Role of *Plasmodium berghei Ubc13 kinase/PK9* in malaria transmission to mosquitoes

&

PbS23 in the egress of liver stage parasites: A reverse genetics approach

Doctor of Philosophy

By

Jyothi Togiri

08LAPH05

DEPARTMENT OF ANIMAL BIOLOGY

SCHOOL OF LIFE SCIENCES

UNIVERSITY OF HYDERABAD

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A thesis submitted to University of Hyderabad for the award of Ph. D. degree in the Department of Animal Biology

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DECLARATION

I, **Jyothi Togiri**, hereby declare that the thesis entitled **"Role of *Plasmodium berghei Ubc13 kinase/PK9* in malaria transmission to mosquitoes & *PbS23* in the egress of liver stage parasites: A reverse genetics approach"** submitted by me under the guidance and supervision of Dr. Kota Arun Kumar is an original and independent research work. 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.

Name : **Jyothi Togiri**

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CERTIFICATE

This is to certify that the thesis entitled **"Role of *Plasmodium berghei Ubc13 kinase/PK9* in malaria transmission to mosquitoes & *PbS23* in the egress of liver stage parasites: A reverse genetics approach"** is a record of bonafide work done by Miss. Jyothi Togiri, 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 **Head of the Department**

(Supervisor)

Dean of the School

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DEDICATED TO MY PARENTS

Table of Contents

Abbreviations

AMA	Apical membrane antigen
ATG	Autophagy related
BSA	Bovine serum albumin
CDK	Cyclin dependent kinase
cDNA	Complementary DNA
CDPK	Calcium dependent protein kinase
CELTOS	Cell traversal protein for ookinetes and sporozoites
CLK	CDK like kinases
CS	Circumsporozoite
DAPI	4', 6' diamidino-2 phenyl indole
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxy ribonucleic acid
DOZI	Development of zygote inhibited
DUB	Deubiquitinating enzyme
EBA	Erythrocyte binding antigen
EBL	Erythrocyte binding like
ECP	Egress cysteine protease
EEF	Exo erythrocytic form
EMP	Erythrocyte Membrane Protein
ePK	Eukaryotic protein kinase
FBS	Fetal bovine serum
FRT	Flippase recognition target site
GAP	Genetically attenuated parasite

GAP	Glideosome associated protein
GFP	Green fluorescent protein
GSK	Glycogen synthase kinase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSPG	Heparin sulfate proteoglycan
HUB	Homologous to ubiquitin
IMC	Inner membrane complex
iRBC	Infected red blood cell
ITN	Insecticide treated net
KO	Knockout
LB broth	Luria-Bertani broth
Lys	Lysine
MAP/MAPK	Mitogen activated protein kinase
mRNA	Messenger RNA
MTIP	Myosin tail interacting protein
NEDD	Neuronal precursor cell expressed, developmentally down regulated
NEK	NIMA related kinase
ng	nanogram
NIMA	Never in mitosis *Aspergillus*
OD	Optical density
P. falciparum	*Plasmodium falciparum*
P. malariae	*Plasmodium malariae*
P.knowlesii	*Plasmodium knowlesii*
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
PTM	Post translational modification
PUF	Pumilio and fem3 transcription binding factor
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuolar membrane
RBC	Red blood cell
RH	Relative humidity
RNA	Ribonucleicacid
RPMI	Roswel Park Memorial Institute medium
SAGE	Serial analysis of gene expression
SAP	Sporozoite asparagine rich protein
SERA	Serine repeat antigen
SIAP	Sporozoite invasion associated protein
SPECT	Sporozoite protein essential for cell traversal
SRPK	Serine arginine rich protein kinase
SSH	Suppression subtractive hybridization
SSP	Sporozoite surface protein
SUB1	Subtilisin like protease
SUMO	Small ubiquitin like modifier
TBS	Tris buffer saline
TRAP	Thrombospondin related anonymous protein
TRSP	Thrombospondin related sporozoite protein

TSR	Thrombospondin related
UBC	Ubiquitin conjugating enzyme
UEV	Ubiquitin E2 variant
UIS	Up regulated in infected salivary glands
UOS	Upregulated in oocyst sporozoites
URM	Ubiquitin related modifier
VTS	Vacuolar translocation signal
WT	Wild type
XA	Xanthurinic acid
µg	Microgram
µL	Microliter
µM	Micrometer

CHAPTER 1

Review of Literature

1.1 Introduction

Malaria is an ancient infectious mosquito borne disease known from 2700 BC. *Plasmodium* is a protozoan protist that causes malaria in vertebrates. *Plasmodium vivax (P. vivax)*, *Plasmodium ovale (P. ovale)*, *Plasmodium malariae (P. malariae)*, *Plasmodium falciparum (P. falciparum)* and *Plasmodium knowlesii (P. knowlesii)* are reported to be the causative agents of malaria in humans. Among the five species, *P. falciparum* is responsible for the majority of malaria deaths globally followed by *P. vivax*, *P. ovale* and *P. malariae*. *P. falciparum* causes a severe form of malaria in the brain, a condition referred to as cerebral malaria which leads to death in majority of the reported malaria cases. Over half a million people die from malaria each year among 207 million estimated cases [1]. Although the vast majority of malaria cases occur in sub-Saharan Africa, the disease is a public health problem in more than 109 countries in the world including India and about 45 countries in Africa (Fig 1) [2]. The disease affects children and pregnant women in majority of the cases. Malaria is associated with poverty and has a major negative affect on economic development of the country and causes poverty [3]. The visible symptoms of malaria include fever, fatigue, chills, vomiting and headache ultimately leading to coma and death if it is not treated properly.

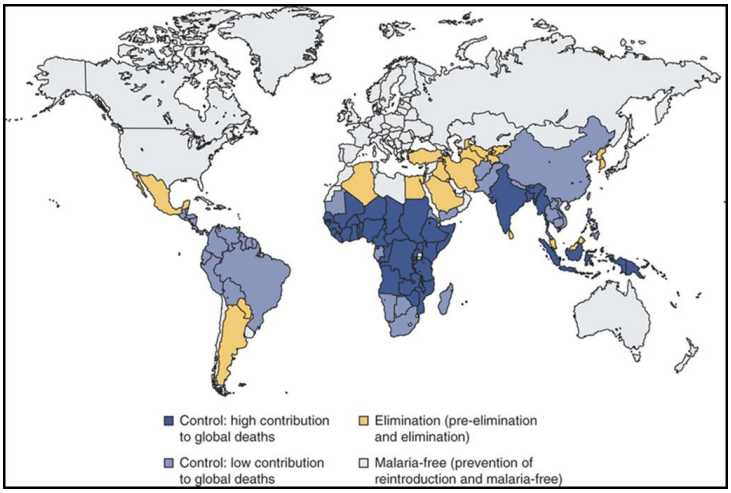

Fig 1. Global distribution of malaria. Most of the countries in Sub-Saharan Africa and India (dark blue) are at the risk of malaria. Malaria is controlled in South America and China (lighter blue) where the global contributions to malaria deaths are low. Few countries (yellow) are free of malaria as of now, but there is a risk of reintroduction of malaria if proper control measures are not implemented. This figure is obtained from Alonso *et al.* [4].

1.2 Life cycle of *Plasmodium*

Transmission of *Plasmodium* requires two hosts, an intermediate invertebrate host (vector) and a definitive vertebrate host (mammals or birds). The life cycle of the malaria parasite is very complex (Fig 2). Malarial transmission to the vertebrate host is initiated by the

injection of sporozoites during the bite of an infected female *Anopheles* mosquito while taking blood meal. The sporozoites infect hepatocytes and develop into exo-erythrocytic forms (EEFs). Fully grown exo-erythrocytic schizont contains 10,000 to 30,000 merozoites which are released into blood circulation and infect erythrocytes. The parasite transforms in the erythrocyte through ring, trophozoite and schizont stages. The erythrocyte containing schizont ruptures and releases merozoites into blood stream which in turn infect new erythrocytes. Concomitantly, a small portion of merozoites develop into male and female gametocytes that constitute the sexual forms in the life cycle. Upon being taken up by the mosquito, male and female gametocytes differentiate into male and female gametes respectively. Fertilization of male and female gametes results in the formation of non-motile zygote which transforms into motile ookinete within 18-24 h of fertilization. The actively moving ookinete traverses through mosquito midgut wall and forms oocyst on the basal lamina of the midgut. Growth and division of each oocyst produces thousands of sporozoites. After 8-15 days, oocyst bursts and releases sporozoites into the haemocoel, from where they travel to and invade salivary glands of the mosquito. The cycle of infection restarts when the infected mosquito takes a blood meal injecting sporozoites into the blood stream of vertebrates.

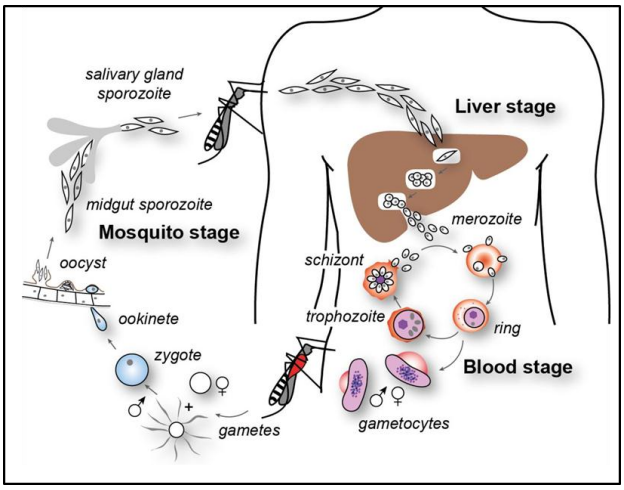

Fig 2. Life cycle of *Plasmodium*. Infection to human (vertebrate) host occurs when female *Anopheles* mosquito injects sporozoites into the dermis. The sporozoites glide through dermal cells and after breaching several cellular barriers, reach blood vessels. Once inside blood circulation, the sporozoites selectively get arrested in hepatocytes of liver. Here the sporozoites transform into liver stages or Exo-Erythrocytic Forms. The EEFs undergo one round of asexual replication and release first generation merozoites. These merozoites initiate the asexual blood stage infection and transforms in to a series of stages called rings, trophozoites, schizonts and gametocytes. Gametocytes are sexually dimorphic forms (male and female) of the parasite that enter the lumen of mosquito midgut after an infective blood meal. Inside the midgut, sexual reproduction occurs resulting in the formation of male and female gametes from respective gametocytes. The gametes fuse to form zygote that transforms into a motile ookinete. The ookinete breaches the midgut epithelium and gets attached on the haemocoel side of the midgut and transforms into oocyst. Sporulation occurs in the oocyst and upon its rupture, sporozoites are released into the haemocoel and migrate to the salivary glands. The sporozoites that lodge in the salivary glands are injected into a vertebrate host, when the infected mosquito attempts to take a blood meal. This figure is obtained from Cowman A.F *et al.* [5].

Each stage of the life cycle expresses a set of proteins that perform unique functions central to that stage. The biology of each of the life cycle stages are described below.

1.2.1 Exo-erythrocytic stages

After release of salivary gland sporozoites into the skin of the vertebrate host, sporozoites actively migrate through skin cells by gliding motility with the help of actin-myosin motor complex present beneath the parasite plasma membrane [6-8]. Sporozoites traverse through different cell types by disrupting plasma membrane, gliding through the cytosol and exiting the host cells [9]. Sporozoite protein essential for cell traversal (SPECT)-1, SPECT-2 and phospholipase are three important proteins known till to date that are shown to be involved in cell traversal of sporozoites [10-12]. Knockout parasites of spect-1 and spect-2 are immobilized in the skin as a consequence of impaired cell traversal ability [13]. Sporozoites then enter the blood circulation and are selectively arrested in the liver by interaction of circumsporozite protein (CSP)-one of the major surface proteins of sporozoite with heparin sulfate proteoglycans (HSPGs) present on the surface of hepatocytes. The extent of sulfation is highest in hepatocytes as compared to other cell types and this acts as signal for the sporozoite to switch from cell traversal activity to productive invasion [14, 15] characterized by the formation of a specialized compartment, the parasitophorous vacuole (PV) [9] inside which the sporozoites transform into EEFs. Mature *Plasmodium* EEFs, as a result of asexual replication form the first generation merozoites, which are contained within the parasitophorous vacuolar membrane (PVM) [16, 17]. These are called pre-erythrocytic or liver stages since the development takes place in the liver, and precedes blood stages infection. The release of hepatic merozoites into the blood stream takes place through specialized host cell derived vesicular structures called merosomes that facilitate a successful evasion from the immune surveillance of highly phagocytic kupffer cells located in the liver lining of sinusoids. Budding of merosomes into hepatic bloodstream releases merozoites by membrane disruption thus allowing merozoites to infect red blood cells (RBC) and initiate erythrocytic cycle [18]. Proteases play a major role in the egress of merozoites [19]. A conserved *Plasmodium* serine protease, subtilisin like protease (SUB1) was reported to be essential for the egress of merozoites both at blood stages and liver stages [20, 21]. SUB1 secretion is triggered by changes in intracellular calcium concentration facilitated by a cGMP dependent protein kinase (PKG) [20, 22]. The role of PKG was revealed initially in the release of blood stage merozoites [22]. Later studies showed that PKG is also essential for merozoite release from hepatic schizonts as *PKG* conditional knockout sporozoites failed to initiate blood stage

infection [23]. Serine repeat antigen (SERA) family proteins act as substrates for SUB1. Cleavage of SERA3 protease by SUB1 triggers a cascade of protease events that are required for the egress process [24, 25]. Transcriptome and proteome of liver stages identified 2000 genes that are active throughout the parasite development in the hepatocytes. Genes selectively upregulated in infected salivary glands (referred to as UIS genes) like *UIS-3, UIS-4* and *P36p* are important for liver stage development. Depletion of *UIS3, UIS4* and *P36p* resulted in the attenuation of the EEF development within the hepatocytes, resulting in an inability to initiate a blood stage infection. Mice immunized with *UIS-3, UIS-4* and *P36p* KO parasites developed long lasting pre-erythrocytic immunity that conferred sterile protection against challenge with wild type parasites [26-28]. The idea of using these genetically attenuated parasites (GAP) as whole organism vaccines is already gaining prominence, as a means to prevent malaria infections [29].

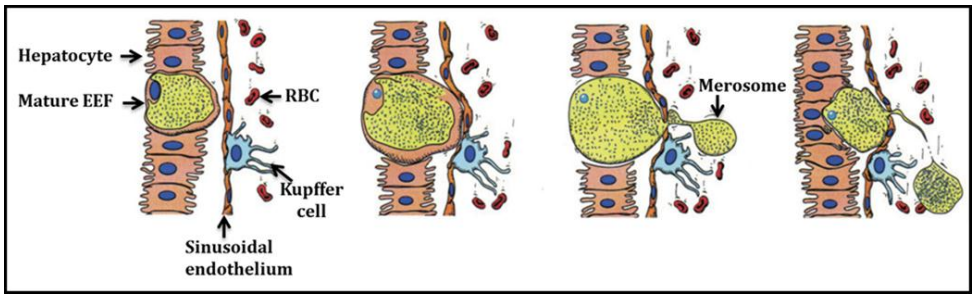

Fig 3. Merozoite release through formation of merosomes. To evade immune clearance, *Plasmodium* liver stages form membrane bound structures called merosomes. Budding of merosomes from fully mature EEFs, results in their release into blood stream that prevents their encounter by kupffer cells residing in liver sinusoids. Rupture of merosome membrane facilitates merozoite release. This figure is obtained from Sturm *et al.* and modified [18].

1.2.2 Erythrocytic stages

Erythrocyte invasion by first generation hepatic merozoites requires multiple receptor-ligand interactions [30]. Invasion of merozoite involves a series of events; attachment of merozoite to RBC followed by reorientation of merozoite apical end to RBC surface and penetration. The invasion process requires two types of proteins; adhesins and invasins. Adhesins are located in the apical organelles of the parasite that binds to receptors on the surface of the erythrocyte [31]. Erythrocyte binding like (EBL) proteins and reticulocyte binding like proteins are two important types of adhesins identified to be localized to micronemes and neck of the rhoptries [32-35]. Erythrocyte binding antigens (EBAs) are the prime examples of adhesins. Invasins play an important role in invasion process that does not involve the direct binding of receptors on the host cell always. Apical membrane antigen -1 (AMA-1) is one of the invasins that is considered to be potential vaccine candidate that has

progressed to clinical trials [36]. The interaction of adhesins with actin myosin motor is necessary for the invasion process. Actin–myosin motor resides in the inner membrane complex (IMC) of the parasite and facilitates the motility [37, 38]. Thrombospondin related anonymous protein in merozoites which is also termed as mTRAP binds to actin filaments through aldolase which further interacts with myosin A (Myo A) tail domain [38-40]. Myosin tail domain interacting protein (MTIP) binds to two IMC proteins referred to as glideosome associated proteins (GAP) - GAP45 and GAP50 along with MyoA. All the components (TRAP-aldolase-actin-MyoA-MTIP-GA45-GAP50) interact together and are referred as motor complex [39]. Drugs that target actin-myosin motor block the invasion process [37]. Inside the erythrocyte, the parasite develops within the parasitophorous vacuole. Immediately after invasion, parasites transform into ring stages, where the cytoplasm appears like a crescent like structure surrounding a vacuole with a distinct nuclei at one end of vacuole. The parasite next progresses to trophozoite stage, where its metabolic and biosynthetic activity is maximal. The parasite grows in size and occupies one third the volume of the host cell [41]. At this stage, late trophozoite enters into schizont stage in which it multiplies asexually and forms daughter merozoites and release into the blood by rupturing of the host cell membrane. The new generation merozoites are capable of infecting other erythrocyte thus continuing the life cycle. A few merozoites differentiate into male and female gametocytes which constitute sexual stages in the life cycle. *Plasmodium* in the erythrocyte uses hemoglobin as energy source and converts free heme which is a non-protein part of the hemoglobin into hemozoin. Hemozoin is the nontoxic pigment and the conversion of free heme to hemozoin is important for the survival of parasites because free heme is toxic to cells [42]. The current antimalarial drugs are designed for targeting the parasites major metabolic pathways. Few anti-malarial drugs like chloroquine and mefloquine are targeted to inhibit hemozoin crystallization. Vaccine approaches are difficult to target erythrocytic stages because of the phenomenon of antigenic variation, a process that involves constant switch in the expression of a family of var genes that are expressed on the surface of the infected erythrocyte membrane [43-46]. The most well studied var gene is *P. falciparum* Erythrocyte Membrane Protein-1 (*Pf*EMP1). *P. falciparum* infected erythrocytes binds to microvascular endothelial cells of human brain and this adhesion is mediated by *Pf*EMP-1 domain [47]. In fact, severe malaria and wide spread endothelial activation is associated with the *Pf*EMP-1 expression [48]. Both pregnant women and children living in malaria endemic areas have shown to generate B cell [49] and CD4+ T cell responses [50] respectively against *Pf*EMP-1 implicating it as a natural immune target in humans. The transport of *Pf*EMP-1 to erythrocyte membrane is facilitated

by an export motif which is conserved among *Plasmodium* species. The export motif includes five amino acid sequence RxLxE/Q/D and termed as *Plasmodium* export element (PEXEL) or vacuolar translocation signal (VTS) [51-53]. More than 300 *Plasmodium* proteins contain PEXEL/VTS motif and participate in protein trafficking from parasite to host [54].

1.2.3 Sexual stages

Schizonts are committed to produce merozoites that either continue asexual cycle or commit to gametogenesis. The schizonts that are committed to gametocyte formation are pre determined with regard to their gender and differentiate either into male or female gametocytes [55, 56]. Gametocytes are ingested by mosquito while taking a blood meal. Gametogenesis occurs in the midgut of the mosquito and is influenced by factors like rise in pH, calcium concentration, decrease in temperature and presence of xanthurinic acid (XA), a mosquito metabolic intermediate [57-59]. Several morphological and functional changes occur during gametogenesis. Male gametocyte undergoes exflagellation and form 8 flagellated male or micro gametes upon activation and are released from the residual body of the erythrocyte. Female gametocyte undergoes nuclear changes and differentiates into female or macro gamete. Fertilization occurs by fusion of micro and macro gamete plasma membrane with the involvement of HAP-2 specific gene product resulting in formation of zygote [60, 61]. Zygote is the only stage in the *Plasmodium* life cycle that is diploid in nature and contains two haploid genome complements. Zygote develops into motile ookinete within 16-25 h post gamete fusion in the lumen of the mosquito midgut. Ookinete moves from the mosquito midgut by gliding. Ookinete is banana shape in structure and contains all apical organelles like rhoptries, micronemes and dense granules. The transition from gamete to zygote and to ookinete is the major bottle neck in the *Plasmodium* life cycle and involves several signaling events involving mainly kinases. Serine arginine rich protein kinase (SRPK) [62], a calcium dependent protein kinase (CDPK4) [59] and a mitogen activated kinase (MAP2) [63] are important for the exflagellation and maturation of flagellated microgametes. NIMA related (never in mitosis *Aspergillus* 1) proteins, NEK-2 and NEK-4 are essential for zygote development [64, 65]. Calcium/calmodulin dependent protein kinases are required for the transformation of round zygote to elongated ookinete [66]. Ookinete traverses through mosquito midgut epithelium by degrading pleotropic matrix of midgut using chintinase [67, 68]. Within the mosquito gut lumen, ookinete is protected by proteolytic activity by two GPI anchored EGF domain containing proteins p25 and p28 [69]. Majority of the genes central to zygote and ookinete development are transcribed in gametocytes and stored as processing bodies (P bodies) or

stress granules which are not subjected to translation. The process is referred as translational repression which is regulated by a RNA helicase that belongs to DDX family, DOZI (development of zygote inhibited) and is common feature in the development of sexual stages of *Plasmodium*. DOZI binds to pre made transcripts and inhibits translation [70, 71]. Translation resumes after the ingestion of gametocytes by the mosquito. After breaching mosquito midgut epithelium, ookinete transforms into a structure called oocyst under the basal lamina. Oocyst development takes 10-14 days depending on *Plasmodium* species and it is the longest developmental stage throughout the *Plasmodium* life cycle [72]. During this process, oocyst grows gradually in size and undergoes several nuclear divisions and produce haploid nuclei [73]. Mature oocysts undergo sporogony and sporozoites are released into the haemocoel upon rupture.

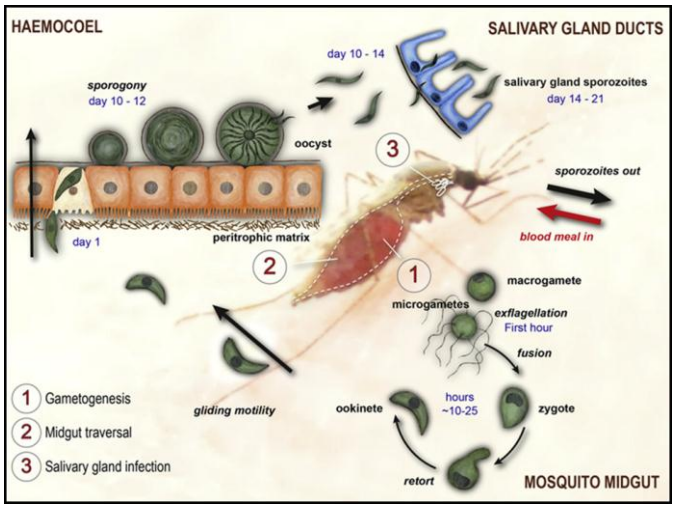

Fig 4. Sexual cycle of *Plasmodium*. Sexual development of *Plasmodium* has a tight temporal regulation. 1) Gametogenesis involves exflagellation of male gametocyte and formation 8 male gametes and female gamete formation. Exflagellation is an important event in sexual development of *Plasmodium* and occurs within 15 min of ingestion of gametocytes. 2) Fertilization, zyote formation and ookinte differentiation take place during 15-25 h post infection. 3) Ookinete is motile and it penetrates midgut epithelium, an event mediated by several ookinete specific proteins. Oocyst formation takes place at basal lamina of midgut. 3) Sporogony takes place within the oocyst and sporozoites invade salivary glands and become ready for next round of infection. This figure is obtained from Angrisano *et al.* [74].

1.2.4 Sporozoites

The development and maturation of sporozoites in oocysts are circumsporozoite protein dependent processes. Deletion of CS gene resulted in oocysts without mature sporozoites [75]. CS protein is secreted from sporozoites and the inner surface of oocyst capsule is covered with this protein [76]. It is a major surface protein of sporozoite that contains signal peptide, a central repeat region that is signature motif for different *Plasmodium* species, two conserved domains referred to as region I and region II plus flanking the repeat region and a TSR (thrombospondin related) domain. Mutation at region II plus inhibits the

egress of sporozoites from oocysts [77]. After release, oocyst derived sporozoites reach salivary glands with the movement of haemolymph and invades salivary glands. Sporozoite is the only stage in the *Plasmodium* life cycle that invades two different types of cells. Midgut sporozoites released from the oocysts invade salivary glands and infectious salivary gland sporozoites invade hepatocytes in mammals. Equally significant as CS protein, is another sporozoite surface protein called as TRAP (thrombospondin related anonymous protein). It contains two modules: A domain of Willebrand factor and a thrombospondin type I repeat (TSR). These two domains ensure the interaction of the sporozoites with different cell types i.e., the salivary gland cells of mosquitoes and hepatocytes of liver in mammals [78]. In salivary glands, sporozoites mature and wait for several days for successful transmission that occurs when the mosquitoes are obtaining a blood meal. While residing in salivary glands, the sporozoites achieve enhanced infectivity. The infectivity of salivary gland sporozoites is higher as compared to the oocyst sporozoites. Suppression subtractive hybridization of oocyst versus salivary gland sporozoites identified 30 genes which are upregulated in salivary gland sporozoites and were designated UIS genes (upregulated in infectious sporozoites) [79]. Genome wide expression analysis identified 47 genes that were specifically upregulated in oocyst sporozoites before they invaded salivary glands. These genes were referred as UOS (upregulated in oocyst sporozoites) genes [80]. It has been reported that sporozoite asparagine rich protein (SAP-1) regulates the differential gene expression associated with infectivity changes in the mosquito as the deletion of SAP1 altered the expression of UIS genes [81, 82]. Few transcripts required for hepatocyte invasion and infectivity are translationally repressed in salivary gland sporozoites and are activated upon entering into mammals during blood meal. Translational repression of transcripts required for hepatocyte infectivity is regulated by a puf (pumilio and fem3 transcription binding factor) family protein puf2 [83-85]. Study of differential gene expression of sporozoites is important for understanding proteins involved in the journey of sporozoites from oocysts to salivary glands and finally to hepatocytes. Very few proteins specific to sporozoites are characterized till to date. The gene products corresponding to most highly upregulated UIS transcripts have a role in liver stage development. Immunization of mice with *uis-3* and *uis-4* KO sporozoites elicited CD8+ immune responses in mice [86]. Thus identifying and functional characterization of proteins involved in sporozoite invasion and hepatocytes infectivity can lead to generation of genetically attenuated sporozoites (GAS) that have potential for inducing sterile immunity.

1.3 Control measures of malaria

Control measures to prevent the spread of malaria include using prophylactic drugs, mosquito eradication and preventing mosquito bites. Residual spraying of insecticides, using mosquito repellants and mosquito nets decreases the transmission of the disease to some extent. Insecticide treated nets (ITNs) are more effective than untreated nets as they simultaneously kill mosquitoes thus reduce the mosquito population and transmission of malaria. Environmental sanitation is an important measure to reduce the mosquito population. Awareness programs to educate about risk of malaria, vector management and symptom recognition can be effective to reduce the socio economic loss. Usage of prophylactic drugs like mefloquine, atovaquone and proguanil in endemic areas can prevent the parasite multiplication in blood. In malaria endemic regions, control measures are important to reduce the mortality and morbidity.

1.4 Prophylaxis and treatment

To prevent the onset of the disease, prophylactic vaccines are more effective than drugs as parasites are acquiring drug resistance. Different stages in the life cycle are targeted to generate a successful vaccine. Pre-erythrocytic stages are attractive targets for vaccine development as parasites that are not able to complete liver stage development have a potential to elicit sterile immunity. Live attenuated parasites obtained by irradiation or through genetic engineering experience a block in liver stage development and are shown to elicit cellular immunity. DNA or protein based subunits vaccines have also shown to trigger immune response albeit with less protective efficacy [87-91]. Genetically attenuated parasites (GAP) range in their attenuation from early liver stage to late liver stages. For example *uis-3* and *uis-4* knockout parasites are arrested at early stages and immunization with *uis-3* and *uis-4* knockout parasites induces CD8 + immunity [86, 92]. Depletion of enzymes involved in fatty acid biosynthesis causes mid to late liver stage developmental arrest before merozoite formation [93]. Target genes involved in late liver stage development could be effective strategy to develop vaccines that confer cross stage protection. PKG [23], SUB1 [21], PALM (*Plasmodium*-specific Apicoplast protein for Liver Merozoite formation) [94], LISP (Liver Specific Protein)-1 [95] are few genes essential for liver merozoite formation and release. To treat malaria, anti-malarial drugs like azithromycin and clindamycin are used which inhibit apicoplast formation and thus give rise to a generation of non-infectious merozoites which are blocked mid-way during intra erythrocytic development, a phenomenon referred as delayed

death phenotype [96]. Chloroquine and mefloquine inhibit hemozoin formation in infected RBC and prevent the growth of asexual erythrocytic parasites but do not affect liver stage development [97]. Artimisinin is an anti-malarial drug that in combination with other drugs is used to treat malaria [98]. In addition to pre-erythocytic vaccines and drugs that target intra erythrocytic parasites, transmission blocking vaccines are gaining importance to prevent transmission of malaria to mosquitoes. Genes that play a major role in gamete, zygote and ookinete formation are targeted to generate transmission blocking vaccines by inhibiting sexual stage development in the mosquito [99].

1.5 Challenges and Current research on malaria

Increasing resistance to available anti-malarial drugs by *Plasmodium* poses a major problem to chemoprophylaxis and treatment leading to mortality and morbidity especially in malaria endemic regions. Chloroquine was widely used drug which diffuses into the infected RBC and inhibits heme crystallization and allows degradation of infected RBC [100]. Chloroquine resistant parasites efflux chloroquine effectively because of the mutation in the chloroquine resistance transporter (CRT) gene [101, 102]. Mutations in the dihydrofolate reductase (DHFR) gene have resulted in parasite resistance to pyrimethamine and sulfadoxine [103, 104]. Artemisinin resistant parasites were also reported for the first time in 2008 [105]. As a result of resistance to conventional anti-malarial drugs, artimisinin in combination with other drugs (artimisinin combination therapy) became first line defense against malaria. Besides drug resistant parasites, *Anopheles* mosquitoes developed resistance to insecticides. According to world malaria report 2012, insecticide resistant *Anopheles* mosquitoes were observed in 64 countries. World health organization is working with governments of malaria endemic countries looping research agencies and industry partners to develop a strategy for insecticide resistance management in malaria vectors. While research is ongoing to understand more about drug resistance, efforts are also underway to identify new gene products against which vaccines can be developed. Considering the complex life cycle of *Plasmodium*, the feasibility to develop vaccines exists at three distinct stages: the pre-erythrocytic stages (sporozoites and liver stages), the erythrocytic stages and the transmission stages. Of all these three stages, vaccines against the pre-erythrocytic stages seem to be more promising as there is a possibility to eliminate the extracellular sporozoites through induction of neutralizing antibodies and the intracellular hepatic forms through CD8+ T cells. Thus the pre-erythrocytic vaccines eliminate parasites before the clinical manifestations of the disease that is associated with the blood stages infections. While radiation attenuated sporozoites remain as

gold standard for pre-erythrocytic vaccines, a limited success has also been obtained through recombinant vaccines aimed at eliciting pre-erythrocytic immunity. For example, RTS, S is one of the recombinant vaccines developed from a central tandem repeats of *P. falciparum* circumsporozoite protein, a major surface antigen of sporozoites. Phase III clinical trials showed that RTS, S/AS01 vaccine provides moderate protection against both clinical and severe malaria in infants [106]. It has been identified that the vaccine gives protection by inducing CD4+ and CD8+ immune response [107]. Owing to the genetic restriction of HLA haplotypes, it is unlikely that the recombinant vaccines elicit a response that is uniform across different individuals. Thus, much of the recent efforts have been towards creating a radiation attenuated *P. falciparum* sporozoite, with an expectation that, when administered to human, a customized immune response based on their genetic make-up would offer the best possible protection from subsequent infections. An equally appealing strategy is the use of genetically attenuated *P. falciparum* parasites obtained through knocking down one or more genes [108-110]. Successful attenuation of the *P. falciparum* liver stages has been convincingly demonstrated in human liver tissues grafted in nude mice [111]. These studies provide ample proof for a pre-erythrocytic vaccine to be reality in near future. Vaccine development against blood stages is compromised by a phenomenon of antigenic variation associated with the mature asexual blood stages. A family of 150 genes grouped in the *Pf* EMP family, are expressed one at a time, in a mutually exclusive way, so as to preclude the induction of neutralizing antibodies against all the variants at the same time [112]. By constantly switching the expression of the variants, the parasites ensures several waves of asexual cycles, each with a distinct antigenic makeup, thus making highly impossible, the development of an effective vaccines against these stages. Genes important for sexual stage development could also be targeted for generating transmission blocking vaccines. For example, Pfs25 transmission blocking vaccine was generated by targeting a 25kDa ookinete surface protein, Pfs25. This vaccine showed poor immunogenicity and failed to completely block the transmission. To improve its immunogenicity, a modified vaccine named Pfs25-EPA was developed by conjugating Pfs25 with nontoxic exoprotein A (EPA) from *Pseudomonas aeruginosa*. Phase 1 clinical trials are currently underway using this vaccine [113]. Several hundred genes are not completely characterized in *Plasmodium* and understanding the function of these genes can lead to the development of more potent anti-malarial vaccine that can target multiple stages of the life cycle.

1.6 *Plasmodium berghei (P. berghei)* – model organism

P. berghei is one of the murine parasites that cause malaria in rodents. It is used in many research laboratories for studies involving basic biology of the parasite and for validation of new drug and vaccines targets. It is the first rodent malarial parasite identified and isolated in 1948 and easily maintained in the laboratory [114]. The morphology, physiology and life cycle of *P. berghei* are similar with human species of malaria with only slight variations making the investigation of human malaria effective. The complete genome of *P. berghei* is sequenced and shows high similarity with *P. falciparum* both in structure and gene content. *P. berghei* can be genetically manipulated by conventional genetic recombination technologies. *P. berghei* offers as a best model organism to study the pre-erythrocytic stages and mosquito stages, as the risk of transmission by mosquitoes to humans is none, when compared to *Plasmodium* species that are infective to humans. Because the *P. berghei* species is easily amenable to genetic manipulation, the functional characterization of several stage specifically expressed genes can be performed by target gene disruption either through double cross over homologous recombination [115, 116], single cross over recombination [117, 118] or by conditional mutagenesis [119]. Further, several transgenic *P. berghei* lines are already available that constitutively express GFP [120], mcherry/red Star [121, 122] and luciferase genes [123]. These reporter lines have been used as effective tools to study and visualize the parasites as they go through mosquito and vertebrate host.

1.7 Post translational modifications

Post translational modification (PTM) is a biochemical mechanism characterized by covalent modification of amino acid residues of a protein. Post translational modifications play a role in many cellular processes such as signal transduction, cellular differentiation, protein degradation, regulation of gene expression and protein-protein interactions. The increase in complexity of proteome from genome is due to post translational modifications. Post translational modifications can be reversible based on the nature of modification. *Plasmodium* transcription is hard wired as there are very few transcriptional changes observed in *Plasmodium* following exposure to environmental stimuli unlike other organisms [124]. So post transcriptional and post translational mechanism likely play a major role in regulation of stage specific functions in *Plasmodium* life cycle [125]. Some of the major PTMs in *Plasmodium* are phosphorylation, ubiquitination, methylation, sumoylation, lipidation and cleavage/processing by proteases [125].

1.7.1 Phosphorylation

Phosphorylation is addition of phosphate group to an amino acid residue catalyzed by kinases and can be reversed by the action of phosphatases. In the human genome 2% of the total number of genes represents kinases. Eukaryotic protein kinases (ePKs) are distributed into 7 clusters based on their phylogeny. These are: 1) Casein kinase 2) CMGC [cyclin dependent kinases (CDK), mitogen activated protein kinases (MAP), glycogen synthase kinase 3(GSK 3) and CDK like kinases (CLK)] kinase 3) tyrosine kinase 4) tyrosine like kinase 5) AGC [protein kinase A (PKA), protein kinase G (PKG), protein kinase C (PKC) and related proteins 6) CamK (calcium/calmodulin dependent kinases) 7) STE (regulatory kinases of MAPKs). *P. falciparum* kinome does not include tyrosine kinases and STE group kinases. Eighty-five putative ePKs were found in the genome of *P. falciparum* 3D7 line [126]. Protein kinases which do not fit into above clusters are described as other protein kinases (OPKs). Four NIMA related kinases (NEKs) are identified in the *P. falciparum* kinome that belongs to OPK group. Kinome of *Plasmodium* contains ePK related kinases that do not cluster with established kinase groups or with any family in the OPK group, named as orphan kinases. Orphan kinases form small satellite clusters and are important in different stages of *Plasmodium* life cycle. PK7 [62] and PK9 [127] are two important examples that belong to orphan kinases with assigned functions. FIKK family of proteins were identified which belongs to orphan kinase group that contains a conserved phenyl alanine-isoleucine-lysine-lysine motif in the N-teminal region of the kinase domain. FIKK family forms a distinct cluster of 21 orphan kinases specific to apicomplexan parasites. This family of kinases possesses a *Plasmodium* export element (PEXEL) and its functions are yet to be identified [128, 129].

1.7.2 Role of kinases in *Plasmodium* life cycle

All stages of *Plasmodium* life cycle express wide variety of protein kinases with diverse functions. The life cycle of *Plasmodium* consists of succession of developmental stages, some of which are characterized by intense cell proliferation and others where the cell cycle is halted. To complete its life cycle successfully, tight regulation of cell cycle is required. Cyclin dependent protein kinases (CDKs) play a major role in regulation of cell cycle in humans. In *P. falciparum*, *Pf*PK5 and *Pf*mrk are two CDKs regulated by binding of *P. falciparum* cyclins 1, 2, 3 and 4 (*Pf*cyc1-4). *Pf*PK5 is necessary to activate or maintain the S phase of the parasite [130, 131]. *Pf*map-1 and *Pf*map-2 play a role in regulation of cellular differentiation and proliferation

in response to environmental stimuli. They belong to MAP kinase family and *Pf*map2 is essential for the completion of asexual erythrocytic cycle [132]. In *P. berghei* deletion of *Pf*map2 orthologue does not have any effect on asexual cycle but exflagellation of male gametocytes is affected [63, 133]. Protein kinase B (PKB) is an important member of phosphatidylinositol-3 kinase dependent pathways in eukaryotes. Human homologue of PKB was identified in *P.falciparum* and the protein is mainly expressed in merozoites and schizonts. Pharmocological inhibitor of *Pf*PKB showed inhibition of parasite growth at late schizont stages. In *in vitro* experiments, the incubation of schizonts with PKB inhibitor showed reduction in parasitemia as the formation of rings were affected as compared to non treated cells [134].

Calcium dependent protein kinases (CDPKs) are important in many signaling pathways as calcium acts as a secondary messenger in signal transduction. Many CDPKs of *Plasmodium* have an essential role in both asexual and sexual stages. CDPKs are a group of serine/threonine protein kinases which contain an N-terminal kinase domain and a C-terminal calmodulin like domain with calcium binding motif. A few kinases that regulate the secretion of microneme proteins play a role in attachment of the parasite to the host cell thus facilitating invasion. *Pf*CDPK1 inhibition altered microneme discharge and erythrocyte invasion [135]. In *in vitro* studies, *Pf*CDPK1 phosphorylates glideosome associated protein 45 (GAP45) and myosin tail interacting protein (MTIP), which are components of the inner membrane complex and are known to be essential for parasite motility [136]. CDPK1 is important for schizogony as small molecule inhibitors of CDPK1 block this asexual replication [137]. Conditional expression of PfCDPK1 by conjugation with destabilization domain showed the role of this kinase in schizont development [138]. *Pb*CDPK1 is expressed in all stages of the life cycle and promoter swapping experiments revealed additionally the role of CDPK1 in sexual stages. The transgenic parasites generated following swapping of CDPK1 promoter with clag promoter resulted in reduction of > 2000 fold oocyst numbers in the mosquito midgut. Differentiation of few zygotes was arrested although majority of them could differentiate into mature ookinetes. These mutant parasites showed downregulation of 65 proteins despite the normal transcript levels. Notably, the levels of MyoA, MTIP and GAP45 which are constituents of motor complex and essential for ookinete formation and motility were strongly downregulated. This dysregulation of ookinete proteins was due to the activation of the corresponding transcripts that were translationally repressed. This translation activation of transcripts required for ookinete development is mediated by CDPK1 [139]. Conditional silencing of *PbCDPK1* using FLP/TRAP transgenic parasites showed that PbCDPK1 is not necessary for pre-erythrocytic stages [140].

Another member of CDPK family, CDPK3 function was revealed to be central to penetration of midgut epithelium and ookinete motility. In these mutants, the number of oocysts are reduced by >90% [141]. CDPK4 is essential for male gametocyte differentiation in *P.berghei* and the deletion of CDPK4 inhibited exflagellation of microgametocytes specifically by inhibiting DNA synthesis which precedes differentiation into microgametes. The cross fertilization of *CDPK4* knockout macrogametes with *P28/P25* knockout microgametes resulted in fully mature ookinetes but its infectivity was compromised thus blocking malaria transmission [59]. *Pf* CDPK5 is important for merozoite egress from mature schizonts in intraerythrocytic development and acts downstream to *Plasmodium* cGMP dependent kinase (PKG) [142]. *Plasmodium* cGMP dependent kinase (PKG) prevents merozoite release by regulating merozoite apical organelle discharge [22]. PKG also has similar function during late liver stage development and inhibits merozoite release from hepatic schizonts [23]. Proteolytic processing of CSP which is triggered by highly sulfated heparin sulfate proteoglycans of hepatocytes activates CDPK6 mediated signaling pathways leading to a switch from sporozoite migratory mode to invasion mode. *CDPK6* mutant parasites produced lesser number of sporozoites and they were unable to cleave CSP when in contact with hepatocytes and inhibitor of CDPK6 decreased sporozoite invasion significantly [15]. *Pf* CDPK7 plays a role in ring to trophozoite transition. *PfCDPK7* KO parasites showed morphologically defective early trophozoites emphasizing its role in blood stages [143].

Kinases also play a role in modulation of splicing. *P.berghei* serine-arginine rich protein kinase (SRPK) phosphorylates a splicing factor (SR1) and inhibits the interaction with pre-mRNA. Attenuation of splicing may be due to the hyper phosphorylation of SR1 by SRPK which causes the inhibition in the interaction of SR1 with other members of spliceosome [144]. Microgamete formation is completely affected by deletion of *SRPK* [62]. SRPK and SR proteins functions as mitotic regulator in cell cycle progression through metaphase. Since single haploid microgametocyte exflagellates and produce eight male gametes within 10 min, deletion of SRPK might be sensitive [145]. NIMA related kinases (NEK) belong to OPK group regulate cell cycle progression and it was reported that NEK-2 and NEK-4 are important to complete sexual cycle of *Plasmodium*. *NEK-4* knockout parasites did not affect asexual stages, gametogenesis and fertilization but the zygote could not transform into ookinetes. Measurement of nuclear content of zygotes showed inhibition in DNA replication prior to meiosis in *NEK-4* KO zygotes [64]. Similar phenotype was observed by deletion of *NEK-2* where ookinete formation was affected. Both kinases are essential for completing sexual cycle because the NEK-2 cannot compensate the function of NEK-4 and vice versa

[65]. In contrast to the role of NEK-2 and NEK-4 in sexual cycle, NEK-1 is essential for completion of the asexual intra erythrocytic development and the protein expression is restricted to male gametocytes [146]. Systematic analysis of *Plasmodium* kinases also revealed the role of cyclin-G associated kinase (GAK) in malaria transmission. As compared to wild type parasites, the GAK mutants produced <10% oocysts. The mutants showed defect in both ookinete formation and sporulation within the reduced oocysts that were formed [62]. The same study also identified an unnamed putative kinase with gene ID PBANKA_040940. The knockout of this kinase affected the ability of sporozoites to reach salivary glands. More than 90% sporozoites could not reach salivary glands and injection of salivary gland sporozoites into mouse did not initiate blood stage infection implicating that this gene may have a role in stages beyond the salivary gland sporozoites [62].

In *Plasmodium*, a small group of kinases could not be assigned to any known kinase groups and hence were referred as orphan kinases. PK7, one of the orphan kinases of *Plasmodium* revealed its role in oocyst development. Growth of PK7 oocysts was halted from day 10 of post infection and exhibited markers of cell death [62]. FIKK family proteins of *Plasmodium* are another group of orphan kinases that possess the PEXEL motif that facilitates its transport into the erythrocyte [147]. However its role during intra erythrocytic development, if any needs further investigation. *PfPK9* is an orphan kinase that phosphorylates ubiquitin conjugating enzyme (UBC) 13 and negatively regulates its conjugating activity [127]. In our present study we targeted orthologue of *Pf*PK9 in *P.berghei* to understand its role in the parasite life cycle.

1.7.3 Kinases as targets to develop novel anti-malarial drugs and malaria vaccines

Protein kinases are now widely recognized as valuable drug targets to treat many diseases [148]. Many kinases are essential for *Plasmodium* throughout its life cycle. Initial experiments with kinase inhibitors revealed that they affected the invasion and development of intra erythrocytic forms, likely pointing that inhibition of kinase activity leads to antimalarial effects [149]. A major challenge in developing an effective anti-malarial kinase inhibitor is to target selectively kinases of *Plasmodium* that do not have homologues in humans. As CDPKs of *Plasmodium* have versatile functions in the parasite life cycle and their domain architecture and mode of regulation is only found in plants, they are considered as excellent targets to design novel drugs [62]. Recent studies with kinase inhibitors revealed that inhibition of a single enzyme may not be sufficient to prevent malaria transmission. For

example, when CDPK1 activity was inhibited, it was observed that CDPK5 can complement the function of CDPK1 [137]. It needs to be tested if inhibition of both enzymes may have synergistic and desired effect to reduce malaria transmission. CDPK4 is considered as master regulator of male specific events during gametogenesis. As gametogenesis is very early requirement for transmission, targeting CDPK4 may be ideal to develop new anti-malarial drugs. Target validation by understanding the essentiality of the kinase and screening different kinase inhibitors for prioritizing the highly specific ones, are perhaps the two crucial steps in the discovery of the potent anti-malarial drugs. In fact, effective inhibitors may be generated against other yet to be identified kinases necessary for sexual cycle such that they block malaria transmission. Likewise, kinases important for sporozoite invasion into hepatocytes, for liver stage development and for the erythrocytic stages can be targeted for developing either pre-erythrocytic or erythrocytic vaccines or drugs. The feasibility of such approaches already exists where small molecule kinase inhibitors have shown to be effective against both liver stages and blood stages of *Plasmodium* [150]. Such kinase inhibitors are excellent chemical probes for dual stage inhibition.

1.7.4 Ubiquitination

Covalent attachment of ubiquitin moiety on to a protein is called ubiquitination and the process is reversible. Ubiquitin is a 76 amino acid peptide found in eukaryotic organisms. Ubiquitination is important for regulation of cell proliferation, cell stress response, DNA repair, signal transduction, transcription, intracellular protein trafficking and endocytosis [151]. Ubiquitination involves three steps involving ubiquitin activation, ubiquitin conjugation and transferring the ubiquitin onto target substrate catalyzed respectively by ubiquitin activating enzyme (E1), ubiquitin conjugating enzymes (E2) and ubiquitin ligases (E3) [152]. Ubiquitin activation is a two-step process; first step is adenylation of ubiquitin molecule by E1 using ATP as energy source and second step transfers ubiquitin to active site of E1 and resulting in formation of thioester bond. Ubiquitin is transferred from E1 to active site of ubiquitin conjugating enzyme (UBC) E2 via thioesterification reaction. Ubiquitin ligase transfers ubiquitin onto target protein by isopeptide linkage between lysine residue of target protein and C-terminal glycine of ubiquitin with the support of E2. Deubiquitinating enzymes (DUBs) reverse ubiquitination by releasing ubiquitin from target protein and replenish free ubiquitin levels. Diverse forms of ubiquitin modifications are reported which have different cellular functions. Mono ubiquitination regulates protein function. Ubiquitin contains seven lysine residues and binding of ubiquitin to more than one lysine residue or multiple ubiquitin

linked chain binding to one lysine residue leads to poly-ubiquitination. Poly-ubiquitination at different lysine residues mediates specific functions. Conjugation of poly-ubiquitin chains linked at lysine-48 residue to target protein act as signal for 26S proteasomal degradation [153]. The proteasomal pathway is essential in many cellular processes including apoptosis, cell cycle, oxidative stress and gene expression. In *Plasmodium*, proteasome is essential for progression of cell cycle. Several studies revealed that proteasome inhibitors inhibited malaria parasite growth and multiplication [154-156]. Poly-ubiquitination via K-63 linkages targets the protein into non proteasomal pathways like DNA repair and endocytosis [157, 158]. In *P. falciparum* ubiquitin genes are expressed throughout the life cycle. *Plasmodium* also expresses ubiquitin like proteins (UBLps) like SUMO (small ubiquitin like modifier), NEDD (neuronal precursor cell expressed, developmentally downregulated) 8, URM (ubiquitin related modifier) 1, HUB (homologous to ubiquitin) 1 and ATG (autophagy related) 1 in all stages of the life cycle [159]. In *Plasmodium* genome 8 putative ubiquitin activating /E1 enzymes and fourteen E2s are identified which are conserved with eukaryotic E2s. However, *Plasmodium* ubiquitin ligases are divergent from eukaryotic ligases unlike E1 and E2. Interaction of different isoforms of E2 with distinct E3s has diverse roles in regulating downstream functions [152]. UBC9 and UBC12 are two isoforms that conjugate SUMO and NEDD8 onto target protein by interacting with E3 respectively. Ubiquitin E2 variant (UEV), lacks a HPN aminoacid motif and active site cysteine in the E2 core and is unable to conjugate ubiquitin. Interaction of UEV2 with UBC13 isoform mediates ubiquitin lysine 63 linkages which in turn participate in DNA post replication error pathway [160]. UBC13 is regulated by PK9/ UBC13 kinase by phosphorylation but the function of UBC13 is not known in *Plasmodium*. Gene expression data for nine of the fourteen *Plasmodium* E2s reveal a diverse pattern of steady state mRNA levels at different stages of the *Plasmodium* intra erythrocytic development. This suggests that during *Plasmodium* life cycle E2s are temporally regulated to deliver ubiquitin or ubiquitin like proteins to different E3s. Ubiquitin ligases are classified by domain type; E3 ligases contain either HECT domain or ring finger domain. RING domain ligases transfer ubiquitin directly onto target protein from E2. Ubiquitin binds to active site of HECT domain ligases prior to transfer to target protein. U-box protein ligases are similar to ring finger protein but lacks metal binding sites. Two U-box domain proteins are identified in *Plasmodium* and are involved in proteasomal degradation pathways [161]. Although functional analysis studies will need to further validate role of ubiquitination pathways in *Plasmodium*, E3 ligases and DUBs may be targets for new therapeutic intervention strategies. As ubiquitin conjugating enzymes (E2s) are well conserved with human E2s it may not be recommended to target E2s but understanding

the regulation of E2s can give an insight into design of new drugs. Our present study is focused on understanding the function of UBC13 kinase/PK9 that regulates the conjugating activity of one of the isoforms of E2, UBC13 throughout *Plasmodium* life cycle.

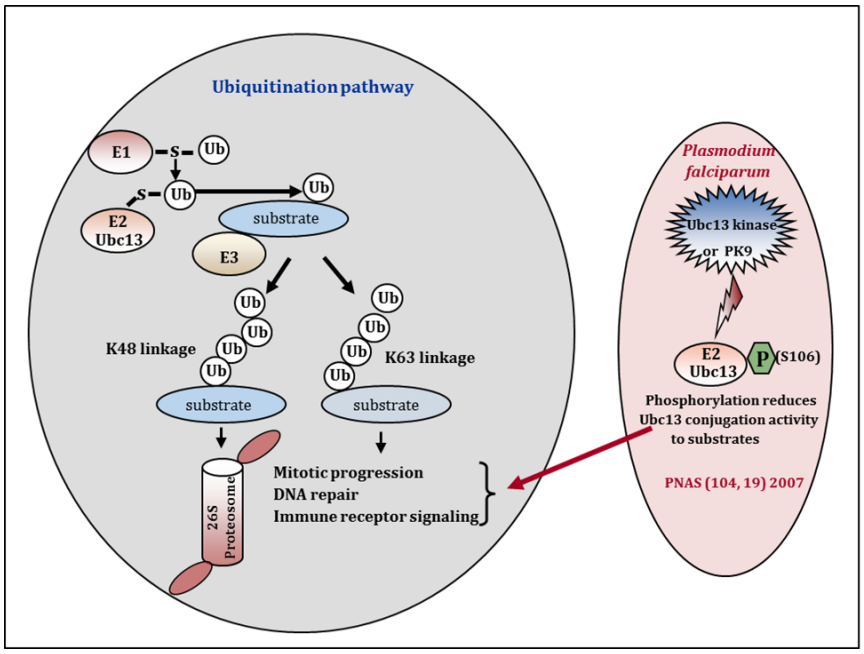

Fig 5. The Ubiquitin system. Ubiquitination is a common post transitional modification of proteins in eukaryotes, bacteria and Archea. It is a process where ubiquitin is covalently attached to a target protein altering its stability, subcellular localization and protein-protein interactions. Central to this pathway are three enzymes that act sequentially. E1 or activation enzyme that forms a reactive thioester bond with the ubiquitin, E2/Ubc13 or conjugation enzyme that accepts ubiquitin on to its active cysteine residue followed by substrate specific ligase or E3 that completes the reaction by formation of an isopeptide bond between the ubiquitin and effector target. There are several lines of evidence that E2 can directly facilitate the ubiquitination of proteins without involvement of E3. There are two distinct fates of ubiquitinated proteins. The assembly of K-48 linked poly ubiquitin chains targets the proteins to non-lysosomal proteolytic degradation through 26S proteasome, whereas the assembly of K-63 linked poly ubiquitinated proteins mediate functions central to mitotic progression, DNA repair and immune receptors signaling.

1.8 High throughput techniques to study global gene expression in *Plasmodium*

The sequencing of *Plasmodium* genome is a major contribution in malaria research. The availability of complete genome of *Plasmodium* provides clues to understand genetic networks and ultimately facilitates in developing new compounds to treat malaria. *Plasmodium* genome contains 14 chromosomes and encodes about 5300 genes. The genome of *Plasmodium* is AT rich and more than 50% genes encode hypothetical proteins without conserved domains and with unknown function. A large portion of genes are important for immune evasion and host-parasite interactions but a smaller portion contributes to enzymes and transporters [163]. For better understanding of *Plasmodium* biology it is necessary to know the function of *Plasmodium* proteins. Recently many techniques have been developed for analysis of the global transcriptome and proteome. Microarray is the most common and widely used technique for both transcriptome and proteome analyses. Using microarrays for whole transcriptome

analysis made a pavement for simultaneous exploration of all parasite genes for their combined and individual function and their regulation. Functions of some of the important stage specifically expressed genes were revealed by reverse genetic approaches, comparative genomics and bioinformatics. Recently, to get deeper understanding of transcriptome, high throughput RNA sequencing was applied as an alternative [164]. Different stages of *Plasmodium* life cycle express different set of proteins which either have same or completely different functions. Techniques like suppression subtractive hybridization (SSH) and serial analysis of gene expression (SAGE) where successfully used for differential gene expression analysis of *Plasmodium* and identified new genes specific for different life cycle stages. SAGE is a powerful technique used for global scale differential transcriptome analysis. Using SAGE analysis, genes upregulated in mosquito salivary glands during sporozoite invasion and genes specific to salivary gland sporozoites were revealed [165, 166]. Using suppression subtractive hybridization of midgut sporozoites versus salivary gland sporozoites, 30 genes which are significantly expressed in salivary gland sporozoites were identified. These UIS (upregulated in salivary gland sporozoites) genes are important for liver stage development [79]. Microarray analysis of oocyst sporozoites and salivary gland sporozoites revealed upregulation of 47 genes in oocyst sporozoites. These genes are called UOS (upregulated in oocyst sporozoites) genes and are important for the invasion of salivary glands [80]. SSH of salivary gland sporozoite versus merozoites identified 25 genes specific for sporozoite stages and are referred as 'S' genes. Circumsporozite protein (CS), TRAP, SPECT1 and SPECT2 are among S genes which are important for sporozoite motility, invasion and infectivity [167]. We selected one of the S genes- *S23* which showed 138 fold induction in microarray studies and our present study revealed the role of *S23* in the egress of liver stages parasites.

1.8.1 Suppression subtractive hybridization

Suppression subtractive hybridization is a PCR based technique that is used to enrich differentially regulated transcripts at specific stage. The method contains two major steps; normalization and subtraction. Target molecules are referred as tester and driver consists of non-target molecules. Double standard cDNA samples are used as substrate for SSH. Tester samples are divided into two groups and two different target sequences (adapter A& adapter B) are attached at 5' ends of the tester cDNA fragments. These two types of tester molecules are denatured and mixed with excessive denatured driver molecules without adapters separately. The first step of hybridization normalizes the abundance of cDNA fragments. Mixing of two types of tester molecules and amplification by primers designed for two

different adapters (A&B) leads to second hybridization involving subtraction and enrichment of target cDNA. Symmetrically formed tester hybrids are not amplified because of PCR suppression (PS) effect due to the suppressed amplification. Asymmetrical hybrids with two different adapters are amplified exponentially leading to enrichment of target transcripts [168]. Kaiser et al., used this technique to enrich sporozoite specific transcripts versus merozoite stages and identified 25 sporozoite genes (S genes) [167].

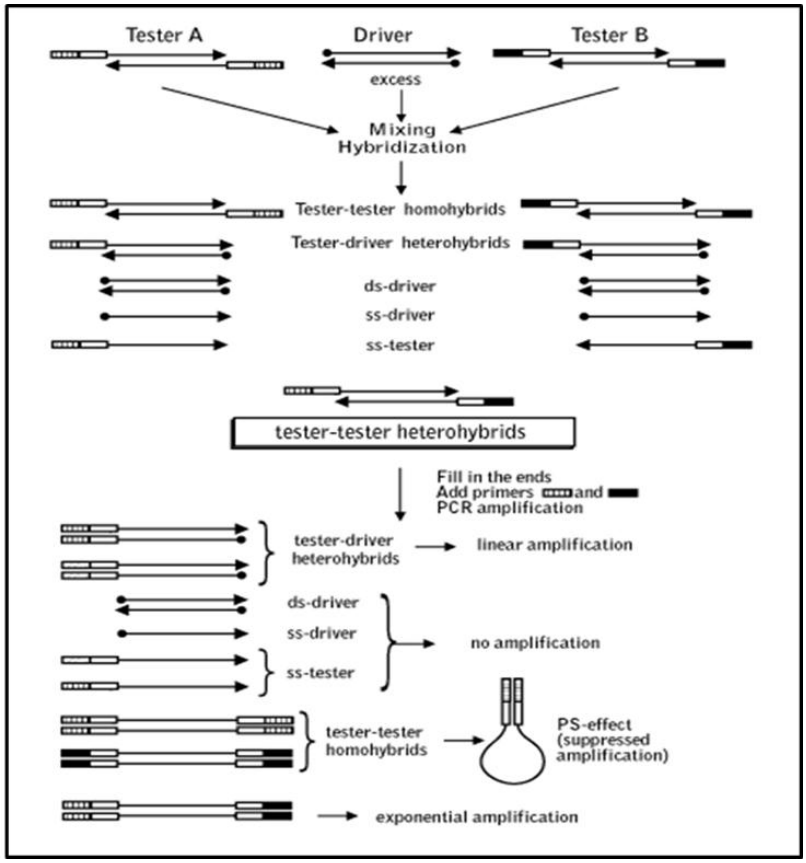

Fig 6. Suppression subtractive hybridization. The technique uses target molecules as tester. Two different types of adapters are used to label tester molecules. Mixing of both types of tester with driver results in the formation of a combination of homo and hetero hybrids. PCR with primers designed for two different adapters resulted in exponential amplification of tester-tester hetero hybrids. This figure is obtained from www.evrogen.com.

1.8.2 Role of Sporozoite (S) genes in motility and invasion of sporozoites

All **s**porozoite genes (S genes) identified by SSH technique are not functionally characterized but majority of these genes are essential for sporozoite motility, invasion and liver stage development and a brief description of their functions is mentioned below. CSP and TRAP are two well-studied proteins among all *Plasmodium* proteins which are included in this category. Circumsporozoite protein is essential for sporozoite formation in the oocysts and sporozoite egress from oocysts [75, 77]. Knockout of *CS* inhibited the egress of sporozoites from oocysts [77]. Circumsporozoite protein binds to HSPGs of hepatocytes and

facilitates the attachment and invasion of sporozoites [169, 170]. S5 is a sporozoite invasion associated protein-1 (SIAP-1) essential for sporozoite egress as the knockout of this gene showed similar phenotype as *CS* knockout parasites [171]. UIS3 and UIS4 were also identified in the screening of SSH library which are reported to be involved in liver stage development [26, 27]. Genetically attenuated parasites obtained through deletion of *uis-3* and *uis-4* where shown to induce CD8+ immunity [86]. A cell traversal protein for ookinetes and sporozoites (Cel Tos) was identified as *S4* and shown to be important for cell traversal activity of ookinetes and sporozoites. In addition to the reduced ookinete infectivity of *Cel Tos* depleted parasites, oocyst formation and sporozoite cell traversal activity were also greatly affected [172]. Thrombospondin related anonymous protein (TRAP)/*S8* is known for its important role in sporozoite gliding motility and invasion [173]. The role of thrombospondin related sporozoite protein (TRSP)/*S21* was shown in sporozoite entry into the host cell. [174]. Hepatocyte infectivity was affected when *spect-2*/*S13* was depleted as the mutant sporozoites were immobilized in the skin fibroblasts [11, 13]. TREP/*S6*, which is a TRAP like protein was shown to be necessary for gliding motility of midgut sporozoites as the number of salivary gland sporozoites were significantly reduced when this gene was deleted [175]. *S22* encodes for an asparagine rich protein of sporozoite and liver stages, also termed as SLARP/ SAP-1 and was shown to be required for liver stage development as injection of *sap-1* knockout sporozoite by bite and intravenous injection failed to initiate blood stage infection [81]. The aforementioned sporozoite genes functionally characterized so far were required for sporozoite motility, invasion and liver stage development. Out studies show for the first time the role of *Pb* S23 beyond liver stage development. Our findings suggested *S23* depletion affects the egress of merozoites from fully grown EEF.

CHAPTER 2

Role of *Plasmodium berghei Ubc13 kinase/PK9* in malaria transmission to mosquitoes

2.1 Introduction

Plasmodium is exposed to different environmental conditions during its life cycle both in vertebrates and mosquitoes but few transcriptional changes are observed in the parasite in response to external stimuli [124]. While microarray studies revealed changes in steady state mRNA levels during its development; very few transcription factors and regulatory motifs are identified so far [176]. This rigidity in transcription of *Plasmodium* suggests that post translational modifications (PTMs) play a major role in the parasite life cycle [125]. Phosphorylation, one of the PTMs, regulates the expression and activity of kinases. Kinases are important targets to treat cancer and many diseases [177]. In *Plasmodium* initial experiments with kinase inhibitors revealed that they negatively impacted the intra erythrocytic development, thus pointing to the central role of kinases in *Plasmodium* development [178, 179]. Following this study, several other kinase inhibitors were reported that were shown to affect the development of *Plasmodium* at different stages of the life cycle [137, 146, 180 and 181]. In *P. falciparum* 3D7 genome, 85 eukaryotic protein kinases were identified but only few kinases have assigned functions [126]. In the *P. berghei* genome, 73 genes with putative protein kinase domains were identified [62]. Phylogenetic analysis of *P. falciparum* and *P. berghei* kinome showed very few differences. Few *Plasmodium* kinases that do not belong to existing kinase groups are referred as "orphan kinases" [182]. Understanding the function of kinases can offer an insight into *Plasmodium* biology which in turn can open venues for development of new intervention strategies.

Plasmodium kinases play important roles throughout the parasite life cycle from asexual reproduction to sexual differentiation and invasion. Among malaria intervention options, transmission blocking strategies are gaining importance by disrupting genes important for sexual cycle. Sexual cycle occurs in the mosquito that is characterized by several distinct events like exflagellation of male gametocyte, differentiation of female gametocyte to female or macrogamete, fertilization of micro and macro gametes, formation of zygote, differentiation of the zygote into ookinete and formation of oocysts. All these events require a variety of kinases for completion of the parasite life cycle. The sexual forms of *Plasmodium* are observed in mammalian host when a small portion of merozoites develop into male or female gametocytes by arresting cell division [55, 56, 183 and 184]. Sexual commitment occurs at schizont stage and merozoites from a single schizont forms either male gametocyte or female gametocyte but not both [185].

Several kinases that regulate the process of sexual reproduction have been identified in the recent past [186]. CDPK4, a calcium dependent protein kinase, was shown to transduce the xanthurenic acid mediated changes in intracellular calcium into cellular responses like cell cycle progression, specifically in the male gametocytes. The NIMA (never in mitosis gene a) family of protein kinases, reported in *Aspergillus nidulans* is known for its activity to regulate centromere functions and entry into mitosis [187, 188]. Two of the NIMA family members- namely nek-2 [65] and nek-4 [64] have been shown to play a role in meiosis in the ookinete stage. While *nek-4* depletion had no effect on the either gamete formation or fertilization, the progression from zygote to ookinete was blocked. An explanation for such phenotype was the inability of *nek-4* deficient zygotes to replicate their genome prior to meiosis. A similar role of nek-2 was demonstrated, where its depletion led to dysregulation of the DNA replication. While both *nek-4* and *nek-2* knockouts failed to form ookinetes and gave identical phenotype resulting in failure of malaria transmission to mosquitoes, both kinases, nonetheless displayed varying substrate specificities, indicating that functionally distinct but related kinases (NIMA family) may mediate same biological function.

The *Plasmodium* kinases that regulate motility of gametes [63] and gliding of ookinetes have also been shown to be critical for malaria transmission to mosquitoes. For example, an atypical mitogen-activated protein kinase of *P. berghei* (PbMAP-2) was shown to be involved in cytokinesis and flagellar motility during formation of male gametes. The *Pbmap-2* deficient parasites were indistinguishable from WT with regard to asexual propagation or formation of gametocytes, including emergence of female macrogametes. However, they were compromised to generate micro gametes that are generated by a process of exflagellation. Interestingly, the manifestation of block in formation of microgametes was after the completion of mitosis and axoneme formation, suggesting a role for PbMAP-2 downstream of the cell cycle check point.

A role of *P. berghei* calcium dependent protein kinase (PbCDPK3) was shown to be essential for motility of the ookinetes through the midgut epithelium [141]. The *PbCDPK3* deficient parasites propagated normally till the ookinete stage, but were unable to glide through the midgut epithelium. While the exact mechanism of how PbCDPK3 might regulate motility is unclear, it is only speculative that transmembrane adhesins may transduce extracellular signals through the parasite membranes, leading to alteration in intracellular calcium levels, influencing calcium dependent protein kinases and hence motility. Thus, in the

absence of such regulation, the *PbCDPK3* KO parasites failed to glide. The specific role of PbCDPK3 in ookinete motility was elegantly revealed by two approaches. Firstly, an episomal complementation restored the gliding through the midgut epithelium in *PbCDPK3 KO* line and secondly, directs microinjection of *PbCDPK3* KO into haemocoel led to the successful completion of sporogony of the KO parasites [141].

In *P. berghei*, calcium dependent protein kinase 1 (CDPK1) was shown to control zygote formation and malaria transmission by translationally activating repressed mRNAs [139]. Earlier studies have reported an essential role of *Pb CDPK1* in asexual stages, as the loci was recalcitrant to gene disruption [62, 138]. However, its role in sexual stage was analyzed by generating promoter swap transgenics, when the *Pb CDPK1* was kept functional by a blood stage specific promoter that was subsequently turned off in the mosquito stages. These conditional *CDPK1* mutants exhibited a developmentally arrested stage of the parasites in the mosquito that blocked transmission. Both transcriptional and translational studies on the *CDPK1* conditional mutants revealed a role of CDPK1 in translationally activating the mRNA species in the zygote stages, which in the macrogametes remain repressed via their 3' and 5' UTR. Thus CDPK1 may have multiple functions across the life cycle stages, and in the sexual stages, they ensure a timely expression of protein required for progression beyond zygote stages.

While the mosquito metabolite, xanthurinic acid (XA) is a known factor that facilitates gametogenesis [57] in mosquito midgut, the precise mechanism of this spontaneous activity is far from being known. The role of cGMP dependent protein kinase was shown to be necessary for XA induced gametogenesis [181]. By using cGMP dependent protein kinase (PKG) inhibitors and transgenic parasites that express mutant version of PKG that is insensitive to inhibitor, it was clearly demonstrated that XA induced gametogenesis was regulated by PKG.

A more comprehensive analysis of all the kinases in *P. berghei* was undertaken, with an objective to identify how many were critical regulators of the malaria transmission to mosquitoes [62]. Towards this end, 66 putative *Plasmodium* kinases were identified based on the presence of the signature domains that commonly occur in the conventional eukaryotic protein kinases. The domain signatures typically contain 12 subdomains that fold to form a catalytic core structure [189]. When the amino acid sequence from the ePK domains of *P.*

falciparum and *P. berghei* were aligned, three invariant amino acids that are catalytically active were found, the first one was the ATP binding lysine in subdomain II, second was the conserved aspartic acid present in the catalytic loop formed by subdomain VIb and third, a conserved aspartic acid in the DGF motif of subdomain VII, that chelates the Mg^{2+} ions. While the aforementioned invariant amino acid residues were found in 86 of the *P. falciparum* ePKs, only 66 ePKs of *P. berghei* contained these residues. All the 66 ePK of *P. berghei* were further functionally characterized by reverse genetics. Of the 66 ePKs, only 23 kinases were amenable to gene disruption revealing their non-essential role in the asexual blood stages. Remaining 43 kinases seem to be essential for the blood stages, as attempts to replace the gene was not feasible. The 23 kinase KOs that were not essential in the blood stages were further transmitted to mosquitoes. Of these 23 kinases, three kinases viz., the SR protein kinase (SRPK), a conserved regulator of clathrin uncoating (GAK) and a likely regulator of energy metabolism (SNF1/KIN) showed a striking phenotype with distinct roles respectively in microgamete formation, ookinete formation and sporozoite development respectively.

To the best of our knowledge, there are no reports of kinases regulating the ubiquitin conjugation activity of cellular proteins thereby affecting the progression of *Plasmodium* life cycle. While Ubc13 kinase/PK9 was discovered for the first time in *P. falciparum*, in the context of negatively regulating the Ubc13/E2 ubiquitin conjugation activity, the functional role of this kinase in parasite life cycle was not elucidated. We investigated the role of this kinase in *P. berghei* by reverse genetics approach and our study describes for the first time the function of Ubc13 kinase/PK9 in regulating the sexual cycle of the parasite, revealing its role as a critical regulator of malaria transmission to mosquitoes.

Ubiquitination is one of the important PTMs like phosphorylation that is essential for many biological functions including DNA repair, cell growth, immune response and apoptosis [190-192]. Recent studies have demonstrated the role of ubiquitination in non-proteolytic pathways in addition to targeting proteins for proteasomal degradation [193]. Ubiquitination involves a series of events catalyzed by ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3). Ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) which mediate the formation of polyubiquitin chains. K48 poly ubiquitination targets the protein for 26S proteasomal degradation. However, K63 linked ubiquitin chains triggers non-proteasomal functions. In the genome of *P. falciparum,* 8 E1 or E1-like proteins, 14 E2 or E2-like conjugating enzymes, 54 E3 or E3-like proteins and 9

ubiquitin or ubiquitin like proteins (UBLs) where identified [194]. Till todate, only Ubc13 is known to mediate formation of K63 linked ubiquitin chains while most E2s activate K-48 ubiquitination [194]. Ubc variant (Uev) act as cofactor for Ubc13 to form K-63 linked ubiquitin chains. Uev is similar to Ubc13 in structure and amino acid sequence except the absence of cysteine residue in the catalytic site that makes Uev catalytically inactive [195]. In humans, Uev1a and mms2 are two Ubc splicing variants that interact with Ubc13 and mediate NFκB activation and DNA damage response respectively [196-198]. In *P. falciparum*, only one homologue of Uev1/Mms2 is identified and the structure is determined by X-ray crystallography. The K-63 polyubiquitination forms a molecular platform for protein- protein interactions involved in kinase signaling and DNA repair.

Although most of the components in ubiquitin system are identified in *Plasmodium* [194, 199] the regulation of ubiquitin pathway is not well studied. One of the likely regulators of cellular ubiquitination was identified as a kinase in *P. falciparum* [127]. The kinase was designated as Ubc13 kinase/PK9 (protein kinase 9) and was shown to phosphorylate Ubc13 at serine-106 residue resulting in negatively regulating the ubiquitin conjugating activity. Since Ubc13 kinase/PK9 did not cluster with any of the known kinase groups, nor did it have any orthologues in other organisms, it was categorized as orphan kinase of *Plasmodium*. This study confirmed the expression of Ubc13 kinase/PK9 in *P. falciparum* blood stages, from late ring stage to schizonts stage [127]. The colocalization of Ubc13 kinase/PK9 with EXP-1 at PVM indicates its likely role in signaling at host-parasite interface. Phosphorylation of Ubc13 by Ubc13 kinase/PK9 reduced its ubiquitin conjugating activity *in vitro*. Serine 106 residue is a highly conserved residue among E2s and it is a surface residue of helix -3 region which is the binding site of ubiquitin [200, 201]. So the phosphorylation by Ubc13 kinase/PK9 could interfere with the binding of ubiquitin to Ubc13 and reducing the conjugating activity. As Ubc13 is essential for formation of K63 poly-ubiquitination, Ubc13 kinase/PK9 can be considered as important regulator for formation K63 ubiquitin chains that may indirectly affect non proteasomal functions of the parasite.

The amino acid sequence alignment of *P. falciparum* and *P. berghei* Ubc13 kinase/PK9 revealed 82% homology possibly suggesting a conserved function of this kinase across these two species. In the present study, we identified the role of Ubc13 kinase/PK9 in sexual cycle of *P. berghei*. We deleted the gene locus using double homologous recombination and the *Ubc13 kinase/PK9* knockout parasites were analyzed for their phenotype throughout

Plasmodium life cycle. *Ubc13 kinase/PK9* knockout parasites failed to form ookinetes and oocysts. The role of *Ubc13 kinase/PK9* beyond oocyst stages was studied by using an FLP/FRT system [202]. Conditional silencing of *Ubc13 kinase/PK9* using transgenic TRAP/FLP parasites revealed the non-essential role of *Ubc13 kinase/PK9* in oocyst sporulation, sporozoite invasion to salivary gland, liver infection, EEF development and subsequent blood stages initiation. These findings reiterated the unique role of *Ubc13 kinase/PK9* only in sexual stages of the life cycle and have relevance for developing inhibitors against this kinase, as possible strategy to prevent malaria transmission.

2.2 Materials and methods

2.2.1 Experimental animals and parasite lines

Eight to ten weeks old female Swiss albino, BALB/c or C57BL/6 mice were purchased from National Institute of Nutrition (NIN) and used for all animal experiments. Animal handling was strictly in accordance to Institutional Animal Ethical Committee protocols. All transgenic parasite clones were generated by transfecting respective targeting plasmid in wild type *P. berghei ANKA* parasite line. Wild type *P. berghei ANKA* GFP line (Surendra KK and Kumar KA, personal communications) was used for comparison of phenotype of knockout parasites throughout the parasite life cycle. Life cycle of all the genetically modified/transgenic parasites were studied by cycling the parasites through Swiss albino or BALB/c and female *Anopheles stephensii (A. stephensii)* mosquitoes. In experiments involving analysis of pre-patent period either by mosquito bite or intravenous injection of sporozoites, C57BL/6 mice were used.

2.2.2 Retrieval of *P. berghei* gene and protein sequences, sequence alignment analysis of Ubc13 kinase/PK9 and Ubc13 (E2)

Two public domain databases, Plasmodb (www.plasmodb.org) and Genedb (www.genedb.org) were used to retrieve all DNA and protein sequences related to *Plasmodium*. Primers were designed based on the sequence information. To check the homology of Ubc13 kinase/PK9 protein between *P. falciparum* and *P. berghei*, we used MULTALIN (http://multalin.toulouse.inra.fr/multalin/). Protein alignment was also performed using MULTALIN for Ubc13 which is the substrate of Ubc13 kinase/PK9.

2.2.3 Primers

Primers used for construction of transfection plasmids and to confirm site specific integration are mentioned in the table 1.

Table 1. Showing primers used for generation and confirmation of *Ubc13 kinase/PK9* KO (Fig11), *Ubc13 kinase/PK9* conditional KO (Fig 14) and *Ubc13* mcherry trangenics (Fig 16).

S.No	Name of the primer	Primer sequence (5'-3')
1	Ubc13 kinase/PK9 5' FP (FP1)	**AGTCTCGAG**TAAAATTAGGAAAAATCCAA
2	Ubc13 kinase/PK9 5`RP (RP1)	**ATAATCGAT**TTGACACTGCCCAGATAC
3	Ubc13 kinase/PK9 3`FP (FP2)	**ATAGCGGCCGC**AACAACTTAGCCCCAACC
4	Ubc13 kinase/PK9 3`RP(RP2)	**ACTGGCGCGCC**TATTTTTATACACATCAATTAA
5	Ubc13 kinase/PK9 confirmation FP (FP3)	TTCCGTATAACCAAACTTAG
6	HSP705' UTR RP (RP3)	TTCCGCAATTTGTTGTACATA
7	DHFR FP (FP4)	GTTGTCTCTTCAATGATTCATAAATAG
8	Ubc13 kinase/PK9 confirmation RP (RP4)	AAAATAACGAAAAAAAAGAT
9	Ubc13kinase/PK9 ORF FP (FP5)	TTATGTATGTCTAATTATGGA
10	Ubc13kinase/PK9 ORF RP (RP5)	TTTAAATCTCTATGTACAATA
11	Ubc13 kinase/PK9 CDS FP (FP1)	**GCAAGATCT**TGCTGTTAATAGTGATTTAAA
12	Ubc13 kinase/PK9 CDS RP(RP1)	**ATAGCGGCCGC*ATATGTTTATTAAAA***TTATGA ATATCGATAGAACCA
13	Ubc13 kinase/PK9 IGS FP(FP2)	**ACAAAGCTT**TTAAACAACTTAGCCCCAAC
14	Ubc13 kinase/PK9 IGS RP(RP2)	**GCAAGATCT**TACAAAAATTCAAAATATGCTATAT
15	Ubc13 kinase/PK9 CDS confimation FP (FP3)	ATACAAACATTAAGAGGTTTAAT
16	Ubc13 kinase/PK9 IGS confimation RP(RP3)	AAGAATCAGAAAATAACGAA
17	Ubc13 CDS FP (FP1)	**TACGGGCCC**AGGTGGCTCCTATAAATTAGA
18	Ubc13 CDS RP (RP1)	**ACACTCGAG**TATGGCACTATTAGCATACAT
19	Ubc13 IGS FP (FP2)	ATA**GCGGCCGC**AATCAAATTTATTGCTACCAT
20	Ubc13 IGS RP (RP2)	**ACTGGCGCGCC**TAGTATTACCATTATTTGAAT
21	Ubc13 confirmation FP (FP3)	ATACTTATTTTAGGTGGTTTT
22	mcherry RP (RP3)	TCGCCCTCGCCCTCGATCT
23	DHFR FP (FP4)	GTTGTCTCTTCAATGATTCATAAATAG
24	Ubc13 confirmation RP (RP4)	ATTCTCTACATATTTTCAGGA

2.2.4 Generation of *Ubc13 kinase/PK9* (PBANKA_141360) knockout construct

2.2.4.1 PCR

To generate *Ubc13 kinase/PK9* knockout construct we used double cross over recombination strategy for target gene replacement. To achieve this, *P. berghei ANKA* genomic DNA was used as a template to amplify 554bp of the 5'flanking region of *Ubc13 kinase/PK9* using primers Ubc13 kinase/PK9 5' forward (FP1) and Ubc13 kinase/PK9 5' reverse primers (RP1). A 507 bp of the 3' flanking region of *Ubc13 kinase/PK9* was amplified using Ubc13 kinase/PK9 3' forward primer (FP2) and reverse primers (RP2) (primer sequences provided in table 1). To amplify 5' and 3' fragments of *Ubc13 kinase/PK9*, 50 µL PCR mix was prepared by adding 1X PCR buffer, 1 mM dNTP mix, 5 mM $MgCl_2$, 0.5 µM FP, 0.5 µM RP, 2.5 units Taq polymerase and 50 ng genomic DNA in final concentration. Volume was made up to 50 µL with nuclease free water. The reaction mix was kept in PCR machine and thermal cycling conditions were 94°C for 2 min for initial denaturation, 94°C for 30 sec followed by annealing at 54°C and extension at 72°C for 1 min, and repeated for 35 cycles with a final extension at 74°C for 10 min. After completion of PCR, 5 µL of the product was analyzed on 1% agarose gel that revealed an expected size of the amplicon (Fig 11D). PCR products were purified using Purelink™ quick gel extraction and PCR purification combo kit (Invitrogen, Cat No.K2200-01).

2.2.4 2 XL-Blue MRF'competent cells preparation and transformation

XL blue MRF' was inoculated in 5 ml of Luria-Bertani broth (LB broth) and incubated at 37°C, 200rpm overnight. Twenty five to fifty ml of LB broth was inoculated with 1-2% inoculum of overnight culture and incubated at 37°C with 200rpm for 2-3 h and measured the absorbance at 650 nm. When optical density (OD) reached 0.5, the culture was incubated in ice for 30 min. Culture was centrifuged at 6000rpm for 10 min. The bacterial pellet was resuspended in 4-8 ml of 0.1 mM $CaCl_2$ solution and incubated for 1 h in ice followed by centrifugation at 6000 rpm for 10 min. The pellet was resuspended in 0.1 mM $CaCl_2$ solution for immediate use. For long time storage the pellet was resuspended in 13% glycerol in 0.1 mM $CaCl_2$ solution, 100 µL was aliquoted in 1.5 ml eppendorff tubes, quickly dipped in liquid nitrogen and stored at -80°C. For transformation, plasmid or ligation mix was added to 100 µL competent cells and incubated for 20 min on ice followed by heat shock at 42°C for 90 sec and further incubation on ice for 5 min. Following this incubation, 1 ml LB

medium was added immediately and incubated in shaker incubator maintained at 37°C and 200rpm for 1 h. Cells were centrifuged and the pellet was resuspended with 100 µL fresh LB medium and spread on the LB agar plate containing appropriate antibiotic for selection of recombinant colonies.

2.2.4.3 Cloning of 5' UTR

Concentration of purified 5' PCR product was quantified using Nanodrop 2000. Sample was prepared for digestion by mixing 3µL of restriction enzyme compatible buffer, 1U of XhoI, 1U of ClaI, 1-2 µg PCR product and nuclease free water made up to 30 µL in volume. The sample was incubated for 3-4 h at 37°C. Transfection vector, pBC-GFP-DHFR was also digested with XhoI and ClaI and incubated at 37°C for 3-4 h. After digestion, samples were purified using Purelink™ quick gel extraction and PCR purification combo kit (Invitrogen, Cat No.K2200-01). After purification, concentration of digested vector and PCR product was quantified using Nanodrop 2000. Ligation was set up by mixing 1 µL of 10X ligation buffer, 1U of ligase, purified vector and insert in 1: 4 ratio in concentration and made up to 10 µL with nuclease free water. The ligation mix was incubated at 22°C for 3 h and transformed into XL-blue MRF' competent cells. After transformation, cells were plated on LB culture plates containing ampicillin. Colony PCR was performed to identify the clones having the insert. Following this preliminary confirmations, plasmid was isolated from positive colony by Sure spin plasmid mini kit (Genetix, Cat No. NP-37105). Restriction digestion by XhoI and ClaI confirmed the presence of 5' fragment in the vector resulting in pBC-GFP-DHFR-Ubc13K KO5'plasmid.

2.2.4.4 Cloning of 3' UTR

Double digestion was performed for purified 3'PCR product and pBC-GFP-hDHFR vecor containing 5' fragment with NotI and AscI and purified using Purelink™ quick gel extraction and PCR purification combo kit (Invitrogen, Cat No.K2200-01). After the purification of digested samples, ligation was set up and the ligation mix was transformed into XL-blue MRF' competent cells. Colony PCR was used to select positive colonies. Plasmid was isolated from a single positive colony. The plasmid was digested with XhoI/ClaI and NotI/AscI to release 5' and 3' fragments (Fig 11E). Sequencing was performed for the plasmid with 5' and 3' fragments with sequence specific primers. After the sequence confirmation, *Ubc13 kinase/PK9* knockout plasmid was isolated in large scale by Sureprep

plasmid MAXI kit (Genetix, Cat No-NP-15162) and digested with XhoI and AscI enzymes (Fig 11F). The fragment containing the targeting construct (5' fragment+GFP-hDHFR cassette+ 3' fragment) was gel excised and purified using Purelink™ quick gel extraction and PCR purification combo kit (Invitrogen, Cat No.K2200-01). The concentration of the targeting plasmid was quantified on Nanodrop 2000. The digested and purified product was used as *Ubc13 kinase/PK9* KO construct for transfection.

2.2.5 *In vitro* culture and purification of schizonts

Blood was collected from *P. berghei* infected mice by cardiac puncture when the parasitemia was 0.5-2% and placed in a 50 ml tube containing 250 U/ml of heparin (Sigma Cat No-H3393-25KU). Ten ml of schizont culture medium (RPMI supplemented with 20% FBS and gentamycin at a concentration of 350 μg/ml of medium) was added to the blood and centrifuged at 200Xg without brakes for 8 min in a swinging bucket rotor. The pellet was resuspended in 1 ml schizont medium and added to 24 ml schizonts medium present in T75 flask. Gasing (5% CO_2, 5% O2, 90% N2) was performed to the culture for 2-3 min and incubated at 36.5°C with 45 rpm for 16-23 h. Nycodenz stock was prepared by adding 100 ml buffer containing 5 mM Tris-HCl, 3 mM KCl and 0.3 mM EDTA to 27.6 g Histodenz (Sigma, Cat No. D2158). 60% Nycodenz was prepared in PBS (pH 7.2) and used for purification of schizonts. Twenty five ml of parasite culture was aliquoted into a 50 ml tube and 10 ml of 60% nycodenz was added slowly under the culture suspension and centrifuged at 370Xg for 20 min without brake in swinging bucket rotor. Purified schizonts were collected from the interface and were washed with 10 ml of culture medium and centrifuged at 1500 rpm for 10 min and used for transfection.

2.2.6 Transfection of *Ubc13 kinase/PK9* knockout construct in *P. berghei* schizonts

Nucleofector solution (Lonza, Cat No-VZB-10001) was prepared by mixing 90 μl mouse T cell nucleofector solution and 20 μl supplement. Ten micrograms of purified *Ubc13 kinase/PK9* knockout construct was mixed with the nucleofector solution. Schizont pellet was resuspended in the DNA/nucleofector solution and transferred into the cuvette. Cuvette was placed in the Amaxa nucleofector device and electroporated using program U-033. Hundred microliters of cold culture medium was added into the cuvette after electroporation and the contents were injected into the mice intravenously. The following day, blood smears were made from mice harboring transfected parasites and stained with Giemsa to observe parasites

under light microscope. Once the parasitemia reached 0.5-1%, the mice were kept on antimalarial drug-pyrimethamine for selection of successful transfectants. Pyrimethamine was prepared by dissolving 7 mg pyrimethamine powder in 1 ml of DMSO. The stock solution was diluted with 100 ml of drinking water and pH was adjusted to 4.0. The mice were treated with pyrimethamine solution, substituted in place of drinking water for 7-10 days. Parasitemia was monitored every day by Giemsa stained blood smears. Once the parasitemia reached 2-3% under drug pressure, parasites were observed under fluorescence microscope for GFP expression. After confirming GFP expression, blood was collected and genomic DNA was isolated. A diagnostic PCR was performed to confirm site specific integration by using primers designed within the targeting cassette and at sites beyond integration. The transgenic KO parasites were subjected to limiting dilution to obtain clonal population. Two clones from independent transfection were obtained by limiting dilution. While both clones exhibited identical phenotype, the detailed phenotypic analysis of one of the clones is described in detail. (Fig 7)

2.2.7 Genomic DNA isolation from infected mouse blood

Infected blood from mouse was collected when the parasitemia reached around 4-5% and centrifuged at 8000 rpm for 5 min. The pellet was resuspended in 1 ml of 0.05% saponin solution in PBS (pH 7.2) to lyse RBC and centrifuged at 13,000 rpm for 10 min. The pellet was washed with 1 ml PBS (pH 7.2) and centrifuged at 13,000 rpm for 10 min. The parasite pellet was used for genomic DNA isolation using DNAsure tissue mini kit (Gnetix, Cat No.NP-61305). The pellet was resuspended in 200 µL lysis (LBT) buffer, 25 µL proteinase K provided with the kit and 200 µL buffer BT3 and incubated at 70°C for 10 min. Following incubation, 210 µL of absolute alcohol was added and vortexed vigorously. The solution was loaded onto the column and centrifuged for 11,000Xg for 1 min. The flow through was discarded and 500µL of WBT buffer was added and centrifuged at 11,000Xg for 1 min. After discarding flow through the column was washed with 650 µL of WBT5 buffer and centrifuged for 11,000Xg for 1 min. Additional 1 min spin was kept to remove residual buffer. To elute DNA the column was kept in new 1.5 ml tube and 30 µL of elution (BET) buffer was added. After addition of elution buffer the column was incubated for 1min at room temperature and centrifuged at 11,000Xg for 1 min. The concentration of eluted genomic DNA was quantified Nanodrop 2000.

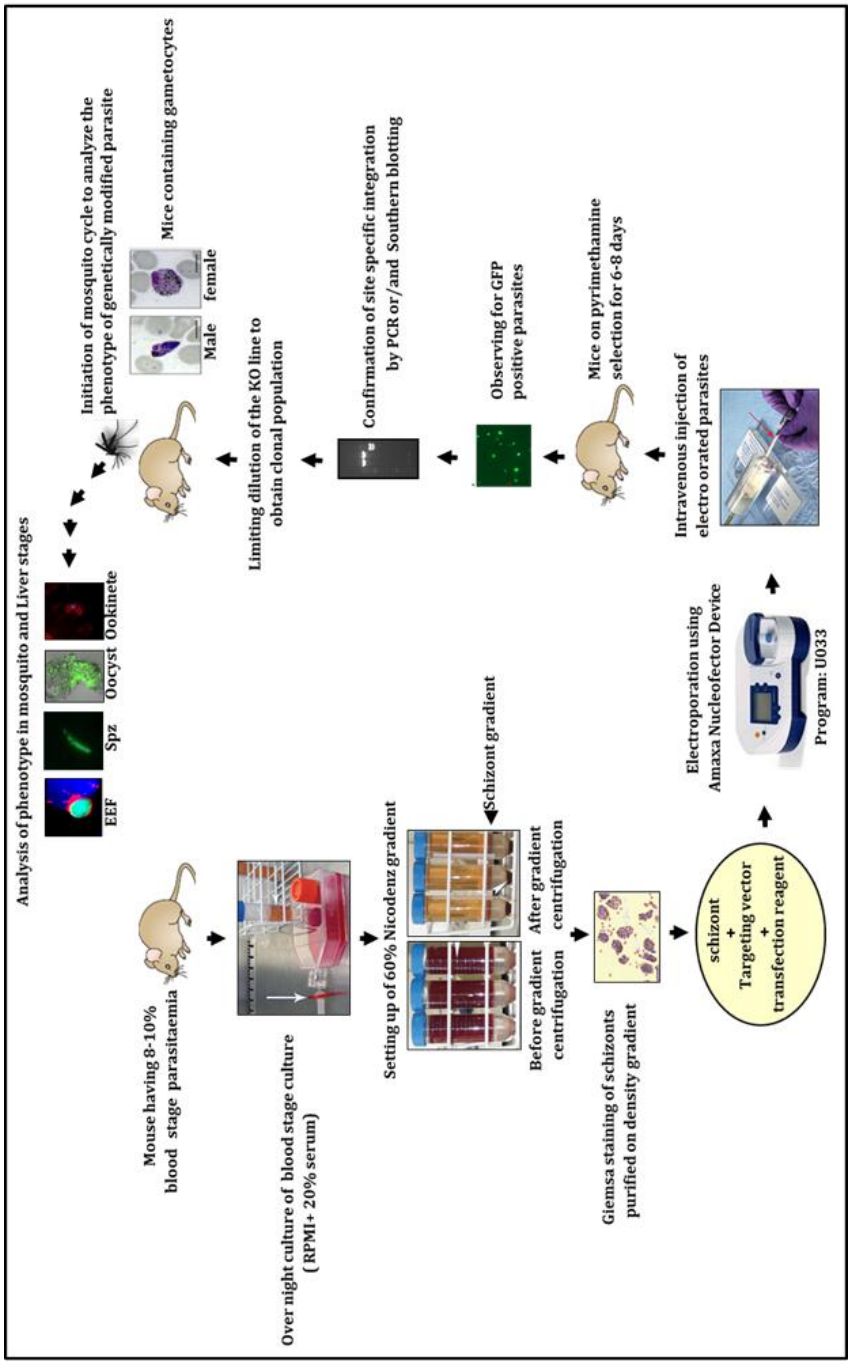

Fig 7. Schematic representation *Plasmodium berghei* transfection, drug selection, confirmation of the site specific integration and phenotypic characterization of the genetically modified transgenic/ knockout/reporter line. Blood is collected from mouse having 8-10% parasitemia and an overnight culture is set up. Next day, the schizonts are enriched on a density gradient. The purified schizonts are collected and electroporated with the targeting construct and immediately injected intravenously into mouse. The mice are kept on a pyramethimine, an antimalarial drug that facilitates selection of the transfectants. The success of stable site specific integration is confirmed either by PCR or Southern. The transfectants are cloned out and passed through mosquito stages to analyze the phenotype in other life cycle stages like oocyst stages, sporozoite stages and liver stage. This figure is obtained from Janse, C.J, Ramesar, J and Water, A.P [203] and modified.

2.2.8 Analysis of asexual blood stage propagation of *Ubc13 kinase/PK9* KO parasites

To determine the effect of *Ubc13 kinase/PK9* knockout on intra-erythrocytic development, $1X10^3$ infected RBC of either WT or *Ubc13 kinase/PK9* KO were intravenously injected into a group of 3 female BALB/c mice and parasitemia was monitored daily by microscopic examination of Giemsa stained blood smears for seven days.

2.2.9 Maintenance of *A. stephensii* colony and transmission of malaria to female mosquitoes for studying mosquito stages of *Plasmodium*

Adult mosquitoes were maintained on 10% sucrose solution at 28°C and relative humidity 80%. For breeding and continuous maintainace of mosquitoes following protocol was used. Rabbit was anaesthesized by injecting 500µL anaesthesia (0.8 ml ketamine (50mg/ml)+ 0.3 ml of xylazine (20mg/ml) + 3.9 ml of 1X PBS) intramuscularly and used for blood meal for two consecutive days. Thirty six to forty eight hours following second blood meal, a bowl of water was placed inside the mosquito cage to facilitate the egg laying. The eggs were collected for 4 consecutive days. The eggs were transferred into environmental chambers maintained at 27°C and 85% RH. During a period of 10-14 days the eggs undergo a series of transformations- early instars, late instars and pupae. The pupae were collected manually and placed in a petridish inside the fresh cage. Adult male and female mosquitoes emerge from pupae and mate within 24-26 hours of emergence. For preparing infection cages, the female mosquitoes were collected by vacuum suction and placed in a new cage. The female mosquitoes were allowed to obtain a blood meal from mice harboring gametocytes from WT or *Ubc13 kinase/PK9* KO line. Infected mosquitoes were maintained at 20-22°C with 80% relative humidity. (Fig 8)

2.2.10 Infection of female mosquitoes with *Ubc13 kinase/PK9* KO parasites

A group of 5-6 BALB/c mice were injected either with WT or *Ubc13 kinase/PK9* knockout parasites. The parasitemia was allowed to progress till 2-3%. When the infected mice were positive for gametocytes, mice were anesthesized and used for infecting mosquitoes. Blood meal was given for 15 min for two consecutive days and the cage was maintained at 20-22°C with 80% relative humidity. At 20 h post blood meal, mosquitoes infected with WT or *Ubc13 kinase/PK9* KO parasites were dissected and blood was collected with 250 U/ml heparin. The collected blood was spotted on microscopic slides and observed

for ookinetes under light microscope or processed for immunofluorescence assay. On 14th day post infection, mosquitoes infected with WT or *Ubc13 kinase/PK9* KO were dissected and midguts were observed for oocysts under fluorescence microscope (Nikon Eclipse NiE AR).

2.2.11 Immunofluorescence assay

Ookinetes were fixed with 4% para formaldehyde for 20 min and washed for 1 min with PBS (pH 7.2). To permeabilize ookinetes, the cells were exposed for 15 min in a solution of 0.1% Triton X -100 made in PBS (pH 7.2). Nonspecific blocking was performed in 3% BSA prepared in PBS (pH 7.2) and was incubated for 1 h at room temperature. This was followed by incubation with mouse monoclonal antibody, p28 (a generous gift from Oliver Billker, Wellcome Sanger Institute, United Kingdom) specific for ookinete surface protein. The primary antibody was used at a dilution of 1:100 and was incubated for 90 min. Following primary antibody incubation, the cells were washed for three consecutive times, with PBS, PBS-tween (0.1% tween in PBS) and PBS for 20 min each. Alexaflour 594 (Molecular probes) conjugated anti mouse secondary antibody was diluted at 1:300 dilution and incubated for 1 h at 37°C followed by washes with PBS, PBS-tween (0.1% tween in PBS) and PBS for 30 min each. Nuclear staining was done with 4', 6' diamidino-2 phenyl indole (DAPI) at 1: 100 dilution which was mixed with secondary antibody solution. Slides were air dried and mounted with an antifade reagent covered with coverslips. The border of the coverslip was coated with nail polish for sealing. Slides were observed under fluorescence microscope (Nikon Eclipse NiE AR).

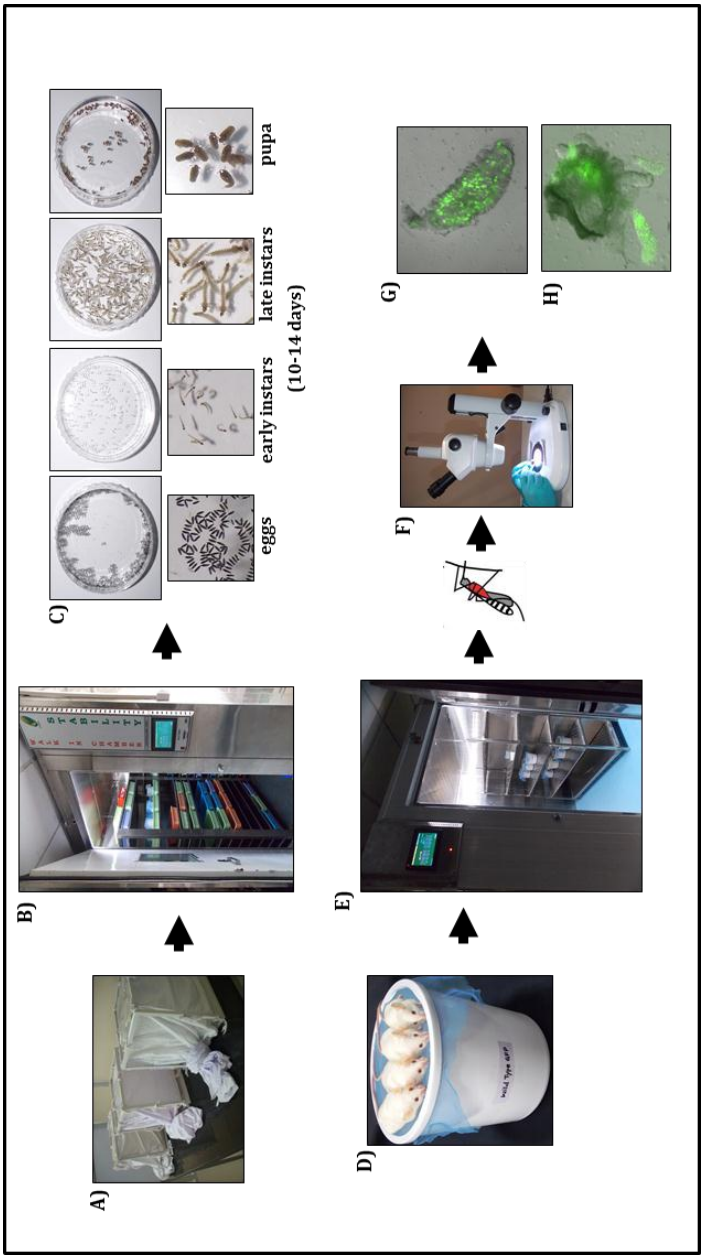

Fig 8. Maintenance of *Anopheles stephansii* colony. Maintenance of *Anopleles stephansii* colony includes activities like preparation of breeding cages, where pupa are placed in water bowls that emerge and mate within 24-36 hours (A). Two successive blood meals from anesthetized rabbit are given to the mated female mosquitoes at an interval of 24 hours. Thirty six hours after second blood meal, a bowl of water is placed inside the breeding cage and eggs are collected for four consecutive days. The eggs are then transferred into environmental chamber maintained at 27°C and RH 80% (B). Under these conditions the eggs hatch and transforms into a series of instars and finally into pupae (C). The pupae and collected and placed inside the breeding cage for emergence of adult 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 *Plasmodium berghei* gametocytes (D). The infected cage was placed in an environmental chamber maintained at 22°C and RH 80% to facilitate the completion of sexual reproduction of *Plasmodium berghei* (E). Mosquitoes were dissected under dissection microscope (F) on D14 (G) and D18 (H) post infection to observe respectively the oocyst and sporozoite in the salivary glands under a fluorescent microscope.

2.2.16 Generation of *Ubc13 kinase/PK9* conditional knockout parasites

A 532 bp product of 3'UTR of Ubc13 kinase/PK9 with flanking restriction sites HindIII and BglII was amplified (using primers Ubc13 kinase/PK9 IGS FP (FP2) and Ubc13 kinase/PK9 IGS RP (RP2)) and cloned into TA vector. The last 537 bp product of Ubc13 kinase/PK9 ORF was amplified (using primers Ubc13 kinase/PK9 CDS FP (FP1) and Ubc13 kinase/PK9 CDS RP (RP1)) cloned with flanking restriction sites BglII and HindIII in tandem to 3' UTR fragment. This construct was released from TA vector using restriction sites HindIII and NotI and cloned into p3'trap-hdhfr-flirte-puc18. The resulting vector was digested using HindIII/BglII and BglII/NotI which released both fragments selected for recombination (Fig 14F). This transfection vector was linearized with BglII and electroporated into FLP transgenic parasites that express FLP recombinase under oocyst specific promoter, TRAP (Surendra KK, Sinnis P and Kumar KA, personal communications). Upon successful double crossover, 3'UTR of TRAP and hDHFR cassette integrates between last 537bp of Ubc13 kinase/PK9 ORF and 3'UTR of Ubc13 kinase/PK9 (Fig 14C). The mice that harbored transfected parasites were kept under pyrimethamine drug pressure. Following drug selection and appearance of drug resistant parasites, genomic DNA was isolated from transgenic parasites and confirmed for correct site specific integration using primers designed beyond sites of recombination (Fig 14G). The *Ubc13 kinase/PK9* conditional KO parasites were subjected to limiting dilution to obtain a clonal population.

2.2.17 Conditional silencing of *Ubc13 kinase/PK9* in mosquito stages

Mouse was injected with *Ubc13 kinase/PK9* conditional knockout parasites intraperitoneally and parasitemia was monitored. When parasitemia reaches 2-3% blood was collected and passaged into 5-6 mice. Gametocyte positive mice were anesthesized and used for feeding of *A. stephensii* mosquitoes for two successive days. Mosquitoes were maintained at 22°C for 16 days, and then transferred and maintained at 25°C till 25th day. Maintenance of mosquitoes at 25°C facilitated the maximal FLP activation thus excising FRTed DNA fragment leading to silencing of the *Ubc13 kinase/PK9* gene expression.

2.2.18 Determination of pre-patent period for *Ubc13 kinase/PK9* conditional KO parasite infected in mouse either through intravenous route or by mosquito bite

On 25th day of post infection, salivary glands were dissected from mosquitoes infected with *Ubc13 kinase/PK9* conditional KO parasites and sporozoites were released by disruption of the salivary glands in RPMI medium. The suspension was a centrifuged at 800 rpm for 3 min at 4°C. The supernatant containing sporozoites was collected and counted using haemocytometer. Twenty thousand sporozoites were intravenously injected per mouse in a group of 3 C57BL/6 mice. For facilitating natural transmission of malaria to mouse, sporozoites were delivered through mosquito bite. To achieve successful transmission, group of 3 anesthesized C57BL/6 mice were placed on a 25 day old cage containing mosquitoes harboring *Ubc13 kinase/PK9* conditional KO parasites. The feeding was done for 21 min with periodic changes in the position of the mice for every 7 min. The mice infected by natural mosquito bite and by intravenous route were monitored for pre patent period by making smears from day 3 onwards and by visualizing parasites by Giemsa staining.

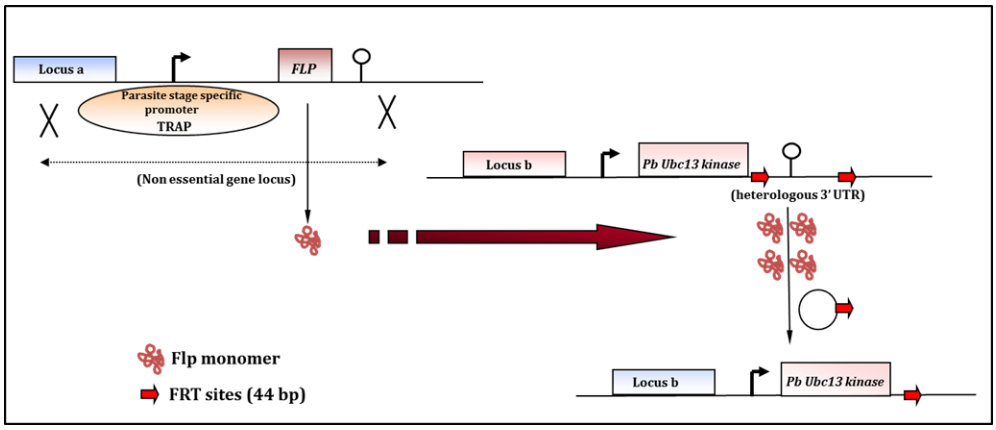

Fig 9.Generation of conditional *Ubc13 kinase /PK9* KO by using yeast FLP/FRT system: For generating a conditional KO line, a transgenic parasite that expresses FLP recombinase in the oocyst stages was utilized. The transgenic was engineered by integrating the FLP expressing ORF under the promoter of TRAP (active in oocyst) and by stably integrating this cassette in a non-essential gene locus. For conditional mutagenesis, a targeting vector p3'trap-hdhfr-flirte-puc18 was utilized. It contained MCS I and MCS II on either side of the trap-hdhfr cassette. The last 537bp of *Ubc13 kinase/PK9* fragment was cloned in MCS-1 and the 532bp fragment of the 3' UTR of *Ubc13 kinase/PK9* was cloned in MCSII. Following linearization of this targeting construct, and upon electroporation, a successful DCO recombination introduces the trap-hdhfr cassette immediately after the ORF of Ubc13 kinase. This integration ensured the stabilization of the gene in the blood stages through the hetrologous 3' UTR sequences. However, upon activation of FLP recombinase in the oocyst stages, the FLP recognizes two FRT sites are engineered in the targeting vector one preceding trap regulator sequence and other after the hdhfr cassette and excises the DNA sequence flanked by FRT. In the conditionally silenced stage, the target gene transcribes and makes an RNA, that lacks a functional 3' UTR resulting in failure to translate and make the protein.

2.2.19 Localization of Ubc13

For localization of Ubc13 or E2 we generated pBC-mcherry-hDHFR vector by replacing HSP70 5'UTR and GFP sequence with mcherry coding sequence in pBC-GFP-hDHFR vector. This modification ensured that pBC-mcherry-hDHFR vector lacked the 5' UTR regulatory sequence, that otherwise will serve as a promoter for mcherry expression (Fig 16B). *P. berghei ANKA* genomic DNA was used as a template to amplify last 522 bp of Ubc13 ORF (without stop codon) using oligonucleotide primers Ubc13 CDS FP (FP1) and Ubc13 CDS RP (RP1) and 506 bp of the 3' UTR region of Ubc13 ORF using Ubc13 IGS FP (FP2) and Ubc13 IGS RP (RP2) (primer sequences provided in table 1) (Fig 16D). After cloning of last 522 bp of Ubc13 ORF using ApaI and XhoI, the 3' UTR fragment was cloned into the resulting vector using NotI and AscI restriction sites and confirmed by restriction digestion. Plasmid was isolated and digested with ApaI and AscI and used as targeting construct for transfection into WT parasites (Fig 16E). *Ubc13* mcherry parasites were generated upon pyrimethamine drug selection. The integration was confimed using primers designed within the vector and beyond sites of recombination (Fig 16F). In successful transfectants, mcherry expression was driven by Ubc13 promoter. The *Ubc13* mcherry transgenic parasites showed red fluorescence in asexual blood stages and mosquito oocyst stage but not in salivary gland sporozoite stages when observed under fluorescence microscope (Nikon Eclipse NiE AR).

2.2.12 Total RNA isolation from schizonts

RNA was isolated from schizonts using Purelink™ RNA mini kit. (Ambion life technologies, Cat No. 121183018A). An overnight culture of asexual blood stages obtained from WT or *Ubc13 kinase/PK9* KO infected mouse was set up. The following day, schizonts were purified using 60% nycodenz gradient centrifugation. Schizonts were washed with PBS followed by resuspension in 300 µL lysis buffer containing 1% β-mercaptoethanol. Insulin syringe was used to lyse schizonts by passing through the needle multiple times. Equal volume of 70% ethanol prepared in RNAse free water was added in 1:1 dilution. After vigorous vortexing, the solution was loaded onto the column provided in the kit and centrifuged at 11,000 rpm for 30 sec. The column was washed with 700 µL PW buffer. The flow through was discarded and the column was washed twice with 500 µL of wash buffer. After discarding the flow through, the column was centrifuged for 1-2 min to remove residual ethanol. Thirty micro liters of nuclease free water was added to the column and incubated at room temp for 1

min and centrifuged for 2 min to elute RNA. Following quantification, 2-4 µg of RNA was treated with DNase I and used for cDNA synthesis using reverse transcriptase. Both WT and *Ubc13 kinase/PK9* KO cDNA was used as a template for semi-quantitative PCR using *Pb*GAPDH primers to check the quality of RNA before sending it for RNA Seq (sequencing) analysis.

2.2.13 Library construction and sequencing

Library preparation was performed at Genotypic Technology's Genomics facility following SureSelect Strand-Specific RNA Library Prep for Illumina Multiplexed Sequencing mRNA Library Preparation Protocol (Cat No. 5500-0116). Briefly, the purified mRNA was fragmented for 4 minutes at elevated temperature (94°C) in the presence of divalent cations followed by the first strand cDNA is synthesis. During the first strand synthesis, strand specificity was maintained by the addition of actinomycin D. Resulting single stranded cDNA was cleaned up using AMPure beads (Agencourt, Cat No-14547000) and second strand cDNA was synthesized and cleaned up using AMPure (Agencourt, Cat No-14547000). Further, cDNA was blunt ended through an end repair reaction using large Klenow fragment, T4 polynucleotide kinase and T4 polymerase provided in the kit. A single 3' adenosine moiety was added to the cDNA using Klenowexo and dATP. The Illumina indexed adapters, containing primer sites for flow cell surface annealing, amplification and sequencing, were ligated onto the repaired ends of the cDNA. The end repaired and adapter ligated cDNA was subsequently amplified by 12 cycles of PCR using the PCR components provided in the kit. Quantification and size distribution of the prepared libraries was performed using Qubitflourometer and Agilent High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturers' instructions. The libraries were sequenced on the Illumina Nextseq500 platform with 150 cycles paired end sequencing chemistry.

2.2.14 Data Analysis

The Illumina NextSeq500 paired end raw reads were quality checked using FastQC, followed by processing using in-house script for adapters and low quality bases trimming towards 3'-end and processed reads were again quality checked. For further analysis Tophat and Cufflinks tools were used. TopHat program was used to align RNA-Seq reads using Bowtie2 to *P. berghei* genome in order to identify exon-exon splice junctions. Alignment was followed by assembly and abundance estimation of transcripts using Cufflinks. Cufflinks

includes a script called Cuffmerge that was used to merge together several Cufflinks assemblies. It also runs Cuffcompare , and automatically filters a number of transfrags that are probably artifacts. Reference GTF file was provided to Cuffmerge in order to gracefully merge novel isoforms and known isoforms and maximize overall assembly quality. The main purpose of this script is to make it easier to make an assembly GTF file suitable for use with Cuffdiff. After merging assembly, significant changes in transcript expression were calculated using Cuffdiff. Merged GTF file produced by Cuffmerge was used as input in Cuffdiff. Finally differentially expressed transcripts were annotated using Ensembl Biomart files (Reference organism: *P. berghei*).

2.2.15 Preparation of WT and *Ubc13 kinase/PK9* KO lysate and western blot with ubiquitin antibody

Parasites lysates were obtained from WT and *Ubc13 kinase/PK9* KO schizonts stages that were synchronized on a 60% nycodenz gradient. The schizonts were lysed with 0.05% saponin in PBS followed a 3 washes in cold PBS. The parasite pellet was resuspended in fresh PBS and denatured in 2X Laemmli sample buffer (120 mM Tris-HCl (pH-6.8), 20% glycerol, 4% SDS and 0.02% (w/v) bromophenol blue). The protein lysates were loaded onto 10% and 15% SDS-PAGE and separated at 100v and electro transferred on to nitrocellulose (NC) membrane at 25v for 16-18 h [204]. After blocking with 5% nonfat milk powder in a solution of Tris buffered saline (TBS, pH 7.2), the membranes were probed with (1) mouse ubiquitin antibody (Santa Cruz Biotechnology, Cat No.sc-8017), (2) mouse Ubc13/E2 antibody (abcam, Cat No. ab38795) and (3) mouse actin antibody (abcam, Cat No. ab3280) used as loading control. After primary antibody treatment, the blot was washed three times for 15min each with TBS, TBST (0.1% Tween in TBS) and TBS respectively followed by incubation for 1h with anti- mouse secondary antibody conjugated with horse radish peroxidase. The blot was again washed for 20min each with TBS, TBST and TBS respectively. Protein bands were detected using AmershamTM ECLTM prime western blotting detection kit (GE healthcare, Cat No. RPN2232). Images were captured in Bio-rad versadoc and analyzed by image lab software.

2.3 Results

2.3.1 Ubc13 kinase/PK9 and Ubc13 are conserved among *Plasmodium* species

Alignment of protein sequences of *P. falciparum* and *P. berghei* Ubc13 kinase/PK9 showed 86% homology. Likewise, Ubc13 protein sequences of *P. falciparum* and *P. berghei* showed 92% homology. (Fig 10)

2.3.2 Homologous recombination using 5' and 3' fragments of *Ubc13 kinase/PK9* results in replacement of the target gene with a GFP-hDHFR cassette

Successful replacement of Ubc13 kinase/PK9 ORF was obtained following a double homologous recombination using 5' UTR and 3' UTR fragments of the *Ubc13 kinase/PK9*. The strategy for gene replacement is shown in Fig 11. Two homologous regions were shown in the *Ubc13 kinase/PK9* knockout plasmid in Fig 11B. Replacement of *Ubc13 kinase/PK9* gene locus with GFP-DHFR cassette is shown in Fig 11C. Primers used for amplification of 5'UTR, 3'UTR and site specific integration PCR are indicated in Fig 11. Double digestion of *Ubc13 kinase/PK9* KO plasmid with XhoI/ClaI and NotI/AscI released 5' and 3' UTRs respectively shown in Fig 11E. Digestion of *Ubc13 kinase/PK9* knockout plasmid with XhoI and AscI released the vector backbone and GFP-DHFR cassette (Fig 11F) bearing two homologous DNA fragments on either side of the cassette, as shown in Fig 11C. Integration PCR with indicated primers designed at sites beyond the recombination event confirmed the 5' and 3' UTR integration (Fig 11G). It also confirmed that *Ubc13 kinase/PK9* gene locus was replaced with GFP-DHFR cassette in *Ubc13 kinase/PK9* knockout parasites. After limiting dilution genomic DNA was isolated from *Ubc13 kinase/PK9* KO clonal population and PCR with gene specific primers confirmed the deletion of Ubc13 kinase/PK9 ORF (Fig 11H). *Ubc13 kinase/PK9* KO parasites express GFP because of the integration of GFP cassette. (Fig 11I)

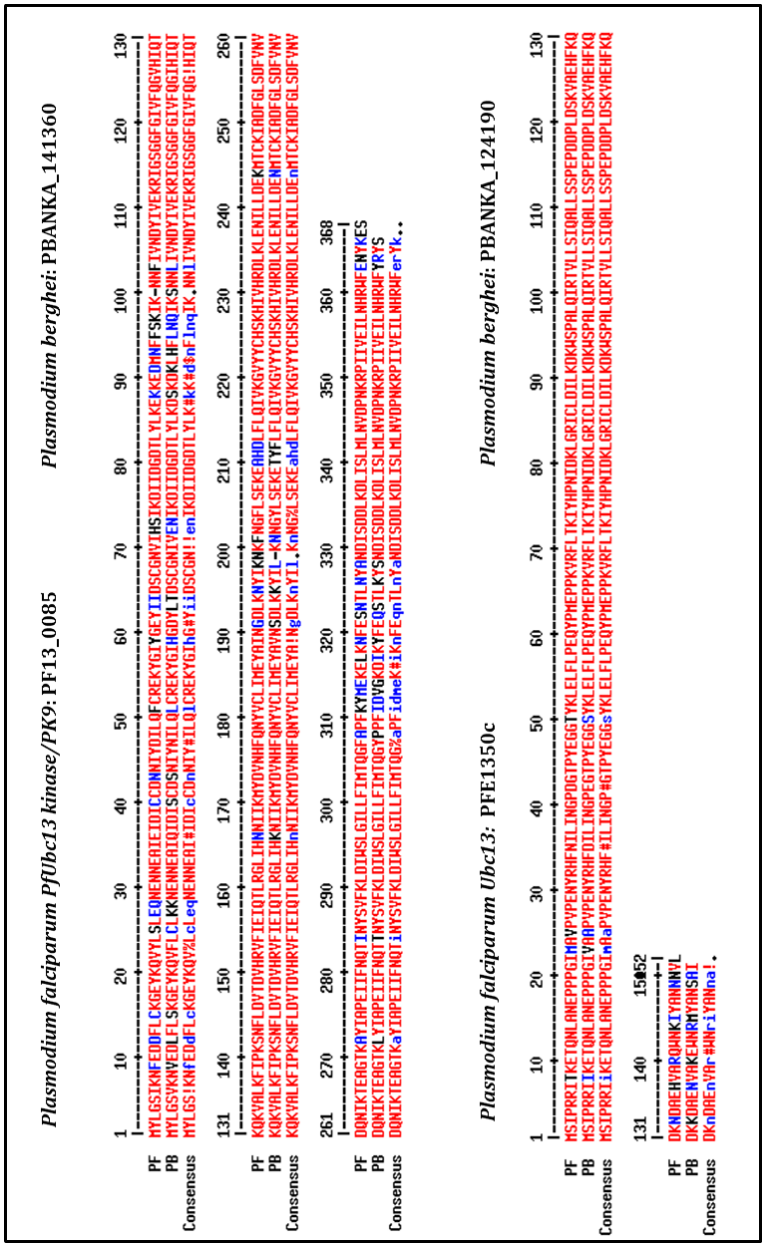

Fig. 10.Amino acid sequence alignment of *Plasmodium berghei* and *Plasmodium falciparum* Ubc13 kinase/PK9 and Ubc13 (E2).

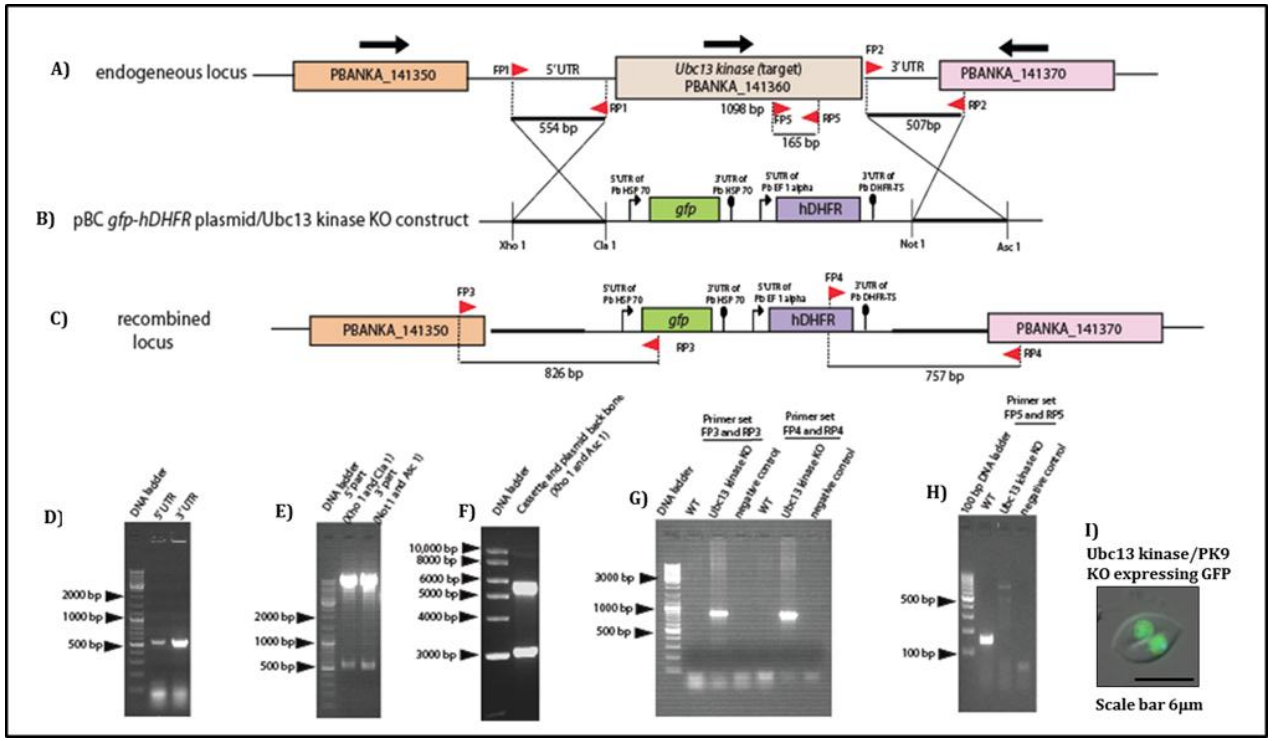

Fig 11. Generation of *Ubc13 kinase/PK9* KO parasite line. A) Genomic locus of *Ubc13 kinase/PK9* (PBANKA_141360) showing 5' and 3' UTR fragments. B) Elements of the targeting vector- pBC-GFP-hDHFR. A 554bp 5' fragment of *Ubc13 kinase/PK9* was cloned in XhoI/ClaI site of the transfection vector. A 507bp 3' fragment was cloned into NotI/AscI site of the targeting vector. C) Recombined locus following successful double crossover resulting in replacement of target gene, *Ubc13 kinase/PK9* by GFP-DHFR cassette. D) A 1% agarose gel showing the PCR product of 5' and 3' fragments. The 5' fragment was amplified with primers Ubc13 kinase 5' FP (FP1) and Ubc13 kinase /PK9 5' RP (RP1) and 3' fragment was amplified with Ubc13 kinase /PK9 3' FP (FP2) and Ubc13 kinase /PK9 3' RP (RP2). E) Release of 5' fragment from transfection vector using restriction enzymes XhoI/ClaI and release of 3' fragment from targeting vector using restriction enzymes NotI/AscI. F) Release of targeting cassette (5'fragment+GFP-DHFR cassette+3' fragment) and vector backbone using restriction enzyme XhoI/AscI. G) Diagnostic PCR using primers within the targeting cassette and beyond sites of recombination reveals correct site specific integration. A PCR product with primers Ubc13 kinase/PK9 5' confirmation FP (FP3) and HSP70 5' UTR RP (RP3) indicate a correct 5' integration and a PCR product with primers DHFR FP (FP4) and Ubc13 kinase/PK9 3' confirmation RP (RP4) indicate a correct 3' integration. H) Genomic DNA isolated from cloned *Ubc13 kinase/PK9* KO parasites does not amplify a PCR product from within Ubc13 kinase /PK9 ORF whereas WT parasites amplify a product of 165bp with primer set FP5 and RP5. I) A merged DIC image showing GFP expressing *Ubc13 kinase/PK9* KO parasite inside RBC.

2.3.3 *Ubc13 kinase/PK9* KO does not exhibit any defect in asexual blood stage propagation

Identical numbers of both wild type and *Ubc13 kinase/PK9* knockout parasites were intravenously injected. Parasitemia was monitored daily for one week post infection. All asexual forms like rings, trophozoites, schizonts and gametocytes were readily observed in *Ubc13 kinase/PK9* KO in similar numbers as compared to WT parasites. This experiment suggested that depletion of *Ubc13 kinase/PK9* did not affect the asexual replication of the parasites (Fig 12).

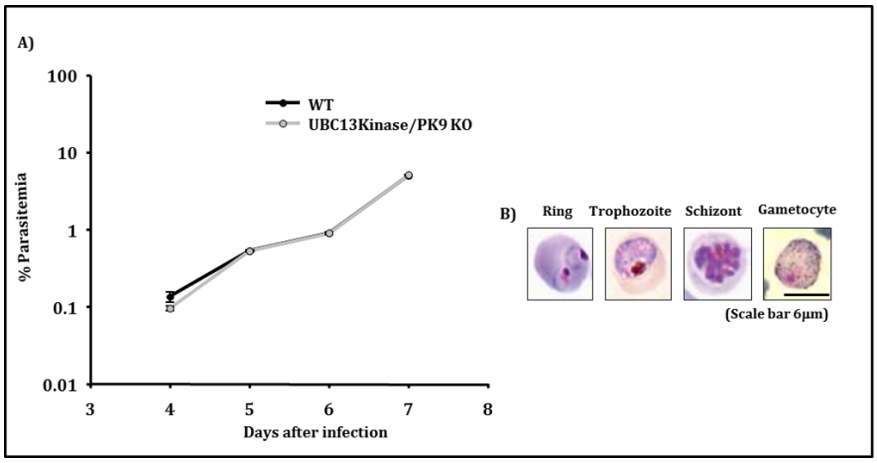

Fig 12. *Ubc13 kinase/PK9* KO asexual parasites propagate identically as WT parasites. A) $1X10^3$ infected RBC of either WT or *Ubc13 kinase/PK9* KO were intravenously injected in two groups of mouse (3 mouse/group) and monitored for propagation of the parasites daily for 7 days by making Giemsa stained smears. B) Representative pictures showing asexual blood stages obtained from Rathore et al., [205].

2.3.4 Production of ookinetes and oocysts is impaired in *Ubc13 kinase/PK9* KO infected mosquitoes

Ubc13 kinase/PK9 knockout parasites were transmitted to mosquitoes and compared with GFP expressing WT parasites to unravel the role of Ubc13 kinase/PK9 in mosquito stages. Ookinete development was analyzed in *Ubc13 kinase/PK9* KO infected mosquitoes by dissecting mosquitoes at 20 h post blood meal. When the blood meal was observed under microscope, no ookinetes were detected in *Ubc13 kinase/PK9* KO parasites, whereas ookinetes were readily observed in blood meal containing WT parasites. These observations were confirmed in three independent mosquito feeding experiments, Exp1, Exp2 and Exp3 (Fig 13A and B). WT ookinetes observed were positive for p28 staining (Fig 13C). Concurrent with the absence of the ookinetes, D14 infected mosquito midguts showed complete absence

of oocysts (Fig 13E) whereas appreciable number of oocysts (Fig 13D) were observed on midguts of mosquitoes that received blood meal containing WT parasites. In three independent experiments where midgut infectivity was evaluated, mosquitoes infected with WT parasites showed infectivity that ranged from 80-92% where as mosquitoes infected with *Ubc13 kinase/PK9* KO showed 0% infectivity. The percentage of infectivity in these experiments was derived from total number of mosquitoes that took blood meal versus the number of mosquitoes that were positive for oocyst. Since the formation of oocysts varies on several parameters like density of the circulating gametocytes in the blood at the time of blood meal, the number of gametocytes taken during blood meal, the rate of formation of gametes and its fertilization etc., infectivity to a mosquito was assigned qualitatively, i.e., based on the presence or absence oocysts rather than on the actual number oocysts (Table 2 in Fig 13).

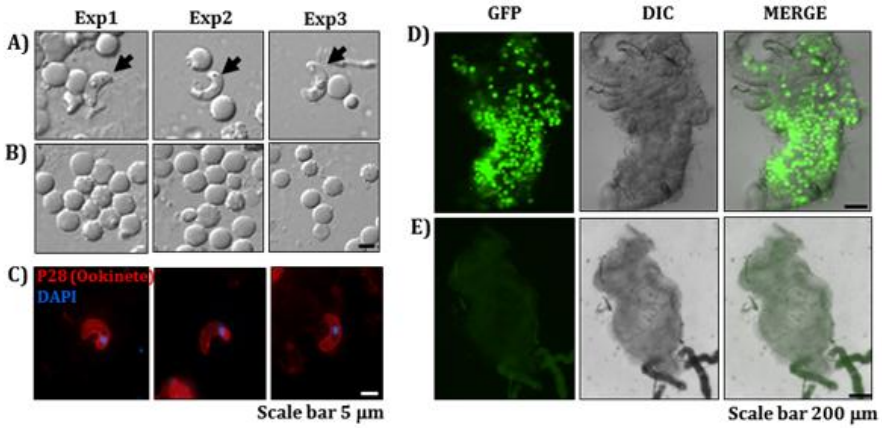

Table 2: Transmission dynamics of Wild Type and *Ubc13 kinase/PK9* KO parasite in *Anopheles stephansii* mosquitoes

Parasite	Experimental group	Number of mosquitoes /group	Number of mosquitoes positive for midgut infection	Percentage of infected mosquitoes*
Wild Type	Group 1	25	21	84%
	Group 2	25	23	92%
	Group 3	25	20	80%
Ubc13 kinase/ PK9 KO	Group 1	25	0	0%
	Group 2	25	0	0%
	Group 3	25	0	0%

*Percentage of infected mosquitoes in each group were scored only on the presence of oocyst but not on actual numbers (burden)

Fig 13. *Ubc13 kinase/PK9* KO do not produce ookinetes and oocyst in mosquitoes: Female *Anopheles* mosquitoes that received blood meal from mouse infected with WT or *Ubc13 kinase/PK9* KO were analyzed for the presence of ookinete at 20 h. Analysis of the blood meal from three independent experiments revealed that *Ubc13 kinase/PK9* KO failed to produce ookintes (B) where as okinetes were readily observed in WT parasite line (A). Ookinetes were detected only in WT (C) but not in *Ubc13 kinase/PK9* KO after performing Immuno Fluorescence Assay (IFA) using antibody against ookinete surface protein p28 and revealed with secondary antibody (anti-rabbit) conjugated to Alexa fluor 594. On D14 post infection oocysts were detected only in WT (D) where as no oocyst were observed in *Ubc13 kinase/PK9* KO parasites (E). Pooled dissected miduts from mosquitoes infected with WT (F) or *Ubc13 kinase/PK9* KO (G). Table 2 indicating the mosquito midgut infectivity in three independent experiments. The mosquitoes infected with WT parasites showed 80%-92% midgut infectivity where as mosquitoes infected with *Ubc13 kinase/PK9 KO* showed 0% infectivity.

2.3.5 *Ubc13 kinase/PK9* is not essential for formation of sporozoites, invasion to hepatocytes and completion of EEF development

Ubc13 kinase/PK9 conditional knockout parasites were transmitted to female *A. stephansii* mosquitoes. Mosquito cages were kept at 25°C and RH 75% for 10 days from D16 post infection (Fig 15A), to achieve maximal activation of FLP. The FLP recombinase activity resulted in the excision of 3' regulatory sequence in both sporulating oocyst and stages beyond oocyst, thus facilitating the study of these stages, under conditions of conditional depletion of *Ubc13 kinase/PK9*. The conditional *Ubc13 kinase/PK9* KO sporozoites when introduced into C57BL/6 mouse, either by natural mosquito bite or by intravenous injection, gave rise to blood stage infection on D4 post infection (Fig 15B, C and D). The blood stages showed both asexual forms as well as gametocytes. Upon obtaining optimal parasitemia, genomic DNA was isolated from the conditional *Ubc13 kinase/PK9* KO blood stages derived from both bite and intravenous injection of sporozoites. Diagnostic PCR was performed by using a set of primers that amplified 3879 bp product that revealed successful excision (Fig 15E). Sequencing of the PCR product confirmed the excision of FRTed sequence (by looping out) and an expected single FRT site in tandem to plasmid backbone (Fig 15F). These results suggested a non-essential role of Ubc13 kinase/PK9 in: 1) formation of salivary glands sporozoites, 2) commitment of sporozoites to hepatocytes and 3) Completion of EEF development.

2.3.6 Ubc13 promoter is active in asexual blood stages and oocyst stages but not in salivary gland sporozoite stages

The *Ubc13* mCherry transgenic parasites were asexually propagated in C57BL/6 mouse. When observed under fluorescent microscope, all asexual blood stages revealed expression of mCherry (Fig 17A). The *Ubc13* mCherry transgenics were transmitted to mosquito to analyze the expression of Ubc13 in mosquito stages. D14 infected midguts showed expression of mCherry (Fig 17B). However, no mCherry epression was observed in the salivary gland sporozoite stages (Fig 17C). We conclude that Ubc13 promoter is active in asexual and oocyst stages.

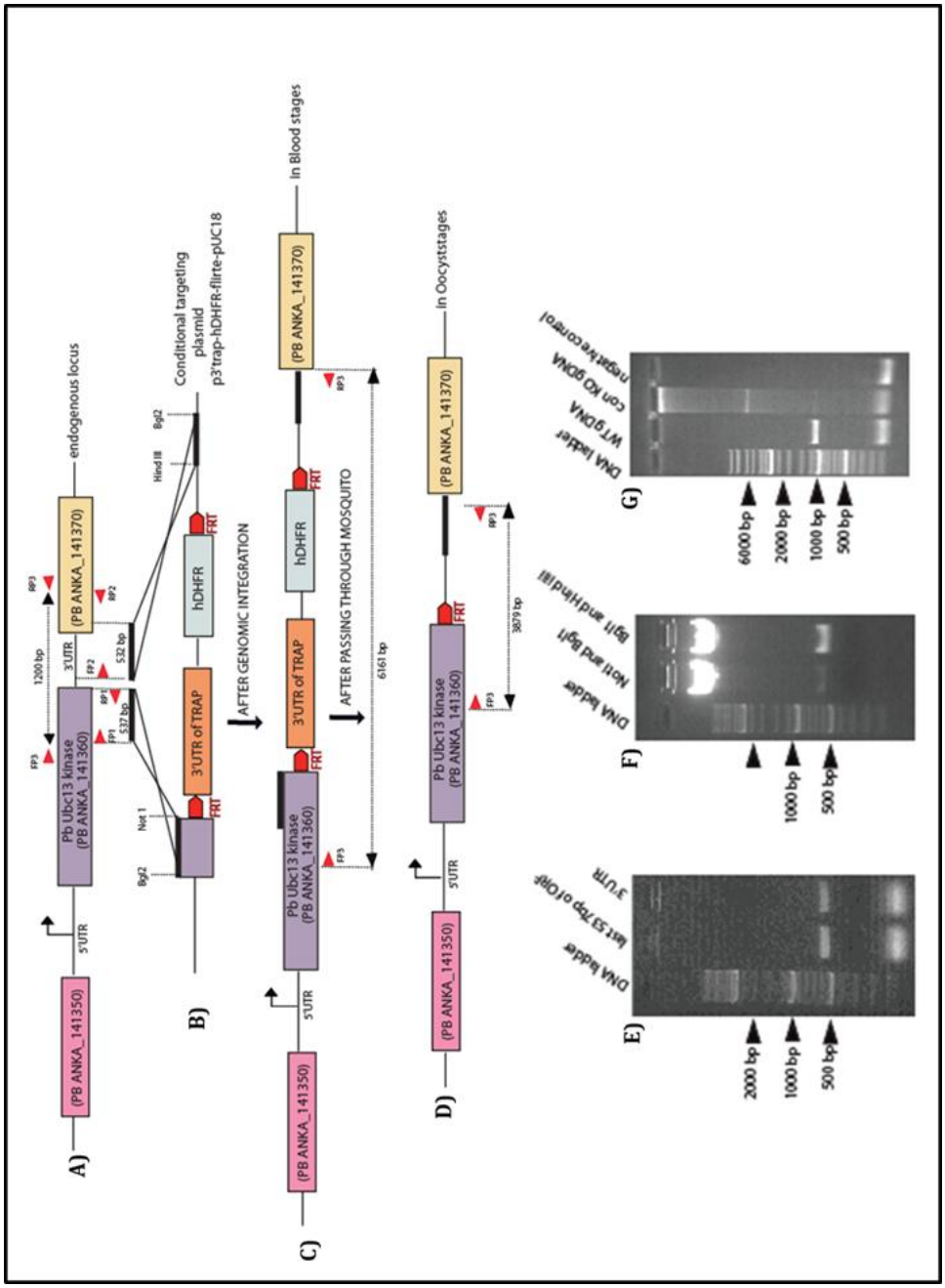

Fig 14.Generation of conditional KO of *Ubc13 kinase/PK9*. A) Genomic locus of *Ubc'13 kinase/PK9* (PBANKA_141360) showing *Ubc13 Kinase/PK9* ORF, 5' and 3' UTRs. B) Elements of the targeting vector showing 3' regulatory sequence of TRAP and hDHFR cassette flanked by two FRT sites. On either ends of the FRT, the last 537bp of *Ubc13 kinase/PK9* ORF and a 532bp of 3' UTR of *Ubc13 kinase/PK9* were cloned. C) Recombined locus after successful double homologous recombination resulting in integration of a heterologous TRAP 3' UTR and hDHFR cassette after *Ubc13 kinase/PK9* ORF. In blood stages, the *Ubc13 kinase/PK9* expression is regulated by heterologous 3' UTR of TRAP. D) After excision in the mosquito oocyst stages, where FLP recombinase is expressed, excision of the FRTed DNA sequence occurs resulting in conditional silencing. E) A 1% agarose gel showing the PCR product of last 537bp of *Ubc13 kinase/PK9* ORF using primers FP1 and RP1 and 532bp 3' UTR of *Ubc13 kinase/PK9* using primers FP2 and RP2 is shown. F) Release of last 537bp of *Ubc13 kinase/PK9* ORF and 532bp 3' UTR of *Ubc13 kinase/PK9* from the targeting vector. G) Diagnostic PCR revealing a successful site specific integration that amplifies a product of 6.1kb in *Ubc13 kinase/PK9* conditional KO and 1.2kb in WT

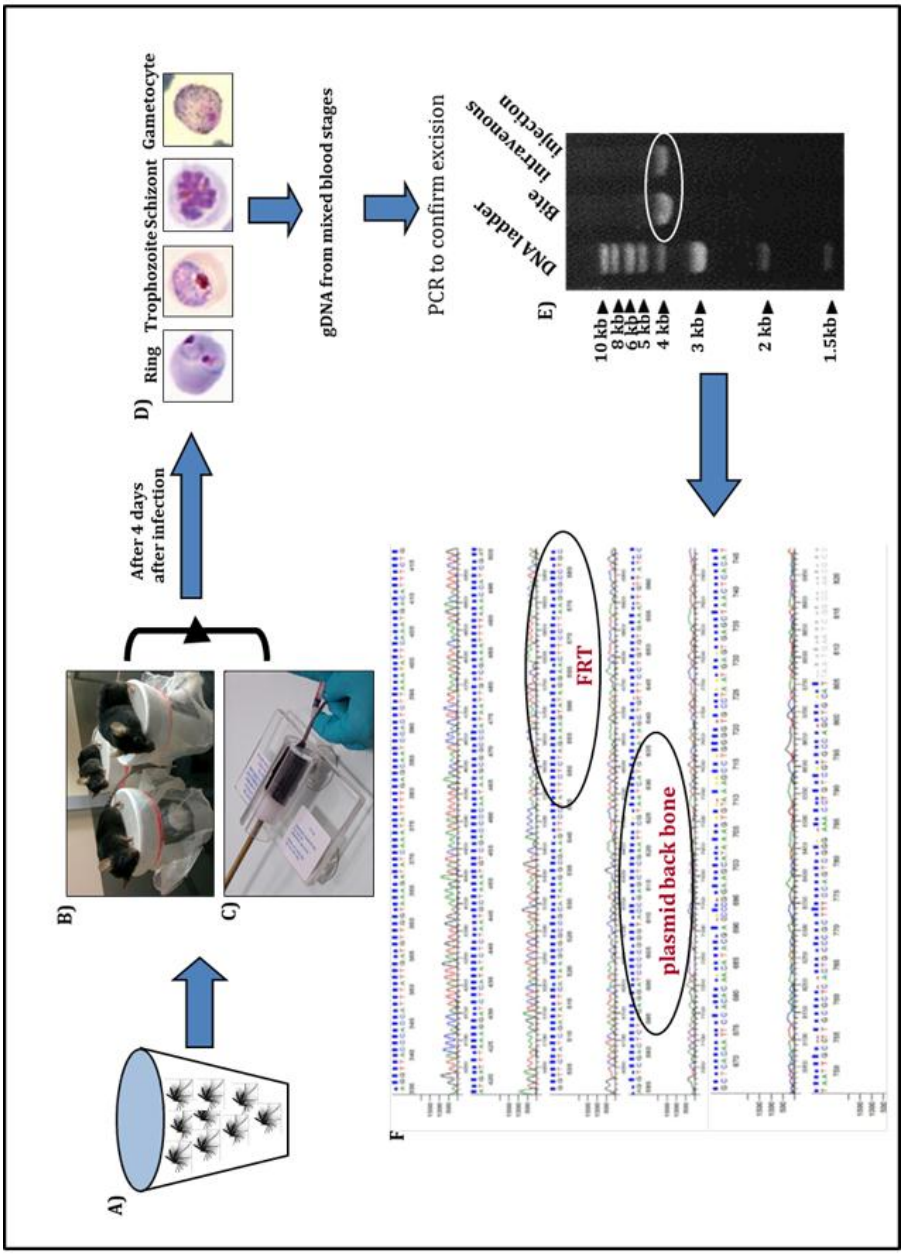

Fig 15. *Ubc13 kinase/PK9* is not required for sporulation of oocyst, for infection of salivary glands, or for invasion of sporozoites to hepatocytes and EEF development. A) Activation of TRAP FLP recombinase by placing infected mosquitoes for 10 days at 25°C and RH75%. B) Inoculation of sporozoites through natural mosquito bite and C) by intravenous injection. D) Monitoring the prepatent period from mice infected through bite and intravenous injection by Giemsa staining. Representative asexual stage figures are taken from [Rathore et al]. E) Genomic DNA PCR revealing the excision of heterologous 3' UTR of *Ubc13 kinase* and hDHFR cassette using primer FP3 and RP3. E) Sequencing of the PCR product that confirmed the excision of heterologous 3' UTR of *Ubc13 kinase* and hDHFR cassette. The sequence showed one FRT site retained after excision of the FRTed DNA following activation of FLP, which brings the plasmid back bone sequence in tandem to FRT site.

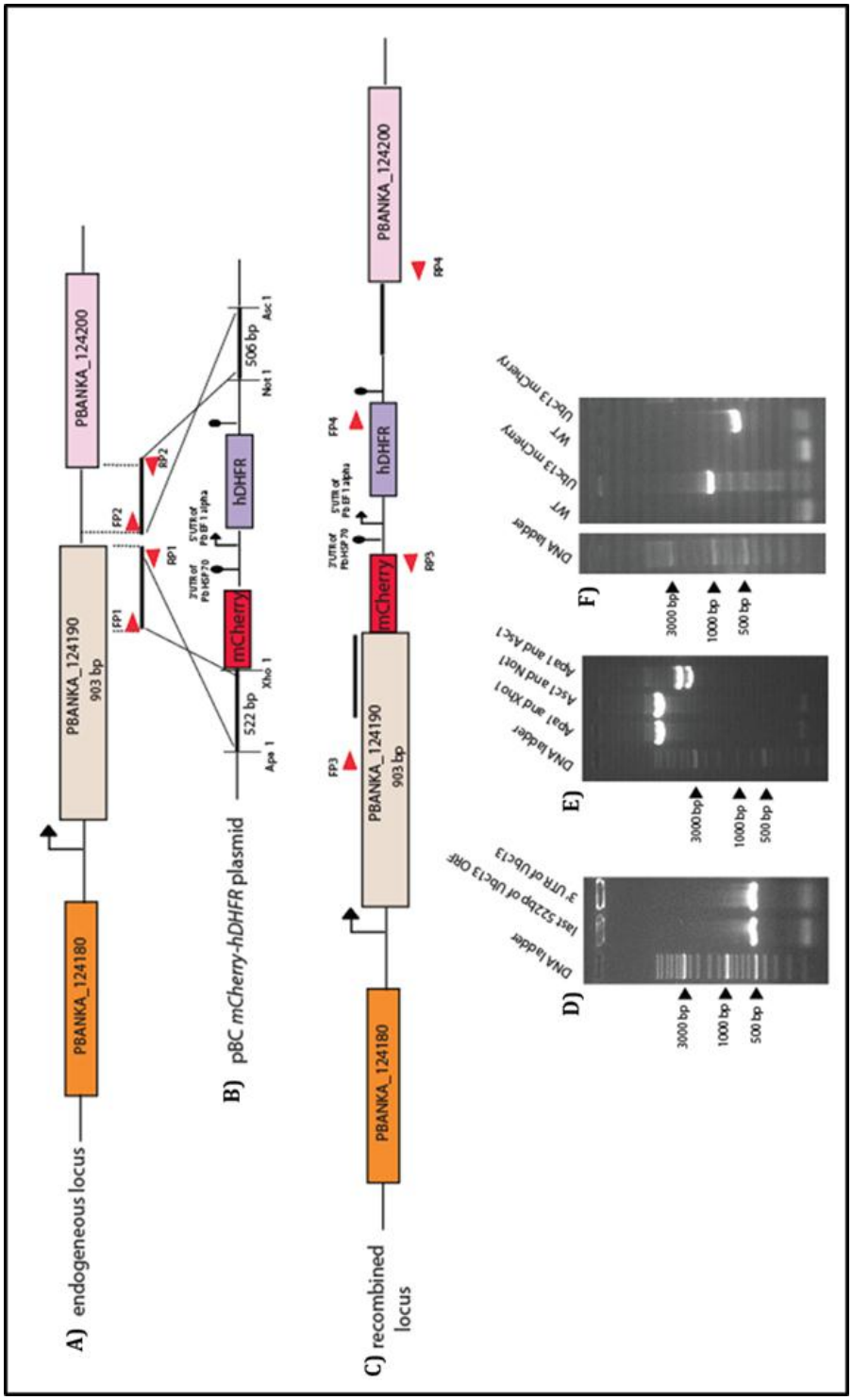

Fig 16. Generation of *Ubc13* mcherry transgenic. A) Genomic organisation of Ubc13 (PBANKA_124190) showing 5' and 3'UTR B) Elements of the targeting vector showing pBC-mcherry-hDHFR. A 522 bp of last 522 bp of *Ubc13* was cloned in ApaI/XhoI site of the targeting vector. A 509 bp of 3' UTR fragment was cloned into NotI/AscI site of the targeting vector. C) Recombined locus following successful double crossover resulting in fusion of Ubc13 ORF in tandem with mcherry ORF. D) A 1% agarose gel showing the PCR product of last 522 bp obtained using primer FP1 and RP1 and 3' UTR of Ubc13 obtained using primers FP2 and RP2. E) Release of last 522 bp of *Ubc13* from targeting vector using restriction enzymes ApaI/XhoI and release of 3' UTR from targeting vector using restriction enzymes NotI/AscI. Release of targeting cassette (CDS+mcherry-DHFR cassette+3' fragment) and vector backbone using restriction enzymes ApaI/AscI. F) Diagnostic PCR using primers within the targeting cassette and beyond sites of recombination revealing correct site specific integration. A PCR product with primers Ubc13 5' confirmation FP (FP3) and mcherry RP (RP3) indicate correct 5' integration and a PCR product with primers DHFR FP (FP4) and Ubc13 3' confirmation RP (RP4) indicate a correct 3' integration.

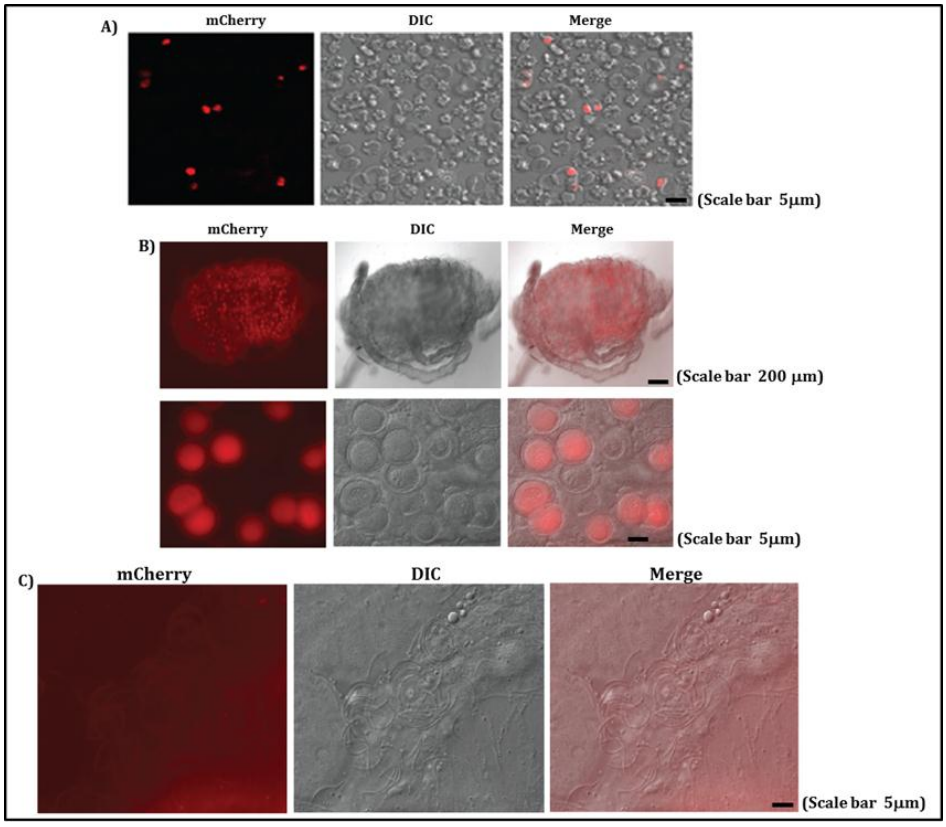

Fig. 17.*P. berghei* Ubc13/E2 is expressed in asexual and oocyst stages. mCherry transgenic of Ubc13/E2 express mCherry in the asexual stages (A), oocyst stages (B and C) but not in salivary gland sporozoite stages (D).

2.3.7 RNA Sequencing revealed global changes in the transcriptome of *Ubc13 kinase/PK9* KO parasites.

To understand the regulation of gene expression that led to complete block of sexual stage development in *Ubc13 kinase/PK9* KO, transcriptomic analysis was performed by RNA sequencing, from synchronized schizonts stages (Fig 18). A total of 8502 transcrips were sequenced and comparison of gene expression between WT and *Ubc13 kinase/PK9* KO revealed 1733 transcripts down regulated and 673 transcripts upregulated. Of the 1733 downregulated transcripts, 521 were significant (Table 3). Out of 673 upregulated transcripts, 150 transcripts were significant (Table 4). The important functional clusters that were down regulated were central to sexual reproduction, kinases other than those involved in sexual reproduction, DNA repair and ubiquitination pathway. The major functional clusters that were upregulated included genes involved in virulence (bir genes) and other transcripts encoding proteins of unknown function. The sexual stage transcripts down regulated ranged from 1-10 fold. These included transcripts that encoded for zygote and ookinete surface

proteins (CPW-WBC family, SOAP and PSOPs), kinases (NEK-2, NEK-4, MAP-2 and CDPK3), transcription factors (AP2-G and AP2-O), cytoskeletal regulatory elements of male gamete motility (MISFIT, actin II, flagellar outer arm dynein associated protein and PF16), gamete specific genes (p48, p47,p230 and MDV-1) and meiotic recombination (DMC-1) (Fig 19).

The other down regulated genes that encoded for kinases (not related to sexual reproduction) were ARK-3, choline kinase, NEK-3, ARK-1, ABC-1, nucleotide diphosphate kinase and calcium/calmodulin dependent protein kinase (Fig 20A). The category of transcripts involved in DNA repair were RAD51, rhp16 and MSH-2 (Fig 20B). The transcripts encoding for ubiquitination pathway were Ubc12, E3, ubiquitin conjugating enzyme, ubiquitin like protein and RPN13 (26S proteasome regulatory subunit) (Fig 20C).

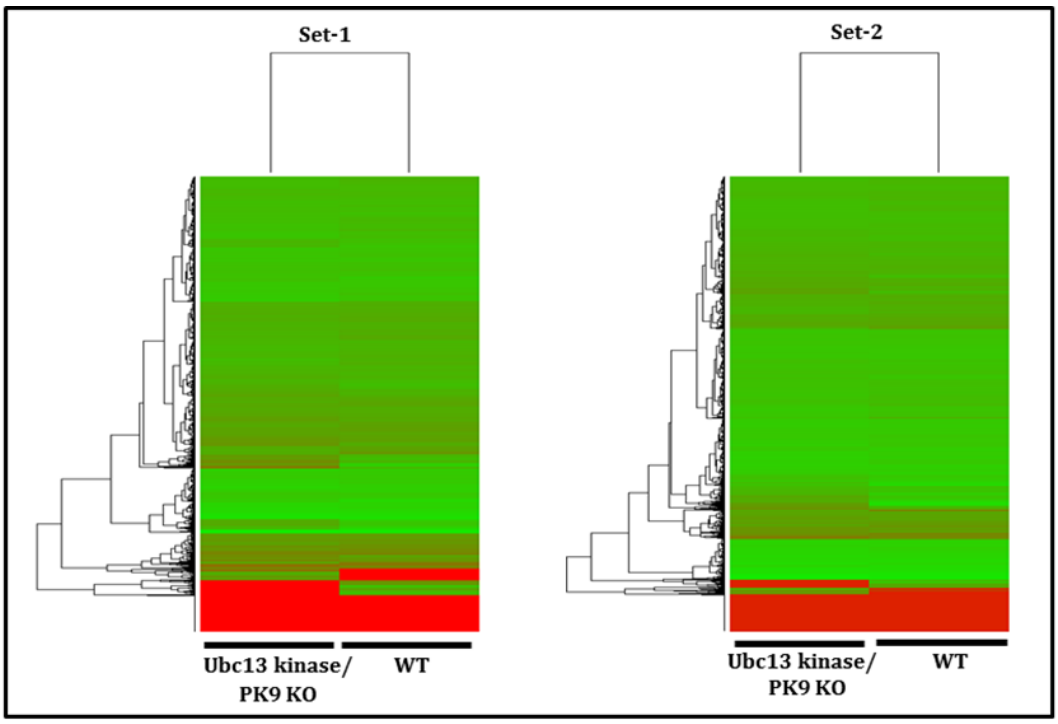

Fig 18. Heat maps revealing global changes in the gene expression of WT versus *Ubc13 kinase/PK9* KO as revealed by RNA Seq analysis. Set 1 and Set 2 indicate two independent biological replicates.

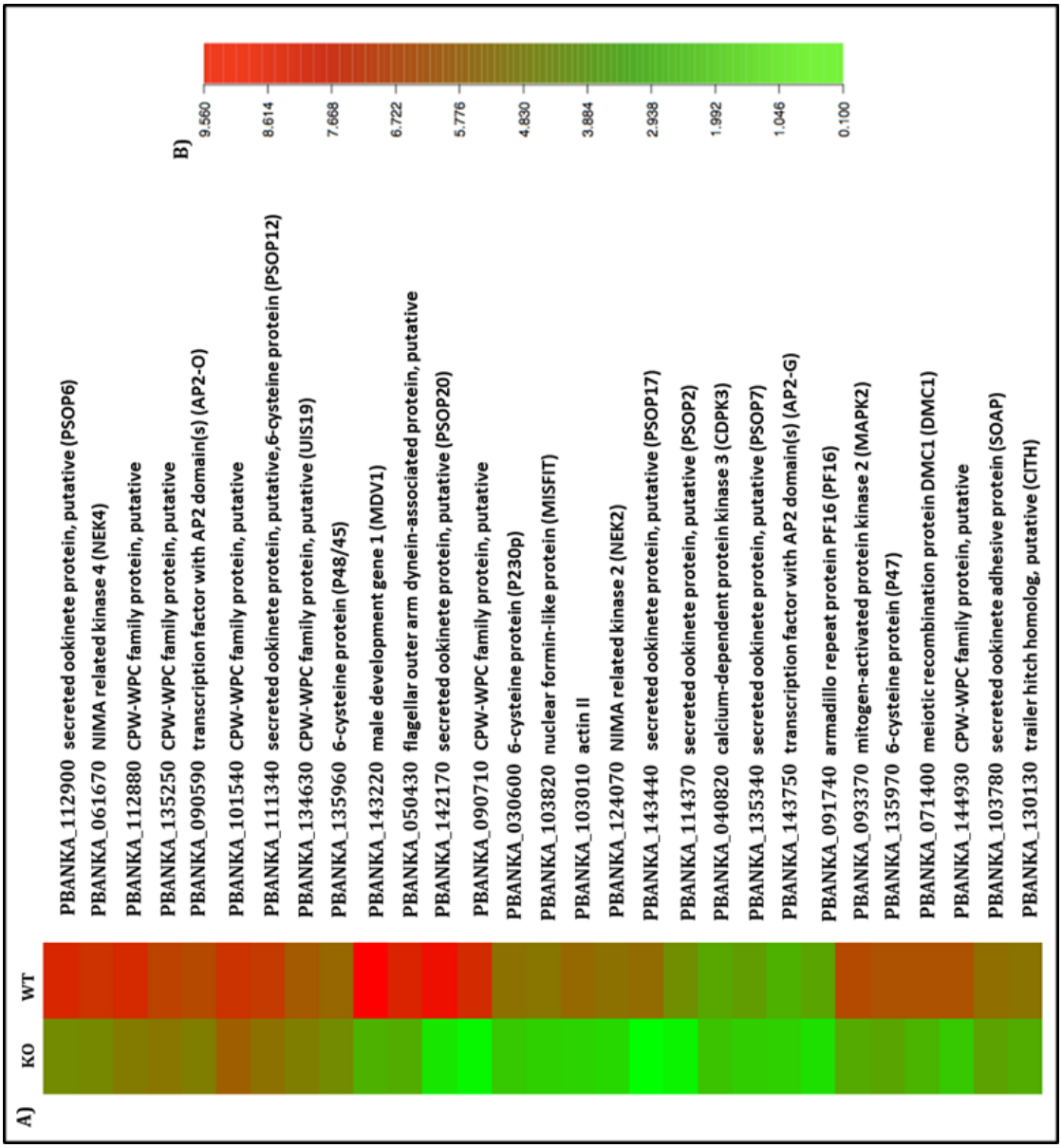

Fig 19. Sexual stage transcripts down regulated in *Ubc13 kinase /PK9* KO. A) List of down regulated sexual stage transcripts indicated with regard to their gene identity and probable functions. B) Scale for assigning fold change values in heat man.

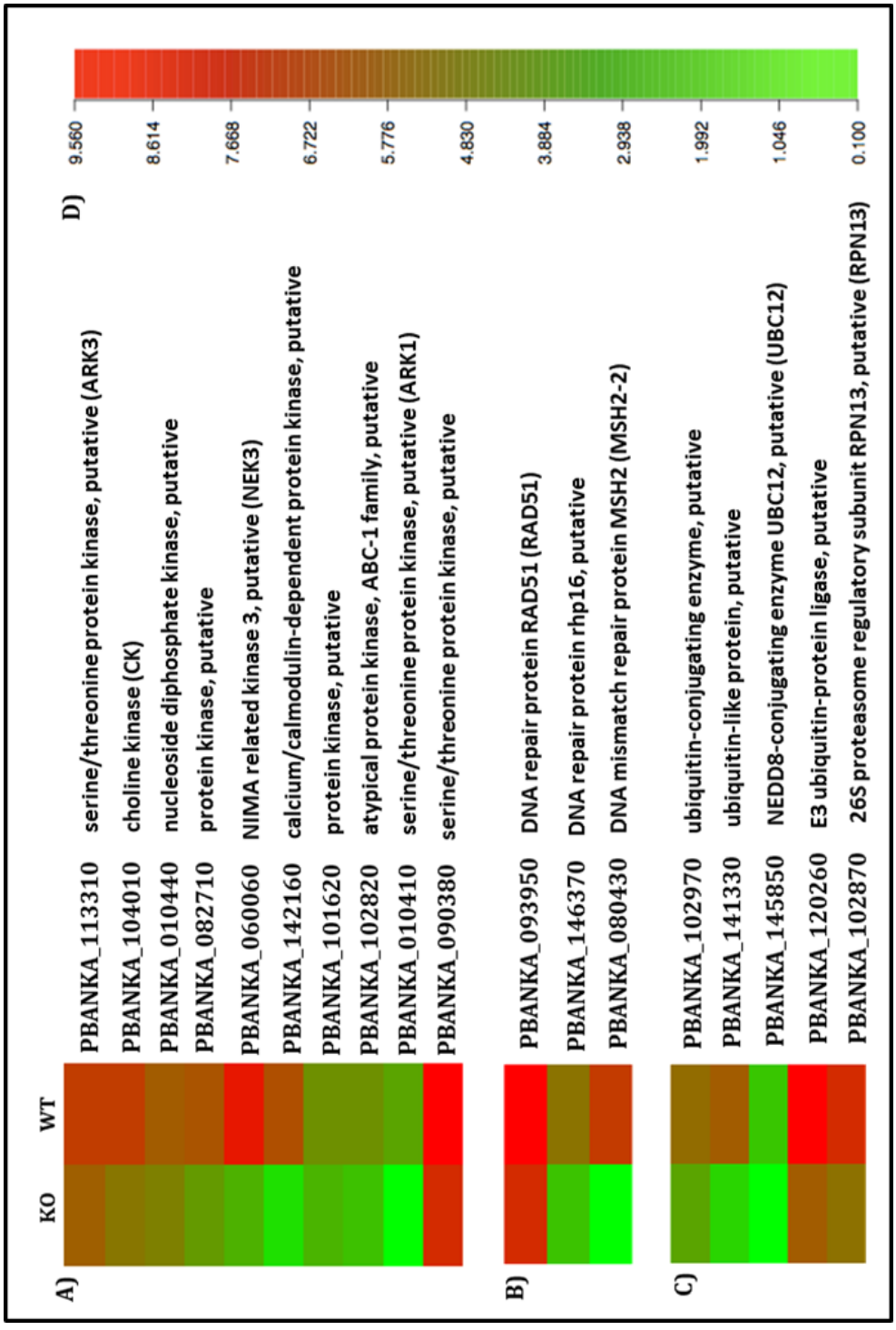

Fig 20. Functional clusters of other down regulated transcripts in *Ubc13 kinase /PK9* KO. A) Downregulated kinases other than those involved in sexual reproduction, B) transcripts involved in DNA repair, C) transcripts involved in ubiquitin pathway. Each of these clusters are indicated with regard to their gene identity and probable functions. D) Scale for assigning fold change values in heat map.

Table 3 showing the list of downregulated transcripts in *Ubc13 kinase/PK9* KO schizonts

Down regulated genes in *Plasmodium berghei Ubc13 kinase/PK9* KO		
S.No	**GeneID**	**Function**
8-10fold		
1	PBANKA_131870	protein phosphatase, putative ppm 3
2	PBANKA_070480	conserved *Plasmodium* protein, unknown function
3	PBANKA_070710	membrane skeletal protein, putative
4	PBANKA_070490	conserved *Plasmodium* protein, unknown function
6-8 fold		
5	PBANKA_134940	conserved *Plasmodium* protein, unknown function
6	**PBANKA_143220**	**male development gene 1 (MDV1) (Ref 1)***
7	PBANKA_140090	conserved *Plasmodium* protein, unknown function
8	PBANKA_133480	conserved *Plasmodium* protein, unknown function
9	PBANKA_010510	conserved *Plasmodium* protein, unknown function
10	PBANKA_110410	conserved *Plasmodium* protein, unknown function
11	PBANKA_111500	conserved *Plasmodium* protein, unknown function
12	PBANKA_113490	zinc finger protein, putative
13	PBANKA_122550	conserved *Plasmodium* protein, unknown function
14	PBANKA_131950	LCCL domain-containing protein (CCP2)
15	PBANKA_133370	phosphodiesterase delta (PDEdelta)
16	PBANKA_136160	E1-E2 ATPase, putative
17	PBANKA_131860	conserved *Plasmodium* protein, unknown function
18	PBANKA_134230	conserved *Plasmodium* protein, unknown function
19	PBANKA_135490	conserved *Plasmodium* protein, unknown function
20	PBANKA_136360	conserved *Plasmodium* protein, unknown function
21	**PBANKA_142170**	**secreted ookinete protein, putative (PSOP20) (Ref 2)***
22	PBANKA_143140	conserved *Plasmodium* protein, unknown function
23	PBANKA_145110	conserved *Plasmodium* protein, unknown function
24	PBANKA_030160	conserved *Plasmodium* protein, unknown function
25	PBANKA_050440	conserved *Plasmodium* protein, unknown function
26	PBANKA_051510	conserved *Plasmodium* protein, unknown function
27	PBANKA_061240	conserved *Plasmodium* protein, unknown function
28	PBANKA_060580	conserved *Plasmodium* protein, unknown function
29	PBANKA_081070	subpellicular microtubule protein 1, putative (SPM1)
30	PBANKA_082890	inner membrane complex protein 1b (IMC1b)
31	**PBANKA_090710**	**CPW-WPC family protein, putative (Ref 3)***
32	PBANKA_094340	conserved *Plasmodium* protein, unknown function
33	PBANKA_090790	conserved *Plasmodium* protein, unknown function
5-6 fold		
34	PBANKA_060580	conserved *Plasmodium* protein, unknown function
35	PBANKA_120940	inner membrane complex sub-compartment protein 1 (ISP1)
36	PBANKA_100790	conserved *Plasmodium* protein, unknown function
37	PBANKA_071980	conserved *Plasmodium* protein, unknown function
38	PBANKA_133620	conserved *Plasmodium* protein, unknown function
39	PBANKA_111030	conserved *Plasmodium* protein, unknown function
40	PBANKA_010980	conserved *Plasmodium* protein, unknown function
41	PBANKA_102960	conserved *Plasmodium* protein, unknown function
42	PBANKA_100790	conserved *Plasmodium* protein, unknown function
43	PBANKA_111020	conserved *Plasmodium* protein, unknown function
44	PBANKA_110930	conserved *Plasmodium* protein, unknown function
45	PBANKA_132990	conserved *Plasmodium* protein, unknown function
46	PBANKA_130070	LCCL domain-containing protein (CCp1)
47	PBANKA_141930	conserved *Plasmodium* protein, unknown function
48	**PBANKA_143440**	**secreted ookinete protein, putative (PSOP17) (Ref 2)***

49	PBANKA_146300	osmiophilic body protein (G377)
50	PBANKA_030160	conserved *Plasmodium* protein, unknown function
51	**PBANKA_050430**	**flagellar outer arm dynein-associated protein, putative**
52	**PBANKA_071400**	**meiotic recombination protein DMC1 (DMC1) (Ref 4)***
53	PBANKA_070470	conserved *Plasmodium* protein, unknown function
54	PBANKA_083160	conserved *Plasmodium* protein, unknown function
55	PBANKA_111410	conserved *Plasmodium* protein, unknown function
4-5 fold		
56	PBANKA_111020	conserved *Plasmodium* protein, unknown function
57	PBANKA_120430	CCR4-NOT transcription complex subunit 5, putative (NOT5)
58	**PBANKA_144930**	**CPW-WPC family protein, putative (Ref 3)***
59	PBANKA_070820	conserved *Plasmodium* protein, unknown function
60	PBANKA_082120	conserved *Plasmodium* protein, unknown function
61	PBANKA_112530	conserved *Plasmodium* protein, unknown function
62	PBANKA_091100	conserved *Plasmodium* protein, unknown function
63	PBANKA_144480	thioredoxin-like associated protein 1, putative (TLAP1)
64	PBANKA_143120	conserved *Plasmodium* protein, unknown function
65	PBANKA_132380	conserved *Plasmodium* protein, unknown function
66	PBANKA_120880	conserved *Plasmodium* protein, unknown function
67	PBANKA_081260	conserved *Plasmodium* protein, unknown function
68	PBANKA_052120	conserved *Plasmodium* protein, unknown function
69	PBANKA_091250	conserved *Plasmodium* protein, unknown function
70	PBANKA_136150	conserved *Plasmodium* protein, unknown function
71	**PBANKA_103820**	**nuclear formin-like protein (MISFIT) (Ref 5)***
72	PBANKA_100960	conserved *Plasmodium* protein, unknown function
73	PBANKA_100990	conserved *Plasmodium* protein, unknown function
74	PBANKA_112380	conserved *Plasmodium* protein, unknown function
75	PBANKA_114520	conserved *Plasmodium* protein, unknown function
76	PBANKA_122530	conserved *Plasmodium* protein, unknown function
77	PBANKA_123130	metabolite/drug transporter, putative
78	PBANKA_124520	conserved *Plasmodium* protein, unknown function
79	PBANKA_124700	BIR protein,PIR protein
80	PBANKA_123350	mRNA-binding protein PUF1 (PUF1)
81	PBANKA_123830	conserved *Plasmodium* protein, unknown function
82	PBANKA_133810	conserved *Plasmodium* protein, unknown function
83	PBANKA_133000	conserved *Plasmodium* protein, unknown function
84	PBANKA_134930	conserved *Plasmodium* protein, unknown function
85	PBANKA_143240	perforin-like protein 2 (PLP2)
86	PBANKA_142160	calcium/calmodulin-dependent protein kinase, putative
87	PBANKA_142150	conserved *Plasmodium* protein, unknown function
88	PBANKA_142910	conserved *Plasmodium* protein, unknown function
89	PBANKA_142920	conserved *Plasmodium* protein, unknown function (S20)
90	PBANKA_143430	conserved *Plasmodium* protein, unknown function
91	PBANKA_144480	thioredoxin-like associated protein 1, putative (TLAP1)
92	PBANKA_145480	RNA-binding protein, putative
93	PBANKA_040620	conserved *Plasmodium* protein, unknown function
94	PBANKA_050790	conserved *Plasmodium* protein, unknown function
95	PBANKA_050440	conserved *Plasmodium* protein, unknown function
96	PBANKA_060120	dynein heavy chain, putative
97	PBANKA_060950	kinesin-7, putative
98	PBANKA_062240	kinesin-19, putative
99	PBANKA_060060	NIMA related kinase 3, putative (NEK3)
100	PBANKA_060620	blood stage antigen 41-3 precursor, putative
101	PBANKA_060860	zinc finger protein, putative

102	PBANKA_071980	conserved *Plasmodium* protein, unknown function
103	PBANKA_070470	conserved *Plasmodium* protein, unknown function
104	PBANKA_080430	DNA mismatch repair protein MSH2 (MSH2-2)
105	PBANKA_080720	conserved *Plasmodium* protein, unknown function
106	PBANKA_081290	conserved *Plasmodium* protein, unknown function
107	PBANKA_090300	conserved *Plasmodium* protein, unknown function
108	PBANKA_092650	petidase, M16 family, putative
109	PBANKA_092760	conserved *Plasmodium* protein, unknown function
110	PBANKA_092770	conserved *Plasmodium* protein, unknown function
111	PBANKA_091360	conserved *Plasmodium* protein, unknown function
112	PBANKA_094230	conserved *Plasmodium* protein, unknown function
2-4 fold		
113	PBANKA_010140	conserved *Plasmodium* protein, unknown function
114	PBANKA_123130	metabolite/drug transporter, putative
115	PBANKA_083030	conserved *Plasmodium* protein, unknown function
116	PBANKA_090940	conserved *Plasmodium* protein, unknown function
117	PBANKA_000030	BIR protein,PIR protein
118	PBANKA_112960	conserved *Plasmodium* protein, unknown function
119	PBANKA_090210	sporozoite asparagine-rich protein (SLARP)
120	PBANKA_093900	ion channel protein, putative
121	PBANKA_121000	conserved *Plasmodium* protein, unknown function
122	PBANKA_136040	conserved *Plasmodium* protein, unknown function
123	PBANKA_123650	conserved *Plasmodium* protein, unknown function, fragment
124	PBANKA_030790	conserved *Plasmodium* protein, unknown function
125	PBANKA_060700	conserved *Plasmodium* protein, unknown function
126	PBANKA_082800	zinc finger protein, putative
127	PBANKA_101300	conserved *Plasmodium* membrane protein, unknown function
128	PBANKA_082710	protein kinase, putative
129	PBANKA_111060	conserved *Plasmodium* protein, unknown function
130	PBANKA_040970	plasmepsin VI
131	PBANKA_082570	telomeric repeat binding factor 1, putative
132	PBANKA_102600	conserved *Plasmodium* protein, unknown function
133	PBANKA_102950	conserved *Plasmodium* protein, unknown function
134	PBANKA_041300	conserved *Plasmodium* protein, unknown function
135	PBANKA_082820	conserved *Plasmodium* protein, unknown function
136	PBANKA_093680	conserved *Plasmodium* protein, unknown function
137	PBANKA_123370	conserved *Plasmodium* protein, unknown function
138	PBANKA_132550	conserved *Plasmodium* protein, unknown function
139	PBANKA_121890	elongation factor 1 (EF-1), putative
140	PBANKA_134040	oxidoreductase, putative
141	PBANKA_112470	conserved *Plasmodium* protein, unknown function
142	PBANKA_133310	conserved *Plasmodium* protein, unknown function
143	PBANKA_110990	stripes inner membrane complex protein, putative (SIP)
144	PBANKA_145080	conserved *Plasmodium* protein, unknown function
145	**PBANKA_103010**	**actin II (Ref 6)***
146	PBANKA_144800	conserved *Plasmodium* protein, unknown function
147	PBANKA_083700	BIR protein,PIR protein
148	**PBANKA_112900**	**secreted ookinete protein, putative (PSOP6) (Ref 2)***
149	PBANKA_145920	delta-aminolevulinic acid synthetase, putative (ALAS)
150	PBANKA_103750	adenylyl cyclase alpha (ACalpha)
151	PBANKA_094370	fam-b protein
152	PBANKA_030160	conserved *Plasmodium* protein, unknown function
153	PBANKA_072230	fam-b protein
154	PBANKA_132910	plasmepsin VIII, putative

155	PBANKA_133500	conserved *Plasmodium* protein, unknown function
156	PBANKA_130450	conserved *Plasmodium* protein, unknown function
157	PBANKA_060560	conserved *Plasmodium* protein, unknown function
158	PBANKA_120350	conserved *Plasmodium* protein, unknown function
159	PBANKA_124140	conserved *Plasmodium* protein, unknown function
160	PBANKA_051350	conserved *Plasmodium* protein, unknown function
161	PBANKA_071290	high mobility group protein B2, putative (HMGB2)
162	PBANKA_123340	conserved *Plasmodium* protein, unknown function
163	PBANKA_010570	MYND finger protein, putative
164	PBANKA_010240	conserved *Plasmodium* protein, unknown function
165	PBANKA_010440	nucleoside diphosphate kinase, putative
166	PBANKA_100620	sporozoite invasion-associated protein 1 (SIAP1)
167	PBANKA_100890	conserved *Plasmodium* protein, unknown function
168	PBANKA_102940	conserved *Plasmodium* protein, unknown function
169	PBANKA_103570	conserved *Plasmodium* protein, unknown function
170	PBANKA_100210	6-cysteine protein (P36)
171	PBANKA_100340	lysine decarboxylase, putative (UIS14)
172	PBANKA_103370	conserved *Plasmodium* protein, unknown function
173	PBANKA_103790	conserved *Plasmodium* protein, unknown function
174	PBANKA_112060	conserved *Plasmodium* protein, unknown function
175	PBANKA_112320	conserved *Plasmodium* protein, unknown function
176	PBANKA_113230	conserved *Plasmodium* protein, unknown function
177	PBANKA_113860	conserved *Plasmodium* protein, unknown function
178	**PBANKA_114370**	**secreted ookinete protein, putative (PSOP2) (Ref 2)***
179	PBANKA_114380	conserved *Plasmodium* protein, unknown function
180	PBANKA_110530	conserved *Plasmodium* protein, unknown function
181	PBANKA_110210	conserved *Plasmodium* protein, unknown function
182	PBANKA_111420	ribose 5-phosphate epimerase, putative
183	PBANKA_111920	conserved *Plasmodium* protein, unknown function
184	PBANKA_112100	conserved *Plasmodium* protein, unknown function
185	PBANKA_112580	conserved *Plasmodium* protein, unknown function
186	**PBANKA_112880**	**CPW-WPC family protein, putative (Ref 3)***
187	PBANKA_120890	conserved *Plasmodium* protein, unknown function
188	PBANKA_113060	conserved *Plasmodium* protein, unknown function
189	PBANKA_120890	conserved *Plasmodium* protein, unknown function
190	PBANKA_123760	zinc finger protein, putative, fragment
191	PBANKA_123770	zinc finger protein, putative, fragment
192	**PBANKA_124070**	**NIMA related kinase 2 (NEK2) (Ref 7)***
193	PBANKA_124490	conserved *Plasmodium* protein, unknown function
194	PBANKA_124520	conserved *Plasmodium* protein, unknown function
195	PBANKA_120040	BIR protein,PIR protein
196	PBANKA_120450	conserved *Plasmodium* protein, unknown function
197	PBANKA_120880	conserved *Plasmodium* protein, unknown function
198	PBANKA_120990	conserved *Plasmodium* protein, unknown function
199	PBANKA_123620	conserved *Plasmodium* protein, unknown function
200	PBANKA_123900	conserved *Plasmodium* protein, unknown function
201	PBANKA_124350	conserved *Plasmodium* protein, unknown function
202	PBANKA_130810	calmodulin-like protein
203	PBANKA_131320	transcription factor with AP2 domain(s), putative (ApiAP2)
204	PBANKA_131840	6-phosphogluconate dehydrogenase, decarboxylating, putative
205	PBANKA_132080	conserved *Plasmodium* protein, unknown function
206	PBANKA_132820	conserved *Plasmodium* protein, unknown function
207	PBANKA_132920	dynein beta chain, putative

208	PBANKA_133130	conserved *Plasmodium* protein, unknown function
209	PBANKA_133160	leucine-rich repeat protein (LRR14.1)
210	PBANKA_135590	phosphoenolpyruvate carboxykinase, putative (PEPCK)
211	**PBANKA_130130**	**trailer hitch homolog, putative (CITH) (Ref 9)***
212	PBANKA_131120	conserved *Plasmodium* protein, unknown function
213	PBANKA_131200	conserved *Plasmodium* protein, unknown function
214	PBANKA_131880	protein tyrosine phosphatase (PTP1)
215	PBANKA_133070	50S ribosomal protein L22, apicoplast, putative (L22)
216	PBANKA_135410	ras-related protein Rab-11B, putative (RAB11b)
217	**PBANKA_135970**	**6-cysteine protein (P47) (Ref 10)***
218	PBANKA_136120	conserved *Plasmodium* protein, unknown function
219	PBANKA_140790	HORMA domain protein, putative
220	PBANKA_140960	conserved *Plasmodium* protein, unknown function
221	PBANKA_142720	protein phosphatase 2C, putative
222	PBANKA_142770	RuvB-like helicase 1, putative (RUVB1)
223	PBANKA_143060	conserved *Plasmodium* protein, unknown function
224	PBANKA_143910	conserved *Plasmodium* protein, unknown function
225	PBANKA_144100	haloacid dehalogenase-like hydrolase, putative (HAD3)
226	PBANKA_144960	PIH1 domain-containing protein, putative (PIH1)
227	PBANKA_145580	GAS8-like protein, putative
228	PBANKA_146070	dipeptidyl aminopeptidase 2 (DPAP2)
229	PBANKA_146310	conserved *Plasmodium* protein, unknown function
230	PBANKA_146590	BIR protein,PIR protein
231	PBANKA_140040	fam-b protein
232	PBANKA_141330	ubiquitin-like protein, putative
233	PBANKA_142280	conserved *Plasmodium* protein, unknown function
234	PBANKA_142370	GDP-mannose 4,6-dehydratase, putative (GMD)
235	PBANKA_142710	aquaporin, putative (AQP2)
236	PBANKA_143490	raf kinase inhibitor, putative (RKIP)
237	PBANKA_143520	conserved *Plasmodium* protein, unknown function
238	PBANKA_144280	leucine-rich repeat protein (LRR13)
239	PBANKA_144600	conserved *Plasmodium* protein, unknown function
240	PBANKA_144790	conserved *Plasmodium* protein, unknown function
241	PBANKA_145480	RNA-binding protein, putative
242	PBANKA_146390	conserved *Plasmodium* protein, unknown function
243	PBANKA_021390	dynein light chain, putative
244	PBANKA_021580	fam-b protein
245	PBANKA_021260	conserved *Plasmodium* protein, unknown function
246	PBANKA_030180	conserved *Plasmodium* protein, unknown function
247	**PBANKA_030600**	**6-cysteine protein (P230p) (Ref 10)***
248	PBANKA_031200	conserved *Plasmodium* protein, unknown function
249	PBANKA_031270	DEAD/DEAH helicase, putative
250	PBANKA_031480	conserved *Plasmodium* membrane protein, unknown function
251	PBANKA_030130	conserved *Plasmodium* protein, unknown function
252	PBANKA_030280	conserved *Plasmodium* protein, unknown function
253	PBANKA_030660	conserved *Plasmodium* protein, unknown function
254	PBANKA_031490	conserved *Plasmodium* protein, unknown function
255	PBANKA_040270	inner membrane complex protein 1e (IMC1e)
256	PBANKA_040430	P-loop containing nucleoside triphosphate hydrolase, putative
257	**PBANKA_040820**	**calcium-dependent protein kinase 3 (CDPK3) (Ref 11)***
258	PBANKA_041340	conserved *Plasmodium* protein, unknown function
259	PBANKA_040060	BIR protein,PIR protein
260	PBANKA_040250	phosphatidylethanolamine-binding protein, putative
261	PBANKA_041130	conserved *Plasmodium* protein, unknown function

262	PBANKA_041580	conserved *Plasmodium* protein, unknown function
263	PBANKA_050030	fam-b protein
264	PBANKA_050190	conserved *Plasmodium* protein, unknown function
265	PBANKA_051030	conserved *Plasmodium* protein, unknown function
266	PBANKA_052240	steroid dehydrogenase, putative
267	PBANKA_050020	BIR protein,PIR protein
268	PBANKA_050350	zinc finger protein, putative
269	PBANKA_050780	conserved *Plasmodium* protein, unknown function
270	PBANKA_051040	conserved *Plasmodium* protein, unknown function
271	PBANKA_052070	conserved *Plasmodium* protein, unknown function
272	PBANKA_060800	porphobilinogen deaminase, putative (PBGD)
273	**PBANKA_061670**	**NIMA related kinase 4 (NEK4) (Ref 8)***
274	PBANKA_062030	conserved *Plasmodium* protein, unknown function
275	PBANKA_062180	3-demethylubiquinone-9 3-methyltransferase, putative
276	PBANKA_062190	mitochondrial import inner membrane translocase subunit TIM14, putative (PAM18)
277	PBANKA_071220	conserved *Plasmodium* protein, unknown function
278	PBANKA_072090	conserved *Plasmodium* protein, unknown function
279	PBANKA_072110	conserved *Plasmodium* protein, unknown function
280	PBANKA_072250	BIR protein,PIR protein
281	PBANKA_070330	conserved *Plasmodium* protein, unknown function
282	PBANKA_070720	alpha/beta hydrolase, putative
283	PBANKA_071130	conserved *Plasmodium* protein, unknown function
284	PBANKA_071970	conserved *Plasmodium* protein, unknown function
285	PBANKA_081450	protease, putative
286	PBANKA_081680	glycolipid transfer protein, putative
287	PBANKA_082290	conserved *Plasmodium* protein, unknown function
288	PBANKA_082580	conserved *Plasmodium* protein, unknown function
289	PBANKA_083690	BIR protein,PIR protein
290	PBANKA_080730	conserved *Plasmodium* protein, unknown function
291	PBANKA_082590	conserved *Plasmodium* protein, unknown function
292	PBANKA_082730	conserved *Plasmodium* protein, unknown function
293	PBANKA_082890	conserved *Plasmodium* protein, unknown function
294	PBANKA_083030	conserved *Plasmodium* protein, unknown function
295	PBANKA_090010	fam-b protein
296	PBANKA_090230	conserved *Plasmodium* protein, unknown function
297	PBANKA_090240	conserved *Plasmodium* protein, unknown function
298	PBANKA_091670	conserved *Plasmodium* protein, unknown function
299	PBANKA_092760	conserved *Plasmodium* protein, unknown function
300	**PBANKA_093370**	**mitogen-activated protein kinase 2 (MAPK2) (Ref 12)***
301	PBANKA_093930	conserved *Plasmodium* protein, unknown function
302	PBANKA_094240	conserved *Plasmodium* protein, unknown function
303	PBANKA_090700	conserved *Plasmodium* protein, unknown function
304	PBANKA_090750	conserved *Plasmodium* protein, unknown function
305	PBANKA_091080	conserved *Plasmodium* protein, unknown function
306	PBANKA_091260	conserved *Plasmodium* protein, unknown function
307	**PBANKA_091740**	**armadillo repeat protein PF16 (PF16) (Ref 13)***
308	PBANKA_093310	conserved *Plasmodium* protein, unknown function
1-2 fold		
309	**PBANKA_090590**	**transcription factor with AP2 domain(s) (AP2-O) (Ref 14)***
310	PBANKA_102700	serine C-palmitoyltransferase, putative
311	PBANKA_134160	translation initiation factor EIF-2B gamma subunit, putative
312	PBANKA_120920	50S ribosomal protein L22, mitochondrial, putative
313	PBANKA_146370	DNA repair protein rhp16, putative

314	PBANKA_142270	superoxide dismutase [Fe], putative (FeSOD)
315	PBANKA_061600	mitochondrial ribosomal protein S8 precursor, putative
316	PBANKA_093780	U3/U14 snoRNA-associated small subunit rRNA processing protein, putative
317	PBANKA_103480	conserved *Plasmodium* protein, unknown function
318	PBANKA_101130	microsomal signal peptidase protein, putative
319	PBANKA_111000	conserved *Plasmodium* protein, unknown function
320	PBANKA_123090	RAP protein, putative
321	PBANKA_090430	apicoplast import protein Tic20, putative (TIC20)
322	PBANKA_134160	translation initiation factor EIF-2B gamma subunit, putative
323	PBANKA_021250	conserved *Plasmodium* protein, unknown function
324	PBANKA_040840	phosphoglycerate mutase, putative
325	PBANKA_070810	dynein light chain 1 (DLC1)
326	PBANKA_070610	conserved *Plasmodium* protein, unknown function
327	PBANKA_092110	methyltransferase, putative
328	PBANKA_091870	A/G-specific adenine glycosylase, putative
329	PBANKA_140500	conserved *Plasmodium* protein, unknown function
330	PBANKA_145850	NEDD8-conjugating enzyme UBC12, putative (UBC12)
331	PBANKA_061160	ribonucleoside-diphosphate reductase, large subunit, putative
332	PBANKA_090380	serine/threonine protein kinase, putative
333	PBANKA_144570	conserved *Plasmodium* protein, unknown function
334	PBANKA_050500	dihydrolipoamide acyltransferase, putative
335	PBANKA_062040	conserved *Plasmodium* protein, unknown function
336	PBANKA_070550	conserved *Plasmodium* protein, unknown function
337	PBANKA_091840	conserved *Plasmodium* protein, unknown function
338	PBANKA_135480	conserved *Plasmodium* protein, unknown function
339	PBANKA_140010	BIR protein,PIR protein
340	PBANKA_070270	rhomboid protease ROM3 (ROM3)
341	**PBANKA_101540**	**CPW-WPC family protein, putative (Ref 3)***
342	PBANKA_050330	conserved *Plasmodium* protein, unknown function
343	PBANKA_081540	conserved *Plasmodium* protein, unknown function
344	PBANKA_021270	zinc finger protein, putative
345	PBANKA_061590	cysteine repeat modular protein 2 (CRMP2)
346	PBANKA_113950	conserved *Plasmodium* protein, unknown function
347	PBANKA_120040	BIR protein,PIR protein
348	PBANKA_020370	phosphatidate cytidylyltransferase, putative
349	PBANKA_061640	conserved *Plasmodium* protein, unknown function
350	PBANKA_061530	conserved *Plasmodium* protein, unknown function
351	PBANKA_103540	glideosome associated protein with multiple membrane spans 3, putative (GAPM3)
352	PBANKA_071310	rhoptry neck protein 5, putative (RON5)
353	PBANKA_091610	ucleic acid binding protein, putative
354	PBANKA_072010	conserved *Plasmodium* protein, unknown function
355	PBANKA_113670	guanylyl cyclase beta (GCbeta)
356	PBANKA_140030	BIR protein,PIR protein
357	PBANKA_111510	amino acid transporter, putative
358	PBANKA_102870	26S proteasome regulatory subunit RPN13, putative (RPN13)
359	PBANKA_132890	potassium channel, putative
360	PBANKA_133130	conserved *Plasmodium* protein, unknown function
361	PBANKA_132610	conserved *Plasmodium* protein, unknown function
362	PBANKA_093810	conserved *Plasmodium* protein, unknown function
363	PBANKA_061530	conserved *Plasmodium* protein, unknown function
364	PBANKA_082740	conserved *Plasmodium* protein, unknown function
365	**PBANKA_135960**	**6-cysteine protein (P48/45) (Ref 15)***

366	PBANKA_124010	conserved *Plasmodium* protein, unknown function
367	PBANKA_102680	zinc finger protein, putative
368	**PBANKA_135340**	**secreted ookinete protein, putative (PSOP7) (Ref 2)***
369	PBANKA_140590	conserved *Plasmodium* protein, unknown function
370	PBANKA_141720	conserved *Plasmodium* protein, unknown function
371	PBANKA_102970	ubiquitin-conjugating enzyme, putative
372	PBANKA_122600	pseudouridylate synthase, putative
373	PBANKA_133470	conserved *Plasmodium* protein, unknown function
374	PBANKA_010630	conserved *Plasmodium* protein, unknown function
375	PBANKA_114640	BIR protein,PIR protein
376	PBANKA_120510	PPPDE peptidase, putative
377	PBANKA_103470	nucleolar preribosomal GTPase, putative
378	PBANKA_101620	protein kinase, putative
379	PBANKA_121590	conserved *Plasmodium* protein, unknown function
380	**PBANKA_143750**	**transcription factor with AP2 domain(s) (AP2-G) (Ref 16)***
381	PBANKA_081330	conserved *Plasmodium* protein, unknown function
382	PBANKA_101700	CorA-like Mg2+ transporter protein, putative
383	PBANKA_070200	conserved *Plasmodium* protein, unknown function
384	PBANKA_146550	BIR protein,PIR protein
385	PBANKA_060010	BIR protein,PIR protein
386	PBANKA_142450	conserved *Plasmodium* protein, unknown function
387	PBANKA_071900	conserved *Plasmodium* protein, unknown function
388	PBANKA_124090	conserved *Plasmodium* protein, unknown function
389	PBANKA_051850	ADP-ribosylation factor, putative
390	PBANKA_141620	conserved *Plasmodium* protein, unknown function
391	PBANKA_135210	conserved *Plasmodium* protein, unknown function
392	PBANKA_031060	conserved *Plasmodium* protein, unknown function
393	PBANKA_082140	ADP-ribosylation factor-like protein
394	PBANKA_133690	conserved *Plasmodium* protein, unknown function
395	PBANKA_102630	conserved *Plasmodium* protein, unknown function
396	PBANKA_142080	conserved *Plasmodium* protein, unknown function
397	PBANKA_090250	kinesin-like protein, putative
398	PBANKA_130050	tubulin epsilon chain, putative
399	PBANKA_010180	conserved *Plasmodium* protein, unknown function
400	PBANKA_010410	serine/threonine protein kinase, putative (ARK1)
401	PBANKA_010780	citrate synthase-like protein, putative
402	PBANKA_010080	conserved *Plasmodium* protein, unknown function
403	PBANKA_100970	ribonuclease H2 subunit B, putative
404	PBANKA_101170	conserved *Plasmodium* protein, unknown function
405	PBANKA_102110	DnaJ protein, putative
406	PBANKA_102310	conserved *Plasmodium* protein, unknown function
407	PBANKA_102820	atypical protein kinase, ABC-1 family, putative
408	PBANKA_101410	exodeoxyribonuclease III, putative
409	PBANKA_101730	conserved *Plasmodium* protein, unknown function
410	PBANKA_103620	RNA-binding protein, putative
411	PBANKA_103670	COBW domain-containing protein 1, putative (CBWD1)
412	**PBANKA_103780**	**secreted ookinete adhesive protein (SOAP) (Ref 17)***
413	PBANKA_104010	choline kinase (CK)
414	PBANKA_110610	conserved *Plasmodium* protein, unknown function
415	**PBANKA_111340**	**secreted ookinete protein, putative,6-cysteine protein (PSOP12) (Ref 2)***
416	PBANKA_111430	conserved *Plasmodium* protein, unknown function
417	PBANKA_112980	tetratricopeptide repeat protein, putative
418	PBANKA_113280	conserved *Plasmodium* protein, unknown function

419	PBANKA_113310	serine/threonine protein kinase, putative (ARK3)
420	PBANKA_113890	conserved *Plasmodium* protein, unknown function
421	PBANKA_114310	conserved *Plasmodium* protein, unknown function
422	PBANKA_110150	anaphase-promoting complex subunit, putative
423	PBANKA_110230	conserved *Plasmodium* protein, unknown function
424	PBANKA_110700	conserved *Plasmodium* protein, unknown function
425	PBANKA_111460	RNA-binding protein, putative
426	PBANKA_113820	RuvB-like helicase 3, putative (RUVB3)
427	PBANKA_121440	conserved *Plasmodium* protein, unknown function
428	PBANKA_121600	iron-sulfur assembly protein, putative
429	PBANKA_121790	conserved *Plasmodium* protein, unknown function
430	PBANKA_122560	alpha/beta hydrolase, putative
431	PBANKA_124640	BIR protein,PIR protein
432	PBANKA_120180	conserved *Plasmodium* protein, unknown function
433	PBANKA_120260	E3 ubiquitin-protein ligase, putative
434	PBANKA_120370	regulator of nonsense transcripts, putative
435	PBANKA_120600	conserved *Plasmodium* protein, unknown function
436	PBANKA_120690	tubulin beta chain, putative
437	PBANKA_121800	nicotinamidase, putative
438	PBANKA_121850	protein phosphatase inhibitor 2, putative
439	PBANKA_122830	conserved *Plasmodium* protein, unknown function
440	PBANKA_123850	conserved *Plasmodium* protein, unknown function
441	PBANKA_131820	nifU protein, putative
442	PBANKA_131970	transcription factor with AP2 domain(s), putative (ApiAP2)
443	PBANKA_132480	conserved *Plasmodium* protein, unknown function
444	PBANKA_133960	MSF1-like protein, putative
445	**PBANKA_134630**	**CPW-WPC family protein, putative (UIS19) (Ref 3)***
446	PBANKA_135790	conserved *Plasmodium* protein, unknown function
447	PBANKA_135780	conserved *Plasmodium* protein, unknown function
448	PBANKA_136570	BIR protein,PIR protein
449	PBANKA_130390	conserved *Plasmodium* protein, unknown function
450	PBANKA_130570	vacuolar-sorting protein SNF7, putative
451	PBANKA_130600	replication factor A protein 3, putative (RPA3)
452	PBANKA_130800	conserved *Plasmodium* protein, unknown function
453	PBANKA_130960	conserved *Plasmodium* protein, unknown function
454	PBANKA_131430	conserved *Plasmodium* protein, unknown function
455	PBANKA_131680	dynactin subunit 4, putative
456	PBANKA_132760	conserved *Plasmodium* protein, unknown function
457	PBANKA_133090	conserved *Plasmodium* protein, unknown function
458	PBANKA_133680	conserved *Plasmodium* protein, unknown function
459	**PBANKA_135250**	**CPW-WPC family protein, putative (Ref 3)***
460	PBANKA_140370	transcription factor with AP2 domain(s), putative (ApiAP2)
461	PBANKA_143760	glideosome-associated protein 45, putative
462	PBANKA_145500	mitochondrial carrier protein, putative
463	PBANKA_145860	mitochondrial ribosomal protein L3 precursor, putative
464	PBANKA_145950	myosin light chain 1, putative,myosin A tail domain interacting protein MTIP, putative (MTIP)
465	PBANKA_140290	conserved *Plasmodium* protein, unknown function
466	PBANKA_140950	protein ISD11, putative (ISD11)
467	PBANKA_141540	conserved *Plasmodium* protein, unknown function
468	PBANKA_141690	chromosome segregation protein, putative
469	PBANKA_144510	ATP-dependent protease subunit ClpQ, putative (ClpQ)
470	PBANKA_145120	p25-alpha family protein, putative
471	PBANKA_145310	sphingomyelin phosphodiesterase, putative

472	PBANKA_020020	fam-a protein
473	PBANKA_020270	kinesin-8, putative
474	PBANKA_020970	zinc carboxy peptidase, putative
475	PBANKA_021400	dynein heavy chain, putative
476	PBANKA_020170	conserved *Plasmodium* protein, unknown function
477	PBANKA_020760	asparagine-rich antigen, putative
478	PBANKA_030140	conserved *Plasmodium* protein, unknown function
479	PBANKA_040560	EB1 homolog, putative
480	PBANKA_040720	dual specificity protein phosphatase (YVH1)
481	PBANKA_041350	conserved *Plasmodium* protein, unknown function
482	PBANKA_041610	dynein heavy chain, putative
483	PBANKA_041380	microneme associated antigen, putative (MA)
484	PBANKA_051810	thioredoxin-like associated protein 2, putative (TLAP2)
485	PBANKA_052270	alpha tubulin 2
486	PBANKA_052360	BSD-domain protein, putative
487	PBANKA_051170	topoisomerase, putative
488	PBANKA_060550	conserved *Plasmodium* protein, unknown function
489	PBANKA_060790	3',5'-cyclic nucleotide phosphodiesterase, putative
490	PBANKA_061470	conserved *Plasmodium* protein, unknown function
491	PBANKA_060090	conserved *Plasmodium* protein, unknown function
492	PBANKA_060300	conserved *Plasmodium* protein, unknown function
493	PBANKA_061070	conserved *Plasmodium* protein, unknown function
494	PBANKA_062170	type 2A phosphatase-associated protein 42, putative (TAP42)
495	PBANKA_070660	conserved *Plasmodium* protein, unknown function
496	PBANKA_070750	RWD domain-containing protein, putative
497	PBANKA_072000	conserved *Plasmodium* protein, unknown function
498	PBANKA_070560	conserved *Plasmodium* protein, unknown function
499	PBANKA_070850	conserved *Plasmodium* protein, unknown function
500	PBANKA_081620	ribonuclease H2 subunit C, putative
501	PBANKA_082440	cyclin-dependent kinases regulatory subunit, putative
502	PBANKA_082660	conserved *Plasmodium* protein, unknown function
503	PBANKA_083390	conserved *Plasmodium* protein, unknown function
504	PBANKA_080550	conserved *Plasmodium* protein, unknown function
505	PBANKA_081510	conserved *Plasmodium* protein, unknown function
506	PBANKA_083270	adenylate kinase-like protein 2, putative (AKLP2)
507	PBANKA_090950	AAA family ATPase, putative
508	PBANKA_092010	cochaperone prefoldin complex subunit, putative
509	PBANKA_092130	deoxyuridine 5'-triphosphate nucleotidohydrolase, putative
510	PBANKA_092290	mitochondrial import inner membrane translocase subunit TIM44, putative (TIM44)
511	PBANKA_092330	conserved *Plasmodium* protein, unknown function
512	PBANKA_092360	GrpE protein homolog, mitochondrial, putative (MGE1)
513	PBANKA_093260	conserved *Plasmodium* protein, unknown function
514	PBANKA_093950	DNA repair protein RAD51 (RAD51)
515	PBANKA_090190	tubulin--tyrosine ligase, putative
516	PBANKA_091300	conserved *Plasmodium* protein, unknown function
517	PBANKA_092740	heat shock factor-binding protein 1, putative (HSBP)
518	PBANKA_093250	SET domain protein, putative
519	PBANKA_093460	conserved *Plasmodium* protein, unknown function
520	PBANKA_094000	conserved *Plasmodium* protein, unknown function
521	PBANKA_094050	conserved *Plasmodium* protein, unknown function

References* 1-17 indicated for genes related to sexual stage functions (highlighted in blue) are indicated in Appendix 1 on Page No 129.

Table 4. Showing the list of upregulated transcripts in *Ubc13 kinase/PK9* KO schizonts

Up regulated genes in *Plasmodium berghei Ubc13 kinase/PK9* KO		
S.No	**GeneID**	**Function**
4-6 fold		
1	PBANKA_000070	fam-b protein
2	PBANKA_104020	BIR protein,PIR protein
3	PBANKA_110070	fam-a protein
2-4 fold		
4	PBANKA_092450	conserved *Plasmodium* protein, unknown function
5	PBANKA_000480	BIR protein,PIR protein
6	PBANKA_136230	conserved *Plasmodium* protein, unknown function
7	PBANKA_050080	BIR protein, pseudogene,PIR protein, pseudogene
8	PBANKA_010140	conserved *Plasmodium* protein, unknown function
9	PBANKA_000480	BIR protein,PIR protein
10	PBANKA_030020	BIR protein,PIR protein
11	PBANKA_081310	conserved *Plasmodium* protein, unknown function
12	PBANKA_062050	tRNAHis guanylyltransferase, putative
13	PBANKA_040640	conserved *Plasmodium* protein, unknown function
14	PBANKA_130035	fam-a protein, pseudogene
15	PBANKA_100180	transcription factor with AP2 domain(s), putative (ApiAP2)
16	PBANKA_100010	reticulocyte binding protein, putative (Pb235)
17	PBANKA_104020	BIR protein,PIR protein
18	PBANKA_104030	fam-b protein
19	PBANKA_120030	BIR protein,PIR protein
20	PBANKA_020030	BIR protein,PIR protein
21	PBANKA_030020	BIR protein,PIR protein
22	PBANKA_050095	BIR protein, pseudogene,PIR protein, pseudogene
23	PBANKA_060020	BIR protein,PIR protein
24	PBANKA_070020	BIR protein,PIR protein
25	PBANKA_092440	splicing factor, putative
1-2 fold		
26	PBANKA_113720	protein transport protein Sec24A, putative (SEC24A)
27	PBANKA_120030	BIR protein,PIR protein
28	PBANKA_021600	conserved *Plasmodium* protein, unknown function
29	PBANKA_030890	conserved *Plasmodium* protein, unknown function
30	PBANKA_031020	conserved *Plasmodium* protein, unknown function
31	PBANKA_041440	formate-nitrite transporter, putative
32	PBANKA_050010	fam-a protein, pseudogene
33	PBANKA_090070	BIR protein,PIR protein
34	PBANKA_113780	conserved *Plasmodium* protein, unknown function
35	PBANKA_041650	copper-transporting ATPase (CuTP)
36	PBANKA_041440	formate-nitrite transporter, putative
37	PBANKA_010930	SNARE associated Golgi protein, putative
38	PBANKA_102010	serine/threonine protein phosphatase 7 (PP7)
39	PBANKA_140410	membrane integral peptidase, M50 family, putative
40	PBANKA_093690	actin-like protein, putative (ALP1)
41	PBANKA_144340	conserved *Plasmodium* protein, unknown function
42	PBANKA_130100	conserved *Plasmodium* protein, unknown function
43	PBANKA_140730	tyrosine recombinase, putative (INT)
44	PBANKA_104040	BIR protein,PIR protein
45	PBANKA_135230	conserved *Plasmodium* protein, unknown function
46	PBANKA_101400	NADP-specific glutamate dehydrogenase, putative (GDH2)
47	PBANKA_021150	proteasome subunit alpha type-5, putative

48	PBANKA_030520	conserved *Plasmodium* protein, unknown function
49	PBANKA_122500	serine/threonine protein kinase, FIKK family
50	PBANKA_071490	lipoamide dehydrogenase, putative
51	PBANKA_103320	DNA damage-inducible protein 1, putative (DDI1)
52	PBANKA_112650	acetyl-CoA synthetase, putative (ACS)
53	PBANKA_020060	fam-a protein
54	PBANKA_050010	fam-a protein, pseudogene
55	PBANKA_050600	schizont egress antigen-1 (SEA1)
56	PBANKA_082720	monocarboxylase transporter, putative
57	PBANKA_093210	palmitoyltransferase (DHHC9)
58	PBANKA_130035	fam-a protein, pseudogene
59	PBANKA_135500	vacuolar ATP synthase subunit d, putative
60	PBANKA_051620	phosphatidylinositol N-acetylglucosaminyltransferase subunit A, putative (PIGA)
61	PBANKA_143790	conserved *Plasmodium* protein, unknown function
62	PBANKA_062345	fam-a protein, pseudogene
63	PBANKA_103260	cytidine diphosphate-diacylglycerol synthase, putative (CDS)
64	PBANKA_122300	conserved *Plasmodium* protein, unknown function
65	PBANKA_050640	conserved *Plasmodium* membrane protein, unknown function
66	PBANKA_120080	RNA-binding protein, putative
67	PBANKA_136290	lysine--tRNA ligase, putative (KRS1)
68	PBANKA_091980	geranylgeranyl pyrophosphate synthase, putative (GGPPS)
69	PBANKA_120020	fam-b protein
70	PBANKA_133970	DEAD box helicase, putative
71	PBANKA_113840	calpain, putative (Pcalp)
72	PBANKA_140710	conserved *Plasmodium* protein, unknown function
73	PBANKA_021220	conserved *Plasmodium* protein, unknown function
74	PBANKA_041080	ubiquitin-protein ligase, putative
75	PBANKA_083630	cytoadherence linked asexual protein 9 (CLAG9)
76	PBANKA_082750	glutamine-dependent NAD(+) synthetase, putative (NADSYN)
77	PBANKA_143470	clathrin heavy chain, putative
78	PBANKA_010200	mitochondrial chaperone BCS1, putative
79	PBANKA_103180	conserved *Plasmodium* protein, unknown function
80	PBANKA_050700	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase, putative (GcpE)
81	PBANKA_091510	LEM3/CDC50 family protein, putative
82	PBANKA_050010	fam-a protein, pseudogene
83	PBANKA_130320	conserved *Plasmodium* protein, unknown function
84	PBANKA_110540	small ubiquitin-related modifier, putative (SUMO)
85	PBANKA_121390	conserved *Plasmodium* protein, unknown function
86	PBANKA_071080	Snf2-related CBP activator, putative (SRCAP)
87	PBANKA_140410	membrane integral peptidase, M50 family, putative
88	PBANKA_040760	asparagine synthetase, putative
89	PBANKA_070240	conserved *Plasmodium* protein, unknown function
90	PBANKA_142780	peptidase family C50, putative
91	PBANKA_092550	calcium-dependent protein kinase 6 (CDPK6)
92	PBANKA_100870	conserved *Plasmodium* protein, unknown function
93	PBANKA_070970	protein kinase 1 (PK1)
94	PBANKA_112760	conserved *Plasmodium* protein, unknown function
95	PBANKA_093290	glycerol-3-phosphate dehydrogenase, putative (G3PDH)
96	PBANKA_040450	conserved *Plasmodium* protein, unknown function

97	PBANKA_134050	conserved *Plasmodium* protein, unknown function
98	PBANKA_132790	conserved *Plasmodium* protein, unknown function
99	PBANKA_146600	BIR protein,PIR protein
100	PBANKA_050520	conserved *Plasmodium* protein, unknown function
101	PBANKA_010540	coatomer alpha subunit, putative
102	PBANKA_010120	elongation factor G, putative (EF-G)
103	PBANKA_010260	conserved *Plasmodium* protein, unknown function
104	PBANKA_100100	conserved *Plasmodium* protein, unknown function
105	PBANKA_101580	conserved *Plasmodium* protein, unknown function
106	PBANKA_102140	conserved *Plasmodium* protein, unknown function
107	PBANKA_103800	cytochrome c, putative
108	PBANKA_103890	conserved *Plasmodium* protein, unknown function
109	PBANKA_100130	conserved *Plasmodium* protein, unknown function
110	PBANKA_101560	apicoplast ribosomal protein L15 precursor, putative
111	PBANKA_102990	AAA family ATPase, putative
112	PBANKA_103340	oxidoreductase, putative
113	PBANKA_103700	trafficking protein particle complex subunit 1, putative (BET5)
114	PBANKA_110270	50S ribosomal protein L28, apicoplast, putative
115	PBANKA_110910	conserved *Plasmodium* protein, unknown function
116	PBANKA_113370	conserved *Plasmodium* protein, unknown function
117	PBANKA_112650	acetyl-CoA synthetase, putative (ACS)
118	PBANKA_123070	conserved *Plasmodium* protein, unknown function
119	PBANKA_124460	phosphoenolpyruvate/phosphate translocator, putative (PPT)
120	PBANKA_130660	conserved *Plasmodium* protein, unknown function
121	PBANKA_133060	1-deoxy-D-xylulose 5-phosphate reductoisomerase, putative (DXR)
122	PBANKA_133590	DNA-directed RNA polymerase, alpha subunit, putative
123	PBANKA_135840	ubiquitin-conjugating enzyme E2, putative
124	PBANKA_136400	conserved *Plasmodium* protein, unknown function
125	PBANKA_130020	BIR protein,PIR protein
126	PBANKA_130730	conserved *Plasmodium* protein, unknown function
127	PBANKA_131930	ferlin, putative
128	PBANKA_133400	conserved *Plasmodium* protein, unknown function
129	PBANKA_134740	apurinic/apyrimidinic endonuclease Apn1, putative
130	PBANKA_134900	MSP7-like protein (MSRP2)
131	PBANKA_135680	conserved *Plasmodium* protein, unknown function
132	PBANKA_141610	conserved *Plasmodium* protein, unknown function
133	PBANKA_142380	ABC transporter, putative
134	PBANKA_143580	conserved *Plasmodium* protein, unknown function
135	PBANKA_144690	dihydrolipoyl dehydrogenase, mitochondrial (LPD1)
136	PBANKA_145370	transcription factor with AP2 domain(s), putative (ApiAP2)
137	PBANKA_145410	ATP-dependent zinc metalloprotease FTSH 1, putative (FTSH1)
138	PBANKA_140060	cytoadherence linked asexual protein, putative
139	PBANKA_140470	conserved *Plasmodium* protein, unknown function
140	PBANKA_140690	conserved *Plasmodium* protein, unknown function
141	PBANKA_140910	ras-related protein Rab-5B, putative (RAB5b)
142	PBANKA_143730	endoplasmin, putative (GRP94)
143	PBANKA_144350	conserved *Plasmodium* protein, unknown function
144	PBANKA_145130	S-adenosyl-methyltransferase, putative
145	PBANKA_021600	BIR protein,PIR protein
146	PBANKA_021180	conserved *Plasmodium* protein, unknown function

147	PBANKA_030480	serine repeat antigen 4 (SERA4)
148	PBANKA_031500	apicoplast RNA methyltransferase precursor, putative
149	PBANKA_041600	high molecular weight rhoptry protein 3, putative (RhopH3)
150	PBANKA_040160	N-ethylmaleimide-sensitive fusion protein, putative (NSF)
15	PBANKA_041360	zinc finger protein, putative
1152	PBANKA_041680	ubiquitin specific protease, putative
153	PBANKA_050470	conserved *Plasmodium* protein, unknown function
154	PBANKA_051160	conserved *Plasmodium* protein, unknown function
155	PBANKA_051890	conserved *Plasmodium* protein, unknown function
156	PBANKA_051940	conserved *Plasmodium* protein, unknown function
157	PBANKA_050260	conserved *Plasmodium* protein, unknown function
158	PBANKA_050610	conserved *Plasmodium* protein, unknown function
159	PBANKA_051980	acetyl-CoA transporter, putative
160	PBANKA_061740	conserved *Plasmodium* protein, unknown function
161	PBANKA_061860	conserved *Plasmodium* protein, unknown function
162	PBANKA_071310	rhoptry neck protein 5, putative (RON5)
163	PBANKA_072280	fam-b protein
164	PBANKA_070010	BIR protein,PIR protein
165	PBANKA_070030	BIR protein,PIR protein
166	PBANKA_081890	heat shock protein 70, putative
167	PBANKA_083020	high molecular weight rhoptry protein 2, putative (RhopH2)
168	PBANKA_083210	conserved *Plasmodium* protein, unknown function
169	PBANKA_082650	histone deacetylase, putative (HDAC1)
170	PBANKA_083630	cytoadherence linked asexual protein 9 (CLAG9)
171	PBANKA_090110	serine/threonine protein kinase, putative
172	PBANKA_091270	3-oxo-5-alpha-steroid 4-dehydrogenase, putative
173	PBANKA_091520	conserved *Plasmodium* protein, unknown function
174	PBANKA_092260	conserved *Plasmodium* protein, unknown function
175	PBANKA_092250	conserved *Plasmodium* protein, unknown function
176	PBANKA_092240	conserved *Plasmodium* protein, unknown function
177	PBANKA_092380	pyruvate dehydrogenase E1 component subunit alpha
178	PBANKA_094250	conserved *Plasmodium* protein, unknown function
179	PBANKA_094310	CCR4-NOT transcription complex subunit 1, putative (NOT1)
180	PBANKA_094350	FeS cluster assembly protein SufD (SufD)

2.3.8 Higher ubiquitination levels of few target proteins and Ubc13/E2 in *Ubc13 kinase/PK9* KO parasite lysates.

The ubiquitination levels of cellular proteins in lysates obtained from synchronized schizont cultures of *Ubc13 kinase/PK9* KO was analyzed by Western blotting (Fig 21). Nearly 4-5 protein bands showed higher ubiquitination level as compared to corresponding proteins in the WT parasite lysates (Fig 21A). Additionally, we also observed higher levels of Ubc13/E2 in *Ubc13 kinase/PK9* KO as compared to WT parasite. Actin immunoreactivity was shown as loading control (Fig 21B).

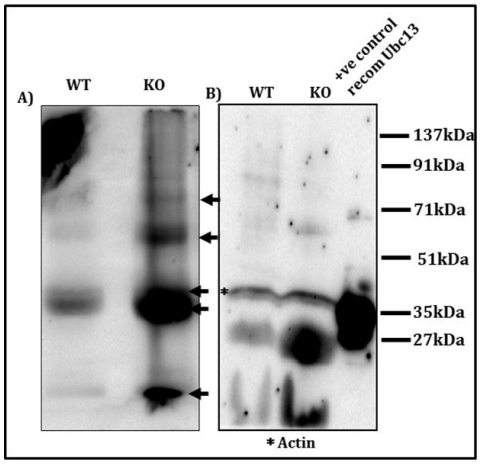

Fig 21. Analysis of cellular proteins reveals higher levels of ubiquination in *Ubc13 kinase/PK9* KO together with higher levels of Ubc13/E2. Total cellular proteins from synchronized WT and *Ubc13 kinase /PK 9* KO schizonts were lysed in SDS-PAGE sample buffer and were resolved on 15% polyacrylamide gels. The resolved proteins were transferred on to nitrocellulose membrane by Western blotting. The bots were probed with (A) anti ubiquitin antibody and (B) anti-Ubc13 or E2 antibody together with anti-actin antibody used as loading control (*). Recombinant human Ubc13/E2 was used as a positive control in B. The immunoreactivity was revealed by using a secondary antibody conjugated to HRP.

2.4 Discussion

Increased risk of drug resistant parasites and insecticide resistant mosquitoes are posing major challenges for malaria prophylaxis and treatment. Understanding the parasite biology and functional proteomics can give an insight into new drug and vaccine development strategies. Current research on *Plasmodium* kinome unraveled the possible intervention strategies by kinase inhibitors in malaria treatment. Inhibition of kinase activity and function was implicated initially in the treatment of cancer [206-208]. Currently kinase specific inhibitors are used to mitigate the activity of kinases to treat a variety of diseases [209, 210]. Studies are ongoing to target apicomplexan parasites including *Plasmodium* by intercepting the protein kinase signaling pathways [211-215]. Few *Plasmodium* kinases have diverged significantly from eukaryotic protein kinases, thus making them as ideal targets for malaria intervention. Several kinases of *Plasmodium* are reported to be essential at different stages of the *Plasmodium* life cycle. Currently screening of kinase inhibitors that affect *Plasmodium* development is underway [150]. Unraveling the function of novel kinases is beneficial for an effective anti-malarial therapy. The current work focuses on unraveling the function of an orphan kinase of *Plasmodium*, PfPK9 or Ubc13 kinase which has been characterized in blood stages of *P. falciparum* [127]. PfPK9/Ubc13 kinase is involved in ubiquitination pathway upstream to one of the E2s or Ubc13. Ubc13 kinase/PK9 regulates Ubc13 activity by phosphorylation. Although the function of Ubc13 kinase/PK9 is assigned in the

ubiquitination pathway the actual role of the Ubc13 kinase/PK9 throughout the parasite life cycle is not known. Towards this objective, we deleted *Ubc13 kinase/PK9* locus in a genetically tractable rodent species of *P. berghei* and analyzed the ability of the knockout parasites to complete the life cycle. Our study unraveled an important and previously unappreciated role of *Pb*Ubc13 kinase/PK9 in sexual development.

In this study, we used double homologous recombination strategy to replace *Ubc13 kinase/PK9* locus. The targeting construct was generated by cloning 5' and 3' flanking regions of Ubc13 kinase/PK9 ORF respectively into the multiple cloning sites MCS-I and MSC-II present on either ends of the GFP and hDHFR (drug selection) cassette. To facilitate the double homologous recombination, the transfection plasmid was linearized and electroporated into the *P. berghei ANKA* WT schizonts. The transgenic parasites generated were confirmed for correct site specific integration by diagnostic PCR that revealed GFP-DHFR cassette at the *Ubc13 kinase/PK9* locus. The transfected parasites were subjected to limiting dilution to obtain clonal population that was further confirmed by PCR. When identical numbers of infected RBC (irbc) of WT or *Ubc13 kinase/PK9* KO were injected into mouse, both parasite lines propagated at identical rates thus revealing a non-essential role of Ubc13 kinase/PK9 in asexual propagation of blood stages. However upon transmission of the *Ubc13 kinase/PK9* KO parasites into female *Anopheles* mosquitoes, the KO parasites failed to form ookinetes, the motile forms of the zygote that normally penetrates the midgut epithelium and gets attached on the haemocoel side of the midgut. The inability of the *Ubc13 kinase/PK9* KO to make ookinetes coincided with the complete absence of oocysts as observed on D14 post infection to female mosquitoes. These studies revealed the essential role of Ubc13 kinase/PK9 in sexual development.

In order to investigate the role of Ubc13 kinase/PK9 beyond oocyst stages a conditional mutagenesis system was employed. The most commonly used conditional gene silencing systems are those of Cre/loxP and Flp/FRT. Cre/loxP system was identified in bacteriophage P and is used for stage specific gene silencing [216]. Flp/FRT system is similar to Cre/loxP system in which Flp recombinase of yeast excises the sequence between FRT sites (flippase recognition target sites) [217, 218]. Flp/FRT system of yeast has been successfully employed in *P. berghei* for conditional mutagenesis of target gene [202]. As *Ubc13 kinase/PK9* knockout exhibited a block in sexual stage development, we generated *Ubc13 kinase/PK9* conditional knockout parasites by transfecting *Ubc13 kinase/PK9* knockout

construct with heterologous regulatory sequence in transgenic parasites that express FLP under TRAP promoter (TRAP/FLP parasite line where FLP is active in sporulating oocyst stages). *Ubc13 kinase/PK9* conditional knockout parasite infected mosquitoes were maintained at 25°C to allow maximal FLP activation. Conditional knockout parasites successfully completed the life cycle in the mosquito which was revealed by formation of salivary gland sporozoites. The *Ubc13 kinase/PK9* conditionally silenced sporozoites when inoculated through mosquito bite or by intravenous injection completed liver stage development and initiated blood stage infection. This revealed the non-essential role of Ubc13 kinase/PK9 in: sporulating oocysts, egress and invasion of sporozoites into salivary glands, infection of hepatocytes and liver stage development followed by infection of RBC. These studies reiterated the specific role of Ubc13 kinase/PK9 only in sexual development and its non-essential role in other life cycle stages.

Ubc13 kinase/PK9 phosphorylates Ubc13 at serine 106 residue and reduces its conjugating activity [127]. We also analyzed Ubc13 protein expression by generating Ubc13 mcherry transgenic parasites in which mcherry expression is regulated by Ubc13 promoter. Visualization of red fluorescence corresponded to mCherry expression that represented Ubc13 promoter activity. These mCherry transgenics revealed Ubc13 expression was prominent in asexual blood stages and oocyst stages. The exclusive role of Ubc13 kinase/PK9 in sexual stages and its substrate localization in asexual blood stages and in oocyst stages may point to the likely role of Ubc13 in transducing Ubc13 kinase/PK9 effects in the parasite. Ubc13 mediates K-63 ubiquitination which targets proteins to non-proteasomal functions including DNA repair, mitotic progression, apoptosis and immune signaling [219-222]. DNA replication is a key step in *Plasmodium* sexual reproduction as it involves both meiotic and mitotic divisions. Inhibition of DNA replication in nek-4 KO zygotes inhibited the formation of ookinetes [64]. As Ubc13 is involved in post replication error repair, the enzyme could play a significant role during formation of zygote and ookinete in *Plasmodium* [219]. The knockout of *Ubc13 kinase/PK9* affects the functions of Ubc13 which impacts the functions of zygote and ookinete formation concurring with the phenotype of *Ubc13 kinase/PK9* knockout parasites.

The fact that Ubc13 kinase/PK9 regulates the E2 conjugation activity and the depletion results in a transmission blocking phenotype is not surprising given that, ubiquitination regulates several important biological functions in the parasite. In *Plasmodium*

ubiquitination represents one of the prominent and most abundant post translational modifications [194] tagging nearly 70% of the *Plasmodium* proteome [223], to mediate a spectrum of biological processes. The existence and importance of ubiquitin system in *Plasmodium* started to receive attention only since 2007 [159, 224] when the components of the ubiquitin proteasomal system (UPS), that included ubiquitin or ubiqutin like modifiers, ubiquitin activating enzyme (E1), ubiquin conjugating enzyme (E2), ubiquitin ligases (E3) and deubiqutinating enzymes (DUBs) were shown to be encoded by the *Plasmodium* genome. These components were present in all species of *Plasmodium*, including other medically important protozoan parasites like *Toxoplasma gondii, Cryptosporidium parvum* and other species like *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Homo sapiens* and *Arabdiopsis thaliana*. Two important aspects of the ubiquitination pathway make them as an ideal target for malaria drug discovery, one being the specificity of certain components involved in this pathway [127], and other being their important biological roles mediated by the action of ubquitin conjugation to effector proteins.

Experimental MudPIT approach identified a total of 437 ubiquitin conjugated proteins following immuno precipitation from three asexual life cycle stages of *P. falciparum* [223]. Amongst these ubiquitin conjugates, the most abundant were from schizont stages where 63 proteins were identified out of which 50 matched the ubiquitin targets as predicted by *insilico* methods. The analysis of the MudPIT data set revelaed other important functional clusters: 12% consisting of chaperones, 11% consisting of proteins involved in translation, RNA metabolism and ubiquitin dependent metabolic processes. Proteins involved in transcription represented 4% of the data set that contained a subunit of RNA polymerase (PFB0715W) (an orthologue of which is shown to be associated with transcriptional arrest in yeast [225, 226] and a putative transcription factor ApiAP2 (PFF0200c) that is mainly expressed and found ubiquitinated only in the schizont stages. Other characteristics of this data set included 8% of proteins involved in process of invasion, 11% of proteins included UPS like 26S proteasome (PF08_0109, PF_130063), one E2 enzyme PFC0225c and 2 ubiquitin ligases: PF08_0020 and PF10_0046.

These interesting findings reveal that ubiquitin targets are involved in a wide variety of biological processes of parasite. The fact that high proportion of *Plasmodium* proteome is a target for ubiquitination in all asexual life cycle stages [223] is consistent with the occurrence of high lysine content in the *Plasmodium* proteins that may act as linkage points for further

addition of ubiquitin moieties. In fact, ubiquitin itself has 7 lysine residues that can offer as scaffolds for linking and extending ubiquitin chains. These occur at position, Lys-6, Lys-11, Lys-27, Lys 29, Lys-33, Lys-48 and Lys 63. Of these, Lys-48 is the pre dominant linkage found in eukaryotes, that facilitates the ubiquitin tagged proteins to 26S proteasome degradation. The roles of Lys-6, Lys-11, Lys-27, Lys 29, Lys-33 are not known completely [227]. However, there is definitive evidence that Lys-6, Lys-11, Lys-48 and Lys-63 linkages exist in *P. falciparum* [223]. Such ubiquitination of diverse Lys residue may play a critical regulatory role in *Plasmodium* parasites that are yet to be discerned.

A logical explanation of the phenotype obtained in our study can be attributed to the absence of the negative regulation of Ubc13 when Ubc13 kinase/PK9 is depleted. We hypothesize that under such conditions, there could be enhanced ubiquitination of the target proteins, leading to its UPS mediated degradation. If such target proteins are transcriptional factors/signaling molecules/enzymes that act as critical switch points, it is likely to impact the transition of parasites from one stage to the other. It is also possible that extent of ubiquitin conjugation may differentially regulate the fate of cellular proteins i.e., a steady state ubiquitin levels could stabilize the target or effector protein and higher levels of ubiquitination may channel target/effectors to UPS mediated degradation. While several regulatory mechanisms may exist to alter the cellular ubiquitin activity, we report here one being regulated by Ubc13 kinase/PK9. We indeed observed 4-5 proteins that are highly ubiquitinated in total cellular lysates obtained from synchronized schizonts of *Ubc13 kinase/PK9* KO as compared to WT. Interestingly, we also observed that levels of Ubc 13/E2 were also enhanced in the *Ubc13 kinase/PK9* KO cellular lysates. While the higher Ubc13/E2 levels may not necessarily mean an increased ubiquitin conjugation activity, the fact that certain cellular proteins in *Ubc13 kinase/PK9* KO are highly ubiquitinated does imply that high Ubc13/E2 levels coincide with its enhanced conjugation activity. In fact there are reports that have shown the role of Ubc/E2 in ubiquitin conjugation, without the involvement of the E3 ligases [228]. The identity of proteins that are comparatively more ubiquitinated in the cellular lysates of *Ubc13 kinase/PK9* KO and their role in blocking the progression of the sexual cycle is currently under investigation.

Consistent with the observation that *Ubc13 kinase/PK9* KO played an essential role in sexual reproduction, we observed several sexual stage transcripts specific to zygote and ookinete surface proteins (CPW-WBC family, SOAP and PSOPs), kinases (NEK-2, NEK-4,

MAPK-2, CDPK3), transcription factors (AP2-G and AP2-O), cytoskeletal regulatory elements of male gamete motility (MISFIT, actin II, flagellar outer arm dynein associated protein and PF16), gamete specific genes (p48, p47, p230 and MDV-1) and meiotic recombination (DMC-1) being down regulated. The absence of ookinete formation may partly be attributed to the down regulation of genes pertaining to ookinete surface proteins like SOAP and PSOPs. However, whether the block in sexual reproduction precedes ookinete formation is not known, but a likely possibility may be that Ubc13 kinase/PK9 may have unique role in male gametogenesis, owing to the down regulation of male specific transcripts in the KO like MISFIT, actin II, flagellar outer arm dynein associated protein, PF16 and MDV-1. Studies are underway to investigate if Ubc13 kinase/PK9 plays a role in male and female gamete formation. This is intended to be done by crossing the *Ubc13 kinase/PK9* KO with either male or female gamete defective lines that express mCherry. If *Ubc13 kinase/PK9* KO generates functional male or female gametes, they complement respectively the male defective and female defective lines resulting in successful completion of sexual reproduction and formation of oocyst that express both GFP and mCherry.

Sexual reproduction is one of the bottlenecks during parasite life cycle in which kinases play a significant role [62]. For example, *P. berghei* CDPK4 is reported to have a necessary role in exflagellation of *Plasmodium* male gametocytes. *Pb CDPK4* knockout male gametocytes could not exflagellate resulted by block in DNA replication [59]. In addition to CDPK4, MAP2 and SRPK are essential for exflagellation [63 and 145]. Other than CDPKs, NIMA related protein kinases of *Plasmodium* are also required for completion of sexual development and thus are targets for transmission blocking drugs or vaccines [55, 56].

Conclusions

The process of sexual reproduction begins with exflagellation of male gametocytes and culminates with the sporulation in oocyst requiring the functions of many genes including kinases. Several *Plasmodium* kinases are necessary for malarial transmission which makes them attractive targets as transmission blocking candidates. Our study adds, to the currently existing list one additional kinase called as Ubc13 kinase/PK9 that belongs to orphan kinases, and regulates *Plasmodium* sexual stage functions. The Ubc13 kinase/PK9 does not cluster with kinase groups though the protein contains necessary domains for kinase activity. Ubc13 kinase/PK9 does not have any homologues in humans; therefore, the kinase inhibitors could

be exclusive in blocking the parasite development without the risk of affecting the host kinases. The exciting possibility of using kinase inhibitors for preventing malaria transmission has recently shown to be feasible [229]. Referred to as bumped kinase inhibitors (BKI-1), these novel class of transmission blocking compounds, specifically designed to act against *Plasmodium* CDPK4, were effective in inhibiting micro gamete exflagellation in both *P. falciparum* and *P. berghei* [229]. Both oocyst formation and sporulation were affected in mosquitoes that received an infective blood meal treated with BKI-1. As Ubc13 kinase/PK9 is essential for sexual stage development and acts as a regulator for transmission of malaria to mosquitoes, small molecule inhibitors developed against Ubc13 kinase/PK9 can be analyzed for its potential to prevent malarial transmission, thus opening avenues for novel antimalarial drug discovery approaches.

CHAPTER 3

Role of *Plasmodium berghei S23* in the egress of liver stage parasites

3.1 Introduction

Plasmodium belongs to phylum apicomplexa characterized by unique organelles present at the anterior end of all invasive forms like sporozoites, merozoites and ookinetes. This apical complex is essential for the parasite invasion [230]. Apical complex of *Plasmodium* consists of three types of membrane bound organelles, rhoptries, micronemes and dense granules. Secretion of proteins from the apical organelles plays a significant role in host cell recognition, invasion and host-parasite interaction. Rhoptries are pear shaped and most prominent secretory organelles consisting of two distinct regions; a duct like structure called rhoptry neck and a bulb like structure called rhoptry bulb region. Both regions contain different set of proteins referred to as rhoptry neck proteins (RONs) and rhoptry bulb proteins (ROPs) that reside in rhoptry neck and bulb respectively [231]. Rhoptry proteins are important for invasion and parasitophorous vacuole formation. Micronemal proteins are important for host cell adhesion and invasion especially in ookinetes, as this stage is deficient in rhoptries and dense granules [232]. Dense granules were first observed in *Sarcocystis tenella* and many dense granule proteins are not characterized in *Plasmodium*. The proteins in dense granules are released after the fusion of dense granule membrane with parasite plasma membrane (PPM) [233]. Along with rhoptry and micronemes proteins, dense granule proteins are also involved in host cell invasion by apicomplexan parasites like *Plasmodium*, *Toxoplasma* and *Cryptosporidium* [233].

Sporozoites are formed within the oocyst and acquire motility before egress. Circumsporozoite protein processing is essential for sporozoite egress from mature oocysts [77]. But the enzyme responsible for CSP cleavage is not known. A putative cysteine protease ECP-1 (egress cysteine protease-1) was reported to be required for egress of sporozoites from oocysts [234]. Knockout of *ecp-1* inhibited the egress of sporozoites though *ecp-1* KO sporozoites showed circular movements inside oocysts. A differential processing of the CSP in *ecp-1* mutant sporozoite likely indicates its direct or indirect role in proteolytic cleavage of CSP [234]. *Plasmodium* sporozoites that are inoculated into the skin of the host during mosquito bite traverses through a variety of cell types and enter into blood circulation and reach liver. A small portion of sporozoites remain at the site of inoculation in the skin and develop into skin exo-erythrocytic forms (EEFs) [235, 236]. Once sporozoites reach liver, major sporozoite surface proteins, CS and thrombospondin related anonymous protein (TRAP) interacts with heparin sulfate proteoglycans of hepatocytes, facilitating sporozoite

attachment to hepatocytes [169, 268 and 269]. The process is followed by formation of a moving junction between hepatocytes membrane and the parasite plasma membrane and involves the activity of an actomyosin motor present beneath the parasite plasma membrane. The generic events associated with the formation of the moving junction seems to be highly conserved across the two stages viz., the entry of merozoites into the RBC and entry of sporozoites into hepatocytes [13, 237]. Sporozoite invasion is followed by formation of parasitophorous vaculole membrane (PVM), which acts as a barrier between the parasite and the host cell [238]. It is speculated that in addition to CS and TRAP, other sporozoite secretory proteins may be involved in invasion and PVM formation. The expression of these proteins may be necessary for the parasite survival within the host cell and for successful liver stage development.

Formation of PVM is obligatory step in the *Plasmodium* life cycle as it plays a significant role in nutrient uptake, protein trafficking and host parasite interactions [239]. The role of PVM in protein export was first revealed in 1996 by showing PVM has ATP dependent protein translocation activity during blood stage development [240]. Later studies reported that proteins are exported to host cell through PVM by a translocon machine called PTEX (*Plasmodium falciparum* Translocon for Exported proteins) in blood stages [241, 242]. PTEX proteins are also expressed in sporozoites and liver stages or EEFs [243]. PTEX is a complex of five proteins which interact with each other. The components include exported protein 2 (EXP2), the ATP binding subunit HSP101, the redox protein thioredoxin 2 (TRX2) and two hypothetical proteins termed PTEX150 and PTEX 88. PTEX proteins are found in dense granules and are transported to newly formed PVM during invasion. Immunoprecipitation studies revealed the interaction of PTEX with *Plasmodium* export element carrying proteins suggesting that PTEX can be transported by these carrier proteins [239, 241]. Proteins should be unfolded before export though PVM [242]. HSP101 plays a role in unfolding of proteins to be exported [241, 242]. EXP2 forms a pore through which protein export takes place. Deletion of TRX2 and PTEX88 in *P. berghei* showed delay in growth of asexual blood stages indicating they are accessary components of PTEX [243, 244]. Deletion of PTEX150 and HSP101 was not successful revealing the essential role of both proteins in intra erythrocytic development. Conditional mutants of HSP101 and PTEX 150 showed inhibition of protein export completely [245]. *Plasmodium* export proteins are classified into PEXEL containing export proteins and PEXEL negative export proteins (PNEPs) based

on presence or absence of an export element called PEXEL motif. PEXEL motif consists of a conserved region with five amino acids RxLxE/Q/D [53, 246, and 247]. *Plasmodium* export proteins play a wide variety of functions necessary for parasite growth, virulence and development. PTEX is essential for export of both PEXEL containing proteins and PNEPs as conditional knockout of PTEX150 and HSP101 affected the export of both types of proteins. Owing to its central role in protein export in *Plasmodium*, PTEX is an attractive target for drug development [245].

PEXEL motif (RxLxE/Q/D) is conserved among *Plasmodium* species and more than 300 *P. falciparum* proteins contain this motif [53, 54 and 246]. PEXEL dependent export was first identified in *Plasmodium* infected erythrocytes and it was revealed that PEXEL containing proteins are exported beyond PVM to erythrocyte cytosol [53, 246]. Later studies unraveled the role of PEXEL dependent protein export to be conserved also in liver stages [248], where it was demonstrated that CSP contains two functional PEXEL motifs that is utilized for the export of CSP into hepatocyte cytosol, that promotes development of liver stages. Both motifs in CSP are functional and mutation in both PEXEL motifs blocked export of CSP [248]. Before export, PEXEL containing proteins are processed as the motif contains a protease cleavage site [249]. The processing takes place at leucine residue and required for protein export [249]. Despite the lack of PEXEL motif, few proteins of *Plasmodium* are reported to be exported to host cell cytosol, referred as PEXEL negative exported proteins [247, 250 and 251]. Mode of transport of PNEPs is clearly not known but experimental evidence shows that PNEPs are exported by translocation through PVM to the host cell cytosol [247].

Another interesting family of membrane proteins that localize to the PVM is the Early transcribed membrane proteins (ETRAMPs) which are specific to *Plasmodium* genus [252, 253]. ETRAMP family consists of 14 members in *P. falciparum*. ETRAMPs are highly transcribed genes in *P. falciparum* and corresponding protein levels are also found in high abundance in PVM [254, 255]. So these proteins are considered as prominent proteins of PVM [256]. Different ETRAMPs show distinct spatial expression in *P. falciparum*. Ring stage ETRAMPs are different from trophozoite ETRAMPs [253]. ETRAMPs contain a most variable and highly charged carboxy terminus that faces towards host cytoplasm. ETRAMPS were also found in *P. berghei* and were referred as small export proteins (SEPs) and show

similar characteristics of *P. falciparum* ETRAMPs [257, 258]. Most of the ETRAMPs are localized to PVM but SEP2 and SEP3 of *P. berghei* were observed as dot like structures in the erythrocyte [258]. Comprehensive study of *P. yoelii* ETRAMPs identified 9 encoded genes. Gene deletion of 7 *P. yoelii* ETRAMPs out of 9 revealed the non-essential role in intra erythrocytic development [259]. Deletion of remaining two genes was not successful. The PyETRAMPS are closely related unlike PfETRAMPs that are more diverse [239]. *Pbsep2* and *Pbsep3* gene knockouts were not successful indicating a significant role of these genes in the parasite. PbSEP2 is highly expressed throughout *Plasmodium* life cycle as compared to PbSEP3 which is a low abundance protein [260]. In the sporozoite stage PbSEP2 is localized to the surface and is secreted during sporozoite gliding motility but the exact role of SEP2 and SEP3 are not known [260]. The expression of PfETRAMPs is not well studied in sexual stages but PfETRAMP10.3 was found in the periphery of gametocytes [261]. PfETRAMP10.3 is the only known ETRAMP that expressed in gametocytes. UIS3 and UIS4 are two PbETRAMPs that expressed in liver stages but are absent in blood stages [26, 27]. These two proteins are localized at PVM and play a very significant role in liver stage development [26, 27 and 86]. Both proteins show characteristic ETRAMP protein structure including highly charged carboxy region facing towards host cell cytoplasm. Knockout of *uis3* and *uis4* arrested liver stage development and both are used as gold standards for genetically attenuated parasites [26, 27 and 86].

In addition to UIS3 and UIS4, many sporozoite proteins are required for sporozoite invasion, establishment of liver stage infection and development into liver stages. To identify other novel sporozoite stage specific proteins, a technique called suppression subtractive hybridization (SSH) was utilized. Screening of the SSH library facilitated the identification of 25 genes in *P. yoelii* [167] that were induced in the sporozoite stage. These genes were named as 'S' genes. The successful enrichment of the sporozoite specific genes was also apparent from the occurrence of gene sequences that encoded for major sporozoite surface proteins like CS and TRAP that were amongst the S genes. Recently, a few of these S genes were functionally characterized and were shown to play important roles in various processes like gliding motility, sporozoite invasion and liver stage development.

CS is the most predominant sporozoite surface protein expressed from oocyst stage to sporozoite stages. CS is a multifunctional protein that contains a signal peptide, amino acid

repeat domain and a hydrophobic c-terminal sequence [262, 263]. Region I, II plus and region III are conserved motifs of CS protein among *Plasmodium* species. Region I is required for the attachment of sporozoites to host cells which contains 5 amino acid sequence KLKQP [263]. Region II plus is a part of thromospondin repeat (TSR) type I domain which involves in adhesion of sporozoites to heparin sulfate proteoglycans of liver sinusoids [169, 170, 264 and 267]. Region III supports the region II plus adhesion motif. CS is required for the formation of sporozoites as the deletion of CS resulted in oocysts lacking sporozoites [265]. Egress of sporozoites from mature oocysts is CS dependent mechanism as CS processing is required for the egress [77]. Mutation at region II plus prevents the egress of sporozoites and the mutated oocysts are resistant to proteolytic activity [77]. Experimental evidence suggests that CS also promotes liver stage development [248].

Another well characterized S gene that plays a very significant role in invasion of sporozoites to host cells in mosquito as well as in vertebrate host is thrombospondin related anonymous protein (TRAP). TRAP is a micronemal protein which is exported on to the surface of the sporozoite and also referred as sporozoite surface protein 2 (SSP2) [266]. TRAP consists of TSR domain and integrin like A domain that helps in adhesion of sporozoite to target cells. TRAP is required for sporozoite gliding motility, invasion and infectivity [173]. Invasion of sporozoites into salivary glands and hepatocytes require TRAP and mutation of TRAP affects gliding motility of sporozoites thus reducing number of the salivary gland sporozoites by 1000 fold. Subsequently TRAP is also necessary for sporozoite invasion into hepatocytes by binding to HSPGs [268, 269 and 78].

TREP is a TRAP related protein that is necessary for sporozoite gliding motility and invasion of salivary glands [175, 270]. Likewise, thrombospondin related sporozoite protein (TRSP) showed similar function as the gene deletion reduced sporozoite invasion efficiency *in vitro* and *in vivo* [174]. A sporozoite stage specific protein, SAP-1 (Sporozoite Aspargine Rich Protein) was shown in independent studies to play a role in post transcriptional regulation of sporozoite infectivity [82], for successful liver stage infection [81] and for initiation of liver stage development [271]. Sporozoites traverse through different cell types prior to liver stage infection [13]. Sporozoite micronemal protein essential for cell traversal (SPECT-1) is necessary for cell traversal of sporozoites and *spect-1* deleted sporozoites completely lost their ability to pass through kupffer cells [10]. SPECT-2/Plasmodium perforin like protein-1 is a

micronemal protein which is also involved in sporozoite invasion. SPECT-2 mutants demonstrated reduced sporozoite infectivity [11]. In addition to the role of S genes central to sporozoite functions, few S genes are also involved in ookinete invasion. Cell traversal protein for ookinetes and sporozoites referred to as CelTOS, plays an important role in ookinete infectivity, as CelTOS deficient parasites have a 1000 fold less oocysts numbers [172]. Sporozoite invasion associated protein (SIAP) 1 is essential for egress of sporozoites from oocysts and also for sporozoite invasion [171, 272].

Although many of S genes are characterized, the function of few S genes remains to be elucidated. Towards this end, we studied the role of S23 in rodent parasite strain, *P. berghei* using reverse genetics approach. We deleted *S23* gene locus by double homologous recombination and analyzed the phenotype of the *S23* KO parasite. While *S23* KO parasites showed no defect in their ability to infect erythrocytes and complete cycle in the mosquito stages, we observed that the KO failed to initiate blood stage infection in mouse when the sporozoites were introduced by natural mosquito bite or by intravenous injection. *In vitro* liver stage development revealed that *S23* KO parasites grew better than WT counterparts with regard to size, though, under *in vivo* conditions, they were not able to initiate a blood stage infection. These observations revealed the role of *S23* in the egress of the late liver stage parasites, a prerequisite for initiation of blood stage infections.

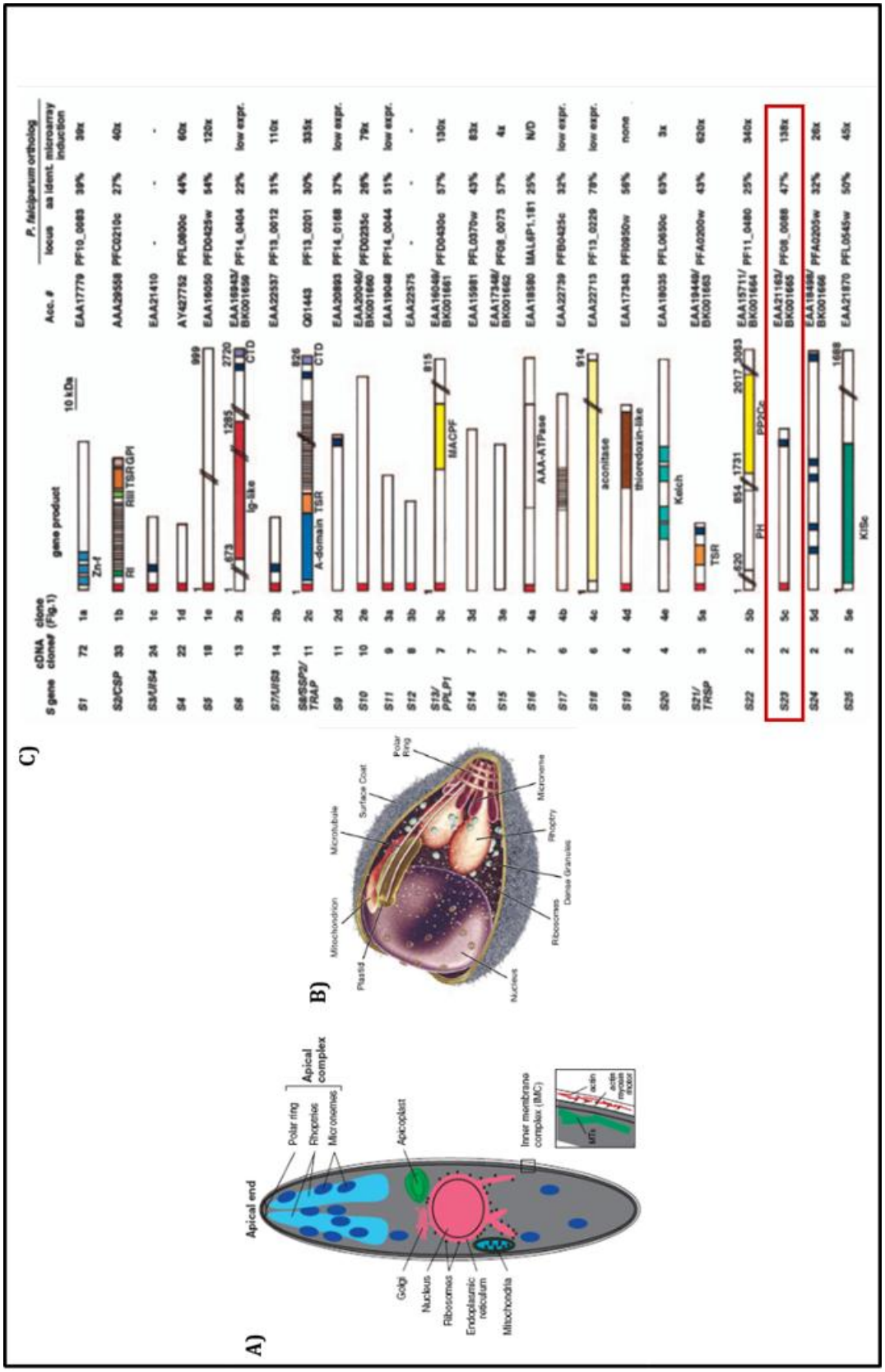

Fig 22. Schematic representation of sporozoite and merozoite stages that invade respectively the hepatocytes and RBC. (A) *Plasmodium* sporozoite stage showing highly organized apical end with polar rings, rhoptries and micronemes. Other cellular organelles include apicoplast, Golgi, nucleus, ribosomes, endoplasmic reticulum and mitochondria. Present underneath the sporozoite plasma membrane is an actin myosin molecular motor and microtubules (Kappe et al., [REF]. (B) Merozoites showing apical end with polar ring, micronemes, rhpotries and dense granules. Other cellular organelles include plastid, nucleus, mitochondria, ribosomes and microtubules. (Jaśkiewicz et al.,). (C) *S23* gene was identified following suppressive subtractive hybridization of sporozoite versus merozoite stages of *Plasmodium yoelli* (Kaiser et al.,). Twenty five S genes were recovered following SSH of sporozoite stages versus merozoite stages. Of the 25 S genes, 12 encoded for signal peptide likely indicating their role in secretory pathway. The expression levels of 25S genes were analysed in *Plasmodium falciparum* through microarray analysis. One of the S genes, S23 showed 138 fold micro array induction. Figure A was obtained from Kappe et al., [263]. Figure B was obtained from Jaskiewicz et al., [273] and Figure C was taken from Kaiser et al.,[167].

3.2 Materials and methods

3.2.1 Experimental animals and parasite lines

Eight to ten weeks old female Swiss albino, BALB/c or C57BL/6 mice were purchased from National Institute of Nutrition (NIN) and used for all animal experiments. Animal handling was strictly in accordance to Institutional Animal Ethical Committee protocols. *S23* KO was generated by transfecting *S23* KO targeting plasmid in wild type *P. berghei ANKA* parasite line. Wild type *P. berghei ANKA* GFP line (Surendra KK and Kumar KA, personal communications) was used for comparison of phenotype of knockout parasites throughout the parasite life cycle. Life cycle of all the genetically modified/transgenic parasites were studied by cycling the parasites through Swiss Albino or BALB/c and female *A. stephensii* mosquitoes. In experiments involving analysis of pre-patent period either by mosquito bite or intravenous injection of sporozoites, C57BL/6 mice were used.

3.2.2 Cell lines

HepG2 cell lines were purchased from National Center for Cell Science (NCCS), Pune. Cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM)(Gibco) containing 2mM L-glutamine, 25mM HEPES and 4.5g/L glucose supplemented with 10% fetal bovine serum (FBS) (Hyclone laboratories), 1X antibiotic-antimycotic (Gibco).

3.2.4 Retrieval of *P. berghei S23* gene and protein sequences, sequence alignment analysis of S23

Two public domain databases, Plasmodb (www.plasmodb.org) and Genedb (www.genedb.org) were used to retrieve all DNA and protein sequences related to *Plasmodium* species. Primers were designed based on the sequence information. To check the homology of S23 protein among *P. berghei, P. yoelii and P. chabaudi* which are rodent *Plasmodium* strains, we used MULTALIN (http://multalin.toulouse.inra.fr/multalin/).

3.2.3 Primers

Primers used for construction of transfection plasmids and to confirm site specific integration are mentioned in the table 5.

Table 5. Showing primers used for generation and confirmation of *S23* KO (Fig 24).

S.No	Name of the primer	Primer sequence (5'-3')
1	S23 5' FP (FP1)	**AGTCTCGAG**ACACTTATTTTATCATATTA
2	S23 5`RP (RP1)	**ATAATCGAT**TTTTAATAGCGTGATGTAA
3	S23 3`FP (FP2)	**ATAGCGGCCGC**TTCCATAAAACATCAAAATA
4	S23 3`RP (RP2)	**ACTGGCGCGCC**AACATGATTCGCACAAAA
5	S23 5' confirmation FP (FP3)	TATAACCCAAATGCTTCG
6	HSP705' UTR RP (RP3)	TTCCGCAATTTGTTGTACATA
7	DHFR FP (FP4)	GTTGTCTCTTCAATGATTCATAAATAG
8	S23 3' confirmation RP (RP4)	TTGAATAACAAAATAAAGAT
9	S23 ORF FP (FP5)	ACATTTAAACAACAATATCC
10	S23 ORF RP (RP5)	TTTAATATTTGTATCGTAAGA

3.2.5 Generation of *S23* (PBANKA_142520) knockout construct

S23 knockout construct was generated for target gene deletion. A 573bp of S23 5'flanking region was amplified by PCR using S23 5'forward primer (FP1) and S23 5' reverse primer (RP1). A 523bp fragment of S23 3'flanking region was amplified by using primers S23 3' FP (FP2) and S23 3' RP (RP2). The PCR products corresponding to 5' and 3' regions were cloned using XhoI/ClaI and NotI/AscI at MCS-I and MCS-II sites respectively. *S23* knockout plasmid was digested with XhoI and AscI and used for transfection.

3.2.6 Generation of *S23* knockout parasites

Linearized *S23* KO targeting construct was electroporated into *P. berghei ANKA* schizonts using U-033 program in Amaxa nucleofector device and injected intravenously into mice. The mice were kept under pyrimetamine drug pressure and parasitemia was monitored daily by Giemsa stained blood smears. Genomic DNA was isolated from drug resistant

parasites and site specific 5' and 3' integration was confirmed by primers designed at beyond sites of recombination. Limiting dilution was performed to obtain clonal population of *S23* KO parasites. S23 ORF specific primers were used to confirm the deletion of *S23* gene locus.

3.2.7 Analysis of *S23* KO parasite asexual and sexual stage propagation

Asexual blood stage propagation of *S23* KO parasites was compared with WT parasites by injecting intravenously, $1X10^3$ infected RBC of either *S23* KO or WT into a group of 3 BALB/c. In a different experiment, female *Anopheles* mosquitoes were allowed to take blood meal either from mice infected with *S23* KO or WT GFP parasites. Mosquitoes were dissected on day 14 to observe the oocyst infectivity and on D18-D21 to observe salivary gland sporozoites.

3.2.8 Isolation of *S23* KO sporozoites from mosquito salivary glands

The infected salivary glands containing *S23* KO sporozoites were dissected on D18 post infection in DMEM. The glands were disrupted and centrifuged at 800 rpm for 3 min at 4°C. Supernatant containing sporozoites were collected and used for *in vitro* and *in vivo* experiments to study the *Plasmodium* liver stage development.

3.2.9 Transmission of *S23* KO sporozoites

To unravel the role of *S23* in liver stage development, sporozoites were inoculated by natural mosquito bite or by intravenous injection. The mice were monitored for blood stage infection daily by Giemsa staining of blood smears.

3.2. 10 *In vitro* EEF development

HepG2 cells were seeded two days prior to the experiment. Approximately $1X10^5$ cells were seeded per each well of Labtek chamber slide that contained 500 µl of DMEM supplemented with 2 mM L-glutamine, 25 mM HEPES and 4.5 g/L glucose supplemented with 10% fetal bovine serum (FBS) and , 1X antibioc-antimycotic. The *S23* KO sporozoites isolated from the disrupted salivary glands were counted using hemocytometer and $2X10^4$sporozoites were added to each well. Labtek chamber slides were centrifuged after addition of sporozoites to facilitate sporozoite adhesion to HepG2 cells. Complete DMEM with 1X antibiotic was replaced with 4X antibiotic-antimycotic containing DMEM throughout

EEF development. At 12 h, 36 h and 65 h time points, EEFs were fixed with 4% paraformaldehyde. EEFs were permeabilized and stained with UIS4 antibody followed by secondary Alexaflour 594. EEFs were observed under fluorescence microscope (Nikon Eclipse NiE AR).

3.3 RESULTS

3.3.1 S23 is conserved among *Plasmodium* rodent species

Alignment of S23 protein sequences of *P. berghei, P.yoelii* and *P. chabaudi* showed 76-85% homology. (Fig 23)

3.3.2 *S23* gene locus is replaced with GFP-hDHFR cassette by double homologous recombination

The strategy for *S23* gene knockout is shown in Fig 24. Genomic organization of *S23* gene locus was shown in Fig 24A. Two regions selected for homologous recombination are shown in the *S23* KO plasmid in Fig 24B. *S23* locus was replaced with GFP-DHFR cassette as shown in Fig 24C. PCR of *S23* 5' and 3' fragments was shown in Fig 24D. Double digestion of *S23* KO plasmid with XhoI/ClaI and NotI/AscI relased 5' and 3' UTRs respectively as shown in Fig 24E. Digestion of *S23* knockout plasmid with XhoI and AscI released the vector backbone and GFP-DHFR cassette bearing two homologous DNA fragments on either side of the cassette, is shown in Fig 24E. Integration PCR with indicated primers designed at sites beyond the recombination event confirmed the 5' and 3' UTR integration (Fig 24F). It also confirmed that *S23* gene locus was replaced with GFP-DHFR cassette in *S23* knockout parasites. After limiting dilution genomic DNA was isolated from *S23* KO clonal population (3 clones) and PCR with gene specific primers confirmed the deletion of S23 ORF (Fig 24G). *S23* KO parasites express GFP because of the integration of GFP cassette. (Fig 24H).

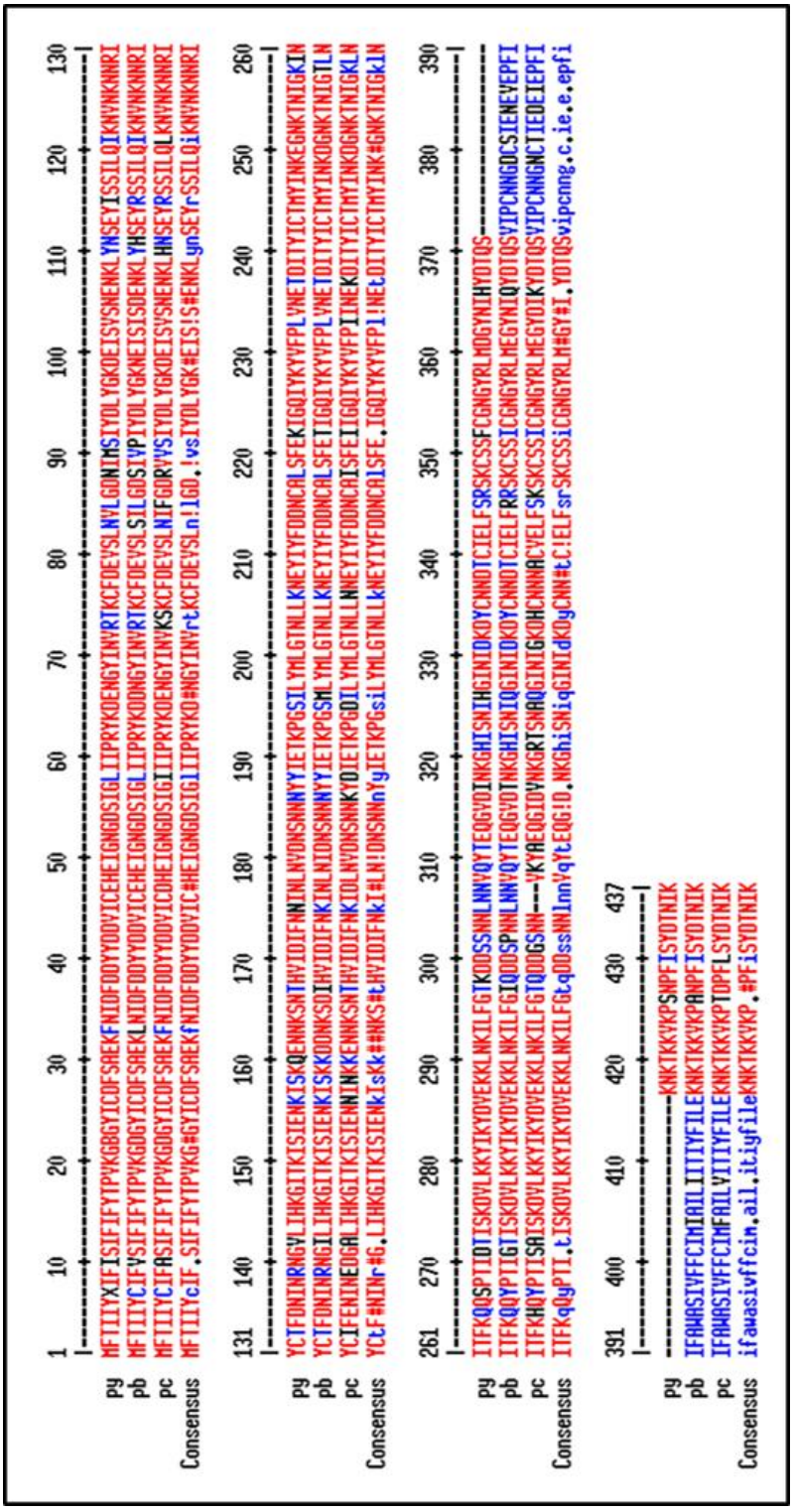

Fig 23. Amino acid sequence alignment of S23 in rodent species of *Plasmodium*. *Plasmodium yoelli (py), Plasmodium berghei (pb)* and *Plasmodium chaubadi (pc)*

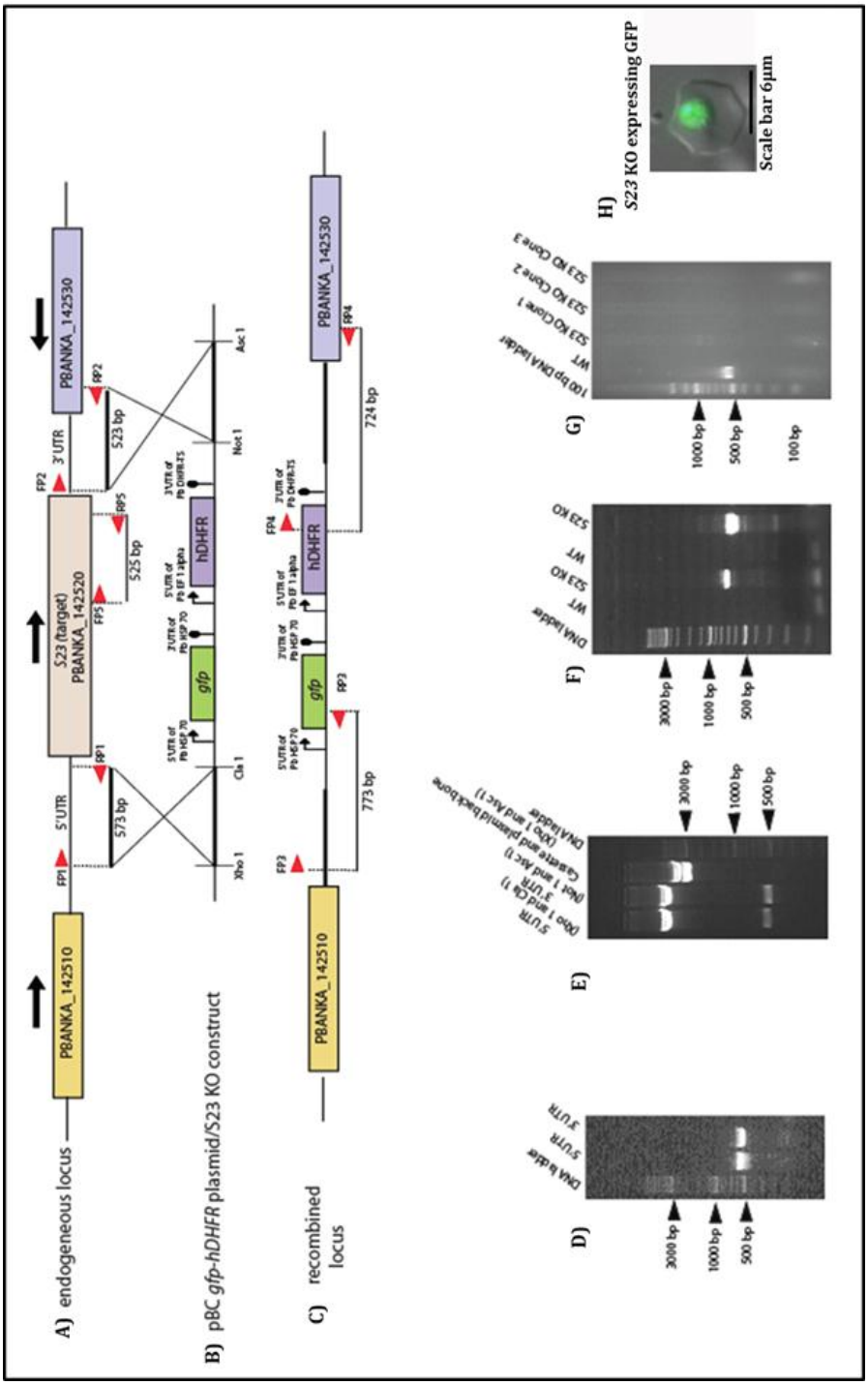

Fig 24. Generation of *S23* KO parasite line. A) Genomic locus of *S23* (PBANKA_142520) showing 5' and 3' UTRs. B) Elements of the targeting vector showing pBC-GFP-hDHFR. A 573 bp 5' fragment of S23 was cloned in XhoI/ClaI site of the targeting vector. A 523 bp 3' fragment was cloned into NotI/AscI site of the targeting vector. C) Recombined locus following successful double cross over recombination resulting in replacement of target gene, *S23* by GFP-DHFR cassette. D) A 1% agarose gel showing the PCR product of 5' and 3' UTRs. The 5' UTR fragment was amplified with primers *S23* 5' FP (FP1) and *S23* 5' RP (RP1) and 3' UTR fragment was amplified with *S23* 3' FP (FP2) and *S23* 3' RP (RP2). E) Release of 5' UTR fragment from transfection vector using restriction enzymes XhoI/ClaI and release of 3'UTR fragment from transfection vector using restriction enzymes NotI/AscI. Release of targeting cassette (5' UTR fragment+GFP-DHFR cassette+3' UTR fragment) and vector backbone using restriction enzymes XhoI/AscI. F) Diagnostic PCR using primers within the targeting cassette and beyond sites of recombination revealing the correct site specific integration. A PCR product with primers *S23* 5' confirmation FP (FP3) and HSP70 5' UTR (RP3) indicated a correct 5' integration and a PCR product with primers DHFR FP (FP4) and *S23* 3' confirmation RP (RP4) indicated a correct 3' integration. G) Genomic DNA isolated from cloned *S23* KO parasites does not amplify a PCR product from the ORF whereas WT parasites amplify a product of 527bp with primer set FP5 and RP5 H) A merged DIC image showing a GFP expressing *S23* KO parasite inside RBC.

3.3.3 S23 is not essential for asexual blood stages

To monitor, if *S23* depletion affected asexual blood stage propagation, two groups of BALB/c mice (3 mice per group) were intravenously injected with $1X10^3$ iRBC (infected RBC) of either WT or *S23* KO and the asexual blood stage replication was monitored for 7 days by making Giemsa stained blood smears. The identical propagation of *S23* KO as that of WT parasites and presence of all stages of asexual forms in *S23* KO revealed its non essential role in asexual blood stages (Fig 25).

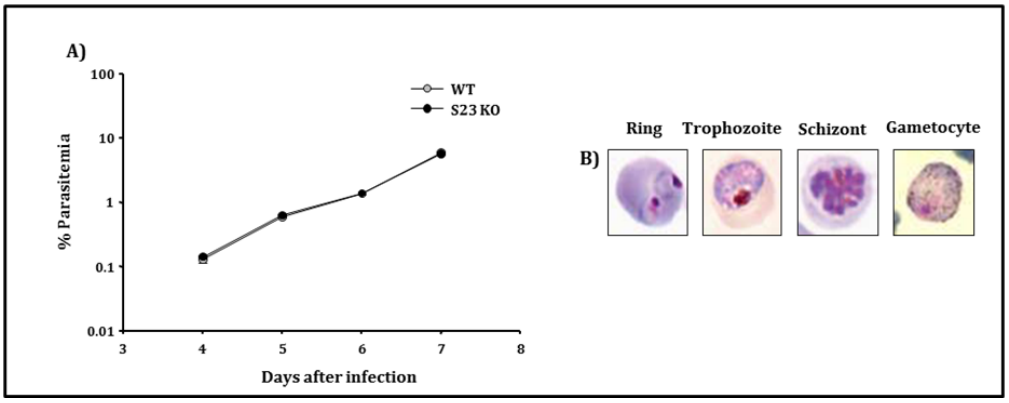

Fig 25. *P. berghei S23* KO asexual parasites propagate identically as WT parasites. A) $1X10^3$ infected RBC of either WT or *S23* KO were intravenously injected in two groups of mouse (3 mouse/group) and monitored for propagation of the parasites daily for 7 days by making Giemsa stained smears. B) Representative pictures showing asexual blood stages obtained from Rathore et al., [205].

3.3.4 S23 is not essential for *P. berghei* mosquito stages

Transmission of *S23* KO parasites to mosquitoes leads to formation of oocysts, whose numbers were comparable to the oocysts derived from the WT parasites (Fig. 26 A and B). The sporulation pattern inside oocyst (Fig 26C and D) and the ability of the egressed sporozoites to reach salivary gland (Fig 26E and F) also were comparable to that of WT parasites suggesting that *S23* KO manifested no defect in the mosquito stages of *P. berghei*.

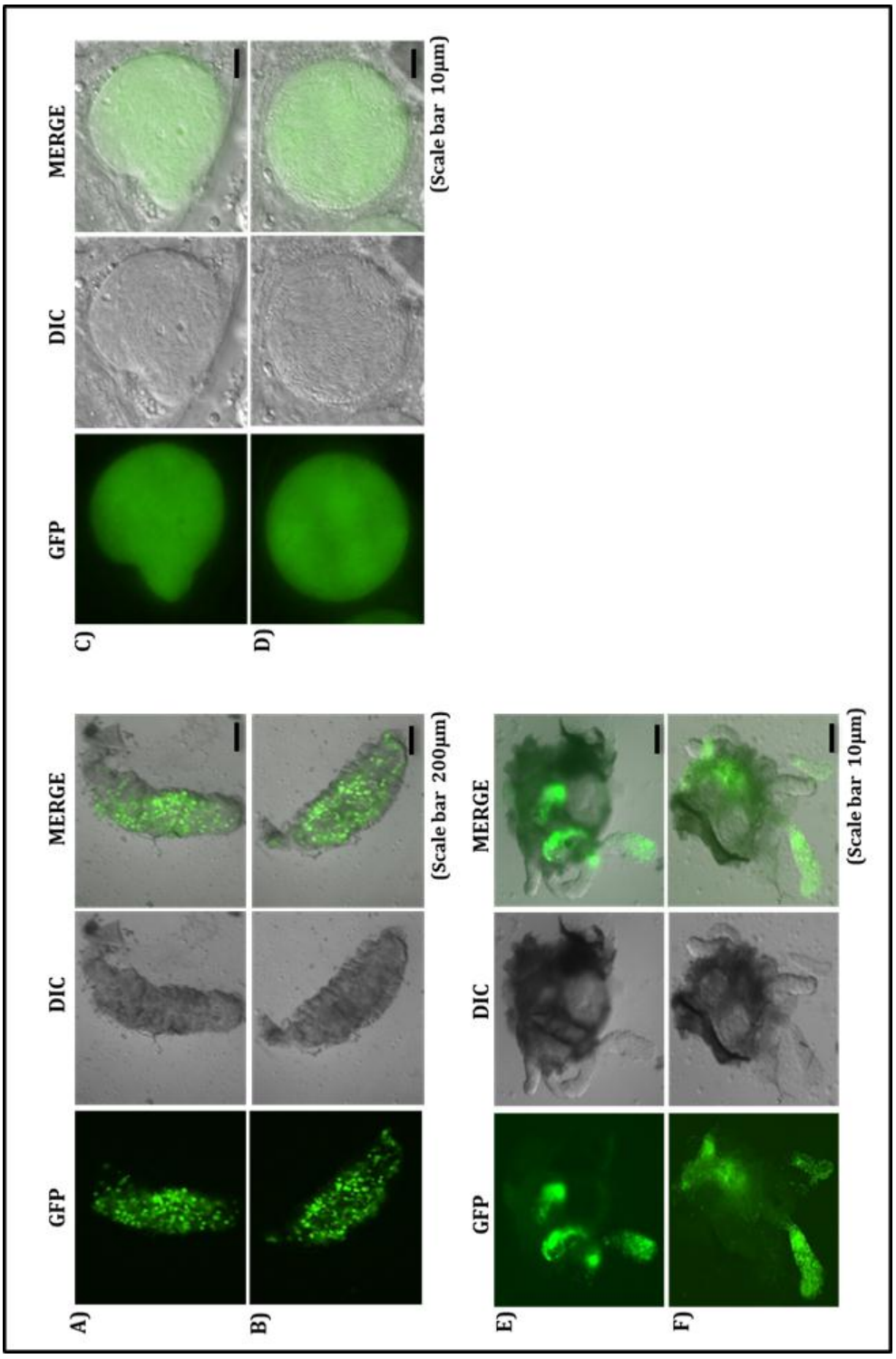

Fig 26. Mosquito stages of *P. berghei S23* KO do not show any defect in sexual development or sporulation. Malaria was transmitted to female *Anopheles* mosquitoes from mouse harboring gametocyte stages of either WT or *PbS23* KO. (A) Midguts showing oocyst derived from WT parasites. (B) Midguts showing oocyst derived from *PbS23* KO parasites. (C) A single magnified oocyst from WT (C) and *PbS23* KO (D). Dissected salivary glands showing WT GFP expressing sporozoites (E) or *PbS23* KO sporozoites (F).

3.3.5 *S23* KO sporozoites failed to initiate blood stage infection

Inoculation of *S23* KO sporozoites through natural mosquito bite or by intravenous injection with $2X10^4$ sporozoites did not initiate blood stage infection in five independent experiments. However WT sporozoites infection through bite or through intravenous injection of $2X10^4$ sporozoites resulted in a break through infection on D4 post infection. (Table 6).

Table 6. Transmission dynamics of WT sporozoites versus *S23* KO sporozoites

Parasite	Experiment no	Total number of mice used per experiment	Mode of Sporozoite infection		Prepatent* period
			By mosquito♣ bite	By intravenous injection	
WT	I	6	3	3	Day 4
	II	5	2	3	Day 4
S23 KO	I	5	1	4	No blood stage infection
	II	5	1	4	No blood stage infection
	III	3	1	2	No blood stage infection
	IV	1	1	0	No blood stage infection
	V	1	1	0	No blood stage infection

♣ All blood meal positive mosquitoes following bite experiment were dissected to collect salivary gland to confirm the presence of GFP expressing sporozoites (WT or S23 KO) under fluorescent microscope

* Prepatent period: Defined as time required for the appearance of blood stages following infection with sporozoites

3.3.6 *S23* KO EEFs exhibit better growth than WT EEFs

S23 knockout sporozoites developed into fully grown EEFs in HepG2 cells. EEF development at 12h (Fig 28A and B) and 36h (Fig 28C and D) was similar to WT EEF development. At 65h, *S23* KO EEFs exhibited better growth than WT EEFs (Fig 28E and F).

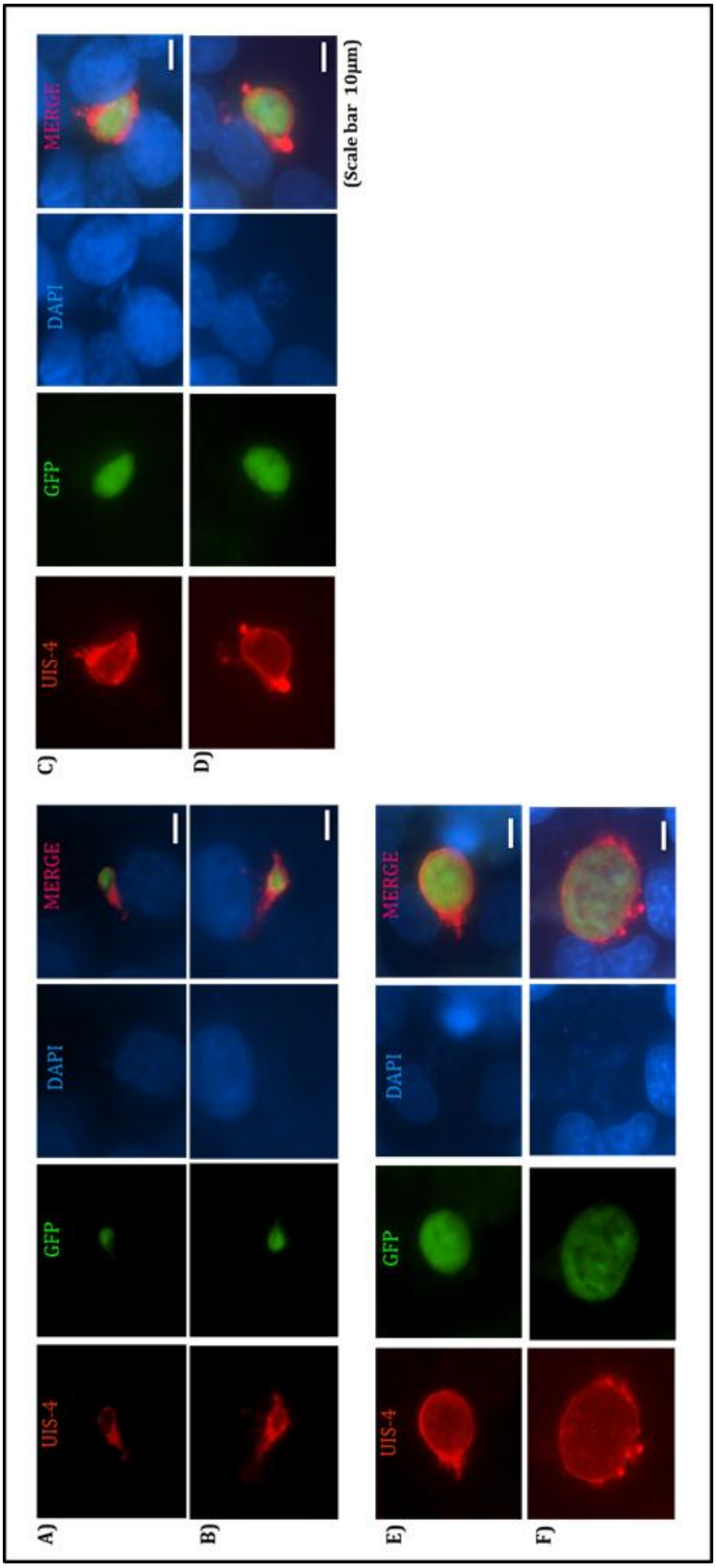

Fig 28. The Exo Erythrocytic Forms (EEF's) of *PbS23* KO reveals better growth at late stages of *in vitro* development (62h). Salivary glands sporozoites were isolated by dissection and $2X10^4$ sporozoites of either WT or *PbS23* KO were added to HepG2 cultures, that supported the complete development of the *P. berghei* EEF's. The cultures were fixed at different time points: 12h, 36h and 62h. The cultures were stained with anti-UIS-4 antibody that reacts with the parasitophorous vacuolar membrane (PVM) of EEF and DAPI (*4', 6'* diamidino-2 phenyl indole) for visualization of HepG2 and parasite nuclei. EEFs derived from *Pb S23* KO sporozoites at 12h (B) and 36h (D) were comparable to that of the WT EEF's at 12h (A) and 36h (C). EEF's derived from *Pb S23* KO sporozoites at 62h (F) was significantly larger as compared to WT EEF's at the same time point (E).

Table 7 Measurement of EEF area at 62h

EEF No	WT area (µm²)	*Pb* S23KO area (µm²)
1	126.52	210.87
2	79.06	179.87
3	121.8	169.3
4	122.91	293.06
5	92.57	181.01
6	75.07	236.72
7	125.66	313.5
8	63.81	270.23
9	105	140.56
10	92.67	149.1
11	114.05	208.63
12	72.25	197.27
13	74.51	126.64
14	59.53	125.96
15	112.15	295.83
Average	95.83	206.57
Std dev	23.1	60.7

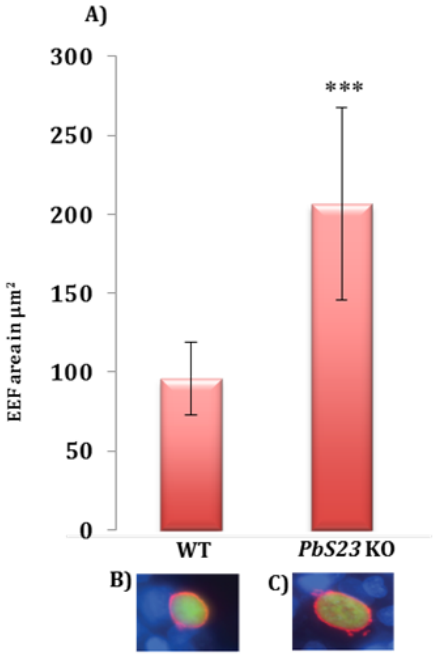

Fig 29. Measurement of EEF size at 62h. Fifteen individual EEFs derived from either *PbS23* KO or WT were measured with regard to their area (Table 7). The average size of the EEF is indicated in the bar graph (A). P value ≤ 0.005. (B) and (C) are representative EEF's derived from WT and *PbS23* KO sporozoites respectively

3.4 Discussion

Genetically attenuated sporozoites are gaining importance in designing live attenuated vaccines to prevent malaria. Towards this end, understanding the sporozoite biology and genes that are essential for sporozoite invasion, infectivity and liver stage development are highly essential. Enrichment of sporozoite transcripts and screening the SSH library by Kaiser et al., resulted in 25 sporozoite stage specific genes [167]. Functional characterization of these sporozoite specific genes is important to unravel the novel candidates for vaccine development. Towards this end, we selected *S23*, one amongst the reported 25 S genes which showed 138 fold induction in microarray studies.

In this study, we generated *S23* knockout parasites by targeting *S23* gene locus by double homologous recombination. The targeting construct was generated by cloning 5' and 3' flanking regions of S23 ORF respectively into the multiple cloning sites (MCS) I and II present on either ends of the GFP (reporter) and hDHFR (drug selection) cassette. Electroporation of linearized S23 knockout construct followed by pyrimethamine drug selection resulted in obtaining drug resistant parasites. The transgenic parasites generated were confirmed for correct site specific integration by diagnostic PCR that revealed GFP-DHFR cassette at the *S23* locus. The transfected parasites were subjected to limiting dilution to obtain clonal population that was further confirmed by PCR. When identical numbers of infected RBC (irbc) of WT or *S23* KO were injected into mouse, both parasite lines propagated at identical rates thus revealing a non-essential role of *S23* in asexual propagation of blood stages. Transmission of *S23* KO parasites to female *Anopheles* mosquitoes revealed a non essential role in mosquito stages, as *S23* KO parasites did not differ from WT parasites in their ability to form oocyst, undergo sporulation and infect the salivary glands.

Inoculation of salivary gland sporozoites by mosquito bite and intravenous injection could not initiate blood stage infection indicating the defect in liver stage development. To study the exo-erythrocytic stages, *S23* KO sporozoites were added to HepG2 cells and development was monitored at different time points. This experiment revealed that development of EEFs at 12h and 36h was identical to that of WT EEF development. However, *S23* KO EEFs at 65 h were significantly larger in size than WT EEFs at the same time point. The size of *S23* KO EEF at 65 h could be the result of defect in merozoite release from fully grown EEF correlating with the inability of S23 KO sporozoites to initiate blood

stage infection when introduced by bite or through intravenous route. These studies revealed the role of *S23* in the egress of merozoites from mature EEFs.

After entering into liver sinusoids, sporozoites invade hepatocytes and develop into exoerythrocytic forms. For the survival in the host, parasite prevents cell death by manipulating hepatocytes. One study reveals the role of circumsporozoite protein (CSP) in this process, where it binds to the host nuclear importins thus preventing the binding NF-κB, which otherwise would be translocated to host nucleus, to bind a set of cognate promoters and to initiate a proinflmmatory response [248]. Following successful transformation into mature liver stage parasite (EEF), egress of merozoites from hepatic schizonts is the most critical event in initiating blood stage infection. However, the merozoite release into liver sinusoidal space may trigger phagocytosis mediated by Kupffer cells residing in liver. To evade host immune system, *Plasmodium* forms membrane bound vesicles filled with infectious merozoites called merosomes [18, 274]. Budding of merosomes from the fully grown EEF detaches it and releases the merosomes into blood steam [18]. In the circulation, merozoites are released by rupture of merosome membrane and infect RBC. The failure to initiate blood stage infection by *PbS23* KO sporozoites could be due to defect in the formation of merosomes which is in agreement with our observations made size at 62h time point of EEF development.

There are several mechanisms that describe the escape of the merozoites from erythrocytes and hepatocytes and this requires both the disruption of PVM and parasite plasma membrane (PPM) [69]. Cysteine protease activity is reported to be essential for PVM rupture as the treatment with a cysteine protease inhibitor, E64 blocked PVM rupture [19]. Serine repeat antigen (SERA) family is a group of cysteine proteases expressed during schizogonic cycle involved in PVM rupture in erythrocytes [275, 276]. In rodent *Plasmodium* strains, five SERA proteins are identified and four proteins among them are upregulated during late liver stage development [275]. During merozoite formation SERA2 and SERA3 proteins are found in the hepatocyte cytoplasm [24]. Very few proteins other than SERA proteins were identified that participate in merozoite egress from hepatic schizonts.

The subtilisin like protease (SUB1) is a serine protease that is essential for the egress of merozoites from liver stages. Role of SUB1 was first identified in merozoite release from infected RBC [277]. SUB1 is important for the maturation of SERA proteins which is an

essential step in the egress of merozoites from infected RBC [277]. Later SUB1 conditional mutagenesis unraveled its role in liver stages. SUB1 mutated parasites failed to initiate blood stage infection confirming the role of SUB1 in hepatic merozoite release [25].

The cGMP (3'-5'-cyclic guanosine monophosphate) signaling mediated by cGMP dependent protein kinase (PKG) plays a very significant role throughout *Plasmodium* life cycle [278]. PKG mediates the initiation of gametogenesis and blood stage schizonts rupture [279, 280]. Conditional knockout of PKG revealed its role in the egress of merozoites from the infected hepatocyte [23]. PKG conditional knockout parasites are defective in merosome formation, merozoite release and subsequent blood stage infection. Injection of PKG conditional knockout sporozoites elicited protective immune response in mice [23].

In our study *S23* KO parasites also showed the similar phenotype as above mentioned gene knockouts. For the first time we identified the role of *PbS23* in the egress of merozoites from liver stages. Very recently, the phenotypic characterization of *P. yoelii* (*Py) S23* by reverse genetics revealed its role in sporozoite gliding motility, surprisingly with no outcome on its ability to infect hepatocytes, transform into EEFs and complete liver stage development and initiate blood stage infection, similar to WT parasites [281]. In contrast to the phenotype of *Py S23* knockout, *Pb S23* KO sporozoites behaved identically as WT parasites during their asexual propagation and during their development in the mosquito, except that they were not able to egress out of the hepatocytes, following EEF development. Infact a phenotype, identical to *PbS23* was reported earlier where depletion of a gene that encodes for Liver specific protein-1 (LISP-1) was shown to generate merozoites in the late liver stages that delayed significantly the initiation of blood stage infection in a timely manner, owing to a defect in the egress [95].

Considering the role of stage specific proteases in egress, a possibility that *PbS23* may be a substrate for these proteases cannot be ruled out. The fact that *PbS23* parasites exhibit better growth characteristics during late liver stage development, may also mean that it exposes these antigens to the immune system likely eliciting a cross stage immunity (blood stage specific), in addition to pre-erythrocytic immunity. Investigation of protective immunity generated by *PbS23* KO parasites could give an insight in using these parasites as genetically attenuated whole organism vaccines.

Summary

The functional role of *P. berghei Ubc13 kinase/PK9* was deciphered by reverse genetics approach that revealed its unique role in sexual stage development. The non-essential role of *Ubc13 kinase/PK9* in salivary gland sporozoite stages, in hepatocyte invasion and in EEF development was documented by generating FLP/FRT based conditional mutants. Localization of Ubc13 kinase/PK9 substrate-Ubc13/E2 was demonstrated by generating an mCherry transgenic that showed Ubc13/E2 promoter activity in asexual stages and in oocyst stages but not in salivary gland stages. Transcriptomic analysis of *Ubc13 kinase/PK9* KO by RNA Seq revealed down regulation of transcripts that belong to functional categories like sexual reproduction, other kinases (not involved in sexual reproduction), DNA repair and ubiquitin pathway. Analysis of cellular proteins from lysates obtained from synchronized schizont cultures revealed certain target proteins being comparatively more ubiquitinated than proteins of the wild type parasites. This coincided with higher levels of E2/Ubc13 in *Ubc13 kinase/PK9* KO. Taken together we provide evidence for the first time the role of Ubc13 kinase/PK9 in regulating *Plasmodium* sexual reproduction.

The functional role of *P. berghei S23* was also unraveled by reverse genetics approach and was shown to be important for the egress of the mature EEFs (exo-erythrocytic forms). The *S23* KO propagated without any defect in asexual blood stages and in mosquito stages. However, when mouse were injected with *S23* KO sporozoites either by natural mosquito bite or by intravenous injection, they failed to initiate blood stage infection. Analysis of *in vitro* grown EEF's at different time points (12 h, 36 h and 62 h) revealed that *S23* KO completed liver stage development and also had a better growth phenotype as compared to EEF's derived from wild type sporozoites. Since completely developed EEF's failed to initiate blood stage infection, we propose a role of *S23* in the egress of the liver stages.

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Appendix 1

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