Investigating the role of Circumsporozoite protein in *Plasmodium berghei (Pb)* mosquito stages using FLP/*FRT* conditional mutagenesis system &

Functional characterization of *Pb* K^+ channel/Adenylyl cyclase α and a conserved protein PBANKA_141700 by reverse genetics approach

Thesis submitted to University of Hyderabad

for the award of Ph.D. degree in

Department of Animal Biology

By

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University of Hyderabad

(A Central University established in 1974 by an Act of Parliament)

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DECLARATION

I, **Surendra Kumar Kolli** hereby declare that this thesis entitled "**Investigating the role of Circumsporozoite protein in *Plasmodium berghei (Pb)* mosquito stages using FLP/*FRT* conditional mutagenesis system & Functional characterization of *Pb* K^+ channel/Adenylyl cyclase α and a conserved protein PBANKA_141700 by reverse genetics approach**" submitted by me under the guidance and supervision of Dr. Kota Arun Kumar is a bonafide research work which is also free from plagiarism. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that this thesis entitled "**Investigating the role of Circumsporozoite protein in *Plasmodium berghei (Pb)* mosquito stages using FLP/*FRT* conditional mutagenesis system & Functional characterization of *Pb* K^+ channel/Adenylyl cyclase α and a conserved protein PBANKA_141700 by reverse genetics approach**" is a record of bonafide work done by Mr. Surendra Kumar Kolli, for the Ph.D. programme in the Department of Animal Biology, University of Hyderabad, under my guidance and supervision. The thesis has not been submitted previously in part or full to this or any other University or Institution for the award of any degree or diploma.

Dr. Kota Arun Kumar
(Supervisor)

Head
Department of Animal Biology

Dean
School of Life Sciences

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Surendra...✍

Dedicated to

My

Beloved Parents

Table of contents

Abbreviations

^{o}C	Degrees Celsius
µg	Microgram(s)
µl	Microliter(s)
µm	Micrometer(s)
µM	Micromolar
ACT	Artemisinin combination therapy
AMA	Apical membrane antigen
bp	Base pairs
BSA	Bovine serum albumin
CD	Cluster of differentiation
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CDPK	Calcium dependent protein kinase
CelTOS	Cell traversal protein for ookinete and sporozoite
CSP	Circumsporozoite Protein
DAPI	4', 6' diamidino-2 phenyl indole
DIC	Differential interference contrast
dNTP	Deoxyribonucleoside triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
DNA	Deoxy ribonucleic acid
DOZI	Development of zygote inhibited
EBA	Erythrocyte binding antigen
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
FRT	FLP recognition target
FP	Forward primer
GAP	Genetically attenuated parasite
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
hDHFR	Human dihydrofolate reductase
HGF	Hepatocyte growth factor
HSPG	Heparin sulfate proteoglycan
i.p.	intraperitoneal
i.v.	intravenous
IMC	Inner membrane complex
iRBC	Infected red blood cell
ITN	Insecticide treated net
K^+	Potassium ion
K/ACα	Potassium channel and Adenylyl cyclase alpha
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
NEK	NIMA related kinase

ng	nanogram
NIMA	Never in mitosis *Aspergillus*
OD	Optical density
ORF	Open reading frame
Pb or P. berghei	*Plasmodium berghei*
P. falciparum	*Plasmodium falciparum*
P. knowlesi	*Plasmodium knowlesi*
P. malariae	*Plasmodium malariae*
P. ovale	*Plasmodium ovale*
P. vivax	*Plasmodium vivax*
PBS	Phosphate buffer saline
PEXEL	*Plasmodium* export element
PK	Protein kinase
PKG	cGMP dependent protein kinase/ protein kinaseG
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
SBP	Skeletal binding protein

SERA	Serine repeat antigen
SPECT	Sporozoite protein essential for cell traversal
SRPK	Serine arginine rich protein kinase
SSR	Site specific recombination
SUB	Subtilisin like protease
TAE	Tris acetate EDTA
TBS	Tris buffer saline
TBV	Transmission blocking vaccines
TE	Tris EDTA
TEA	Tetraethylammonium
TgDHFR	*Toxoplasma gondii* dihydrofolate reductase
TRAP	Thrombospondin related anonymous protein
TRSP	Thrombospondin related sporozoite protein
TSR	Thrombospondin related
TVN	Tubulovesicular network
UIS	Up regulated in infected salivary glands
UOS	Upregulated in oocyst sporozoites
UTR	Untranslated region
UV	Ultraviolet
VTS	Vacuolar translocation signal
v/v	Volume per volume
WHO	World health organisation
WT	Wild type
w/v	Weight per volume
XA	Xanthurinic acid

Chapter 1

Review of Literature

1.1 History of malaria

Malaria is one of the ancient diseases, and the incidents were recorded in the Chinese medical writings from 2700BC and in Egyptian texts from 1570BC (Cox, 2010). In the 6th century BC, *Susruta Samhita*, a Sanskrit medical literature described the symptoms of malaria and attributed to the bites of some insects (CDC, 2016). The word malaria originated from Italian word - "Mal-aria" which means "bad air". It was named so because of the belief that malaria originated from winds of swamps and rivers in marshy lands, an idea that was in sway until the parasite was discovered (AMCA, 2014). In 1880, a French Army surgeon, Charles Alphonse Laveran identified the causative agent of malaria, while observing the blood of a patient suffering from the disease. He named the organism as *Oscillaria malariae*. In 1897, Sir Ronald Ross, a British Medical doctor had demonstrated that mosquitoes are the vectors for transmitting malaria (Cox, 2010). Both Sir Ronald Ross and Charles Alphonse Laveran were awarded Nobel Prize in years 1902 and 1907 respectively for their seminal contribution towards discovering the *Plasmodium* life cycle stages. By 1900, the complete life cycle of *Plasmodium* was elucidated except its pre-erythrocytic development before the appearance in blood. In 1948, two British scientists Henry Edward Shortt and Percy Cyril Garnham, have discovered the pre-erythrocytic development of *Plasmodium cynamolgi* in the liver of rhesus monkey (Shortt & Garnham, 1948).

1.2 Epidemiology of malaria

Malaria remains as a devastating disease in most tropical and subtropical regions (Fig. 1). In the year 2015, 214 million malaria cases were reported that accounted for 438,000 deaths worldwide. Nearly 90% of the deaths were reported in sub-Saharan Africa of which 70% are the children under the age of 5 years. Today, malaria is a public health concern in about 97 countries, and 50% of the world's population is at the risk of getting malaria (WHO, 2015b). In addition to the threat to health, malaria also has a severe impact on socioeconomic development in endemic countries by impacting on long term demographics and the acquisition of human and physical capital (Sachs & Malaney, 2002). The symptoms of malaria include fever, chills, headache, nausea, vomiting and anemia ultimately leading to coma and death if left untreated.

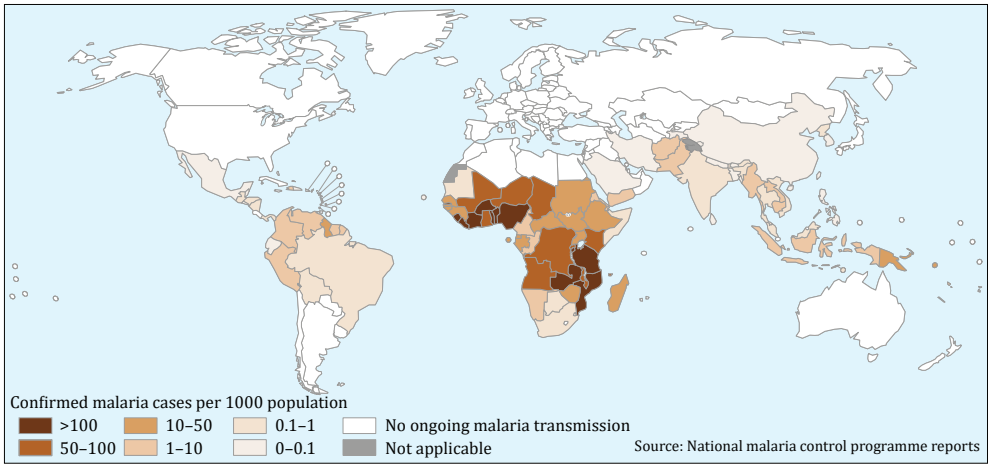

Fig.1 Global malaria transmission and countries at the risk of inflicting malaria. Many countries in the sub-Saharan Africa (dark brown and brown) have more malaria cases. Malaria is controlled in South America and China (pale brown) where number of cases per year are low. Few countries (white) are free of malaria as of now, but there is a risk of reintroduction if proper control measures are not implemented. Image adapted from WHO, 2014.

The malaria burden has decreased steadily in the last decade in the areas of Southern Africa and in the Horn of Africa as the intervention strategies became more widespread and efficacy improved. Increased funding, an improvement in the procurement and distribution of effective means of prevention are the major causes of decline. However, in the central part of Africa, little progress is documented. Change in the drug treatment from chloroquine to a cocktail of sulphadoxine and pyrimethamine or artemisinin combination and scale up in the insecticide treated bed nets and indoor residual spraying are the key reasons for steady decline (O'Meara *et al.*, 2010).

Plasmodium belongs to the phylum Apicomplexa. Four species of *Plasmodium* are known to infect humans - *Plasmodium falciparum* (*P. falciparum*), *Plasmodium vivax* (*P. vivax)*, *Plasmodium malariae* (*P. malariae*) and *Plasmodium ovale* (*P. ovale)*. Among these, *P. falciparum* causes a severe form of malaria in the brain called cerebral malaria and is responsible for more malaria deaths globally. Recently, a fifth *Plasmodium* species, *Plasmodium knowlesi (P. knowlesi)*, known to infect macaques, has also been shown to infect human hosts (Collins, 2012).

1.3 Overview of *Plasmodium* lifecycle

The life cycle of *Plasmodium* is very complex that switches between sexual and asexual developments and requires two hosts - an invertebrate vector and a definitive vertebrate host (Fig. 2). The sexual development (gamogony) as well as sporogony takes place in the female *Anopheles* mosquito while the parasite undergoes asexual development (schizogony) in the vertebrate host (mammals or birds).

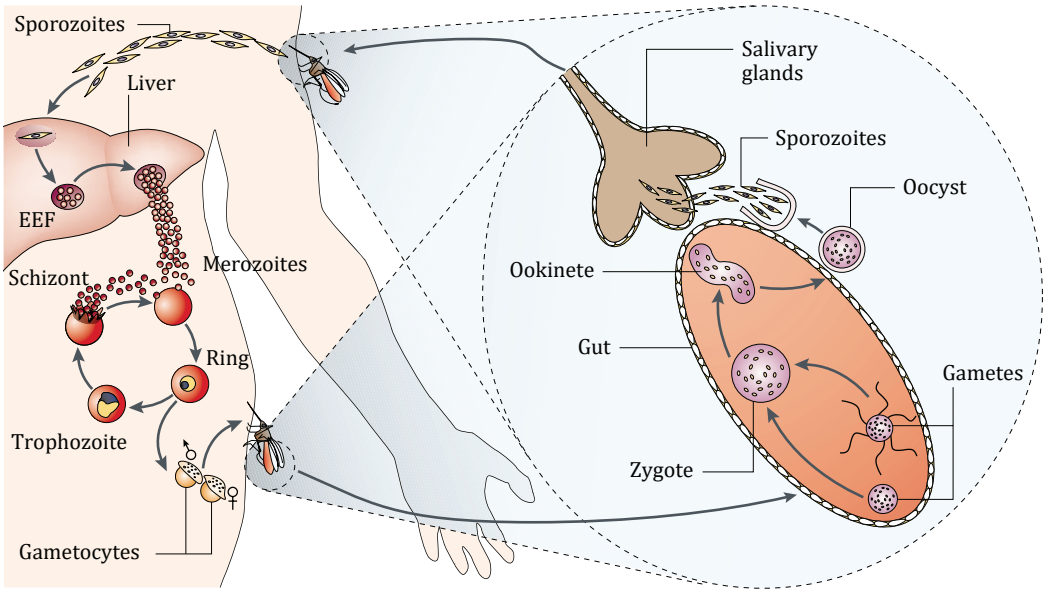

Fig.2 Life cycle of *Plasmodium*. Infection of *Plasmodium* to human host starts when an infected female *Anopheles* mosquito injects sporozoites into the skin. The sporozoites glide through the dermal cells and reach the blood vessels. Through circulation, sporozoites make their way to liver, infects hepatocytes and develop into Exo-erythrocytic forms (EEFs) or liver stages. EEFs undergoes schizogony forming thousands of hepatic merozoites. These merozoites are released into the blood and infect RBC and undergo transition through stages like ring, trophozoite, and schizont by asexual reproduction. Few merozoites that infect RBC undergo transformation into sexually dimorphic stages called gametocytes (male and female). These gametocytes circulate in the peripheral blood and are taken up by the mosquito at the time of blood meal. Inside the mosquito gut, the male and female gametocytes get activated into male and female gametes. A zygote is formed by the fusion of male and female gametes, that transforms into a motile ookinete and traverses through the midgut epithelium and settles on the basal side of midgut forming an oocyst. Sporulation occurs within the oocyst forming thousands of sporozoites. Upon rupture of oocyst, sporozoites get released into the hemocoel. The sporozoites migrate to the salivary glands and settles in the lobes. These sporozoites infect a new host when mosquito obtain a blood meal. Image adapted and modified from Wimmer, 2003.

Sporozoites are injected into the vertebrate host by an infected female *Anopheles* mosquito at the time of obtaining a blood meal. These sporozoites migrate through the skin cells, find the nearby capillaries and reach liver via the blood stream and infect

hepatocytes. Inside hepatocytes, they develop into mature exoerythrocytic forms (EEF), which are clinically silent. The EEF grow and differentiate into thousands of new parasitic forms called merozoites that are subsequently released into the blood circulation and initiate erythrocytic cycle. The merozoites invade erythrocytes and transform through ring, actively feeding trophozoite and multinucleated schizont stages forming 16-32 merozoites by erythrocytic schizogony. The schizonts rupture releasing merozoites into the blood which invade new RBC thus continuing the erythrocytic cycle. A small portion of the erythrocyte invaded merozoites withdraw from proliferation and differentiate into sexual stages - male and female gametocytes. During a blood meal these gametocytes are taken up by the mosquito and in the gut lumen, the factors like reduced temperature, change in pH and xanthurenic acid (mosquito derived factor) trigger the male gametogenesis (by exflagellation) and female gametogenesis from male and female gametes respectively. The male and female gametes fuse to form a zygote which transforms into a motile, elongated ookinete. Ookinete traverses through the midgut epithelium and develops into an oocyst between the basal lamina and midgut epithelia. Sporulation within the oocyst yields thousands of sporozoites. Upon rupture of the oocyst, sporozoites are released into the hemocoel and make their way to salivary glands of the mosquito and are ready to infect a new host. Inoculation of these sporozoites into a new vertebrate host reinitiates the life cycle of the parasite.

Plasmodium shows a tight stage specific expression of proteins that perform unique functions central to each stage of life cycle. The biology of each life cycle stage is described in detail below.

1.4 *Plasmodium* life cycle in the mammalian host in detail

1.4.1 Sporozoite inoculation by mosquito bite and the "dermis stage"

A female *Anopheles* mosquito while probing for a blood meal injects few nanolitres of saliva into the dermis of the vertebrate host that contains anticoagulants and vasodilators. If the mosquito harbors the *Plasmodium* sporozoites in the salivary gland lobes, sporozoites get carried along with the saliva and are deposited in the dermis of vertebrate host (Ribeiro & Francischetti, 2003). This stage of infection is termed as "dermis stage" (Fig. 3A) and has been shown to trigger the host immune

response following deposition of sporozoites at the time of mosquito bite (Sinnis & Zavala, 2008).

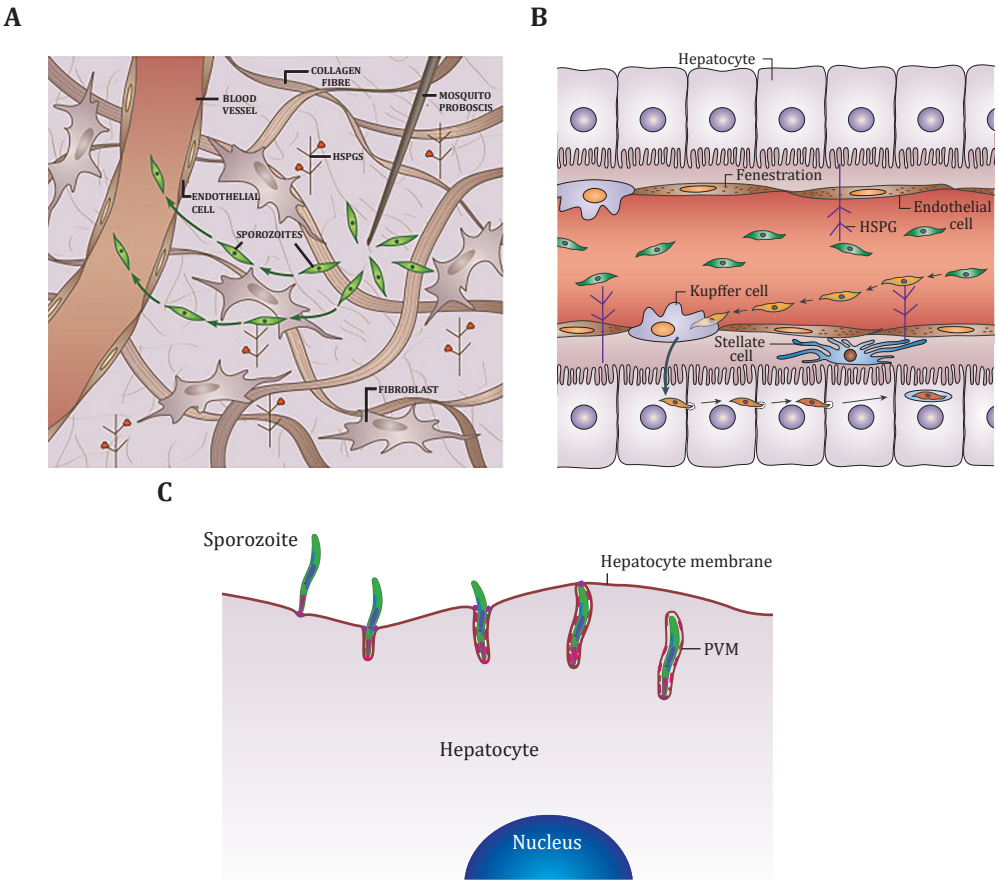

Fig. 3 Sporozoite journey from injection site (dermis) to infection site (liver) and successful invasion of hepatocyte. A) *Plasmodium* sporozoites are injected into the host dermis when an infected female *Anopheles* mosquito probes for a blood meal. The sporozoites glide and traverse through the dermal cells to access the blood vessel. B) Through circulation sporozoites reach hepatocytes and this process is aided by Kupffer cells that act as portals of entry. Sporozoites are activated by process of migration through many hepatocytes before productive invasion – a process during which the sporozoite entry is mediated by the formation of an invagination in the hepatocyte plasma membrane that deepens and finally subsumes resulting in the formation of a parasitophorous vacuolar membrane (PVM) that harbors the intracellular sporozoite. C) Steps associated with internalization of extracellular sporozoite resulting in the formation of PVM. Image adapted and modified from Prudencio & Mota, 2007.

Sporozoites are highly motile and intravital microscopy showed that in order to find dermal blood vessels, they migrate through the neighboring skin cells by gliding motility with the assistance of powerful actomyosin molecular motor present beneath the parasite plasma membrane (Vanderberg & Frevert, 2004). Around 20-30% of the sporozoites injected into the dermis can enter a lymphatic vessel and reach the draining lymph node, where they infect the dendritic cells and partially develop into EEF (Amino

et al., 2006). The proteins essential for the gliding motility are released from the secretory organelles called micronemes and rhoptries in a sequential and coordinated manner (Gubbels & Duraisingh, 2012). In the vertebrate host, sporozoites traverse through different cell types, a process that involves damaging the host cell membrane, gliding through the cytosol and finally exiting the host cell.

The dermis stage of infection has been explored recently revealing few interesting findings. The sporozoites that were injected through mosquito bite into the mice immunized with γ–irradiated sporozoites were rapidly immobilized and did not appear to reach the dermal blood vessels and died in the skin within several hours (Kebaier *et al.*, 2009). These results indicate that protective antibodies against the sporozoite in the skin may help in reducing further infection by blocking their movement into blood vessels and ultimately the liver, which could aid in mounting immunity to pre-erythrocytic stages and form a basis for malaria vaccine design (Sinnis & Zavala, 2008).

Sporozoite surface proteins play a key role in the migration through different cell types. Circumsporozoite protein (CSP), a major sporozoite surface protein and thrombospondin related anonymous protein (TRAP), a primary motor protein of sporozoite are essential for motility (Myung *et al.*, 2004, Sultan *et al.*, 1997). A surface phospholipase (Pb PL) of sporozoite hydrolyzes phosphatidylcholine and other phospholipids present on the host cell membrane and allows the sporozoite to enter and exit the host cells. Sporozoites that lack Pb PL have shown impaired ability to cross the epithelial monolayers and the infectivity was significantly reduced through mosquito transmission (Bhanot *et al.*, 2005). Two additional sporozoites surface proteins SPECT (sporozoite microneme protein essential for cell traversal) and SPECT2 are also critical to host cell traversal. The mutant parasites lacking either SPECT or SPECT2 exhibited *in vitro* gliding motility but were unable traverse through host dermal cells (Ishino *et al.*, 2005a, Ishino *et al.*, 2004).

1.4.2 Exo-erythrocytic stages

Once in circulation, the sporozoites reach liver sinusoid and traverse through the Kupffer cells (resident macrophages in the liver) and enter the liver parenchyma to access the hepatocytes (Fig. 3B). Sporozoite specificity to target hepatocytes is achieved

by the interaction between CSP, one of the major sporozoite surface protein and heparin sulfate proteoglycans (HSPGs) present on the surface of hepatocytes (Cerami *et al.*, 1992, Frevert *et al.*, 1993). Compared to other cell types, hepatocytes have the highest degree of sulphation and sporozoites are activated via calcium dependent protein kinase 6 (CDPK6) to shift from traversal mode to productive invasion (Coppi *et al.*, 2007, Pradel *et al.*, 2002). For the pre-erythrocytic cycle of *Plasmodium* life cycle, the preference of hepatocytes as target cells is still a mystery. Compared to other cell types, hepatocytes are rich in nutrients, express the detoxifying enzymes and are immune tolerant (Protzer *et al.*, 2012). The rapidly proliferating *Plasmodium* parasites in the hepatocyte are at a high demand for lipids, glucose and nucleotides for the organelle biosynthesis and DNA replication. The mammalian hepatocytes are ideal to meet these requirements. The parasite lacks sterol biosynthetic pathway and the genes encoding enzymes involved in sterol biosynthesis and lipid metabolism are upregulated in the infected hepatocytes (Albuquerque *et al.*, 2009) suggesting that the parasite manipulates the host transcription for its survival. During EEF development, several of the parasite proteins are exported into the host cytosol. These parasite effectors modulate several host processes and facilitate: acquisition of nutrients, suppression of host immunity (Epiphanio *et al.*, 2008, Singh *et al.*, 2007), alterations in post translational modifications (Maruthi M and Kumar KA, personal communication) and inhibition of host cell apoptosis (van de Sand *et al.*, 2005).

It was demonstrated that host factors like hepatocyte growth factor (HGF) renders neighboring hepatocytes susceptible to infection (Carrolo *et al.*, 2003, Mota *et al.*, 2002). Exposure of sporozoites to intracellular concentration of [K^+] (Kumar *et al.*, 2007) and uracil derivatives (Ono *et al.*, 2008) activate them to shift from migratory mode to invasion mode. The activated sporozoites expose adhesive surface proteins through apical regulated exocytosis (Mota *et al.*, 2002). These adhesive proteins interact with the receptors on the hepatocyte to form a tight connection between the apical end of sporozoite and hepatocyte plasma membrane that moves progressively forming a circular ring that propels the parasite into the parasitophorous vacuole (PV) (Amino *et al.*, 2008). Molecular composition of this junction (between sporozoites and hepatocyte) is poorly understood and only few proteins were identified so far. Members of conserved 6 cysteine family proteins of *P. berghei*, P52 (Pbs36p) and Pbs36 are

specifically produced in liver-infective sporozoites and infectivity, but not cell traversal was abolished when the proteins were depleted (Ishino *et al.*, 2005b). A micronemal protein- apical membrane antigen 1 (AMA1) and rhoptry neck proteins RON2, RON4 and RON5 are involved in the formation of tight connection between sporozoites and hepatocyte. RON4 was shown to be essential for all the invasive stages of *Plasmodium* and *Toxoplasma* whereas AMA1 was critical in few invasive stages (Giovannini *et al.*, 2011). The moving junction proteins of the *Toxoplasma* play a crucial role in defining the host protein composition in the PV membrane that contributes to the non-fusogenic nature of parasitophorous vacuole thereby preventing the fusion with host lysosomes (Morisaki *et al.*, 1995).

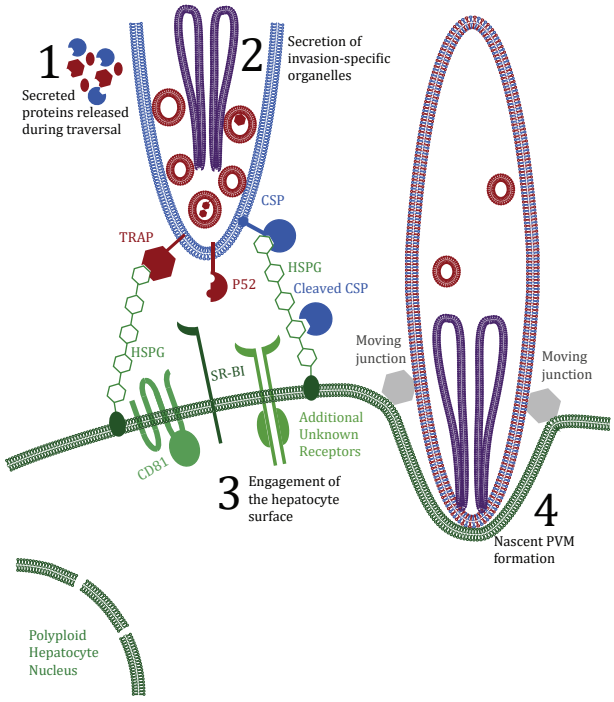

Fig. 4 Model showing the attachment of *Plasmodium* sporozoite to hepatocyte and its subsequent invasion. Gliding and traversal of sporozoites though dermis and blood involves the secretion of micronemal proteins (events 1, 2). After reaching the liver, sporozoite enters the liver parenchyma through kupffer cell and engage a hepatocyte for invasion (event 3). The binding of sporozoite involves the interaction of CSP and HSPGs. Upon attachment, a moving junction is initiated by the sporozoite forming a nascent PVM (event 4). Image adapted from Kaushansky and Kappe, 2015.

In addition to sporozoite proteins, hepatocyte receptors are also important for invasion. Two hepatocyte proteins, scavenger receptor SR-B1 and the tetraspanin CD81, have shown to have a role in parasite invasion (Fig. 4). SR-B1 is high density lipoprotein receptor on the hepatocyte surface along with CD81 present in cholesterol rich microdomains (Rodrigues *et al.*, 2008, Yalaoui *et al.*, 2008). CD81 is associated with

other proteins and lipids to form membrane microdomains on the surface of hepatocytes. CD81 is essential for the host cell invasion by *P. yoelii* (Silvie *et al.*, 2006) but *P. berghei* shows CD81 independent invasion (Silvie *et al.*, 2007).

The parasite resides in a vacuole within the hepatocyte called parasitophorous vacuole (PV). The membrane of the PV separates the host and parasite milieu. The PV membrane (PVM) is derived from the host cell during the process of invasion and is significantly modified with the lipids and proteins of the parasite origin, but not much is known about the process of remodeling (Bano *et al.*, 2007). The parasite proteins that are essential for the formation of PV is not known, but in *P. yoelii*, double knockouts of *P36* and *P52* resulted in a defect in PVM formation thereby arrest at the early liver stage development (Labaied *et al.*, 2007). *P52* mutants of *P. berghei* and *P. falciparum* are also unable to form PVM and showed early liver stage arrest (van Dijk *et al.*, 2005, van Schaijk *et al.*, 2008). The PVM resident proteins –UIS3 (upregulates in infected sporozoites, UIS), UIS4, EXP1 (exported protein1), ETRAM (early transcribed membrane protein) were also shown to play a key role in liver stage development. Targeted disruption of *UIS3, UIS4* or *P36p* have shown to be defective in the EEF development within the hepatocytes, and are unable to initiate blood stage infection. Mice immunized with *UIS3, UIS4* or *P36p* KO parasites were able to generate long lasting pre-erythrocytic immunity that conferred sterile protection against challenge with wild type sporozoites (Mueller *et al.*, 2005a, Mueller *et al.*, 2005b, van Dijk *et al.*, 2005). UIS3 and UIS4 reside in the PVM exposing their C-terminal domains to the hepatocyte as shown for EXP1 (Ansorge *et al.*, 1997) and ETRAM (Spielmann *et al.*, 2003) in erythrocytic stages, thereby involving in host pathogen interaction. UIS3 has been shown to interact with the fatty acid binding proteins of the host and involved in the uptake of host fatty acids to the parasite compartment (Mikolajczak *et al.*, 2007). Thus the PVM specific proteins are involved in the host parasite interactions like nutrient uptake, host cell remodeling, etc. Exploration of PVM composition may help in understanding the survival mechanism of the parasite inside hepatocyte and immune evasion strategies employed by the parasite.

Plasmodium exports a repertoire of proteins into the host cytosol by a recognizing a pentameric amino acid signal sequence called PEXEL/HT (*Plasmodium* export element/host targeting) motif (RxLxE/Q/D). This motif is present in the N-

terminus of the protein and occurs at approximately 35 amino acids after the signal peptide (Hiller *et al.*, 2004, Marti *et al.*, 2004). Both the membrane bound and soluble proteins have the PEXEL motif and an endoplasmic reticulum (ER) specific aspartyl protease referred to as Plasmepsin V. Plasmepsin V cleaves the PEXEL on C-terminal side of (RxL ↓ xE/D/Q) conserved Leucine (Boddey *et al.*, 2010, Russo *et al.*, 2010) and protein is N-acetylated after cleavage (Chang *et al.*, 2008). These proteins transport through the PTEX (*Plasmodium* translocon of exported proteins) complex in the PVM which constitutes PTEX 150, HSP101, PTEX 88, thioredoxin and Exp2 (de Koning-Ward *et al.*, 2009). The proteins that are cleaved by the protease plasmepsin V followed by N-acetylation get unfolded in the PV and transported through the PTEX complex and gets transported into the host cytosol where the heat shock proteins refold the exported proteins. However few exported proteins do not have a PEXEL motif and the parasite operates PEXEL independent protein export system (Heiber *et al.*, 2013). Not much is known about liver stage exportome. CSP was the first parasite protein detected in the infected liver cell (Hollingdale *et al.*, 1983). It has two PEXEL motifs, one at the N-terminus and other at the cleavable glycosylphosphatidylinositol (GPI) anchor in the C-terminus. CSP is present in the host cell cytoplasm till 24 hrs of liver stage development and two functional roles were attributed based on the cellular localization: (i) inhibition of protein synthesis by binding to host ribosomes (Frevert *et al.*, 1998) and (ii) inhibition of nuclear translocation of host NF-κB thereby reprogramming the host cell gene expression (Singh *et al.*, 2007). Apart from CSP, there are only few liver exported proteins studied so far - a transmembrane protein IBIS1 which is exported into the host cell during both liver stage and blood stage development (Ingmundson *et al.*, 2012), Sporozoite and liver stage expressed tryptophan rich protein (SLTRiP) that is exported into the host cell during early hours of liver stage infection (Jaijyan *et al.*, 2015) and liver specific protein 2 (LISP2) that is exported into the host cell cytoplasm and nucleus during mid to late liver stage development. Gene deletion of LISP2 resulted in a defect in the formation of merozoites (Orito *et al.*, 2013).

Upon successful invasion, the sporozoite gets dedifferentiated into an active replication form, the liver stage trophozoite (Jayabalasingham *et al.*, 2010). During this process it retains only organelles that are needed for multiplication viz., apicoplast, mitochondria and ER, disassembling the molecular and cellular components necessary

for motility and invasion (Bano *et al.*, 2007). The dedifferentiated trophozoite undergoes schizogony by multiplication of its genome by 10^4-10^5 times. The cell organelles also get multiplied as branched and intertwining structures forming a multinuclear syncytium that occupies the maximum space of hepatocyte. The organelle segregation followed by cytokinesis yields thousands of hepatic merozoites (Sturm *et al.*, 2006). The merozoites formed gets released into the host cytoplasm damaging the PVM. The degradation of PVM is mediated by *Plasmodium* derived cysteine proteases and blocked completely by E64, a cysteine protease inhibitor (Sturm *et al.*, 2006). Serine repeat antigen (SERA) proteases are the likely candidates of the cysteine proteases that are conserved across the *Plasmodium* species and are shown to be essential for the egress of merozoites from the erythrocyte and egress of sporozoites from the oocyst in mosquito (Blackman, 2008). Members of SERA family, such as PbSERA1 and PbSERA2 are expressed abundantly in the final stages of merozoite formation and are targeted to the PVM. In *P. berghei*, loss of function mutants of either SERA1 or SERA2 progressed normally throughout the parasite life cycle although SERA3 was upregulated suggesting that members of this family can compensate for each other (Putrianti *et al.*, 2010). A subtilisin like protease (SUB1), a conserved serine protease of *Plasmodium* was shown to be essential for merozoite egress from the host cell in both blood and liver stages (Agarwal *et al.*, 2013, Tawk *et al.*, 2013). Increase in intracellular calcium concentration (Agarwal *et al.*, 2013) and cGMP dependent protein kinase (PKG) (Falae *et al.*, 2010, Collins *et al.*, 2013) triggers the SUB1 secretion from exonemes into the PV. Serine repeat antigen (SERA) family proteins act as substrates for SUB1. SUB1 cleaves and activates the SERA proteases which initiate a cascade of protease events that are required for the egress process (Schmidt-Christensen *et al.*, 2008, Suarez *et al.*, 2013).

Following the PVM breakdown, few to several thousand merozoites are packed into vesicles surrounded by host cell derived membranous structure called merosomes (Fig. 5) that are extruded from the infected hepatocyte into the liver sinusoid (Sturm *et al.*, 2006, Tarun *et al.*, 2006). Formation of merosomes facilitates a successful evasion of host cell immunity from the highly phagocytic kupffer cells located in the liver sinusoids. Budding of merosomes into hepatic bloodstream facilitates the release of merozoites by membrane disruption thus allowing merozoites to infect red blood cells (RBC) and initiate erythrocytic cycle (Sturm *et al.*, 2006).

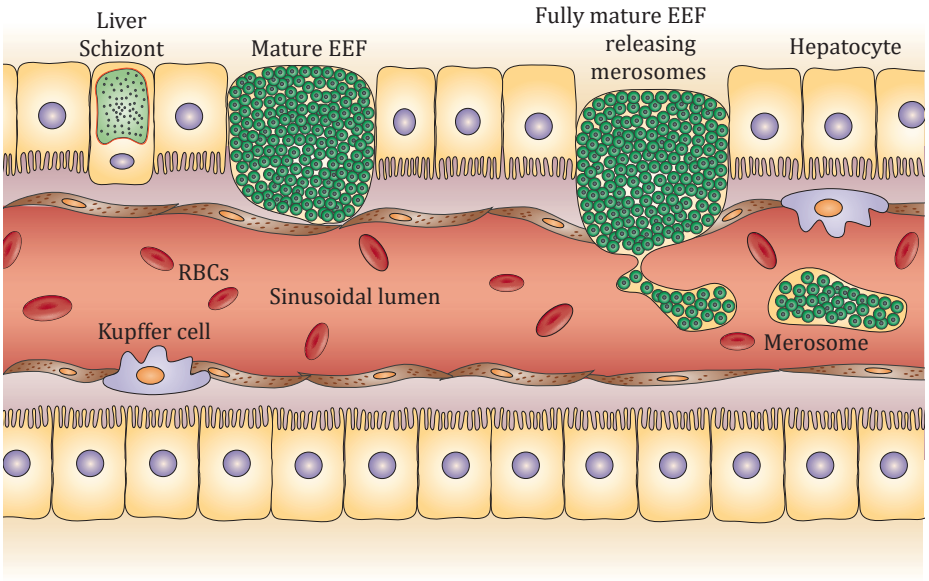

Fig. 5 Hepatic merozoite release by formation of merosomes. The EEF undergoes schizogony to form thousands of hepatic merozoites. To evade immune clearance by liver resident macrophages, *Plasmodium* liver stage merozoites form membrane bound structures called merosomes. Budding of merosomes from fully mature EEFs results in their release into bloodstream that prevents their encounter by kupffer cells residing in liver sinusoids. Rupture of merosome membrane facilitates merozoite release into the circulation thereby allowing them to infect RBCs. Image adapted and modified from Prudêncio *et al.*, 2006.

1.4.3. Erythrocytic stages

The malaria disease symptoms are associated with the erythrocytic proliferation of *Plasmodium*. The hepatic merozoites generated during exo-erythrocytic cycle are released into the blood stream to initiate the erythrocytic cycle. The merozoites are small elliptical cells with a conical protuberance at the apical end that contains a polar ring and specialized secretory organelles- rhoptries, micronemes, and dense granules. Blood stage infection is initiated when the extracellular merozoite invades the RBC (Fig. 6). The initial attachment of merozoite to RBC is mediated by merozoite surface protein (MSP) mainly MSP1 with band 3 on RBC membrane (Goel *et al.*, 2003). The next step, reorientation of merozoite occurs where the apical end points towards the erythrocyte membrane and this is mediated by AMA1 that is considered to be a potential vaccine candidate that has progressed to clinical trials (Mitchell *et al.*, 2004). The interaction of merozoite surface proteins with actomyosin motor present in the inner membrane complex (IMC) is crucial for the invasion process. (Cowman & Crabb, 2006). Drugs that target actin-myosin motor block the process of parasite invasion (Pinder *et al.*, 1998). SUB2, a micronemal serine protease mediates the shedding of fuzzy coating on the merozoite surface (Harris *et al.*, 2005). Just like sporozoites, the merozoite also invades host cell forming a parasitophorous

vacuole. Mature erythrocytes lack the intracellular organelles and thereby standard biosynthetic pathways are absent. In erythrocyte, a tubulovesicular network (TVN) extends from PVM to erythrocyte membrane (Elmendorf & Haldar, 1994) likely facilitates the acquisition of nutrients (mostly lipids, proteins and purines) from blood plasma since *de novo* synthesis of these are lipids, purines and proteins is absent in mammalian RBC (de Koning-Ward *et al.*, 2016). *Plasmodium* exports hundreds of proteins into erythrocyte cytosol and membrane for remodeling the erythrocyte to increase the permeability, rigidity and cytoadherence for its survival (Elsworth *et al.*, 2014). The export motif includes five amino acid sequence RxLxE/Q/D and termed as *Plasmodium* export element (PEXEL) or vacuolar translocation signal (VTS). More than 300 *Plasmodium* proteins contain PEXEL/VTS motif and participate in protein trafficking from parasite to host, which likely play an important role in virulence and survival of the asexual blood stages (Goldberg & Cowman, 2010). Just like in hepatocytes the proteins with PEXEL motif are transported into the erythrocyte by PTEX complex.

Some of the exported proteins do not have a PEXEL motif and are called PEXEL negative proteins (PNEPs). These include the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), Ring Exported proteins 1 and 2 (REX1 and REX2) and skeletal binding protein (SBP1) (Dixon *et al.*, 2008, Haase *et al.*, 2009, McMillan *et al.*, 2013). Cytoadherence of *P. falciparum* infected RBC (iRBC) is mediated by PfEMP1 that is inserted into the erythrocyte membrane and facilitates sequestration of iRBCs in capillaries of brain and the placenta during pregnancy. (Baruch *et al.*, 1995). PfEMP1, along with Knob associated histidine rich proteins (KAHRP) form electron dense regions on the iRBC membrane called knobs (Waller *et al.*, 1999). In the loss of function mutants of *kharp*, knobs were absent on the iRBC membrane and PfEMP1 is diffused in the cytoplasm (Crabb *et al.*, 1997). PfEMP1 family encodes around 60 variable (*var*) genes and show monoallelic expression controlled by switching between the variants of the antigen thereby evading the host immunity (Scherf *et al.*, 2008). Other var gene families that show antigenic variation in *Plasmodium* are STEVOR and RIFIN, which are the gametocyte surface proteins. Because of the shift in expression of these *var* genes in each cycle, the parasite escapes from the host immune system (Scherf *et al.*, 2008).

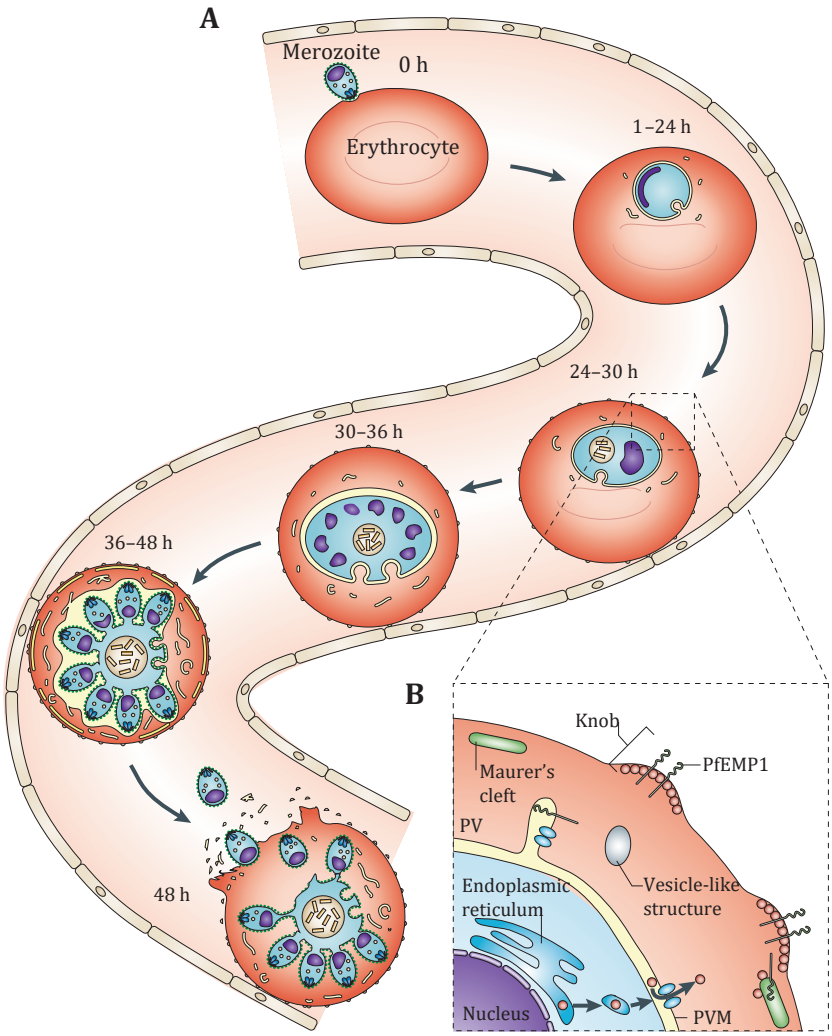

Fig. 6 Erythrocytic cycle of *Plasmodium*. The hepatic merozoites released from merosomes enter the circulation and infect erythrocytes. The merozoite binds to the erythrocyte and reorients to form a tight junction with the erythrocyte membrane and invades it forming a PVM. In erythrocyte, it undergoes a series of transformation stages. The first stage is a ring stage (0-24 hours) followed by trophozoite (24-36 hours), an active feeding stage and in which the parasite DNA undergoes replication. The final stage is schizont stage (36-48 h), during which cytokinesis occurs forming merozoites. The schizont ruptures releasing the merozoites that can infect fresh RBCs. Once the parasite is inside erythrocyte, it exports many proteins into the host cytosol across the PVM. Few of the parasite proteins like PfEMP1 (*P. falciparum* erythrocyte membrane protein 1) are targeted to the erythrocyte membrane and forms knob like structures. PV: parasitophorous vacuole, PVM: parasitophorous vacuolar membrane. Image adapted and modified from Goldberg and Cowman, 2010.

Plasmodium goes through different transition stages inside an erythrocyte. Immediately after invasion, it transforms into ring stage with a thick rim of cytoplasm surrounding a vacuole, with internal organelles and a nucleus. The parasite then progresses to trophozoite stage, an active feeding stage during which the metabolic and biosynthetic activities of the parasite are maximal. During this period, parasite grows substantially in size and occupies one-third the volume of the erythrocyte (Saliba *et al.*,

1998). *Plasmodium* trophozoite uses hemoglobin as energy source and converts the non-protein, toxic heme part of the hemoglobin into non-toxic hemozoin crystals (Egan, 2008). Majority of the antimalarial drugs are designed against major metabolic pathways of the parasites. Few anti-malarial drugs like chloroquine and mefloquine are targeted to inhibit hemozoin crystallization (Foley & Tilley, 1998). The trophozoite undergoes erythrocytic schizogony forming a schizont stage that has 16-32 merozoites and these get released into the blood by rupturing of the RBC membrane. The molecular events in the merozoite egress from the infected erythrocytes is mediated by proteases that belong to the serine rich antigens (SERA) family. SERA5 and SERA6 are essential in blood stage propagation (Miller *et al.*, 2002, McCoubrie *et al.*, 2007). *P. falciparum* has three subtilisin like serine proteases (subtilases) SUB1, 2 and 3. Of these three SUB proteins, SUB3 is not essential in blood stages, SUB2 is involved in the shedding of merozoite surface proteins during erythrocyte invasion (Harris *et al.*, 2005). SUB1 plays a key role in the merozoite egress from infected erythrocyte. The SERA proteins are in zymogen form and SUB1 released into the PV just before egress, activates SERA 4, 5 and 6. Inhibition of PfSUB1 blocked SERA processing. These activated SERA proteins especially SERA5 damages the PVM thereby mediating the egress of mature merozoites from the iRBC (Yeoh *et al.*, 2007, Arastu-Kapur *et al.*, 2008). The new generation merozoites infect fresh RBC and continue the life cycle. Few of the merozoites in the iRBCs differentiate into male and female gametocytes which are the sexual forms of parasite life cycle.

1.4.4 Sexual stages

Life cycle of *Plasmodium* has a phase of sexual reproduction that takes place in the mosquito vector and is initiated when male and female gametes emerge from respective gametocytes. The formation of gametocytes (referred as gametocytogenesis), occurs in only small population of blood stage (~0.2-1%) parasites both *in vitro* and *in vivo* and is a prerequisite for disease transmission (Sinden, 1983, Taylor & Read, 1997). Single haploid malaria parasite replicating asexually can produce both male and female gametocytes. The gender is not chromosomal specific as there are no sex chromosomes in *Plasmodium* and the gametocyte gender is governed by differential gene expression (Alano & Carter, 1990). By expressing GFP under *Pfg377* (female specific marker) and α-tubulin-II (male specific marker), it was demonstrated

that the merozoites from a single schizont develop into the gametocytes of the same sex (Silvestrini *et al.*, 2000, Smith *et al.*, 2000). The sexual commitment of a merozoite takes place at a point before schizogony. The early commitment is explained by the targeted disruption of an asexual stage specific gene *Pfgig* (*Plasmodium falciparum* gene implicated in gametocytogenesis) that resulted in the production of less number of gametocytes and the phenotype got restored by gene complementation (Gardiner *et al.*, 2005). Factors like high parasitemia, anti-malarial drugs like chloroquine increase the gametocytogenesis *in vitro* (Bruce *et al.*, 1990, Buckling *et al.*, 1999). The decision of which cell to commit for gametocyte is stochastic. A transcription factor AP2-G, which is a master regulator of gametocytogenesis was discovered recently in *P. falciparum* and *P. berghei*. The loss of function mutation of *ap2-g* resulted in total loss of gametocytogenesis that resulted in the downregulation of many transcripts that are expressed in early gametocytes (Kafsack *et al.*, 2014, Sinha *et al.*, 2014). Binding of AP2-G to a specific DNA motif (GTAC) present upstream to many gametocyte specific genes like *pfs16*, *pfg27*, *pfg14.774*, etc. was confirmed by gel shift assays. AP2-G specific DNA motif was further confirmed by driving the reporter expression by the promoter with GTAC motif and the expression was blocked by mutating the motif (Campbell *et al.*, 2010, Kafsack *et al.*, 2014).

Transcriptomic and proteomic studies have identified around 200-300 gametocyte-specific transcripts and proteins for sexual development (Eksi *et al.*, 2005, Khan *et al.*, 2005, Silvestrini *et al.*, 2005, Young *et al.*, 2005) with small subset of transcripts including those of *P25* and *P28* which are translationally repressed in gametocytes stored for gametogenesis and fertilization (Paton *et al.*, 1993, Hall *et al.*, 2005). Separate male and female gametocyte proteomic analysis revealed that both the gametocytes are different at their molecular level and of 406 gametocyte specific proteins, both share 69 proteins. The share of sex specific proteins was more for male gametocyte (69%) having the proteins involved in different events of exflagellation (Khan *et al.*, 2005).

Gametocytes are the crucial stages in the *Plasmodium* life cycle as these are essential for the transmission to mosquito and subsequent infection to other humans. So, it is important to study gametocyte biology to prevent the transmission to mosquito thereby eliminating and eradicating malaria. The current antimalarial drugs including

chloroquine and artemisinin combinational therapies are not against the mature gametocytes. Primaquine is the only approved antimalarial drug that is active against gametocyte stages. But it causes hemolysis in the patients with glucose-6-phosphate dehydrogenase deficiency (White, 2013).

The time required for gametocytes to mature varies between rodent and human malaria parasites. *P. berghei* and *P. yoelii* gametocytes require around 24-26 hours to mature, whereas the gametocytes of *P. falciparum* takes 8-10 days for maturation (Carter & Miller, 1979, Mons *et al.*, 1985). *P. falciparum* gametocytes can be divided into 5 different morphologically recognizable stages (Stage I-V) (Carter & Miller, 1979). Stage I parasite resembles the trophozoite of asexual reproduction and most of the gametocytes get sequestered in the bone marrow to avoid splenic clearance. Only Stage I and V gametocytes circulate in the peripheral blood (Farfour *et al.*, 2012) and the mature gametocytes are arrested at G0 stage of cell cycle (Sinden, 1983). The mature gametocytes, when taken up by mosquito during blood meal, gets activated by the change in pH, reduced temperature, and mosquito derived factors like Xanthurenic acid (XA).

1.5. *Plasmodium* life cycle in the *Anopheles* mosquito

The sexual cycle of *Plasmodium* is very complex and takes place in the gut of female *Anopheles* mosquito. The zygote formed as a result of sexual reproduction transforms into an ookinete and moves to basal side of the gut and develops into an oocyst stages forming thousands of sporozoites that migrate to the salivary glands of mosquito and are ready to transmit to vertebrate host (Fig. 7).

1.5.1 Activation of gametocytes

Female *Anopheles* mosquito takes up the malaria gametocytes at the time of blood feeding from an infected human. In the mosquito midgut, all the stages of malaria parasites get digested except mature gametocytes. The gametocytes egress from the red blood cell and develops into gametes (gametogenesis). The female gametocyte transforms into a single female gamete whereas the male gametocyte produces 8 microgametes by a rapid process called exflagellation, which takes 10-20 minutes to complete. Male gametogenesis is associated with three rounds of DNA replication and

axoneme assembly (Pradel, 2007) whereas female gametogenesis involves release of the transcripts from the DOZI-mediated translational repression (Mair *et al.*, 2006).

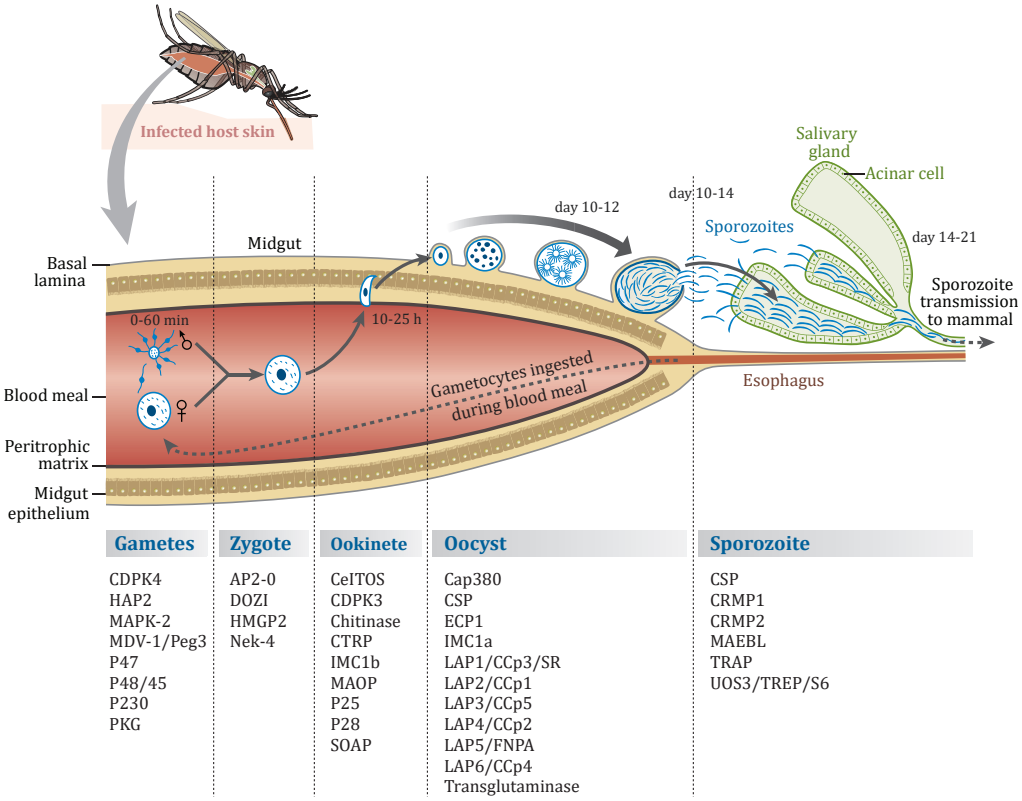

Fig. 7 Development of *Plasmodium* inside mosquito. When a female *Anopheles* mosquito probes a malaria infected person having circulating gametocytes in the peripheral blood, the gametocytes are ingested along with the blood. The mosquito gut environment favors the activation of male and female gametocytes leading to the emergence of gametes within the first hour of ingestion. Male gametocyte undergoes exflagellation forming 8 male gametes. The male and female gametes fuse to form a zygote. The zygote differentiates into a motile ookinete (during 15-25 hours post feeding) and penetrates through the gut epithelium and settles on the basal lamina towards hemocoel and develops into oocyst. Sporogony takes place within the oocyst forming thousands of sporozoites (day 10-12 post feeding). Sporozoites egress by rupturing the oocyst capsule and are released into the hemocoel (day 10-14 post feeding). The journey from hemocoel to salivary glands (day 14-21 post feeding) is likely mediated by motility of sporozoites and movement of hemolymph. Once in salivary glands, the sporozoites are ready to infect a new host when mosquito obtains next blood meal. Image adapted and modified from Aly et al., 2009.

In the mosquito midgut, gametogenesis is influenced by the factors like decrease in temperature, rise in pH and the presence of mosquito derived factors like xanthurenic acid (XA) (Billker *et al.*, 1997, Billker *et al.*, 1998). The molecular events that take place during gametogenesis have been understood considerably over the past few decades. Gametogenesis is measured by the formation of exflagellation centers which takes

around 15 min from the time of blood meal (Sinden *et al.*, 1978). Several events precede the process of exflagellation that include an increase in the intracellular calcium, the activation of phospholipase C and guanylyl cyclase which in turn raises the intracellular cGMP levels. In *P. falciparum*, XA activates two membrane guanylyl cyclases (GCα and GCβ) (Carucci *et al.*, 2000). In *P. berghei*, GCβ disruption showed the normal exflagellation, but motility was impaired in ookinetes (Hirai *et al.*, 2006). The increase in cGMP activates cGMP dependent protein kinase, PKG that leads to the rounding up of gametocytes (McRobert *et al.*, 2008). In *P. berghei*, calcium dependent protein kinase (CDPK4) plays a key role in sexual stage signaling. The loss of function mutant of CDPK4 showed a defect in the activation of male gametocyte. CDPK4 gets activated by increase in the calcium levels in the cytosol following XA activation that results in the replication of microgametocyte genome (Billker *et al.*, 2004).

The conserved 6-cysteine family proteins have shown to be essential in the *Plasmodium* gamete stages. These proteins show the surface localization and are important in cell to cell interactions. P48/45, P47, P230, and P230p are the important 6 cysteine family members that get transcribed in gametocyte stages and expressed exclusively in the gamete stages. P230 and P48/45 are male gamete specific proteins and have shown to be essential for fertilization. The P48/45 deletion mutants produce a fertile female gamete (van Dijk *et al.*, 2001). A paralog of P48/45, P47 gets expressed exclusively in the female gametocyte and the targeted disruption of *p47* yielded defective female gametes (van Dijk *et al.*, 2001).

Upon exposure to mosquito factors, female gametocyte undergoes nuclear changes and differentiates into female or macrogamete and exits from the RBC allowing the male gamete to attach and fuse. Male gametocyte express a large number of proteins essential for their motility and fertility including pf16, hap2/gcs1, and actin II. Pf16 is a flagellar protein and a null mutant of pf16 showed reduced fertility (Straschil *et al.*, 2010). Actin II is expressed only in the male gamete and its targeted disruption led to a defect in male gamete exflagellation (Deligianni *et al.*, 2011).

1.5.2 Fertilization and ookinete formation

During fertilization, the plasma membranes of male and female gamete get fused with the involvement of HAP-2 resulting in the formation of zygote (Liu *et al.*, 2008). In

Plasmodium life cycle, zygote is the only stage that is diploid and all the other stages are haploid in nature. Within 3 hours of zygote formation, it undergoes meiosis and becomes tetraploid (Janse *et al.*, 1986). NIMA (never in mitosis Aspergillus 1) related proteins, NEK-2 and NEK-4 play a key role in zygote development (Reininger *et al.*, 2005, Reininger *et al.*, 2009). In the lumen of the mosquito midgut, within 16-25 hours post gamete fusion, the zygote transforms into banana shaped ookinete. Ookinete traverses through mosquito midgut epithelium and chitinase secreted by the parasite facilitate the degradation of peritrophic matrix of midgut (Huber *et al.*, 1991, Shahabuddin *et al.*, 1993). Two parasite surface proteins P25 and P28 having GPI anchor and EGF domain protect the ookinetes from the proteolytic activity in the mosquito gut lumen (Tomas *et al.*, 2001). Majority of the genes that are essential for zygote and ookinete development are transcribed in the gametocytes and stored as P-bodies (processing bodies) or stress granules. The 3' UTRs of these transcripts have a nucleotide motif that binds to Pumilio (Puf) proteins that repress translation and regulate mRNA stability (Cui *et al.*, 2002, Braks *et al.*, 2008). These translationally repressed mRNAs accumulate in cytoplasmic granules that contain an RNA binding helicases that belongs to DDX family, called DOZI (development of zygote inhibited). The central role of DOZI was revealed by its targeted deletion which showed normal blood stage cycle but absence of female gametocyte resulting in developmental arrest of the parasite in the mosquito (Mair *et al.*, 2006).

Calcium dependent protein kinase 3 (CDPK3) and Circumsporozoite and TRAP related protein (CTRAP) are essential for the motility and infectivity of ookinetes. Deletion of CDPK3 produced immotile ookinetes that were not able to invade the midgut epithelium (Siden-Kiamos *et al.*, 2006). Targeted disruption of CTRAP led to reduced gliding motility of ookinetes and oocyst formation was abolished (Yuda *et al.*, 1999). The parasite remains in tetraploid stage until the syncytium formation in the oocyst which eventually gives rise to haploid sporozoites (Janse *et al.*, 1986).

1.5.3 Oocyst development and sporulation

After migration through midgut epithelium, ookinete transforms into oocyst beneath the basal lamina. It has been postulated that oocyst is the only longest developmental stage of *Plasmodium* and takes around 10-14 days to mature depending on species (Hillyer *et al.*, 2007). After settling beneath the basal lamina, the ookinete

becomes round and the apical complex is absorbed into the cytoplasm of the developing oocyst (Garnham *et al.*, 1969).

From second day after the formation of oocyst, the parasite secretes an amorphous capsule that surrounds the entire surface of oocyst. The capsule separates the parasite from the mosquito tissues. The capsule consists of parasite specific transglutaminase (Adini *et al.*, 2001) and some mosquito-derived laminins (Nacer *et al.*, 2008). The mosquito immune system recognizes and eliminates the parasites during the transit from the lumen of midgut towards the basal lamina. However subsequent to formation of a non-motile oocyst its capsule protects the parasite from mosquito defense molecules. The oocyst grows in size and the nucleus undergoes several mitotic divisions forming a multinuclear syncytium (Canning & Sinden, 1973). The nutrients required for the oocyst are presumably acquired from the hemolymph and the mechanism by which the parasite absorbs nutrients through the basal lamina is not yet known (Aly *et al.*, 2009).

CSP plays a key role in the process of oocyst formation and sporulation. CSP expression is detected few days after oocyst formation (Thathy *et al.*, 2002). CSP has an N-terminal signal peptide and binds to the parasite plasma membrane with the help of C-terminal GPI anchor (Moran & Caras, 1994). Within the oocyst, sporoblasts are formed by the invagination of the oocyst plasma membrane forming lobes of cytoplasm with the dividing nuclei (Sinden & Strong, 1978). In sporoblasts, CSP is present on the plasma membrane, in the cytoplasm, and endoplasmic reticulum (Yoshida *et al.*, 1980, Santoro *et al.*, 1983, Tsuji *et al.*, 1992). Deletion of CSP resulted in the defect in sporozoites formation (Menard *et al.*, 1997). Just beneath the sporoblast plasma membrane, microtubule organization centers (MTOCs) are located which leads to the formation of apical complex and nuclei that are incorporated into the daughter sporozoites. It is assumed that CSP plays a key role in the formation and organization of MTOCs within the sporoblasts (Thathy *et al.*, 2002). After subcellular organelle assembly and with the formation of triple layer pellicular structure, sporozoites bud off from the sporoblasts (Vanderberg & Rhodin, 1967, Meszoely *et al.*, 1989). Inner membrane complex 1 (IMC1) is an important protein of the inner membrane complex and targeted disruption revealed its essential role in maintaining the crescent shape and its critical role in sporozoites motility (Khater *et al.*, 2004).

At the end of sporulation, the oocyst is completely filled with sporozoites. The mechanism of sporozoite release from the oocyst is not well understood. The sporozoites are released from mature oocysts by forming small fenestrae in the oocyst capsule. Sporozoite egress is an active process mediated by the parasite (Sinden, 1974). Targeted disruption of *Plasmodium* cysteine protease, ECP1 (egress cysteine protease) that specifically expressed in oocyst resulted in viable sporozoites that are unable to egress from oocyst (Aly & Matuschewski, 2005). Mutation in the Region II plus of CSP also resulted in defective sporozoite egress from the oocyst (Wang *et al.*, 2005a). This suggests that sporozoite release is an active process involving more than one protein and not just by the mechanical force resulting within the fully developed oocyst.

1.5.4 Migration of sporozoites to salivary glands

The oocyst sporozoites are released into the hemolymph - the circulatory system of the mosquito and have access to all the tissues of the mosquito. The sporozoite surface ligands specifically recognize and bind to the receptors on the basal lamina of salivary glands. After binding, the sporozoite breaches the basal lamina and invade the distal, lateral and medial lobes of the salivary gland (Sinden & Matuschewski, 2005). Studies using antibodies raised against whole salivary glands identified Saglin, a surface protein as one of the receptors for sporozoite invasion (Brennan *et al.*, 2000).

Numerous studies have revealed the involvement of few parasite proteins in attachment of sporozoites to the basal lamina of salivary gland. Region I of CSP has been shown to be involved in the attachment of sporozoites to the salivary glands. (Sidjanski *et al.*, 1997). Injection of full length recombinant CSP or N-terminal peptide of CSP into the hemocoel of infected mosquitoes reduced the migration of sporozoites to salivary glands (Myung *et al.*, 2004). Similarly, anti-CSP antibodies injected into the hemocoel of infected mosquitoes blocked the salivary gland invasion of sporozoites (Warburg *et al.*, 1992). Disruption of region II abolished the ability of sporozoites to invade salivary glands as well as infect the vertebrate host (Tewari *et al.*, 2002). The mutant *P. berghei* parasites in which the endogenous CSP is replaced with *P. gallinaceum* CSP (primarily differs in the region I lacking central lysine residue) yielded sporozoites that are unable to invade salivary glands showing the important role of Region I in attachment of sporozoites to the salivary glands (Tewari *et al.*, 2005).

Thrombospondin related anonymous protein (TRAP), an important component of the actomyosin molecular motor is type I transmembrane domain protein that contains von Willebrand A and thrombospondin type I domains (Robson *et al.*, 1995). Gene deletion of TRAP resulted in the accumulation of sporozoites in the hemolymph, and the parasites were unable to glide. They do not invade the salivary glands and cannot infect the vertebrate host (Sultan *et al.*, 1997). Mutational analysis revealed that the extracellular adhesive domain and thrombospondin repeat region of TRAP are essential for invasion of salivary gland in mosquito as well as hepatocytes of vertebrate host, but not for motility of the sporozoite (Matuschewski *et al.*, 2002a). TRAP specifically binds to saglin on the mosquito salivary glands and the interaction is crucial for sporozoite invasion of mosquito salivary glands (Ghosh *et al.*, 2009). The C-terminal cytoplasmic tail of TRAP interacts with the actomyosin motor and is essential for sporozoite gliding and host cell invasion (Kappe *et al.*, 1999).

MAEBL is a micronemal protein that is secreted on to the sporozoite surface before invading the salivary glands (Kariu *et al.*, 2002). Targeted disruption of MAEBL resulted in defective sporozoites that failed to get attached to basal lamina of mosquito salivary glands. Nonetheless, the sporozoites in the mosquito hemocoel showed normal gliding motility and were able to infect the mammalian host (Kariu *et al.*, 2002). MAEBL is regulated by alternative splicing and post translational processing at different life cycle stages and the variants of MAEBL manifest different functions at different life cycle stages of the parasite (Matuschewski, 2006).

Within the salivary glands, the sporozoite achieves enhanced infectivity by changing its transcriptional repertoire and wait for its successful transmission to the vertebrate host at the time of blood meal. Salivary gland sporozoites show enhanced infectivity when compared to oocyst sporozoites. Transcriptomic studies have identified 30 genes that are uniquely upregulated in salivary gland sporozoites that were designated as UIS genes (upregulated in infectious sporozoites) (Matuschewski *et al.*, 2002b). UIS gene products are required for the hepatocyte invasion and development. UIS1 is a kinase that negatively regulates eukaryotic initiation factor-2α by phosphorylation thereby inhibiting the translation of mRNA stored in granules. Targeted disruption of UIS1 resulted in the premature transformation of sporozoites into EEFs in the mosquito thereby losing their infectivity (Zhang *et al.*, 2010). UIS3 and

UIS4 are essential for early liver stage development and targeted disruption of either UIS3 or UIS4 resulted in developmental arrest at early liver stage (Mueller *et al.*, 2005a, Mueller *et al.*, 2005b). Sporozoite asparagine rich protein (SAP1) is shown to be essential for the expression of UIS genes and the targeted deletion of SAP1 leads to depletion of the UIS transcripts (Aly *et al.*, 2008, Aly *et al.*, 2011). SAP1 knockout parasites show normal traversal and hepatocyte invasion but are unable to initiate liver stage development (Aly *et al.*, 2008). Mice immunized with *uis3* and *uis4* knockout sporozoites elicited $CD8^+$ T cell immunity (Kumar *et al.*, 2009). Thus identification and characterization of key proteins involved in sporozoite infection of hepatocytes is important to generate genetically attenuated sporozoites (GAS) that have potential for inducing sterile immunity.

1.6 Control measures of malaria

The major approaches for malaria control include the prevention of new incidents and effective treatment of the reported cases. The methods to prevent the transmission of parasites in the endemic areas include using prophylactic drugs, eradication of mosquito, and prevention of mosquito bites. Intervention of malaria transmission is a combined effort, hitting several of the *Plasmodium* life cycle stages. Several antimalarial drugs can be taken as prophylactic medicine daily or weekly at lower doses to prevent the disease transmission in the endemic regions. These include chloroquine, mefloquine, doxycycline, and malarone (combination of atovaquone and proguanil hydrochloride). Vector control would protect people against infective mosquito bites and to reduce the malaria transmission at the community level. Indoor residual insecticide spraying (IRS) involves the application of residual insecticides on surfaces where the mosquitoes dwell, thus effectively controlling malaria transmission. Insecticide treated nets (ITN) and beds protect people from the infected mosquito bites thereby reducing the disease transmission. The spread of insecticide resistance is a major threat to vector control programs. Environmental sanitation is an important measure to control the mosquito population. Awareness programs to educate about risk of malaria, vector management and symptom recognition can be effective to bring down the socioeconomic loss.

1.7 Prophylaxis and treatment

As *Plasmodium* tend to attain resistance against drugs, prophylactic vaccines are most effective than drugs to prevent the onset of malaria. However, there are several problems associated with *Plasmodium* vaccine development. To maintain the levels of acquired immunity, the infected individuals must be continuously exposed to low levels of *Plasmodium* antigens as the infected individuals lacks immunological memory (Struik & Riley, 2004). This suggests that the protection through vaccine is short lived and needs several boosters. *Plasmodium* life cycle is extremely complex and many of the antigens expressed are polymorphic, suggesting that an effective vaccine may have to be comprised of several antigens expressed at various life-cycle stages. The malaria vaccines that are currently being developed target pre-erythrocytic, erythrocytic or the transmissible stages of *Plasmodium.*

Pre-erythrocytic stage vaccines aim to target sporozoites to prevent hepatocyte invasion with neutralizing antibodies or target the infected hepatocytes by triggering a cell mediated immune responses. This strategy prevents either hepatocyte infection or erythrocytic cycle (Girard *et al.*, 2007). The most successful sporozoite vaccine, RTS,S, targets the sporozoite surface protein CSP. In phase I/II trials in Gambia, RTS,S elicited protective immunity with 70% efficacy for 9 weeks and the level of protection decreased after this time (Stoute *et al.*, 1998). Other CSP based vaccines are not successful in protecting individuals against a challenge infection (Girard *et al.*, 2007). Live sporozoites attenuated by irradiation successfully invade the hepatocytes and prevent the parasite development inside hepatocytes. The parasites that are not able to complete the liver stage development elicit protective immunity and hence pre-erythrocytic stages are the attractive targets for the vaccine development. Genetically attenuated parasites (GAP) range in their degree of attenuation from early liver stage to late liver stages. *uis3* and *uis4* knockout parasites showed arrest in the early liver stage development and elicit CD8+ T cell immunity (Mueller *et al.*, 2007, Kumar *et al.*, 2009). Depletion of *FabL*, that codes for an enzyme involved in fatty acid biosynthesis caused mid to late EEF arrest prior to the formation of liver stage merozoites (Yu *et al.*, 2008). Targeting the late liver stage developmental arrest is an effective strategy to develop vaccines that confer cross stage protection.

The main aim of erythrocytic vaccines is to protect against the clinical disease by inducing high antibody titers against few antigens which can protect the host efficiently by inhibiting the invasion or multiplication of merozoites. The important candidates used in erythrocytic malaria vaccine are Apical Membrane Antigen 1, (AMA1), Erythrocyte-Binding Antigen-175 (EBA-175), Glutamate-Rich Protein (GLURP), Merozoite Surface Protein 1 (MSP1), MSP2, MSP3 and Serine-Repeat Antigen 5 (SERA5) which are highly expressed on the merozoite surface (Crompton *et al.*, 2010). To date, none of these candidates have demonstrated protection against clinical outcomes (Miura, 2016).

The transmission blocking vaccines target the sexual stages of the parasite to prevent development in the *Anopheles* mosquito. These vaccines do not protect the individual from infection but reduce transmission within a community. So these must be used along with the pre-erythrocytic or erythrocytic vaccines (Girard *et al.*, 2007). Four antigens of *P. falciparum* have attracted the attention as mosquito stage vaccines. Gametocyte or gamete surface proteins Pfs230 and Pfs48/45 and zygote or ookinete surface proteins Pfs25 and Pfs28. These antigens show very limited sequence diversity compared to blood stage antigens. Phase I trials with Pvs25, a homologue of Pfs25 revealed low immunogenicity (Malkin *et al.*, 2005). It is important to identify novel gamete or the ookinete surface proteins to increase the repertoire of potential transmission blocking vaccine candidates.

As there is no effective malaria vaccine, chemotherapy is the only mainstay of controlling malaria. The chemotherapeutic drugs target cellular processes or disrupt the metabolic pathways within different subcellular organelles of the parasite. Today, different classes of antimalarials like quinoline derivatives, antifolate drugs, artemisinin compounds and antibiotics are in use.

Quinine is a prominent example in the class of quinolones which are heterocyclic organic compounds. Quinine is extracted from the bark of *Cinchona* plant and is one of the oldest antimalarial drugs (Pasvol, 2005). It's derivative chloroquine became the first drug of choice for many decades in treatment of severe malaria and prophylaxis because it was safe, highly effective and inexpensive (Hyde, 2007a). Chloroquine inhibits the polymerization of toxic haem into hemozoin within the food vacuole in blood stage

parasites (Muller & Hyde, 2010). Resistance to chloroquine is obtained by a point mutations in *P. falciparum* chloroquine resistance transporter (PfCRT) and *P. falciparum* multidrug resistance transporter 1 (PfMDR1), both located in the membrane of the food vacuole that mediates the efflux of chloroquine from the food vacuole (Fidock *et al.*, 2000, Lakshmanan *et al.*, 2005, Sidhu *et al.*, 2006). 8-aminoquinolone derivative, primaquine actively eliminate the *P. vivax* hypnozoites and is used in combination therapy with other antimalarials (Winstanley & Ward, 2006, Galappaththy *et al.*, 2007). Other quinolone derivatives used to treat malaria are amodiaquine, piperaquine, mefloquine, halofantrine, and lumefantrine.

Antifolates inhibit the *de novo* synthesis of folate by inhibiting dihydropteroate synthase (of HPPK/DHPS) and dihydrofolate reductase (of DHFR/TS). Pyrimethamine inhibits the enzyme DHFR and sulfonamides inhibit DHPS enzyme (Hyde, 2007b). Antifolate compounds used in monotherapy led to development of resistant parasites quickly (Gregson & Plowe, 2005), however combination of DHPS and DHFR inhibitors showed synergistic effect in malaria treatment. Thus a combination of pyrimethamine and sulfadoxine is used for the treatment of *P. falciparum* malaria (Gregson & Plowe, 2005, Hyde, 2007b). Other antifolate drugs used in antimalarial therapy are proguanil, trimethoprim, dapsone, sulfalene, sulfamethoxazole, sulfadoxine, etc.

Artemisinin (Chinese name-Qinghaosu) is a natural compound isolated from the plant *Artemisia annua* that has been used in traditional Chinese medicine for centuries to treat fevers and malaria. It is a sesquiterpene lactone with a unique peroxide linkage, which is thought to be responsible for antimalarial activity (Hien & White, 1993). It acts on asexual blood stage parasites as well as on young gametocytes. Artemisinin is now the first line antimalarial drug discovered by Tu Youyou and colleagues in 1981 and is used with the combination of other classes of antimalarials in the form of Artemisinin combination therapy (ACT). Dihydroartemisinin, artesunate, and artemether are the synthetic derivatives of artemisinin that have higher antimalarial activity and better solubility (O'Neill & Posner, 2004). Because of the widespread of resistance against antimalarials like chloroquine, antifolates, etc., ACTs are recommended by the WHO for effective treatment of malaria (Nosten & White, 2007, White, 2008). Recent studies have revealed that the mutations in the Klech 13 propeller domain of *Plasmodium* are

associated with resistance also to artemisinin both *in vitro* and *in vivo* (Ariey *et al.*, 2014).

Many antibiotics exhibit antimalarial activities. Doxycycline, tetracycline and clindamycin are potent antimalarials that are used for both treatment and prophylaxis along with other antimalarial drugs (Pradel & Schlitzer, 2010). It is believed that antibiotics affect apicoplast or mitochondrial metabolic pathways due to their bacterial origin (Goodman *et al.*, 2007). Tetracycline and doxycycline inhibit protein translation in mitochondrion (Prapunwattana *et al.*, 1988) as well as in the apicoplast (Dahl *et al.*, 2006, Goodman *et al.*, 2007). Clindamycin inhibits protein translation in the apicoplast, as it does same in *Toxoplasma gondii* (Camps *et al.*, 2002).

1.8 Challenges and current research on malaria

The problems of the severity of malaria are further intensified by a continuous evolution of parasite and vector resistance to antimalarial drugs and insecticides respectively. The antimalarial drug resistance by *Plasmodium* emerged as a greatest challenge to control malaria in endemic regions as discussed in preceding section. Drug resistance may have implications in re-introduction of malaria in the eradicated regions. Along with the drug resistant parasites, insecticide resistant mosquitoes is the biggest challenge in control and elimination of malaria. According to WHO world malaria report, insecticide resistant *Anopheles* mosquitoes were observed in 64 countries. WHO is working with the federal agencies and industrial partners in malaria endemic countries to develop strategies to deal with insecticide resistance in malaria vectors. Along with antimalarial drugs and vector control strategies, an effective vaccine is a key tool to control, eliminate and eradicate malaria. Infectious diseases can be prevented effectively by vaccination and till now a licensed malaria vaccine is not available. This is because of the complexity of parasite life cycle, antigenic variation exhibited by the parasite and the poor understanding of host pathogen interactions. In recent years many malaria research laboratories are trying to identify new vaccine candidates that target different stages of parasite life cycle. An ideal malaria vaccine should induce protection against different antigens that are expressed at various life cycle stages of parasite and should induce both cell mediated and humoral immune responses (Arama & Troye-Blomberg, 2014). The only malaria vaccine that has entered phase III clinical

trials is RTS, S/AS01 and was shown to protect moderately against both clinical and severe malaria in infants (Rts, 2014).

1.9 *P. berghei* as model organism

Working with human *Plasmodium* sp. for *in vivo* investigation is very difficult as the accessibility to liver and other organs is not possible. Laboratory research on human species of *Plasmodium* is restricted as it is not feasible to reproduce the complete parasite life cycle *in vitro*. To overcome these limitations, avian, primate and rodent malaria models complement the studies on human malaria. Among these, rodent malaria models are easier to handle and are closely related to the human species (Martinsen *et al.*, 2008). The rodent *Plasmodium* species *P. berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei* were initially isolated from thicket rats in Central Africa and have been adapted to grow in laboratory mice and rats (Carlton *et al.*, 2005). The rodent models are widely used to successfully complement the research on *P. falciparum*. Rodent malaria models, particularly, *P. berghei* have been extremely valuable for understanding parasite biology and studying host-parasite interactions. *P. berghei* shows similarities with human malaria species in morphology, physiology and life cycle with only slight variations making the investigation of human malaria effective. The genomes of different species of *Plasmodium* is conserved, and almost 80% of around 5300 genes coded by the *Plasmodium* genome are orthologous (Janse *et al.*, 2011). The genome of *P. berghei* is 18-20Mb in size with 14 chromosomes and shows 62.9% protein identity, 70.3% nucleotide identity and has 3,890 orthologous genes when compared with *P. falciparum* (Hall *et al.*, 2005). *P. berghei* genome can be manipulated efficiently by conventional recombinant technologies with the possibility of analysing parasites throughout their complete life cycle, both *in vitro* and *in vivo*. This has made *P. berghei* the most frequently used malaria model for gene function analysis (Carvalho & Menard, 2005, Janse *et al.*, 2006a). The ease of completing the entire life cycle of the parasite under laboratory conditions offers a great opportunity to study the complex parasite biology and gene functions in many aspects (Balu & Adams, 2007, Kooij *et al.*, 2012). Epitope tagging of parasite proteins or obtaining certain target proteins as translational fusions with fluorescent proteins enable the investigation of stage specific expression, localization and protein-protein interactions. The reporter lines expressing fluorescent

proteins under constitutive promoters have been used as effective tools to study and track the parasites as they go through mosquito and vertebrate host.

1.10 Research Objectives

Complete eradication of malaria offers several challenges that include a complex life cycle of *Plasmodium*, antigenic variation associated with the blood stage parasites, the ability of parasites to develop resistance against the current antimalarials and the emergence of insecticide resistant *Anopheline* strains.

Unraveling the functions of parasite stage specific genes demands an efficient gene manipulation system. In *Plasmodium* genetic manipulation is well established in *P. berghei* with very high efficiency and the knockout (KO) mutants are excellent tools to study the function of the target gene by cycling through the mammalian host and mosquito vector. The KO is obtained by replacing the target gene by double crossover recombination with fluorescent reporter and a drug resistance cassette that facilitates the selection of the successful transfectants. A block in propagation of life cycle in the absence of target gene product provides evidence for its function in a stage specific manner. In the event of target gene being essential at the selection stages, thus precluding the study of its function, a conditional mutagenesis system of yeast FLP (flippase, a recombinase)/*FRT* (flippase recognition target) can be employed where FLP expressed exclusively at the mosquito stages of *Plasmodium* achieves excision of FRTed target gene. Employing the aforementioned genetic approaches, we have investigated the role of

(i) Circumsporozoite protein (CSP) in mosquito stages using FLP/*FRT* conditional mutagenesis system,

(ii) A bifunctional K^+ channel/adenylyl cyclase α (*K^+/ACα*) protein in sporozoite stage and

(iii) A hypothetical protein PBANKA_141700 in *Plasmodium* life cycle stages.

Chapter 2

Investigating the role of Circumsporozoite protein (CSP) in mosquito stages by using FLP/FRT based conditional mutagenesis system

2.1 Introduction

Plasmodium is a digenetic parasite that requires a mammalian and a mosquito host to complete its life cycle. Infection to mammalian host occurs following inoculation of the sporozoites that infect hepatocyte and transform into exoerythrocytic forms (EEFs). The merozoites are released from fully mature EEFs and initiate the erythrocytic cycle that allows rapid asexual replication of the parasite leading to most of the clinical symptoms of the disease. These asexual forms are the only stages that are amenable for genetic manipulation because they are produced in very large numbers owing to the several rounds of multiplication inside erythrocytes. The rapid replication efficiency at these stages makes them suitable for electroporation and endows with the advantage of successfully selecting the recombinant parasites. However, one of the limitations of this approach is that the parasite genes having essential roles in erythrocytic stages cannot be studied because gene inactivation in the haploid stages impairs growth and precludes selection of the recombinant clones.

The pre-erythrocytic stages of *Plasmodium* include the sporozoite stages that are inoculated by female *Anopheles* mosquito and EEFs that develop in hepatocytes following invasion of sporozoites. The rodent species of *Plasmodium* is routinely used for analysis of pre-erythrocytic stages as cyclic passage of parasites through mouse and mosquito can be performed with ease under laboratory conditions. However, studying the functions of certain genes that are active at pre-erythrocytic stages may also pose limitations as genetic manipulation of parasite in blood stages may hamper the development in mosquito stage if the gene has a vital function at that stage. Alternatively, the parasites may experience a block in progression of life cycle following depletion of gene that has conserved function at multiple life cycle stages. For example, the genes that govern parasite motility (Menard *et al.*, 2008), egress from host cells (Blackman & Carruthers, 2013) and parasite multiplication (Vaughan *et al.*, 2008) are essential and thus are recalcitrant to genetic modification. Therefore, for effective investigation of protein function at various life cycle stages, temporal inactivation of the target gene is highly essential.

Conditional silencing of gene function can be achieved in eukaryotes by different strategies. The most widely used method employs the tetracycline resistance

operon that functions through a Tet repressor (TetR) and prevents transcription by binding to *tet*O operator, a 19 bp repeat sequence engineered around the transcriptional start site of the target gene. In the presence of tetracycline, the TetR-tetO interaction dissociates and leads to activation of target gene expression. In *Toxoplasma*, the essential role of myosin A in parasite motility (Meissner *et al.*, 2002), AMA1 in host cell invasion (Mital *et al.*, 2005) and MIC2 in invasion and virulence (Huynh & Carruthers, 2006) was shown by using tet regulated conditional system. In all these studies, the transcriptional transactivator (tTA) protein was stably expressed in the WT parasites, and the line was transfected with episomal construct carrying anhydrotetracycline-responsive target gene, whose expression was regulatable in the absence of the endogenous copy of the target gene. Using this system, constitutive transcription of genes was ensured until the addition of tetracycline that subsequently releases the tTA from the operator site and preventing transcription (Tet-off system). While conditional depletion of endogenous genes can be obtained in *Toxoplasma* using this system, recapitulating the same in *Plasmodium* imposes several challenges. Notably, the stages of *Plasmodium* that occur in mosquito cannot be subjected to tet-regulation owing to its inaccessibility to tetracycline. However, in *Plasmodium*, TetR was engineered to act as a transcriptional activator that upon interaction with *tet*O activates transcription and its activity is repressed in the presence of anhydrotetracycline (Meissner *et al.*, 2005). While this system allows studying the function of essential genes in *P. falciparum* and for expression of transgenes and dominant negative mutants only in blood stages, it does not conditionally repress endogenous genes.

The second conditional approach is by regulating the stability of the endogenous protein by expressing it as a fusion product with mutants of the human FKBP12 protein, which act as a destabilising domain. This destabilising affect can be reversed by addition of a synthetic ligand Shield-1, which binds to the destabilizing domains and shields it from degradation (Banaszynski *et al.*, 2006). Thus by obtaining target protein as a recombinant fusion product with destabilising domain, the endogenous protein is rapidly degraded in the absence and stabilised in the presence of Shield-1. This approach was successfully used in *P. falciparum* for rescuing the cysteine protease falcipain-2 KO line (Armstrong & Goldberg, 2007) and in

Toxoplasma for conditional stabilisation of dominant negative mutants of *Rabs* (Herm-Gotz *et al.*, 2007, Agop-Nersesian *et al.*, 2010). One of the apparent limitations of this system is the fusion of 107 amino acid residues of destabilising domain to target protein that may alter the function of the protein.

The third conditional approach described recently in *Plasmodium* is promoter swap strategy, where the promoter of the target gene essential in blood stages is swapped or exchanged by a heterologous promoter that is normally active only in blood stages. By this strategy, the expression of essential genes in blood stages is ensured. However, when promoter swapped line is passed through mosquito, the heterologous promoter is silenced at these stages thus allowing study of target protein feasible in mosquito stages and possibly in pre-erythrocytic stages, if target protein is not essential for *Plasmodium* mosquito stages. Using this elegant strategy, the role of MyoA and CDPK1 were shown to be essential for ookinete motility (Siden-Kiamos *et al.*, 2011) and differentiation (Sebastian *et al.*, 2012) required for successful completion of parasite life cycle in mosquito stages.

The fourth approach for conditional depletion of target proteins is by excision of target DNA sequence using site specific recombination (SSR) system. SSR employs recombinases like Cre (from bacteriophage P1) and Flp (from *Saccharomyces cerevisiae*) that recognizes a 34bp sequence called as *loxP* or *FRT* respectively (Branda & Dymecki, 2004). Depending on the orientation of the recognition sequence, the recombinase mediates excision, inversion, insertion or exchange of DNA.

The first SSR system was developed in *P. berghei* (Carvalho *et al.*, 2004) where expression of FLP and engineering of *FRT* sites at target gene locus was achieved in two different lines. Following cross of both lines in mosquito, a fraction of haploid sporozoite population that carried both FLP gene and the *FRT*ed copy of the target gene were shown to undergo efficient excision. Further modifications of this system involved engineering *FRT* sites on to the target gene locus in parental transgenic lines that express FLP or its thermolabile variant FLPL (Combe *et al.*, 2009) under *Plasmodium* promotors active in the mosquito stages. Both systems (Carvalho *et al.*, 2004, Combe *et al.*, 2009) necessitates the passage of recombinase expressing parasites through mosquitoes for recycling of the marker. Using this system, the

previously unappreciated roles of some of the essential genes like MSP-1 (Combe *et al.*, 2009) and SUB1 (Suarez *et al.*, 2013) in formation of merozoites in hepatic stages, the role of RON4 in hepatocyte invasion, the role of cGMP in formation of merosomes (Falae *et al.*, 2010) was established.

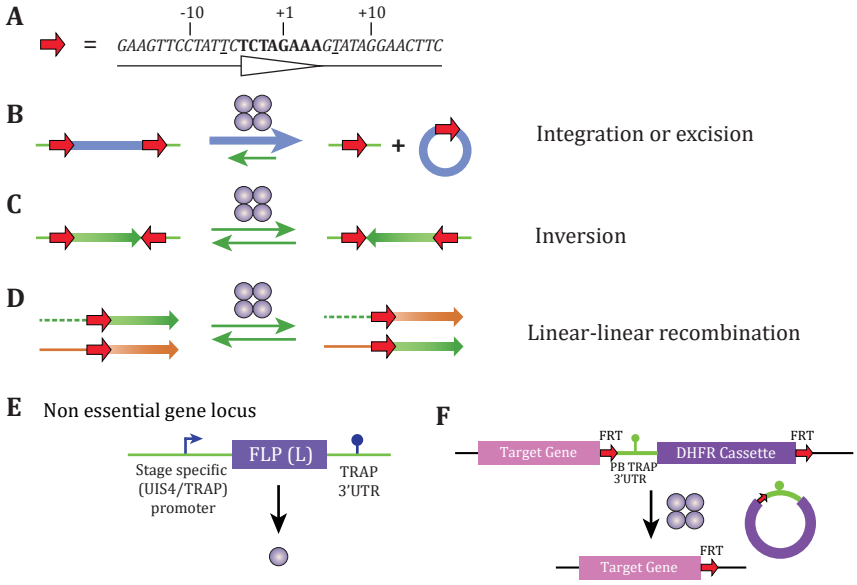

Fig. 8 Mechanism of FLP/*FRT* site specific recombination and conditional silencing of the target gene using this system. A) The nucleotide sequence of *FRT* site, a 34 bp sequence is represented as a red arrow. It has two palindromic sequences (thin lines) of 13 bp length separated by 8 bp spacer sequence that determines the direction of *FRT* sequence (small open arrow head). FLP binds to these palindromic sequence and breaks in the spacer sequence for strand exchange and ligation. B) FLP acts as a tetramer and excises the DNA present between two *FRT* sites as a circular molecule, if two *FRT* sequences orient in the same direction. FLP mediates the integration of excised DNA in the reverse direction but this reaction is kinetically less favorable when compared with excision. C) FLP flips the DNA present between two *FRT* sites, if they are in the opposite direction. D) FLP mediates the exchange of DNA between the two chromosomes, if *FRT* sites are present in different chromosomes. E) Generation of FLP or FLPL transgenic that is driven by UIS4 or TRAP promoter at non-essential gene locus. F) FLP mediated excision of heterologous 3'UTR flanked by *FRT* sites there by making the mRNA derived from the target gene unstable. Image adapted and modified from Lacroix *et al.*, 2011.

During *Plasmodium* life cycle, the expression of CSP is developmentally regulated and is first detected in immature oocyst (Posthuma *et al.*, 1988) and later localizes to inner surface of mature oocyst capsule, membranes of sporoblast, budding and free sporozoite thus implicating its role at multiple stages of oocyst development (Cross & Langhorne, 1998, Nagasawa *et al.*, 1987). Following egress from oocyst, colonisation of sporozoite to salivary glands is also a function of CSP (Myung *et al.*, 2004). CSP enters the sporozoite secretory pathway, undergoes processing on parasite surface (Coppi *et al.*, 2005) and is shed continuously during gliding motility. Shortly after the invasion of sporozoite into hepatocytes, CSP is also detected on the

membrane of early EEFs and in the cytoplasm of the infected hepatocytes (Hamilton *et al.*, 1988). CSP crosses the parasitophorous vacuolar membrane (PVM) (Singh *et al.*, 2007) during EEF maturation, enabling the efficient presentation of its T cell epitopes on infected hepatocytes.

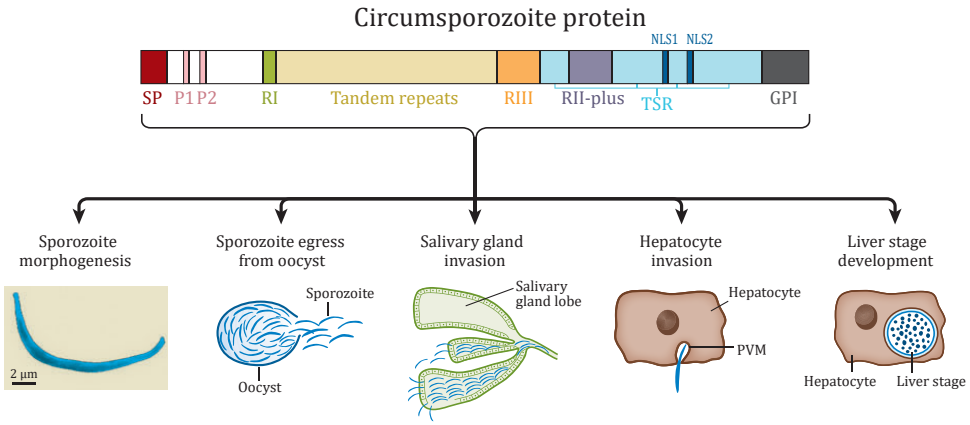

Fig. 9 Schematic representation of Circumsporozoite protein (CSP) structure and its functions. CSP has a signal peptide (SP) at the N-terminal region and two PEXEL motifs 1 and 2 (P1 and P2). CSP has a central conserved tandem repeat region and the amino acids in the repeat region are species specific. It has region I (RI) and region II plus (R II plus), region III and thrombospondin related region (TSR) domains. The amino acids in the region I and region II plus are conserved in CSP across all the species of *Plasmodium*. There are 2 nuclear localization signals - NLS1 and NLS2, and C-terminal glycosyl phosphatidylinositol (GPI) anchor signal. CSP is a multifunctional protein that is essential for the maintenance of sporozoite morphology, egress of sporozoites from oocysts and their invasion of salivary glands. In the vertebrate host, CSP is essential for the hepatocyte invasion and in the liver stage development. PVM: parasitophorous vacuolar membrane. The image adapted and modified from Aly et al., 2009.

However, a KO line of CSP could not reiterate the several of the above functions owing to its essential role in sporulation with the oocyst. Thus to overcome this limitation, we utilised a yeast based FLP/*FRT* conditional mutagenesis system and generated parental lines expressing FLP and FLPL using *Plasmodium* promotors TRAP (TRAP@FLPL) and UIS4 (UIS4@FLP) active respectively at oocyst and salivary gland sporozoite stage. By replacing endogenous 3' UTR of *csp* with *trap* 3' UTR and engineering the *FRT* sites flanking the heterologous 3' UTR in these parental lines, we show that this system can be efficiently excise the flirted DNA thus achieving depletion of CSP at oocyst stage. We report that conditional silencing of *CSP* gene leads to compromised sporulation and lack of egress leading to the inability of sporozoites to reach salivary glands in both lines - an observation that can be explained only in the light of an overlapping promotor activity of UIS4 and TRAP at the oocyst development stage.

2.2 Materials and Methods

2.2.1 Experimental animals

Female inbred Swiss, BALB/c or C57BL/6 mice and male New Zealand White rabbit were purchased from National Centre for Laboratory Animal Science (NCLAS), NIN, Hyderabad and used for the experiments. The animals were maintained at 22-24^{o}C and 50-60% relative humidity with 12 hr light and 12 hr dark cycle, housed at animal facility at University of Hyderabad. Mice and rabbit were fed with pelleted diet, and both food and water were administered on an *ad libitum* basis. All mice used for experiments wereaged 8-10 weeks old and weighed approximately 25g. For maintenance of *Anopheles stephensi* colony, rabbits aged 2-3 months were used as source of blood meal. All the experiments in which animals were involved have been performed according to the regulations of the University of Hyderabad Institutional Animal Ethical Committee (IAEC).

2.2.2 Parasite lines

Plasmodium berghei is a rodent malaria species, originally isolated from thicket rats in Central Africa. *P. berghei* is one of the four common murine models and is popular for reverse genetics because of its relative ease in transfection. All the transgenic or knockout parasite lines were generated using *P. berghei* ANKA strain. For conditional silencing of CSP, *P. berghei* ANKA TRAP@FLPL and UIS4@FLP transgenic parasites were used.

2.2.3 Bacteria

E. coli XL-1 Blue MRF cells (Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1* gyrA96 relA1 lac *[F′* proAB lacI^{q}ZΔM15 *Tn*10 *(Tet^{r})]*) were used for cloning and propagation of the targeting constructs used for *Plasmodium* transfections.

2.2.4 Retrieval of target gene and protein sequences

Two *Plasmodium* gene databases namely plasmoDB (http://plasmodb.org/plasmo/), and GeneDB database (http://www.genedb.org/Homepage/Pberghei) hosted by Sanger Institute were used to retrieve the protein, gene, and the untranslated sequences of the target genes.

S. No.	Primer Name	Primer Sequence (5' – 3')
1	FP1	GA*GAATTC*ATGAAGAAGTGTACCATTTTAGTTGT
2	RP1	AA*GCGGCCGC*TGTTTATTAAAATTAATTAAAGAATACTAA TACTAATA
3	FP2	AT*GGCGCGCC*ATAAACATTACGCATGATTATAAATATTT
4	RP2	GC*GAATTC*TAAAGATCTTATACATCCAGATAAATTATC
5	FP3	ACCCTCACATAAGACAATCCTTATAAAAGGAGTT
6	RP3	TATATAATTGAATAAATAACATAAAAAGATGGCA
7	FP4	GGAAACAGCTATGACCATG
8	RP4	TTCGACAAAGGATAACGAATATTGTAT
9	FP5	TAGTATCACAGAGGAATGGTCTCAATGTAACG
10	RP5	TGATTACTTTCACATTTCATCCAAAGTGGA
11	TRAP RT FP	CTCCCAAACAATCTACCAGAAAGTCCATCTGAC
12	TRAP RT RP	TATATGGGTTATCATCCGTTGGTGGTACATGCTT
13	UIS4 RT FP	GAACCCTGAAGTTCGAGAAAAATTTAGAATTGG
14	UIS4 RT RP	TCTGGTGAATTTTCTGGTGAATTTTCTGGTGA
15	18S RT FP	GGGGATTGGTTTTGACGTTTTTTG
16	18S RT RP	AAGCATTAAATAAAGCGAATACATCCTTA

Table 1: List of primers and their sequences used for the construction of transfection plasmids, to confirm the site specific integrations, to check the FLPL/FLP mediated excision in CScKO TRAP@FLPL and UIS4@FLP parasites, and real time PCR analysis.

2.2.5 Construction of the *CSP* conditional silencing vector

CSP conditional silencing vector was constructed by cloning the full length (1023 bp) *open reading frame* (*ORF*) and 545 bp of the *3' untranslated region* (*UTR*) of *CSP* gene (PBANKA_0403200) in the conditional silencing vector pSKC-TRAP-hDHFR-GFP (Appendix - Fig. A1) for targeting *CSP* locus by double homologous recombination. To clone ORF, 50µl Polymerase Chain Reaction (PCR) was setup using 40ng *P. berghei* ANKA genomic DNA, 0.25µM FP1 containing *EcoRI* restriction site, 0.25µM RP1 containing *NotI* restriction site, 1mM deoxyribonucleoside triphosphates (dNTPs) (Invitrogen, Cat# R72501), 0.5U of Platinum Pfx DNA polymerase (Invitrogen, Cat# 11708021), and 1X PCR buffer containing 2.5mM $MgSO_4$. *CSP ORF* was amplified using thermal cycler conditions:

94°C for 2min
94°C for 30 sec
54°C for 30 sec
68°C for 1min 15 sec } 30 cycles
68°C for 10min

Similarly, *3' UTR* was amplified using 0.25µM FP2 containing *AscI* restriction site and 0.25µM RP2 containing *EcoRI* restriction site. The thermal cycler conditions used to amplify *3' UTR* were

94°C for 2min
94°C for 30 sec
56°C for 30 sec } 30 cycles
68°C for 45 sec
68°C for 10min

Amplified products of both *CSP ORF* and *3' UTR* were excised from the gel and extracted following agarose gel electrophoresis using Purelink quick gel extraction and PCR purification kit (Invitrogen, Cat# K220001). The purified products were adenylated by adding 1µl of 10X PCR buffer with $MgCl_2$, 0.2µl of 10mM dATP, and 0.1U Taq DNA polymerase (Invitrogen, Cat# 10342053) and incubated at 72°C for 15min. The adenylated PCR product was ligated in TA vector pTZ57R/T in 1:3 vector insert ratio with 1X ligation buffer, and 2U ligase (Thermo Scientific, Cat# EL0011) and incubated overnight at 16°C. The ligation mixture was transformed into XL-1 Blue MRF competent cells and plated on ampicillin (Sigma, Cat# A9518) and tetracycline (Himedia, Cat# CMS219) plate to select the recombinant clones. The plates were incubated overnight at 37°C, and the positive clones were screened by colony PCR with M13 primers present in the vector. The positive clones were inoculated in 5ml LB broth and after overnight incubation, the plasmid was isolated by miniprep and the clones were further confirmed by double digestion. *CSP ORF* in TA vector was confirmed by digesting 2µg of the plasmid with 2U of EcoRI (Thermo Scientific, Cat# ER0271) and 2U of NotI (Thermo Scientific, Cat# ER0593) in the presence of compatible 1X BamHI buffer with 0.1mg/ml BSA in 30µl reaction mixture. Similarly, *CSP 3'UTR* in TA vector was confirmed by digesting 2µg of the plasmid with 2U of AscI (Thermo Scientific, Cat# ER1892) and 2U of EcoRI in the presence of compatible 1X BamHI buffer with 0.1ml/ml BSA in 30µl reaction mixture. The reaction mixtures were incubated at 37°C for 3 hr, and release of inserts was confirmed by agarose gel electrophoresis.

CSP ORF and *3' UTR* sequences were confirmed by sequencing the respective TA vectors with M13 sequencing primers. *CSP 3' UTR* and *ORF* were cloned in tandem in TA vector using EcoRI and one of the restriction enzymes present in the TA vector

backbone and confirmed by digestion with AscI and NotI restriction enzymes. This was subcloned into pSKC-TRAP-hDHFR-GFP plasmid to obtain *CSP* conditional KO plasmid (Appendix – Fig. A2). Approximately 3μg each of *CSP 3'UTR+ORF* TA plasmid (to release insert) and pSKC-TRAP-hDHFR-GFP plasmid were digested with AscI and NotI, insert and vector were gel extracted following agarose gel electrophoresis, and ligation reaction was set in 1:3 vector insert ratio. Approximately 5-6μl of this ligation mixture was transformed into XL-1 Blue competent cells and plated on LB plate with chloramphenicol (Sigma, Cat# C0378) and tetracycline antibiotics. The positive clones were screened for the insert by colony PCR, and the plasmid was isolated from the positive colony by miniprep. The presence of insert was confirmed by digestion with AscI and NotI. The *CSP* conditional silencing plasmid was isolated in large scale by maxiprep, linearized with EcoRI restriction enzyme, DNA was purified and transfected in the transgenic lines - TRAP@FLPL and UIS4@FLP parental lines.

2.2.6 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to separate DNA molecules, according to their charge, size, and conformation. A 1% agarose (w/v) in 1x TAE buffer diluted from 50X stock (24.2 g - tris base, 5.7 mL - acetic acid and 10mL - 0.5 M EDTA made up to 100ml with double distilled H_2O to get 50X TAE) was boiled in a microwave to dissolve the agarose. The solution was cooled, ethidium bromide (0.4μg/ml) was added and poured into gel tank with the comb slotted to provide the wells for loading DNA samples. The DNA samples were mixed with 6X loading dye and loaded to the wells. Three microliters of 1kb DNA ladder (Thermo Scientific, Cat # SM0333) was loaded along with the samples, and DNA fragments were separated in 1X TAE buffer at 100V for 40-60 min. Gels were visualized using ultraviolet illumination in gel documentation system.

2.2.7 Preparation of *E. coli* XL-1 Blue MRF competent cells and transformation

Cryopreserved glycerol stock of *E. coli* XL-1 Blue MRF was streaked on the LB agar plate containing tetracycline and incubated overnight at 37°C. A single colony was inoculated in 3ml LB broth containing tetracycline and incubated overnight at 37°C with 180 rpm shaking. Next day morning, 1% of the overnight culture was inoculated in 20ml broth containing tetracycline and incubated at 37°C with shaking for 2-3 hrs,

and the absorbance was measured at 600nm. When the optical density (OD) reached 0.5, the culture was incubated on ice for 30 min and centrifuged at 6000 rpm for 10 min at 4^{o}C. The bacterial cell pellet was resuspended in 3ml of cold 0.1M $CaCl_2$ solution and incubated on ice for 1 hr. Following incubation, the cells were centrifuged at 6000 rpm for 10 min at 4^{o}C. The bacterial pellet was resuspended in 1ml cold 0.1M $CaCl_2$ and 100µl was used for immediate transformation. For the long-term storage of competent cells, the pellet was resuspended in 1 ml of cold 13% glycerol (v/v) in 0.1M $CaCl_2$, and 100µl was aliquoted into 1.5ml eppendorf tubes, were snap frozen in liquid nitrogen and stored at -80^{o}C. For transformation, 5-6µl of the ligation mixture or 50-100 ng of the plasmid was added to the competent cells, tapped the bottom of the tube 3-4 times and incubated on ice for 20min and given heat shock at 42^{o}C for 60 sec followed by incubation on ice for 5min. One ml of fresh LB broth was added and incubated at 37^{o}C with shaking for 1hr followed by centrifugation at 3000 rpm for 5min. The pellet was resuspended in 100µl fresh LB broth and spread on the LB agar plate with the appropriate antibiotics and plates were incubated overnight at 37^{o}C in an upright position.

2.2.8 Small scale isolation of plasmids by miniprep

Plasmid was isolated from 3-5ml of overnight bacterial culture using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Cat# K0503) according to the manufacturer's protocol. In brief, the overnight culture was centrifuged at 8000 rpm for 5min and the pellet was resuspended in 250µl of resuspension solution and lysed with 250µl of lysis solution. Following this, 350µl of neutralization solution was added and centrifuged at 13000 rpm for 5min. The supernatant was loaded onto GeneJet spin column and centrifuged at 8000 rpm for 1min. The column was washed twice with 500µl of wash solution and the plasmid DNA was eluted in 30µl of elution buffer. The plasmid concentration was estimated by nanodrop and stored at -20^{o}C.

2.2.9 Large scale isolation of transfection plasmid by maxiprep

Plasmodium targeting construct used for performing transfection was obtained in large scale by maxiprep. Around 20-30ng of the targeting plasmid was transformed into XL-1 Blue competent cells and plated on LB plate with corresponding antibiotics and incubated overnight at 37^{o}C. A single colony was picked and inoculated in 5ml LB

broth containing antibiotics used for primary culture. Approximately 1% of the primary culture was inoculated in 500ml LB both with antibiotics and incubated at 37^{o}C overnight. The culture was harvested after incubation and centrifuged at 8000 rpm for 10 min at 4^{o}C. This pellet was used to isolate the plasmid by Sureprep plasmid Maxi kit (Genetix, Cat# NP-15162) according to manufacturer's protocol. In brief, the pellet was resuspended in 12ml resuspension buffer (PRB1) and lysed with 12ml lysis buffer (PLB2), inverted 5-6 times and incubated at room temperature for 2 min. This was neutralized by adding 12ml of precooled neutralization buffer (PNB3) and inverted 5-6 times and incubated on ice for 5 min. Bacterial lysate was filtered with a filter supplied with the kit. The SurePrep maxi column was equilibrated with 6 ml of equilibration buffer (PQB2), and the filtrate was loaded onto the SurePrep column. The column was washed twice with 32 ml of wash buffer (PWB3) and DNA was eluted in 15ml of elution buffer (PEB5). Plasmid DNA eluted was precipitated with 11 ml of isopropanol and centrifuged at 10,000 rpm for 40 min at 4^{o}C. The pellet was washed with 5ml of 70% ethanol and centrifuged at 10,000 rpm for 20 min, the supernatant was discarded, and the pellet was air dried to remove residual ethanol. The pellet was dissolved in 100µl of 1X TE. The concentration of the plasmid was estimated using nanodrop and the plasmid was digested with the specific restriction enzymes present on either side of the targeting construct. The digested DNA was run on 0.5% agarose gel and the corresponding band was gel excised and its concentration was estimated. DNA was gel extracted, and concentration was estimated. Around 4-5µg of the targeting construct was used for transfection.

2.2.10 *In vitro* culture of *P. berghei* asexual stages

Transfection of *P. berghei* mature schizonts purified from parasite overnight cultures and selection of recombinant parasites was performed as described previously (Janse *et al.*, 2006b).

Swiss mice were injected intraperitoneally with either *P. berghei* ANKA (WT ANKA), *P. berghei* TRAP@FLPL or UIS4@FLP transgenic parasites from the frozen stock. When the parasitemia was around 0.5-2%, the blood was collected and passaged to four mice such that each mouse received 10^7 iRBCs. When the parasitemia of the infected mice reached around 4-5%, the blood was collected by cardiac puncture

using heparinized syringes from mice anesthetized with ketamine-xylazine (200µl of mouse anesthesia per mouse, prepared by mixing 0.8ml Ketamine (50mg/ml) + 0.3ml Xylazine (20mg/ml) + 3.9 ml 1X PBS). The blood from all the four infected mice was collected in a 50ml tube containing 250µl of 200U/ml Heparin (Sigma, Cat# H3393). Nearly 10ml of schizont medium (Schizont medium was prepared by mixing 396.5 ml Roswell Park Memorial Institute (RPMI) medium (Lonza BioWhittaker, Cat# 12-115F), 100 ml Fetal Bovine Serum (Hyclone, Cat# SH30071.03) and 3.5 ml of gentamicin (Sigma, Cat# G1397) and filter sterilized.) was added to blood and centrifuged (Eppendorf 5810R) using swinging bucket rotor at 200 g for 8 min without break or deceleration. The cell pellet was gently resuspended in 5 ml fresh schizont medium by swirling the tube and was split into four T75 flasks containing 20ml schizont medium. The cultures were gassed gently (5% CO_2, 5%O_2, 90%N_2) for 2-3 min and incubated overnight (16-20 hrs) at 36.5^{o}C with a gentle shaking of around 55 rpm in a shaker incubator. The next day morning, 0.5 ml of the culture was centrifuged and a thin smear was made from the concentrated cell pellet. The smear was stained with Giemsa and checked for the mature schizonts using a light microscope under 100X oil immersion objective lens (Lawrence and Mayo XSZ-N107T).

2.2.11 Purification of Schizonts

Purification of schizonts was achieved by density gradient centrifugation using 60% Nycodenz solution. Nycodenz solution is prepared by mixing 27.6g Nycodenz powder, 500µl of 5mM Tris-HCl, 300µl of 3mM KCl, and 60µl of 0.5M EDTA and making up the volume to 100ml with double distilled water. The solution was autoclaved and stored at 4^{o}C. To enrich schizonts, 25ml of the cultured schizonts were gently transferred to a 50 ml tube followed by addition of 10 ml of the 60% nycodenz solution (diluting nycodenz solution with 1X phosphate buffered saline (PBS), pH 7.4 (Gibco, Cat # 10010)) at the bottom of the tube without disturbing the upper schizont culture. The tubes were centrifuged at 380g for 20 min using a swing bucket rotor at room temperature with no break. The brown interphase of the schizonts was carefully collected in a fresh 50 ml tube using a Pasteur pipette (Fisher Scientific, Cat#13-678) and centrifuged at 1500 rpm for 7 min at room temperature. The pellet was washed with 10 ml schizont medium and centrifuged at 1500 rpm for 7 min at room

temperature. The supernatant was removed and the schizont pellet was used for electroporation.

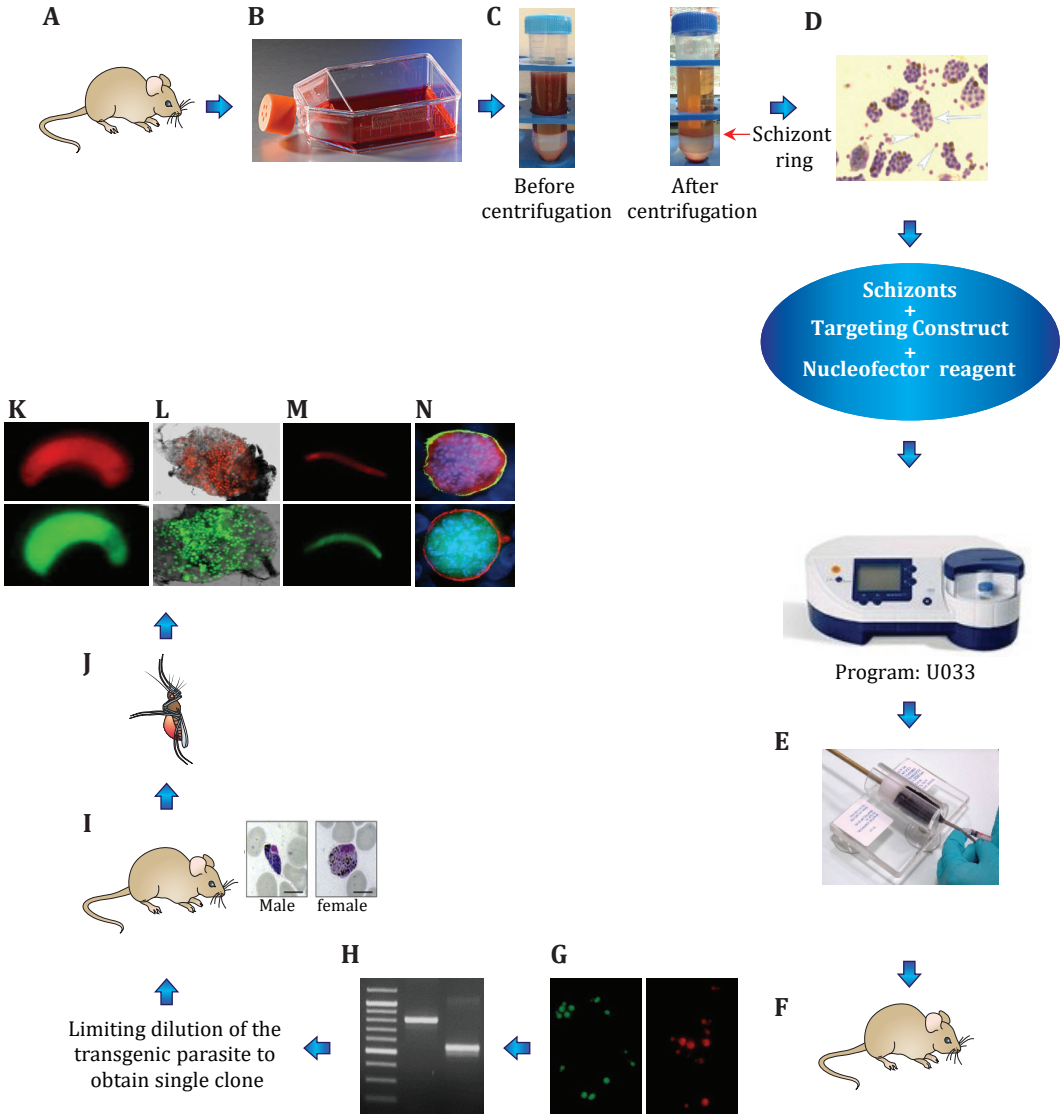

Fig. 10 Schematic representation of *P. berghei* transfection, drug selection, confirmation of site specific integration and phenotypic characterization of genetically modified parasites. *P. berghei* infected blood was collected from the mouse having 2-5% parasitemia (A) and an overnight *in vitro* culture was set up (B). Next day, schizonts were enriched on a 60% nycodenz density gradient by centrifugation (C). The purified schizonts (D) were collected and electroporated with the targeting construct using Amexa nucleofector device and immediately injected intravenously into mouse (E). Pyrimethamine, an antimalarial drug was provided orally in drinking water to mouse harbouring transfected parasites that facilitate selection (F). The transfected parasites were observed for either GFP or mCherry fluorescence (G) depending on the reporter cassette used in the targeting construct. Site specific integration was confirmed by PCR (H). Clonal population of successful transfectants was obtained by limiting dilution and the cloned line was passed through mosquitoes (J) to analyze the phenotype in stages like ookinete (K), oocyst (L), sporozoite (M) in mosquito and liver stages (N) in the vertebrates. Images adapted and modified from Janse *et al*, 2006b.

2.2.12 Electroporation of purified schizonts

The schizont pellet was resuspended in 500µl schizont medium and the number of schizonts were counted using hemocytometer. Nearly 2-3 x 10^7 schizonts were aliquoted into a 1.5ml eppendorf and centrifuged at maximum speed (around 10,000-12,000 rpm) for 5 sec. Ninety microliters of Mouse T-cell nucleofector reagent (Lonza, Cat#VPA-1006) was mixed with 20µl of supplement 2 solution (provided in the kit) and 5-6µg of targeting DNA construct. This mixture was added to the schizonts and transferred to an electroporation cuvette and electroporated using Amaxa Nucleofector II device applying the manufacturers' pre-defined program U-033. Immediately after the electroporation, 100µl of the schizont medium was added and the electroporated parasites were injected intravenously into the tail vein of a naive mouse.

2.2.13 Selection of the recombinant parasites

The day following transfection, blood smear was made from mice harbouring transfected parasites and parasitemia was monitored by Giemsa staining. When the parasitemia reached around 0.5-1%, the recombinant parasites were selected by oral administration of antifolate drug pyrimethamine (Sigma, Cat#46706) given at a concentration of 7 mg/100 ml through drinking water to the mice. Pyrimethamine is insoluble in water at neutral pH. Therefore it was initially dissolved in 1ml DMSO and then diluted to 30-40ml with drinking water and pH was adjusted to 3.5-4.0 with 1N HCl and made to 100ml. Parasitemia was monitored daily by making blood smears. The parasitemia decreased gradually and no parasites were observed on day 7 post transfection. On day 8 pyrimethamine resistant parasites started to appear and the parasitemia increased gradually. When the parasitemia was around 3-4%, the parasites were observed for the presence of GFP/mCherry fluorescence based on the reporter cassette in the targeting DNA construct. The pyrimethamine resistant parasites were cryopreserved, and genomic DNA was isolated for genotyping. For each targeting construct, two independent transfections were done and labeled as T1 and T2. The phenotypic characterization of mutants was performed with clonal lines obtained after limiting dilution of two independent transfections.

2.2.14 Giemsa Staining

The course of parasitemia was determined by Giemsa (Sigma, Cat#GS1L) staining of smears made from caudal vein puncture. The thin smear was air dried and fixed with methanol. Giemsa stain was diluted to 5 times with tap water, and the smear was stained for 10min. After 10 minutes, the stain was discarded, the slide was washed carefully with tap water and the slides were left in an upright position to air-dry. The stained smear was examined under a light microscope with immersion oil and 100X objective. Parasites were identified as bodies stained within the red blood cells and were counted in fields of uniformly spread out RBCs to avoid overlapping and duplication. At least 10 fields each containing around 300 RBC were examined for parasitemia and the final parasitemia was noted as the percent of red blood cells infected. The life-cycle stage of the parasites were identified by the distinctive morphological features of each stage.

2.2.15 Cryopreservation of the blood stage parasites

The blood stages were cryopreserved in liquid nitrogen for long term storage. When the parasitemia is around 3-5%, the blood was collected in heparin (10Units for 1 ml blood). For cryopreservation, 350µl of blood was added to 650µl of freezing solution prepared by adding 1ml Glycerol (Invitrogen, Cat# 15514011) to 9ml Alsever's solution (Sigma, Cat# A3551) and mixed gently. The solution containing blood and cryopreservative were aliquoted as 250µl samples, in 1.2 ml cryogenic vials (Corning, Cat# CLS430658). The samples were cooled gradually using a freezing container (Nalgene, Cat# 5100), kept at -80^{o}C and the vials were later shifted to liquid nitrogen.

2.2.16 Isolation of parasites from infected blood

Approximately 400µl of blood was collected from *P. berghei* infected mice in 1.5ml eppendorf containing 40µl of heparin (200U/ml) and centrifuged at 1500 rpm for 8 min. The cell pellet was washed twice with 1X PBS and centrifuged at 1500 rpm for 8 min. To the pellet, 400µl of 0.02% saponin (Sigma, Cat# 47036) prepared in 1X PBS was added and incubated at room temperature for 2-3 min to lyse the RBC. After incubation, the sample was centrifuged at 6000 rpm for 4 min and the pellet was washed with 400µl of 1X PBS. All centrifugations were performed at 4^{o}C. The

supernatant was discarded and the parasite pellet was used for either genomic DNA isolation (gDNA), RNA isolation or for making protein lysates.

2.2.17 Isolation of genomic DNA from parasites

The genomic DNA was isolated from blood stage parasites for the amplification of target genes or for genotyping the transgenic parasite lines. Blood was collected from infected mice and parasites were isolated as described in earlier section. The gDNA was then isolated using Nucleospin tissue kit (MACHEREY-NAGEL) according to the manufacturer's protocol. In brief, the parasite pellet was resuspended in 200µl of buffer T1. To this, 25µl of proteinase K (20mg/ml) and 200µl of buffer B3 were added, vortexed vigorously and incubated at 70°C for 10-15 min. After incubation, DNA was precipitated with 210µl of 100% ethanol and vortexed. This solution was loaded onto the nucleospin column and centrifuged at 11000 g for 1 min. Flow through was discarded and the column was washed with 500µl of buffer BW followed by 600µl of buffer B5 and centrifuged at 11000 g for 1 min. The column was dried by spinning at 13000 rpm for 2 min. Nucleospin column was placed in a fresh 1.5 ml eppendorf and 50µl buffer BE preheated at 70°C was added at the center of the disk and incubated at room temperature for 1 min followed by centrifugation at 13000 rpm for 1 min. The concentration of DNA was estimated by nanodrop spectrophotometer using buffer BE as blank.

2.2.18 Cloning the recombinant parasites by limiting dilution

The transfected parasites were selected on pyrimethamine to enrich integrants. To eliminate the wild type parasites that survived drug treatment, homogeneous parasite population was obtained from a single parent. To achieve this, limiting dilution was done in 15 mice by injecting 0.5 parasite per mouse (one parasite per two mice) resulting in 15-20% infection rate in mice. A donor mouse was infected with cryopreserved mixed population parasites obtained after transfection. When the parasitemia was around 0.5%, blood was collected from the mouse and diluted to 1:10,000 using incomplete RPMI and the number of RBC were counted using hemocytometer. The number of iRBC/µl were calculated and the sample was further diluted to 0.5 parasites/200µl using the incomplete medium. The diluted blood was injected intravenously into 15 mice and each mouse received 200µl. Blood smears

were made from day 9 post infection to check for the parasites. Around 15-20% of the mice became positive for infection. Blood was collected both for cryopreservation and genomic DNA isolation. The clonal population was confirmed by PCR with integration specific primers.

2.2.19 Confirmation of the recombinant parasites by integration specific PCRs

To check the correct integration in the recombinant parasites, a set of primers were designed on either side of the two homology arms, one in the genomic region and other in the replacement cassette. The correct 5' and 3' integrations were confirmed by designed primers respectively in the genomic region before the homology arm and in the replacement cassette after homology arm. The products of correct integration were analysed on 1% agarose gel along with a 1Kb DNA ladder.

2.2.20 Phenotypic analysis of mixed blood-stage parasite *in vivo*

The cloned parasite was injected into a naive mouse and parasitemia was checked by Giemsa stained blood smears. When the parasitemia was around 0.5%, blood was drawn and iRBCs/µl were counted using hemocytometer. The blood was diluted to get 1000 iRBCs in 200µl. A group of 5 mice were injected with 1000 iRBCs and blood stage propagation of the cloned parasite line was monitored. Similarly, 1000 iRBCs of WT GFP/mCherry were injected intravenously into a group of 5 mice as a control. Smears were made from day 4 to 8 post infection, and the mean percentage of parasitemia was calculated and plotted as a graph.

2.2.21 Rearing of *Anopheles stephensi* (*A. stephensi*) mosquitoes

To study the developmental stages of *P. berghei* in mosquito and in mammalian host, *A. stephensi* colony was maintained. Freshly emerged 3-8 day old male and female mosquitoes were allowed to mate in the breeding cages. For the production of eggs, female mosquitoes were fed on blood meal. Mosquitoes were starved for 5-6 hrs and a rabbit was anesthetized with a combination of 0.3ml of xylazine (20mg/ml) and 0.6ml of ketamine (50mg/ml) injected intramuscularly, and placed on the adult mosquito cage for 15-20 min and the female mosquitoes were allowed to take a blood meal. Two successive blood meals were given with an interval of 24 hrs. After 36 hrs of the first blood meal, a water bowl was placed in the breeding cage to allow the female

mosquitoes to lay eggs. Eggs were collected for 4 consecutive days and transferred to trays maintained at 27°C and 70-80% relative humidity (RH) to facilitate hatching into larva. Hatched larvae were fed with powdered food made from fat-free special K (Kellogs) and Wheat germ (Avees) in 60 and 40 ratio. The water was changed once in every 2-3 days and the trays were maintained with optimal larval density. From day 6-7 post-hatching, the larvae transformed to pupae. These pupae were collected and transferred to the new cages where the mosquitoes emerged from the pupae. Adult mosquitoes were fed with 10% sucrose solution using cotton balls.

2.2.22 Transmission of malaria to *Anopheles* mosquitoes

To transmit malaria to mosquitoes, an infection cage was made with around 600-700 female *Anopheles* mosquitoes. The male and female mosquitoes were separated by placing palm on the outside of the adult mosquito cage that attracted only female mosquitoes by sensing the body temperature. The female mosquitoes that gathered in the palm region of the cage were collected using a tube connected to a vacuum pump. The females collected were transferred to a new cage and shifted to an environmental chamber maintained at 20°C and 80% RH. The female mosquitoes separated for preparing an infection cage were starved for 2 hrs. Five *P. berghei* infected BALB/c mice that were positive for gametocytes were anesthetized by injecting 200µl of mouse anesthesia. The sedated mice were kept on the infection cage harbouring starved mosquitoes and allowed to take blood meal for 15 min with three changes in the position of mice on the cage to ensure all the mosquitoes received blood from gametocyte positive mice. Second successive blood meal was given at around 24 h of first blood meal. The cage was maintained in the environmental chamber for 18-21 days to facilitate the completion of the sexual development, formation of sporozoites in the oocysts and their migration to the salivary gland. During this period, mosquitoes were fed with cotton pads soaked in 10% sucrose solution that was replaced on alternate days.

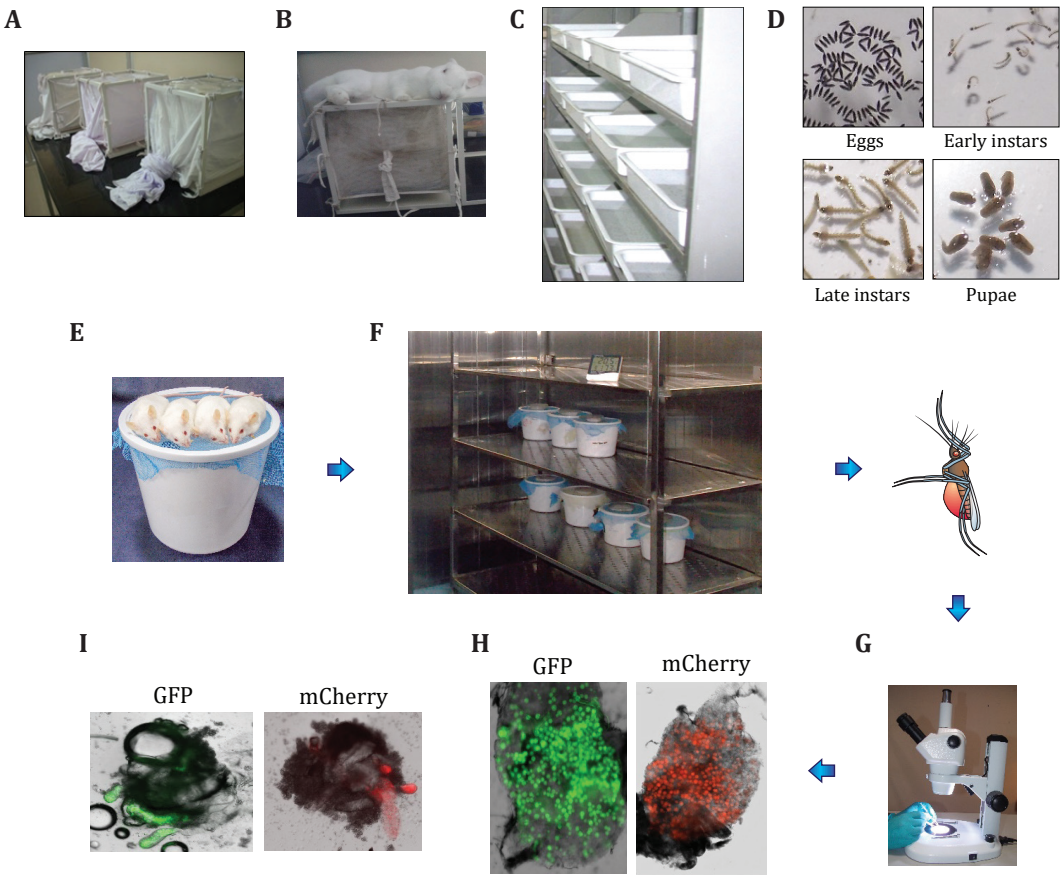

Fig. 11 Maintenance of *A. stephensi* colony, and transmission of *P. berghei* to mosquito to study the developmental stages and to isolate sporozoites. To maintain the *A. stephensi* colony breeding cages were prepared by placing pupae in water bowl to allow the mosquitoes to emerge. The adult mosquitoes mate within 24-36 hours after they emerge (A). Anesthetized rabbit was placed on the breeding cage and mated female mosquitoes were allowed to take blood meal (B). Two successive blood meals were given at an interval of 24 hours. Thirty six hours after second blood meal, a water bowl was placed inside the breeding cage and eggs were collected for four consecutive days. The eggs were then transferred into environmental chamber maintained at 27°C and RH 80% (C). Under these conditions the eggs hatch and transforms into a series of instars and finally into pupae (D). The pupae were collected and placed inside cages for emergence of mosquitoes. For preparation of infection cages, female mosquitoes are separated using a vacuum pump. The cage containing female *Anopheles* mosquitoes were allowed to obtain an infected blood meal from mice carrying *P. berghei* gametocytes (E). The infected cage was placed in an environmental chamber maintained at 22°C and RH 80% to facilitate the completion of sexual reproduction and development of *P. berghei* (F). Mosquitoes were dissected under dissection microscope (G) on day 14 (H) and day18 (I) post infection to observe respectively the oocyst sporulation in the midgut, and sporozoites in the salivary glands under a fluorescent microscope. Photo credits Ravi Jillapalli.

2.2.23 Monitoring the formation of oocysts in the mosquito

Successful transmission of malaria to mosquitoes was monitored by observing the oocysts on the mosquito midgut. Since WT GFP and both the CSP conditional KO lines constitutively express GFP, all the mosquito stages of the *Plasmodium* were

monitored under a fluorescent microscope. On day 14 and day 21 post infection, around 30-40 mosquitoes were dissected to isolate the midguts that provided an idea of the parasite burden in the mosquito. The number of GFP expressing oocysts and the sporulation within the oocyst was observed by using 100X objective lens with immersion oil under a fluorescent microscope. On day 14 and 21, oocyst sporulation was quantified by crushing the dissected midguts and the released sporozoites were counted using a hemocytometer.

2.2.24 Dissection of salivary glands and purification of sporozoites

To isolate the sporozoites from salivary glands, day 21 post infection mosquitoes were collected, anesthetized by keeping on ice for 5-10min. The mosquitoes were washed thrice with 70% ethanol followed by 3 washes in incomplete RPMI containing antibiotic and antimycotic solution (Gibco, Cat# 15240062). The salivary glands of the mosquito were dissected under a dissection microscope and collected in 1.5ml eppendorf tube in a small volume of about 20-30µl. The dissected salivary glands were disrupted by crushing with a plastic pestle to release the sporozoites. The crushed sample was centrifuged at 800 rpm for 3 min at 4^{o}C (Eppendorf centrifuge, Cat# 5415R). The supernatant was collected in a fresh 1.5ml tube. For sporozoite enumeration 2µl of the supernatant was diluted to 20µl (1:10 dilution). Ten microliters of sample was placed in Neubauer's chamber. The number of sporozoites from 4 quadrants were recorded and averaged. The sporozoites in the main stock were calculated using the formula:

No. of Sporozoites= Average no. of sporozoites from 4 quadrants X 10 (dilution factor) X 10^4 (Hemocytometer correction)/ml

2.2.25 Immunofluorescence assay of sporozoites

Day 21 midguts were disrupted by crushing to release sporozoites. The crushed sample was centrifuged at 800 rpm for 3 min to remove the cell debris. The sporozoites in the supernatant were counted and around 5000 sporozoites were spotted on a spotted slide (Thermo Scientific, Cat#1014326412WHITE), fixed with 4% paraformaldehyde for 20 min at room temperature and washed for 1 min with TBS (pH 7.4). Nonspecific blocking was done with 3% BSA prepared in TBS (pH 7.4) and was incubated for 1 h at 37^{0}C. This was followed by incubation with 3D11 mouse

monoclonal antibody specific for the repeat region of *P. berghei* circumsporozoite protein (Yoshida *et al.*, 1980). The primary antibody (1:1000 dilution in 3% BSA) was incubated for 60 min at 37°C. Following incubation, the cells were washed with TBS, TBST (0.1% tween 20 in TBS) and TBS for 15 min each. The slide was next incubated with an anti-mouse Alexa Flour 594 secondary antibody (Molecular probes, Cat# A11005) at 1:250 dilution in 3% BSA for 1 h at 37°C along with DAPI at 1:1000 dilution (Sigma, Cat#D9564). Slides were again washed with TBS, TBST and TBS each for 15 min, air dried and mounted with ProLong gold antifade mountant (Molecular Probes, Cat#P36930) and covered with coverslips. The border of the coverslip was sealed with transparent nail polish and preserved in dark. The samples were visualized using Nikon Eclipse upright fluorescent microscope and images were acquired and analyzed with NIS elements advanced research software.

2.2.26 Total RNA isolation

Around 30 mosquitoes infected with *P. berghei* wild type GFP were collected on day 8, day 10, day 12 and day 14 post infection and midguts were dissected in incomplete DMEM and RNA was isolated using Purelink RNA Minikit (Ambion, 12183018A). The guts were centrifuged and the pellet was crushed with a plastic pestle, lysed with 300µl of lysis buffer containing 1% β-mercaptoethanol, vortexed and passed through insulin syringe for 10 times. Approximately, 300µl of 70% ethanol was added to the lysed cells and vortexed. The mix was applied to the spin column and centrifuged at 8000 rpm for 30 sec at room temperature. Flow through was discarded and the column was washed with 700µl of wash buffer I. The column was washed twice with 500µl of wash buffer II. All the washes were done at room temperature at 8000 rpm for 30 sec. Spin column was dried by centrifugation at 13000 rpm for 2min. RNA was eluted in 50µl of nuclease free water and its concentration was estimated using nanodrop using nuclease free water as blank. Two micrograms of RNA was subjected to DNase treatment prior to cDNA synthesis. Remaining RNA was stored at -80°C for further use.

2.2.27 DNase I treatment and cDNA generation

Two micrograms of RNA was used for reverse transcription following treatment with DNase I (Invitrogen, 18068015) to eliminate the genomic DNA

contamination. DNase treatment was performed in a reaction mixture consisting of 1µl 10X DNaes I reaction buffer, 1µl DNaseI (1U/µl), 2µgm RNA sample and volume was made up to 10µl with DEPC treated water and incubated at room temperature for 20 min. After incubation, the DNase I was inactivated by the adding 1µl of 25mM EDTA and heated for 10 min at 65°C. The DNaseI treated RNA was used for cDNA generation and the reaction components used were

10X PCR buffer	- 3.0µl
dNTPs (2.5mM each)	- 1.5µl
Random Hexamers	- 1.5µl
RNase Inhibitor	- 1.5µl
Reverse transcriptase	- 1.5µl
RNA	- 2.0µgm

The reaction volume was made up to 30µl with DEPC treated water. The thermal cycler conditions used for cDNA generation are 25°C for 10 min, 42°C for 20 min and 98°C for 5 min. The cDNA generated was stored at -20°C.

2.2.28 Real Time PCR

The gene expression of *TRAP* and *UIS4* during the oocyst development was quantified by real time PCR using absolute quantification method. A 150 bp of *UIS4* and 148bp of *TRAP* fragments were amplified from *P. berghei* genomic DNA using the primers indicated in the table and cloned in pTZ57R/T vector. The clones were confirmed and log dilutions of plasmids were generated and used as gene specific standards with a range that covered from 10^8 copies/µl to 10^2 copies/µl. Similarly, *18S rRNA* standards were generated for internal control. Real time PCR was set up in triplicates using iQ SYBR Green Supermix (BioRad, Cat# 1708882) along with 0.25µM each of forward and reverse primers and cDNA or the standards as a template. A ratio of absolute copy numbers of *TRAP* or *UIS4* versus absolute copy number of *18S rRNA* was obtained and the normalized values were plotted in a graph.

2.2.29 Preparation of protein lysates, SDS-PAGE and Western blotting

Protein levels of CSP in day 21 oocysts of WT GFP and both the CSP conditional lines was analyzed by Western blotting. Around 30 mosquitoes infected with either WT GFP, CScKO TRAP@FLPL or UIS4@FLP were dissected and midguts were isolated. The midguts were lysed using 30µl of 2X SDS loading buffer (100 mM Tris-HCl pH 6.8;

4% SDS; 0.2% (w/v) bromophenol blue; 20% glycerol) and heated at 100°C for 8 min. The samples were resolved in 12% SDS-PAGE at 100V and electrotransferred onto nitrocellulose (NC) membrane at 25V for 16 hrs (Towbin *et al.*, 1979). The NC membrane was first blocked with 3% (w/v) fat free milk powder in 1X TBS (pH 7.2) for 1 hr with rocking at room temperature. The primary antibodies were diluted in 3% nonfat milk in TBS (1:1000 of both 2E6: anti-HSP 70 mouse monoclonal and 3D11: anti-CSP mouse monoclonal) and the membrane was incubated overnight at 4°C (with rocking) with diluted antibody in milk solution. The membrane was washed with TBS, TBST (0.1% tween 20) and TBS, each for 15 min respectively with rocking at room temperature. This was followed by incubation with horseradish peroxidase (HRP) labeled anti-mouse secondary antibody used in 1:2000 dilution in 3% nonfat milk in TBS at room temperature. The blot was washed with TBS, TBST and TBS for 15 min at room temperature. Protein bands were detected using AmershamTM ECLTM prime Western blotting detection kit (GE healthcare, Cat# RPN2232). Images were captured in Bio-rad versadoc and analysed by image lab software.

2.2.30 Confirmation of 3'UTR excision by PCR

FLP/FLPL mediated excision of the *FRT*ed DNA (*TRAP 3' UTR* and *hDHFR* cassette) was confirmed by PCR using a primer set FP5/RP5 that differentially amplified either 2838bp (non-excised) or 588bp (excised) product. Genomic DNA was isolated from day 21 midguts post infected with either WT GFP, CScKO TRAP@FLPL or UIS4@FLP. PCR reaction was set up with 0.25µM each of FP5 and RP5, 1X PCR buffer with 2.5mM $MgCl_2$, 1mM each DNTPs and 0.4U of iProof DNA polymerase (BioRad, Cat# 1725301) in a 20µl reaction volume. The amplified products were run on 1% agarose gel along with DNA marker, and the bands were visualized in gel documentation system.

2.3 Results

2.3.1 Successful targeting of *CSP 3' UTR* with *FRT*ed *TRAP 3' UTR* to generate CSP conditional knockout (CScKO) parasites in FLP@UIS4 and TRAP@FLPL parental lines

The organisation of *CSP* locus is shown in Fig 12A. For successful replacement of the endogenous *3' UTR* of *CSP*, the *CSP ORF* and *3' UTR* regions were used in double crossover recombination to introduce a *FRT*ed *TRAP 3' UTR, hDHFR* and *GFP* cassettes. The complete *ORF* and *3' UTR* of *CSP* were cloned in tandem in the conditional plasmid pSKC-TRAP-hDHFR-GFP. The plasmid linearized with unique restriction enzyme *EcoRI* introduced between *CSP 3'UTR* and *ORF* and the linearized construct was used for transfection (Fig 12B). The genomic organisation at *CSP* locus in both TRAP@FLPL and UIS4@FLP lines following the successful double homologous recombination is shown in Fig 12C. The *ORF* and *3' UTR* fragments of *CSP* were amplified by PCR using primer sets FP1/RP1 and FP2/RP2 respectively and products were resolved on 1% agarose gel (Fig 12D). Both the fragments were cloned into the targeting vector and their successful ligations were confirmed by release of *3' UTR* and *ORF* using restriction enzymes AscI/EcoRI and EcoRI/NotI respectively (Fig 12E). The linearized *CSP* conditional construct was electroporated into *P. berghei* ANKA TRAP@FLPL and UIS4@ FLP transgenic parasites using Amexa nucleofector using predefined program U033 and the parasites were injected intravenously into mice. The recombinant parasites were selected using pyrimethamine and the parasitemia was monitored daily by blood smears stained with Giemsa. Genomic DNA was isolated from the drug resistant parasites and used as template for performing diagnostic PCR that confirmed the presence of two *FRT* sites at the recombined locus. First *FRT* was in tandem to *CSP ORF*, prior to the *TRAP 3' UTR* and second *FRT* between the *hDHFR* and *GFP* cassettes. The correct 5' and 3' site specific integration at the *CSP* locus was verified by integration PCR using primer sets FP3/RP3 and FP4/RP4 in TRAP@FLPL (Fig 12F (a)) and UIS4@FLP transgenic lines (Fig 12F (b)). Limiting dilution was performed to obtain clonal population of CScKO TRAP@FLPL and UIS4@FLP parasite lines. The cloned lines of CScKO TRAP@FLPL (Fig 12G (a)) and CScKO UIS4@FLP (Fig 12G (b)) expressed GFP constitutively.

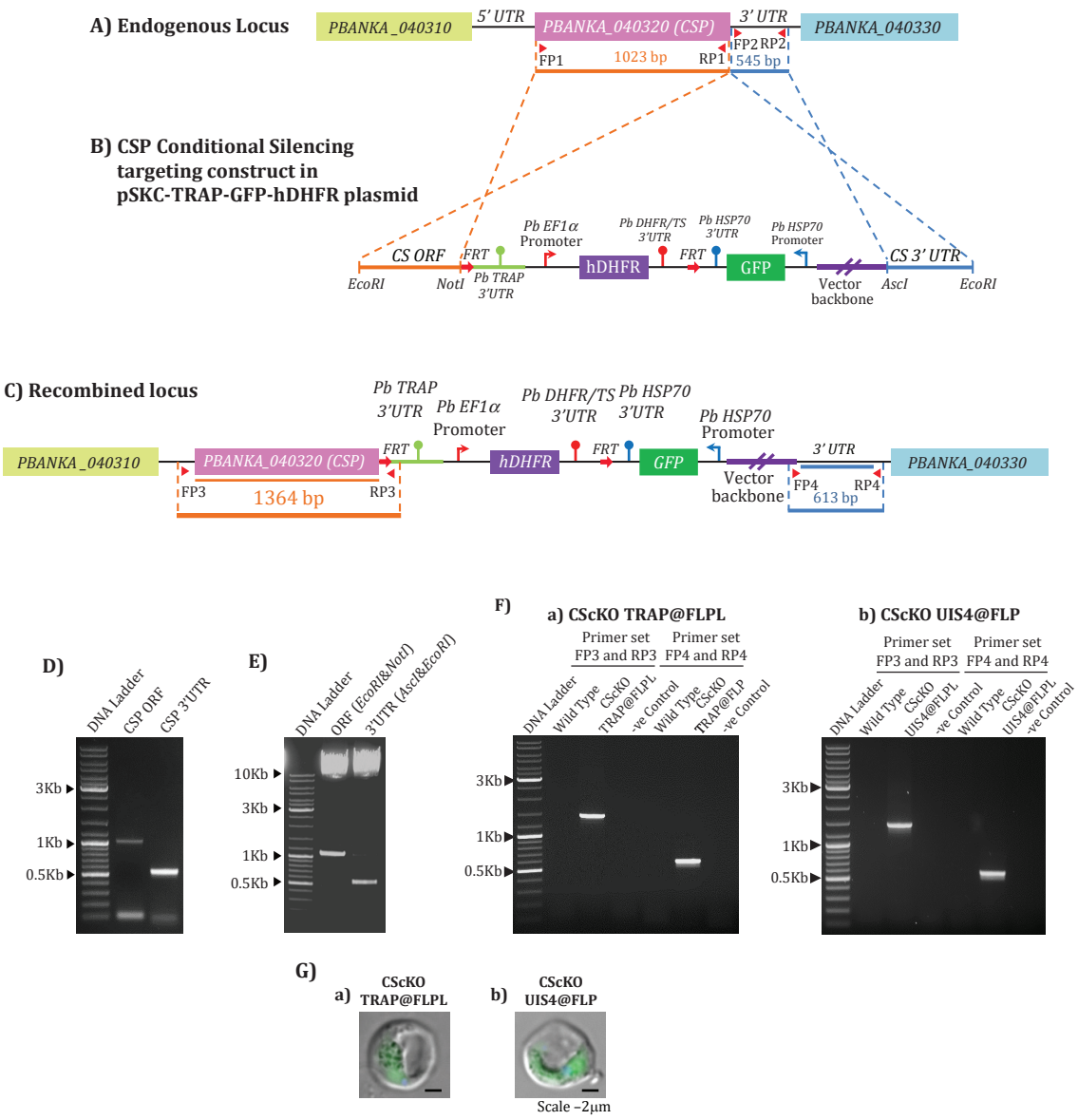

Fig. 12 Generation of conditional KO of *CSP* (CScKO) in TRAP@FLPL line and UIS4@FLP lines A) Genomic locus of *CSP* (*PBANKA_040320*) showing *CSP ORF, 5'* and *3' UTRs*. B) Elements of the targeting vector showing regions selected for homology, *3' UTR* of *TRAP*, *hDHFR* cassette and *GFP* cassette. First *FRT* site is present in between *CSP ORF* and *TRAP 3'UTR*, and the second one after *hDHFR* cassette. *CSP 3'UTR* and *ORF* were cloned in tandem introducing a unique restriction site *EcoRI*, which was used for linearization of the targeting construct. C) Target gene locus after successful double homologous recombination. In the recombined locus, an *FRT* site and 3' regulatory sequence of TRAP is present in tandem to the *CSP ORF*. D) A 1% agarose gel showing the PCR product of 1035 bp corresponding to 1023bp *CSP ORF*+12bp of *TRAP 3' UTR* and 545bp corresponding to *3' UTR* of *CSP* amplified using the primer sets FP1/RP1 and FP2/RP2 respectively. E) A 1% agarose gel showing digestion products of 1035bp of *CSP ORF* and 545bp of *CSP 3' UTR* following release with EcoRI/Not1 and Asc1/EcoRI respectively. F) Diagnostic PCR to confirm site specific integration in a) CScKO TRAP@FLPL line and b) CS*c*KO UIS4@FLP line. Correct integration was confirmed by using primer sets FP3/RP3 and FP4/RP4 designed beyond sites that amplified respectively 1364bp and 613bp products. G) Merged DIC image showing GFP expressing CScKO made in a) TRAP@FLPL line and b) UIS4@FLP line.

2.3.2 CScKO in TRAP@FLPL and UIS4@FLP lines showed normal asexual blood stage propagation

Following limiting dilution, the asexual propagation rates of both the conditional lines (CScKO TRAP@FLPL and UIS4@FLP lines) were compared with WT GFP by injecting 10^3 infected RBC (iRBC) in a group of 3 mice, and parasitemia was monitored daily for 7 days by Giemsa staining. The blood stage parasites propagated at similar rates in all the groups of mice (Fig 13). Thus the replacement of endogenous *3'UTR* of *CSP* with *TRAP 3' UTR* did not affect the propagation of the asexual stages.

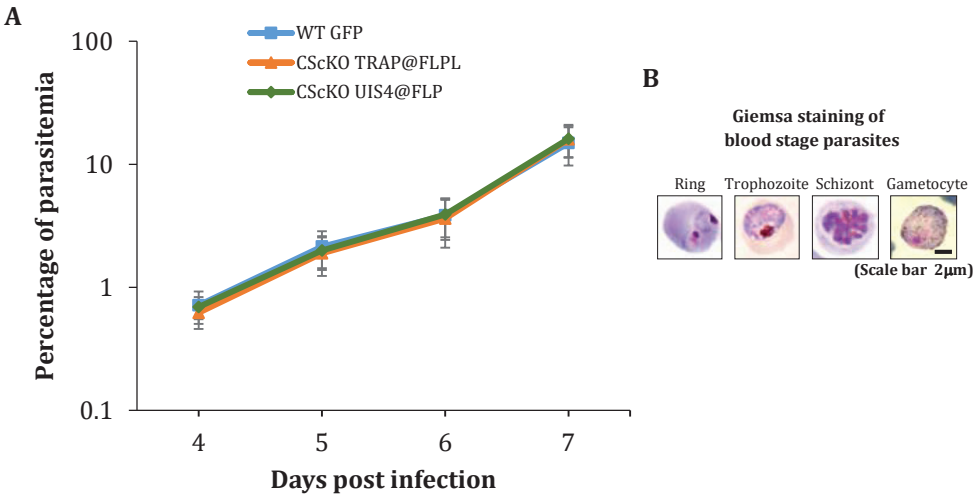

Fig. 13 CScKO parasites obtained in TRAP@FLPL and UIS4@FLP lines propagate asexually at similar rates as WT GFP parasites. A) $1X10^3$ infected RBC of either WT or CScKO lines obtained in TRAP@FLPL and UIS4@FLP lines were intravenously injected in three groups of mice (3 mice/group) and monitored for propagation of the parasites daily for 7 days by making Giemsa stained smears. B) Representative pictures showing blood stages of parasites. Images adapted and modified from (Rathore *et al.*, 2010).

2.3.3 Both CScKO parasite lines showed normal oocyst burden compared to WT GFP

The CScKO obtained in both lines were validated for the efficiency of 3' UTR excision of CSP gene by passing through *Anopheles* mosquitoes. The mosquito midguts were dissected from both CScKO mutant lines and the oocyst burden on day 14 was analysed. We noted no difference in the frequency of the oocysts in both CScKO lines as compared to WT GFP (Fig 14).

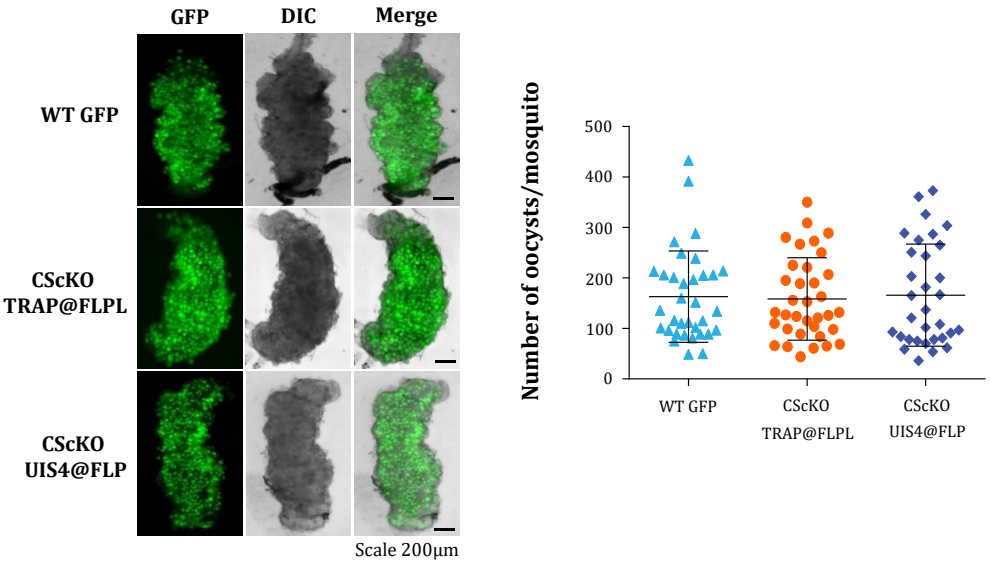

Fig. 14 CScKO parasites obtained in TRAP@FLPL and UIS4@FLP lines form oocyst in similar number as WT GFP parasites. The gametocyte positive mouse harboring WT parasites or CScKO parasites obtained in TRAP@FLPL and UIS4@FLP lines were allowed to transmit malaria to female *Anopheles* mosquitoes. For each parasite line, 5 mice were used per cage of mosquitoes to initiate sexual cycle in mosquito. A) On D14 post infection, the mosquito midguts were dissected and observed for the frequency of the oocyst from WT GFP, CScKO TRAP@FLPL, and CScKO UIS4@FLP. B) Dot plot showing number of oocyst per mosquito. Number of mosquitoes used per group: WT GFP=34, CScKO TRAP@FLPL=36 and CScKO UIS4@FLP=32. Data represented as Mean+SD.

2.3.4 CScKO TRAP@FLPL and UIS4@FLP lines manifest compromised sporulation, fail to egress oocyst and infect salivary glands

We next observed the oocyst sporulation in both lines and noted a dramatic decrease in sporulation in both lines as compared to WT GFP (Fig 15A). In order to quantify the sporulation efficiency, the sporozoites were isolated by mechanical disruption of oocyst and their numbers were enumerated. We observed 26 fold and 14.5 fold decrease in the sporulation respectively in TRAP@FLPL and UIS4@FLP CSP conditional mutants as compared to WT GFP (Fig 15B). We next analysed the sporozoite burden in intact salivary glands obtained from mosquitoes infected with WT GFP or both the conditional KO lines as indicated in Fig 15C. We observed that in both the conditional KO lines, the sporozoites failed to reach salivary glands. We again analysed the oocyst sporulation (Fig 15D) and counted the sporozoite numbers on day 21 p.i and noted a 50 fold and 10 fold decrease respectively in sporozoite counts in TRAP@FLPL and UIS4@FLP CSP conditional mutants (Fig 15E) as compared to WT GFP. Thus while both CSP conditional mutant lines manifested an impaired

sporulation in oocyst, the CScKO TRAP@FLPL were affected more severely as compared to CScKO UIS4@FLP. Interestingly, decrease in the oocyst sporulation from 26 fold on day 14 to 50 fold on day 21 in CScKO TRAP@FLPL may indicate more extensive degeneration of sporozoites within oocyst of CScKO TRAP@FLPL as compared to CScKO UIS4@FLP. We conclude that conditional depletion of CSP in both lines affects parasite functions central to oocyst sporulation, albeit to different extent.

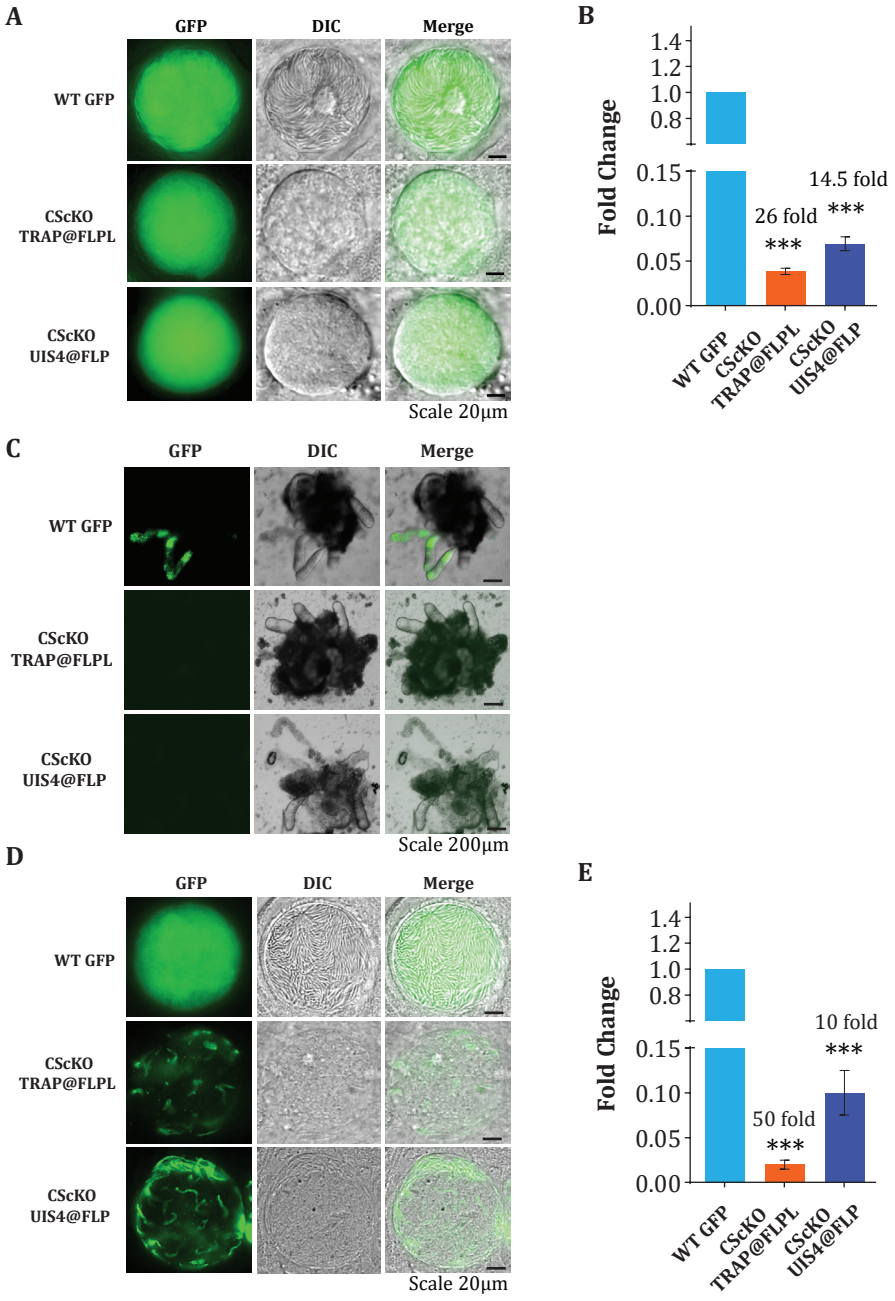

Fig. 15 Analysis of oocyst sporulation pattern on Day 14 and on Day 21 and sporozoite migration to salivary gland on Day 21 in CScKO parasites obtained in TRAP@FLPL and UIS4@FLP lines as

compared to WT. A) On day 14 p.i, the sporulation was highly compromised in TRAP@FLPL line as compared to UIS4@FLP line. Sporulation in WT GFP oocyst was used as control (magnification 100X, scale bar 20μm). B) Quantification of oocyst sporozoites on day 14. The sporozoites numbers in CScKO TRAP@FLPL line and UIS4@FLP lines were represented as fold reduction as compared to WT. Number of experiments, n=3. Each group contains 25 mosquitoes (***p<0.001). C) Salivary glands were dissected on day 21 p.i from mosquitoes infected with CScKO TRAP@FLPL line, UIS4@FLP line and WT GFP. Intact salivary glands were observed under fluorescent microscope. Salivary glands obtained from mosquitoes infected with WT GFP parasites showed appreciable sporozoite loads where as those obtained from CScKO TRAP@FLPL and UIS4@FLP infected lines showed no sporozoites (magnification 10X, scale bar 200 μm). D) On day 21 p.i, the sporulation was further compromised in TRAP@FLPL line as compared to UIS4@FLP line. Sporulation in WT GFP oocyst was used as control (magnification 100X, scale bar 20 μm). E) Quantification of oocyst sporozoites on day 21. The sporozoites numbers in CScKO TRAP@FLPL line and UIS4@FLP lines were represented as fold reduction as compared to WT. Number of experiments, n=3. Each group contains 20 mosquitoes (***p<0.001).

2.3.5 Excision PCR in CScKO sporozoites

The extent of FLPL/FLP mediated excision in both CScKO lines was confirmed by diagnostic PCR that differentially amplifies a product of 2838 bp or 588 bp from the genomic DNA of non-excised and excised population respectively using primer set FP5/RP5 (Fig. 16A). The recombinase mediated excision was more robust in TRAP@FLPL line (Fig. 16B (a)) as compared to UIS4@FLP (Fig. 16B (b)). Since the FLPL/FLP excises the *FRT*ed DNA sequence by formation of a circular loop with one *FRT* sequence, the other *FRT* site is retained in the parasite genome following successful excision. In sequencing result, *FRT* site and plasmid backbone sequence was in tandem to *CSP ORF* in both CScKO lines, a possibility that arises only when the DNA sequence flanked by *FRT* sites is successfully excised (Fig. 16C (a) and (b)).

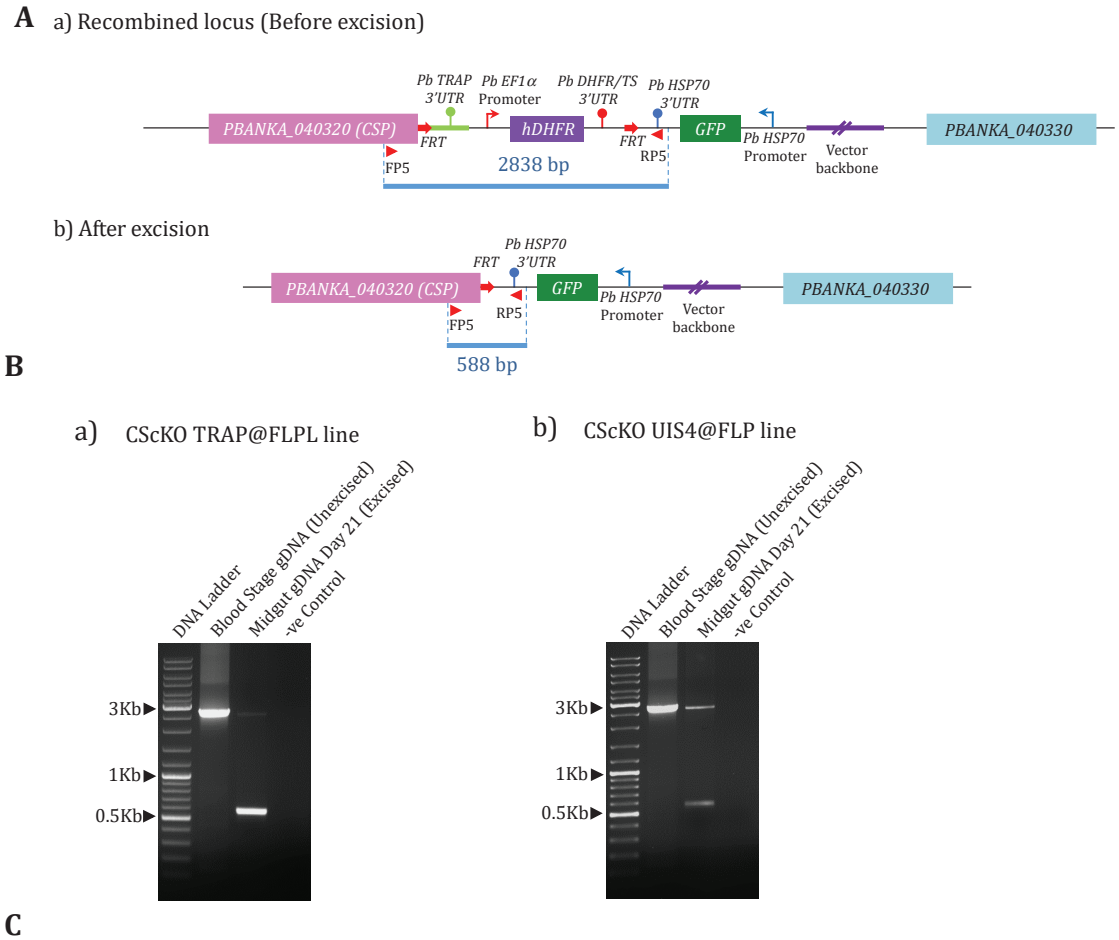

Fig. 16 PCR based confirmation of CSP 3' UTR excision in CScKO TRAP@FLPL and UIS4@FLP line. A) Schematic representation of the target gene locus before (a) and after (b) excision. Genomic DNA was isolated from midgut on day 21 post infection. A diagnostic PCR was set up using primers FP5/RP5 that differentially amplified 2838bp in non-excised population and 588p in excised population. B) Diagnostic PCRs using blood stage gDNA (non-excised) and day 21 genomic DNA (excised) from the midguts in both CScKO TRAP@FLPL line (a) and UIS4@FLP line (b). Excision was confirmed by presence of 588bp PCR product. C) Sequencing of PCR product confirmed after excision. Successful excision places the Pb *HSP70 3'UTR* of *GFP* cassette in tandem to *CSP ORF* and *FRT* site. This was confirmed by sequencing the 588bp product amplified from both CScKO TRAP@FLPL line (a) and UIS4@FLP line (b).

2.3.6 CSP is depleted in CScKO oocysts and sporozoites

Transcription of *CSP* gene following *3' UTR* excision results in mRNA that lacks a functional *3' UTR* that is unstable and prone to degradation. This leads to temporal silencing of *CSP* leading to diminished levels of protein. To reiterate the same, we performed immunoblotting with lysates of day 14 midgut sporozoites and observed a significant reduction in the levels of CSP in both CScKO lines as compared to WT GFP oocyst sporozoites (Fig 17A). The results were concurrent with IFA of day 21 oocyst sporozoites that showed aberrant and non-uniform distribution of CSP on sporozoite surface in both CScKO mutant lines (Fig 17B). We conclude that the *3' UTR* silencing of *CSP* gene indeed depletes the cellular levels of CSP thus compromising both sporulation efficiency and ability of sporozoites to invade salivary gland.

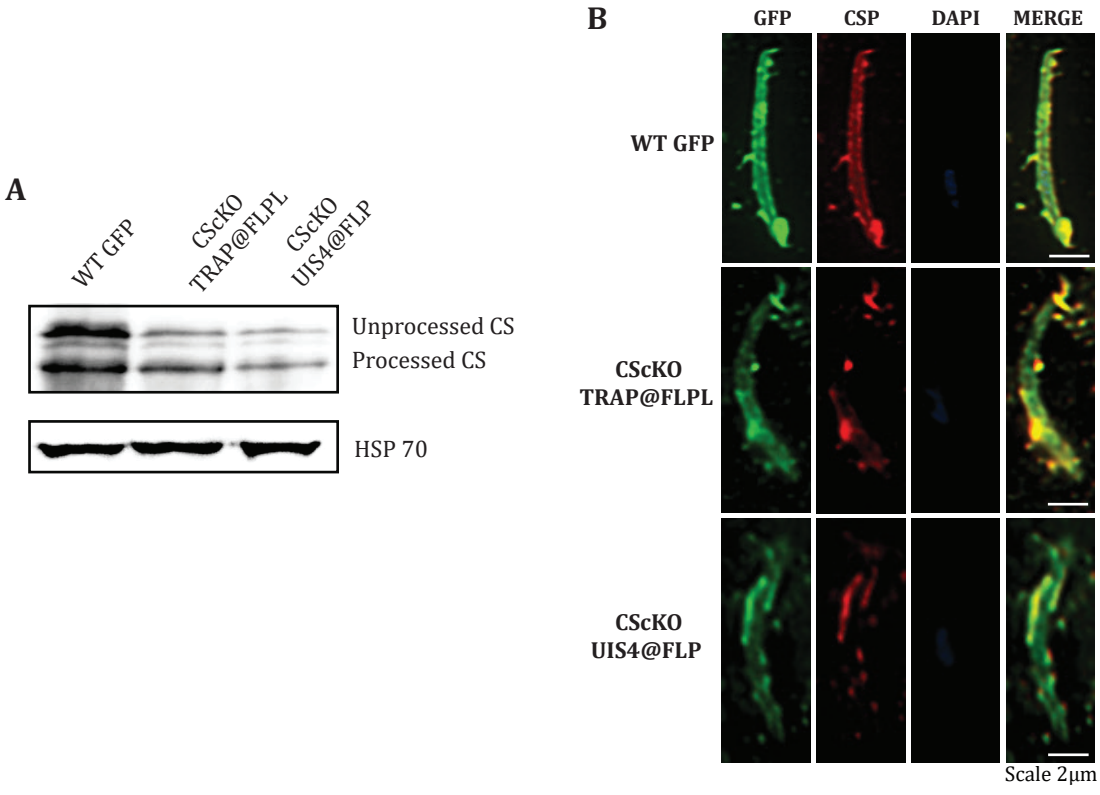

Fig. 17. Confirmation of CSP depletion in CScKO TRAP@FLPL and UIS4@FLP lines by western blotting and Immunostaining in Day 21 sporozoites using anti-CSP monoclonal antibody. A) Expression of CSP levels in WT GFP, CScKO TRAP@FLPL and UIS4@FLP lines assessed by Western blotting using sporozoite lysates. The blots were revealed with α-CS antibody and HSP70 was used as loading control. B) The midgut sporozoites obtained from D21 oocyst of CScKO TRAP@FLPL line, UIS4@FLP line and WT GFP were fixed in 4% PFA and permeabilized in chilled absolute methanol. Following nonspecific blocking the sporozoites were incubated with 3D11 monoclonal antibodies and the immunoreactivity was revealed with anti-mouse secondary antibody conjugated to Alexa Fluor 594. The parasite nuclei were stained with DAPI.

2.3.7 Quantitative gene expression of *TRAP* and *UIS4* from WT parasites

Inability of oocyst sporozoites to reach salivary glands in CScKO TRAP@FLPL line was in good agreement with the predominant activity of TRAP during oocyst development stages that results in maximal excision of *CSP 3' UTR*. However, a similar non egressing phenotype was unexpected for CScKO UIS4@FLP line as the expected promoter activity of *UIS4* is more predominant in salivary gland sporozoites than midgut sporozoites. While both CScKO lines grossly manifested a block in sporozoite egress, nonetheless as discussed earlier the sporulation was more severely impaired in TRAP@FLPL line as compared to UIS4@FLP line. These observations were concurrent with higher TRAP expression over UIS4 in midgut sporozoites. We looked at differential expression of TRAP and UIS4 during oocyst development (day 8 – day 14) by qRT PCR. The levels of TRAP and UIS4 were indistinguishable on days 8, 10 and 12 (Fig 18). While a sudden burst of TRAP expression was noted on day 14, interestingly, the levels of UIS4 was appreciably higher at this point compared to the day 8-12 levels (Fig. 18). Our gene expression analysis revealed a 4.5 fold higher TRAP activity at D14 oocyst stages as compared to UIS4, that could possibly explain more recombinase expression and hence drastic reduction in sporulation in CScKO TRAP@FLPL line as compared to CScKO UIS4@FLP line. We conclude that the minimal activity of UIS4 promotor during day 8-14 of oocyst development was sufficient to achieve the excision of the *FRT*ed DNA sequence, thus yielding a phenotype nearly similar to TRAP@FLPL.

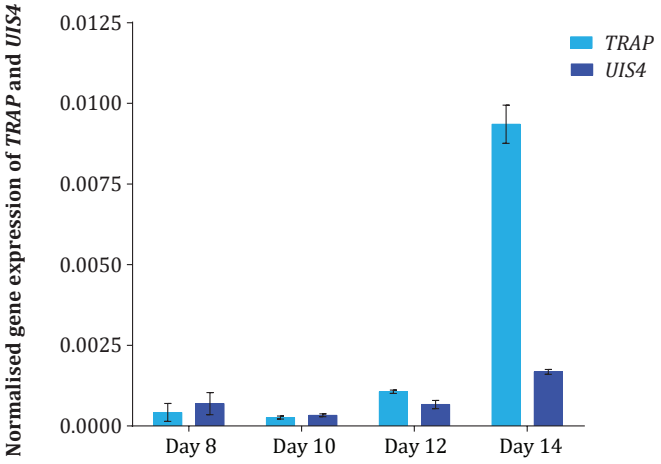

Fig. 18 Quantifying the expression of *TRAP* and *UIS4* during oocyst development stages in WT *P. berghei*. RNA was isolated from mosquito midguts during oocyst stages of development (day 8, 10, 12 and 14) and cDNA was generated. The cDNA was used as template to quantify the expression of *TRAP* and *UIS4*. The gene expression values were normalized by taking ratio of the target gene (TRAP or UIS4) copy number and *P. berghei 18S rRNA* copy number.

2.4 Discussion

Several studies have aimed at identifying the structure-function aspects of CSP in order to account for its functions during parasite development in mosquito stages and at liver stage. These approaches have elucidated in great detail the importance of CSP in regulating sporozoite stage specific functions. The region I bears the cleavage site where the CSP is processed when sporozoite contacts HepG2 cells. Processing of CSP is critical for sporozoite infectivity and is a function of cysteine protease (Coppi *et al.*, 2011). The region II-plus consists of a conserved motif of positively charged amino acids that likely facilitates sporozoite invasion of hepatocytes via interaction with heparan sulfate proteoglycan on the host cells (Pinzon-Ortiz *et al.*, 2001). A mutant of region II-plus generated by substituting this motif with alanines resulted in the production of oocyst that showed normal sporulation but were unable to exit the oocysts (Wang *et al.*, 2005a). Mutants of repeat region alone or N-terminal sequence along with the repeat region resulted in a phenotype that was unaffected in oocyst formation but had defective sporozoite development (Ferguson *et al.*, 2014). More recent studies demonstrated the existence of CSP in two conformational states, an adhesive conformation in which the C-terminal cell-adhesive domain is exposed and a nonadhesive conformation in which the N-terminus masks this domain. The cell-adhesive domain is required for sporozoite development and hepatocyte invasion while N-terminal masking of the cell-adhesive domain maintains the sporozoite in a migratory state required for sporozoites to reach salivary glands and mammalian liver (Coppi *et al.*, 2005). Mutation analysis of GPI addition sequence of CSP revealed that the mutants fail to sporulate (Coppi *et al.*, 2005, Wang *et al.*, 2005b). Thus while genetic analysis of different motifs in CSP allowed dissecting the basis of the protein function in greater detail, attempts to study the role of CSP in mosquito and liver stage has been hampered because CSP mutants failed to sporulate. Thus the vital questions that remain speculative are whether CSP is the sole antigen that functions central to egress, hemocoel migration, colonization to salivary gland, and whether depletion of CSP affects the transmission and navigation of the sporozoites within the mammalian host. Further, CSP being the prime liver stage antigen critical for protective immunity (Kumar *et al.*, 2006), it will be interesting to study if EEFs derived from temporally silenced CSP can still induce an effective protective immunity. Owing to the essential role of CSP in oocyst sporulation (Menard *et al.*, 1997), only conditional system will

help address these vital questions.

To address the role of CSP in mosquito stages and during EEF development, we generated two transgenic lines expressing FLP and its thermolabile variant FLPL in *Pb*A GIMO mother line (Lin *et al.*, 2011) and used these lines for conditional depletion of CSP gene. In line with already proven role in *Plasmodium* development in mosquito stage like sporozoite budding and development (Menard *et al.*, 1997, Thathy *et al.*, 2002, Coppi *et al.*, 2011), exit from oocyst (Wang *et al.*, 2005a) and migration to salivary gland (Myung *et al.*, 2004, Coppi *et al.*, 2011), our findings recapitulated the previously described phenotype where diminishing the cellular levels of CSP in sporozoites affected their morphology, ability to egress and migrate to salivary glands (Thathy *et al.*, 2002) thus confirming efficient excision of 3'UTR of CSP in both lines. In contrast to the observed higher activity of TRAP@FLPL at 25^{0}C (Lacroix *et al.*, 2011, Panchal *et al.*, 2012) and an almost exclusive UIS4@FLP activity in salivary gland sporozoites (Lacroix *et al.*, 2011), we observed (i) appreciable activity of TRAP@FLPL even at 21^{o}C that led to excision and phenotype indistinguishable from that obtained by maintaining the infected mosquitoes at 25^{o}C and (ii) also noted appreciable activity of UIS4 in the midgut stages that contributed to excision. The nearly similar phenotype of both lines with regard to reduced sporulation and non-egress phenotype, when maintained all through at 21^{o}C can be explained as follows: Firstly, CSP is synthesized in immature oocyst from day 6 onwards even before the formation of sporozoites (Posthuma *et al.*, 1988). CSP localizes to inner surface of mature oocyst capsule, peripheral cytoplasmic vacuoles and membranes of sporoblast, budding and free sporozoite implicating its role at multiple stages of oocyst development (Nagasawa *et al.*, 1987, Gonzalez-Ceron *et al.*, 1998). Thus the non-egress phenotype manifested by TRAP@FLPL CScKO may be a consequence of prolonged suboptimal activity at 21^{o}C during oocyst developmental stages that ensures sufficient window time for excision. Secondly, a low levels of UIS4 promoter activity during oocyst development may yield active FLP recombinase albeit at levels much lesser as compared to FLPL but sufficient for efficient excision. Thus optimal activity rather than duration of exposure to active recombinase may be key to rapid excision of *FRT*ed DNA. In line with such assumption, *in vitro* recombination reactions performed in the presence of FLP and its substrate at 23°C resulted in more than 50% and nearly 70% excision at 30 min and 60 min

respectively (Buchholz *et al.*, 1996). Also, the minimum requirement for excision of *FRT*ed single copy gene are four recombinase molecules (Chen & Rice, 2003). It is likely that parasites can efficiently synthesize such levels of recombinase even under conditions of lower promotor activity as observed for UIS4. In support of such assumption, we provide evidence for expression of UIS4 during oocyst development stages from day 8 to day 14.

Our diagnostic PCR results revealed maximal excision of *FRT*ed DNA in TRAP@FLPL line as compared to UIS4@FLP line. While the complete absence of salivary gland sporozoites in UIS4@FLP line may not account for the existence of non-excised population as detected in PCR of day 21 oocyst (Fig 16Bb), it may be possible that a small number of non-excised sporozoites may exist within oocyst that are unable to egress due to insufficient levels of CSP. Lack of tight regulation of UIS4 exclusive to salivary glands sporozoite stage precluded our attempts to track the fate of CScKO sporozoites during their journey to liver and its subsequent transformation into EEFs. The activity of UIS4@FLP promotor at oocyst stages was independently confirmed in *PbSufS* conditional KO generated in UIS4@FLP line (S. Mishra, personal communication). In conclusion, our work provides evidence that the expression of UIS4 is not exclusive to salivary glands sporozoite stages. These findings call for identification of novel promoters that will aid in more specific temporal gene silencing at salivary gland sporozoite stages to study its effects in liver stages.

Chapter 3

Functional Characterization of Pb K+ Channel Adenylyl Cyclase α by reverse genetics approach

3.1 Introduction

Plasmodium is an obligate intracellular parasite that completes its life cycle in two hosts–a female Anopheline vector and a mammalian host. Sporozoites are the infective forms of parasites to the mammalian host and are injected into the dermis of the skin, when the female *Anopheles* mosquito probes for a blood meal (Vanderberg & Frevert, 2004, Amino *et al.*, 2006). Shortly after their deposition in the dermis, the sporozoites migrate through several cellular barriers utilizing an actin-myosin based molecular motor present beneath the plasma membrane and linked to the target cell surface receptors by the transmembrane protein TRAP (Sultan *et al.*, 1997, Buscaglia *et al.*, 2003). After crossing the endothelial cells of dermal vessels, the sporozoites gain a swift momentum within the circulating blood allowing its selective arrest in the liver sinusoids (Frevert *et al.*, 2005). This event is mediated by specific interactions between the major surface protein of sporozoites, the Circumsporozoite protein (CSP) and the heparin sulphate proteoglycans secreted by the stellate cells (Frevert *et al.*, 1993, Pradel *et al.*, 2004). The sporozoites further glide through sinusoidal endothelial fenestrae or through the Kupffer cells and finally reach the hepatocytes (Baer *et al.*, 2007).

Sporozoite migration is an essential step for completion of parasite life cycle. Sporozoites are deposited in dermis which is far from the site of actual infection – the hepatocyte. Sporozoite migration leads to breaching of cell barriers when they move from skin to hepatocytes. While this process seems to be highly essential for infection of hepatocytes *in vivo*, interestingly sporozoites also migrate while infecting HepG2 cells that are permissive for complete development of *P. berghei* EEFs. The sporozoite traversal through cells that are readily permissive for transformation likely points to the need for a brief process of activation likely by exposure to host intracellular factors. During traversal, sporozoites enter and exit several hepatocytes by breaching the plasma membrane which is repaired rapidly both *in vitro* and *in vivo* conditions as revealed by cell wounding assay (Mota *et al.*, 2001). Breaching the host cell membranes by sporozoites is an active process that involves many parasite proteins like SPECT, SPECT2, phospholipase (*Pb* PL) and Cell Traversal protein for Ookinete and Sporozoite (CelTOS). SPECT and SPECT2 are the sporozoite proteins secreted by micronemes that are involved in migration and targeted disruption of genes encoding

these proteins yielded sporozoites that invade hepatocyte normally and developed into EEFs *in vitro*, however both mutants exhibited poor infectivity *in vivo* (Ishino *et al.*, 2004, Ishino *et al.*, 2005a). Phospholipase (Pb PL) is a sporozoite surface protein that hydrolyses the phosphatidylcholine and other phospholipids present on the host cell membrane forming a pore there by allowing the sporozoite to enter and exit the host cells. Pb PL mutant sporozoites showed defect in transmigration *in vitro* and the infectivity was significantly reduced through mosquito transmission (Bhanot *et al.*, 2005). CelTOS is a micronemal protein that is involved in the traversal activity of ookinete and sporozoite. Reverse genetics approach revealed that CelTOS is essential for the movement of sporozoites through the host cell cytosol (Kariu *et al.*, 2006).

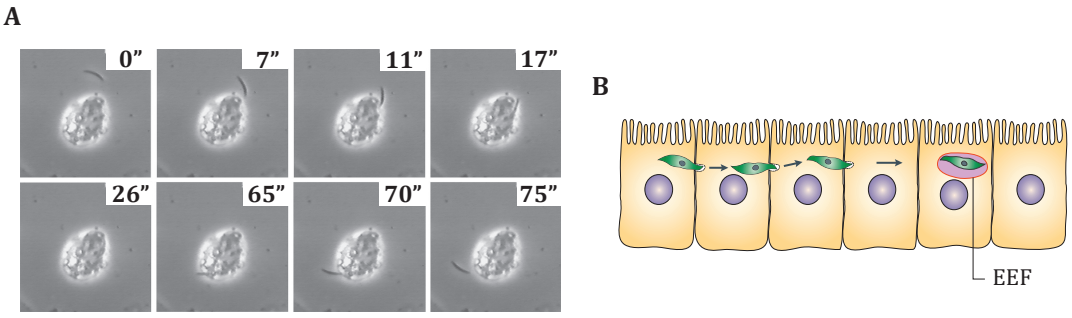

Fig. 19 Sporozoite migrates through many cells before it productively invades a hepatocyte through formation of PVM. A) Time lapse microscopy images of *P. berghei* sporozoite entering and exiting a hepatocyte. Sporozoite enters and exits a host cell with in a minute. The images are in sequence with top right corner showing time (in seconds). B) The representative image of sporozoite migration through many hepatocytes during which the sporozoites get activated for productive invasion forming a PVM to establish liver stages or EEFs. Images were adapted and modified from Mota *et al.*, 2001 and Prudencio *et al.*, 2006.

The sporozoite migration through hepatocytes inflicts cell wounding that releases hepatocyte growth factor (HGF) that in turn potentiates the infectivity of neighbouring hepatocytes by recruiting HGF receptor called MET (Carrolo *et al.*, 2003). The increase in susceptibility of these cells is due to reorganisation of host cell cytoskeletal actin (Leiriao *et al.*, 2005). Contrary to this observation, another independent study demonstrated that the cellular contents released from ruptured cells induces the host innate immune responses through activation of NFκB, which is unfavourable for the sporozoite and limits the infection partially (Torgler *et al.*, 2008). While keeping the observation of these two contrasting findings in place, a fundamental question that emerges from this scenario is why sporozoites resort to the traversal activity in the first place. One likely explanation is that a range of host

cytosolic factors may be used as a cue for sporozoites to enhance productive invasion. Many ions have a concentration gradient across the cell membrane. Example – [K^+] is at high concentration inside as compared to outside of plasma membrane. Ions like [Na^+] and [Cl^-] are at high concentration in the extracellular region and occur in low concentration with in the cell. Thus, there is a possibility that high concentration of intracellular [K^+] may influence parasite infectivity. In line with such speculation, a shift in ionic concentration of [K^+] facilitated synchronised invasion of host cells by *Toxoplasma* tachyzoites (Kafsack *et al.*, 2004). These observations present a possible regulation of parasite switch to invasive mode by varying [K^+] concentration. Similarly, *P. berghei* sporozoites exposed to 142mM [K^+] showed enhanced infectivity by 8-10 times *in vitro* (Fig. 20A) with simultaneous decrease in host cell traversal (Kumar *et al.*, 2007).

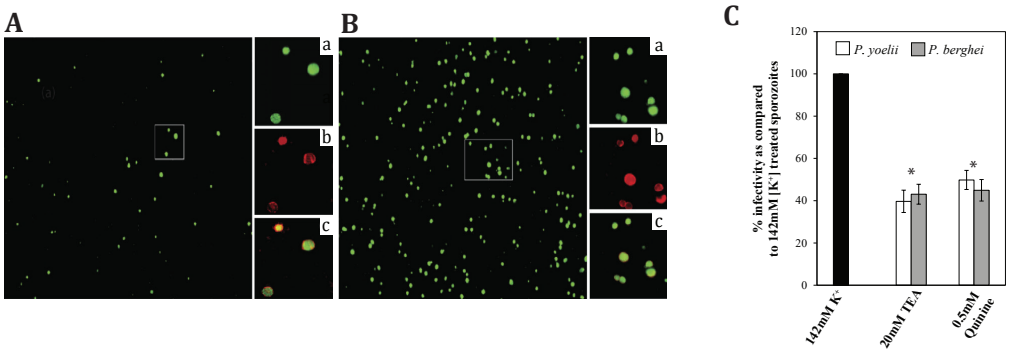

Fig. 20 Sporozoite infectivity increases when exposed to 142mM [K^+] ions before infection. A) EEFs derived from sporozoites treated with control medium. B) EEFs derived from sporozoites treated with 142mM [K^+] medium. The number of EEFs are more when the sporozoites were exposed to 142mM [K^+] before infection. The inset images a, b and c are HSP 70 (green), UIS4 (Red) and merged EEFs respectively. C) Bar graph showing the specificity of 142mM [K^+] in enhancing the infectivity of sporozoites in *P. berghei* and *P. yoelii* by using potassium channel blockers tetraethyl ammonium (TEA) and quinine. The infectivity was reduced in both *P. berghei* and *P. yoelii* when the sporozoites were treated with potassium channel blockers in the presence of 142mM [K^+]. Images were adapted from Kumar *et al.*, 2007.

Towards addressing as how the *Plasmodium* sporozoites sense the [K^+] concentration during intracellular migration, we searched *Plasmodium* genome for possible existence of K^+ channel encoding genes. We found that *Plasmodium* genome has 3 potassium ion channel genes - *Kch1*, *Kch2* and third one was a bifunctional gene with domains for K^+ channel and adenylyl cyclase alpha (*K^+/ACα*). Targeted disruption of *P. berghei Kch1* showed almost 98% reduction in the transmission of malaria to mosquitoes showing its essential role in the sexual development (Ellekvist *et al.*,

2008). Immunofluorescence studies with antibodies raised against products of *PfKch1* and *PfKch2* revealed that the former was expressed in trophozoites and schizonts stages while the latter was restricted to mature schizonts with minimal expression in trophozoites (Waller *et al.*, 2008). In *P. falciparum*, *Kch1* and *Kch2* are essential for the asexual blood stage development as attempts for targeted disruption of both these genes were unsuccessful (Waller *et al.*, 2008). The bifunctional K^+ channel/adenylyl cyclase alpha was first identified in *Paramecium* and is conserved in unicellular protozoans (Schultz *et al.*, 1992). Purified *K^+/ACα* when incorporated into the lipid bilayer exhibited ion channel properties and cyclase activity. Both the activities of *K^+* channel domain and *ACα* were physiologically linked to hyperpolarization in *Paramecium* leading to elevated levels of intracellular cAMP, which was inhibited by K^+ channel inhibitors - tetraethylammonium (TEA), Cs^+ ions, and quinine. *Paramecium* mutant with a defect in K^+ conductance failed to regulate their cyclic AMP activity (Schultz *et al.*, 1992).

The likely advantage of migration seems to be in regulating the exposure of secretory antigens required for sporozoite commitment to hepatocyte attachment and invasion. Sporozoite proteins like TRAP, CSP, AMA1, etc. essential for hepatocyte infection and are released from the secretory organelles of sporozoites by apical regulated exocytosis (Mota *et al.*, 2002). Exocytosis can be measured by release of TRAP at the apical end or into the medium. Sporozoite exocytosis can be induced when the cytosolic cyclic AMP (cAMP) levels are raised which can be achieved *in vitro* with the treatment of 8-bromo cAMP, a permeable structural analogue of cAMP (Ono *et al.*, 2008). When *P. yoelii* sporozoites were treated with 8-bromo cAMP, the traversal activity of sporozoites was decreased with concomitant increase in productive invasion and formation of EEFs. This was further confirmed following treatment of sporozoites with an adenylyl cyclase (AC) inhibitor, MDL 12.330A that inhibits cAMP synthesis and consequently the inhibition of apical regulated exocytosis (Ono *et al.*, 2008). *Plasmodium* genome codes for two adenylyl cyclase genes - *AC alpha* (*ACα*) and *AC beta* (*ACβ*). Targeted deletion of *ACα* domain of bifunctional *K^+/ACα* gene in *P. berghei* resulted in mutant that showed normal blood stage development and mosquito development. However, *Pb ACα* KO sporozoites manifested a 50% reduction

in sporozoite infectivity than wild type both under *in vitro* and *in vivo* conditions because of reduced apical exocytosis (Ono *et al.*, 2008).

In the current study we provide functional evidence for the role of K^+ channel domain of *Pb K⁺/ACα* in mediating the sporozoite activation. To address this, we generated *Pb K⁺/ACα* knockout parasites and tested these mutants for their ability to infect HepG2 cells with or without prior exposure to 142mM [K^+]. Our study clearly demonstrated that the effect of intracellular concentration of [K^+] enhanced productive invasion of WT sporozoites, a phenomenon that was highly compromised in *Pb K+/ACα* KO parasite.

3.2 Materials and methods

All the methods used in functional characterization of *Pb K⁺/ACα* have also been described in great detail in chapter 2 - "Investigating the role of Circumsporozoite protein in *Plasmodium berghei* (*Pb*) mosquito stages by using FLP/*FRT* conditional mutagenesis system". Since many methods are common, only brief description is made here.

3.2.1 Experimental animals and parasite lines

Female BALB/c or C57BL/6 mice aged 8-10 weeks old, were purchased from NCLAS, Hyderabad and used for all animal experiments. Animal handling was strictly in accordance with Institutional Animal Ethical Committee protocols of University of Hyderabad. *Pb K⁺/ACα* KO was generated by transfecting *Pb K⁺/ACα* KO targeting construct in wild type *P. berghei ANKA* strain. *P. berghei ANKA* GFP parasite line was generated by targeting the non-essential gene locus *P230p* in *P. berghei* ANKA and used as control line. Phenotypic analysis of the genetically modified/transgenic parasites was carried out by cycling the parasites through Swiss Albino or BALB/c and female *A. stephensi* mosquitoes. C57BL/6 mice were used for the analysis of pre-patent period either by mosquito bite or intravenous injection of sporozoites. New Zealand White Rabbit was used for feeding the mosquitoes to maintain the *A. stephensi* colony.

3.2.2 Cell lines

Human hepatoma (HepG2) cell line was preserved in liquid nitrogen as frozen stock suspended in freezing medium. The composition of mammalian cell freezing medium was -70% incomplete Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Cat# 11965092), 20% fetal bovine serum (FBS) (Gibco, Cat# 10500064) and 10% dimethyl sulphoxide (DMSO) (Sigma, Cat# D2650). To initiate the culture, the frozen vial was quickly thawed by placing in a water bath at 37°C. The thawed cells were transferred to a 15ml tube and 5ml of pre-warmed complete DMEM (prepared by mixing 445 ml of DMEM, 50ml FBS, and 5ml 100X antibiotic antimycotic solution (Gibco, Cat# 15240062) and filtered through 0.2µm filter and stored at 4°C) and centrifuged at 1500 rpm for 3 min. The supernatant was discarded, and the pellet was resuspended in 3ml of complete DMEM and transferred to a T-25 culture flask (Corning, Cat# 430639). The flask was shifted to CO_2 incubator maintained at 5% CO_2 and 37°C. The cells were subcultured before they reached 100% confluency by adding 0.25% Trypsin-EDTA (Gibco, Cat# 25200056) and incubated at 37°C for 2 min. Following trypsinization, the cells were washed in 5ml of complete DMEM by centrifuging at 1500 rpm for 5 min. The viable cells were counted and seeded at the desired density based on the experimental needs.

3.2.3 Retrieval of target gene sequences

Two public domain databases namely Plasmo DB (http://www.plasmodb.org/plasmo) and Sanger gene database (http://www.genedb.org/Homepage/Pberghei) were used to retrieve ORF, 5' and 3' untranslated regions of *Pb K⁺/ACα* and *Pb P230p*.

S. No.	Primer	Primer Sequence (5' – 3')
1	K FP1	CCC*CTCGAG*GAACGTTGTATTTTATTTTAACTCATAA
2	K RP1	ATAAT*ATCGAT*TACAAATGTCTTTAAAATCAGGCAT
3	K FP2	ATAAG*GCGGCCGC*TTTTTTAATTATAAGAAAAAATAAA
4	K RP2	CCC*GGCGCGCC*TCAAAAAAAAAAAATTAAAAAATTAACT CTTAACG
5	K FP3	GTTTGGTTTGTTCTTTTGATTTGTTGGAATAT
6	H RP	TTCCGCAATTTGTTGTACATAAAATAGGCA
7	D FP	GTTGTCTCTTCAATGATTCATAAATAGTTG
8	K RP3	AAATGCGGCACGCTTTGAGTATAAATATTCA
9	K FP4	TCGGTCGAGCTAAATATTCTCTTGTTTC
10	K RP4	AATCGAATACACAAATTGTTATAGTATAATATAC

11	K FP5	GATGGGTCGATAAACAAAAATATAGGAG
12	K RP5	GGATGTAACTCCTGTTTTGGTATATTCTTGA
13	N FP1	AA*CTCGAG*TCATGGATCATATCCACTAACAAT
14	N RP1	AAT*ATCGAT*TGTGTTTTATTTGGATGTGCAAT
15	N FP2	AT*GCGGCCGC*TTCTCTTGAGCCCGTTAATGAA
16	N RP2	AT*GGCGCGCC*TAGGAAATTTGTTTATTTTTAT
17	N FP3	AGAATTGTATATGGTAAAGAACCTACTAACACAA
18	N RP3	ATAGTGACTTTCAGTGAAATCGCAAACATAAG
19	N FP4	ATTTCCAAAACCGTTAGAATATGTAGCATTACA
20	N RP4	TTGGCGTCCCATCTATGCTACTCACTT
21	*Pb 18S* FP	GGGGAGATTGGTTTTGACGTTTATG
22	*Pb 18S* RP	AAGCATTAAATAAAGCGAATACATCCTTA
23	*hGAPDH* FP	CCTCAACTACATGGTTTACAT
24	*hGAPDH* RP	GCTCCTGGAAGATGGTGATG

Table 2: List of primers and their sequences used for the construction of transfection plasmids, to confirm the site specific integrations, to check wild type contamination from cloned lines of *Pb K⁺/ACα* KO and WT GFP, and real time PCR analysis.

3.2.4 RNA isolation from different life cycle stages of *P. berghei* to study expression of *Pb K⁺/ACα* and synthesis of cDNA

To study the gene expression of *Pb K⁺/ACα*, RNA was isolated from various life cycle stages. In brief, mice were infected with WT *P. berghei* asexual blood stages and after obtaining 10-12% parasitemia, the blood was harvested by cardiac puncture. The blood was lysed using 0.5% saponin and pelleted at 15,000 rpm at 4°C. Following 3-4 washes with sterile RNAse free PBS, the sample was pelleted. The midguts and salivary glands were obtained on D14 and D18 respectively following dissection of infected *A. stephensi* mosquitoes. Different time points of developing liver stages were harvested from HepG2 culture, following trypsinization. The cells were washed 3-4 times with sterile RNAse free PBS and samples were pelleted. The samples obtained from different stages were subjected to RNA extraction using PureLink RNA isolation kit according to manufacturer's protocol. Approximately 2µg of isolated RNA was reverse transcribed to generate cDNA.

3.2.5 Expression analysis of *Pb K+/ACα* by quantitative real time PCR

Gene expression of *Pb K⁺/ACα* was quantified by absolute method of qRT PCR. A 120bp fragment of *Pb K⁺/ACα* was amplified by PCR using the primers K FP5 and K RP5, adenylated and ligated in pTZ57R/T. The clone was confirmed by sequencing and

gene specific standards were made from 10^8 to 10^2 copies/µl range by log dilution. Similarly, standards were generated for *Pb 18S rRNA* (135bp) to be used as internal control. For real time PCR, cDNA generated from different stages of *P. berghei* and gene specific standards were used as a template along 0.25µM each of forward and reverse primers of either *Pb K⁺/ACα* or *Pb 18S rRNA* and iQ SYBR Green supermix. The data normalization was done by obtaining the ratio of absolute copy numbers of *Pb K⁺/ACα* and *Pb 18S rRNA* for each sample.

3.2.6 Construction of *Pb K⁺/ACα* knockout vector

For disruption of *Pb K⁺/ACα* gene, 929 bp of 5' UTR was amplified using primer pair K FP1 (*XhoI* restriction site) and K RP1 (*ClaI* restriction site) and 506 bp of 3' UTR was amplified with primer pair K FP2 (*NotI* restriction site) and K RP2 (*AscI* restriction site) from *P. berghei* ANKA genomic DNA. Both fragments were amplified with Platinum Pfx polmerase (invitrogen) using thermal cycler conditions 94°C for 2min, 30 cycles of 94°C for 30 sec, 56°C for 30 sec and 68 °C for 1 min and a final extension of 68 °C for 10 min. The amplified products were gel extracted, adenylated and cloned in pTZ57R/T vector. The clones were confirmed by double digestion with respective restriction enzymes, followed by sequencing. Sequence confirmed *5' and 3' UTRs* were subcloned into the targeting vector pBC-GFP-hDHFR (Appendix - Fig. A3) at *XhoI*/*ClaI*, and *NotI*/*AscI* restriction sites respectively to obtain *Pb K+/ACα* KO plasmid (Appendix - Fig. A4). The targeting construct was released using XhoI and AscI, gel extracted and used for *Plasmodium* transfection to replace the genomic locus, thus generating a "knock-out" parasite expressing GFP.

3.2.7 Construction of *P230p* targeting plasmid for generating *Pb* ANKA WT GFP (WT GFP) parasites

For successful replacement of nonessential gene locus - *P230p* (*PBANKA_030600*), *5'* and *3'* fragments of the gene were amplified and cloned into the targeting vector pBC-GFP-hDHFR. To achieve this, 537 bp of *5'* fragment was amplified using the primers N FP1 (containing *XhoI*) and N RP1 (containing *ClaI*) and 918 bp of *3'* fragment was amplified using the primers N FP2 (containing *NotI*) and N RP2 (containing *AscI*), and cloned in 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 respective restriction enzymes and subcloned into the targeting vector pBC-GFP-hDHFR to obtain GFP@*P230p* targeting plasmid (Appendix - Fig. A5). 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.

3.2.8 *Plasmodium* transfection and selection of stable integrants

Plasmodium transfections were done as described earlier. In brief, the overnight cultured schizonts were enriched by nycodenz and the targeting construct was electroporated into schizonts. Immediately after electroporation, transfected schizonts were injected intravenously into mice. Two independent transfections were done for each construct and labeled as T1 and T2. After 24 hrs, the mice were subjected to pyrimethamine treatment for 5-7 days to select the stably transfected parasite population. Stable integration was confirmed by the presence of GFP parasites in the blood smears of mice. The clonal population was obtained from both the transfected lines and the phenotypic characterization was done independently with the two lines.

3.2.9 Confirmation of correct integrations by PCRs

Site specific integrations of targeting construct was confirmed by PCR using genomic DNA as a template. A forward primer K FP3 or N FP3, designed upstream of the recombined fragment and a reverse primer H RP designed in HSP70 promoter of the replacement cassette amplified a 1166 bp and 883 bp respectively from *Pb K+/ACα* KO and WT GFP. Similarly, a forward primer D FP designed in 3'UTR region of DHFR cassette in the targeting construct and a reverse primer K RP3 or N RP3 flanking beyond the site of integration amplified 831 bp and 1139 bp respectively from *Pb K+/ACα* and KO WT GFP.

3.2.10 Limiting dilution of transfected parasites

The drug resistant population confirmed for correct site specific integration was propagated in a naïve BALB/c mouse and the parasitemia was monitored daily. When the parasitemia was around 1-2%, the blood was diluted to 1 infected RBC/200µl in incomplete RPMI medium. Approximately 200µl each of this diluted

blood was injected intravenously into 20 BALB/c mice to achieve clonal dilution of transfected parasites. Blood smears were made from day 8 post infection and parasitemia was monitored daily. When the parasitemia reached around 3-5%, blood was collected from mice and a portion of the blood was taken for genomic DNA isolation while remaining was frozen for future use. Diagnostic PCR was performed using primer sets K FP4/K RP4 or N FP4/N RP4 that will amplify respectively products from ORF of *Pb K+/ACα* or *Pb P230p*. A positive PCR from WT genomic DNA while a complete absence of the product from the clonal population confirmed that the transfected parasites were successfully cloned.

3.2.11 Analysis of *Pb K⁺/ACα* KO propagation in blood and mosquito

Blood stage propagation of *Pb K+/ACα* KO parasites was compared with WT GFP parasites by intravenous injection of $1x10^3$ infected RBC of either *Pb K+/ACα* KO or WT GFP into a group of 3 BALB/c mice. Progression of parasitemia was monitored by Giemsa stained smears from day 3 post infection. *A. stephensi* mosquitoes were fed on *Pb K+/ACα* KO or WT GFP infected BALB/c mice having 1-2% gametocytes. Following two successive feedings, the infected mosquitoes were maintained at 20°C and 80% RH. On day 14 post infection, the mosquitoes were dissected to separate midguts and observe the oocyst burden and its sporulation pattern. On day 18-22 post infection with blood meal, sporozoites were obtained by disrupting dissected salivary glands. Dissections of mosquito salivary glands were performed in incomplete RPMI. The glands were crushed with plastic pestle and centrifuged at 800 rpm for 3min at 4°C to remove the gland debris. The supernatant was separated, and sporozoites were counted using Neubauer's chamber.

3.2.12 Treatment of sporozoites with K⁺ channel inhibitors

Twenty thousand WT GFP or *Pb K⁺/ACα* KO sporozoites were exposed to 0.5mM quinine or 20mM Tetraethylammonium (TEA) either in the presence or absence of 142mM K^+ buffer for 20 minutes and added to HepG2 cultures maintained in labtek chamber slides. The infectivity of the cultures was evaluated at the end of 48 h by measuring the *P. berghei* specific *18S rRNA* copy number by real time PCR. Normalization was done with the host GAPDH to obtain the parasite burden.

3.2.13 *In vivo* infectivity *Pb K⁺/ACα* KO sporozoites

To study the *in vivo* sporozoite infectivity of *Pb K⁺/ACα* KO towards hepatocytes, transmission was allowed through mosquito bite and intravenous injections. Nearly 15-20 mosquitoes harbouring *Pb K⁺/ACα* KO parasites (day 18 to 21 post infection) were transferred to a new cage and allowed to bite anesthetized C57BL/6 mice for 15 min. Two independent experiments were done, with 3 mice in each group. As a control, we also transmitted WT GFP sporozoites through similar routes. Salivary glands from all blood meal positive mosquitoes were dissected to confirm the presence of GFP expressing sporozoites in both lines, which was used as a correlate of successful malaria transmission to mice. In an independent experiment, $2x10^4$ sporozoites of either *Pb K⁺/ACα* KO or WT GFP parasites were intravenously injected into 4 mice each and at 40 hours post infection, the mice were sacrificed and dissected to obtain liver tissue. The liver was washed twice in PBS and homogenized in 10ml TRIzol (Invitrogen, Cat# 15596026). Approximately 200µl of liver homogenate was transferred into fresh tube and diluted to 1 ml in TRIzol. RNA was isolated according to the manufacturer's instructions. cDNA was generated from 2 µg of RNA and the parasite burden was quantified by real time PCR analysis as described earlier (Bruna-Romero *et al.*, 2001) using *Pb 18S rRNA* primers and iQ SYBR Green Supermix. Host GAPDH gene expression was quantified in parallel as an internal control. Parasite burden was expressed as normalized values obtained by taking the ratio of *Pb 18S rRNA*/host *GAPDH* copy numbers.

3.2.14 *In vitro* infectivity of *Pb K⁺/ACα* KO sporozoites treated with 142mM [K⁺]

To ascertain the role of *Pb K⁺/ACα* in sporozoite infectivity, $2x10^4$ sporozoites of either WT GFP or *Pb K⁺/ACα* KO were incubated in control medium or 142mM [K⁺] medium for 35 min at room temperature and were added to HepG2 cells. RNA was extracted from 48h EEF cultures using Purelink RNA isolation kit following manufacturer's instructions. cDNA was generated using 2 µg RNA and parasite burden was quantified using *Pb 18S rRNA* primers. Parasite burden was expressed as normalized values obtained by taking the ratio of *Pb 18S rRNA*/human *GAPDH* copy numbers.

3.2.15 *In vitro* EEF development

To study the liver stage or exo erythrocytic form (EEF) development *in vitro*, human liver carcinoma (HepG2) cells that support complete development of *P. berghei* were used. Briefly, $1x10^5$ HepG2 cells were seeded per well in an eight well glass LabTek chamber slide (Nunc, Cat# 177402) that was pretreated with Collagen (Corning, Cat# 354236) and maintained in a CO_2 incubator at 5% CO_2 and 37°C. When the HepG2 cells were 60-70% confluent, $2x10^4$ sporozoites were added to each well and the chamber slide was centrifuged at 1500 rpm for 4 min using a swing bucket rotor to facilitate instant attachment of sporozoites to HepG2 cells. The cultures were placed in a CO_2 incubator and medium was replaced after 2-3 hours of sporozoite addition with fresh complete DMEM supplemented with 10% FBS and 1X antibiotic antimycotic solution. To prevent contamination of the cultures, the medium was changed every 8 hours until 67 h time point. Cultures were fixed at different stages of EEF development like 12 h, 24 h, 36 h and 67 h with 4% paraformaldehyde and stored at 4°C for immunofluorescence assay (IFA). Alternatively, cells were harvested at different time points for RNA isolation to study the gene expression by qRT PCR.

3.2.16 Immunofluorescence assay

To monitor the progressive development of EEFs in HepG2 cells, the fixed cultures were washed with 1X TBS (pH-7.4) and permeabilized with ice cold methanol and acetone (1:1 ratio) for 20 min at 4°C. The cultures were washed with 1X TBS and nonspecific blocking was done with 3% BSA (SRL, Cat# 0140105) for one hour at 37°C. In the next step, the cultures were incubated for 1h at 37°C with a rabbit primary antibody (1:1000 dilution) generated against UIS4, a parasitophorous vacuolar membrane (PVM) specific protein. The cultures were washed with TBS, TBST and TBS 15 min each at 37°C. To reveal the immunoreactivity, the cultures were incubated with anti-rabbit secondary antibody (1:300 dilution) conjugated to Alexa Fluor 594 (Life Technologies, Cat# A11005) and DAPI (Sigma, Cat#9564) diluted in 3% BSA solution and incubated additionally for 1h at 37°C. The cultures were washed again with TBS, TBST and TBS for 15 min each at 37°C. After final wash, the cultures were air dried and mounted with ProLong Gold antifade mountant (Molecular Probes, Cat#P36930) and covered with a coverslip that was sealed with transparent nail polish and slides were preserved in dark. The samples were visualized using Nikon Eclipse upright

fluorescence microscope and images were acquired and analyzed with NIS Elements advanced research software.

3.2.17 Determination of pre-patent period

Pre patent period is defined as time required for the appearance of blood stages following infection with sporozoites. To study the pre-patent period following delivery of *Pb K⁺/ACα* KO sporozoites, transmission of malaria was allowed through mosquito bite and intravenous route. Nearly, 15-20 mosquitoes (day 18-20 post infection) carrying either *Pb K⁺/ACα* KO or WT GFP sporozoites in their salivary glands were transferred to a new cage, and allowed to bite anesthetized C57BL/6 mice for 15 min. Three independent experiments were performed, with 3 or 4 mice in each group. As control, pre patent period was also monitored for mice that received WT GFP sporozoites, through bite in two independent experiments (3 mice in each group). Salivary glands from all the mosquitoes that received blood meal were dissected to confirm the presence of GFP expressing WT or *Pb K⁺/ACα* KO sporozoites, which was used as a correlate of successful malaria transmission to mice. In separate experiment, high dose ($2x10^4$) of either *Pb K⁺/ACα* KO or WT GFP sporozoites isolated from salivary glands were intravenously injected into C57BL/6 mice (3 mice in each group) and pre patent periods were determined by Giemsa stained blood smears.

3.3 Results

3.3.1 Gene expression analysis of *Pb K⁺/ACα* by quantitative real time PCR revealed maximal expression in 16hr EEF

In order to quantify the gene expression of *Pb K⁺/ACα*, gene specific standards were prepared for *Pb K⁺/ACα* and *Pb 18S rRNA*. The cDNA samples generated from different stages of the life cycle stages were run alongside with standards. At the end of PCR, the gene expression was expressed as copy numbers of either *Pb K⁺/ACα* or *Pb 18S rRNA*. Normalisation was done by obtaining a ratio of *Pb K⁺/ACα* copy number versus *Pb 18S rRNA* copy number. The normalised data revealed highest expression of *Pb K⁺/ACα* in 16hr EEF (Fig. 21).

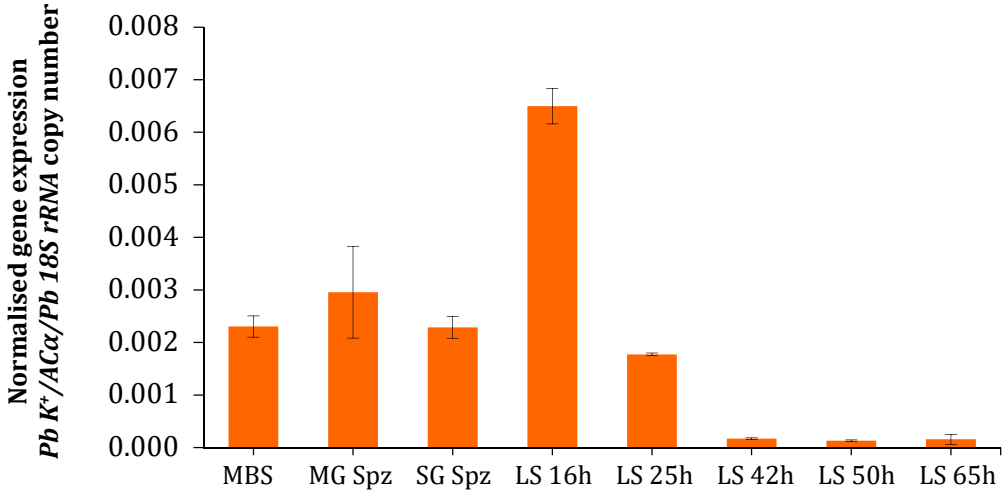

Fig. 21 Normalized gene expression of *Pb K⁺/ACα* across the life cycle stages of *P. berghei*. Gene expression analysis by quantitative real time PCR revealed highest gene expression in the 16hr EEF (LS 16hr) followed by midgut sporozoites (MG Spz). The normalised data was expressed as a ratio of absolute copy numbers of *Pb K⁺/ACα* versus *Pb 18S rRNA* (internal control) for each stage of *Plasmodium* life cycle. MBS: mixed blood stages, MG Spz: Midgut sporozoites, SG Spz: salivary gland sporozoites, LS 16h: Liver stage 16h, LS 25h: Liver stage 25h, LS 42h: Liver stage 42h, LS 50h: Liver stage 50h, LS 65h: Liver stage 65h.

3.3.2 Successful replacement of *Pb K⁺/ACα* by GFP-DHFR cassette by double crossover recombination

The gene locus of *Pb K⁺/ACα* is shown in Fig. 22A. To achieve successful double crossover recombination for replacing the *Pb K⁺/ACα ORF*, *5'* and *3' UTRs* of *Pb K⁺/ACα* were cloned in multiple cloning sites present on either side of *GFP-hDHFR* cassettes in pBC-GFP-hDHFR plasmid (Fig. 22B). The genomic organisation at *Pb K⁺/ACα* locus following the successful double homologous recombination is shown in Fig. 22C. The *5' UTR* and *3' UTR* of *Pb K⁺/ACα* were amplified by PCR using primer sets K FP1/K RP1 and K FP2/K RP2 respectively that amplified products of 929 bp and 506 bp that were resolved on 1% agarose gel (Fig. 22D). These fragments were cloned into the targeting vector and confirmed by the release of 5' UTR using the restriction enzymes *XhoI*/*ClaI* and 3' UTR by using restriction enzymes *NotI*/*AscI* (Fig. 22E (a)). The complete cassette was released with the restriction enzymes *XhoI*/*AscI* (Fig. 22E (b)), and it was purified by gel extraction. The purified *Pb K⁺/ACα* KO construct was electroporated into *P. berghei* schizonts using predefined program U033 in Amexa nucleofector and the parasites were injected intravenously into mice. The recombinant parasites were selected using pyrimethamine (given to mice through oral route) and the parasitemia was monitored daily by blood smears stained with Giemsa. Genomic DNA was isolated from the drug resistant parasites and correct site specific integration of 5' UTR was confirmed by the primer set K FP3 designed beyond the region of recombination and H RP designed in the GFP cassette that amplified 1166 bp product. Similarly, the correct integration of 3' UTR was confirmed by the primer set D FP designed in the hDHFR cassette and K RP3 designed in the 3' UTR beyond the region of recombination that amplified 831 bp product. These confirmations indicated correct integrations at 5' and 3' UTRs (Fig. 22F (a)). Limiting dilution was performed to obtain clonal population of *Pb K⁺/ACα* KO parasites. *Pb K⁺/ACα* ORF specific primers K FP4 and K RP4 were used to confirm deletion of *Pb K⁺/ACα* locus that specifically amplify a product of 886 bp only in WT but not in *Pb K⁺/ACα* KO parasites (Fig. 22F (b)). The cloned line of *Pb K⁺/ACα* KO expressed GFP constitutively (Fig. 22G).

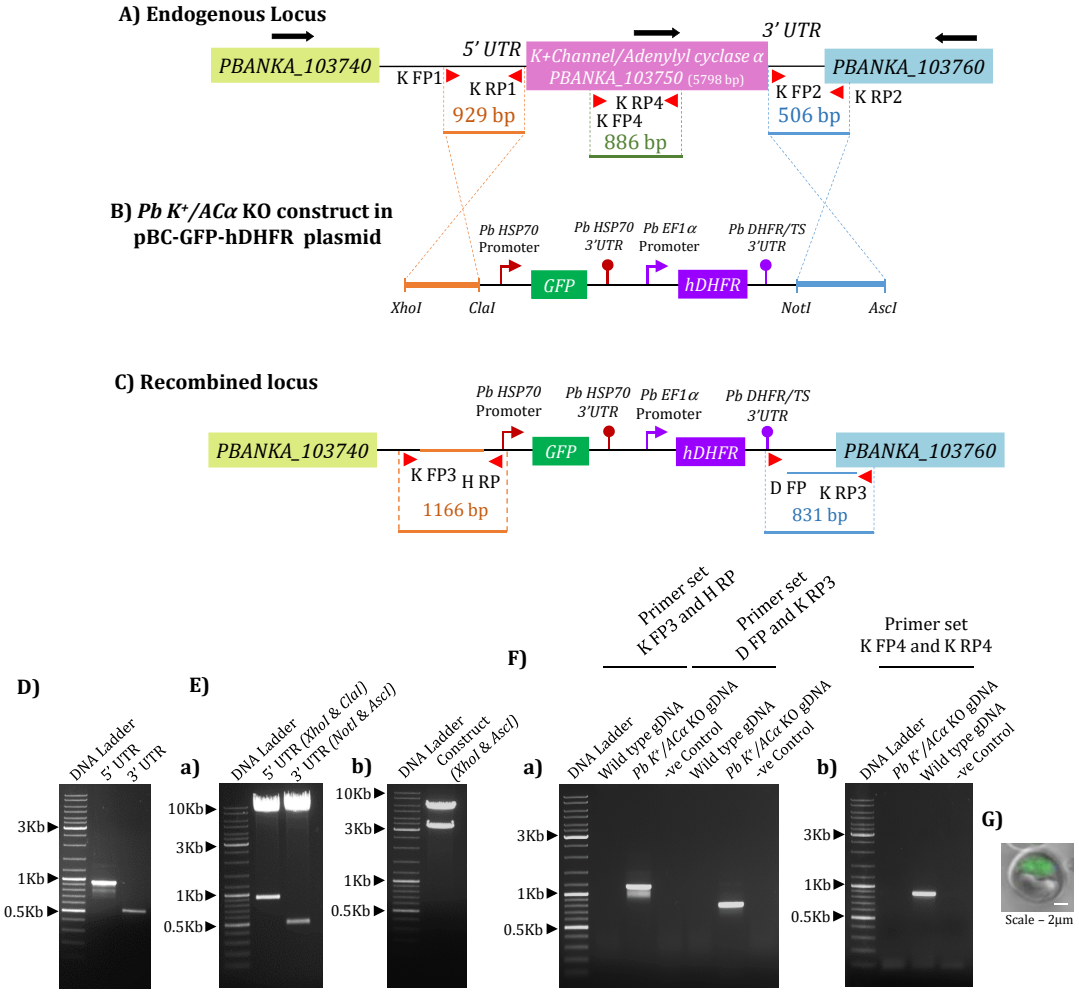

Fig. 22 Generation of *Pb K⁺/ACα* KO parasite line. A) Genomic locus of *Pb K⁺/ACα* (PBANKA_103750) showing 5' and 3' UTRs. B) Elements of the targeting vector showing regions selected for homology, *GFP* and *hDHFR* cassettes. A 929 bp *5' UTR* fragment of *Pb K⁺/ACα* was cloned in XhoI/ClaI sites of the targeting vector. A 506 bp of *3' UTR* fragment was cloned into NotI/AscI site of the targeting vector. C) Recombined locus following successful double cross over recombination resulting in replacement of target gene, *Pb K⁺/ACα* by *GFP-hDHFR* cassettes. D) A 1% agarose gel showing the PCR products of *5' UTR* and *3' UTR* fragments. The *5' UTR* fragment was amplified with primers K FP1 and K RP1. The *3' UTR* fragment was amplified with primers K FP2 and K RP2. E) a) Release of *5'* and *3' UTR* fragments from transfection vector using restriction enzymes XhoI/ClaI and NotI/AscI. b) Release of targeting cassette (*5' UTR* fragment+*GFP* and *hDHFR* cassettes+*3' UTR* fragment) from the vector using restriction enzymes XhoI/AscI. F) a) Diagnostic PCR using primers within the targeting cassette and beyond sites of recombination revealing the correct site specific integration. PCR products of 1166 bp with primers K FP3 and H RP and 831 bp with primers D FP and K RP3 indicated correct 5' and 3' integrations respectively. b) A PCR product of 886 was amplified from the genomic DNA of WT parasites but not from cloned *Pb K⁺/ACα* KO line with primer set K FP4/K RP4. G) A merged DIC image showing a GFP expressing *Pb* WT GFP parasite inside RBC.

3.3.3 Successful disruption of *Pb P230p* locus by GFP-DHFR cassette by double crossover recombination to generate WT GFP parasite line

The gene locus of *Pb P230p* is shown in Fig. 23A. To integrate the *GFP* and *hDHFR* cassettes in the *Pb P230p* locus, *5'* and *3'* fragments of *Pb P230p* ORF selected for recombination were cloned in restriction sites present on either side of *GFP-hDHFR* cassettes in the plasmid pBC-GFP-hDHFR (Fig. 23B). The genomic organisation at *Pb P230p* locus following the successful double homologous recombination is shown in Fig. 23C. The *5'* and *3'* fragments of *Pb P230p* were amplified by PCR using primer sets N FP1/N RP1 and NFP2/N RP2 respectively that amplified products of 537 bp and 918 bp that were resolved on 1% agarose gel (Fig. 23D). Both the fragments were cloned into the targeting vector and confirmed by the release of *5'* fragment using the restriction enzymes *XhoI*/*ClaI* and *3'* fragment by using restriction enzymes *NotI*/*AscI* (Fig. 23E (a)). The complete cassette was released with the restriction enzymes *XhoI*/*AscI* (Fig. 23E (b)), and it was purified by gel extraction. The purified *Pb P230p* targeting construct was electroporated into *P. berghei* schizonts using predefined program U033 in Amexa nucleofector and the parasites were injected intravenously into mice. The recombinant parasites were selected using pyrimethamine (given to mice through oral route) and the parasitemia was monitored daily by blood smears stained with Giemsa. Genomic DNA was isolated from the drug resistant parasites and site specific integrations of *5'* fragment was confirmed by the primer set N FP3 designed beyond the region of recombination and H RP designed in the GFP cassette that amplified a product of 883 bp. Similarly, the correct integration of *3'* fragment was confirmed by the primer set D FP designed in the hDHFR cassette and N RP3 designed beyond the region of recombination that amplified a product of 1139 bp. These confirmations indicated correct integrations at *5'* and *3'* regions (Fig. 23F (a)). Limiting dilution was performed to obtain clonal population of *Pb P230p* GFP (WT GFP) parasites. *Pb P230p* ORF specific primers N FP4 and N RP4 were used to confirm disruption of *Pb P230p* locus that specifically amplified a product of 896 bp only in WT but not in WT GFP parasites (Fig. 23F (a)). The cloned line of WT GFP expressed GFP constitutively (Fig. 23G).

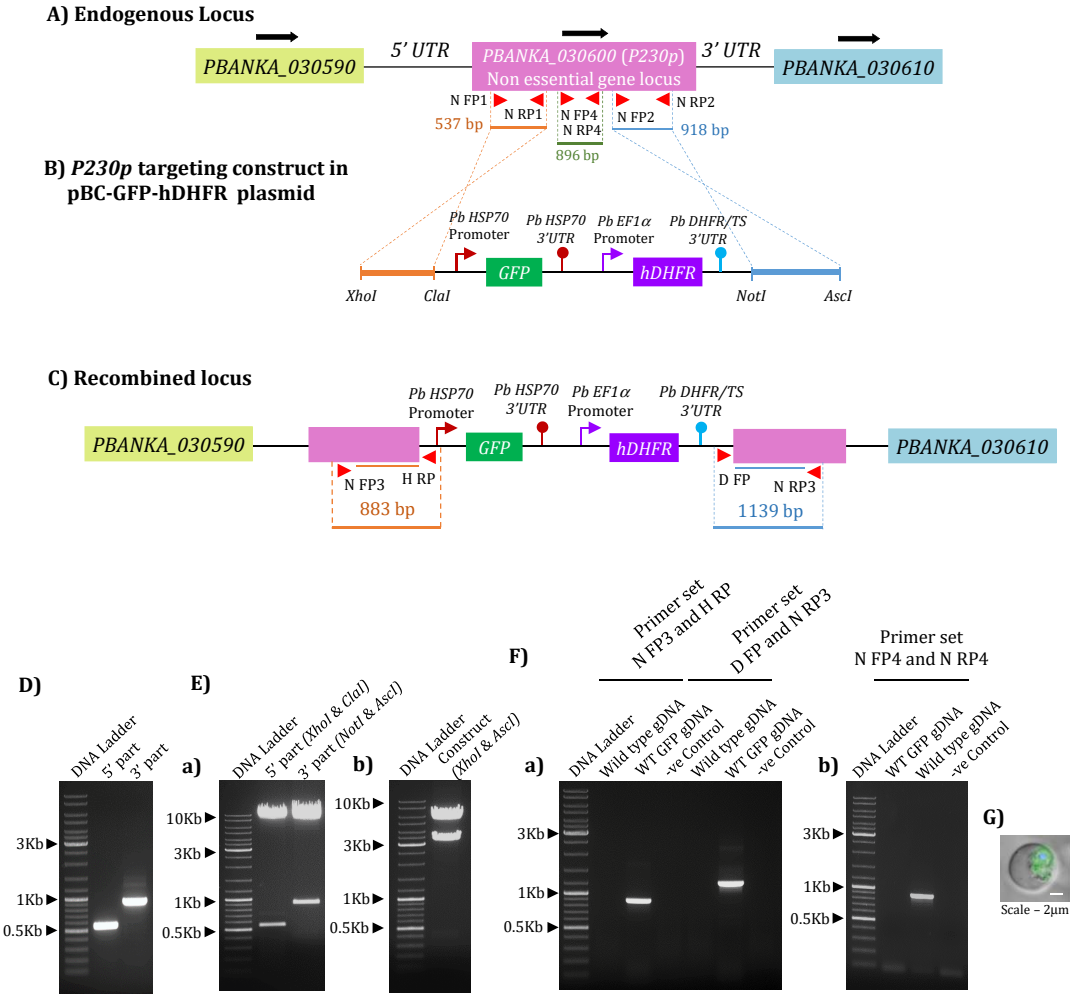

Fig. 23 Generation of WT GFP parasite line by targeting the non-essential gene locus *P230p*. A) Genomic locus of *Pb P230p* (PBANKA_030600) showing 5' and 3' UTRs. B) Elements of the targeting vector showing regions selected for homology, *GFP* and *hDHFR* cassettes. A 537 bp 5' fragment and 918 bp of 3' fragment of *Pb P230p* were cloned at *XhoI*/*ClaI* and *NotI*/*AscI* sites respectively in the targeting vector. C) Recombined locus following successful double cross over recombination resulting in replacement of target gene, *Pb P230p* by *GFP-hDHFR* cassettes. D) A 1% agarose gel showing the PCR products of *5'* and *3'* fragments. The *5'* and *3'* fragments were amplified with primer sets N FP1/N RP1and N FP2/N RP2 respectively. E) a) Release of *5'* and *3'* fragment from transfection vector using restriction enzymes XhoI/ClaI and NotI/AscI respectively. b) Release of targeting cassette (*5'* fragment+ *GFP* and *hDHFR* cassettes+*3'* fragment) from the vector using restriction enzymes XhoI/AscI. F) a) Diagnostic PCR using primers within the targeting cassette and beyond sites of recombination revealing the correct site specific integration. A PCR product of 883 bp with primers N FP3 and H RP and a product of 1139 bp with primer set D FP and N RP3 indicated correct 5' and 3' integrations. b) A PCR product of 896 was amplified from the genomic DNA of WT parasites but not from cloned WT GFP line with primer set N FP4/N RP4. H) A merged DIC image showing a GFP expressing WT GFP parasite inside RBC.

3.3.4 *Pb K⁺/ACα* is not essential for blood stage propagation

To monitor, if *Pb K⁺/ACα* disruption affected the blood stage propagation, two groups of BALB/c mice (3-4 mice/group) were intravenously injected with 1 x 10^3 iRBCs per mice of either *Pb K⁺/ACα* KO or WT GFP parasites. The blood stage propagation was monitored for 7 days by making Giemsa stained blood smears. The propagation of *Pb K⁺/ACα* KO parasites at similar rates as WT GFP parasites and the presence of all asexual stages and gametocyte stages in *Pb K⁺/ACα* KO infected mouse revealed its non essential role during intra erythrocytic development (Fig. 24).

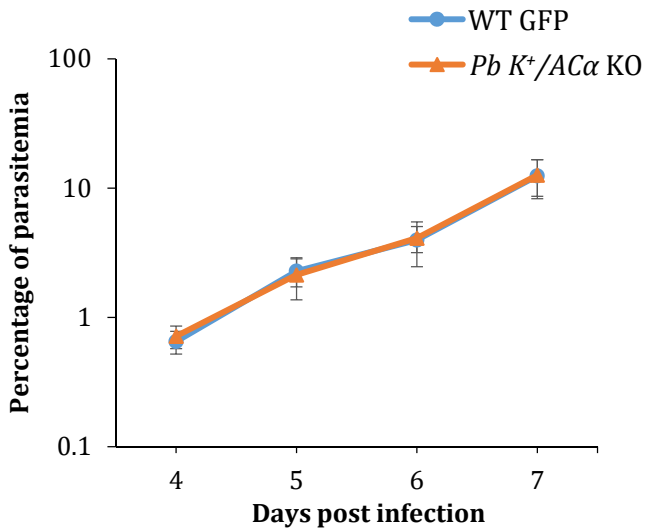

Fig. 24 *Pb K⁺/ACα* KO blood stage parasites propagate identically as WT GFP parasites. A) 1X10^3 iRBCs of either WT GFP or *Pb K⁺/ACα* KO parasites were intravenously injected in two groups of mouse (3 mouse/group) and monitored for the blood stage propagation of parasites daily for 7 days by making Giemsa stained smears and plotted in a logarithmic graph.

3.3.5 *Pb K⁺/ACα* is not essential for mosquito stage development

Pb K⁺/ACα KO parasites were transmitted to the *A. stephensi* mosquitoes to monitor the mosquito stage development. *Pb K⁺/ACα* KO parasites developed oocysts in numbers comparable to WT GFP (Fig. 25A and 25B). Oocyst sporulation (Fig. 25C and 25D) and ability of sporozoites to egress and migrate to salivary glands (Fig. 25E and 25F) were also comparable with that of WT GFP parasites suggesting that deletion of *Pb K⁺/ACα* manifested no defect in the developmental stages in mosquito.

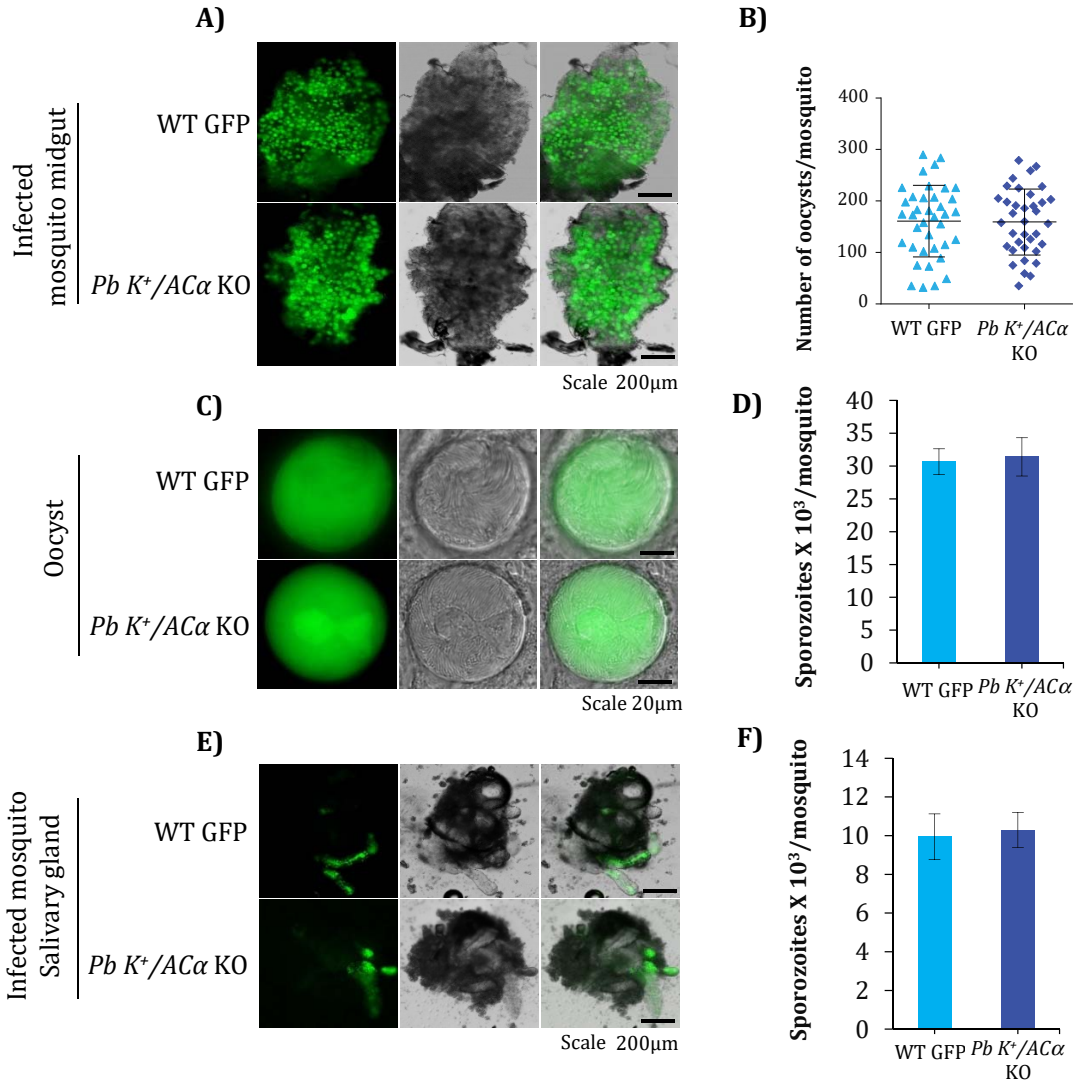

Fig. 25 Mosquito stages of *Pb K⁺/ACα* KO do not show any defect in sexual development, formation and sporulation of oocysts and the migration of sporozoites to salivary glands. Malaria was transmitted to female *Anopheles* mosquitoes from mice harboring gametocytes stages of either WT GFP or *Pb K⁺/ACα* KO parasites. A) Microscopic images of day 14 midguts (post feeding) showing the oocysts derived from WT GFP and *Pb K⁺/ACα* KO parasites. B) Dot plot showing the quantification of oocysts in an infected mosquito midgut on day 14 post feeding. C) Microscopic images of oocyst sporulation on day 14 post feeding in WT GFP and *Pb K⁺/ACα* KO parasites. D) Bar graph showing the average number of midgut sporozoites per mosquito on day 14 post feeding. E) Microscopic images of day 18 (post feeding) salivary glands showing the WT GFP and *Pb K⁺/ACα* KO sporozoites. F) Bar graph showing the average number of salivary gland sporozoites per mosquito on day 18 (post feeding). All the quantifications were represented as Mean+SD.

3.3.6 *Pb K⁺/ACα* KO sporozoites have compromised *in vivo* infectivity

In vivo infectivity of *Pb K⁺/ACα* KO sporozoites was analysed by quantifying parasite burden at 40 hours of EEF development in the liver of C57BL/6 mice delivered by either i.v., or mosquito bite. RNA was isolated from the liver, cDNA was generated and the parasite burden was obtained by taking ratio of *Pb 18S rRNA* copy number versus host GAPDH copy number. Similar experiments were done with WT GFP parasites for comparison. Analysis of parasite liver stage burden in mice that received *Pb K⁺/ACα* KO sporozoites either by i.v., or by mosquito bite showed a fold reduction of 4.81 and 5.92 respectively as compared to WT GFP.

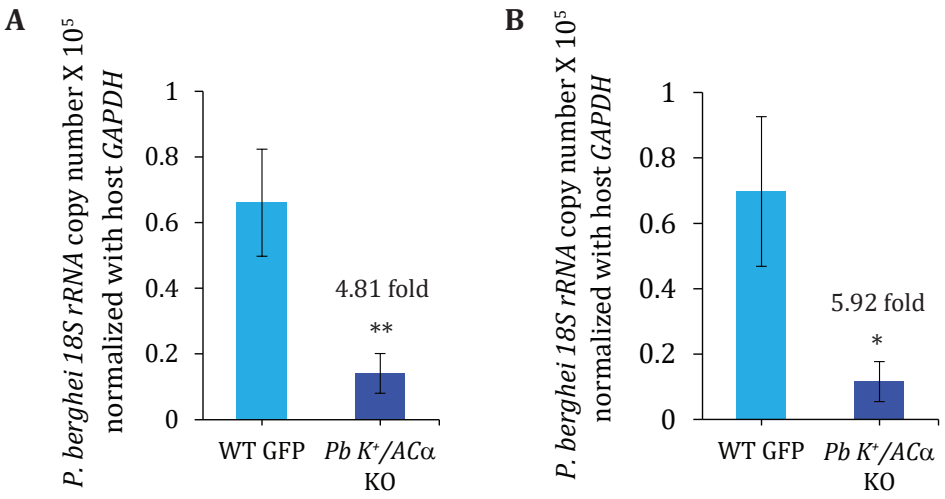

Fig. 26 *Pb K⁺/ACα* KO sporozoites have reduced *in vivo* infectivity. A) C57BL/6 mice were injected intravenously with 2x10⁴ sporozoites and the livers were isolated at 40h time point. The parasite burden was quantified by obtaining a ratio of *Pb 18S rRNA* copy number and host GAPDH copy number. A 4.81 fold reduction in parasite burden was observed in mice infected with *Pb K⁺/ACα* KO sporozoites as compared to WT GFP. B) Quantificatin of parasite burden in liver of C57BL/6 mice that received *Pb K⁺/ACα* KO sporozoites by mosquito bite showed 5.92 fold reduction in parasite burden as compared to WT GFP.

3.3.7 *Pb K⁺/ACα* KO sporozoites are less responsive to 142mM [K⁺]

Since sporozoites migrate through several cells prior to productive invasion, we reasoned that the sporozoites that are ready for productive invasion (referred to as "activated sporozoites") may be qualitatively different from those of the non-traversed sporozoites (freshly isolated salivary glands). Since it is difficult to obtain sporozoites that are ready for productive invasion, we mimicked conditions, as close as possible to intracellular conditions. We incubated $2x10^4$ sporozoites at room temperature in the medium containing 142mM [K⁺] for 35 minutes, the optimal time required for the sporozoites to reach hepatocytes from the site of natural infection (Sidjanski & Vanderberg, 1997). Following this incubation the sporozoites were considered qualitatively similar to those that are ready for productive invasion. The treated sporozoites were tested for their ability to infect HepG2 cells. Sporozoite infectivity was quantified by taking the ratio of parasite *18S rRNA* copy number to host GAPDH copy number by absolute method of real time PCR. The fold increase in the infectivity of WT GFP sporozoites was around 9.94 fold (Fig. 27A) as compared to 2.58 fold (Fig. 27B) for *Pb K⁺/ACα* KO sporozoites. We conclude that exposure of sporozoites in the presence of 142mM [K⁺] facilitates maximum activation of sporozoites for productive invasion and that this activation is mediated by K⁺ channel protein.

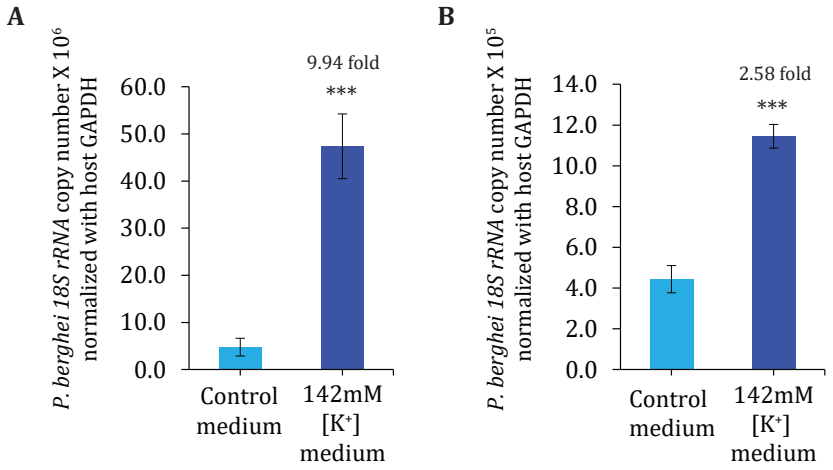

Fig. 27 *Pb K⁺/ACα* KO sporozoites show diminished infectivity to HepG2 cells following exposure to 142mM [K⁺] medium. HepG2 cells were infected with $2x10^4$ WT GFP or *Pb K⁺/ACα* KO sporozoites exposed to control or 142mM [K⁺] medium for 35 minutes at room temperature and the *in vitro* infectivity was quantified by gene expression analysis of *Pb 18S rRNA* copy number by real time PCR. A) Exposure of WT sporozoites to 142mM [K⁺] medium as compared to control medium at room temperature enhanced sporozoite infectivity by 9.94 fold. B) Identical experiment performed with *Pb K⁺/ACα* KO enhanced sporozoite infectivity only by 2.58 fold. Number of experiments, n=3 (***p<0.001)

3.3.8 *Pb K⁺/ACα* KO is not essential for EEF development

For analysis of *in vitro* EEF development, the salivary glands of mosquitoes harbouring *Pb K⁺/ACα* KO or WT GFP sporozoites were dissected, crushed to release the sporozoites. These sporozoites were added to HepG2 cultures to study the *in vitro* transformation into EEFs. *Pb K⁺/ACα* KO sporozoites developed into fully grown EEFs in HepG2 cells. EEF development of *Pb K⁺/ACα* KO parasites were comparable in size and development with WT GFP at 12 h, 24 h, 36 h and 67 h that revealed its non-essential role during EEF development (Fig. 28).

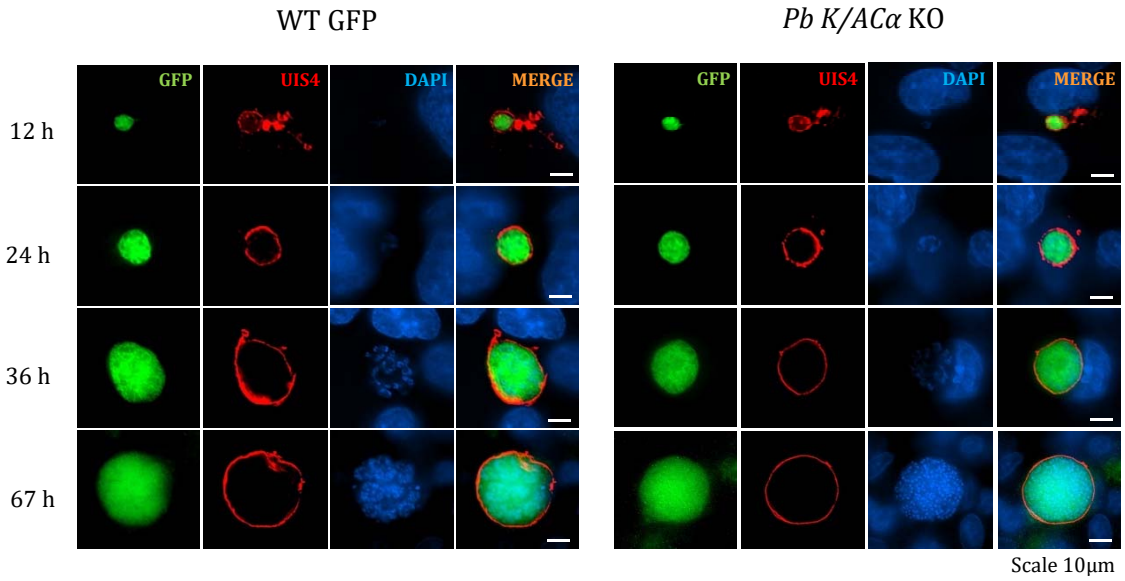

Fig. 28 The EEFs of *Pb K⁺/ACα* KO reveals no growth defect in liver stage development *in vitro*. Approximately 2x10⁴ sporozoites of either WT GFP or *Pb K⁺/ACα* KO were added to HepG2 cultures that support the complete *in vitro* EEF development of *P. berghei*. The cultures were fixed at indicated time points. The cultures were stained with anti UIS4 antibody that reacts with the PVM protein of EEF and DAPI was used to visualize the host and parasite nuclei. The EEFs derived from *Pb K⁺/ACα* KO sporozoites at all the time points were comparable to that of WT GFP EEFs.

3.3.9 *Pb K⁺/ACα* KO disruption had no effect on initiation of blood stage infection after exo-erythrocytic cycle

Inoculation of *Pb K⁺/ACα* KO sporozoites to C57BL/6 mice through mosquito bite or by i.v., of $2X10^4$ sporozoites successfully initiated blood stage infection on D4 post infection in three independent experiments. As a control, WT GFP sporozoite were infected in C57BL/6 mice by both routes (Table 3).

Route of infection	Parasite Strain	Expt. No.	No. of animals used	No. of animals positive for blood stage infection	Pre-patent period
Mosquito bite	Wild Type GFP	I	3	3/3	D3.5
		II	3	3/3	D3.5
	Pb K⁺/ACα KO	I	4	4/4	D4
		II	3	3/3	D4
Intravenous injection of $2x10^4$ sporozoites per mouse	Wild Type GFP	I	3	3/3	D3.5
		II	3	3/3	D3.5
	Pb K⁺/ACα KO	I	4	4/4	D4
		II	3	3/3	D4
		III	4	4/4	D4

Table 3: Showing the kinetics of malaria transmission by mosquito bite and i.v. routes, the details of number of experiments performed, number of animals used in each experiment, the number of animals that became positive for blood stage infection and the prepatent period. All blood meal positive mosquitoes following bite experiment were dissected to collect salivary glands to confirm the presence of GFP expressing sporozoites (WT GFP or *Pb K⁺/ACα* KO) under fluorescent microscope.

3.3.10 *Pb K⁺/ACα* KO sporozoites are refractory to K⁺ channel inhibitors

The effect of K⁺ channel inhibitors on the sporozoite infectivity was analysed by exposing WT GFP or *Pb K⁺/ACα* KO sporozoites to 142mM K⁺ in presence or absence of two classical K⁺ channel inhibitors, quinine and TEA for 30 minutes at room temperature. The WT GFP sporozoites treated with 0.5mM quinine and 20mM TEA showed 2.3 fold and 2.12 fold decrease in infectivity respectively as compared to untreated control (Fig. 29A). *Pb K⁺/ACα* KO sporozoites had no effect on infectivity when treated with inhibitors as compared to untreated group (Fig. 29B). These results concur with the lack of channel activity in the *Pb K⁺/ACα* KO sporozoites.

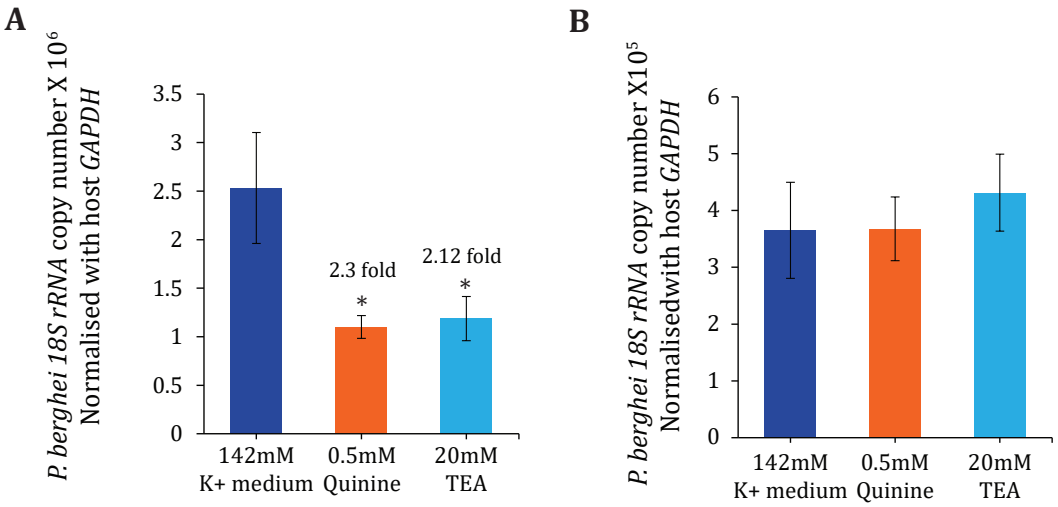

Fig. 29 *Pb K⁺/ACα* KO sporozoites do not respond to K⁺ channel inhibitors quinine and TEA. A) WT GFP or *Pb K⁺/ACα* KO sporozoites were either exposed 142mM K⁺ medium in presence or absence of K⁺ channel inhibitors, quinine (0.5mM) or TEA (20mM) and added to HepG2 cells. After 48 hours, the infectivity was quantified by gene expression analysis of *Pb 18S rRNA* copy number by real time PCR. The infectivity of WT GFP sporozoites exposed to quinine and TEA were reduced by 2.3 and 2.12 fold respectively as compared to 142mM K⁺ exposed WT sporozoites. B) Identical experiments were performed with *Pb K⁺/ACα* KO sporozoites. Exposure to either quinine or TEA showed no inhibitory effect on infectivity as compared to the 142mM K⁺ exposed *Pb K⁺/ACα* KO sporozoites. Number of experiments, n=3 (** $p<0.02$)

3.4 Discussion

Since *Plasmodium* sporozoites migrate through cells both under *in vivo* and *in vitro* conditions, we reasoned if changes in the concentration of ionic milieu could have a profound effect on sporozoite infectivity. It was already demonstrated that intracellular concentrations of [K^+] enhances the infectivity of the sporozoites. To prove that the observed effect of sporozoite activation was specifically mediated by the K^+ channel protein, we generated *Pb K^+/ACα* KO replacing the ORF with GFP and hDHFR cassettes employing regular double homologous recombination method. These mutants were analysed for their ability to complete *Plasmodium* life cycle. While the depletion did not affect the development of parasite in blood and mosquito stages, the *Pb K^+/ACα* mutant exhibited a dramatic decrease in sporozoite infectivity to hepatocytes. Interestingly, the fraction of sporozoites that successfully infected hepatocytes showed normal EEF development. We conclude that only sporozoite stages are responsive to high [K^+] concentrations that mediates its effects through the bifunctional *K^+/ACα* protein. Further studies are required to elucidate how K^+ influences the gene expression of sporozoites thus rendering them highly infectious to hepatocytes. One approach will be to look at global transcriptional changes that results from exposure to 142mM [K^+] in WT and *Pb K^+/ACα* mutant lines. Such studies may enable identification of pathways uniquely activated following K^+ exposure. It will be interesting to see if these mutants fail to activate such pathways.

Previous studies have demonstrated the role of cAMP in *Plasmodium* sporozoite apical exocytosis and stimulation of this process by agents like uracil derivatives and membrane permeant analogue of cAMP like 8Br-cAMP or forskolin, an activator of adenylyl cyclase (Ono *et al.*, 2008). In order to test whether extracellular K^+ regulates sporozoites apical exocytosis, *P. yoelii* sporozoites were exposed to either regular medium or in K^+ free medium followed by exposure to uracil derivatives. Exocytosis stimulated with uracil derivatives was inhibited in K^+ free medium. Concomitant with these findings, exocytosis was inhibited when sporozoites were pre incubated with different K^+ channel inhibitors. In contrast, extracellular K^+ was not required if sporozoite exocytosis was induced by 8 Br-cAMP or forskolin, suggesting that extracellular K^+ played a role only upstream to cAMP in the signaling cascade. However, an important consideration limits the direct implications of K^+ channel

activity on sporozoites exocytosis in this study. The authors have reported that ACα is required for exocytosis, a process stimulated by migration through hepatocytes. In such scenario, a choice of 142mM [K^+] will be a closer correlate to intracellular ionic mileau as compared to 5mM used by the authors to imply for the proposed effect of the K^+ channel mediated exocytosis. A direct comparison of our results with the earlier reported results of A*Cα* KO (Ono *et al.*, 2008) indicate that the our *Pb K⁺/ACα* KO had more dramatic phenotype as compared to *ACα* KO. One likely explanation for the observed difference can be attributed for the complete replacement of the K^+ channel gene as compared to the partial knock down of the *ACα* KO as generated by the authors.

However, sporozoite activation for productive invasion could be more complex and regulated process than envisaged, involving other parasite and host molecules. For instance the remarkable tropism exhibited by *Plasmodium* sporozoites for hepatocytes is possible because of the occurrence of specific interaction between the sporozoite and the target hepatocytes (Coppi *et al.*, 2007). This was shown in an elegant study that linked degree of HSPG sulphation as a molecular signal for switch in the parasite behaviour from migratory to invasive mode, a trait associated with proteolytic cleavage of CSP initiated when sporozoites contacted highly sulphated HSPGs of hepatocytes.

If sporozoites are retained at the site of injection for sustained periods (due to constrain of not encountering the dermal vessels) can prolonged migration through the avascular dermal tissue promote transformation and if so, what is the minimal host factors favouring this transformation in non-permissive cells? We have reported earlier that exposure to 142mM [K^+] facilitates the complete development of EEFs in various non-permissive cell lines leading to the formation of functional merozoites capable of causing blood stage infection (Kumar *et al.*, 2007). However, owing to transformed nature of the non-permissive cell lines, a direct extrapolation of these observations to the *in vivo* settings is highly questionable. A recently study however has demonstrated that ~ 10% of *P. berghei* sporozoites injected intradermally develop into EEFs that are functional equivalents of those derived from hepatocytes (Gueirard *et al.*, 2010). These EEFs were found to be associated with the epidermis and dermal hair follicles, sites that are highly immune-privileged and deprived of Class-I antigen

presentation. A likely prediction for the occurrence of skin EEFs may be the failure of sporozoites to reach the dermal vessels. This prompts the hypothesis that migration through skin cells also can promote initiation of sporozoite transformation, an event that can overcome or compensate the requirement for hepatocyte HSPGs.

In the light of emerging scenario unravelling the complex hierarchies of sporozoite-hepatocyte interactions, we provide evidence that the intracellular concentrations of [K^+] provide stimulus to dramatically alter the sporozoite responsiveness to productive invasion. The sporozoites utilize this cue likely in a time dependent (Kumar *et al.*, 2007) and progressive manner and migration ensures this requirement is met. Once a threshold level of activation is achieved through K^+ exposure, it remains a matter of chance if the sporozoites are in vicinity of dermal vessels for its efficient delivery to liver sinusoides. Alternatively, failure to leave the site of injection can also trigger EEF transformation in non-permissive cells.

Chapter 4

Functional Characterization of a Conserved Protein PBANKA_141700 by reverse genetics approach

4.1 Introduction

Plasmodium is a digenetic parasite that requires a vertebrate host and a female Anopheline mosquito to complete its life cycle. It has a complex life cycle and several stage specifically expressed proteins play important roles supporting its development or assisting in host-parasite interactions. Identification of proteins that are obligatory for parasite stage specific function can be vulnerable targets for either vaccines or drug development.

The current challenges in controlling malaria include emergence of drug resistant parasites and the insecticide resistant mosquitoes. Progression of drug resistant parasites can be limited by preventing their transmission to mosquitoes. Most of the antimalarial drugs target the asexual blood stages and reduce the parasite burden. These antimalarials have little or no effect on the gametocyte stages of the parasite. The gametocytes are the only stages that are responsible for the parasite transmission from vertebrate host to mosquito. *P. falciparum* gametocytes have an average circulation time of 3.4-6.8 days. The gametocytes circulate in the blood even after the efficacious clearance of the asexual stages by chemotherapy owing to the release of sequestered early stage gametocytes into the circulation. This ensures that the diseased individuals carry gametocytes for a weeks' time even after the complete cure of infection (Smalley & Sinden, 1977, Eichner *et al.*, 2001), thus acting as reservoirs for the transmission of malaria to mosquitoes.

In *P. falciparum*, 0.2-1% of the asexual blood stage population yields gametocytes and it takes around 8-10 days for their maturity. The development of *P. falciparum* gametocytes is divided into five different stages (Stage I to V), that can be microscopically distinguished and only stage V gametocytes are capable of mosquito transmission (Carter & Miller, 1979). In fact, an increase in gametocyte conversion rate is observed in rodent as well as in human *P. falciparum* malaria when exposed to antimalarial drugs. The early gametocyte stages (Stage I to III) are susceptible to the schizonticidal antimalarials while stage IV and V gametocytes are insensitive to most of the antimalarial drugs except primaquine (Shekalaghe *et al.*, 2007). This is because stage V gametocytes are developmentally arrested and their metabolic activity is minimal (Sinden & Smalley, 1979, Sinden, 1983). These quiescent stage V male and

female gametocytes enter the mosquito midgut during the blood meal and undergo rapid development and differentiation into respective gametes upon sensing environmental changes like drop in temperature and the presence of mosquito derived xanthurenic acid (XA). Male gametocytes undergo exflagellation forming eight male gametes following three rounds of DNA replication and axoneme assembly which takes around 20 min (Ponzi *et al.*, 2009, Raabe *et al.*, 2009). Female gametogenesis involves the egress from erythrocyte and increased translation of mRNAs repressed by RNA helicase DOZI (Mair *et al.*, 2006). Male gamete fuses with the female gamete forming a zygote that subsequently transforms into a motile ookinete. The ookinete migrates through the gut epithelium and develops into an oocyst beneath the basal lamina. Extensive sporulation occurs within oocysts. Mature oocyst sporozoites eventually egress into the hemocoel and migrate to salivary glands and await to infect a new vertebrate host.

Understanding and blocking the parasite transmission from vertebrate host to mosquito is the effective and key step in complete eradication of malaria. This includes the inactivation of parasite transmission stages either in the vertebrate host or the vector by using drugs or vaccines. Alternative strategies include genetic modification of *Anopheles* resulting in reduced susceptibility to *Plasmodium* (Sinden *et al.*, 2012). Attempts to reduce malaria transmission include administration of formulations that have combined transmission blocking and schizonticidal effects. These transmission blocking drugs may act against gametocytes or they may prevent the activation of gametocytes in the mosquito midgut when taken along with the blood meal. To be active against the gametocytes, the drug should have the matching half-life to that of gametocytes as these can circulate for weeks in the human host (Sinden *et al.*, 2012). The only gametocidal drug currently available is primaquine and WHO recommends single dose of 0.25mg/Kg along with artemisinin combination therapy (artemisinin-piperaquine) (WHO, 2015a). Primaquine should not be used to treat malaria in pregnant women and infants under the age of 6 months as they are at the risk of gastrointestinal intolerance and hemolysis due to deficiency of glucose-6-phosphate dehydrogenase (G6PD). A novel transmission blocking compound - bump kinase inhibitor (BKI-1) was developed to target calcium dependent protein kinase-4 (CDPK4) of the parasite that is essential for exflagellation of male gametocyte (Ojo *et*

al., 2012). Pyrazolopyrimidine-based inhibitors were tested *in vitro* against PfCDPK1 and these inhibitors blocked *P. falciparum* exflagellation *in vitro* (Ojo *et al.*, 2014, Vidadala *et al.*, 2014).

The efficacy of transmission blocking antibodies in vertebrates was first established in bird malaria model by immunizing avian hosts with killed mixed blood stages of *P. gallinaceum* (Huff *et al.*, 1958). In birds, repeated immunizations with purified gametocytes of *P. gallinaceum* induced transmission blocking antibodies against gametocyte proteins (Carter & Chen, 1976, Gwadz, 1976). *Ex vivo* studies revealed that the monoclonal antibodies against some of the sexual stage proteins have reduced or completely blocked the transmission of *P. gallinaceum* to mosquitoes (Kaushal *et al.*, 1983, Grotendorst *et al.*, 1984). Transmission blocking vaccines (TBV) can only prevent the transmission of malaria to mosquito and are not able to induce protective immunity. TBVs induce antibodies against the surface antigens of sexual stages in humans which can act in the blood meal preventing the disease transmission to mosquito thereby reducing the clinical cases and subsequent deaths in the endemic areas (Carter, 2001). The promising transmission blocking candidates that are expressed on the surface of mature gametocytes are Pfs230 and Pfs48/45 that belong to the family of proteins having 6 cysteine motif domain (Templeton & Kaslow, 1999). Both these genes are expressed from the mid-gametocyte development stages in both male and female gametocytes until fertilisation. Pfs230 binds to the RBC during exflagellation and targeted disruption revealed the inability of male gamete to fertilize there by a drastic reduction in the formation of oocysts by more than 90% (Williamson, 2003, Eksi *et al.*, 2006). Even though Pfs48/45 is expressed in both male and female gametocytes, targeted deletion studies revealed its role exclusively in male gametocytes (van Dijk *et al.*, 2001). Post fertilisation antigens of *Plasmodium*, Pfs25 and Pfs28 are also attractive targets for generating transmission blocking immunity. The transcripts of both Pfs25 and Pfs28 could be seen in the female gametocyte, but they get translated only after fertilisation. The expression of Pfs25 starts from the gamete stage and the expression continues through ookinete and oocyst whereas Pfs28 expression starts from ookinete stage (Hisaeda *et al.*, 2000). Pfs25 is an important TBV candidate that has entered the clinical trials. In pre-clinical studies, the antibodies raised against Pfs25 have inhibited transmission to mosquito and achieved

high transmission blocking activity (Nikolaeva *et al.*, 2015). There is a need to identify new transmission blocking agents that target the parasite proteins efficiently and prevent the transmission.

Complete genome sequence of *Plasmodium* was done in 2002, and the 23Mb parasite genome encodes for nearly 5021 genes and only 10% of these genes have been functionally investigated till to date (de Koning-Ward *et al.*, 2015), and nearly 2175 genes still remain unannotated (Fig. 30). In depth functional analysis of parasite genome on a gene to gene basis will yield valuable information for developing effective drugs and vaccines against the malaria disease. Several transcriptomic and proteomic studies provide a snapshot of molecular changes that occur in parasite during stage-specific transitions. Identifying the key parasite proteins involved in sexual cycle will enable development of effective transmission blocking interventions to eradicate malaria completely.

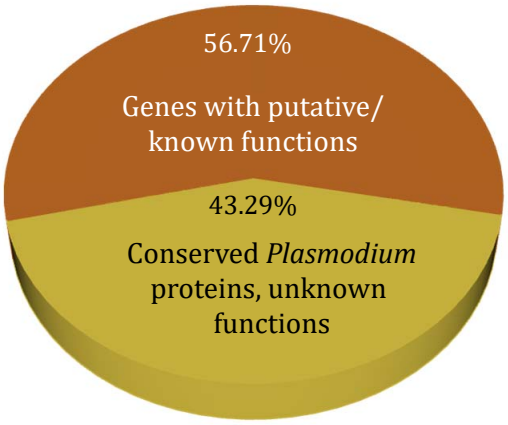

Fig. 30 Nearly 43% of the *Plasmodium* genes encode conserved proteins of unknown function. *Plasmodium* genome codes for around 5021 genes. PlasmoDB database search revealed 56.71% genes with known or putative functions and the other 43.29% genes are annotated as conserved proteins with unknown functions.

Owing to the critical role of certain highly transcribed genes in transmission stages, we wanted to investigate the functional role of *PBANKA_141700* by reverse genetics approach. The selection of the candidate was based on two important criterion: 1. This transcript was recovered at high frequency in RNA sequencing data of gametocyte stages (Otto *et al.*, 2014) and 2. The proteomic analysis detected peptide corresponding to this protein in oocyst and salivary gland sporozoite stages (Lasonder *et al.*, 2008). *PBANKA_141700* has no orthologues or functional motifs in other

organisms and is exclusive for *Plasmodium.* Considering its unique expression in *Plasmodium* and its likely role in the transmission stages, we investigated the gene function across all *Plasmodium* life cycle stages.

Blast search revealed that the orthologues of *PBANKA_141700* were unique to *Plasmodium* and not present in other species. Detailed amino acid analysis of the protein sequence did not reveal any conserved domains. Therefore it is an interesting candidate to investigate if it has a likely role in transmission stages. In order to investigate its functional role, we employed a reverse genetics approach. We disrupted the promoter of *PBANKA_141700* by inserting mCherry and TgDHFR cassettes by double homologous recombination method and analysed the phenotype of *PBANKA_141700* promoter disrupted (PD) parasites. Surprisingly, the disruption of this locus had no effect on parasite development in the mouse or in mosquito suggesting that the product encoded by PBANKA_141700 was not essential for parasite life cycle.

4.2 Materials and Methods

All the methods used in functional characterization of *PBANKA_141700* have also been described in detail in chapter 2 - "Investigating the role of Circumsporozoite protein in *Plasmodium berghei* (*Pb*) mosquito stages by using FLP/*FRT* conditional mutagenesis system", and chapter 3 - "Functional characterization of *Pb* K^+ channel/ adenylyl cyclase α by reverse genetics approach". Since many of these are common, we present only a brief description here.

4.2.1 Experimental animals and parasite lines

Female BALB/c or C57BL/6 mice aged 8-10 weeks old were used for all animal experiments. Animal handling was strictly in accordance with Institutional Animal Ethical Committee protocols of University of Hyderabad. *PBANKA_141700* gene disrupted parasite line was generated by transfecting *PBANKA_141700* targeting construct in wild type *P. berghei ANKA* strain. *P. berghei ANKA* mCherry parasite line was generated by targeting the non-essential gene locus *P230p* in WT *P. berghei* ANKA. Phenotypic characterisation of *PBANKA_141700* PD line was performed across all life cycle stages in comparison to WT mCherry line. Phenotypic analysis of the genetically modified/transgenic parasites was carried out by cycling the parasites through Swiss

Albino or BALB/c and female *A. stephensi* mosquitoes. C57BL/6 mice were used for the analysis of pre-patent period either by mosquito bite or intravenous injection of sporozoites. New Zealand White rabbit was used for feeding the mosquitoes to maintain the *A. stephensi* colony.

4.2.2. Cell lines

Human hepatoma (HepG2) cell line was maintained in complete DMEM and used for *in vitro* EEF development.

4.2.3 Software and databases

Two public domain databases namely PlasmoDB (http://www.plasmodb.org/plasmo) and Sanger gene database (http://www.genedb.org/Homepage/Pberghei) were used to retrieve ORF and 5' untranslated region of *PBANKA_141700* or to retrieve the ORF of *Pb P230p*. Orthologous protein sequences of *PBANKA_141700* in other *Plasmodium* species were obtained from PlasmoDB. Multiple sequence alignment of *PBANKA_141700* orthologues from other species of *Plasmodium* were done using Clustal Omega (Sievers *et al.*, 2011).

S. No.	Primer Name	Primer Sequence (5' – 3')
1	P FP1	CCC*CTCGAG*GAACGTTGTATTTTATTTTAACTCATAA
2	P RP1	ATAAT*ATCGAT*TACAAATGTCTTTAAAATCAGGCAT
3	P FP2	ATAAG*GCGGCCGC*TTTTTTAATTATAAGAAAAAATAAA
4	P RP2	CCC*GGCGCGCC*TCAAAAAAAAAAAATTAAAAAATTAAC TCTTAACG
5	P FP3	GTTTGGTTTGTTCTTTTGATTTGTTGGAATAT
6	H RP	TTCCGCAATTTGTTGTACATAAAATAGGCA
7	TD FP	TTACCAACTCAATTTAATAGATGTGTTATG
8	P RP3	AAATGCGGCACGCTTTGAGTATAAATATTCA
9	P FP4	CAGCGGCCGCATGGAATATTTAATATTAGAAG
10	P RP4	CAGACATTCGATATGTCCTTTACACGA
11	N FP1	AA*CTCGAG*TCATGGATCATATCCACTAACAAT
12	N RP1	AAT*ATCGAT*TGTGTTTTATTTGGATGTGCAAT
13	N FP2	AT*GCGGCCGC*TTCTCTTGAGCCCGTTAATGAA
14	N RP2	AT*GGCGCGCC*TAGGAAATTTGTTTATTTTTAT
15	N FP3	AGAATTGTATATGGTAAAGAACCTACTAACACAA
16	D FP	GTTGTCTCTTCAATGATTCATAAATAGTTG
17	N RP3	ATAGTGACTTTCAGTGAAATCGCAAACATAAG
18	N FP4	ATTTCCAAAACCGTTAGAATATGTAGCATTACA

19	N RP4	AAGTGAGTAGCATAGATGGGACGCCAA
20	P FP5	GGAAAGTAAAATACATGAAATAACCGAGGAA
21	P RP5	TTTTCATTAAGTTTGGAAATATGTTCTTTTAAATC
22	*Pb 18S* RT FP	GGGGAGATTGGTTTTGACGTTTATG
23	*Pb 18S* RT RP	AAGCATTAAATAAAGCGAATACATCCTTA

Table 4: List of primers and their sequences used for the construction of transfection plasmids, to confirm the site specific integrations, to check wild type contamination from cloned lines of *PBANKA_141700* PD line and WT mCherry, and real time PCR analysis.

4.2.4 Expression analysis of *PBANKA_141700* from different life cycle stages of *P. berghei*

To study the gene expression of *PBANKA_141700*, RNA was isolated from various life cycle stages and 2µg of RNA was reverse transcribed to generate cDNA. Gene expression of *PBANKA_141700* was quantified by the absolute method of qRT PCR. A 114bp fragment with in the coding sequence of *PBANKA_141700* was amplified using the primers P FP5 and P RP5 and ligated in pTZ57R/T. The clone was confirmed by sequencing and gene specific standards were made in the range of 10^8 to 10^2 copies/µl by log dilution. Similarly, standards were generated for *Pb 18S rRNA* to be used as internal control. For real time PCR, cDNA samples generated from different stages of *P. berghei* and gene specific standards were used as a template along with 0.25µM each of forward and reverse primers of either *PBANKA_141700* or *Pb 18S rRNA* and iQ SYBR Green supermix. The data normalization was done by obtaining the ratio of absolute copy numbers of *PBANKA_141700* and *Pb 18S rRNA* for each sample.

4.2.5 Construction of *PBANKA_141700* promoter disruption plasmid

For promoter disruption of *PBANKA_141700*, a 538 bp of *5' UTR* was amplified using the primer pairs P FP1 (*KpnI* restriction site) and P RP1 (*ClaI* restriction site) and 604 bp of *ORF* was amplified using the primer pairs P FP2 (*NotI* restriction site) and P RP2 (*AscI* restriction site) from *P. berghei* ANKA genomic DNA using Platinum Pfx polymerase using thermal cycler conditions 94°C for 2 min, 30 cycles of 94°C for 30 sec, 56°C for 30 sec and 68 °C for 1 min and a final extension of 68 °C for 10 min. The amplified products were run on 1% agarose gel, the bands were excised, gel extracted, adenylated and cloned into pTZ57R/T vector. The clones were confirmed by double digestion with respective restriction enzymes, followed by sequencing. Sequence confirmed fragments of *5' UTR* and *ORF* were subcloned in pSKC-mCherry-TgDHFR

(Appendix - Fig. A6) at *KpnI*/*ClaI*, and NotI/AscI restriction sites respectively to obtain *PBANKA_141700* promoter disruption plasmid (Appendix - Fig. A7). The targeting construct was released using KpnI and AscI and separated on 1% agarose gel. The gel extracted product was used for *Plasmodium* transfection to generate *PBANKA_141700* PD line.

4.2.6 Construction of *P230p mCherry* targeting construct for generating *Pb* ANKA WT mCherry parasites (WT mCherry)

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 (Appendix - Fig. A8). To achieve this, 537 bp of *5'* fragment was amplified using the primers N FP1 (containing *XhoI*) and N RP1 (containing *ClaI*) and 918 bp of *3'* fragment was amplified using the primers N FP2 (containing *NotI*) and N 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 (Appendix – Fig. A9). 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.

4.2.7 *Plasmodium* transfection and selection of stable integrants

Plasmodium transfections were done as described earlier (Janse *et al.*, 2006b). In brief, the overnight cultured schizonts were enriched by nycodenz and the targeting construct was mixed with nucleofector reagent and electroporated into schizonts. Immediately after electroporation, transfected schizonts were injected intravenously into mice. Two independent transfections were done for each construct and labeled as T1 and T2. After 24 hours, the mice were subjected to pyrimethamine treatment for 5-7 days to select the stably transfected parasite population. Stable integration was confirmed by the presence of mCherry parasites in the blood smears of mice. The clonal population was obtained from both the transfected lines and the phenotypic characterization was done independently with the two lines.

4.2.8 Confirmation of correct integrations by PCRs and limiting dilution to obtain clonal line

To confirm correct site specific integration of targeting construct, PCR was performed using genomic DNA as template. A forward primer P FP3 or N FP3 designed upstream of the recombined fragment and a reverse primer H RP designed in the HSP 70 promoter of the replacement cassette amplified a product of 822 bp and 883 bp respectively from *PBANKA_141700* PD line and WT mCherry. Similarly, primer sets TD FP/P RP3 and D FP/N RP3 amplified respectively 906 bp and 1139 bp from *PBANKA_141700* PD line and WT mCherry. Genomic DNA was isolated from the cloned lines obtained after limiting dilution of *PBANKA_141700* PD line and WT mCherry. PCR was performed with *PBANKA_141700* PD line and WT mCherry genomic DNA using the primer sets P FP4/P RP4 and N FP4/N RP4 that amplified respectively a products of 697 bp and 896 bp from loci of *PBANKA_141700* and *Pb P230p*. Complete absence of PCR product from genomic DNA of cloned lines confirmed successful cloning.

4.2.9 Analysis of blood stage propagation of *PBANKA_141700* PD line

Asexual blood stage propagation of *PBANKA_141700* PD line parasites was compared with WT mCherry by intravenously injecting $1x10^3$ iRBCs of either *PBANKA_141700* PD line or WT mCherry into a group of 3 BALB/c mice. Progression of parasitemia was monitored by Giemsa stained smears from day 3 post infection.

4.2.10 Transmission of *PBANKA_141700* PD line through *A. stephensi* mosquitoes

A. stephensi female mosquitoes were fed on anaesthetised mice infected with either *PBANKA_141700* PD line or WT mCherry having 1-2% gametocytes. Following two successive blood feedings, the mosquitoes were transferred to an environmental chamber maintained at 22^{o}C and 80% RH. On day 14 post infection, the mosquitoes were dissected to observe the midgut infectivity and on day 18-22 post infection, salivary glands were dissected to observe the sporozoite loads. To obtain sporozoites, glands were disrupted by crushing with a plastic pestle and centrifuged at 800 rpm for 3min at 4^{o}C to remove the gland debris. The supernatant was separated, and sporozoite numbers were enumerated using Neubauer's chamber. Approximately $2x10^4$ sporozoites were added to HepG2 cells to study the exoerythrocytic development.

4.2.11 *In vitro* EEF development and Immunofluorescence assay to reveal growth of EEFs

Twenty thousand sporozoites were added to HepG2 cells that were 60-70% confluent and maintained in complete DMEM in a labtek chamber slide. After addition of sporozoites to HepG2 cells, the cultures were fixed in 4% paraformaldehyde at 12 h, 24 h, 36 h and 67 h. The cultures were incubated with UIS4 (PVM specific protein) primary antibody raised in rabbit. Anti-rabbit secondary antibody conjugated to Alexa Flour 488 was used to reveal UIS4 immunoreactivity. DAPI was used to stain the nuclei of host and the parasite. The slides were observed and documented with a Nikon upright fluorescent microscope.

4.2.12 Determination of pre-patent periods of *PBANKA_141700* PD line

To study the pre-patent period of *PBANKA_141700* PD line in C57BL/6 mice, transmission was allowed through mosquito bite and intravenous injection. Nearly 15-20 mosquitoes harbouring *PBANKA_141700* PD line (day 18 to 21 post infection) were transferred to a new cage and allowed to bite anesthetized C57BL/6 mice for 15 min. Three independent experiments were done, with 3 or 4 mice in each group. As a control, the pre-patent period was also monitored for mice that received WT mCherry sporozoites, through bite in two independent experiments (3 mice in each experiment). Salivary glands from all the blood meal positive mosquitoes were dissected to confirm the presence of mCherry expression in *PBANKA_141700* PD and WT mCherry and sporozoites, which was used as a correlate of successful malaria transmission to mice. In a separate experiment, high dose ($2x10^4$) of either *PBANKA_141700* PD or WT mCherry salivary gland sporozoites were intravenously injected into C57BL/6 mice (3 mice in each group) and pre-patent periods were determined by Giemsa stained blood smears.

4.3 Results

4.3.1 *PBANKA_141700* is conserved across the *Plasmodium* species

Amino acid sequence alignment of *PBANKA_141700* with its orthologue in *P. yoelii* showed 98.24% similarity and with *P. falciparum,* it showed 85.52% similarity and is conserved across the *Plasmodium* species (Fig. 31).

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PBANKA MEYLILEEKYKNLLNKSNHEKTVLKKETEALQKKIENLECAYIEKESKIHEITEEKEKLK
PY17X  MEYLILEEKYKNLLNKSNYEKTVLKKETEALQKKIENLESAYIEKESKINEITEEKEKLK
PF3D7  MEYLILEEKYKNLLNKSNYENRLLKKETEILNKKLENLESAYIDTENKITEFIKDKEELE
PCHAS  MEYLILEEKYKNLLNKSNYEKTVLKKETEALQKKIENLESSYIEKESKINEITEEKEKLK
PKH    MEYLILEEKYKNLLNKSNHEKAVLKKESQALRKKLQNLEGAYIEKEKEVADILGEKENLE
PVX    MEYLILEEKYKNLLNKSNHEKAVLKKESEALRKKLQNLEGAYIEKEKEVAEILGEKESLE
       ******************:*: :****:: *.**::*** :**:.*.:: ::  :**.*:

PBANKA DNLFEIKKENKDLKEHISKLNEKIVDISNVCKTYRRMIKIRNTELQETEILISENMNLRK
PY17X  DNLFEIKKENKDLKEHISKLNEKIVDISNVCKTYRRMIKIRNTELQETEILISENINLRK
PF3D7  DYLYKIKRENLDLKDEVSKLNEKIQDLKGLTKTYRKMIKNRNKELFESEILMAENINLRN
PCHAS  DELL-IKKENKDLKEHISKLNERIVDISNVCKTYRRMIKIRNTELQETEILISENISLRK
PKH    NRLSIIGKENESLEEEIIKLNEKIVDLTDLSKTYRQMIKSRNKELQHSHFLVAENMHLRN
PVX    DRLSKMGRENESLEEEIVKLNEKIVDLTDLSKTYRQMIRSRNKELQHAHFLVAENMNLRS
       : *  : :** .*::.: ****:* *:..: ****:**: **.** .:.:*::**: **.

PBANKA NIEDIEKDKMYLESELKEKTKIINLIKNKYKKNISRLLENYNEKDKNIYEFQNFIIQELN
PY17X  NIEDIEKDKMYLESELKEKINIINLIKNKYKKNISRLLENYNEKDKNIYEFQNFIIQELN
PF3D7  NIQVVNNEKLSLESELNKKKKIINVIKDKYKKNIGRLLEKFNQKDRHIYEFQSFIIDELN
PCHAS  NIEDIEKDKIYLESQLKEKTYIINLIKNKYKKNISRLLENYNEKDKNIYEFQNFIIQELN
PKH    SLELAHSEKLEMESELGKKKNIIRLIKDKYKNNIGRLLEKFNEKDRHFYEFQTSVVKELN
PVX    SLELAQSEKIELENELGKKKNIIQLIKDKYKNNIGRLLDKFNEKDRHFYEFQTSVVKELH
       .::  ..:*: :*.:* :*  **.:**:***:**.***:::*:**:::****. ::.**:

PBANKA NLKIDINEENENQYCDQSVMNNKIMNICFYIDTLTKKLEEKMNISLMR----
PY17X  NLKIDINEENENQYCDQSVMNNKIMNICFYIDTLAKKLEEKMNISLTR----
PF3D7  NLKEVILRENENMHFDETLMNNKFMNISFHLDILTKKLEEKMTISIIE----
PCHAS  NLKIDINEENENQYCDQSVMNNKIMNICFYIDTLAKKLEEKMNISLTDREII
PKH    NLKMAIRREQENTFYDDSIRDDTIFNISHHLDVLIKKMEEKMTISVTK----
PVX    NLKLAIRREKENTFYDDSVRDDTILNISLHLDVLIKKMEEKMTIPVPK----
       ***  * .*:** . *::: ::.::**. ::* * **:****.* :
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Fig. 31 Amino acid sequence alignment of *PBANKA_141700* orthologues among the *Plasmodium* species. PBANKA: *P. berghei* ANKA (*PBANKA_141700*), PY17X: *P. yoelii* 17X (*PY17X_141870*), PF3D7: *P. falciparum* 3D7 (*PF3D7_131850*), PCHAS: *P. chabaudi chabaudi* AS (*PCHAS_141880*), PKH, *P. knowlesi* H (*PKNH_141930*), PVX: *P. vivax* (*PVX_122742*).

4.3.2 Gene expression analysis of *PBANKA_141700* by quantitative real time PCR revealed maximal expression in mixed blood stages and in day 14 midgut sporozoites

To quantify the gene expression of *PBANKA_141700*, gene specific standards were generated for *PBANKA_141700* and *Pb 18S rRNA*. The cDNA samples generated from various life cycle stages were used as template for real time PCR along with the gene specific standards. At the end of PCR, gene expression was expressed as absolute copy numbers for both *PBANKA_141700* and *Pb 18S rRNA*. Normalization was done by obtaining the ratio of *PBANKA_141700* copy number versus *Pb 18S rRNA* copy number. The normalized data revealed the highest expression in mixed blood stages and in day 14 midgut sporozoites (Fig. 32).

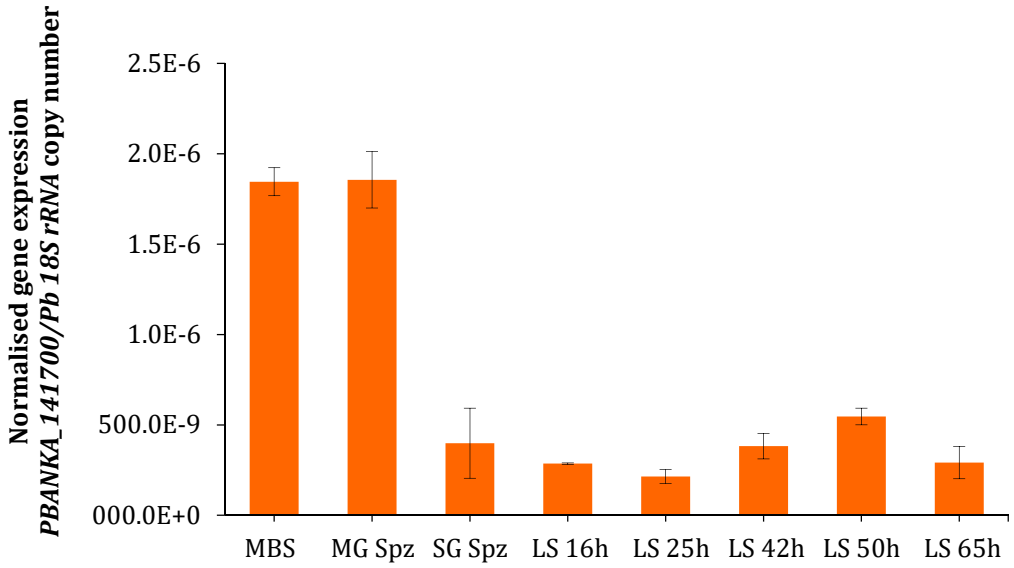

Fig. 32 Normalized gene expression of *PBANKA_141700* across the life cycle stages of *P. berghei*. Normalised gene expression showing highest expression of *PBANKA_141700* in mixed blood stages (MBS) and midgut sporozoite (MG Spz) stages. MBS: Mixed blood stages, MG Spz: Midgut Sporozoites, SG Spz: Salivary gland Sporozoites, LS 16h: Liver stage 16 h, LS 25 h: Liver stage 25 h, LS 42 h: Liver stage 42 h, LS 50 h: Liver stage 50 h, LS 65 h: Liver stage 65 h.

4.3.3 Successful disruption of *PBANKA_141700* promoter by *mCherry-TgDHFR* cassette by double homologous recombination

The gene locus of *PBANKA_141700* is shown Fig. 33A. For disrupting the promoter of *PBANKA_141700* by double crossover, the *5' UTR* and *ORF* of *PBANKA_141700* were cloned on either side of *mCherry-TgDHFR* cassettes in the plasmid pSKC-mCherry-TgDHFR (Fig. 33B). The organization of recombined locus following the successful double homologous recombination is shown in Fig. 33C. The *5' UTR* and *ORF* of *PBANKA_141700* were amplified by PCR using primer sets P FP1/P RP1 and P FP2/P RP2 respectively that amplified and products of 538 bp and 604 bp that were resolved on 1% agarose gel (Fig. 33D). Both the fragments were cloned into the targeting vector and confirmed by the release of *5' UTR* using the restriction enzymes KpnI/ClaI and *ORF* by using restriction enzymes NotI/AscI (Fig. 33E (a)). The complete cassette was released with the restriction enzymes KpnI/AscI (Fig. 33E (b)) and it was purified by gel extraction. The purified *PBANKA_141700* targeting construct was electroporated into *P. berghei* schizonts using predefined program U033 in Amexa nucleofector and the parasites were injected intravenously into mice. The recombinant parasites were selected using pyrimethamine and the parasitemia was monitored daily by blood smears stained with Giemsa. Genomic DNA was isolated from the drug resistant parasites and correct site specific integrations of *5' UTR* was confirmed by the primer set P FP3 designed beyond the region of recombination and H RP designed in *HSP70* promoter of *mCherry* cassette that amplified a product of 882 bp. Similarly, the correct integration of ORF was confirmed by the primer set TD FP designed in the *TgDHFR* cassette and P RP3 designed in the ORF beyond the region of recombination that amplified a product of 906 bp (Fig. 33F (a)). Limiting dilution was performed to obtain a clonal population of *PBANKA_141700* PD parasites. To confirm the promoter disruption by PCR, primer set P FP4/P RP4 was used that specifically amplified a product of 697 bp only in WT but not in *PBANKA_141700* PD line (Fig. 33F (b)). The cloned *PBANKA_141700* PD line expressed mCherry constitutively (Fig. 33G).

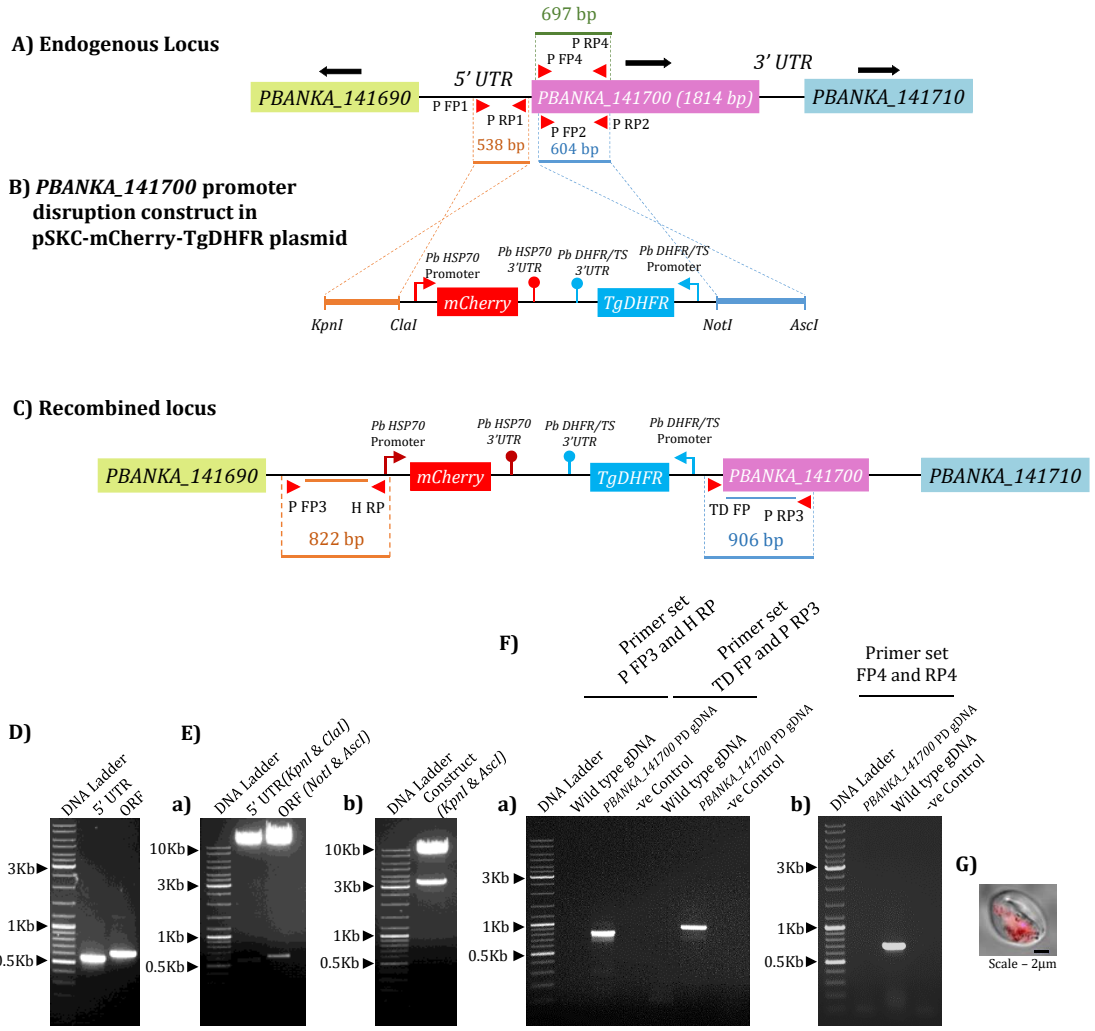

Fig. 33 Generation of *PBANKA_141700* PD line. A) Genomic locus of *PBANKA_141700* showing *5' UTR, ORF* and *3' UTR*. B) Elements of the targeting vector showing regions selected for homology, *mCherry* and *TgDHFR* cassettes. A 538 bp of *PBANKA_141700* promoter was cloned at *KpnI*/*ClaI* sites and 604 bp of *ORF* was cloned at *NotI*/*AscI* sites of the targeting vector. C) Recombined locus following successful double crossover recombination resulting in disruption of the target gene, *PBANKA_141700* by *mCherry-TgDHFR* cassettes. D) A 1% agarose gel showing the PCR products of *5' UTR* and *ORF*. The *5' UTR* fragment was amplified with primers P FP1 and P RP1. The *ORF* fragment was amplified with primers P FP2 and P RP2. E) a) Release of *5' UTR* and *ORF* fragments from transfection vector using restriction enzymes KnpI/ClaI and NotI/AscI respectively. b) Release of targeting construct (*5' UTR* fragment+*mCherry* and *TgDHFR* cassettes+*ORF* fragment) from the vector using restriction enzymes KpnI/AscI. F) a) Diagnostic PCR using primers within the targeting cassette and beyond the sites of recombination revealing the correct site specific integration. PCR products of 822 bp and 906 bp with primer sets P FP3/H RP and TD FP/P RP3 indicated correct 5' and 3' integrations respectively. b) A PCR product of 697 bp was amplified using primer set P FP4/ P RP4 from the genomic DNA of WT parasites but not from cloned *PBANKA_141700* PD line. G) A merged DIC image showing an mCherry expressing *PBANKA_141700* PD parasite inside RBC.

4.3.4 Successful disruption of *Pb P230p* locus by *mCherry-hDHFR* cassette by double crossover recombination to generate WT mCherry parasite line

The gene locus of *Pb P230p* is shown in Fig. 34A. To integrate the *mCherry* and *hDHFR* cassettes in the *Pb P230p* locus, two fragments 5' part and 3' part of *Pb P230p ORF* were cloned on either side of *mCherry-hDHFR* cassettes in the plasmid pSKC-mCherry-hDHFR (Fig. 34B). The genomic organization of *Pb P230p* locus following the successful double homologous recombination is shown in Fig. 34C. The *5'* and *3'* fragments of *Pb P230p ORF* were amplified by PCR using primer sets N FP1/N RP1 and NFP2/N RP2 respectively that amplified products of 537 bp and 918 bp that were resolved on 1% agarose gel (Fig. 34D). Both the fragments were cloned into the targeting vector and confirmed by the release of 5' part using the restriction enzymes XhoI/ClaI and 3' part by using restriction enzymes NotI/AscI (Fig. 34E (a)). The complete cassette was released with the restriction enzymes XhoI and AscI (Fig. 34E (b)), and it was purified by gel extraction. The purified *Pb P230p* targeting construct was electroporated into *P. berghei* schizonts using predefined program U033 in Amexa nucleofector and the parasites were injected intravenously into mice. The recombinant parasites were selected using pyrimethamine and the parasitemia was monitored daily by blood smears stained with Giemsa. Genomic DNA was isolated from the drug resistant parasites and site specific integrations of 5' part was confirmed by the primer set N FP3 designed beyond the region of recombination and H RP designed in the mCherry cassette. Similarly, the correct integration of 3' part was confirmed by the primer set D FP designed in the hDHFR cassette and N RP3 designed beyond the region of recombination that indicates the correct integrations of 5' and 3' parts (Fig. 34F (a)). Limiting dilution was performed to obtain a clonal population of *Pb P230p* mCherry (WT mCherry) parasites. *Pb P230p* ORF specific primers N FP4 and N RP4 were used to confirm disruption of *Pb P230p* locus that specifically amplifies a product only in WT but not in WT mCherry parasites (Fig. 34F (b)). The cloned line of WT mCherry expressed mCherry constitutively (Fig. 34G).

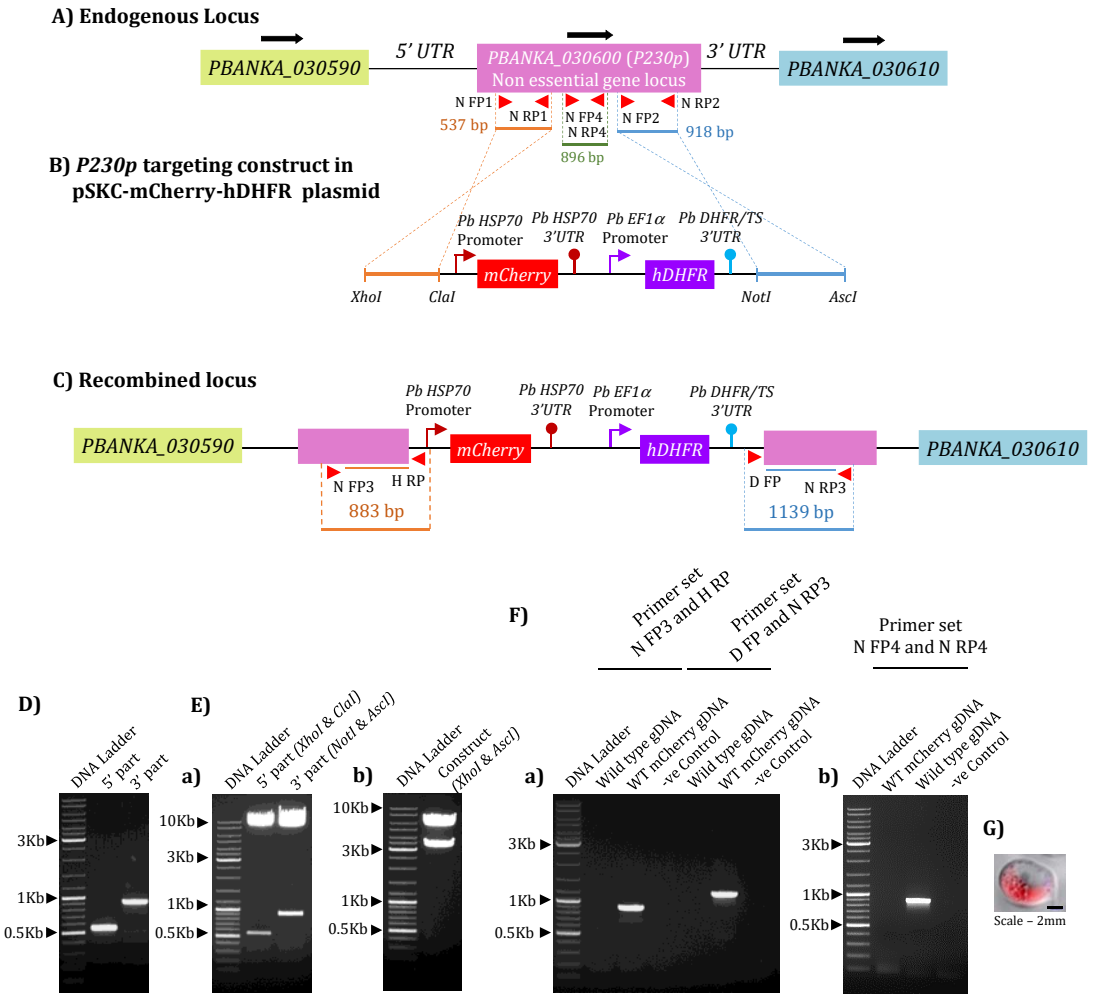

Fig. 34 Generation of *P. berghei* WT mCherry line targeting the non-essential gene locus P230p. A) Genomic locus of *Pb P230p* (*PBANKA_030600*) showing *5'* and *3' UTRs*. B) Elements of the targeting vector showing regions selected for homology, *mCherry* and *hDHFR* cassettes. A 537 bp 5' fragment and 918 bp 3' fragment of *Pb P230p ORF* were cloned at *XhoI*/*ClaI* and *NotI*/*AscI* sites of the targeting vector respectively. C) Recombined locus following successful double crossover recombination resulting in disruption of target gene, *Pb P230p* by *mCherry-hDHFR* cassettes. D) 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. E) a) Release of 5' and 3' fragments from transfection vector using restriction enzymes XhoI/ClaI and NotI/AscI respectively. b) Release of targeting cassette (*5'* fragment+*mCherry-hDHFR* cassettes+*3'* fragment) from the vector using restriction enzymes XhoI/AscI. F) a) 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 b) 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. H) A merged DIC image showing an mCherry expressing WT mCherry parasite inside RBC.

4.3.5 *PBANKA_141700* is not essential for blood stage propagation

To monitor, if *PBANKA_141700* PD line affected the blood stage propagation, BALB/c mice (3-4 mice/group) were intravenously injected with $1x10^3$ iRBCs of either *PBANKA_141700* PD line or WT mCherry per mouse. The blood stage propagation was monitored for 7 days by making Giemsa stained blood smears. The identical propagation of *PBANKA_141700* PD line as that of WT mCherry and presence of all asexual stages and gametocytes in *PBANKA_141700* PD line revealed its non-essential role in blood stage development (Fig. 35).

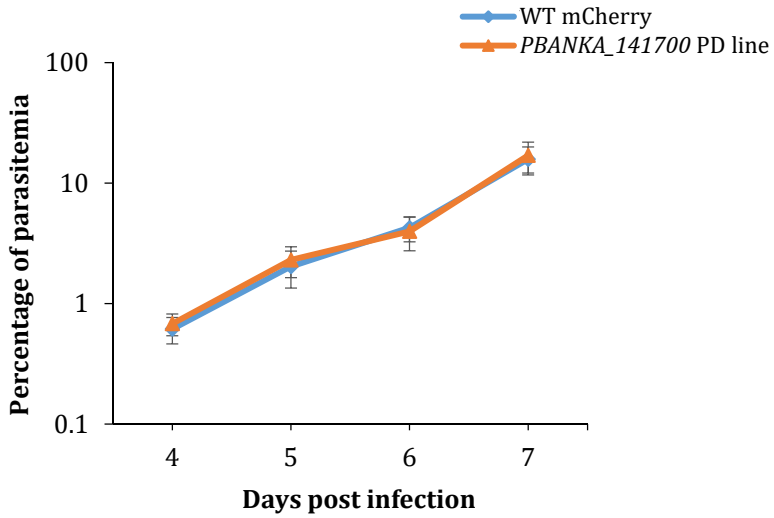

Fig. 35 Blood stages of *PBANKA_141700* PD line propagate identically as WT mCherry line. A) $1X10^3$ iRBCs of either WT mCherry or *PBANKA_141700* PD line were intravenously injected in two groups of mice (3 mice/group) and monitored for the blood stage propagation of parasites daily for 7 days by making Giemsa stained smears and plotted on a logarithmic graph.

4.3.6 *PBANKA_141700* is not essential for mosquito stage development

PBANKA_141700 PD parasites were transmitted to the *A. stephensi* mosquitoes to monitor the mosquito stage development. *PBANKA_141700* PD parasites developed oocysts in comparable numbers with that of WT mCherry (Fig. 36A and 36B). Oocyst sporulation (Fig. 36C and 36D) and the ability of sporozoites to egress and migrate to salivary glands (Fig. 36E and 36F) were also comparable with that of WT mCherry parasites suggesting that disruption of *PBANKA_141700* manifested no defect in the developmental stages of *P. berghei* in the mosquito.

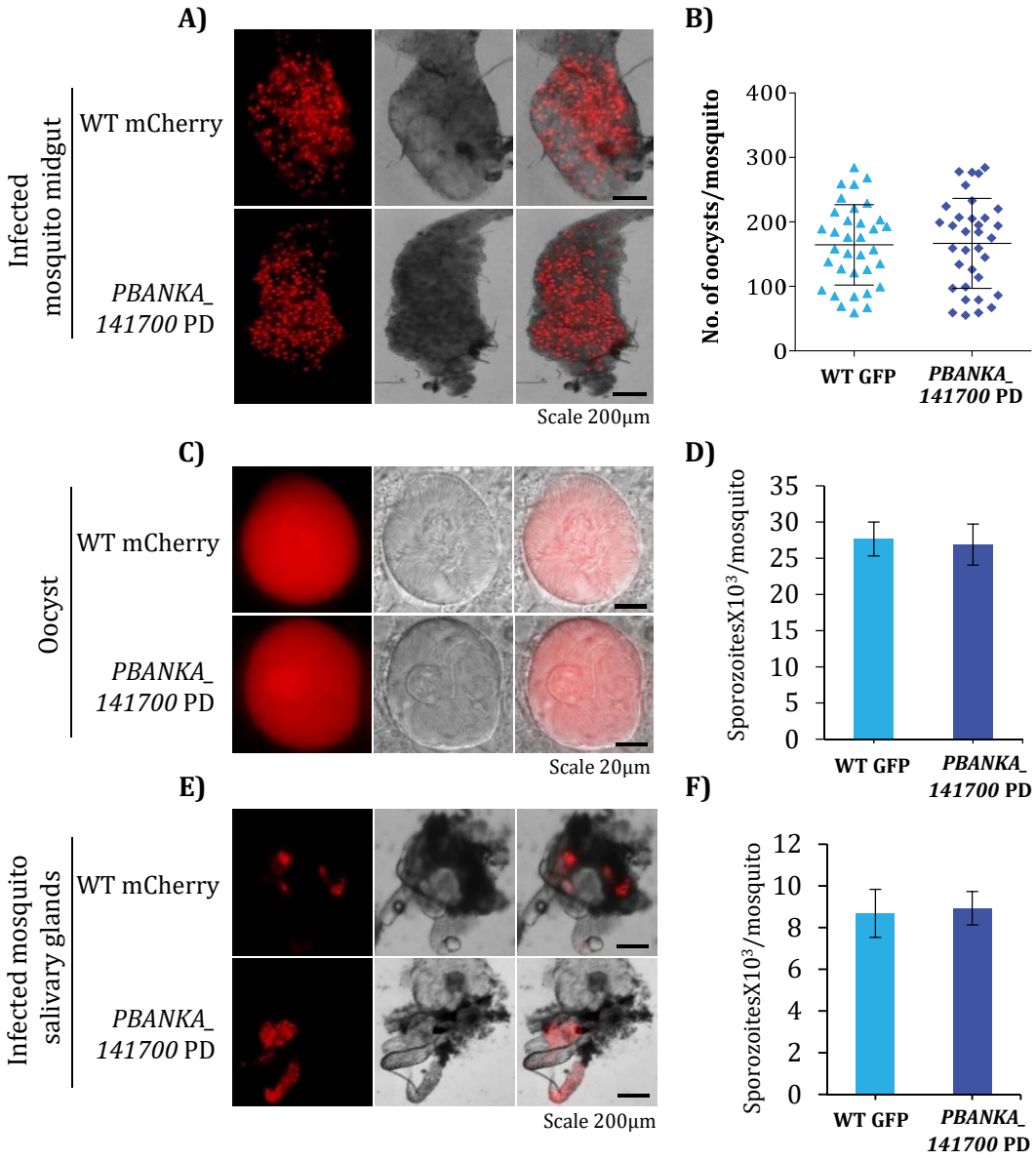

Fig. 36 Mosquito stages of *PBANKA_141700* PD line do not show any defect in sexual development, oocyst formation, sporulation and migration of sporozoites to salivary glands. Malaria was transmitted to female *Anopheles* mosquitoes from the mice harboring gametocytes stages of either WT mCherry or *PBANKA_141700* PD line. A) Microscopic images of day 14 midguts oocysts post feeding derived from WT mCherry and *PBANKA_141700* PD line. B) Dot plot showing the quantification of oocysts in an infected mosquito midgut on day 14 post feeding. C) Microscopic images of oocyst sporulation on day 14 post feeding in WT mCherry and *PBANKA_141700* PD line. D) Bar graph showing the average number of midgut sporozoites per mosquito on day 14 post feeding. E) Salivary glands showing sporozoites on day 18 post feeding from WT mCherry and *PBANKA_141700* PD line. F) Bar graph showing the average number of salivary gland sporozoites per mosquito on day 18 post feeding. All the quantifications were represented as mean+SD.

4.3.7 *PBANKA_141700* is not essential for invasion of sporozoites to hepatocytes and subsequent EEF development

Analysis of sporozoite transformation in HepG2 cells at 12h, 24h, 36h and 67h revealed that *PBANKA_141700* PD line was comparable to WT mCherry with regard to size and development thus revealing its non-essential role in EEF development (Fig. 37).

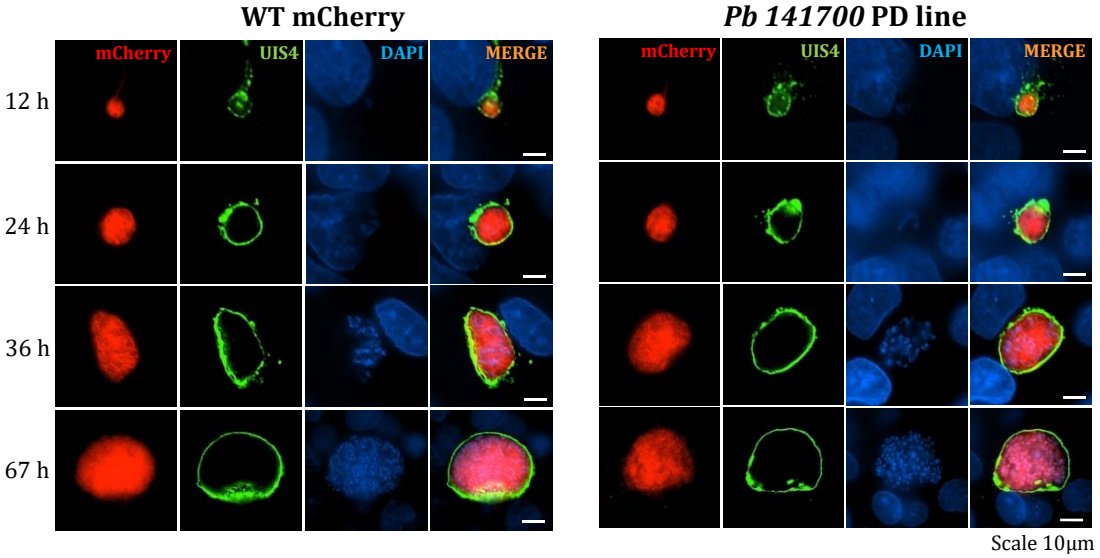

Fig. 37 The EEFs of *PBANKA_141700* PD line revealed no growth defect in *in vitro* liver stage development. Sporozoites derived from WT mCherry and PBANKA_141700 PD line were added to HepG2 cells and were fixed at different time points as indicated. The cultures were incubated with anti-UIS4 antibody that reacts with the PVM protein of EEF and revealed with anti-rabbit secondary antibody conjugated to AlexaFlour 488. DAPI was used to visualize the host and parasite nuclei. The EEFs derived from *PBANKA_141700* PD sporozoites were comparable to that of WT mCherry EEFs at all indicated time points.

4.3.8 *PBANKA_141700* PD line has same patency as WT

Inoculation of *PBANKA_141700* PD sporozoites to C57BL/6 mice through mosquito bite or by i.v. injection of $2X10^4$ sporozoites successfully initiated blood stage infection on day 3.5 - day4 post infection in three independent experiments. As a control, WT mCherry sporozoites were infected in C57BL/6 mice through either mosquito bite or i.v., route (Table 5).

Route of infection	Parasite Strain	Expt. No.	No. of animals used	No. of animals positive for blood stage infection	Pre-patent period
Mosquito bite	Wild Type mCherry	I	3	3/3	D3.5
		II	3	3/3	D3.5
	PBANKA_141700 PD line	I	4	4/4	D4
		II	3	3/3	D3.5
		III	3	3/3	D3.5
Intravenous injection of $2x10^4$ sporozoites per mouse	Wild Type mCherry	I	3	3/3	D3.5
		II	3	3/3	D3.5
	PBANKA_141700 PD line	I	3	3/3	D3.5
		II	4	4/4	D3.5
		III	4	4/4	D3.5

Table 5: Kinetics of malaria transmission by mosquito bite and i.v. routes, the details of number of experiments performed, number of animals used in each experiment, the number of animals that became positive for blood stage infection and the prepatent period. All blood meal positive mosquitoes following bite experiment were dissected to collect salivary glands to confirm the presence of mCherry expressing sporozoites (WT mCherry or *PBANKA_141700* PD line) under fluorescent microscope.

4.4 Discussion

Transmission blocking is an important strategy in eliminating malaria in the endemic regions. This can be achieved by targeting the proteins that are expressed in sexual stages of the parasite like gametocytes, gametes, zygote, and ookinete stages. There is a need to understand pathways uniquely operated at these stages that govern the biology of the parasite. As a means of understanding the biology of transmission stages, proteomic analysis of gametocytes were performed that identified 406 proteins expressed in gametocytes of which 69 proteins were shared by both male and female gametocytes (Khan *et al.*, 2005). Male and female gametocytes share 302 proteins with asexual stages. Male gametocytes exclusively express 36% of specific proteins that are involved in DNA replication, axoneme assembly, and flagellar motility, whereas 19% of the proteins are unique to female gametocytes (Khan *et al.*, 2005). Once gametocytes sense the mosquito environment, the mosquito derived XA activates gametogenesis. This process engages many signaling pathways which include kinases and phosphatases. Proteomic study has identified 11 protein kinases and 10 protein phosphatases unique to gametocytes of *P. berghei* (Khan *et al.*, 2005). Identifying and blocking the unique surface proteins, signaling molecules and metabolic enzymes of the sexual stages may offer novel means of blocking transmission. Activation of gametocytes in the mosquito gut is an active process that involves many essential signaling molecules. For example, Pfmap2 is essential for male gametogenesis (Rangarajan *et al.*, 2005), NIMA (Never in Mitosis Gene A) related kinases like nek-2 and nek-4 are involved in the zygote development and meiosis (Reininger *et al.*, 2005, Reininger *et al.*, 2009), calcium dependent protein kinase CDPK4 is essential for exflagellation (Billker *et al.*, 2004) and PfPDEδ, a cyclic dinucleotide phosphodiesterase is involved in cGMP signaling in gametocytes (Taylor *et al.*, 2008).

Gene targeting by approaches in our own laboratory has revealed two important proteins that are essential for *Plasmodium* sexual development. One is nicotinamidase, a metabolic enzyme unique to the parasite that is involved in the biosynthesis of NAD+ and is essential for the gametogenesis. (Segireddy R and Kumar KA, personal communication). Another, a kinase referred to as PbPK9 (UBC13 kinase) which negatively regulates the ubiquitination pathway and was essential for completion of sexual development. Targeted deletion of PbPK9 resulted in the

downregulation of many transcripts involved in sexual reproduction. PbPK9 likely acts as a master regulator upstream to the signaling pathways that induce transcription of genes involved in sexual reproduction of *P. berghei* (Togiri J and Kumar KA, personal communication). Both nicotinamidase and PbPK9 are unique to *Plasmodium* and orthologues are absent in the host. Thus structure based drug designing against such unique *Plasmodium* targets may indeed be a realistic option to curtail transmission.

PBANKA_141700 had several interesting features like 1) increased mRNA expression (from RNA Seq data) during sexual differentiation, 2) protein detected in the developmental stages in mosquito, and 3) absence of orthologues in other organisms that prompted us to pursue this candidate for functional investigation.

Towards this end, we disrupted the promoter of *PBANKA_141700* by inserting mCherry and TgDHFR cassettes in between the promoter and ORF. The PD line was analysed for completion of parasite development by cyclic passage through mouse and mosquito. Surprisingly, the PD mutants did not manifest defect in any life cycle stage. We conclude that *PBANKA_141700* is a dispensable locus and its product is not required for completion of *Plasmodium* life cycle.

Though we could not demonstrate any significant role of PBANKA_141700 in transmission stages, nonetheless an important outcome of this investigation was the lack of correlation between abundance of gene expression, detection of gene product at a particular stage and complete lack of function for the encoded product. While most dramatic mutants that exhibited a block at certain stage of parasite life cycle were generated by knocking out the corresponding highly upregulated gene (Mueller *et al.*, 2005b, Mueller *et al.*, 2005a, van Dijk *et al.*, 2005), this study is a precedent that brings to forefront an observation that not all genes highly transcribed yield products indispensable for parasite stage specific functions. Thus a thorough investigation of upregulated genes which are not annotated yet must precede prioritisation of valid targets for drug or vaccine development. Since *PBANKA_141700* locus is dispensable for *Plasmodium*, it can be utilised for expression of stable transgenes. The previously described dispensable loci as confirmed by gene deletion studies include *P230p* (Janse *et al.*, 2006a), *S1* (Jacobs-Lorena *et al.*, 2010) and *Plasmepsin VII* (Mastan *et al.*, 2014). Together with these, *PBANKA_141700* locus can also be additionally used to create transgenic *P. berghei* expressing *P. falciparum* genes to elucidate their functions.

Summary

Summary

The focus of the present work was to investigate the functional role of three *Plasmodium* proteins: Circumsporozoite protein (CSP), K^+ channel/Adenylyl cyclase α and a hypothetical protein PBANKA_141700 in parasite life cycle. By using approaches of reverse genetics, these studies unravelled some interesting and previously unappreciated functions that significantly advance our understanding of the parasite biology.

CSP is a major surface protein of *Plasmodium* sporozoite. Shortly after invasion of sporozoite into hepatocytes, CSP is also detected on EEF membrane and in the cytoplasm of the infected hepatocytes. A compelling role of CSP in sporulation at oocyst stages has impeded the investigation of its functions at salivary glands stages and at liver stages. To address this lacuna, we generated two conditional knock out lines of CSP in *P. berghei* using the yeast FLP/*FRT* system. For effective depletion of CSP expression in mosquito stages, a heterologous *3' UTR sequence* of *TRAP* was engineered with flanking *FRT* sites and targeted to the endogenous *3' UTR* of *CSP*. Gene targeting was done in two parental lines expressing recombinases - FLPL or FLP under *TRAP* and *UIS4* promoters active respectively in sporulating oocyst and in salivary glands sporozoites stages. Both conditional lines formed oocysts whose frequency was similar to that of WT parasites. We next evaluated their ability to: undergo sporulation within oocyst, egress and reach salivary glands. We observed that CSP conditional KO lines generated in TRAP@FLPL line were more severely compromised in sporulation as compared with UIS4@FLP line. These results though were in agreement with the high expression of *TRAP* as compared to *UIS4* in sporulating oocyst, surprisingly both lines failed to egress from oocyst and reach salivary glands. The overall implications of these observations revealed that *UIS4* expression, though predominant at salivary gland sporozoite stage than at midgut oocyst stages, still manifested fairly sufficient FLP activity leading to excision of *FRT*ed DNA. The inability of these conditional mutants to invade salivary glands precluded our attempts to study the role of CSP at liver stages. However, these studies highlight the need for identifying *Plasmodium* promoters that are exclusively active at salivary gland sporozoite stage in order to restrict temporal silencing at these stages. Such approaches will allow functional investigation of proteins having important roles both in mosquito and liver stages.

The ability of Apicomplexan parasites to breach cellular barriers facilitate them to arrive at their target cell that is critical for completion of their life cycle. During this process, the parasites traverse through different cell types and very likely the host factors activate their infection competence. Our previous studies have shown that *Plasmodium* sporozoites are activated following a brief exposure to 142mM [K^+], a concentration encountered during migration. In this study, we present evidence that this response is mediated by a bifunctional protein having domains for K^+ channel and adenylyl cyclase activity. Targeted disruption of *K^+/AC α* yielded mutant sporozoites that were significantly compromised in hepatocyte infectivity and were non responsive to K^+ channel inhibitors. Our studies reveal that regulation of infectivity in response to intracellular concentration of [K^+] is a cue only used by sporozoite while gaining entry to hepatocytes and lack of *K+/AC α* does not affect the propagation of parasite at other life cycle stages.

Gametocytes are the terminal stages of malaria parasite in blood of the vertebrate host and they re-program their transcriptional repertoire in order to successfully transmit to mosquito and complete the process of sexual reproduction. Transcriptional studies provide a snapshot of the molecular changes that occur in expression of genes that influence stage specific transitions. In this study we selected a gene *PBANKA_141700* that was upregulated in the gametocyte transcriptome and interestingly, its product was also detected in proteome analysis of the oocyst stages. Owing its likely role in the sexual stages, we resorted to a targeted disruption of this gene and analysed the mutant across all life cycle stages. Contrary to its anticipated role in malaria transmission, we observed a dispensable role of this gene product in parasite life cycle. These studies reiterate the need for a thorough genetic investigation of the transcriptionally upregulated genes to ensure that such targets are not prioritised for vaccine and drug development.

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Appendix

List of Plasmids and their maps used in the study

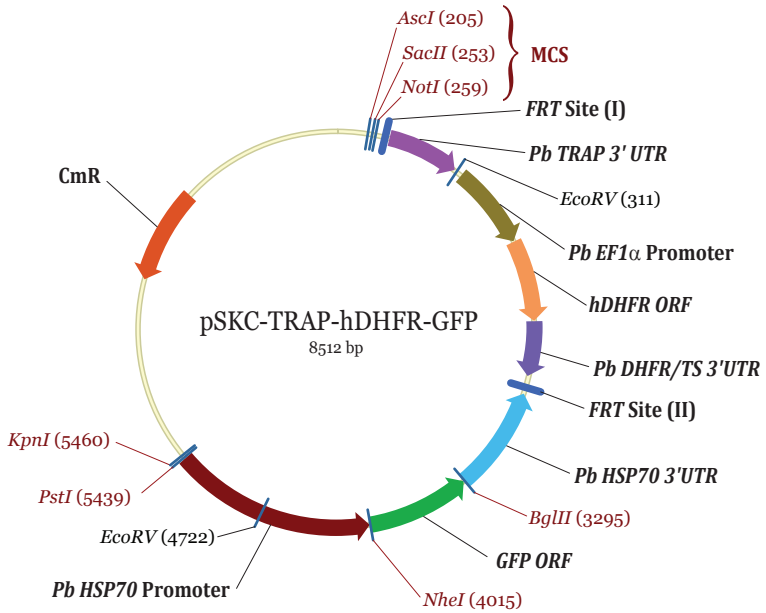
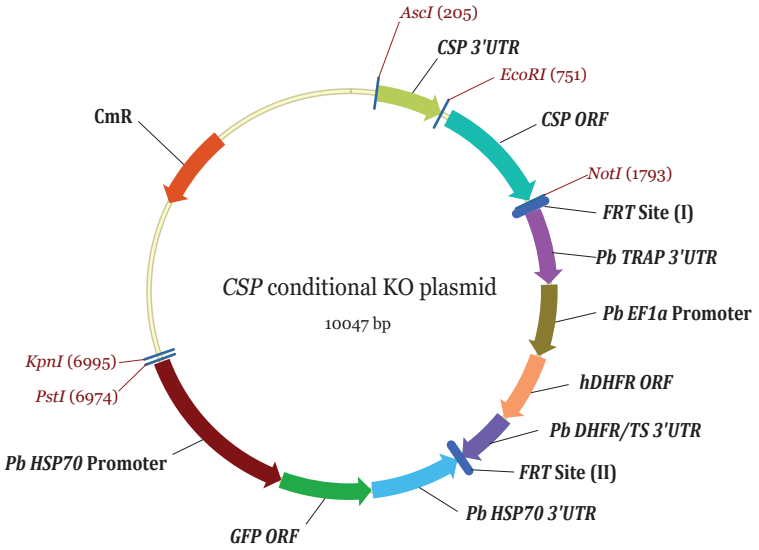


Fig. A1: Map of pSKC-TRAP-hDHFR-GFP plasmid. Schematic showing the structural features of pSKC-*TRAP-hDHFR-GFP* plasmid (8512 bp) used for conditional silencing in *P. berghei* (*Pb*). The plasmid contains chloramphenicol resistance cassette (CmR, 660 bp) for selection in *E.coli*. It has two *FRT* sites (34 bp) flanking the *TRAP 3'UTR* (599 bp) and human dihydrofolate reductase (hDHFR) cassette that is used for selection in *P. berghei*. It has a GFP cassette outside the two *FRTs*, therefore the recombinants generated using this plasmid express GFP independent of excision. *Pb EF1α* promoter (578 bp) drives the expression of *hDHFR ORF* (564 bp) and *Pb HSP70* promoter (1388 bp) drives expression of *GFP* (735 bp). *hDHFR* and *GFP ORF*s are flanked on their 3' side by *Pb DHFR/TS* (455 bp) and *HSP70* (730 bp) 3'UTRs respectively. MCS: multiple cloning sites.



Appendix 1

Fig. A2: Map of *CSP* conditional KO plasmid. For homologous recombination, *CSP 3'UTR* (539 bp) and *ORF* (1023 bp) were cloned at *AscI* and *NotI* restriction sites of pSKC-*TRAP-hDHFR-GFP* plasmid introducing a unique restriction site *EcoRI* between them. The plasmid was linearised with EcoRI and used for transfection.

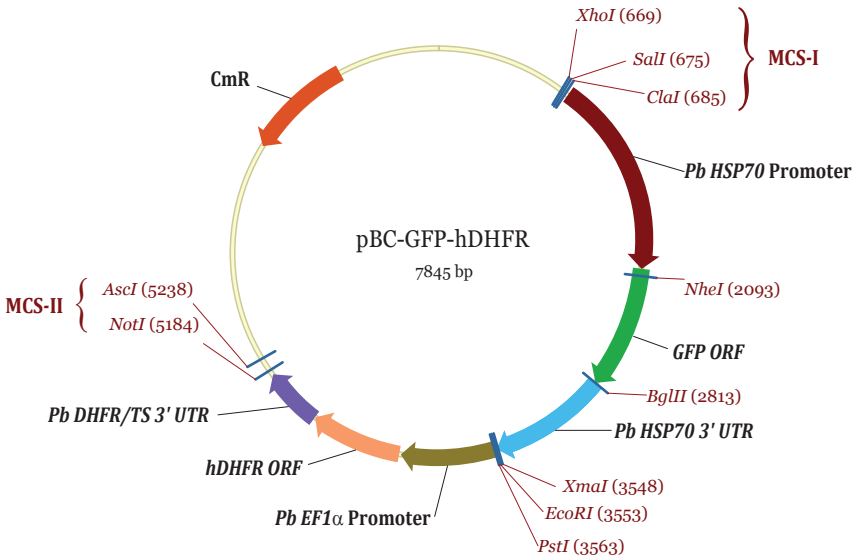
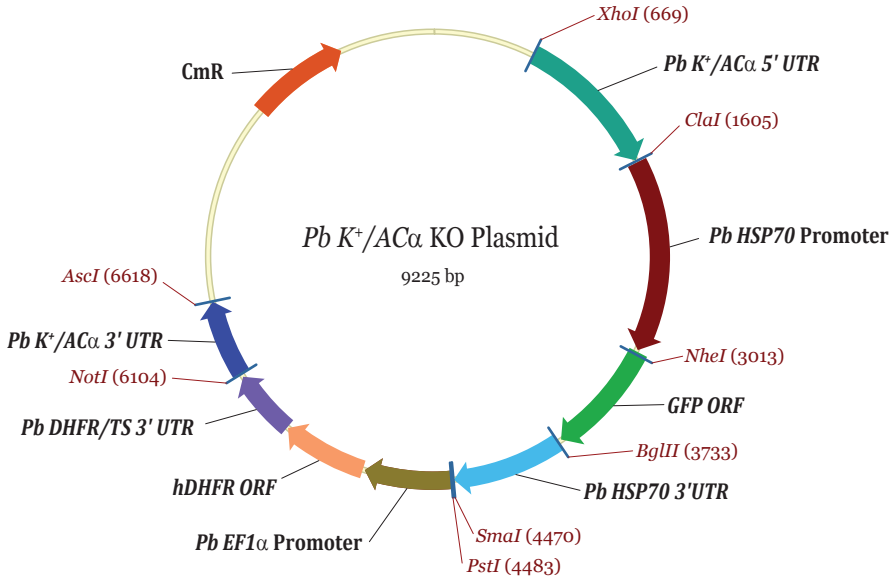


Fig. A3: Map of pBC-GFP-hDHFR plasmid. pBC-*GFP-hDHFR* plasmid (7845 bp) is used to generate targeting constructs for gene knockout studies in *P. berghei*. It contains a chloramphenicol resistance cassette (*CmR*, 660 bp) for selection in *E.coli* and *hDHFR* cassette for selection in *P. berghei*. Two multiple coning sites (MCS-I and MCS-II) flank the *GFP* and *hDHFR* cassettes for cloning the homologous sequences for recombination. It is a kind gift from Dr. Robert Menard, Pasteur Institute, Paris



Appendix 1

Fig. A4: Map of *Pb K⁺/ACα* KO plasmid. For homologous recombination, *Pb K⁺/ACα* 5' UTR (929 bp) and 3' UTR (506 bp) were cloned at *XhoI/ClaI and NotI/AscI* respectively at MCS I and MCS II of pBC-*GFP-hDHFR*. The targeting construct was released with restriction enzymes XhoI/AscI, gel extracted and used for transfection.

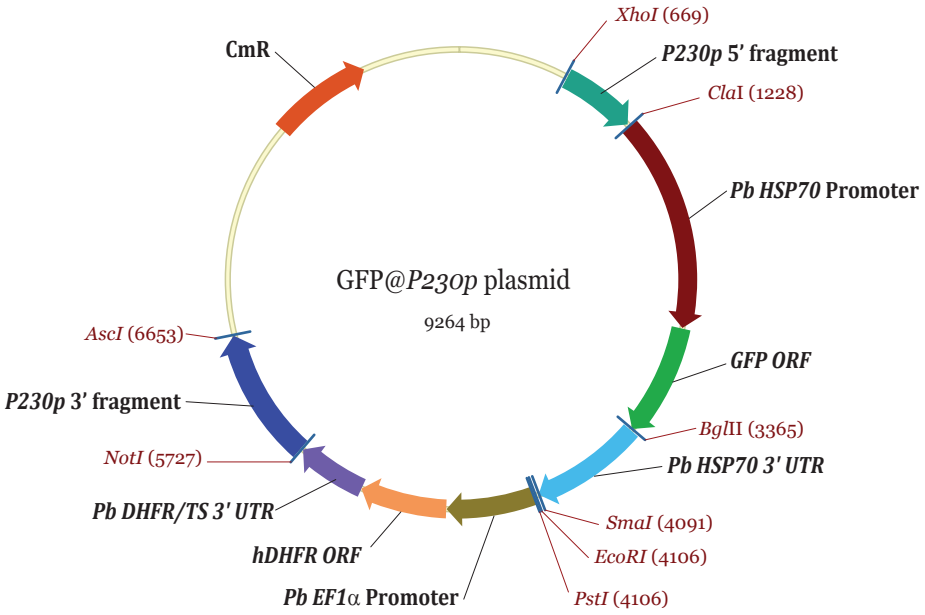


Fig. A5: Map of GFP@*P230p* plasmid. For homologous recombination, *Pb P230p* 5' (537 bp) and 3' (918 bp) fragments were cloned at *XhoI/ClaI and NotI/AscI* respectively at MCS I and MCS II of pBC-*GFP-hDHFR*. The targeting construct was released with restriction enzymes XhoI/AscI, gel extracted and used for transfection.

Appendix 1

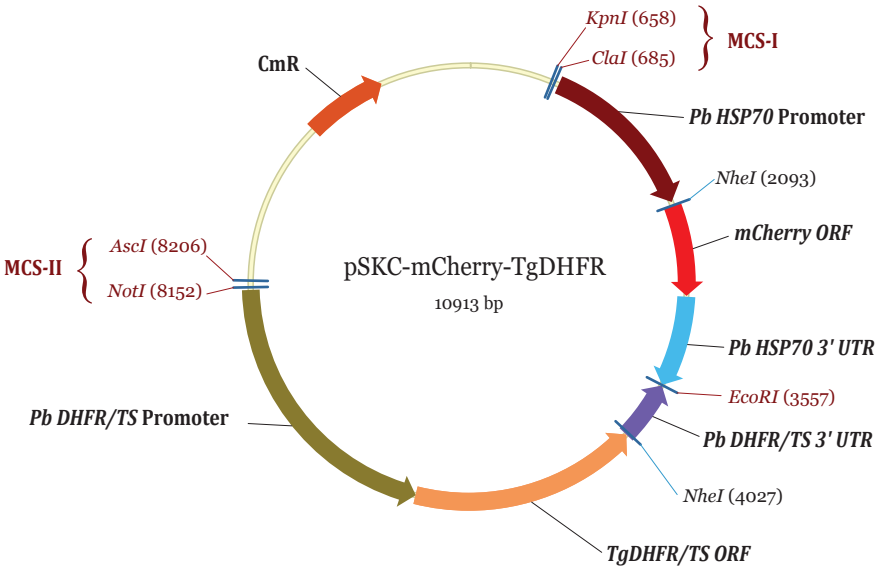


Fig. A6: Map of pSKC-mCherry-TgDHFR plasmid. pSKC-*mCherry-TgDHFR* plasmid (10913 bp) is used to generate targeting constructs for gene knockout studies in *P. berghei*. It contains a chloramphenicol resistance cassette (CmR, 660 bp) for selection in *E.coli* and TgDHFR cassette for selection in *P. berghei*. Two multiple coning sites (MCS-I and MCS-II) flank the mCherry and TgDHFR cassettes for cloning the homologous sequences for recombination.

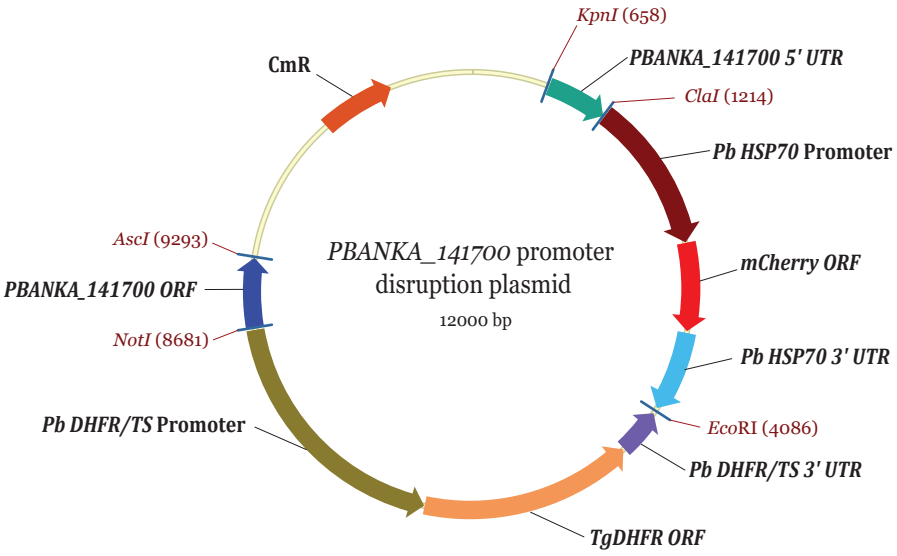


Fig. A7: *PBANKA_141700* promoter disruption plasmid. For homologous recombination, *PBANKA_141700* 5' UTR (538 bp) and ORF (604 bp) were cloned at *KpnI/ClaI* and *NotI/AscI* respectively at MCS I and MCS II of pSKC-mCherry-TgDHFR. The targeting construct was released with restriction enzymes KpnI/AscI, gel extracted and used for transfection.

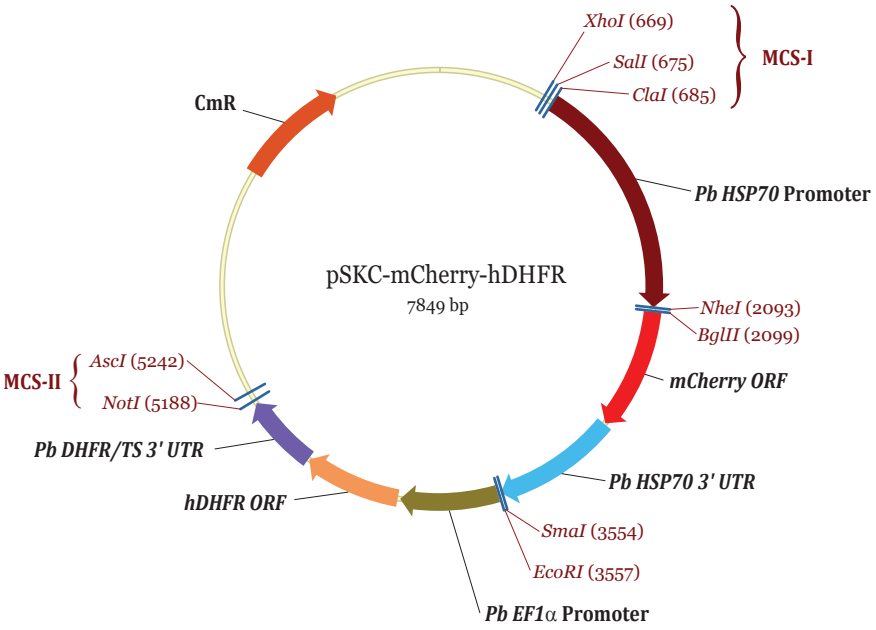


Fig. A8: Map of pSKC-mCherry-hDHFR plasmid. pSKC-mCherry-hDHFR plasmid (7849 bp) is used to generate targeting constructs for gene knockout studies in *P. berghei*. It contains a chloramphenicol resistance cassette (CmR, 660 bp) for selection in *E.coli* and hDHFR cassette for selection in *P. berghei*. Two multiple coning sites (MCS-I and MCS-II) flank the mCherry and hDHFR cassettes for cloning the homologous sequences for recombination.

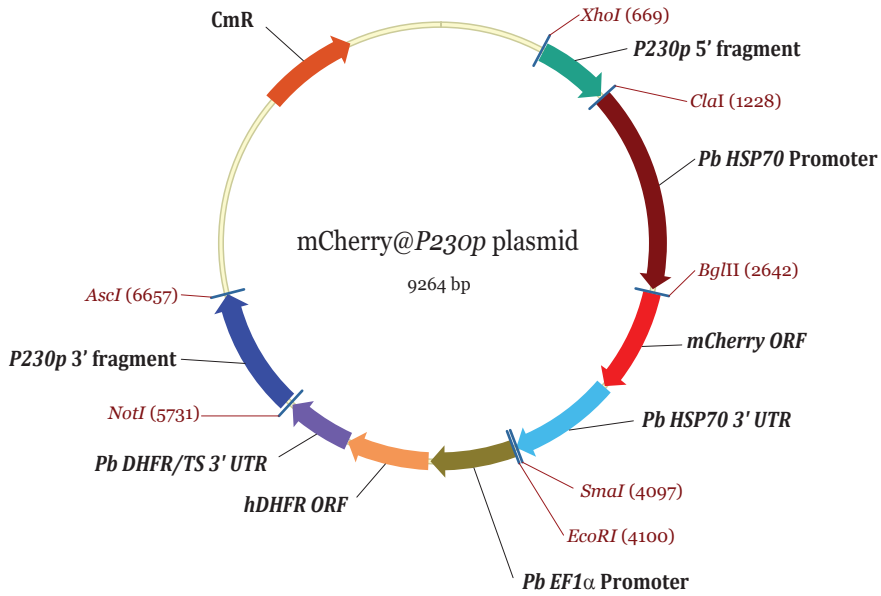


Fig. A9: Map of mCherry@*P230p* plasmid. For homologous recombination, *Pb P230p* 5' (537 bp) and 3' (918 bp) fragments were cloned at *XhoI/ClaI and NotI/AscI* respectively at MCS I and MCS II of pSKC-mCherry-hDHFR. The targeting construct was released with restriction enzymes XhoI/AscI, gel extracted and used for transfection.

Anti-plagiarism Certificate

INVESTIGATING THE ROLE OF CIRCUMSPOROZOITE PROTEIN IN PLASMODIUM BERGHEI

by Surendra Kumar Kolli

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3	Mastan, Babu S., Anchala Kumari, Dinesh Gupta, Satish Mishra, and Kota Arun Kumar. "Gene disruption reveals a dispensable role for Plasmepsin VII in the Plasmodium berghei life cycle", Molecular and Biochemical Parasitology, 2014. Publication	<%1
4	Kappe, Stefan H.I., Carlos A. Buscaglia, and Victor Nussenzweig. "PLASMODIUM SPOROZOITE MOLECULAR CELL BIOLOGY", Annual Review of Cell and Developmental Biology, 2004. Publication	<%1