally silenced *PbHDP* mutant parasites are successfully colonising the mosquito midgut. Process of Hb uptake and break down is an absolute survival mechanism of *Plasmodium* during asexual blood stage growth. During the crucial process of Hb catabolism, heme is generated as a byproduct which compromises survival of the parasite. It has been earlier reported that heme detoxification protein is involved in conversion of toxic heme to inert bio-crystals known as hemozoin (296). Though sufficient number evidences have been documented yet the sequence of events and factors involved to accomplish the heme biosynthetic pathway is poorly understood (107,308). ~200-kDa protein complex is present in food vacuole, which is required for degradation of Hb and Hz formation. This complex contains several parasitic proteins such as endopeptidases, cysteine proteases (falcipain 2/2′), and aspartic proteases (plasmepsin II & IV) and heme detoxification protein.

To decipher the role in malaria pathogenesis, *PfHDP* (PF14_0446/PF3D7_1446800) was one of the hypothetical genes selected for a functional genomics study with the help of *in silico* approaches. It revealed that though *Pf*HDP lacks a transmembrane domain and a classical signal sequence yet it encodes for a domain known as fasciclin (309,310). To understand the involvement of the precise domain in Hz formation, two truncated versions of *Pf*HDP protein was generated out of which one had C-terminus fasciclin domain. The recombinantly expressed truncated proteins were successful in heme binding activity but failed to produce Hz, inferring the inability of unaided fasciclin domain in Hz formation (293). With the help of anti-*Pf*HDP antibody, the protein was localised both in cytoplasm of infected RBC and in transit vesicles responsible for host Hb transport to food vacuole. As reported, *Pf*HDP is known to be exported into the cytosol of infected erythrocytes and takes a convoluted trafficking

pathway to finally reach the food vacuole and catalyses the synthesis of Hz. It shows that trafficking of *Pf*HDP in RBC cytosol happens via cytostomal uptake in vesicles having Hb, instead of the classical secretory pathway (296). Subsequently each molecule of recombinant *Pf*HDP protein is involved in conversion of 1566 molecules of heme to hemozoin per hour, elucidating a robust interaction of the protein during the process of Hz formation. It is well known that 75% of the host haemoglobin is endocytosed and delivered to the FV as a cytosolic component. A similar proportion of HDP is being delivered to the food vacuole and distributed homogenously in this environment to facilitate the formation of Hz. This concludes the importance of HDP for asexual growth of parasite in blood stages. As shown in our investigation, the gene expression data reveals a considerable expression of HDP in mixed blood stages. Moreover, when we inspected RMgmDB for information related to disruption of *Pb*HDP, it exhibited that the attempt of modifying the gene was unsuccessful, likely suggesting its indispensable role in blood stages.

The existence of multienzyme hemoglobin complex along with HDP in food vacuole refers to the fact that degradation of Hb followed by formation of Hz is not taken care of by a single enzyme or protein. Moreover, with the help of *in vitro* coimmunoprecipitation and ELISA-based interaction studies, it has been reported that falcipain-2 directly interacts with HDP proteins in the multi protein complex. These proteins are confirmed to be involved during Hz synthesis as shown by immunoelectron microscopy. Number of bio-physiochemical techniques such as X-ray diffraction, infrared spectroscopy and scanning electron microscopy has suggested that Hb incubated with falcipain-2 and HDP results in degradation and conversion of Hb to Hz, suggesting an essential interaction required during the blood stages of the parasite. 85% of Hb hydrolysis is done by the cysteine protease falcipain-2 alone. In addition to that, involvement of aspartic proteases such plasmepsins aids enhancement in the production of heme during the process along with *Pf*HDP (311).

Though HDP has been found out to be the major generator of Hz, to figure out the function of other enzymes involved in Hb degradation a loss of function analysis was conducted. This included generation of gene deletion mutants of 8 selected *Pf* orthologous proteases present in FV and involved significantly in Hb hydrolysis such as berghepain-2 (BP2), falcipains (FP-2 and FP-3), dipeptidyl peptidase 1 (DPAP1),

aminopeptidase P [APP], M1-family alanyl aminopeptidase [AAP], M17-family leucyl aminopeptidase [LAP], and M18-family aspartyl aminopeptidase [DAP], plasmepsin 4 (PM4), bergheilysin (BLN) along with heme detoxification protein (HDP) and 3 other enzymes with indeterminate roles in Hb hydrolysis viz. berghepain 1, dipetidyl peptidases (DPAP2 and DPAP3). Several attempts to disrupt BLN, AAP and HDP were failed because of their essential roles during blood stage propagation. This data concurs with our prediction related to HDP as shown via RMgmDB where HDP has been referred to be indispensable as several attempts to delete the gene has been failed. This led us to directly adapt the conditional approach to investigate its role beyond blood stages. Parasites lacking PM4, DPAP1, BP1, LAP or APP showed a drastic reduction in growth rates of blood stages parasites when compared with WT. Interestingly mutants lacking both PM4 and BP2 showed a slow growth of the parasite initially but later the parasitemia increased by 50%, leaving the mouse anemic; while most of the RBCs were restricted to reticulocytes in the double mutant parasites. These parasites were producing an insignificant number of schizonts and merozoites as compared to WT, occupying a small space as confirmed by Giemsa-stained images and live ImageStream flow cytometry. Though uptake and degradation of Hb was not affected but a negligible amount of Hz was seen to be deposited via reflection contrast polarized light-microscopy (305). Taken together HDP plays an essential role along with other interacting proteins and enzymes for growth and survival of the parasite during the asexual blood stage.

While analysing the STRING predicted interactions, we found out the important proteases present in the protein complex in FV along with HDP. However, we have postulated that as hydrolysis of Hb and formation of Hz is a hemostatic event during the parasite life cycle, probably the above-mentioned enzymes and their reported roles would unveil the interaction of HDP with the proteases. It can also be inferred that HDP and its interacting partners tightly regulate the progressive Hb degradation and conversion of heme to Hz. Moreover, this would also help to decipher the pathway involved to complete the critical process of Hz formation. This might pave the path towards promising drug or vaccine candidate, targeting the asexual blood stages of the parasite. This explains why HDP is indispensable in blood stages.

As HDP is essential in intraerythrocytic growth of the parasite, this limited our investigation in other life cycle stages. Because of which we adapted a stage specific

conditional mutagenesis system which occurs by controlling the excision of endogenous target DNA sequence. FLP (the site-specific recombinase) recognises the 34-bp long target sequence known as FRT. The reciprocal conservative recombination is catalysed by the position and relative orientation of the two identical target sequences leading towards either excision, inversion, insertion, or exchange of DNA. This system allows to place the recognition sequences (FRT) on both sides of the HDP gene (312). The engineered plasmid having a FRTted locus was linearised and transfected into a parental line containing Flp recombinase which is under the TRAP promoter. The TRAP promoter gets activated during the oocyst stage followed by activation of the recombinase which recognises the FRTed locus and excises the DNA sequence. This leads towards conditionally silencing the gene beyond oocyst stages. The advantage of the system is, it gives a liberty to choose the desired length DNA sequence flanked by FRT sites for excision. Moreover, the conditional mutagenesis of the targeted gene is very useful during the study of multi-functional proteins (312).

As the FRTed copy of the gene replaces the endogenous locus in the parasite genome, it survives in the blood stages, allowing us to charecterise the phenotype of the gene in later life cycle stages. Owing to the successful transfection we obtained the drug resistant population. Integration PCR, GFP fluorescing parasites in blood stages and DNA sequencing showing FRT sequences in the genome, confirms correct integration. Further a typical asexual and sexual blood stage growth by the *PbHDP* cKO parasites similar to WT, establishes the fact that replacement of the endogenous locus with a FRTed locus was successful, allowing the parasites to survive in blood stages.

As the gene expression data shows *PbHDP* has the highest expression in salivary gland stage, which may imply to some important moonlighting functions of this protein in mosquito stages of *Plasmodium* which needs to be further investigated.

It was long been expected that only degradation of haemoglobin results in synthesis of heme but Nagraj *et.al.* in 2013 reported for the first time that parasite can also synthesise heme via *de novo* pathway which in tun converts to Hz in FV (303). It is still ambiguous whether the Hb degraded heme and the parasite synthesized heme are functionally comparable yet it can be postulated that the parasite synthesized heme could be a survival strategy when the original pathway is abrupted. Apart from intraerythrocytic stages heme was seen to be required in mosquito and liver stages as well. As investigated

by Nagraj *et. al.* the intraerythrocytic stages were unharmed when the first enzyme δ-aminolevulinate synthase (ALAS) and the last enzyme- ferrochelatase (FC) of heme-biosynthetic pathway were disrupted. Further upon transmission to mosquitos, the mutant parasites manifested a notable decrease in oocyst colonisation and a complete absence of sporozoites in the salivary gland. Upon treatment of infected mosquitos with aminolevulinic-acid (ALA) supplement, the infectivity of the sporozoites were completely restored either injected via intravenous injection or delivered via natural route, giving a breakthrough infection and a similar prepatency period as compared to WT. The above data inferred, HDP not only plays a vital role in blood stages but also is important during other stages of *Plasmodium* life cycle. Owing to this above-mentioned study, our stage specific expression data shows similar kind of pattern where a mild expression of the *PbHDP* can be seen in midgut sporozoites whereas the highest expression can be seen in the salivary gland sporozoites.

For the first time our data shows that the blood stage growth of *PbHDP* cKO mutant parasites was absolutely normal and the mutant parasites were successfully able to colonise in mosquito midgut and form sporulated oocysts similar to WT parasites. To understand the role of *PbHDP* in salivary gland stages and liver stages further investigation is required which will shed some light on the crucial function of this protein in different life cycle stages of *Plasmodium.*

Summary

4.1 Summary

The focus of the current study was to investigate the functional role of two *Plasmodium* genes/proteins (1) PbPhIP, an IMC specific protein that is essential for sexual stages of the parasite and (2) PbHDP, an important protein that is highly expressed in salivary gland sporozoites and is required for heme to Hz conversion in intraerythrocytic stages. With help of reverse genetics approaches, we have functionally charecterise the role of these proteins in different stages of the parasite, which greatly facilitated important aspects in parasite biology by unravelling some interesting and significant functions. This could be beneficial for the development of therapeutic interventions against this lethal disease.

We have successfully generated deletion mutants of *PbPhIP* in *Plasmodium berghei* (*Pb*) and investigated the KO parasites across different life cycle stages in both vertebrate and insect host. Our study reveals for the first time that *PbPhIP* is indispensable during sexual development of the parasite both in the mammalian and *Anopheline* host. Our localisation studies using anti-PhIP antibody, indicated PhIP to be an IMC resident protein. Since the proteins present in IMC interactome are required for maintaining the cytoskeletal stability and involved in gliding motility, loss of PhIP has highly affected other interacting partners by causing a dramatic change in global gene expression pattern and has majorly impaired the growth of the parasite in mosquito stages. Our gene expression data shows a huge expression of *PbPhIP* in salivary gland stages which compelled us investigate the role of this protein beyond sexual stages. Further, a block in sexual development led us to generate conditional mutants of *PbPhIP* by adapting Yeast FLP/FRT based conditional mutagenesis approach. The endogenous locus has been successfully replaced with FRTed locus and the integration of the engineered cassette in the genome of mutant parasites has been confirmed via Integration PCR and DNA sequencing. We are eager to find out the role of PhIP in sporozoites and liver stages by transmitting the *PbPhIP cKO* parasites through female *Anopheles* mosquitos which might provide ample amount of information for our quest towards developing inhibitors for PhIP to block its clientship with IMC. This may have a pivotal significance in the prevention of malaria transmission.

Since *Pb*HDP is essential during blood stages, we have directly taken the conditional approach to generate *PbHDP cKO* parasites using FLP/FRT site specific

recombination system. The thermolabile variant of Flp, known as FlpL expresses under TRAP promoter which gets activated during oocyst stage of the parasite and excises the FRTed DNA sequence. Our quest of generating a conditional line for *PbHDP* initiated after observing its strong expression in salivary gland sporozoites in gene expression analysis and unsuccessful attempts of disrupting the gene in blood stages. Though several reports have established the role HDP in conversion of toxic heme to Hz during asexual blood stage growth of parasite yet its role beyond blood stages is only speculative. With the existing reports of HDP, having a role in midgut and salivary gland sporozoites concurring with our gene expression analysis data, we generated *PbHDP* conditionally silenced parasites and confirmed integration of the FRTed HDP locus in the genome. We observed a normal growth of the parasites during intraerythrocytic cycles likely suggesting a successful replacement of the endogenous locus with the FRTed sequence. Owing to the previously reported literature, STRING analysis data revealing interaction of HDP with several proteases and our stage specific expression analysis report, we have transmitted the parasites in mosquitos. The mutant parasites successfully generated midgut oocyst and the further stages are under investigation which might propose other functional roles of *PbHDP* beyond blood stages.

References

5.1 References

1. Carter, R., and Mendis, K. N. (2002) Evolutionary and historical aspects of the burden of malaria. *Clinical microbiology reviews* **15**, 564-594

2. Chow, C. (2002) [A brief history of the early stage of malaria control in China]. *Zhongguo ji sheng chong xue yu ji sheng chong bing za zhi = Chinese journal of parasitology & parasitic diseases* **20**, 63-64

3. Bordbar, B., Tuikue Ndam, N., Renard, E., Jafari-Guemouri, S., Tavul, L., Jennison, C., Gnidehou, S., Tahar, R., Gamboa, D., Bendezu, J., Menard, D., Barry, A. E., Deloron, P., and Sabbagh, A. (2014) Genetic diversity of VAR2CSA ID1-DBL2Xb in worldwide *Plasmodium falciparum* populations: impact on vaccine design for placental malaria. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases* **25**, 81-92

4. <World Malaria report 2021.pdf>.

5. Greenwood, B. M., Bojang, K., Whitty, C. J., and Targett, G. A. (2005) Malaria. *Lancet* **365**, 1487-1498

6. O'Meara, W. P., Mangeni, J. N., Steketee, R., and Greenwood, B. (2010) Changes in the burden of malaria in sub-Saharan Africa. *The Lancet. Infectious diseases* **10**, 545-555

7. Tavares, J., Formaglio, P., Thiberge, S., Mordelet, E., Van Rooijen, N., Medvinsky, A., Menard, R., and Amino, R. (2013) Role of host cell traversal by the malaria sporozoite during liver infection. *The Journal of experimental medicine* **210**, 905-915

8. Venugopal, K., Hentzschel, F., Valkiunas, G., and Marti, M. (2020) *Plasmodium* asexual growth and sexual development in the haematopoietic niche of the host. *Nature reviews. Microbiology* **18**, 177-189

9. Sturm, A., Amino, R., van de Sand, C., Regen, T., Retzlaff, S., Rennenberg, A., Krueger, A., Pollok, J. M., Menard, R., and Heussler, V. T. (2006) Manipulation of host

hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science* **313**, 1287-1290

10. De Niz, M., Helm, S., Horstmann, S., Annoura, T., Del Portillo, H. A., Khan, S. M., and Heussler, V. T. (2015) In vivo and in vitro characterization of a *Plasmodium* liver stage-specific promoter. *PloS one* **10**, e0123473

11. Silvie, O., Mota, M. M., Matuschewski, K., and Prudencio, M. (2008) Interactions of the malaria parasite and its mammalian host. *Current opinion in microbiology* **11**, 352-359

12. Amino, R., Giovannini, D., Thiberge, S., Gueirard, P., Boisson, B., Dubremetz, J. F., Prevost, M. C., Ishino, T., Yuda, M., and Menard, R. (2008) Host cell traversal is important for progression of the malaria parasite through the dermis to the liver. *Cell host & microbe* **3**, 88-96

13. Sinnis, P., and Zavala, F. (2008) The skin stage of malaria infection: biology and relevance to the malaria vaccine effort. *Future microbiology* **3**, 275-278

14. Vanderberg, J. P., and Frevert, U. (2004) Intravital microscopy demonstrating antibody-mediated immobilisation of *Plasmodium berghei* sporozoites injected into skin by mosquitoes. *International journal for parasitology* **34**, 991-996

15. Yamauchi, L. M., Coppi, A., Snounou, G., and Sinnis, P. (2007) *Plasmodium* sporozoites trickle out of the injection site. *Cellular microbiology* **9**, 1215-1222

16. Ribeiro, J. M., and Francischetti, I. M. (2003) Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Annual review of entomology* **48**, 73-88

17. Kappe, S. H., Buscaglia, C. A., Bergman, L. W., Coppens, I., and Nussenzweig, V. (2004) Apicomplexan gliding motility and host cell invasion: overhauling the motor model. *Trends in parasitology* **20**, 13-16

18. King, C. A. (1981) Cell surface interaction of the protozoan Gregarina with concanavalin A beads - implications for models of gregarine gliding. *Cell biology international reports* **5**, 297-305

19. Russell, D. G., and Sinden, R. E. (1981) The role of the cytoskeleton in the motility of coccidian sporozoites. *Journal of cell science* **50**, 345-359

20. Ejigiri, I., Ragheb, D. R., Pino, P., Coppi, A., Bennett, B. L., Soldati-Favre, D., and Sinnis, P. (2012) Shedding of TRAP by a rhomboid protease from the malaria sporozoite surface is essential for gliding motility and sporozoite infectivity. *PLoS pathogens* **8**, e1002725

21. Sultan, A. A., Thathy, V., Frevert, U., Robson, K. J., Crisanti, A., Nussenzweig, V., Nussenzweig, R. S., and Menard, R. (1997) TRAP is necessary for gliding motility and infectivity of *Plasmodium* sporozoites. *Cell* **90**, 511-522

22. Amino, R., Thiberge, S., Martin, B., Celli, S., Shorte, S., Frischknecht, F., and Menard, R. (2006) Quantitative imaging of *Plasmodium* transmission from mosquito to mammal. *Nature medicine* **12**, 220-224

23. Chakravarty, S., Cockburn, I. A., Kuk, S., Overstreet, M. G., Sacci, J. B., and Zavala, F. (2007) CD8+ T lymphocytes protective against malaria liver stages are primed in skin-draining lymph nodes. *Nature medicine* **13**, 1035-1041

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

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

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

27. Kariu, T., Ishino, T., Yano, K., Chinzei, Y., and Yuda, M. (2006) CelTOS, a novel malarial protein that mediates transmission to mosquito and vertebrate hosts. *Molecular microbiology* **59**, 1369-1379

28. Moreira, C. K., Templeton, T. J., Lavazec, C., Hayward, R. E., Hobbs, C. V., Kroeze, H., Janse, C. J., Waters, A. P., Sinnis, P., and Coppi, A. (2008) The *Plasmodium* TRAP/MIC2 family member, TRAP-Like Protein (TLP), is involved in tissue traversal by sporozoites. *Cellular microbiology* **10**, 1505-1516

29. Bhanot, P., Schauer, K., Coppens, I., and Nussenzweig, V. (2005) A surface phospholipase is involved in the migration of *Plasmodium* sporozoites through cells. *The Journal of biological chemistry* **280**, 6752-6760

30. Pradel, G., and Frevert, U. (2001) Malaria sporozoites actively enter and pass through rat Kupffer cells prior to hepatocyte invasion. *Hepatology* **33**, 1154-1165

31. Mota, M. M., Pradel, G., Vanderberg, J. P., Hafalla, J. C., Frevert, U., Nussenzweig, R. S., Nussenzweig, V., and Rodriguez, A. (2001) Migration of *Plasmodium* sporozoites through cells before infection. *Science* **291**, 141-144

32. Mota, M. M., Hafalla, J. C., and Rodriguez, A. (2002) Migration through host cells activates *Plasmodium* sporozoites for infection. *Nature medicine* **8**, 1318-1322

33. Carrolo, M., Giordano, S., Cabrita-Santos, L., Corso, S., Vigario, A. M., Silva, S., Leiriao, P., Carapau, D., Armas-Portela, R., Comoglio, P. M., Rodriguez, A., and Mota, M. M. (2003) Hepatocyte growth factor and its receptor are required for malaria infection. *Nature medicine* **9**, 1363-1369

34. Royal, I., Lamarche-Vane, N., Lamorte, L., Kaibuchi, K., and Park, M. (2000) Activation of cdc42, rac, PAK, and rho-kinase in response to hepatocyte growth factor differentially regulates epithelial cell colony spreading and dissociation. *Molecular biology of the cell* **11**, 1709-1725

35. Mota, M. M., and Rodriguez, A. (2004) Migration through host cells: the first steps of *Plasmodium* sporozoites in the mammalian host. *Cellular microbiology* **6**, 1113-1118

36. Menard, R., Tavares, J., Cockburn, I., Markus, M., Zavala, F., and Amino, R. (2013) Looking under the skin: the first steps in malarial infection and immunity. *Nature reviews. Microbiology* **11**, 701-712

37. Kappe, S., Bruderer, T., Gantt, S., Fujioka, H., Nussenzweig, V., and Menard, R. (1999) Conservation of a gliding motility and cell invasion machinery in Apicomplexan parasites. *The Journal of cell biology* **147**, 937-944

38. Sinnis, P., and Coppi, A. (2007) A long and winding road: the *Plasmodium* sporozoite's journey in the mammalian host. *Parasitology international* **56**, 171-178

39. Coppi, A., Pinzon-Ortiz, C., Hutter, C., and Sinnis, P. (2005) The *Plasmodium* circumsporozoite protein is proteolytically processed during cell invasion. *The Journal of experimental medicine* **201**, 27-33

40. Coppi, A., Tewari, R., Bishop, J. R., Bennett, B. L., Lawrence, R., Esko, J. D., Billker, O., and Sinnis, P. (2007) Heparan sulfate proteoglycans provide a signal to *Plasmodium* sporozoites to stop migrating and productively invade host cells. *Cell host & microbe* **2**, 316-327

41. Gantt, S., Persson, C., Rose, K., Birkett, A. J., Abagyan, R., and Nussenzweig, V. (2000) Antibodies against thrombospondin-related anonymous protein do not inhibit *Plasmodium* sporozoite infectivity in vivo. *Infection and immunity* **68**, 3667-3673

42. Matuschewski, K., Nunes, A. C., Nussenzweig, V., and Menard, R. (2002) *Plasmodium* sporozoite invasion into insect and mammalian cells is directed by the same dual binding system. *The EMBO journal* **21**, 1597-1606

43. Wengelnik, K., Spaccapelo, R., Naitza, S., Robson, K. J., Janse, C. J., Bistoni, F., Waters, A. P., and Crisanti, A. (1999) The A-domain and the thrombospondin-related motif of *Plasmodium falciparum* TRAP are implicated in the invasion process of mosquito salivary glands. *The EMBO journal* **18**, 5195-5204

44. Silvie, O., Franetich, J. F., Charrin, S., Mueller, M. S., Siau, A., Bodescot, M., Rubinstein, E., Hannoun, L., Charoenvit, Y., Kocken, C. H., Thomas, A. W., Van Gemert, G. J., Sauerwein, R. W., Blackman, M. J., Anders, R. F., Pluschke, G., and Mazier, D. (2004) A role for apical membrane antigen 1 during invasion of hepatocytes by *Plasmodium falciparum* sporozoites. *The Journal of biological chemistry* **279**, 9490-9496

45. Vaughan, A. M., Aly, A. S., and Kappe, S. H. (2008) Malaria parasite pre-erythrocytic stage infection: gliding and hiding. *Cell host & microbe* **4**, 209-218

46. Silvie, O., Rubinstein, E., Franetich, J. F., Prenant, M., Belnoue, E., Renia, L., Hannoun, L., Eling, W., Levy, S., Boucheix, C., and Mazier, D. (2003) Hepatocyte CD81 is required for *Plasmodium falciparum* and *Plasmodium yoelii* sporozoite infectivity. *Nature medicine* **9**, 93-96

47. Yalaoui, S., Huby, T., Franetich, J. F., Gego, A., Rametti, A., Moreau, M., Collet, X., Siau, A., van Gemert, G. J., Sauerwein, R. W., Luty, A. J., Vaillant, J. C., Hannoun, L., Chapman, J., Mazier, D., and Froissard, P. (2008) Scavenger receptor BI boosts hepatocyte permissiveness to *Plasmodium* infection. *Cell host & microbe* **4**, 283-292

48. Cha, S. J., Park, K., Srinivasan, P., Schindler, C. W., van Rooijen, N., Stins, M., and Jacobs-Lorena, M. (2015) CD68 acts as a major gateway for malaria sporozoite liver infection. *The Journal of experimental medicine* **212**, 1391-1403

49. Kaushansky, A., Douglass, A. N., Arang, N., Vigdorovich, V., Dambrauskas, N., Kain, H. S., Austin, L. S., Sather, D. N., and Kappe, S. H. (2015) Malaria parasites target the hepatocyte receptor EphA2 for successful host infection. *Science* **350**, 1089-1092

50. Meis, J. F., Verhave, J. P., Jap, P. H., Sinden, R. E., and Meuwissen, J. H. (1983) Malaria parasites--discovery of the early liver form. *Nature* **302**, 424-426

51. Jayabalasingham, B., Bano, N., and Coppens, I. (2010) Metamorphosis of the malaria parasite in the liver is associated with organelle clearance. *Cell research* **20**, 1043-1059

52. Labaied, M., Harupa, A., Dumpit, R. F., Coppens, I., Mikolajczak, S. A., and Kappe, S. H. (2007) *Plasmodium yoelii* sporozoites with simultaneous deletion of P52 and P36 are completely attenuated and confer sterile immunity against infection. *Infection and immunity* **75**, 3758-3768

53. Mueller, A. K., Labaied, M., Kappe, S. H., and Matuschewski, K. (2005) Genetically modified *Plasmodium* parasites as a protective experimental malaria vaccine. *Nature* **433**, 164-167

54. Mikolajczak, S. A., Jacobs-Lorena, V., MacKellar, D. C., Camargo, N., and Kappe, S. H. (2007) L-FABP is a critical host factor for successful malaria liver stage development. *International journal for parasitology* **37**, 483-489

55. Mueller, A. K., Camargo, N., Kaiser, K., Andorfer, C., Frevert, U., Matuschewski, K., and Kappe, S. H. (2005) *Plasmodium* liver stage developmental arrest by depletion of a protein at the parasite-host interface. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 3022-3027

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

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

58. Boddey, J. A., Hodder, A. N., Gunther, S., Gilson, P. R., Patsiouras, H., Kapp, E. A., Pearce, J. A., de Koning-Ward, T. F., Simpson, R. J., Crabb, B. S., and Cowman, A. F. (2010) An aspartyl protease directs malaria effector proteins to the host cell. *Nature* **463**, 627-631

59. Russo, I., Babbitt, S., Muralidharan, V., Butler, T., Oksman, A., and Goldberg, D. E. (2010) Plasmepsin V licenses *Plasmodium* proteins for export into the host erythrocyte. *Nature* **463**, 632-636

60. Chang, H. H., Falick, A. M., Carlton, P. M., Sedat, J. W., DeRisi, J. L., and Marletta, M. A. (2008) N-terminal processing of proteins exported by malaria parasites. *Molecular and biochemical parasitology* **160**, 107-115

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

62. Heiber, A., Kruse, F., Pick, C., Gruring, C., Flemming, S., Oberli, A., Schoeler, H., Retzlaff, S., Mesen-Ramirez, P., Hiss, J. A., Kadekoppala, M., Hecht, L., Holder, A. A.,

Gilberger, T. W., and Spielmann, T. (2013) Identification of new PNEPs indicates a substantial non-PEXEL exportome and underpins common features in *Plasmodium falciparum* protein export. *PLoS pathogens* **9**, e1003546

63. Plassmeyer, M. L., Reiter, K., Shimp, R. L., Jr., Kotova, S., Smith, P. D., Hurt, D. E., House, B., Zou, X., Zhang, Y., Hickman, M., Uchime, O., Herrera, R., Nguyen, V., Glen, J., Lebowitz, J., Jin, A. J., Miller, L. H., MacDonald, N. J., Wu, Y., and Narum, D. L. (2009) Structure of the *Plasmodium falciparum* circumsporozoite protein, a leading malaria vaccine candidate. *The Journal of biological chemistry* **284**, 26951-26963

64. Ingmundson, A., Nahar, C., Brinkmann, V., Lehmann, M. J., and Matuschewski, K. (2012) The exported *Plasmodium berghei* protein IBIS1 delineates membranous structures in infected red blood cells. *Molecular microbiology* **83**, 1229-1243

65. Jaijyan, D. K., Singh, H., and Singh, A. P. (2015) A Sporozoite- and Liver Stage-expressed Tryptophan-rich Protein Plays an Auxiliary Role in *Plasmodium* Liver Stage Development and Is a Potential Vaccine Candidate. *The Journal of biological chemistry* **290**, 19496-19511

66. Bano, N., Romano, J. D., Jayabalasingham, B., and Coppens, I. (2007) Cellular interactions of *Plasmodium* liver stage with its host mammalian cell. *International journal for parasitology* **37**, 1329-1341

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

68. Putrianti, E. D., Schmidt-Christensen, A., Arnold, I., Heussler, V. T., Matuschewski, K., and Silvie, O. (2010) The *Plasmodium* serine-type SERA proteases display distinct expression patterns and non-essential in vivo roles during life cycle progression of the malaria parasite. *Cellular microbiology* **12**, 725-739

69. Tawk, L., Lacroix, C., Gueirard, P., Kent, R., Gorgette, O., Thiberge, S., Mercereau-Puijalon, O., Menard, R., and Barale, J. C. (2013) A key role for *Plasmodium* subtilisin-like SUB1 protease in egress of malaria parasites from host hepatocytes. *The Journal of biological chemistry* **288**, 33336-33346

70. Schmidt-Christensen, A., Sturm, A., Horstmann, S., and Heussler, V. T. (2008) Expression and processing of *Plasmodium berghei* SERA3 during liver stages. *Cellular microbiology* **10**, 1723-1734

71. Suarez, C., Volkmann, K., Gomes, A. R., Billker, O., and Blackman, M. J. (2013) The malarial serine protease SUB1 plays an essential role in parasite liver stage development. *PLoS pathogens* **9**, e1003811

72. Tarun, A. S., Baer, K., Dumpit, R. F., Gray, S., Lejarcegui, N., Frevert, U., and Kappe, S. H. (2006) Quantitative isolation and in vivo imaging of malaria parasite liver stages. *International journal for parasitology* **36**, 1283-1293

73. Belachew, E. B. (2018) Immune Response and Evasion Mechanisms of *Plasmodium falciparum* Parasites. *Journal of immunology research* **2018**, 6529681

74. Kerlin, D. H., and Gatton, M. L. (2013) Preferential invasion by *Plasmodium* merozoites and the self-regulation of parasite burden. *PloS one* **8**, e57434

75. Cowman, A. F., Tonkin, C. J., Tham, W. H., and Duraisingh, M. T. (2017) The Molecular Basis of Erythrocyte Invasion by Malaria Parasites. *Cell host & microbe* **22**, 232-245

76. Salinas, N. D., Tang, W. K., and Tolia, N. H. (2019) Blood-Stage Malaria Parasite Antigens: Structure, Function, and Vaccine Potential. *Journal of molecular biology* **431**, 4259-4280

77. Goel, V. K., Li, X., Chen, H., Liu, S. C., Chishti, A. H., and Oh, S. S. (2003) Band 3 is a host receptor binding merozoite surface protein 1 during the *Plasmodium falciparum* invasion of erythrocytes. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 5164-5169

78. Casares, S., and Richie, T. L. (2009) Immune evasion by malaria parasites: a challenge for vaccine development. *Current opinion in immunology* **21**, 321-330

79. Mitchell, G. H., Thomas, A. W., Margos, G., Dluzewski, A. R., and Bannister, L. H. (2004) Apical membrane antigen 1, a major malaria vaccine candidate, mediates the close attachment of invasive merozoites to host red blood cells. *Infection and immunity* **72**, 154-158

80. Cowman, A. F., and Crabb, B. S. (2006) Invasion of red blood cells by malaria parasites. *Cell* **124**, 755-766

81. Pinder, J. C., Fowler, R. E., Dluzewski, A. R., Bannister, L. H., Lavin, F. M., Mitchell, G. H., Wilson, R. J., and Gratzer, W. B. (1998) Actomyosin motor in the merozoite of the malaria parasite, *Plasmodium falciparum*: implications for red cell invasion. *Journal of cell science* **111 (Pt 13)**, 1831-1839

82. Keeley, A., and Soldati, D. (2004) The glideosome: a molecular machine powering motility and host-cell invasion by Apicomplexa. *Trends in cell biology* **14**, 528-532

83. Haynes, J. D., Dalton, J. P., Klotz, F. W., McGinniss, M. H., Hadley, T. J., Hudson, D. E., and Miller, L. H. (1988) Receptor-like specificity of a *Plasmodium* knowlesi malarial protein that binds to Duffy antigen ligands on erythrocytes. *The Journal of experimental medicine* **167**, 1873-1881

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

85. Mayer, D. C., Cofie, J., Jiang, L., Hartl, D. L., Tracy, E., Kabat, J., Mendoza, L. H., and Miller, L. H. (2009) Glycophorin B is the erythrocyte receptor of *Plasmodium falciparum* erythrocyte-binding ligand, EBL-1. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 5348-5352

86. Mayer, D. C., Jiang, L., Achur, R. N., Kakizaki, I., Gowda, D. C., and Miller, L. H. (2006) The glycophorin C N-linked glycan is a critical component of the ligand for the *Plasmodium falciparum* erythrocyte receptor BAEBL. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 2358-2362

87. Crosnier, C., Bustamante, L. Y., Bartholdson, S. J., Bei, A. K., Theron, M., Uchikawa, M., Mboup, S., Ndir, O., Kwiatkowski, D. P., Duraisingh, M. T., Rayner, J. C., and Wright, G. J. (2011) Basigin is a receptor essential for erythrocyte invasion by *Plasmodium falciparum*. *Nature* **480**, 534-537

88. Tham, W. H., Wilson, D. W., Lopaticki, S., Schmidt, C. Q., Tetteh-Quarcoo, P. B., Barlow, P. N., Richard, D., Corbin, J. E., Beeson, J. G., and Cowman, A. F. (2010) Complement receptor 1 is the host erythrocyte receptor for *Plasmodium*

falciparum PfRh4 invasion ligand. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 17327-17332

89. Triglia, T., Tham, W. H., Hodder, A., and Cowman, A. F. (2009) Reticulocyte binding protein homologues are key adhesins during erythrocyte invasion by *Plasmodium falciparum*. *Cellular microbiology* **11**, 1671-1687

90. Chen, L., Lopaticki, S., Riglar, D. T., Dekiwadia, C., Uboldi, A. D., Tham, W. H., O'Neill, M. T., Richard, D., Baum, J., Ralph, S. A., and Cowman, A. F. (2011) An EGF-like protein forms a complex with PfRh5 and is required for invasion of human erythrocytes by *Plasmodium falciparum*. *PLoS pathogens* **7**, e1002199

91. Dreyer, A. M., Matile, H., Papastogiannidis, P., Kamber, J., Favuzza, P., Voss, T. S., Wittlin, S., and Pluschke, G. (2012) Passive immunoprotection of *Plasmodium falciparum*-infected mice designates the CyRPA as candidate malaria vaccine antigen. *Journal of immunology* **188**, 6225-6237

92. Reddy, K. S., Amlabu, E., Pandey, A. K., Mitra, P., Chauhan, V. S., and Gaur, D. (2015) Multiprotein complex between the GPI-anchored CyRPA with PfRH5 and PfRipr is crucial for *Plasmodium falciparum* erythrocyte invasion. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 1179-1184

93. Weiss, G. E., Gilson, P. R., Taechalertpaisarn, T., Tham, W. H., de Jong, N. W., Harvey, K. L., Fowkes, F. J., Barlow, P. N., Rayner, J. C., Wright, G. J., Cowman, A. F., and Crabb, B. S. (2015) Revealing the sequence and resulting cellular morphology of receptor-ligand interactions during *Plasmodium falciparum* invasion of erythrocytes. *PLoS pathogens* **11**, e1004670

94. Volz, J. C., Yap, A., Sisquella, X., Thompson, J. K., Lim, N. T., Whitehead, L. W., Chen, L., Lampe, M., Tham, W. H., Wilson, D., Nebl, T., Marapana, D., Triglia, T., Wong, W., Rogers, K. L., and Cowman, A. F. (2016) Essential Role of the PfRh5/PfRipr/CyRPA Complex during *Plasmodium falciparum* Invasion of Erythrocytes. *Cell host & microbe* **20**, 60-71

95. Srinivasan, P., Beatty, W. L., Diouf, A., Herrera, R., Ambroggio, X., Moch, J. K., Tyler, J. S., Narum, D. L., Pierce, S. K., Boothroyd, J. C., Haynes, J. D., and Miller, L. H. (2011) Binding of *Plasmodium* merozoite proteins RON2 and AMA1 triggers commitment

to invasion. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 13275-13280

96. Yeoh, S., O'Donnell, R. A., Koussis, K., Dluzewski, A. R., Ansell, K. H., Osborne, S. A., Hackett, F., Withers-Martinez, C., Mitchell, G. H., Bannister, L. H., Bryans, J. S., Kettleborough, C. A., and Blackman, M. J. (2007) Subcellular discharge of a serine protease mediates release of invasive malaria parasites from host erythrocytes. *Cell* **131**, 1072-1083

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

98. de Koning-Ward, T. F., Dixon, M. W., Tilley, L., and Gilson, P. R. (2016) *Plasmodium* species: master renovators of their host cells. *Nature reviews. Microbiology* **14**, 494-507

99. Elmendorf, H. G., and Haldar, K. (1994) *Plasmodium falciparum* exports the Golgi marker sphingomyelin synthase into a tubovesicular network in the cytoplasm of mature erythrocytes. *The Journal of cell biology* **124**, 449-462

100. Goldberg, D. E., and Cowman, A. F. (2010) Moving in and renovating: exporting proteins from *Plasmodium* into host erythrocytes. *Nature reviews. Microbiology* **8**, 617-621

101. Dixon, M. W., Hawthorne, P. L., Spielmann, T., Anderson, K. L., Trenholme, K. R., and Gardiner, D. L. (2008) Targeting of the ring exported protein 1 to the Maurer's clefts is mediated by a two-phase process. *Traffic* **9**, 1316-1326

102. Haase, S., Herrmann, S., Gruring, C., Heiber, A., Jansen, P. W., Langer, C., Treeck, M., Cabrera, A., Bruns, C., Struck, N. S., Kono, M., Engelberg, K., Ruch, U., Stunnenberg, H. G., Gilberger, T. W., and Spielmann, T. (2009) Sequence requirements for the export of the *Plasmodium falciparum* Maurer's clefts protein REX2. *Molecular microbiology* **71**, 1003-1017

103. McMillan, P. J., Millet, C., Batinovic, S., Maiorca, M., Hanssen, E., Kenny, S., Muhle, R. A., Melcher, M., Fidock, D. A., Smith, J. D., Dixon, M. W., and Tilley, L. (2013) Spatial

and temporal mapping of the PfEMP1 export pathway in *Plasmodium falciparum*. *Cellular microbiology* **15**, 1401-1418

104. Scherf, A., Lopez-Rubio, J. J., and Riviere, L. (2008) Antigenic variation in *Plasmodium falciparum*. *Annual review of microbiology* **62**, 445-470

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

106. Saliba, K. J., Horner, H. A., and Kirk, K. (1998) Transport and metabolism of the essential vitamin pantothenic acid in human erythrocytes infected with the malaria parasite *Plasmodium falciparum*. *The Journal of biological chemistry* **273**, 10190-10195

107. Egan, T. J. (2008) Recent advances in understanding the mechanism of hemozoin (malaria pigment) formation. *Journal of inorganic biochemistry* **102**, 1288-1299

108. Foley, M., and Tilley, L. (1998) Quinoline antimalarials: mechanisms of action and resistance and prospects for new agents. *Pharmacology & therapeutics* **79**, 55-87

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

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

111. Arastu-Kapur, S., Ponder, E. L., Fonovic, U. P., Yeoh, S., Yuan, F., Fonovic, M., Grainger, M., Phillips, C. I., Powers, J. C., and Bogyo, M. (2008) Identification of proteases that regulate erythrocyte rupture by the malaria parasite *Plasmodium falciparum*. *Nature chemical biology* **4**, 203-213

112. Yeoh, S., O'Donnell, R. A., Koussis, K., Dluzewski, A. R., Ansell, K. H., Osborne, S. A., Hackett, F., Withers-Martinez, C., Mitchell, G. H., Bannister, L. H., Bryans, J. S., Kettleborough, C. A., and Blackman, M. J. (2007) Subcellular discharge of a serine protease mediates release of invasive malaria parasites from host erythrocytes. *Cell* **131**, 1072-1083

113. Sinden, R. E. (1982) Gametocytogenesis of *Plasmodium falciparum* in vitro: an electron microscopic study. *Parasitology* **84**, 1-11

114. Sinden, R. E. (1983) Sexual development of malarial parasites. *Advances in parasitology* **22**, 153-216

115. Silvestrini, F., Alano, P., and Williams, J. L. (2000) Commitment to the production of male and female gametocytes in the human malaria parasite *Plasmodium falciparum*. *Parasitology* **121 Pt 5**, 465-471

116. Smith, T. G., Lourenco, P., Carter, R., Walliker, D., and Ranford-Cartwright, L. C. (2000) Commitment to sexual differentiation in the human malaria parasite, *Plasmodium falciparum*. *Parasitology* **121 (Pt 2)**, 127-133

117. Kafsack, B. F., Rovira-Graells, N., Clark, T. G., Bancells, C., Crowley, V. M., Campino, S. G., Williams, A. E., Drought, L. G., Kwiatkowski, D. P., Baker, D. A., Cortes, A., and Llinas, M. (2014) A transcriptional switch underlies commitment to sexual development in malaria parasites. *Nature* **507**, 248-252

118. Sinha, A., Hughes, K. R., Modrzynska, K. K., Otto, T. D., Pfander, C., Dickens, N. J., Religa, A. A., Bushell, E., Graham, A. L., Cameron, R., Kafsack, B. F. C., Williams, A. E., Llinas, M., Berriman, M., Billker, O., and Waters, A. P. (2014) A cascade of DNA-binding proteins for sexual commitment and development in *Plasmodium*. *Nature* **507**, 253-257

119. Brancucci, N. M. B., Bertschi, N. L., Zhu, L., Niederwieser, I., Chin, W. H., Wampfler, R., Freymond, C., Rottmann, M., Felger, I., Bozdech, Z., and Voss, T. S. (2014) Heterochromatin protein 1 secures survival and transmission of malaria parasites. *Cell host & microbe* **16**, 165-176

120. Coleman, B. I., Skillman, K. M., Jiang, R. H. Y., Childs, L. M., Altenhofen, L. M., Ganter, M., Leung, Y., Goldowitz, I., Kafsack, B. F. C., Marti, M., Llinas, M., Buckee, C. O., and Duraisingh, M. T. (2014) A *Plasmodium falciparum* histone deacetylase regulates antigenic variation and gametocyte conversion. *Cell host & microbe* **16**, 177-186

121. Eksi, S., Morahan, B. J., Haile, Y., Furuya, T., Jiang, H., Ali, O., Xu, H., Kiattibutr, K., Suri, A., Czesny, B., Adeyemo, A., Myers, T. G., Sattabongkot, J., Su, X. Z., and Williamson, K. C. (2012) *Plasmodium falciparum* gametocyte development 1 (Pfgdv1) and gametocytogenesis early gene identification and commitment to sexual development. *PLoS pathogens* **8**, e1002964

122. Rea, E., Le Roch, K. G., and Tewari, R. (2018) Sex in *Plasmodium falciparum*: Silence Play between GDV1 and HP1. *Trends in parasitology* **34**, 450-452

123. Smalley, M. E., Abdalla, S., and Brown, J. (1981) The distribution of *Plasmodium falciparum* in the peripheral blood and bone marrow of Gambian children. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **75**, 103-105

124. Smalley, M. E., and Sinden, R. E. (1977) *Plasmodium falciparum* gametocytes: their longevity and infectivity. *Parasitology* **74**, 1-8

125. Nixon, C. P. (2016) *Plasmodium falciparum* gametocyte transit through the cutaneous microvasculature: A new target for malaria transmission blocking vaccines? *Human vaccines & immunotherapeutics* **12**, 3189-3195

126. Mair, G. R., Braks, J. A., Garver, L. S., Wiegant, J. C., Hall, N., Dirks, R. W., Khan, S. M., Dimopoulos, G., Janse, C. J., and Waters, A. P. (2006) Regulation of sexual development of *Plasmodium* by translational repression. *Science* **313**, 667-669

127. Colegrove-Otero, L. J., Minshall, N., and Standart, N. (2005) RNA-binding proteins in early development. *Critical reviews in biochemistry and molecular biology* **40**, 21-73

128. Quenault, T., Lithgow, T., and Traven, A. (2011) PUF proteins: repression, activation and mRNA localization. *Trends in cell biology* **21**, 104-112

129. Edwards, T. A., Pyle, S. E., Wharton, R. P., and Aggarwal, A. K. (2001) Structure of Pumilio reveals similarity between RNA and peptide binding motifs. *Cell* **105**, 281-289

130. del Carmen Rodriguez, M., Gerold, P., Dessens, J., Kurtenbach, K., Schwartz, R. T., Sinden, R. E., and Margos, G. (2000) Characterisation and expression of pbs25, a sexual and sporogonic stage specific protein of *Plasmodium berghei*. *Molecular and biochemical parasitology* **110**, 147-159

131. Paton, M. G., Barker, G. C., Matsuoka, H., Ramesar, J., Janse, C. J., Waters, A. P., and Sinden, R. E. (1993) Structure and expression of a post-transcriptionally regulated malaria gene encoding a surface protein from the sexual stages of *Plasmodium berghei*. *Molecular and biochemical parasitology* **59**, 263-275

132. Mair, G. R., Lasonder, E., Garver, L. S., Franke-Fayard, B. M., Carret, C. K., Wiegant, J. C., Dirks, R. W., Dimopoulos, G., Janse, C. J., and Waters, A. P. (2010) Universal features of post-transcriptional gene regulation are critical for *Plasmodium* zygote development. *PLoS pathogens* **6**, e1000767

133. Cui, L., Fan, Q., and Li, J. (2002) The malaria parasite *Plasmodium falciparum* encodes members of the Puf RNA-binding protein family with conserved RNA binding activity. *Nucleic acids research* **30**, 4607-4617

134. Fan, Q., Li, J., Kariuki, M., and Cui, L. (2004) Characterization of PfPuf2, member of the Puf family RNA-binding proteins from the malaria parasite *Plasmodium falciparum*. *DNA and cell biology* **23**, 753-760

135. Gomes-Santos, C. S., Braks, J., Prudencio, M., Carret, C., Gomes, A. R., Pain, A., Feltwell, T., Khan, S., Waters, A., Janse, C., Mair, G. R., and Mota, M. M. (2011) Transition of *Plasmodium* sporozoites into liver stage-like forms is regulated by the RNA binding protein Pumilio. *PLoS pathogens* **7**, e1002046

136. Muller, K., Matuschewski, K., and Silvie, O. (2011) The Puf-family RNA-binding protein Puf2 controls sporozoite conversion to liver stages in the malaria parasite. *PloS one* **6**, e19860

137. Miao, J., Li, J., Fan, Q., Li, X., Li, X., and Cui, L. (2010) The Puf-family RNA-binding protein PfPuf2 regulates sexual development and sex differentiation in the malaria parasite *Plasmodium falciparum*. *Journal of cell science* **123**, 1039-1049

138. Miao, J., Fan, Q., Parker, D., Li, X., Li, J., and Cui, L. (2013) Puf mediates translation repression of transmission-blocking vaccine candidates in malaria parasites. *PLoS pathogens* **9**, e1003268

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

140. Billker, O., Shaw, M. K., Margos, G., and Sinden, R. E. (1997) The roles of temperature, pH and mosquito factors as triggers of male and female gametogenesis of *Plasmodium berghei* in vitro. *Parasitology* **115 (Pt 1)**, 1-7

141. Nofal, S. D., Patel, A., Blackman, M. J., Flueck, C., and Baker, D. A. (2021) *Plasmodium falciparum* Guanylyl Cyclase-Alpha and the Activity of Its Appended P4-ATPase Domain Are Essential for cGMP Synthesis and Blood-Stage Egress. *mBio* **12**

142. Hirai, M., Arai, M., Kawai, S., and Matsuoka, H. (2006) PbGCbeta is essential for *Plasmodium* ookinete motility to invade midgut cell and for successful completion of parasite life cycle in mosquitoes. *Journal of biochemistry* **140**, 747-757

143. Billker, O., Dechamps, S., Tewari, R., Wenig, G., Franke-Fayard, B., and Brinkmann, V. (2004) Calcium and a calcium-dependent protein kinase regulate gamete formation and mosquito transmission in a malaria parasite. *Cell* **117**, 503-514

144. Rangarajan, R., Bei, A. K., Jethwaney, D., Maldonado, P., Dorin, D., Sultan, A. A., and Doerig, C. (2005) A mitogen-activated protein kinase regulates male gametogenesis and transmission of the malaria parasite *Plasmodium berghei*. *EMBO reports* **6**, 464-469

145. Janse, C. J., van der Klooster, P. F., van der Kaay, H. J., van der Ploeg, M., and Overdulve, J. P. (1986) DNA synthesis in *Plasmodium berghei* during asexual and sexual development. *Molecular and biochemical parasitology* **20**, 173-182

146. Reininger, L., Billker, O., Tewari, R., Mukhopadhyay, A., Fennell, C., Dorin-Semblat, D., Doerig, C., Goldring, D., Harmse, L., Ranford-Cartwright, L., Packer, J., and Doerig, C. (2005) A NIMA-related protein kinase is essential for completion of the sexual cycle of malaria parasites. *The Journal of biological chemistry* **280**, 31957-31964

147. Reininger, L., Tewari, R., Fennell, C., Holland, Z., Goldring, D., Ranford-Cartwright, L., Billker, O., and Doerig, C. (2009) An essential role for the *Plasmodium* Nek-2 Nima-related protein kinase in the sexual development of malaria parasites. *The Journal of biological chemistry* **284**, 20858-20868

148. Bennink, S., Kiesow, M. J., and Pradel, G. (2016) The development of malaria parasites in the mosquito midgut. *Cellular microbiology* **18**, 905-918

149. Guttery, D. S., Roques, M., Holder, A. A., and Tewari, R. (2015) Commit and Transmit: Molecular Players in *Plasmodium* Sexual Development and Zygote Differentiation. *Trends in parasitology* **31**, 676-685

150. Huber, M., Cabib, E., and Miller, L. H. (1991) Malaria parasite chitinase and penetration of the mosquito peritrophic membrane. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 2807-2810

151. Shahabuddin, M., Toyoshima, T., Aikawa, M., and Kaslow, D. C. (1993) Transmission-blocking activity of a chitinase inhibitor and activation of malarial parasite chitinase by mosquito protease. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 4266-4270

152. Tomas, A. M., Margos, G., Dimopoulos, G., van Lin, L. H., de Koning-Ward, T. F., Sinha, R., Lupetti, P., Beetsma, A. L., Rodriguez, M. C., Karras, M., Hager, A., Mendoza, J., Butcher, G. A., Kafatos, F., Janse, C. J., Waters, A. P., and Sinden, R. E. (2001) P25 and P28 proteins of the malaria ookinete surface have multiple and partially redundant functions. *The EMBO journal* **20**, 3975-3983

153. Aly, A. S., Vaughan, A. M., and Kappe, S. H. (2009) Malaria parasite development in the mosquito and infection of the mammalian host. *Annual review of microbiology* **63**, 195-221

154. Yuda, M., Sakaida, H., and Chinzei, Y. (1999) Targeted disruption of the *Plasmodium berghei* CTRP gene reveals its essential role in malaria infection of the vector mosquito. *The Journal of experimental medicine* **190**, 1711-1716

155. Siden-Kiamos, I., Ecker, A., Nyback, S., Louis, C., Sinden, R. E., and Billker, O. (2006) *Plasmodium berghei* calcium-dependent protein kinase 3 is required for ookinete gliding motility and mosquito midgut invasion. *Molecular microbiology* **60**, 1355-1363

156. Tremp, A. Z., Khater, E. I., and Dessens, J. T. (2008) IMC1b is a putative membrane skeleton protein involved in cell shape, mechanical strength, motility, and infectivity of malaria ookinetes. *The Journal of biological chemistry* **283**, 27604-27611

157. Wang, X., Qian, P., Cui, H., Yao, L., and Yuan, J. (2020) A protein palmitoylation cascade regulates microtubule cytoskeleton integrity in *Plasmodium*. *The EMBO journal* **39**, e104168

158. Harding, C. R., and Meissner, M. (2014) The inner membrane complex through development of Toxoplasma gondii and *Plasmodium*. *Cellular microbiology* **16**, 632-641

159. Lhomme, S., Nicot, F., Jeanne, N., Dimeglio, C., Roulet, A., Lefebvre, C., Carcenac, R., Manno, M., Dubois, M., Peron, J. M., Alric, L., Kamar, N., Abravanel, F., and Izopet, J. (2020) Insertions and Duplications in the Polyproline Region of the Hepatitis E Virus. *Frontiers in microbiology* **11**, 1

160. Hillyer, J. F., Barreau, C., and Vernick, K. D. (2007) Efficiency of salivary gland invasion by malaria sporozoites is controlled by rapid sporozoite destruction in the mosquito haemocoel. *International journal for parasitology* **37**, 673-681

161. Adini, A., Krugliak, M., Ginsburg, H., Li, L., Lavie, L., and Warburg, A. (2001) Transglutaminase in *Plasmodium* parasites: activity and putative role in oocysts and blood stages. *Molecular and biochemical parasitology* **117**, 161-168

162. Nacer, A., Walker, K., and Hurd, H. (2008) Localisation of laminin within *Plasmodium berghei* oocysts and the midgut epithelial cells of *Anopheles stephensi*. *Parasites & vectors* **1**, 33

163. Canning, E. U., and Sinden, R. E. (1973) The organization of the ookinete and observations on nuclear division in oocysts of *Plasmodium berghei*. *Parasitology* **67**, 29-40

164. Moran, P., and Caras, I. W. (1994) Requirements for glycosylphosphatidylinositol attachment are similar but not identical in mammalian cells and parasitic protozoa. *The Journal of cell biology* **125**, 333-343

165. Sinden, R. E., and Strong, K. (1978) An ultrastructural study of the sporogonic development of *Plasmodium falciparum* in *Anopheles* gambiae. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **72**, 477-491

166. Santoro, F., Cochrane, A. H., Nussenzweig, V., Nardin, E. H., Nussenzweig, R. S., Gwadz, R. W., and Ferreira, A. (1983) Structural similarities among the protective antigens of sporozoites from different species of malaria parasites. *The Journal of biological chemistry* **258**, 3341-3345

167. Yoshida, N., Nussenzweig, R. S., Potocnjak, P., Nussenzweig, V., and Aikawa, M. (1980) Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasite. *Science* **207**, 71-73

168. Menard, R., Sultan, A. A., Cortes, C., Altszuler, R., van Dijk, M. R., Janse, C. J., Waters, A. P., Nussenzweig, R. S., and Nussenzweig, V. (1997) Circumsporozoite protein is required for development of malaria sporozoites in mosquitoes. *Nature* **385**, 336-340

169. Thathy, V., Fujioka, H., Gantt, S., Nussenzweig, R., Nussenzweig, V., and Menard, R. (2002) Levels of circumsporozoite protein in the *Plasmodium* oocyst determine sporozoite morphology. *The EMBO journal* **21**, 1586-1596

170. Vanderberg, J., and Rhodin, J. (1967) Differentiation of nuclear and cytoplasmic fine structure during sporogonic development of *Plasmodium berghei*. *The Journal of cell biology* **32**, C7-10

171. Khater, E. I., Sinden, R. E., and Dessens, J. T. (2004) A malaria membrane skeletal protein is essential for normal morphogenesis, motility, and infectivity of sporozoites. *The Journal of cell biology* **167**, 425-432

172. Aly, A. S., and Matuschewski, K. (2005) A malarial cysteine protease is necessary for *Plasmodium* sporozoite egress from oocysts. *The Journal of experimental medicine* **202**, 225-230

173. Frischknecht, F., and Matuschewski, K. (2017) *Plasmodium* Sporozoite Biology. *Cold Spring Harbor perspectives in medicine* **7**

174. Sidjanski, S. P., Vanderberg, J. P., and Sinnis, P. (1997) *Anopheles stephensi* salivary glands bear receptors for region I of the circumsporozoite protein of *Plasmodium falciparum*. *Molecular and biochemical parasitology* **90**, 33-41

175. Tewari, R., Spaccapelo, R., Bistoni, F., Holder, A. A., and Crisanti, A. (2002) Function of region I and II adhesive motifs of *Plasmodium falciparum* circumsporozoite protein in sporozoite motility and infectivity. *The Journal of biological chemistry* **277**, 47613-47618

176. Singh, D., Patri, S., Narahari, V., Segireddy, R. R., Dey, S., Saurabh, A., Vijay, M., Prabhu, N. P., Srivastava, A., Kolli, S. K., and Kumar, K. A. (2022) A conserved *Plasmodium* structural integrity maintenance protein (SIMP) is associated with sporozoite membrane and is essential for maintaining shape and infectivity. *Molecular microbiology* **117**, 1324-1339

177. Robson, K. J., Frevert, U., Reckmann, I., Cowan, G., Beier, J., Scragg, I. G., Takehara, K., Bishop, D. H., Pradel, G., Sinden, R., and et al. (1995) Thrombospondin-related adhesive protein (TRAP) of *Plasmodium falciparum*: expression during sporozoite ontogeny and binding to human hepatocytes. *The EMBO journal* **14**, 3883-3894

178. Kariu, T., Yuda, M., Yano, K., and Chinzei, Y. (2002) MAEBL is essential for malarial sporozoite infection of the mosquito salivary gland. *The Journal of experimental medicine* **195**, 1317-1323

179. Matuschewski, K. (2006) Getting infectious: formation and maturation of *Plasmodium* sporozoites in the *Anopheles* vector. *Cellular microbiology* **8**, 1547-1556

180. Matuschewski, K., Ross, J., Brown, S. M., Kaiser, K., Nussenzweig, V., and Kappe, S. H. (2002) Infectivity-associated changes in the transcriptional repertoire of the malaria parasite sporozoite stage. *The Journal of biological chemistry* **277**, 41948-41953

181. Zhang, M., Fennell, C., Ranford-Cartwright, L., Sakthivel, R., Gueirard, P., Meister, S., Caspi, A., Doerig, C., Nussenzweig, R. S., Tuteja, R., Sullivan, W. J., Jr., Roos, D. S., Fontoura, B. M., Menard, R., Winzeler, E. A., and Nussenzweig, V. (2010) The *Plasmodium* eukaryotic initiation factor-2alpha kinase IK2 controls the latency of sporozoites in the mosquito salivary glands. *The Journal of experimental medicine* **207**, 1465-1474

182. Aly, A. S., Mikolajczak, S. A., Rivera, H. S., Camargo, N., Jacobs-Lorena, V., Labaied, M., Coppens, I., and Kappe, S. H. (2008) Targeted deletion of SAP1 abolishes the expression of infectivity factors necessary for successful malaria parasite liver infection. *Molecular microbiology* **69**, 152-163

183. Kumar, K. A., Baxter, P., Tarun, A. S., Kappe, S. H., and Nussenzweig, V. (2009) Conserved protective mechanisms in radiation and genetically attenuated uis3(-) and uis4(-) *Plasmodium* sporozoites. *PloS one* **4**, e4480

184. Steffen, R., and Behrens, R. H. (1992) Travellers' malaria. *Parasitology today* **8**, 61-66

185. Frean, J., Poonsamy, B., Shandukani, B., Moonasar, D., and Raman, J. (2013) Case management of malaria: diagnosis. *South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde* **103**, 789-793

186. Adesanmi, T. A., Okafor, H. U., Okoro, A. B., and Mafe, A. G. (2011) Diagnosis of malaria parasitemia in children using a rapid diagnostic test. *Nigerian journal of clinical practice* **14**, 195-200

187. Howard, R. J., Uni, S., Aikawa, M., Aley, S. B., Leech, J. H., Lew, A. M., Wellems, T. E., Rener, J., and Taylor, D. W. (1986) Secretion of a malarial histidine-rich protein (Pf HRP II) from *Plasmodium falciparum*-infected erythrocytes. *The Journal of cell biology* **103**, 1269-1277

188. Girard, M. P., Reed, Z. H., Friede, M., and Kieny, M. P. (2007) A review of human vaccine research and development: malaria. *Vaccine* **25**, 1567-1580

189. Stoute, J. A., Kester, K. E., Krzych, U., Wellde, B. T., Hall, T., White, K., Glenn, G., Ockenhouse, C. F., Garcon, N., Schwenk, R., Lanar, D. E., Sun, P., Momin, P., Wirtz, R. A., Golenda, C., Slaoui, M., Wortmann, G., Holland, C., Dowler, M., Cohen, J., and Ballou, W. R. (1998) Long-term efficacy and immune responses following immunization with the RTS,S malaria vaccine. *The Journal of infectious diseases* **178**, 1139-1144

190. Crompton, P. D., Pierce, S. K., and Miller, L. H. (2010) Advances and challenges in malaria vaccine development. *The Journal of clinical investigation* **120**, 4168-4178

191. Malkin, E. M., Durbin, A. P., Diemert, D. J., Sattabongkot, J., Wu, Y., Miura, K., Long, C. A., Lambert, L., Miles, A. P., Wang, J., Stowers, A., Miller, L. H., and Saul, A. (2005) Phase 1 vaccine trial of Pvs25H: a transmission blocking vaccine for *Plasmodium vivax* malaria. *Vaccine* **23**, 3131-3138

192. Hyde, J. E. (2007) Drug-resistant malaria - an insight. *The FEBS journal* **274**, 4688-4698

193. Galappaththy, G. N., Tharyan, P., and Kirubakaran, R. (2013) Primaquine for preventing relapse in people with *Plasmodium vivax* malaria treated with chloroquine. *The Cochrane database of systematic reviews*, CD004389

194. O'Neill, P. M., and Posner, G. H. (2004) A medicinal chemistry perspective on artemisinin and related endoperoxides. *Journal of medicinal chemistry* **47**, 2945-2964

195. White, N. J. (2008) Qinghaosu (artemisinin): the price of success. *Science* **320**, 330-334

196. Prapunwattana, P., O'Sullivan, W. J., and Yuthavong, Y. (1988) Depression of *Plasmodium falciparum* dihydroorotate dehydrogenase activity in in vitro culture by tetracycline. *Molecular and biochemical parasitology* **27**, 119-124

197. Camps, M., Arrizabalaga, G., and Boothroyd, J. (2002) An rRNA mutation identifies the apicoplast as the target for clindamycin in Toxoplasma gondii. *Molecular microbiology* **43**, 1309-1318

198. Tizifa, T. A., Kabaghe, A. N., McCann, R. S., van den Berg, H., Van Vugt, M., and Phiri, K. S. (2018) Prevention Efforts for Malaria. *Current tropical medicine reports* **5**, 41-50

199. Coleman, M., Hemingway, J., Gleave, K. A., Wiebe, A., Gething, P. W., and Moyes, C. L. (2017) Developing global maps of insecticide resistance risk to improve vector control. *Malaria journal* **16**, 86

200. <ITNS.pdf>.

201. Raouf, S., Mpimbaza, A., Kigozi, R., Sserwanga, A., Rubahika, D., Katamba, H., Lindsay, S. W., Kapella, B. K., Belay, K. A., Kamya, M. R., Staedke, S. G., and Dorsey, G. (2017) Resurgence of Malaria Following Discontinuation of Indoor Residual Spraying of Insecticide in an Area of Uganda With Previously High-Transmission Intensity. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **65**, 453-460

202. Tusting, L. S., Thwing, J., Sinclair, D., Fillinger, U., Gimnig, J., Bonner, K. E., Bottomley, C., and Lindsay, S. W. (2013) Mosquito larval source management for controlling malaria. *The Cochrane database of systematic reviews*, CD008923

203. Fillinger, U., and Lindsay, S. W. (2011) Larval source management for malaria control in Africa: myths and reality. *Malaria journal* **10**, 353

204. Diabate, A., and Tripet, F. (2015) Targeting male mosquito mating behaviour for malaria control. *Parasites & vectors* **8**, 347

205. Greenwood, B. (2017) Elimination of malaria: halfway there. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **111**, 1-2

206. Thomson, R., Parr, J. B., Cheng, Q., Chenet, S., Perkins, M., and Cunningham, J. (2020) Prevalence of *Plasmodium falciparum* lacking histidine-rich proteins 2 and 3: a systematic review. *Bulletin of the World Health Organization* **98**, 558-568F

207. Price, R. N., Douglas, N. M., and Anstey, N. M. (2009) New developments in *Plasmodium vivax* malaria: severe disease and the rise of chloroquine resistance. *Current opinion in infectious diseases* **22**, 430-435

208. Patel, P., Bharti, P. K., Bansal, D., Ali, N. A., Raman, R. K., Mohapatra, P. K., Sehgal, R., Mahanta, J., Sultan, A. A., and Singh, N. (2017) Prevalence of mutations linked to antimalarial resistance in *Plasmodium falciparum* from Chhattisgarh, Central India: A malaria elimination point of view. *Scientific reports* **7**, 16690

209. Ashley, E. A., Dhorda, M., Fairhurst, R. M., Amaratunga, C., Lim, P., Suon, S., Sreng, S., Anderson, J. M., Mao, S., Sam, B., Sopha, C., Chuor, C. M., Nguon, C., Sovannaroth, S., Pukrittayakamee, S., Jittamala, P., Chotivanich, K., Chutasmit, K., Suchatsoonthorn, C., Runcharoen, R., Hien, T. T., Thuy-Nhien, N. T., Thanh, N. V., Phu, N. H., Htut, Y., Han, K. T., Aye, K. H., Mokuolu, O. A., Olaosebikan, R. R., Folaranmi, O. O., Mayxay, M., Khanthavong, M., Hongvanthong, B., Newton, P. N., Onyamboko, M. A., Fanello, C. I., Tshefu, A. K., Mishra, N., Valecha, N., Phyo, A. P., Nosten, F., Yi, P., Tripura, R., Borrmann, S., Bashraheil, M., Peshu, J., Faiz, M. A., Ghose, A., Hossain, M. A., Samad, R., Rahman, M. R., Hasan, M. M., Islam, A., Miotto, O., Amato, R., MacInnis, B., Stalker, J., Kwiatkowski, D. P., Bozdech, Z., Jeeyapant, A., Cheah, P. Y., Sakulthaew, T., Chalk, J., Intharabut, B., Silamut, K., Lee, S. J., Vihokhern, B., Kunasol, C., Imwong, M., Tarning, J., Taylor, W. J., Yeung, S., Woodrow, C. J., Flegg, J. A., Das, D., Smith, J., Venkatesan, M., Plowe, C. V., Stepniewska, K., Guerin, P. J., Dondorp, A. M., Day, N. P., White, N. J., and Tracking Resistance to Artemisinin, C. (2014) Spread of artemisinin resistance in *Plasmodium falciparum* malaria. *The New England journal of medicine* **371**, 411-423

210. Hien, A. S., Soma, D. D., Hema, O., Bayili, B., Namountougou, M., Gnankine, O., Baldet, T., Diabate, A., and Dabire, K. R. (2017) Evidence that agricultural use of pesticides selects pyrethroid resistance within *Anopheles* gambiae s.l. populations from cotton growing areas in Burkina Faso, West Africa. *PloS one* **12**, e0173098

211. Lourenco, C., Tatem, A. J., Atkinson, P. M., Cohen, J. M., Pindolia, D., Bhavnani, D., and Le Menach, A. (2019) Strengthening surveillance systems for malaria elimination: a global landscaping of system performance, 2015-2017. *Malaria journal* **18**, 315

212. Yang, W. Z., and Zhou, X. N. (2016) [New challenges of malaria elimination in China]. *Zhonghua yu fang yi xue za zhi [Chinese journal of preventive medicine]* **50**, 289-291

213. Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., Zhang, L., Fan, G., Xu, J., Gu, X., Cheng, Z., Yu, T., Xia, J., Wei, Y., Wu, W., Xie, X., Yin, W., Li, H., Liu, M., Xiao, Y., Gao, H., Guo, L., Xie, J., Wang, G., Jiang, R., Gao, Z., Jin, Q., Wang, J., and Cao, B. (2020) Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* **395**, 497-506

214. <Rates of Coinfection between SARSCoV2 and other respiratory Pathogens.pdf>.

215. Chanda-Kapata, P., Kapata, N., and Zumla, A. (2020) COVID-19 and malaria: A symptom screening challenge for malaria endemic countries. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases* **94**, 151-153

216. Zawawi, A., Alghanmi, M., Alsaady, I., Gattan, H., Zakai, H., and Couper, K. (2020) The impact of COVID-19 pandemic on malaria elimination. *Parasite epidemiology and control* **11**, e00187

217. Carlton, J., Silva, J., and Hall, N. (2005) The genome of model malaria parasites, and comparative genomics. *Current issues in molecular biology* **7**, 23-37

218. Martinsen, E. S., Perkins, S. L., and Schall, J. J. (2008) A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): evolution of life-history traits and host switches. *Molecular phylogenetics and evolution* **47**, 261-273

219. Kooij, T. W., Rauch, M. M., and Matuschewski, K. (2012) Expansion of experimental genetics approaches for *Plasmodium berghei* with versatile transfection vectors. *Molecular and biochemical parasitology* **185**, 19-26

220. Balu, B., and Adams, J. H. (2007) Advancements in transfection technologies for *Plasmodium*. *International journal for parasitology* **37**, 1-10

221. Janse, C. J., Kroeze, H., van Wigcheren, A., Mededovic, S., Fonager, J., Franke-Fayard, B., Waters, A. P., and Khan, S. M. (2011) A genotype and phenotype database of genetically modified malaria-parasites. *Trends in parasitology* **27**, 31-39

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

223. Carvalho, T. G., and Menard, R. (2005) Manipulating the *Plasmodium* genome. *Current issues in molecular biology* **7**, 39-55

224. Janse, C. J., Franke-Fayard, B., Mair, G. R., Ramesar, J., Thiel, C., Engelmann, S., Matuschewski, K., van Gemert, G. J., Sauerwein, R. W., and Waters, A. P. (2006) High efficiency transfection of *Plasmodium berghei* facilitates novel selection procedures. *Molecular and biochemical parasitology* **145**, 60-70

225. Saini, E., Zeeshan, M., Brady, D., Pandey, R., Kaiser, G., Koreny, L., Kumar, P., Thakur, V., Tatiya, S., Katris, N. J., Limenitakis, R. S., Kaur, I., Green, J. L., Bottrill, A. R., Guttery, D. S., Waller, R. F., Heussler, V., Holder, A. A., Mohmmed, A., Malhotra, P., and Tewari, R. (2017) Photosensitized INA-Labelled protein 1 (PhIL1) is novel component of the inner membrane complex and is required for *Plasmodium* parasite development. *Scientific reports* **7**, 15577

226. <Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases 8th Edition.pdf>.

227. Shibeshi, M. A., Kifle, Z. D., and Atnafie, S. A. (2020) Antimalarial Drug Resistance and Novel Targets for Antimalarial Drug Discovery. *Infection and drug resistance* **13**, 4047-4060

228. Milner, D. A., Jr. (2018) Malaria Pathogenesis. *Cold Spring Harbor perspectives in medicine* **8**

229. <malaria-transmission-blocking_2.pdf>.

230. Chawla, J., Oberstaller, J., and Adams, J. H. (2021) Targeting Gametocytes of the Malaria Parasite *Plasmodium falciparum* in a Functional Genomics Era: Next Steps. *Pathogens* **10**

231. Dearnley, M. K., Yeoman, J. A., Hanssen, E., Kenny, S., Turnbull, L., Whitchurch, C. B., Tilley, L., and Dixon, M. W. (2012) Origin, composition, organization and function of the inner membrane complex of *Plasmodium falciparum* gametocytes. *Journal of cell science* **125**, 2053-2063

232. Ferreira, J. L., Heincke, D., Wichers, J. S., Liffner, B., Wilson, D. W., and Gilberger, T. W. (2020) The Dynamic Roles of the Inner Membrane Complex in the Multiple Stages of the Malaria Parasite. *Frontiers in cellular and infection microbiology* **10**, 611801

233. Kono, M., Herrmann, S., Loughran, N. B., Cabrera, A., Engelberg, K., Lehmann, C., Sinha, D., Prinz, B., Ruch, U., Heussler, V., Spielmann, T., Parkinson, J., and Gilberger, T. W. (2012) Evolution and architecture of the inner membrane complex in asexual and sexual stages of the malaria parasite. *Molecular biology and evolution* **29**, 2113-2132

234. Bullen, H. E., Tonkin, C. J., O'Donnell, R. A., Tham, W. H., Papenfuss, A. T., Gould, S., Cowman, A. F., Crabb, B. S., and Gilson, P. R. (2009) A novel family of Apicomplexan glideosome-associated proteins with an inner membrane-anchoring role. *The Journal of biological chemistry* **284**, 25353-25363

235. Frenal, K., Dubremetz, J. F., Lebrun, M., and Soldati-Favre, D. (2017) Gliding motility powers invasion and egress in Apicomplexa. *Nature reviews. Microbiology* **15**, 645-660

236. Sinnis, P., and Sim, B. K. (1997) Cell invasion by the vertebrate stages of *Plasmodium*. *Trends in microbiology* **5**, 52-58

237. Saini, E., Sheokand, P. K., Sharma, V., Agrawal, P., Kaur, I., Singh, S., Mohmmed, A., and Malhotra, P. (2021) *Plasmodium falciparum* PhIL1-associated complex plays an essential role in merozoite reorientation and invasion of host erythrocytes. *PLoS pathogens* **17**, e1009750

238. Tremp, A. Z., and Dessens, J. T. (2011) Malaria IMC1 membrane skeleton proteins operate autonomously and participate in motility independently of cell shape. *The Journal of biological chemistry* **286**, 5383-5391

239. Poulin, B., Patzewitz, E. M., Brady, D., Silvie, O., Wright, M. H., Ferguson, D. J., Wall, R. J., Whipple, S., Guttery, D. S., Tate, E. W., Wickstead, B., Holder, A. A., and Tewari, R. (2013) Unique apicomplexan IMC sub-compartment proteins are early markers for apical polarity in the malaria parasite. *Biology open* **2**, 1160-1170

240. Gomez-Diaz, E., Yerbanga, R. S., Lefevre, T., Cohuet, A., Rowley, M. J., Ouedraogo, J. B., and Corces, V. G. (2017) Epigenetic regulation of *Plasmodium falciparum* clonally variant gene expression during development in *Anopheles* gambiae. *Scientific reports* **7**, 40655

241. Lopez-Barragan, M. J., Lemieux, J., Quinones, M., Williamson, K. C., Molina-Cruz, A., Cui, K., Barillas-Mury, C., Zhao, K., and Su, X. Z. (2011) Directional gene expression and antisense transcripts in sexual and asexual stages of *Plasmodium falciparum*. *BMC genomics* **12**, 587

242. Zanghi, G., Vembar, S. S., Baumgarten, S., Ding, S., Guizetti, J., Bryant, J. M., Mattei, D., Jensen, A. T. R., Renia, L., Goh, Y. S., Sauerwein, R., Hermsen, C. C., Franetich, J. F., Bordessoulles, M., Silvie, O., Soulard, V., Scatton, O., Chen, P., Mecheri, S., Mazier, D., and Scherf, A. (2018) A Specific PfEMP1 Is Expressed in P. *falciparum* Sporozoites and Plays a Role in Hepatocyte Infection. *Cell reports* **22**, 2951-2963

243. Gilk, S. D., Raviv, Y., Hu, K., Murray, J. M., Beckers, C. J., and Ward, G. E. (2006) Identification of PhIL1, a novel cytoskeletal protein of the Toxoplasma gondii pellicle, through photosensitized labeling with 5-[125I]iodonaphthalene-1-azide. *Eukaryotic cell* **5**, 1622-1634

244. Barkhuff, W. D., Gilk, S. D., Whitmarsh, R., Tilley, L. D., Hunter, C., and Ward, G. E. (2011) Targeted disruption of TgPhIL1 in Toxoplasma gondii results in altered parasite morphology and fitness. *PloS one* **6**, e23977

245. Parkyn Schneider, M., Liu, B., Glock, P., Suttie, A., McHugh, E., Andrew, D., Batinovic, S., Williamson, N., Hanssen, E., McMillan, P., Hliscs, M., Tilley, L., and Dixon, M. W. A. (2017) Disrupting assembly of the inner membrane complex blocks *Plasmodium falciparum* sexual stage development. *PLoS pathogens* **13**, e1006659

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

247. Wichers, J. S., Wunderlich, J., Heincke, D., Pazicky, S., Strauss, J., Schmitt, M., Kimmel, J., Wilcke, L., Scharf, S., von Thien, H., Burda, P. C., Spielmann, T., Low, C., Filarsky, M., Bachmann, A., and Gilberger, T. W. (2021) Identification of novel inner membrane complex and apical annuli proteins of the malaria parasite *Plasmodium falciparum*. *Cellular microbiology* **23**, e13341

248. Zhang, M., Wang, C., Otto, T. D., Oberstaller, J., Liao, X., Adapa, S. R., Udenze, K., Bronner, I. F., Casandra, D., Mayho, M., Brown, J., Li, S., Swanson, J., Rayner, J. C., Jiang, R. H. Y., and Adams, J. H. (2018) Uncovering the essential genes of the human malaria parasite *Plasmodium falciparum* by saturation mutagenesis. *Science* **360**

249. McIntosh, M. T., Drozdowicz, Y. M., Laroiya, K., Rea, P. A., and Vaidya, A. B. (2001) Two classes of plant-like vacuolar-type H(+)-pyrophosphatases in malaria parasites. *Molecular and biochemical parasitology* **114**, 183-195

250. Birnbaum, J., Scharf, S., Schmidt, S., Jonscher, E., Hoeijmakers, W. A. M., Flemming, S., Toenhake, C. G., Schmitt, M., Sabitzki, R., Bergmann, B., Frohlke, U., Mesen-Ramirez, P., Blancke Soares, A., Herrmann, H., Bartfai, R., and Spielmann, T. (2020) A Kelch13-defined endocytosis pathway mediates artemisinin resistance in malaria parasites. *Science* **367**, 51-59

251. Wall, R. J., Zeeshan, M., Katris, N. J., Limenitakis, R., Rea, E., Stock, J., Brady, D., Waller, R. F., Holder, A. A., and Tewari, R. (2019) Systematic analysis of *Plasmodium* myosins reveals differential expression, localisation, and function in invasive and proliferative parasite stages. *Cellular microbiology* **21**, e13082

252. Green, J. L., Wall, R. J., Vahokoski, J., Yusuf, N. A., Ridzuan, M. A. M., Stanway, R. R., Stock, J., Knuepfer, E., Brady, D., Martin, S. R., Howell, S. A., Pires, I. P., Moon, R. W., Molloy, J. E., Kursula, I., Tewari, R., and Holder, A. A. (2017) Compositional and expression analyses of the glideosome during the *Plasmodium* life cycle reveal an additional myosin light chain required for maximum motility. *The Journal of biological chemistry* **292**, 17857-17875

253. Mitchell, A. L., Attwood, T. K., Babbitt, P. C., Blum, M., Bork, P., Bridge, A., Brown, S. D., Chang, H. Y., El-Gebali, S., Fraser, M. I., Gough, J., Haft, D. R., Huang, H., Letunic, I., Lopez, R., Luciani, A., Madeira, F., Marchler-Bauer, A., Mi, H., Natale, D. A., Necci, M., Nuka, G., Orengo, C., Pandurangan, A. P., Paysan-Lafosse, T., Pesseat, S., Potter, S. C., Qureshi, M. A., Rawlings, N. D., Redaschi, N., Richardson, L. J., Rivoire, C., Salazar, G. A., Sangrador-Vegas, A., Sigrist, C. J. A., Sillitoe, I., Sutton, G. G., Thanki, N., Thomas, P. D., Tosatto, S. C. E., Yong, S. Y., and Finn, R. D. (2019) InterPro in 2019: improving coverage, classification and access to protein sequence annotations. *Nucleic acids research* **47**, D351-D360

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

255. Afroz, T., Cienikova, Z., Clery, A., and Allain, F. H. T. (2015) One, Two, Three, Four! How Multiple RRMs Read the Genome Sequence. *Methods in enzymology* **558**, 235-278

256. Maris, C., Dominguez, C., and Allain, F. H. (2005) The RNA recognition motif, a plastic RNA-binding platform to regulate post-transcriptional gene expression. *The FEBS journal* **272**, 2118-2131

257. Chaudhury, A., Chander, P., and Howe, P. H. (2010) Heterogeneous nuclear ribonucleoproteins (hnRNPs) in cellular processes: Focus on hnRNP E1's multifunctional regulatory roles. *Rna* **16**, 1449-1462

258. Lunde, B. M., Moore, C., and Varani, G. (2007) RNA-binding proteins: modular design for efficient function. *Nature reviews. Molecular cell biology* **8**, 479-490

259. Muto, Y., and Yokoyama, S. (2012) Structural insight into RNA recognition motifs: versatile molecular Lego building blocks for biological systems. *Wiley interdisciplinary reviews. RNA* **3**, 229-246

260. Khan, S. M., Franke-Fayard, B., Mair, G. R., Lasonder, E., Janse, C. J., Mann, M., and Waters, A. P. (2005) Proteome analysis of separated male and female gametocytes reveals novel sex-specific *Plasmodium* biology. *Cell* **121**, 675-687

261. Shears, M. J., Sekhar Nirujogi, R., Swearingen, K. E., Renuse, S., Mishra, S., Jaipal Reddy, P., Moritz, R. L., Pandey, A., and Sinnis, P. (2019) Proteomic Analysis of *Plasmodium* Merosomes: The Link between Liver and Blood Stages in Malaria. *Journal of proteome research* **18**, 3404-3418

262. Hamada, S., Pionneau, C., Parizot, C., Silvie, O., Chardonnet, S., and Marinach, C. (2021) In-depth proteomic analysis of *Plasmodium berghei* sporozoites using trapped ion mobility spectrometry with parallel accumulation-serial fragmentation. *Proteomics* **21**, e2000305

263. Birney, E., Kumar, S., and Krainer, A. R. (1993) Analysis of the RNA-recognition motif and RS and RGG domains: conservation in metazoan pre-mRNA splicing factors. *Nucleic acids research* **21**, 5803-5816

264. Sonnhammer, E. L., von Heijne, G., and Krogh, A. (1998) A hidden Markov model for predicting transmembrane helices in protein sequences. *Proceedings. International Conference on Intelligent Systems for Molecular Biology* **6**, 175-182

265. Rudlaff, R. M., Kraemer, S., Streva, V. A., and Dvorin, J. D. (2019) An essential contractile ring protein controls cell division in *Plasmodium falciparum*. *Nature communications* **10**, 2181

266. Al-Khattaf, F. S., Tremp, A. Z., and Dessens, J. T. (2015) *Plasmodium* alveolins possess distinct but structurally and functionally related multi-repeat domains. *Parasitology research* **114**, 631-639

267. Sologub, L., Kuehn, A., Kern, S., Przyborski, J., Schillig, R., and Pradel, G. (2011) Malaria proteases mediate inside-out egress of gametocytes from red blood cells

following parasite transmission to the mosquito. *Cellular microbiology* **13**, 897-912

268. Dixon, M. W., Dearnley, M. K., Hanssen, E., Gilberger, T., and Tilley, L. (2012) Shape-shifting gametocytes: how and why does P. *falciparum* go banana-shaped? *Trends in parasitology* **28**, 471-478

269. Dearnley, M. K., Yeoman, J. A., Hanssen, E., Kenny, S., Turnbull, L., Whitchurch, C. B., Tilley, L., and Dixon, M. W. A. (2012) Origin, composition, organization and function of the inner membrane complex of *Plasmodium falciparum* gametocytes. *Journal of cell science* **125**, 2053-2063

270. <Bookshelf_NBK2261.pdf>.

271. Orjih, A. U., and Fitch, C. D. (1993) Hemozoin production by *Plasmodium falciparum*: variation with strain and exposure to chloroquine. *Biochimica et biophysica acta* **1157**, 270-274

272. Barry, D. N. (1982) Metabolism of Babesia parasites in vitro. amino acid production by Babesia rodhaini compared to *Plasmodium berghei*. *The Australian journal of experimental biology and medical science* **60**, 175-180

273. Saiki, E., Nagao, K., Aonuma, H., Fukumoto, S., Xuan, X., Bannai, M., and Kanuka, H. (2013) Multivariable analysis of host amino acids in plasma and liver during infection of malaria parasite *Plasmodium yoelii*. *Malaria journal* **12**, 19

274. Divo, A. A., Geary, T. G., Davis, N. L., and Jensen, J. B. (1985) Nutritional requirements of *Plasmodium falciparum* in culture. I. Exogenously supplied dialyzable components necessary for continuous growth. *The Journal of protozoology* **32**, 59-64

275. Francis, S. E., Sullivan, D. J., Jr., and Goldberg, D. E. (1997) Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. *Annual review of microbiology* **51**, 97-123

276. Rosenthal, P. J. (1995) *Plasmodium falciparum*: effects of proteinase inhibitors on globin hydrolysis by cultured malaria parasites. *Experimental parasitology* **80**, 272-281

277. Rosenthal, P. J., McKerrow, J. H., Rasnick, D., and Leech, J. H. (1989) *Plasmodium falciparum*: inhibitors of lysosomal cysteine proteinases inhibit a trophozoite proteinase and block parasite development. *Molecular and biochemical parasitology* **35**, 177-183

278. Coronado, L. M., Nadovich, C. T., and Spadafora, C. (2014) Malarial hemozoin: from target to tool. *Biochimica et biophysica acta* **1840**, 2032-2041

279. Egan, T. J., Combrinck, J. M., Egan, J., Hearne, G. R., Marques, H. M., Ntenteni, S., Sewell, B. T., Smith, P. J., Taylor, D., van Schalkwyk, D. A., and Walden, J. C. (2002) Fate of haem iron in the malaria parasite *Plasmodium falciparum*. *The Biochemical journal* **365**, 343-347

280. Kannan, R., Sahal, D., and Chauhan, V. S. (2002) Heme-artemisinin adducts are crucial mediators of the ability of artemisinin to inhibit heme polymerization. *Chemistry & biology* **9**, 321-332

281. Parhizgar, A. R., and Tahghighi, A. (2017) Introducing New Antimalarial Analogues of Chloroquine and Amodiaquine: A Narrative Review. *Iranian journal of medical sciences* **42**, 115-128

282. Sullivan, D. J., Jr., Gluzman, I. Y., Russell, D. G., and Goldberg, D. E. (1996) On the molecular mechanism of chloroquine's antimalarial action. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 11865-11870

283. Hien, T. T., and White, N. J. (1993) Qinghaosu. *Lancet* **341**, 603-608

284. Asawamahasakda, W., Ittarat, I., Pu, Y. M., Ziffer, H., and Meshnick, S. R. (1994) Reaction of antimalarial endoperoxides with specific parasite proteins. *Antimicrobial agents and chemotherapy* **38**, 1854-1858

285. Asawamahasakda, W., Ittarat, I., Chang, C. C., McElroy, P., and Meshnick, S. R. (1994) Effects of antimalarials and protease inhibitors on plasmodial hemozoin production. *Molecular and biochemical parasitology* **67**, 183-191

286. Meshnick, S. R., Taylor, T. E., and Kamchonwongpaisan, S. (1996) Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. *Microbiological reviews* **60**, 301-315

287. Dorn, A., Stoffel, R., Matile, H., Bubendorf, A., and Ridley, R. G. (1995) Malarial haemozoin/beta-haematin supports haem polymerization in the absence of protein. *Nature* **374**, 269-271

288. Pisciotta, J. M., Coppens, I., Tripathi, A. K., Scholl, P. F., Shuman, J., Bajad, S., Shulaev, V., and Sullivan, D. J., Jr. (2007) The role of neutral lipid nanospheres in *Plasmodium falciparum* haem crystallization. *The Biochemical journal* **402**, 197-204

289. Egan, T. J., Chen, J. Y., de Villiers, K. A., Mabotha, T. E., Naidoo, K. J., Ncokazi, K. K., Langford, S. J., McNaughton, D., Pandiancherri, S., and Wood, B. R. (2006) Haemozoin (beta-haematin) biomineralization occurs by self-assembly near the lipid/water interface. *FEBS letters* **580**, 5105-5110

290. Tekwani, B. L., and Walker, L. A. (2005) Targeting the hemozoin synthesis pathway for new antimalarial drug discovery: technologies for in vitro beta-hematin formation assay. *Combinatorial chemistry & high throughput screening* **8**, 63-79

291. Sullivan, D. J. (2002) Theories on malarial pigment formation and quinoline action. *International journal for parasitology* **32**, 1645-1653

292. Fong, K. Y., and Wright, D. W. (2013) Hemozoin and antimalarial drug discovery. *Future medicinal chemistry* **5**, 1437-1450

293. Sullivan, D. J., Jr., Gluzman, I. Y., and Goldberg, D. E. (1996) *Plasmodium* hemozoin formation mediated by histidine-rich proteins. *Science* **271**, 219-222

294. Fitch, C. D., Cai, G. Z., Chen, Y. F., and Shoemaker, J. D. (1999) Involvement of lipids in ferriprotoporphyrin IX polymerization in malaria. *Biochimica et biophysica acta* **1454**, 31-37

295. de Villiers, K. A., and Egan, T. J. (2021) Heme Detoxification in the Malaria Parasite: A Target for Antimalarial Drug Development. *Accounts of chemical research* **54**, 2649-2659

296. Jani, D., Nagarkatti, R., Beatty, W., Angel, R., Slebodnick, C., Andersen, J., Kumar, S., and Rathore, D. (2008) HDP-a novel heme detoxification protein from the malaria parasite. *PLoS pathogens* **4**, e1000053

297. Pagola, S., Stephens, P. W., Bohle, D. S., Kosar, A. D., and Madsen, S. K. (2000) The structure of malaria pigment beta-haematin. *Nature* **404**, 307-310

298. Rifaie-Graham, O., Pollard, J., Raccio, S., Balog, S., Rusch, S., Hernandez-Castaneda, M. A., Mantel, P. Y., Beck, H. P., and Bruns, N. (2019) Hemozoin-catalyzed precipitation polymerization as an assay for malaria diagnosis. *Nature communications* **10**, 1369

299. Schneider, E. L., and Marletta, M. A. (2005) Heme binding to the histidine-rich protein II from *Plasmodium falciparum*. *Biochemistry* **44**, 979-986

300. Nakatani, K., Ishikawa, H., Aono, S., and Mizutani, Y. (2014) Identification of essential histidine residues involved in heme binding and Hemozoin formation in heme detoxification protein from *Plasmodium falciparum*. *Scientific reports* **4**, 6137

301. Gupta, P., Mehrotra, S., Sharma, A., Chugh, M., Pandey, R., Kaushik, A., Khurana, S., Srivastava, N., Srivastava, T., Deshmukh, A., Panda, A., Aggarwal, P., Bhavesh, N. S., Bhatnagar, R. K., Mohmmed, A., Gupta, D., and Malhotra, P. (2017) Exploring Heme and Hemoglobin Binding Regions of *Plasmodium* Heme Detoxification Protein for New Antimalarial Discovery. *Journal of medicinal chemistry* **60**, 8298-8308

302. Surolia, N., and Padmanaban, G. (1992) de novo biosynthesis of heme offers a new chemotherapeutic target in the human malarial parasite. *Biochemical and biophysical research communications* **187**, 744-750

303. Nagaraj, V. A., Sundaram, B., Varadarajan, N. M., Subramani, P. A., Kalappa, D. M., Ghosh, S. K., and Padmanaban, G. (2013) Malaria parasite-synthesized heme is essential in the mosquito and liver stages and complements host heme in the blood stages of infection. *PLoS pathogens* **9**, e1003522

304. Bushell, E., Gomes, A. R., Sanderson, T., Anar, B., Girling, G., Herd, C., Metcalf, T., Modrzynska, K., Schwach, F., Martin, R. E., Mather, M. W., McFadden, G. I., Parts, L., Rutledge, G. G., Vaidya, A. B., Wengelnik, K., Rayner, J. C., and Billker, O. (2017) Functional Profiling of a *Plasmodium* Genome Reveals an Abundance of Essential Genes. *Cell* **170**, 260-272 e268

305. Lin, J. W., Spaccapelo, R., Schwarzer, E., Sajid, M., Annoura, T., Deroost, K., Ravelli, R. B., Aime, E., Capuccini, B., Mommaas-Kienhuis, A. M., O'Toole, T., Prins, F., Franke-Fayard, B. M., Ramesar, J., Chevalley-Maurel, S., Kroeze, H., Koster, A. J., Tanke, H. J., Crisanti, A., Langhorne, J., Arese, P., Van den Steen, P. E., Janse, C. J., and Khan, S. M. (2015) Replication of *Plasmodium* in reticulocytes can occur without hemozoin formation, resulting in chloroquine resistance. *The Journal of experimental medicine* **212**, 893-903

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

307. Gupta, P., Pandey, R., Thakur, V., Parveen, S., Kaur, I., Panda, A., Bishi, R., Mehrotra, S., Akhtar, A., Gupta, D., Mohmmed, A., and Malhotra, P. (2022) Heme Detoxification Protein (PfHDP) is essential for the hemoglobin uptake and metabolism in *Plasmodium falciparum*. *FASEB bioAdvances* **4**, 662-674

308. Egan, T. J. (2008) Haemozoin formation. *Molecular and biochemical parasitology* **157**, 127-136

309. Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *Journal of molecular biology* **305**, 567-580

310. Schultz, J., Milpetz, F., Bork, P., and Ponting, C. P. (1998) SMART, a simple modular architecture research tool: identification of signaling domains. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 5857-5864

311. Chugh, M., Sundararaman, V., Kumar, S., Reddy, V. S., Siddiqui, W. A., Stuart, K. D., and Malhotra, P. (2013) Protein complex directs hemoglobin-to-hemozoin formation in *Plasmodium falciparum*. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 5392-5397

312. Lacroix, C., Giovannini, D., Combe, A., Bargieri, D. Y., Spath, S., Panchal, D., Tawk, L., Thiberge, S., Carvalho, T. G., Barale, J. C., Bhanot, P., and Menard, R. (2011) FLP/FRT-mediated conditional mutagenesis in pre-erythrocytic stages of *Plasmodium berghei*. *Nature protocols* **6**, 1412-1428

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Succinctus

Plasmodium berghei plasmepsin VIII is essential for sporozoite gliding motility

Babu S. Mastan [a], Sunil Kumar Narwal [b], Sandeep Dey [a], Kota Arun Kumar [a,*], Satish Mishra [b,*]

[a] Department of Animal Biology, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India
[b] Division of Parasitology, CSIR-Central Drug Research Institute, Lucknow 226031, India

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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

* Corresponding authors.
E-mail addresses: kaksl@uohyd.ernet.in (K.A. Kumar), satish.mishra@cdri.res.in (S. Mishra).

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Plasmodium berghei sporozoite specific genes- *PbS10* and *PbS23/SSP3* are required for the development of exo-erythrocytic forms

Jyothi Togiri[a,1], Rameswara Reddy Segireddy[a,1], Babu S. Mastan[a], Dipti Singh[a], Surendra Kumar Kolli[a], Ankit Ghosh[b], Faisal Mohammed Abdul Al-Nihmi[a], Mulaka Maruthi[a], Hadi Hasan Choudhary[b], Sandeep Dey[a], Satish Mishra[b], Kota Arun Kumar[a,*]

[a] Department of Animal Biology, School of Life Sciences, University of Hyderabad, Hyderabad, 500046, India
[b] Division of Parasitology, CSIR-Central Drug Research Institute, Lucknow, 226031, India

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ABSTRACT

Plasmodium sporozoites are infective forms of the parasite to mammalian hepatocytes. Sporozoite surface or secreted proteins likely play an important role in recognition, invasion and successful establishment of hepatocyte infection. By approaches of reverse genetics, we report the functional analysis of two *Plasmodium berghei* (*Pb*) sporozoite specific genes- *PbS10* and *PbS23/SSP3* that encode for proteins with a putative signal peptide. The expression of both genes was high in oocyst and salivary gland sporozoite stages as compared to other life cycle stages and *PbS23/SSP3* protein was detected in salivary gland sporozoites. Both mutants were indistinguishable to wild-type parasites with regard to asexual growth in RBC, ability to complete sexual reproduction and form sporozoites in vector host. While the sporozoite stage of both mutants were able to glide and invade hepatocytes normally in vitro and in vivo, *PbS10* mutants suffered growth attenuation at an early stage while *PbS23/SSP3* mutants manifested defect during late exo-erythrocytic form maturation. Interestingly, both mutants gave rare breakthrough infections, suggesting that while both were critical for liver stage development, their depletion did not completely abrogate blood stage infection. These findings have important implications for weakening sporozoites by multiple gene attenuation towards the generation of a safe whole organism vaccine.

1. Introduction

Malaria is a deadly infectious disease caused by a protozoan parasite that belongs to the genus *Plasmodium.* The parasite kills nearly half a million people each year, mostly young children in sub-Saharan Africa [1]. These parasites have a complex life cycle that alternates between a mammalian host and a female *Anopheles* mosquito. Infection to the mammalian host is initiated when infected mosquito injects sporozoites into the skin while obtaining a blood meal [2]. The injected sporozoites glide through several cellular barriers using an actin-myosin based molecular motor present beneath its plasma membrane [3]. This movement referred to as gliding motility facilitates the sporozoite to reach the blood vessels. Once inside the capillaries, the sporozoites are selectively arrested in the liver sinusoids. Within the liver, the sporozoites actively migrate through few hepatocytes prior to the productive invasion, characterized by invagination of hepatocyte plasma membrane and formation of a parasitophorous vacuole (PV) [4]. Within the PV, the sporozoite transforms into exo-erythrocytic forms (EEFs) or liver stages and undergo several rounds of asexual replication producing few thousand hepatic merozoites. Upon complete maturation of EEFs, the hepatic merozoites are packaged in host cell-derived membrane-bound structures called merosomes [5]. The merosomes facilitate the delivery of the hepatic merozoites into the blood and initiate an erythrocytic cycle (EC). During the EC, the parasites transform within the RBC into different forms like the rings, trophozoites and schizonts. The rapid multiplication of parasites at this stage is associated with all clinical manifestations of malaria. The schizonts contain 16–32 merozoites and upon egress from RBC, reinitiate the EC or alternatively form sexually dimorphic stages called the gametocytes. The gametocytes are the terminal stages of the parasite in the vertebrate host and undergo further development only in the gut of the female *Anopheles* mosquito. When a mosquito ingests these gametocytes during a blood meal, the male and female gametes emerge from respective gametocytes. The gametes fuse and form a zygote. The zygote transforms into a

* Corresponding author.
E-mail address: kaksl@uohyd.ernet.in (K.A. Kumar).
[1] These authors contributed equally for the work.

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Biochemical and physiological investigations on adenosine 5′ monophosphate deaminase from *Plasmodium spp.*

Lakshmeesha Kempaiah Nagappa,[1†] Dipti Singh,[2†] Sandeep Dey,[2] Kota Arun Kumar[2*] and Hemalatha Balaram[1*]

[1] *Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bengaluru, India.*

[2] *Department of Animal Biology, School of Life Sciences, University of Hyderabad, Hyderabad, India.*

Summary

The interplay between ATP generating and utilizing pathways in a cell is responsible for maintaining cellular ATP/energy homeostasis that is reflected by Adenylate Energy Charge (AEC) ratio. Adenylate kinase (AK), that catalyzes inter-conversion of ADP, ATP and AMP, plays a major role in maintaining AEC and is regulated by cellular AMP levels. Hence, the enzymes AMP deaminase (AMPD) and nucleotidases, which catabolize AMP, indirectly regulate AK activity and in-turn affect AEC. Here, we present the first report on AMPD from *Plasmodium*, the causative agent of malaria. The recombinant enzyme expressed in *Saccharomyces cerevisiae* was studied using functional complementation assay and residues vital for enzyme activity have been identified. Similarities and differences between *Plasmodium falciparum* AMPD (PfAMPD) and its homologs from yeast, *Arabidopsis* and humans are also discussed. The AMPD gene was deleted in the murine malaria parasite *P. berghei* and was found to be dispensable during all stages of the parasite life cycle. However, when episomal expression was attempted, viable parasites were not obtained, suggesting that perturbing AMP homeostasis by over-expressing AMPD might be lethal. As AMPD is known to be allosterically modulated by ATP, GTP and phosphate, allosteric activators of PfAMPD could be developed as anti-parasitic agents.

Abbreviations: ADSS, adenylosuccinate synthetase; ASL, adenylosuccinate lyase; GMPS, guanosine monophosphate synthetase; HGXPRT, hypoxanthine-guanine-xanthine phosphoribosyltransferase; IMPDH, inosine monophosphate dehydrogenase; ISN1, inosine monophosphate specific nucleotidase; PNP, purine nucleoside phosphorylase; IFA, immunofluorescence assay; EEFs, exo-erythrocytic forms.

Accepted 22 May, 2019. *For correspondence: E-mail: kaksl@uohyd.ernet.in (K.A. Kumar); Tel. (+91) 40 23134530; Fax (+91) 40 23010120 and E-mail: hb@jncasr.ac.in (H. Balaram); Tel. (+91) 80 22082812; Fax (+91) 80 22082766.†Have contributed equally to this work.

Introduction

Malaria caused by the parasitic protozoan *Plasmodium* is one of the deadliest diseases causing 445,000 deaths per annum worldwide (WHO, 2017). Although the PlasmoDB database is replete with information on the genome and proteome of the organism, most proteins are uncharacterized or have putative status (Bahl *et al.*, 2002, 2003; Aurrecoechea *et al.*, 2008). This inadequacy of proper understanding of the biochemistry of the organism has been a major impediment to the efforts put forth towards mitigation of this menacing disease. With genetic manipulation being highly challenging in *P. falciparum*, understanding the physiological significance of these uncharacterized/putative proteins becomes a non-trivial task (de Koning-Ward *et al.*, 2000). As the parasite is completely dependent on the salvage of precursors (predominantly hypoxanthine) for its purine nucleotide bio-synthesis (Downie *et al.*, 2008), the enzymes involved in this pathway have been sought after as potential drug targets. Although many enzymes of the purine nucleotide metabolism in *Plasmodium* have been studied in great detail (Hypoxanthine-guanine-xanthine phosphoribosyltransferase (HGXPRT), adenylosuccinate synthetase (ADSS), adenylosuccinate lyase (ASL), purine nucleoside phosphorylase) (Jayalakshmi *et al.*, 2002; Eaazhisai *et al.*, 2004; Madrid *et al.*, 2008; Bulusu *et al.*, 2009; Mehrotra *et al.*, 2010; Roy *et al.*, 2015a,b), a few more remain uncharacterized (AMPD, IMP dehydrogenase, IMP-specific nucleotidase and other purine nucleotidases). Through this study, we add further knowledge to the existing understanding of this protozoan

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RESEARCH ARTICLE

A *Plasmodium berghei* putative serine-threonine kinase 2 (*PBANKA_0311400*) is required for late liver stage development and timely initiation of blood stage infection

Ravi Jillapalli[1,*], Sunil Kumar Narwal[2,*], Surendra Kumar Kolli[1,*], Babu S. Mastan[1], Rameswara Reddy Segireddy[1], Sandeep Dey[1], Pratik Narain Srivastava[2], Satish Mishra[2,‡] and Kota Arun Kumar[1,‡]

ABSTRACT

In *Plasmodium*, protein kinases govern key biological processes of the parasite life cycle involved in the establishment of infection, dissemination and sexual reproduction. The rodent malaria model *Plasmodium berghei* encodes for 66 putative eukaryotic protein kinases (ePKs) as identified through modelling domain signatures and are highly conserved in *Plasmodium falciparum*. We report here the functional characterisation of a putative serine-threonine kinase *PBANKA_0311400* identified in this kinome analysis and designate it as *Pbstk2*. To elucidate its role, we knocked out *Pbstk2* locus and performed a detailed phenotypic analysis at different life cycle stages. The *Pbstk2* knockout (KO) was not compromised in asexual blood stage propagation, transmission and development in the mosquito vector. The *Pbstk2* KO produced viable salivary gland sporozoites that successfully transformed into exo-erythrocytic forms (EEFs) and were morphologically indistinguishable from wild-type GFP (WT GFP) with regard to size and shape until 48 h. An intravenous dose of 1×10^3 *Pbstk2* KO sporozoites in C57BL/6 mice failed to establish blood stage infection and a higher dose of $5X10^3$ showed a 2–3 day delay in prepatency as compared to WT GFP parasites. Consistent with such an observation, analysis of *in vitro* EEF development at 62 h revealed that the hepatic merozoite numbers were reduced to nearly 40% as compared to WT GFP and showed meagre expression of MSP1. Our studies provide evidence for the role of *Pb*STK2 in late liver stage development and for the successful establishment of a timely blood stage infection.

KEY WORDS: *Plasmodium*, Serine-threonine kinases, *Uis* genes, Exo-erythrocytic forms, Hepatic schizogony, Pre-patent period, MSP1

INTRODUCTION

Malaria is a mosquito-borne infectious disease caused by a protozoan parasite that belongs to the genus *Plasmodium*. The parasite kills nearly half a million people annually with deaths predominantly occurring in sub-Saharan Africa (WHO, 2017). The parasite infects a mammalian host via the bite of a female *Anopheles* mosquito that inoculates sporozoites into the skin during a blood meal (Sinnis and Zavala, 2008). The sporozoites make their way to the liver and develop into exoerythrocytic forms (EEFs) inside hepatocytes. After several rounds of asexual reproduction, the hepatic merozoites are released into bloodstream (Prudencio et al., 2006) to initiate an erythrocytic cycle, a phase that is responsible for all clinical manifestations of malaria. Gametocytes are the terminal stages of a parasite developing within erythrocytes and do not undergo further development in the mammalian host until they arrive in the mosquito gut. Within the mosquito midgut, the parasites undergo sexual reproduction, culminating in the production of thousands of infectious sporozoites. The sporozoites migrate to salivary glands and reside there to initiate new infection cycle in the mammalian host (Matuschewski, 2006).

Plasmodium parasites have evolved distinct kinase families with novel domain structures and biochemical features (Ward et al., 2004). These signalling molecules play a key role in the regulation of several physiological processes (Solyakov et al., 2011). In general, phosphorylation of specific amino acid residues like serine (Ser), threonine (Thr), tyrosine (Tyr), histidine (His), and aspartate (Asp) affects the activity of target proteins either by bringing a conformational change in its active site or regulating protein–protein interactions (Pereira et al., 2011). The systematic functional investigation of *Plasmodium berghei* kinome by reverse genetic approach revealed that nearly two-thirds of the *P. berghei* kinases were essential (Tewari et al., 2010). While the possibility of targeting kinases essential for *Plasmodium* development in vector host may not be feasible, nonetheless several kinases seem to regulate the transmission of malaria to mosquitoes and the forms of parasite that are infective to hepatocytes can only be obtained from mosquito stage (Tewari et al., 2010). Thus it is imperative that an in-depth functional investigation of kinase mutants be done at all life cycle stages for all 'possibly essential kinases' such that the importance of the same kinase playing a role at multiple life cycle stages of the parasite is not overlooked and those critical for establishment of malaria infection in a mammalian host is not undermined. To date, only a few protein kinases have been identified that are required for *Plasmodium* liver stage development. The lipid kinase, phosphatidylinositol-4-OH kinase [PI(4)K] is required for hypnozoite formation in a *Plasmodium cynomolgi* (McNamara et al., 2013). Two mitogen-activated protein kinases (MAPKs) have also been identified in *P. berghei* and are designated as *Pb*MAPK1 and *Pb*MAPK2. While both the *Pb*MAPK genes are transcribed during *P. berghei* liver stage development, and *Pb*MAPK1 localises to the cytomere stage, depleting its locus did not affect the parasite viability in the liver stages (Wierk et al.,

[1]Department of Animal Biology, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India. [2]Division of Parasitology, CSIR-Central Drug Research Institute, Lucknow 226031, India.
*These authors contributed equally to this work

‡Authors for correspondence (satish.mishra@cdri.res.in; kaksl@uohyd.ernet.in)

S.K.N., 0000-0001-5476-9753; S.K.K., 0000-0001-6125-0036; P.N.S., 0000-0001-9273-675X; S.M., 0000-0002-8942-6416; K.A.K., 0000-0002-9451-2917

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RESEARCH ARTICLE

Bloom Helicase Along with Recombinase Rad51 Repairs the Mitochondrial Genome of the Malaria Parasite

Payal Jha,[a] Abhilasha Gahlawat,[a] Sunanda Bhattacharyya,[b] Sandeep Dey,[c] Kota Arun Kumar,[c] Mrinal Kanti Bhattacharyya[a]

[a]Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad, India
[b]Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, Hyderabad, India
[c]Department of Animal Biology, School of Life Sciences, University of Hyderabad, Hyderabad, India

ABSTRACT The homologous recombination (HR) pathway has been implicated as the predominant mechanism for the repair of chromosomal DNA double-strand breaks (DSBs) of the malarial parasite. Although the extrachromosomal mitochondrial genome of this parasite experiences a greater number of DSBs due to its close proximity to the electron transport chain, nothing is known about the proteins involved in the repair of the mitochondrial genome. We investigated the involvement of nucleus-encoded HR proteins in the repair of the mitochondrial genome, as this genome does not code for any DNA repair proteins. Here, we provide evidence that the nucleus-encoded "recombinosome" of the parasite is also involved in mitochondrial genome repair. First, two crucial HR proteins, namely, *Plasmodium falciparum* Rad51 (PfRad51) and *P. falciparum* Bloom helicase (PfBlm) are located in the mitochondria. They are recruited to the mitochondrial genome at the schizont stage, a stage that is prone to DSBs due to exposure to various endogenous and physiologic DNA-damaging agents. Second, the recruitment of these two proteins to the damaged mitochondrial genome coincides with the DNA repair kinetics. Moreover, both the proteins exit the mitochondrial DNA (mtDNA) once the genome is repaired. Most importantly, the specific chemical inhibitors of PfRad51 and PfBlm block the repair of UV-induced DSBs of the mitochondrial genome. Additionally, overexpression of these two proteins resulted in a kinetically faster repair. Given the essentiality of the mitochondrial genome, blocking its repair by inhibiting the HR pathway could offer a novel strategy for curbing malaria.

IMPORTANCE The impact of malaria on global public health and the world economy continues to surge despite decades of vaccine research and drug development efforts. An alarming rise in resistance toward all the commercially available antimalarial drugs and the lack of an effective malaria vaccine brings us to the urge to identify novel intervention strategies for curbing malaria. Here, we uncover the molecular mechanism behind the repair of the most deleterious form of DNA lesions on the parasitic mitochondrial genome. Given that the single-copy mitochondrion is an indispensable organelle of the malaria parasite, we propose that targeting the mitochondrial DNA repair pathways should be exploited as a potential malaria control strategy. The establishment of the parasitic homologous recombination machinery as the predominant repair mechanism of the mitochondrial DNA double-strand breaks underscores the importance of this pathway as a novel druggable target.

Keywords PfBlm, PfRad51, mitochondria, DNA repair, homologous recombination, *Plasmodium falciparum*

Malaria continues to be one of the deadliest infectious diseases of mankind in the modern era. Half of the world's population is at risk of malaria: children and pregnant women are severely affected by this pathogen (1). *Plasmodium falciparum*, one

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RESEARCH ARTICLE

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A conserved *Plasmodium* structural integrity maintenance protein (SIMP) is associated with sporozoite membrane and is essential for maintaining shape and infectivity

Dipti Singh[1] | Smita Patri[1] | Veeda Narahari[1] | Rameswara R. Segireddy[1] | Sandeep Dey[1] | Archi Saurabh[2] | Macha Vijay[3] | N. Prakash Prabhu[2] | Anand Srivastava[3] | Surendra Kumar Kolli[1] | Kota Arun Kumar[1]

[1]Department of Animal Biology, School of Life Sciences, University of Hyderabad, Hyderabad, India

[2]Department of Biotechnology & Bioinformatics, School of Life Sciences, University of Hyderabad, Hyderabad, India

[3]National Institute of Animal Biotechnology, Hyderabad, India

Correspondence
Surendra Kumar Kolli and Kota Arun Kumar, Department of Animal Biology, School of Life Sciences, University of Hyderabad, Gachibowli, Hyderabad, Telangana – 500046, India.
Email: skk@uohyd.ac.in and kaksl@uohyd.ac.in

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Abstract

Plasmodium sporozoites are extracellular forms introduced during mosquito bite that selectively invade mammalian hepatocytes. Sporozoites are delimited by a cell membrane that is linked to the underlying acto-myosin molecular motor. While membrane proteins with roles in motility and invasion have been well studied, very little is known about proteins that maintain the sporozoite shape. We demonstrate that in *Plasmodium berghei (Pb)* a conserved hypothetical gene, *PBANKA_1422900* specifies sporozoite structural integrity maintenance protein (SIMP) required for maintaining the sporozoite shape and motility. Sporozoites lacking SIMP exhibited loss of regular shape, extensive membrane blebbing at multiple foci, and membrane detachment. The mutant sporozoites failed to infect hepatocytes, though the altered shape did not affect the organization of cytoskeleton or inner membrane complex (IMC). Interestingly, the components of IMC failed to extend under the membrane blebs likely suggesting that SIMP may assist in anchoring the membrane to IMC. Endogenous C-terminal HA tagging localized SIMP to membrane and revealed the C-terminus of the protein to be extracellular. Since SIMP is highly conserved among *Plasmodium* species, these findings have important implications for utilizing it as a novel sporozoite-specific vaccine candidate.

KEYWORDS
gliding motility, inner membrane complex, *Plasmodium*, sporozoites

1 | INTRODUCTION

The life cycle of *Plasmodium* in mammalian host starts with the bite of a female *Anopheles* mosquito that delivers sporozoites in the skin of the vertebrate host (Gueirard et al., 2010). The sporozoites make their way to the liver, where they transform into exoerythrocytic forms. Following exoerythrocytic schizogony, first-generation merozoites are packaged in membrane-bound structures called merosomes (Sturm et al., 2006) that are extruded into sinusoids. Rupture of merosome releases parasites into the bloodstream, which initiates an erythrocytic cycle. In RBC, the parasites replicate asexually and go through a series of transformations resulting in rings, trophozoites, schizonts, and gametocytes.

Gametocytes are sexual forms and the terminal stages of the parasite in the mammalian host. These forms enter gut of a female *Anopheles* mosquito during a blood meal. Sexual reproduction occurs in the lumen of gut, resulting in a zygote that transforms into motile ookinete. The ookinete breaches midgut epithelium and settles on

DNA damage-induced nuclear import of HSP90α is promoted by Aha1

Nupur Fangaria[a], Khushboo Rani[a], Priyanka Singh[a], Sandeep Dey[b], Kota Arun Kumar[b], and Sunanda Bhattacharyya[a,*]

[a]Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, Hyderabad 500046, Telangana, India; [b]Department of Animal Biology, School of Life Sciences, University of Hyderabad, Hyderabad 500046, Telangana, India

ABSTRACT **The interplay between yHSP90α (Hsp82) and Rad51 has been implicated in the DNA double-strand break repair (DSB) pathway in yeast. Here we report that nuclear translocation of yHSP90α and its recruitment to the DSB end are essential for homologous recombination (HR)-mediated DNA repair in yeast. The HsHSP90α possesses an amino-terminal extension which is phosphorylated upon DNA damage. We find that the absence of the amino-terminal extension in yHSP90α does not compromise its nuclear import, and the non-phosphorylatable-mutant HsHSP90α^{T7A} could be imported to the yeast nucleus upon DNA damage. Interestingly, the flexible charged-linker (CL) domains of both yHSP90α and HsHSP90α play a critical role during their nuclear translocation. The conformational restricted CL mutant yHSP90α$^{\Delta(211\text{-}259)}$, but not a shorter deletion version yHSP90α$^{\Delta(211\text{-}242)}$, fails to reach the nucleus. As the CL domain of yHSP90α is critical for its interaction with Aha1, we investigated whether Aha1 promotes the nuclear import of yHSP90α. We found that the nuclear import of yHSP90α is severely affected in Δ*aha1* strain. Moreover, Aha1 is accumulated in the nucleus during DNA damage. Hence Aha1 may serve as a potential target for inhibiting nuclear function of yHSP90α. The increased sensitivity of Δ*aha1* strain to genotoxic agents strengthens this notion.**

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INTRODUCTION

Eukaryotic cells are susceptible to a wide variety of genotoxic stresses which cause several types of DNA lesions including DNA double-strand breaks (DSBs). If DSBs remain unrepaired in the cell, they can cause genomic instability (Aylon and Kupiec, 2004). One of the commonly employed mechanisms to repair DSB is by homologous recombination (HR). Unlike the mammalian system, this mode of DNA repair is preferred in budding yeast over the error-prone nonhomologous end joining pathway. In HR-mediated DNA repair, Rad52 and Rad51 are assembled at the 3′ ssDNA tail in an organized manner (Li and Heyer, 2008). These proteins are recruited during the end resection of DNA DSB and catalyze the formation of DNA (Andriuskevicius *et al.*, 2018). Studies in the model eukaryote, *Saccharomyces cerevisiae*, have shown that yHSP90α, the budding yeast ortholog of human HSP90α (HsHSP90α), provides stability to Rad51 protein and plays a regulatory role in the effective recruitment of Rad51 to the broken DNA (Suhane *et al.*, 2014, 2019).

HSP90α is associated with a wide variety of cochaperones that are essential for its reaction cycle. Sba1 binds to HSP90α in the presence of ATP and stabilizes the ATP bound conformation which is required for the client protein activation (Ali *et al.*, 2006). Cdc37 aids in folding and activation of specific client protein kinases. It suppresses the ATP turnover by HSP90α and thereby helps in client protein loading (Kimura *et al.*, 1997; Siligardi *et al.*, 2002). Aha1

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Author contributions: S.B. conceived the idea, designed the experiments, guided, and wrote the paper; N.F. conducted experiments and wrote the paper; K.R. and P.S. conducted experiments; N.F., S.D., and K.A.K. conducted the fluorescence microscopic studies.

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All supporting data in relation to the studies reported here are provided in this manuscript.

*Address correspondence to: Sunanda Bhattacharyya (sdeb70@gmail.com; sbtsl@uohyd.ac.in).

Abbreviations used: Aha1, activator of hsp90 ATPase; Cdc37, cell division cycle 37; HOcs, homothallic endonuclease cut site; Hsp90, heat shock protein 90; Sba1, sensitivity to benzoquinone ansamycins.

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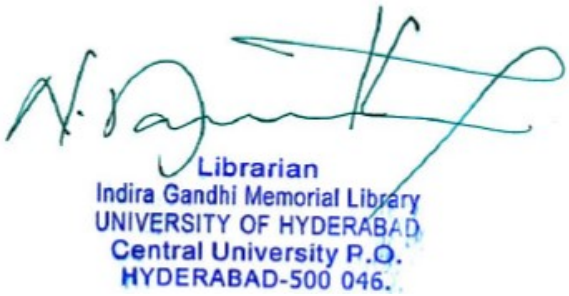
Role of Plasmodium PhIL-1 Interacting Protein (PhIP) in malaria transmission

by Sandeep Dey



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