“Using reverse genetics to elucidate the role of sporozoite specific proteins S14 and SCOT1 in *Plasmodium*”

Thesis

submitted to

Jawaharlal Nehru University

New Delhi, India

For the Degree of

Doctor of Philosophy

By

Ankit Ghosh

Division of Molecular Parasitology and Immunology

CSIR-Central Drug Research Institute

Lucknow-226031, India

2020

Dedicated to
My Beloved Parents

सी.एस.आई.आर.-केन्द्रीय औषधि अनुसंधान संस्थान, लखनऊ
(वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद्)
सेक्टर 10, जानकीपुरम विस्तार, सीतापुर रोड, लखनऊ – 226 031 (भारत)
CSIR - Central Drug Research Institute
(Council of Scientific & Industrial Research)
Sector 10, Janakipuram Extension, Sitapur Road, Lucknow - 226 031 (India)
CSIR - Central Drug Research Institute, Lucknow

Date 31.01.2020.

Dr. Satish Mishra
Principal Scientist & Associate Professor (AcSIR)
Division of Molecular Parasitology and Immunology

Certificate

This is to certify that the thesis entitled "**Using reverse genetics to elucidate the role of sporozoite specific proteins S14 and SCOT1 in *Plasmodium***" being submitted to the Jawaharlal Nehru University (JNU), New Delhi in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy, embodies the research work done by **Ankit Ghosh**, under my supervision at CSIR- Central Drug Research Institute, Lucknow. This is further certified that work embodied in the thesis has not been submitted for any other degree, and unless otherwise stated, is all original.

(Dr. Satish Mishra)
Supervisor

Declaration

I declare that the research work embodied in this thesis entitled **"Using reverse genetics to elucidate the role of sporozoite specific proteins S14 and SCOT1 in *Plasmodium*"** submitted to Jawaharlal Nehru University (JNU), New Delhi for the degree of Doctor of Philosophy (Ph.D) is the outcome of investigations carried out by me under the supervision of **Dr. Satish Mishra, Principal Scientist,** CSIR-Central Drug Research Institute, Lucknow. I hereby declare that the work incorporated is original and has not been submitted elsewhere for any degree or diploma to this or any other university.

Date: 31.01.2020
Place: Lucknow

(Ankit Ghosh)

Acknowledgement

First and foremost I wish to express my heartfelt gratitude to my mentor and supervisor Dr Satish Mishra, Principal scientist division of Molecular Parasitology and Immunology, for giving me the opportunity to pursue the study under his supervision. His pursuits in scientific endeavors were with utmost diligence and his approach to a problem is inspirational. He helped me with the mosquito experiments especially with his amazing mosquito dissecting skills. I would like to thank for supporting me through my work and training me with patience.

I would like to express my sincere gratitude to Dr. Tapas K Kundu, Director, and former directors Dr.Alok Dhawan, Dr.Madhu Dixit, Dr. Ram Vishwakarma and Dr. S. K. Puri for providing all the support, facilities during the course of doctoral study. I sincerely thank Council of Scientific and Industrial Research, New Delhi for providing me financial assistance.

I am highly thankful to Dr.Renu Tripathi, Head Division of Parasitology; faculty members of Parasitology division: Dr.Shailja Bhattacharya, Dr.Anuradha Dube, Dr.Kumkum Srivastava, Dr.Mrigank Srivastava, Dr.Sushanta Kar, Dr.Niti Kumar and Dr.Bidyut Purkait for their kind help. I owe my sincere thanks to Mr.Sumit Parihar and Mr .Dhananjay for confocal and intravital microscopy.

I sincerely thank the rest of my thesis committee Dr Mriganka Srivastava and Dr Sidharth Chopra for encouragement and their insightful comments.

I would like to thank my seniors Dr.Hadi Hasan Choudhary for his help and support throughout my work tenure.

My heartfelt thanks to my colleague Sunil Narwal for helping me with all aspects of my work and his unconditional support. My heartfelt thanks to my juniors Pratik, Akansha, Bandita, Himadri and Aastha who always helped me in my time of need and supporting me.

I would like to thank my friends Swetarka and Vaibhave for their support throughout my tenure.

I would like to thank all my division members for their help and support.

I am grateful to the tremendous assistance provided by the insectary staff, particularly Shivbaran and Rohit. My thanks also go to members of Molecular Parasitology and Immunology division: Mr. Kamlesh Singh, Mr. Ahshan Manhas, Ms.Shikha Mishra, Prembhaiya, Iqbal, Rohit and Basant for their help in division.

My acknowledgement will never be complete without the special mention of entire staff members of animal house, library, computer section, administration, instrumentation & maintenance, stores &purchase, account, security staff and lab attendant of CDRI for their kind assistance and cooperation to carry out the entire work smoothly.

I dedicate my work to my lovely parents Shri Prabir Kanti Ghosh and Shrimati Sumita Ghosh without their support none of this would have been possible

Ankit

Contents

Abbreviations

	%	Percentage
°C		Degree Centigrade/Celsius
bp		Base pairs
p.i.		post infection
RBC		Red Blood Cells
rcf		Relative Centrifugal Force
RNA		Ribonucleic Acid
RP		Reverse Primer
sec		Seconds(s)
TRAP		Thrombospondin Related Anonymous Protein
UTR		Un Translated Region
WHO		World Health Organization
µ		Micro
KO		Knock Out
LB		Luria Bertani
M		Molarity
mg		Milligram
Min		Minute(s)
mM		Milli Molar
ng		Nanogram
o/n		Overnight
ORF		Open Reading Frame
DCO		Double Crossover Recombination
DNA		Deoxyribonucleic Acid
EEFs		Exo- erythrocytic forms
FP		Forward Primer
GKO		General Knock Out
h		hours/hour
i.p.		Intraperitoneally
i.v.		Intravenously
Kb		Kilobases
KO		Knock Out

Chapter 1
Review of Literature & Introduction

1.1 History

The earliest account found in the literature for malaria was made by Hippocrates in ancient Greece. He made an observation that places around marshes or stagnant water had a higher prevalence of the disease. The Romans also made similar observations, and the word malaria derives from the Latin words *mal aria* which translates to bad air (Oaks S.C Jr 1991). These early observations drove several futile efforts to isolate the causative agent from the soil and water samples from malaria-endemic areas. In 1897 a British military doctor, Ronald Ross who was working in India, was able to decipher that the parasite was transmitted to the invertebrate host by the mosquito vector. Ross made his groundbreaking discovery using the avian malaria model. He was closely followed by Italians, Giovani Battista Grassi, Amico Bignami and Guiseppe Bastianelli.

Malaria has been the scourge of the tropics for millennia and was most likely present in the earliest human populations (Brier, 2004). Studies of ancient Egyptian mummified human remains led to the identification of specimens with porotic hyperostosis (cranial thickening which is indicative of severe anemia) and splenomegaly (enlarged spleen), common symptoms of malaria (Brier, 2004). The advancement in the feild of molecular biology, investigators were able to establish that Tutankhamun (c. 1333-1324 BC) suffered from malaria but were unsure that it was the cause of his death (Hawass *et al.*, 2010, Timmann & Meyer, 2010). It is believed that malaria was spread along the shipping routes from Africa to South East Asia, India, China, and to Europe. Written accounts of malaria are found from ancient Greece and Rome by Hypocrates (460 – 370 BC) and Celsus (25 BC – 54 AD) respectively. Romans were the first to name the disease ‘mal aria’ meaning ‘bad air’, they believed the disease spread due to the noxious air of Roman marshlands. Active measures such as draining marshlands correlated to reduction of malaria episodes adding validation to their hypothesis. It took over 2 millennia in uncovering the real cause responsible for the disease.

Although several theories were put forward since the fall of the Roman Empire, few major scientific breakthroughs were made regarding malaria until 1880, when a French clinician named Alfonse Laveran working in Algeria noted the presence of

black pigment in the spleen and brain of his patients that died of malaria. He consequently studied the blood of malaria patients until finally witnessing the exflagellation of a gametocyte leading to the confirmation for parasitic origin of the disease (Garnham, 1966). This particular observation was called 'filariae of the blood' until its classification as genus *Plasmodium*. The identification of the parasite sparked interest in the mechanism of transmission and vector involved. Despite suspicion throughout history by physicians throughout the world direct evidence linking malaria to mosquitos was not available. In the year 1897 Surgeon-Major Ronald Ross and Dr. Patrick Manson were able to identify malarial parasite in midguts of Anopheles mosquitos in after feeding on a malaria patients. Other mosquito types fed on patients lacked the parasite. They observed 'germinal rods' (sporozoites) in infected mosquito salivary glands which led them to the conclusion that parasite transfer occurs via mosquito bite (Ross & Smyth, 1897). Ross successfully demonstrated transmission from insect to host in birds but evidence of mosquito to man transmission came in 1899 by Bignami and Grassi. They were able to follow the parasite's lifecycle to completion and their experiments were repeated and confirmed by Dr. Patrick Manson in London a year later (Grassi & Noe, 1900, Manson, 1900). Since then, malaria control has focused on insecticides for destroying vector breeding grounds, mosquito nets and repellents. Using these measures has resulted in a reduction of malaria incidents in the developing world and eliminated malaria from much of the developed world where countries boast the infrastructure and capital to effectively control the spread of disease. Several advancements in research methodology such as in vitro culture of *Plasmodium falciparum* (Jensen & Trager, 1978), and the sequencing of the *P. falciparum* genome (Gardner *et al.*, 2002) and development of various animal models have aided drug and vaccine research immensely. Despite all this, malaria persists and devastates the tropics to this day.

1.2 Malaria epidemiology

Malaria remains a major health problem in terms of morbidity and mortality. In 2018 it caused 228 million cases and 405,000 deaths across the globe (W.H.O, 2019). Africa accounts for more than 213 million and also the highest number of deaths due to malaria. The prevalence of the disease can be mapped primarily in tropical, sub-tropical and temperate zones. *P. falciparum* causes the most severe form of malaria.

The highest number of cases of *P. falciparum* infection is reported in Sub Saharan Africa. According to the latest WHO report, India along with Sub Saharan Africa contributes to 85% of the global malaria burden (Figure 1). The reason for the high incidence of *P. falciparum* cases in Africa revolves around the fact that *Anopheles gambiae* is the most prevalent malaria vector in the region and prefers to feeds on humans rather than other vertebrates. *A. gambiae* to a large extent feeds and rests indoors, this increases its chances of transmitting malaria among humans (Beier, 1998). Also, the temperature and weather of Africa present exceedingly hospitable conditions for the mosquitoes to bloom. Compared to *P. falciparum*, *P. ovale* which is found in the Pacific islands exhibits lower pathogenicity. *P. vivax* found in Asia and Latin America has a unique stage known as the hypnozoite stage, the hypnozoites can persist for long periods of time without any symptoms and have been found to cause disease relapse (White *et al.*, 2016). *P. malariae* is a species that is spread throughout the globe and causes an unceasing form of malaria thereto. *P. knowlesi* can briskly progress from a cinch to severe malaria infection. There are findings that indicate that *P. knowlesi* is a zoonotic infection. It is supposed to be transmitted from macaque monkeys to humans by the vector Anopheles leucosphyrus (Coatney, 1968) (Cox-Singh *et al.*, 2008).

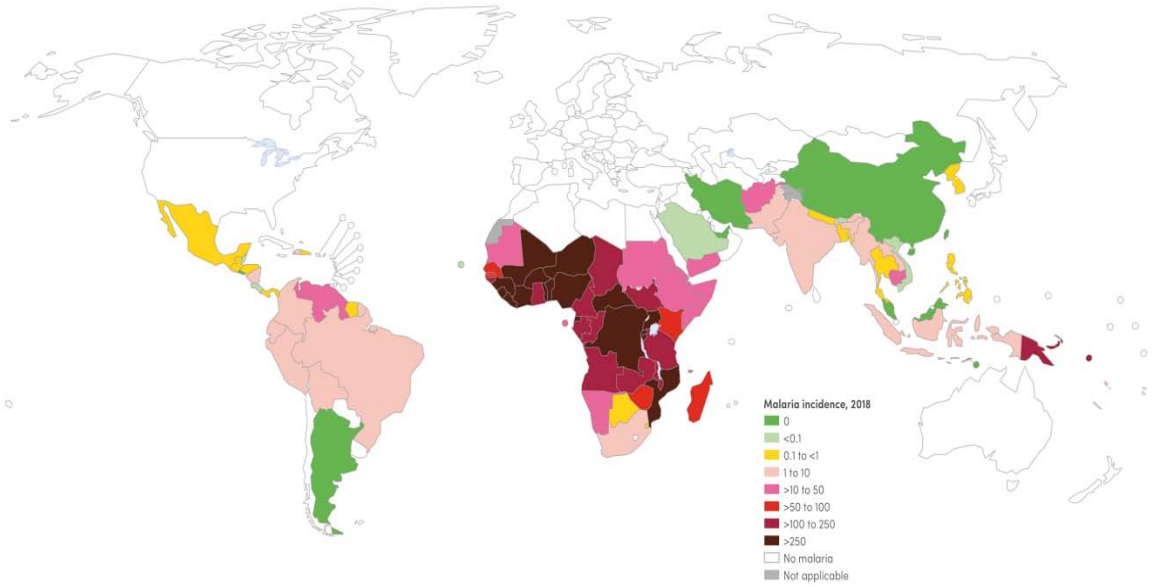


Figure 1. Cases of malaria reported in 2018 worldwide.

1.3 The life cycle of *Plasmodium*

Plasmodium life cycle initiates with female mosquito taking a blood meal from an infected host, blood of the mammalian host contains sexual gametocytes. Once inside the mosquito midgut gametocytes exit infected red blood cells and fuse to form a zygote. The zygote undergoes transformation to form ookinete. Ookinetes penetrate the midgut epithelium, transforms into sessile oocysts. The oocysts develop in the mosquito midgut undergo rapid DNA replication and division (Shahabuddin, 1998) after 10-14 days release sporozoites into the mosquito hemolymph. The sporozoites in the hemocoel are motile and are carried to the mosquito salivary glands by the hemolymph cycling. The sporozoites invade the salivary gland lobes by traversing basal lamina (Ghosh *et al.*, 2009) creating a reservoir, which can be injected into host while probing for a blood meal.

These infected mosquitos deposit sporozoites in the skin of the host during a blood meal (Vanderberg, 2014). These sporozoites use gliding motility to move through and penetrate the epithelial cells. Once the sporozoites encounter capillary they enter the blood stream and migrate to the host liver (Vanderberg, 2014), (Amino *et al.*, 2008). On reaching liver sinusoids sporozoites exit circulation and start traversing through either liver sinusoidal endothelial cells (LSECs) or Kupffer cells reaching the space of disse (Tavares *et al.*, 2013, Pradel *et al.*, 2004).

Here the sporozoites start traversing through hepatocytes (Mota & Rodriguez, 2004) (Ishino *et al.*, 2004) and pass through several hepatocytes before productively invading one. A productive invasion eads to the formation of a parasitophorous vacuole (PV) membrane (Meis *et al.*, 1983) which harbours the parasite providing a niche for growth and development. The PV though derived from the host membrane is modified by parasite proteins. Inside the PV the sporozoite transforms into the sessile liver-stage also known as exo-erythrocytic forms (Mota & Rodriguez, 2002). In this stage, the parasite undergoes the process termed as exo-erythrocytic schizogony, where rapid replication takes place leading to the formation of a syncytium which on completion of development form exo-erythrocytic merozoites (Vaughan *et al.*, 2008). A single productive invasion leads to the formation of thousands of progeny. In the human parasites *P. vivax and P. ovale*, as well as a non-human primate parasite *P. cynomolgi*, some liver stages halt replication and develop into hypnozoites (Krotoski

et al., 1982). Hypnozoites are parasite form that can persist for months before reverting to normal liver-stage schizont, the factors triggering this are not understood (Cogswell, 1992). The merozoites exit the host cells packed in host cell-derived vesicles termed as merosomes (Sturm *et al.*, 2006). These merosomes disintegrate when they reach pulmonary vasculature (Baer *et al.*, 2007) releasing merozoites that infect red blood cells initiating the asexual blood stages (Figure 2).

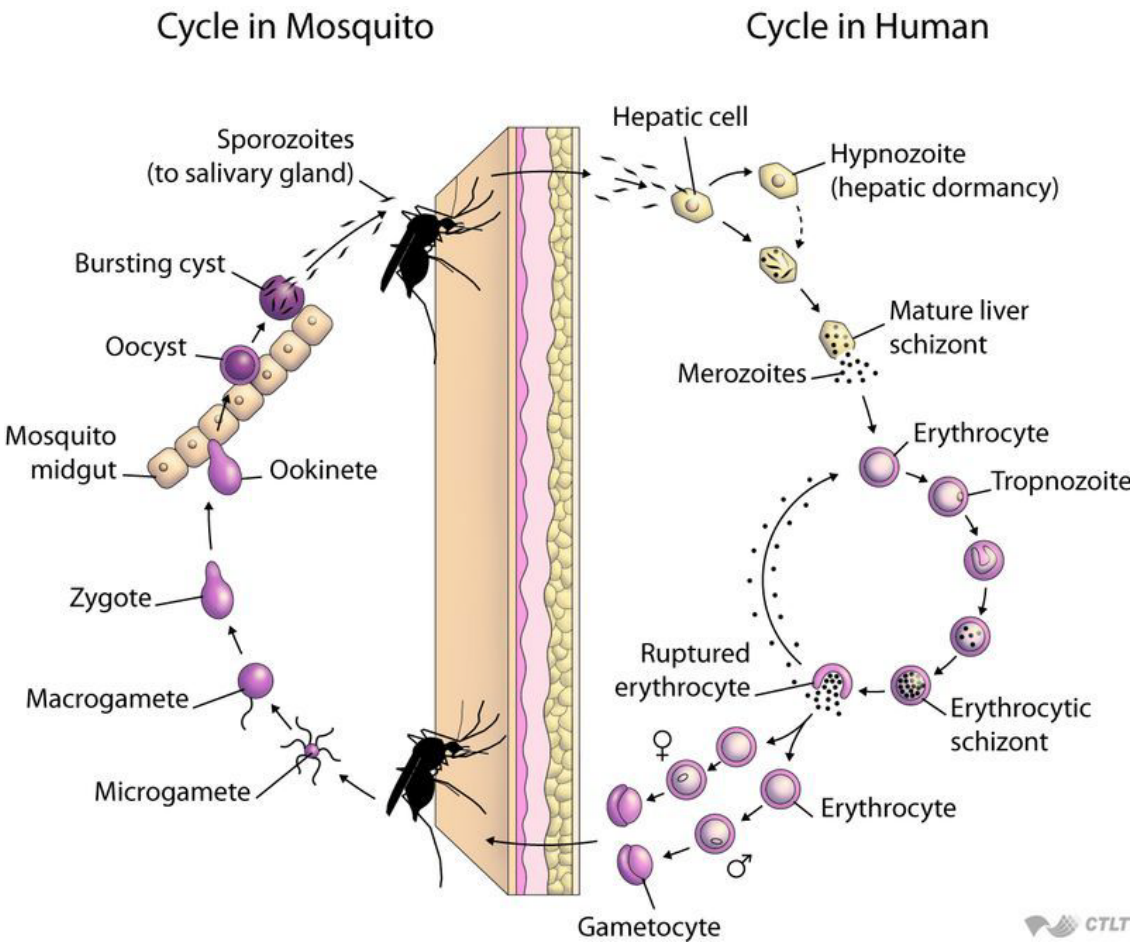


Figure 2. The life cycle of the malaria parasite in mammalian and mosquito hosts. It starts when an infected female Anopheles mosquito bites a mammalian host and inoculates sporozoites in the skin during a blood meal. The sporozoites reach the liver, develop and start hepatic schizogony and forms hepatic schizonts. These hepatic schizonts bud in the form of merosomes, infect the RBCs and starts the erythrocytic cycle. During the erythrocytic cycle, the schizonts develop into different forms such as immature trophozoites, mature trophozoites which either form gametocytes or schizonts. The gametocytes are taken up by mosquitoes during blood meal where the male gametocytes undergo exflagellation and result in the formation of male microgametocytes. Male microgametocytes fuse with female macrogametocyte to form a zygote which further leads to the formation of ookinetes. Ookinetes further transform into oocysts where sporozoite formation takes place. The oocysts rupture and release the sporozoites into the hemocoel and invade the salivary glands to continue the further cycle (CDC, 2018).

Asexual blood stages are responsible for the clinical symptoms of malaria. After infecting an erythrocyte the merozoite changes drastically into several different morphological stages namely ring stage then trophozoite and finally schizont stage. In contrast to exo-erythrocytic schizogony, the blood-stage schizont can produce approximately few dozen merozoites (Bannister & Mitchell, 2003), (Arnot & Gull, 1998, Bannister *et al.*, 2000). Finally mature infective merozoites are released into the blood stream after rupturing of the iRBC membrane where they reinitiate the infection cycle (Cowman & Crabb, 2006). In a subset of iRBCs, the merozoites develop into new sexual stage gametocytes (Inselburg, 1983). These gametocytes ensure the transmission of malaria.

1.3.1Mosquito stage development

1.3.1.1 Gametogenesis and ookinete formation

The process of gametocyte formation is one of the least understood amongst *Plasmodium* parasite biology (Kooij & Matuschewski, 2007). Several physical factors that have been found to play a role in stimulating the process. The male gametogenesis and exflagelation is stimulated by the change in pH, temperature and exposure to xanthurenic acid (Billker *et al.*, 1998, Billker *et al.*, 1997). Several 6-cysteine proteins exert crucial roles in fertilization, P48/P45 are proteins of this family and the knockouts of P48/P45 male gametes were unable to fertilize female macrogametes (van Dijk *et al.*, 2001). P47 is present on the surface of female macrogamete is a paralog to the P48/P45 though *p47* deletion had no effect on fertilization (van Schaijk *et al.*, 2006). P230 another 6-cys protein whose targeted deletion revealed its role in the process of gamete formation and fertilization (Eksi *et al.*, 2006). The fertilization of the gametes leads to zygote formation which in midgut lumen transforms into ookinete. The *P. berghei* calcium-dependent protein kinase 4 (CDPK4) and mitogen-activated protein kinase (MAPK2) both play an important role in the development and maturity of male gametocyte (Billker *et al.*, 2004, Rangarajan *et al.*, 2005). In the case of *P. falciparum* contrastingly MAPK2 was not essential for male gametocyte formation but found to be essential for asexual blood-stage (Dorin-Semblat *et al.*, 2007). *P. falciparum* cGMP dependent protein kinase (PKG) along with xanthurenic acid was essential for male gametocyte exflagelation.

P. berghei HAP2 was essential for gametocyte fusion i.e. essential for fusion of gamete membranes (Liu *et al.*, 2008), gametocyte maturation is unaffected but zygote formation is completely abolished, it was also found to localize at the site of gamete fusion. Transcriptional control plays a crucial role in zygote formation and a robust example was the deletion of development of zygote inhibited (DOZI) which is a RNA helicase that leads to degradation of over 370 transcripts which were repressed and zygote development was terminated (Mair *et al.*, 2006).

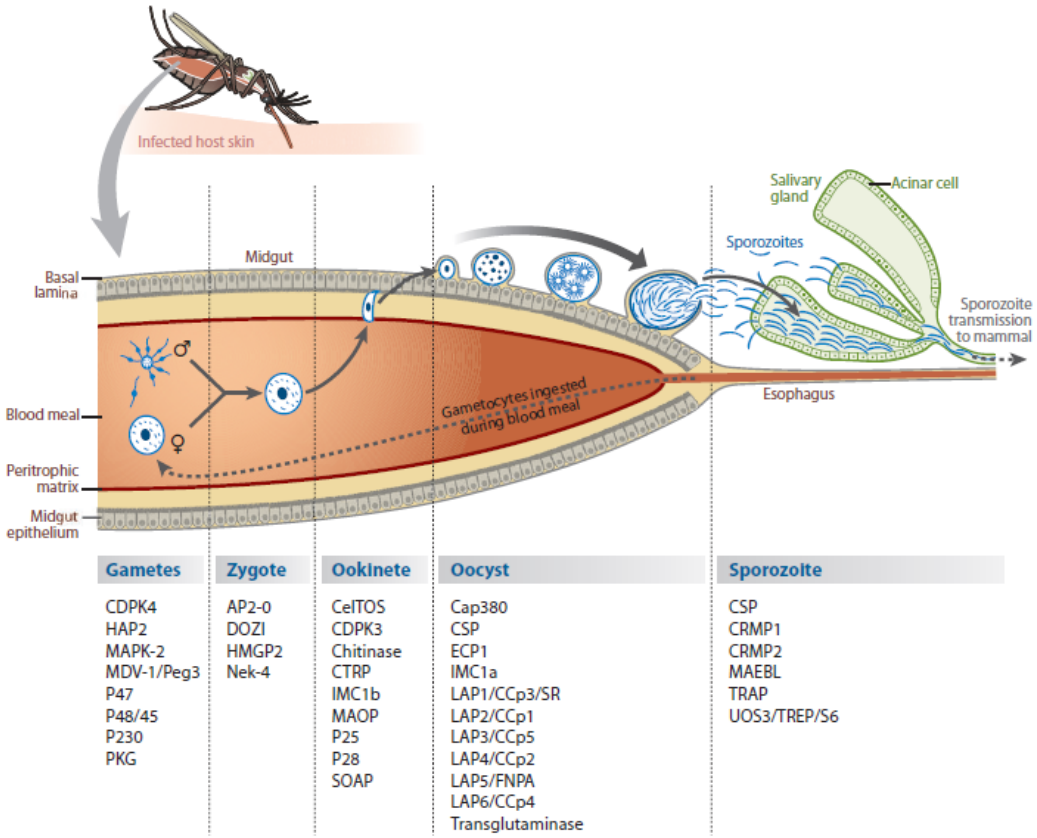


Figure 3. Development and progression of malaria parasite in mosquito vector. Gametocytes ingested by female *Anopheles* mosquito probing for a blood meal. Male and female gametes emerge from gametocytes and fertilize to form zygote. Zygote transforms into ookinete. Ookinetes breach peritrophic matrix that surrounds the blood meal entering into the apical end of the midgut epithelium transform into sessile oocyst once they reach the basal end. Oocyst grows for 10-14 days producing thousands of sporozoites. Sporozoites exit the oocyst, travel to the salivary glands through the hemolymph. They attach to the basal side of the salivary glands acinar cells. They enter the salivary gland ducts and are transmitted to the host during the next blood meal. List of proteins implicated during this stage is provided.

The completion of the sexual cycle requires high motility protein group 2 (HMGP2) (Gissot *et al.*, 2008) which is responsible for the expression of 35 genes which are

part of cascade responsible for the sexual development in the parasite (Gissot *et al.*, 2008). Analysis of the *Plasmodium* genome has revealed probable transcriptional factors in *Plasmodium* (Aravind *et al.*, 2003), (Gardner *et al.*, 2002). The studies have revealed a plant-like transcriptional factor Apatella 2 (AP2) (Balaji *et al.*, 2005). AP2 like domains bind DNA (De Silva *et al.*, 2008) and the deletion of AP2 does not affect ookinete formation but the maturation is abolished leading to ookinetes being immature and non-motile (Figure 3) (Yuda *et al.*, 2009).

1.3.1.2 Ookinete invasion of the midgut epithelium

Meiosis in the *Plasmodium* life cycle takes place just after zygote formation. Zygote is a spherical structure that transforms into elongated ookinete which contains specialized organelles possessed by all invasive stages which are micronemes and rhoptries. The NIMA (never in mitosis /asparagus) related protein kinase (NeK4) is indispensable for the transformation of the zygote into ookinete (Reininger *et al.*, 2005). Chitinase in *P. falciparum* has been found to be essential for crossing the peritrophic matrix which surrounds the blood meal bolus (Dessens *et al.*, 2001, Vinetz *et al.*, 1999, Vinetz *et al.*, 2000). Micronemes which are characteristic for invasive stages, secrete several proteins that play a crucial role in invasion. Micronemal calcium-dependent protein kinase 3 (CDPK3) and circumsporozoite and TRAP related proteins (CTRP) both of which when subjected to targeted deletion led to abolition in the invasion of the mosquito midgut (Ishino *et al.*, 2006, Siden-Kiamos *et al.*, 2006). CDPK3 is responsible for the motility of the ookinete (Ishino *et al.*, 2006). CTRP was found to interact with mosquito lamins (Mahairaki *et al.*, 2005) and its deletion led to the complete abolition of oocyst formation (Dessens *et al.*, 1999). Another micronemal secreted protein contains a membrane attack complex perforin like domain called the membrane attack ookinete protein (MAOP) as the name of the protein suggests it disrupts the host cell membrane and has a role in invasion (Limviroj *et al.*, 2002). Cell traversal is a common feature displayed by all motile stages of the parasite before a successful infection be it ookinete or sporozoite in different hosts. Cell traversal protein for ookinete and sporozoite (CelTOS) is crucial for the ookinete traversal through the midgut epithelium but its targeted deletion does not completely disrupt the proc (Kariu *et al.*, 2006) . The parasite switch to the sessile mode from motile mode is believed to be triggered by transmigration. Ookinete

finally exits from the basal side of the epithelium and becomes sessile starts oocyst formation.

1.3.1.3 Ookinete transformation into oocyst

The ookinete transformation into oocyst can be triggered in *vitro* on matrigel in the presence of lamin like substrate (Al-Olayan *et al.,* 2002). Lamin is believed to be one of the host factors responsible for the transformation (Adini & Warburg, 1999). The parasite proteins that interact with lamins include well-identified GPI-linked ookinete surface proteins *P25/P28* and their interaction with vector lamins facilitates ookinete to oocyst transformation (Vanderberg *et al.,* 1990). Targeted deletions for both genes individually have been found to cause mild disruption in the formation of the oocyst whereas the double knockout for *P25/P28* leads to complete disruption of ookinete invasion and oocyst formation (Tomas *et al.,* 2001). Other ookinete proteins identified which have been found to interact with lamins include surface proteins with adhesion domains include secreted ookinete adhesion protein (SOAP). SOAP knocked out ookinetes were unable to transform into oocyst with compromised invasion (Dessens *et al.,* 2003) .

1.3.1.4 Development of oocysts and sporozoites

Ookinete invades and colonizes the midgut transforming into oocyst. oocysts take a long time to develop which is around 10-12 days and are the only parasite stage that develops extracellularly. LAP (LCCL/lectin adhesive- like proteins) protein expresses in female gametocyte and the ookinete which is crucial for sporulation. (Carter *et al.,* 2008, Claudianos *et al.,* 2002, Trueman *et al.,* 2004) It is speculated that the growth of the oocyst syncytium requires the host-derived nutrients but distinct mechanisms are yet to be investigated. Mosquito lamins constitute the outer layer of the oocyst capsule also consists of parasite Cap380 (oocyst capsule protein) (Srinivasan *et al.,* 2011, Srinivasan *et al.,* 2008). The mature oocyst could attain sizes upto 50-60 µm finally leading to the production of sporozoites. Prior to sporozoite formation, CSP production and accumulation begins in the developing oocyst plasma membrane (Thathy *et al.,* 2002). Sporozoite formation involves the invagination of the oocyst plasma membrane into lobular structures which contain nuclei still undergoing division (Sinden, 1974), which are termed as sporoblasts. The distinct feature of

sporoblast is peripheral localization of daughter nuclei and the CSP marking the membrane (Thathy *et al.,* 2002). CSP is indispensable for sporoblast formation (Menard *et al.,* 1997). It is believed CSP also plays an important role in the organization of the microtubule organizing centers (MTOC) which localize below the sporoblast membrane. As in other organisms, MTOC determines polarity, specifically, here MTOC are responsible for the formation of apical complex and distribution of genetic material in the developing sporozoites (Thathy *et al.,* 2002). The initial apical ring formation is followed by the formation of the IMC and its attachment to the nascent polymerized subpellicular microtubules and finally budding off from the sporoblast (Vanderberg & Rhodin, 1967). An IMC localised protein conveniently called IMC1 has been found to be crucial for the sporozoite motility and shape (Khater *et al.,* 2004). The oocyst after completion of the sporulation acts as a reservoir for thousands of newly developed sporozoites (Sinden & Garnham, 1973) which later egress from the midgut into the hemocoel.

1.3.1.5 Egress of sporozoites from the midgut into the hemolymph

Fully developed sporozoites in the oocysts have minimal motility and do not exhibit full-scale gliding similar to salivary gland sporozoites (Sultan *et al.,* 1997), (Vanderberg, 1974). Earlier it was believed that the growth in oocyst sporozoite number and their accumulation results in rupture of the oocysts (Sinden, 1974). Egress cysteine protease1 (ECP1) has been implicated in this process and studies have deemed ECP1 essential for sporozoite egress into hemocoel (Aly & Matuschewski, 2005). The ECP KO oocyst and sporozoite development were complete but sporozoites failed to egress oocysts hemolymph (Aly & Matuschewski, 2005). A similar phenotype was observed with mutations in the CSP region II plus where the positively charged amino acids were replaced with neutral residues correlating ECP1 with CSP processing (Wang *et al.,* 2005). Oocyst hypothetical protein in *P. berghei* showed similar phenotype as ECP1 (Lasonder *et al.,* 2008) which demonstrates that the egress of sporozoites from the oocyst is a complex process and requires multiple proteins.

1.3.1.6 Sporozoite cellular structure

Inside the mosquito, the sporozoite exists in two forms. The mosquito midgut harbour oocyst's where the initial development of the sporozoite begins before it egresses and reaches the mosquito salivary glands. The two forms show little or no morphological difference but a marked difference in phenotypes particularly when liver-stage infectivity of the two forms are compared. When it comes to liver-stage infectivity, salivary gland sporozoites are 100 to 10,000 times more infective than the midgut oocyst sporozoites (Vanderberg, 1975). Transcriptomes of the two different sporozoite types were compared and more than 120 genes were found to be upregulated in salivary gland sporozoites (Matuschewski *et al.*, 2002, Mikolajczak *et al.*, 2008), (Kappe *et al.*, 2001). The comparative transcriptomics between sporozoites and merozoites revealed set of genes enriched in sporozoites, also collectively referred to as up-regulated in infective sporozoites (UIS) have been found to play a critical role in the infectivity of the sporozoites (Mueller *et al.*, 2005a), (Labaied *et al.*, 2007), (van Dijk *et al.*, 2005), (Zhang *et al.*, 2016)

The products encoded by UIS genes are essentially required for the hepatocyte stage development, yet they express in the salivary gland sporozoite stage prematurely and maintained as transcript reservoirs (Müller *et al.*, 2011). Premature translation of these genes could lead to early transformation into Exo Erythrocytic Forms (EEF) and loss of infectivity (Gomes-Santos *et al.*, 2011). The maintenance of these pre transcribed mRNA is made possible by its sequestration by RNA binding protein *Puf*2 which stops them from premature transcription, which gets transcribed in the liver stage (Gomes-Santos *et al.*, 2011).

The sporozoites are comparatively elongated cells, crescent-shaped looks like an eyelash under a light microscope. The sporozoites are polarized as the apical end contains a complex set of organelles from which the phylum Apicomplexa derives its name (Baum et al., 2008). This complex consists of polar rings which tether the cytoskeletal components and the secretory organelles such as micronemes, rhoptries and dense granules (Figure 4). The proteins that constitute and contained in these organelles are responsible for diverse functions such as motility, cell adhesion, cell traversal and cell invasion as well as the proteins responsible for the parasitophorous vacuole formation and modification. Motility of the parasite is driven by adhesive

micronemal proteins which are secreted at the apical end and are translocated along the length of the sporozoite to the basal end (Kappe *et al.*, 2004), which is driven by an actin-myosin motor located in between the sporozoite outer membrane and set of internal membranes (Kappe *et al.*, 2004, Vaughan *et al.*, 2008). The structural and functional aspects of this complex are reviewed in greater detail later.

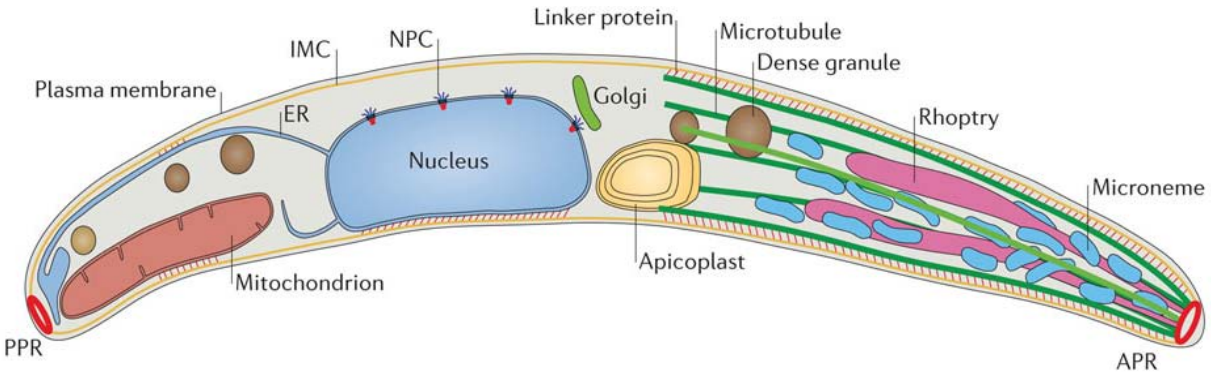


Figure 4. *Plasmodium* sporozoite showing internal structure and features as identified by cryoelectron tomography. Organelles associated with sporozoite are posterior polar ring (PPR), mitochondrion, endoplasmic reticulum (ER), inner membrane complex (IMC), nuclear pore complexes (NPCs) and nucleus, Golgi apparatus, apicoplast, dense granules, microtubules, rhoptries, micronemes and apical polar ring (APR). Adapted from de Niz 2017 (De Niz *et al.*, 2017).

As sporozoites egress from the midgut, they use gliding motility to invade the salivary gland ducts ensuring their transmission to mammalian host through a mosquito bite. Inside the mammalian host, sporozoites glide continuously through the skin to reach the blood vessels as seen by *in vivo* imaging of mouse skin (Amino *et al.*, 2008). Through the blood vessels the sporozoite reaches the liver where it penetrates the space of Disse and traverses through several hepatocytes before establishing infection (Amino *et al.*, 2008), (Ishino *et al.*, 2004).

1.3.1.7 Sporozoite invasion of the salivary gland

The circulation in the mosquito hemolymph is responsible for the translocation of sporozoites to all tissues in mosquito including wings and legs (Sinden & Matuschewski, 2005). Host-parasite ligand-receptor interactions mediate the attachment of sporozoites to the salivary gland basal lamina which they breach (Sinden & Matuschewski, 2005). They also breach the basal plasma membrane to

reach the acinar cells (Pimenta *et al.*, 1994). The invasion requires a host-derived vacuole which is transient in nature and plasma membrane disruption is not involved during salivary gland invasion (Pimenta *et al.*, 1994). CSP arguably the most important protein throughout the sporozoite life plays an important role in sporozoite salivary gland binding, specifically the central conserved region I (Sidjanski *et al.*, 1997). CSP N-terminal region and full-length CSP both compete and inhibit sporozoite attachment to salivary glands (Myung *et al.*, 2004, Sidjanski *et al.*, 1997). Thrombospondin Related Anonymous Protein (TRAP) deletion caused the abolition of sporozoite invasion and accumulation in the hemolymph (Sultan *et al.*, 1997). The extracellular adhesive A domain in TRAP and thrombospondin repeat domain both are required for salivary gland invasion (Matuschewski *et al.*, 2002). Upregulated in oocyst sporozoites 3 (UOS3) or S6 are similar to TRAP, the deletion of UOS3 resulted in the inhibition of salivary gland invasion by hemocoel sporozoites and compromised sporozoite motility through contrastingly these hemocoel sporozoites were successful in causing liver-stage infection(Combe *et al.*, 2009, Mikolajczak *et al.*, 2008). MAEBL a micronemal protein expressed in all sporozoites forms is also essential for salivary gland infection even though it is not required for gliding and hepatocyte invasion, sporozoites devoid of MAEBL are unable to attach to salivary gland basal lamina (Preiser *et al.*, 2004, Kariu *et al.*, 2002, Kappe *et al.*, 1998),

1.3.2 Development in mammals

1.3.2.1 Sporozoite migration to the liver

Majority of the mosquito salivary gland sporozoites that are deposited in the mammalian host skin selectively invade the hepatocyte. A distinguishing feature about the liver is the presence of specialized immune cells which responsible for inhibiting inflammation and T- cell activation (Tiegs & Lohse, 2010). This is necessary to prevent detrimental immune activation by harmless antigens present in intestinal blood (Weiner, 1997). Hepatocyte has an intrinsic tolerogenic MHCII signaling pathway (Wiegard *et al.*, 2007) which suppresses host immune response to an extent, along with a unique intracellular milieu which includes metabolites essential for parasite development that can be directly scavenged from the hepatocyte cytoplasm making hepatocyte an ideal host cell type (Figure 5).

Unlike closely related Apicomplexan parasites, *Toxoplasma* and *Theileria* which can invade a range of host cells, *Plasmodium* sporozoites exhibit host cell specificity (Dadimoghaddam *et al.*, 2014, Zöller *et al.*, 2013). This specificity can be attributed to circumsporozoite protein (CSP) which is also the dominant surface protein. CSP interacts with the Heparan surface proteoglycans (HSPGs), present on the hepatocyte surface (Cerami *et al.*, 1992), (Pinzon-Ortiz *et al.*, 2001). HSPGs are found throughout the body, but when expressed on the hepatocyte they have additional level of sulfations. The highly sulfated HSPGs (hsHSPGs) are negative charged and interact with the positively charged amino acids on both N-terminal (Rathore et al., 2002) and main thrombospondin like repeat region (TSR) of CSP (Frevert *et al.*, 1993), (Sinnis *et al.*, 1994) to target the liver. During sporozoites traversal through Kupffer cells, CSP is responsible for the prevention of the respiratory burst thereby safeguarding sporozoite in the macrophage during traversal (Usynin *et al.*, 2007).

hsHSPG's interaction with CSP is responsible for switching from traversal to productive invasion phenotype (Coppi *et al.*, 2007). This has been attributed to the cleavage of CSP by parasite cysteine protease which leads to the exposure of TSR (Coppi *et al.*, 2005, Coppi *et al.*, 2011) and conformational change in CSP triggered by calcium signaling cascade which is initiated due to CSP and hsHSPG interaction (Coppi *et al.*, 2007). The overexpression of pre cleaved CSP leads to increased level of infection by *P. berghei* in mice (Coppi *et al.*, 2011). Also, treatment with either protease inhibitors that inhibit this cleavage (Coppi *et al.*, 2005) or antibodies leads to the abolishment of the infection (Espinosa *et al.*, 2015).

Another sporozoite surface protein that contains TSR and integrin like A domain possibly interacts with the hsHSPG's is Thrombospondin Related Anonymous Protein (TRAP). TRAP has been found nonessential for adhesion but is indispensable for sporozoite invasion of hepatocytes. Recombinant TRAP has been shown to interact with hsHSPG's on hepatocyte surface (Frevert *et al.*, 1993), (Matuschewski *et al.*, 2002, Müller *et al.*, 1993) and recent studies show that TRAP interacts with host integrin αvβ3 (Dundas *et al.*, 2018).

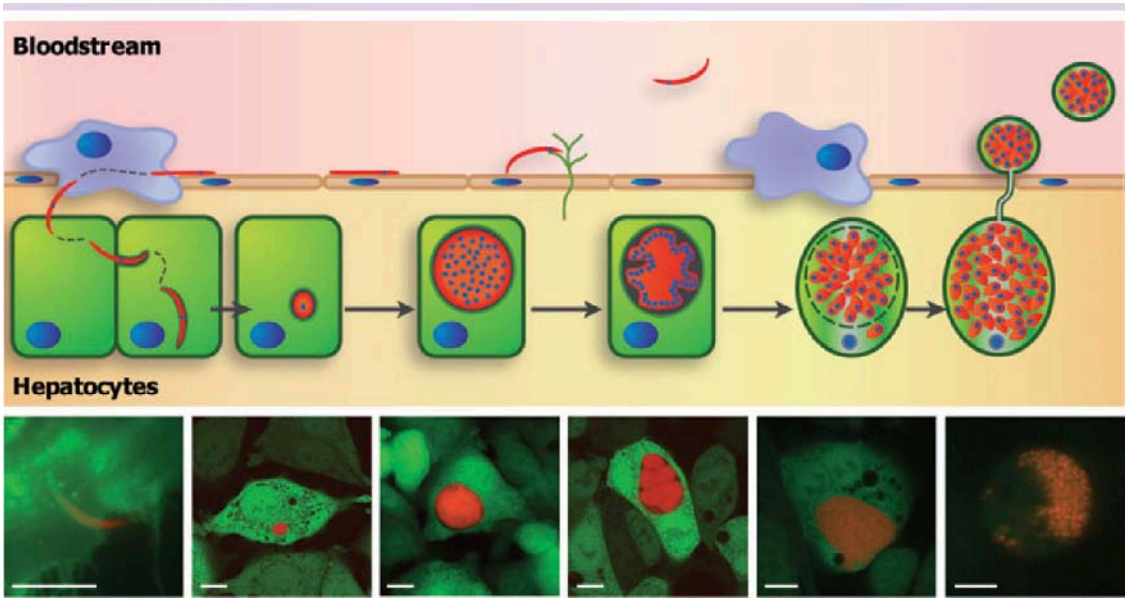


Figure 5. ***Plasmodium*** **parasite pre-erythrocytic stage development.** On reaching the liver through the bloodstream the sporozoite binds the highly sulfated heparan sulfate proteoglycans (hsHSPGs) present on the liver cell surface and glide along the endothelium and pass through Kupffer cells. They begin transmigration, after passing through several cells and finally invade hepatocyte. Productive invasion is marked by parasitophorous vacuole formation into a spherical structure that matures into a large schizont. Finally, the parasitophorous vacuole membrane dissociates and merozoites are free inside the host cell. They are released in the blood stream in the form of merosomes (infective merozoites packed vesicles surrounded by host cell membrane) (Rankin *et al.*, 2010).

1.3.2.2 Sporozoites invasion of the hepatocyte

There are two distinct types of cellular entry that a sporozoite performs. In the first case, sporozoite enters the cell but does not infect it, rather it exits the cell and leaving it wounded and this process has been termed as cell traversal (Mota *et al.*, 2001). The wounding of the host cell membrane is due to the parasite proteins that induce pore formation which includes cell traversal protein for ookinetes and sporozoites (CelTOS), CelTOS is essential for traversal in both mosquitos and mammalian host (Kariu *et al.*, 2006). SPECT 1/2 which are sporozoite micronemal proteins has also been found to be indispensable for cell traversal. The downregulation of SPECT results in the complete abolition of cell traversal but does not affect the ability of sporozoite to form liver stages (Ishino *et al.*, 2004) (Ishino *et al.*, 2005a). Studies suggest the phenomenon of wounding caused by the sporozoite only occurs during sporozoite exit, at the time of sporozoite entry formation of a transient vacuole has been observed (Risco-Castillo *et al.*, 2015a). This vacuole merges with the lysosome

resulting in the lowering of pH, SPECT2 is responsible for the disruption of this membrane and release of the sporozoite into the host cell cytoplasm, the sporozoite then exits the cell by membrane wounding. *Spect* 2 knockout parasites fail to exit the transit vacuole which leads to parasite degradation (Risco-Castillo *et al.*, 2015a).

As mentioned earlier, the process of traversal helps in the process of proteolytic processing of CSP (Coppi *et al.*, 2005) which triggers the secretion of other proteins involved in the process of invasion (Mota & Rodriguez, 2002). Export of CSP to hepatocytes inhibits NF-κB signaling and reprogramming host cell gene expression (Singh *et al.*, 2007), though NF-κB mediated inflammatory response triggered by traversal has also been observed to affect parasite development . Studies suggest rhoptry secretions are not triggered by traversal (Risco-Castillo *et al.*, 2014, Risco-Castillo *et al.*, 2015a). The wounding of hepatocytes due to traversal releases hepatocyte growth factor which has been shown to enhance infection in the case of *P. berghei (Carrolo et al., 2003)* specifically and not the other species (Kaushansky & Kappe, 2011). Also, phenotype exhibited by SPECT deficient sporozoites raise questions on the essentiality of the process of cell traversal. Though opinions remain divided on the benefits of cell traversal, the fact remains that a sporozoite traverses several hepatocytes before it productively invades a cell as observed using fluorescent microscopy (Mota *et al.*, 2001), the degree of traversal may vary *in vivo* as intravenously injected sporozoite traverse to a greater degree than sporozoites transmitted through a mosquito bite (Mota *et al.*, 2001).

CD81 which is a part of the complex of proteins present together on hepatocyte surface are required for several homeostatic processes such as cell signaling and adhesion (Levy *et al.*, 1998) was implicated in the entry of Hepatitis C virus by interacting to viral protein E2 (Pileri et al., 1998). CD81 was amongst the first host factors recognized which was essential for sporozoite infection of the hepatocyte. Gene deletion and anti-CD81-antibodies both demonstrate the abrogation of hepatocyte infection by sporozoites in *P. yoelii* and *P. falciparum* (Silvie *et al.*, 2003, Silvie *et al.*, 2006) (Silvie *et al.*, 2007)but not in *P. berghei* (Silvie *et al.*, 2007). CD81 though incosequential to host cell traversal but is necessary for the parasitophorous vacuole membrane (PVM) formation as it triggers the discharge from the rhoptries (Risco-Castillo *et al.*, 2014).

Scavenger receptor B1 (SR-B1) which is a lipid transporter present on the hepatocyte also known as hepatitis C entry factor plays a crucial role in productive *Plasmodium* infection. The absence of SR-B1 does not abolish but leads to a significant reduction in the infection (Yalaoui et al., 2008). This partial control on infection could be exerted through CD81, as maintenance of the CD81 tetraspanin microdomain requires SR-B1 (Yalaoui *et al.,* 2008). Another host factor aiding parasite survival in the host cell by altering intracellular milieu is hepatocyte growth factor c-met which is released on hepatocyte injury by traversing *P. berghei* sporozoites (Carrolo *et al.,* 2003). It also has an anti-apoptotic role further helping in maintaining infection (Leiriao *et al.*, 2005). Hepatocyte EphA2 like CD81 is critical for the formation of PVM and it interacts with previously identified 6-cysteine proteins which derive their name from fold domain present in the protein-containing 6-cysteins are a feature of many membrane proteins (Kaushansky *et al.,* 2015).

Plasmodium micronemal proteins P52 and P36 are amongst 6-cystein proteins and P36 shown to interact with EPhA2 and this process is responsible for the PVM formation (Kaushansky *et al.*, 2015). Both these proteins have been found to be critical for PVM formation(Ishino *et al.*, 2005b). The importance of these micronemal proteins had been previously established as individual KOs for both genes show complete abolition of PVM formation (van Dijk *et al.*, 2005). Double KOs of *P36* and *P52* in both *P. yoelii* and *P. falciparum* show complete abolition of liver stage development (Labaied *et al.*, 2007, VanBuskirk *et al.*, 2009).

Plasmodium 6-Cys fold proteins show structural similarity to Ephrin-like fold found on many membrane proteins found in metazoans (Arredondo *et al.,* 2012). As mentioned above EphA2 of this family interacts with P36 for PVM formation and mice devoid of EphA2 do not develop infection (Kaushansky *et al.*, 2015).

Rhoptry and micronemal proteins are common to all parasite invasive stages which indicate the indispensable role of their secretion for the process of invasion. The secretion from these organelles along with actin-myosin motor drives the process of invasion. The precise mechanism is not fully understood, though it is known to be driven by the same parasite actin-myosin motor that drives gliding motility, rather than internalization by the host cell (Amino *et al.,* 2008). It is thought that after secretory organelles discharge, the excreted proteins attach to ligands on the

hepatocyte surface, which leads to the formation of a tight ring around the parasite (Baum *et al.*, 2006, Bano *et al.*, 2007). The complex is then translocated away from the apical end using an actin-myosin motor similar to that used in sporozoite gliding motility (Baum *et al.*, 2006) creating a moving junction which excludes host surface proteins, creating the PVM (Bano *et al.*, 2007). Though host F-actin localizes to the moving junction (Gonzalez et al., 2009), but it does not participate in active internalization of the parasite; it is believed it helps to stabilize the complex. Interestingly, host actin and the actin-related protein 2/3 complex (Arp2/3) appears to be recruited to the moving junction, though what signals may promote this recruitment is unknown (Gonzalez *et al.*, 2009).

When merozoites invade the red blood cell two proteins namely rhoptry neck protein RON2 and micronemal apical membrane antigen 1 (AMA1) form the moving junction (Florens *et al.*, 2002, Srinivasan *et al.*, 2011). RON4 (rhoptry neck protein) a similar protein in found in sporozoites, facilitates invasion, and AMA1 is also expressed in sporozoites (Florens *et al.*, 2002). Antibodies against AMA1 inhibit invasion of *P. falciparum* sporozoites *in vitro* (Silvie *et al.*, 2004) as well as *P. yoelii in vivo* (Schussek *et al.*, 2013). However, in *P. berghei*, AMA1 is not involved in sporozoite invasion (Giovannini *et al.*, 2011). Also, the formation of RBC tight junction seems to be independent of host proteins, contrastingly hepatocyte membrane proteins seem to be essential for sporozoite invasion, thus invasion processes of both invasive stages differ from each other. Elucidating these interactions could aid in vaccine development and consorted efforts to block malaria at the liver stage.

1.3.2.3 Liver stage development and host cell interactions

As and when a sporozoite completes a productive invasion i.e. after transcytosis the elongated sporozoite changes morphology (de-differentiation) into spherical shape ejecting from the host cell protein complexes involved in the process of invasion and motility placing itself in close proximity to the host nucleus (Bano *et al.*, 2007, VanBuskirk *et al.*, 2009). Serum factors (albumin or bicarbonate) and change in temperature from 22□C (mosquito body temperature) to 37^0C (human body temperature) can stimulate isolated sporozoites into EEF like spherical morphogenic forms in *vitro* (Hegge *et al.*, 2010, Kaiser *et al.*, 2003), these morphogenic changes require proteasomal degradation which is initiated by a surge of calcium ions (Doi *et*

al., 2011, Coppens, 2011). Time taken for this process could vary and generally, it takes 20-28 hours in rodents and 3 days in human parasites (VanBuskirk *et al.*, 2009, Hegge *et al.*, 2010, March *et al.*, 2013).

The PVM undergoes modification by the parasite via the insertion of parasite-derived proteins. The main function of PVM is that it acts as the main interface between host and parasite and protection from host cellular defenses. The B-cell lymphoma (Bcl-2) derived pathway has been implicated in the clearance of infected liver stages (Kaushansky *et al.*, 2013), a host-specific resistance from clearance has also been observed in certain liver-stage parasites (van de Sand *et al.*, 2005). *P36-/P52-* parasite where no PVM formation takes place is also cleared rapidly (Labaied *et al.*, 2007), (van Dijk *et al.*, 2005). PVM formation is compromised in Cells with diminished EphA2 receptor expression and infection does not persist beyond 24 hrs (Kaushansky *et al.*, 2015) .

Intracellular clearance of the parasite could be a result of autophagy by the host. Well-characterized autophagy marker LC3 decorates the PVM but this gradually subsides with the progression of time (Thieleke-Matos *et al.*, 2016, Prado *et al.*, 2015), which could be a result of parasite-mediated PVM modification. Targeted deletion of host Atg5 results in reduced clearance of infected hepatocytes in *P. berghei* (Prado *et al.*, 2015).

When compared to blood-stage parasites the liver stage remodeling of the PVM does not appear to be drastic. During late time points, liver stage development trans vesicular network (TVN) is seen (Grützke *et al.*, 2014) and the growth of the liver stage schizont supersedes the size of the host cell many fold and deforms it physically (Vaughan *et al.*, 2012). There are rarely any proteins reported which cross PVM entering into the host cell cytosol (Pei *et al.*, 2010) (Orito *et al.*, 2013). A possible reason is the exposure of parasite proteins could result in their presentation via Major histocompatibility complex leading to cytotoxic CD8 T-cell mediated destruction (Montagna *et al.*, 2014, Bongfen *et al.*, 2007). LISP2 expresses in the mid to late liver stages is amongst the proteins exported to the hepatocyte and is essential for merozoite formation (Orito *et al.*, 2013).

The PV and TVN are the major interfaces separating host and parasite, these are major sites for nutrient scavenging. UIS3 which localizes at the PVM interacts with liver fatty acid binding protein (L-FABP) (Mikolajczak *et al.*, 2007) which is probably involved in the uptake of essential lipids required for parasite growth such as cholesterol, precise mechanism of transport is unclear (Favretto *et al.*, 2013). Incorporation of host phospholipids into PV and TVN by the parasite such as phosphatidylcholine (Itoe *et al.*, 2014) and phosphatidylinositol phosphate [PI(3,5)P2] (Thieleke-Matos *et al.*, 2014) has also been observed. Both late endosomes and lysosomes are sequestered around the PVM (Thieleke-Matos *et al.*, 2014), (Lopes da Silva *et al.*, 2012) possibly providing nutrients to the parasite through non-specific autophagy of host cellular components (Thieleke-Matos *et al.*, 2016, Prado *et al.*, 2015). A close association between parasite PVM and the host nuclear membrane and endoplasmic reticulum have also been observed (Bano *et al.*, 2007), though the function of this association is unknown. Molecules up to 855 kilodaltons can pass through PVM passively (Bano *et al.*, 2007) which might allow uptake of host metabolites. The egress of merozoite from the cell requires PVM rupture several proteases in *Plasmodium* have been identified which facilitate this process. *Plasmodium* protease *Sub*1 is essential for PVM rupture as the targeted disruption of *Sub*1 stops the egress of hepatic merozoites. During liver stage development synthesis of organellar and merozoite membranes creates a high demand for lipids. Initially, it was speculated that parasite scavenges lipids from the host (Vial & Ancelin, 1992), later apicoplast based de novo synthesis of fatty acids through fatty acid pathway II (FAS II) was discovered (Ralph *et al.*, 2004). The FAS II pathway enzymes FabB/F, FabG, FabI, and FabZ are essential for late liver stage development but not for blood-stage (Vaughan *et al.*, 2009) making them attractive targets for chemotherapeutic intervention.

Sporozoite surface protein essential for liver stage development (SPELD) which localizes on the sporozoite plasma membrane in *P. berghei* has been found to be essential for EEF development (Al-Nihmi *et al.*, 2017). Gene regulation at the transcriptional level plays a critical role in liver stage development, RNA binding protein meiosis inhibited (Mei 2) has been found to be essential for merozoite development. *Mei2* KO exhibit abnormal DNA segregation and impaired merozoite formation (Dankwa *et al.*, 2016).

The rupture of the parasite PV membrane causes major alterations in the host cell membrane these include separation from the actin cytoskeleton and loss of PIP_2 (phosphatidylinositol 4,5-bisphosphate). PIP_2 dependent protein Ezrin links actin to the plasma membrane and several transmembrane proteins which include E-cadherin and lipid-linked proteins. These alterations destabilize the host cell membrane playing a crucial role in merozoite egress from the infected hepatocyte (Burda *et al.*, 2017).

1.3.2.4 Asexual blood stages

Merozites released in the blood sense low K^+ ion concentration which triggers a signaling cascade leading to a rise in intracellular cAMP level. cAMP-mediated activation of PKAc leads to the secretion of micronemal proteins which facilitate the invasion process (Dawn *et al.*, 2014). The merozoite orients itself to erythrocyte membrane using adhesins and initiates the process of invasion by using invasins which happens in under a minute (Cowman & Crabb, 2006). The reorientation and recognition of erythrocyte surface markers by invasins are followed by the formation of the tight junction. Parasite formin FRM1 localizes to the moving junction is responsible for actin polymerization at the site (Baum *et al.*, 2006). The invasion related proteins are stored in micronemes which are secreted during host cell invasion (Cowman & Crabb, 2006). The merozoite consists of specialized membranes, the external plasma membrane and inner membrane which in complex with the cytoskeleton maintain merozoite shape which provides rigidity and strength and also facilitates the process of cell division. The merozoite surface contains several proteins including MSP1 with a cohort of GPI anchored and other associated proteins which include MSP1, MSP2, MSP4, MSP5, and MSP10 (Sanders *et al.*, 2005) MSP6 and MSP7 form a functional complex (Kauth *et al.*, 2006, Kauth *et al.*, 2003). The blood-stage adhesins can be of two major category's erythrocyte binding like (EBL) and reticulocyte binding like (RBL) proteins which are involved in the invasion of either RBCs (mature RBCs) or reticulocytes (young RBCs) (Sim *et al.*, 1994, Rayner *et al.*, 2000, Triglia *et al.*, 2001, Duraisingh *et al.*, 2003). Erythrocyte binding antigens (EBAs) another group of adhesion proteins such as EBA-175, Eb11 and EBA-140 is involved in interaction with glycophorin A, B and C respectively (Sim *et al.*, 1994, Lobo *et al.*, 2003, Maier *et al.*, 2003) on the RBC membrane.

Blood stage parasites are devoid of locomotory organelles; hence they reach the erythrocytes passively in blood stream. *Plasmodium* merozoites are 1.5 µm long ovoid structures (Bannister & Mitchell, 2003) consisting of polar rings, and secretory organelles: rhoptries, dense granules, exonemes and micronemes (Yeoh *et al.*, 2007) (Fig). The Rhoptries localize at the apical end of the parasite in a pair wise arrangement and store Rh family proteins (Bannister *et al.*, 2000). Dense granules store and secrete proteins that are exported to the PVM completing the final step of invasion (Bannister *et al.*, 1975, Aikawa *et al.*, 1990), the exonemes secrete subtilisin 1 (SUB1) which mediate merozoite rupturing (Yeoh *et al.*, 2007). Another invasin essential for the invasion process is Apical membrane antigen 1 (AMA1) (Mitchell *et al.*, 2004). During the merozoite release AMA1 undergoes proteolytic cleavage for activation (Narum & Thomas, 1994). Merozoite thrombospondin-related anonymous protein (mTRAP) with actin-myosin motor forms a complex through aldolase (Figure 6) (Pinder *et al.*, 1998, Jewett & Sibley, 2003).

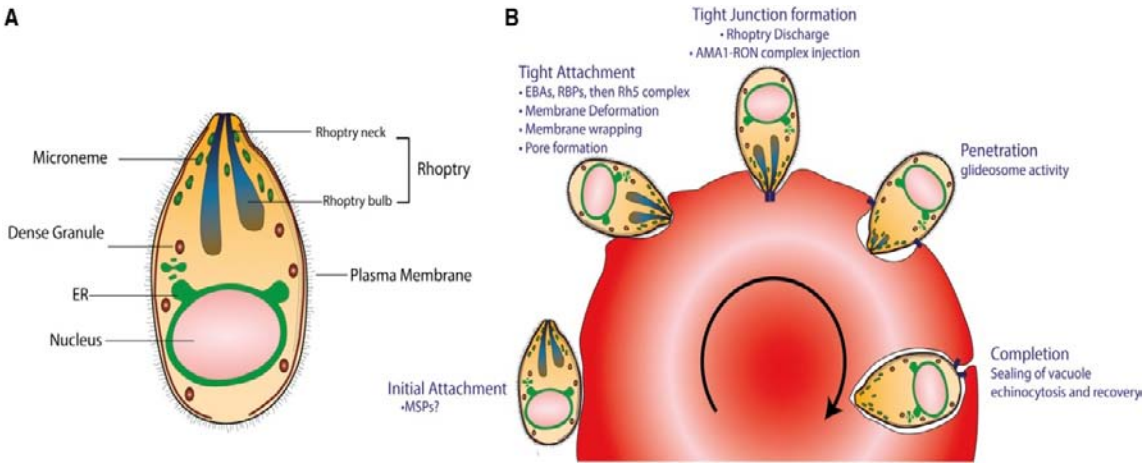


Figure 6. The internal structure of *P. falciparum* blood-stage merozoites and host erythrocyte invasion mechanism. A. Subcellular structure of merozoites, showing micronemes and rhoptries at the apical end. Micronemes and rhoptries secrete proteins like AMA1 and Rhoptry Neck (RON) proteins. B. Mechanism of RBC invasion by merozoites. Apical reorientation leads to the apical end of merozoite coming towards the RBC membrane, forming a tight attachment. RON complex moves in the host cell membrane and forms a tight junction with AMA1 on merozoites surface and RON2 inserted in the erythrocyte membrane. The tight junction then moves towards the posterior end. It is powered by parasite actin-myosin motor. The surface coat is shed and after reaching the posterior end, adhesive proteins at the tight junction are proteolytically removed by a resident protease (Cowman *et al.*, 2012).

1.4 Malaria vaccines

Though *Plasmodium* demonstrates an innate ability of immune evasion, frequent exposure to the parasite can lead to the development of natural immunity against it (Longley *et al.,* 2017). The modern approach to vaccine development against malaria started with the immunization of irradiated sporozoites in mice (Nussenzweig *et al.*, 1967). Since then there has been steady influx of data, and several breakthroughs have contributed to enhancing our understanding of cellular and molecular pathways that mediate protection in animal and human models. There is no effective vaccine against malaria, it is important to consider though the humongous scientific and technical challenge for those who attempt to develop a vaccine against such a complex eukaryotic parasite. The revised malaria vaccine road map 2030 (Moorthy *et al.,* 2013) calls for a next-generation vaccine aiming for 75% efficacy over 2 years against *P. falciparum* and/or *P.vivax* , while also fulfilling goals set in 2015 of first-generation vaccine which requires to provide >50% efficacy which can last for more than 1 year. To achieve these goals a concerted effort has to be made in combining the successes of the current pre-erythrocytic subunit vaccine and whole sporozoite based vaccine along with new strategies to integrate blood-stage and transmission-blocking immunity to combat the disease.

1.4.1 Sporozoite subunit vaccine

RTS,S/AS01 has been the most extensively tested sub-unit vaccine against *P. falciparum* malaria, which directs immune response against major sporozoite surface protein the circumsporozoite protein (*Pf*CSP). The vaccine contains a virus-like particle with two components- 18 copies of central repeat region and C-terminal region of the major sporozoite surface antigen circumsporozoite protein (*Pf*CSP) fused with Hepatitis B surface antigen (HBsAg). GlaxoSmithKline formulated this with a potent liposomal adjuvant system AS01. Though it is the only vaccine has demonstrated protective efficacy against clinical malaria phase III trial the protection is partial and shows differences in efficacy based on the age (protection was lower in 6-12 weeks old infants compared to 5-17-month-old young children). A smaller phase II study conducted on a few hundred children suggests that there may be shifting and rebound cases in 5 years post-vaccination (Olotu *et al.*, 2016). The exact immune mechanism responsible for RTS,S efficacy is yet to be determined. The existing data

suggest that the antibody concentration against NANP amino acid repeats is closely linked to protection and the reduction in anti-NANP antibody titers could possibly be responsible for decreased efficacy (White *et al.*, 2015). The C-terminal region of *Pf*CSP which is also a component of the RTS,S vaccine the antibody response against this region is unclear as monoclonal antibodies (MAbs) obtained from humans by immunization of sporozoites does not confer resistance in mice (Scally *et al.*, 2018). As the C-terminus region of *Pf*CSP contains two well-defined epitopes for CD4+ T cells, studies suggest that CD4+ T cells response also plays some role in protection (Kazmin *et al.*, 2017).

Administration of AS01 adjuvant along with RTS,S has been found to induce high antibody titers as the observed anti-NANP polyclonal titers were as high as 150 µg/ml in the serum (Kester *et al.*, 2009). Dose modification and administration schedules have been found to be the most effective ways of increasing the efficacy of RTS,S/AS01. Volunteers demonstrated protection 3 weeks after the last dose under standard regimen 0-1-2 months were against Controlled Human Malaria Infection (CHMI). In a variation to this dosing regimen, the volunteers with delayed 3rd dose (6 months of the second dose) with only one-fifth of the original dose achieved enhanced immunological parameters such as somatic hyper mutation and isotype switching (Regules *et al.*, 2016).

Future variants of subunit vaccines being tested as a part of the next-generation vaccine initiative contain R21 vaccine. This variant comprises a single subunit vaccine but lacks four-fold excess HBsAg (Collins *et al.*, 2017). The proportionate increase of the *Pf*CSP component to HBsAg when compared to RTS,S could possibly improve anti-NANP IgG responses. Clinical testing is underway for full-length *Pf*CSP containing N terminal non repeat region. Structure-based vaccine designing is the latest approach for vaccine development and several viral proteins have been subjected to this approach (Kwong, 2017). In this approach monoclonal antibodies (mAb's) from people previously exposed to an infection are isolated that initiate neutralization with the highest level of potency. The structure of the epitope bound to the Ab is determined, this information is further utilized to designing immunogens. This approach is being used for *Pf*CSP to find characteristics of anti-NANP mABs to aid next-generation vaccine designing (Oyen *et al.*, 2017).

A novel class of anti-*Pf*CSP antibodies has been identified that target the junction between R1 cleavage site and the repeat region. These have also been found to be more effective against *P. falciparum* infection in humanized liver chimeric mice (Kisalu *et al.*, 2018, Tan *et al.*, 2018). These new insights might help in the generation of a potent anti-CSP response as this contains the junctional epitope that RTS,S lacked. *Pf*CelTOS, a micronemal secreted protein has been used as a recombinant vaccine conjugated with GLA-SE as an adjuvant. Although this combination showed modest immunogenicity and inefficient CHMI. The use of genomic, transcriptomic and proteomic techniques with advanced computational biological tools has led to a long list of potential vaccine targets. Thus the challenge in hand is screening these targets to obtain a potent vaccine that assimilates the qualities previously existing candidates with enhanced clinical protection (Figure 7).

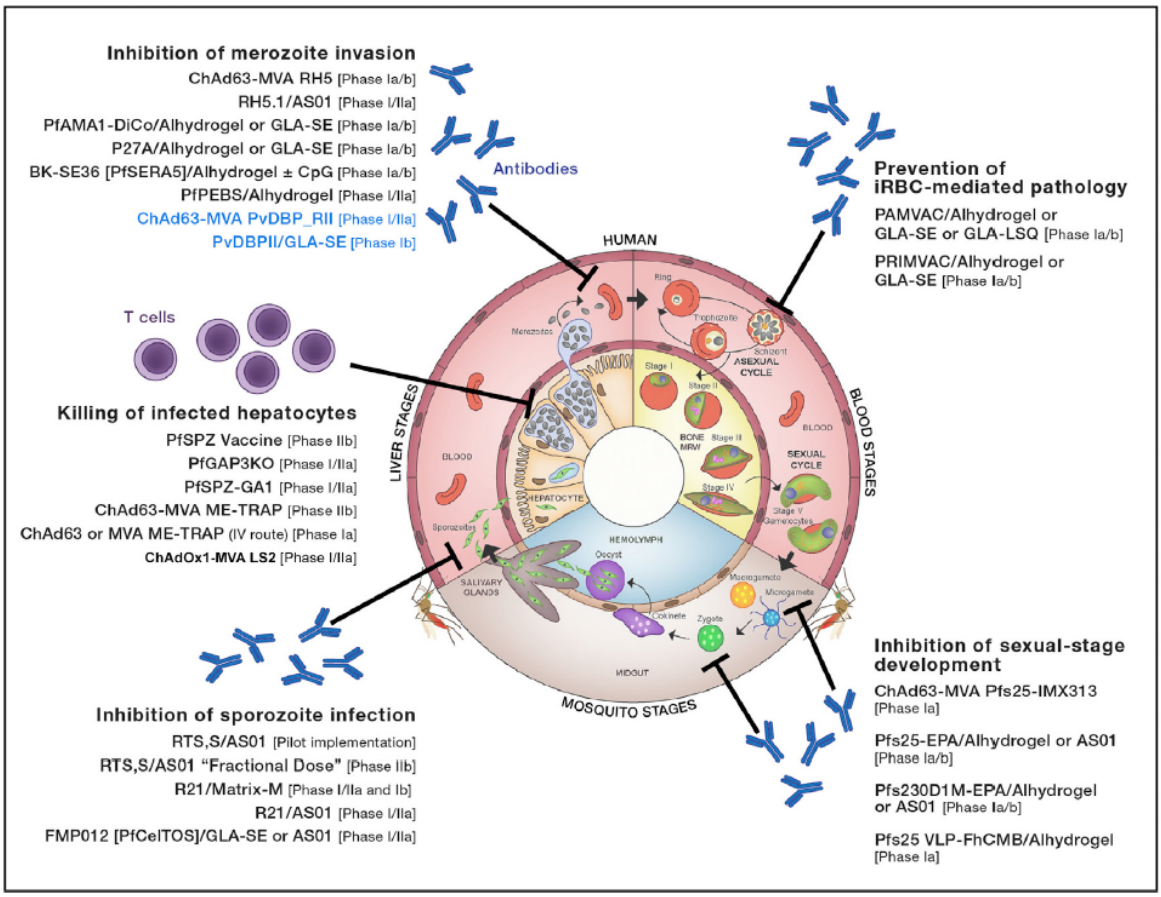


Figure 7. The vaccine candidates in the stage of clinical development. The data sources include the WHO malaria vaccine rainbow table and Clinical trials.gov.

1.4.2 Whole sporozoite Vaccines

The whole sporozoite vaccine (WSV) as the name indicates are live attenuated sporozoites that infect hepatocytes normally but cease to develop in between failing to reach the blood stages. WSVs have been proven to elicit high-level protection in animal models and similar results were obtained in CHMI. The challenge in vaccine designing remains to treat malaria-endemic population with a vaccine with high efficacy and also keeping in mind the duration of protection. Studies on rodent models have shown that the stage at which the parasite ceases development has a direct correlation to the potency and duration for which the immune response exists.

Two variants of WSV are Radiation attenuated sporozoites (RAS) and Genetically attenuated parasite (GAP) vaccine. Developmental arrest exhibited by RAS is random in nature and they cease development at early liver stages whereas GAP which has targeted gene deletions arrest at specific time points based on the gene being targeted. Radiation attenuated *P. falciparum* sporozoites (*Pf*SPZ vaccine) have been used for conducting clinical trials and major advances in vaccine research have made possible the use of cryopreserved *Pf*SPZ vaccine. The intravenous administration of *Pf*SPZ in non-human primates has been found to elicit strong tissue-resident $CD8^+$ T-cell response which is not observed in subcutaneous administration (Epstein *et al.*, 2011) similar studies in humans also suggest that IV administration compared to subcutaneous administration demonstrated a greater potency . The administration of *Pf*SPZ to pre-exposed individuals has been found to elicit anti-CSP IgM responses which inhibit sporozoite invasion in vitro (Zenklusen *et al.*, 2018). *Pf*SPZ will also be tested in a large scale field trial which will be done on 2,800 individuals in Africa beginning in 2020.

First clinical trial conducted using *p52⁻/p36⁻ P. falciparum* GAP resulted in breakthrough (Spring *et al.*, 2013), Spring et al., 2013). A triple knockout GAP with *p52⁻/p36⁻/sap⁻* (PfGAP3KO) demonstrates complete attenuation at the early liver stage and is immunogenic when administered through mosquito bite in US adults. Conceptually parasites that arrest at late liver stages should provide superior protection compared to an early stage arresting GAP as they would express a larger variety of liver-stage antigens exposing the host immune system to a greater immunogenic repertoire. A comparative study conducted in mice demonstrates late-

stage arresting GAP exhibit superior protection and broader range of CD8 T-cell responses than early-stage arresting GAP. Late liver-stage arresting GAPs also provide stage transcending immune protection in mice (Sack *et al.*, 2015). A double knockout in *P. yoelii* of *plasmei2*$^{-}$*/lisp2*$^{-}$ arrests at late liver stage shows complete attenuation and a robust antibody and T cell response is being conceptualized for GAP development in *P. falciparum* (Vaughan *et al.*, 2018).

Another strategy for WSV utilizes drug prophylaxis in combination with sporozoite immunization. Sporozoites inoculation along with administration of chloroquine permits complete liver stage development complemented by chloroquine which prevents blood stage development. This approach has shown 100% protection in CHMI but reduced protection when two different parasite strains were used. Development of WSV is crucial as they target a bottleneck in the parasite lifecycle as the stage it targets is also asymptomatic and a successful liver-stage vaccine could bring mankind one step closer to malaria eradication.

1.4.3 Blood stage vaccines

As mentioned above repeated exposure to blood-stage parasite leads to naturally acquired immunity against malaria. Blood stage parasite vaccines aim to target the parasites machinery involved in erythrocyte invasion hence several major merozoite antigens have been used for the development of an efficient blood-stage vaccine. Attempts at generating vaccines that mimic naturally acquired immunity could be futile as certain individuals exhibiting natural immunity to malaria and have asymptomatic parasitemia can transmit malaria to non-immune individuals. Hence newer goals in blood-stage malaria vaccine development attempt a more robust inhibition of merozoite invasion. This approach targets merozoite antigens that are not subject to significant natural immune evasion. The previously used immuno dominant merozoite antigens preferred as vaccine targets have been inefficient in clinical trials due to several different reasons, antibody response could be strain-specific due to broad range of antigenic polymorphism displayed by the parasite and targeting invasion pathways which are redundant.

An appropriate example to elucidate points mentioned above is the Phase1 clinical trial for JAIVAC-1, a vaccine that combines conserved region from *Pf*MSP-1 and

receptor binding F2 domain of erythrocyte binding antigen 175 (EBA175). Results indicate strain-specific immune response with poor response to PfMSP1 and the protection was driven by *Pf*F2 serum antibodies (Mueller *et al.*, 2015). *Pf*AMA1 is a well-characterized merozoite protein, as it displays ample polymorphic variation in new efforts to target this gene uses several different allelic variants and identifying antibodies that target several epitopes increasing the spectrum of susceptible parasite strains (Sirima *et al.*, 2017). An in vivo study conducted in *Aotus* monkeys a peptide ligand from PfRON2 was used complexed with *Pf*AMA1 which demonstrated improved protection (Srinivasan *et al.*, 2017). The reticulocyte binding protein homolog 5 (PfRH5) in *P. falciparum* which interacts with the basigin (CD147) present on the erythrocyte surface is conserved amongst strains and an attractive target to combat problems of redundancy as it is essential for merozoite invasion (Crosnier *et al.*, 2011, Draper *et al.*, 2015). Vaccination with full-length PfRH5 conferred protection against challenge with heterologous wild type strains also the purified serum IgG was tested in vitro for growth inhibition activity in a cell-independent assay which demonstrated protective outcome (Douglas *et al.*, 2015). Similar growth inhibition assays were used to identify anti-*Pf*RH5 human monoclonal antibodies and a study conducted in humanized mice showed in vivo protection on passive transfer of these antibodies (Foquet *et al.*, 2018).

A new soluble multiprotein complex has been identified containing *Pf*RH5-*Pf*Ripr-*Pf*CyRPA which could address a lot of key problems of blood vaccine design mentioned above. This complex is conserved. It is also essential for invasion, involved in Ca^{++} ion release and ultimately for the formation of tight junction along with *Pf*113 merozoite surface protein (Chen *et al.*, 2011, Volz *et al.*, 2016, Galaway *et al.*, 2017). Targeting multi-protein complexes could be the way forward in designing vaccines that target processes such as invasion as they could act in a synergy resulting in improved inhibition.

1.4.4 Transmission blocking vaccines

This variant of malaria vaccines targets the sexual stages of malaria parasites, it is unique in malaria vaccines as it does not combat with the symptomatic stages of parasite infection and the person immunized does not benefit from the vaccine. It aims to disrupt the parasite life cycle by inhibiting its development in the mosquito stages

hence preventing transmission. This class of malaria vaccines is beneficial to the populations endemic to the disease and addresses problems of malaria transmission through asymptomatic carriers of the parasite.

The obvious targets for transmission-blocking vaccines are ookinete and gametocyte proteins, amongst these *Pfs*25 which is a GPI linked ookinete surface protein (Stowers *et al.*, 2000). *Pfs*230 is responsible for the RBC binding of the gametocyte and oocyst development (Eksi *et al.*, 2006). *Pfs*48/45 another gametocyte surface protein implicated in fertilization. In a comparative preclinical study between them, all three have shown promising results individually (Kapulu *et al.*, 2015). A recent study conducted in mice immunized with several fusion chimeras of *Pfs*230 and *Pfs*48/45 demonstrated a threefold increase in transmission-blocking in one of the variants in comparison to each individual antigen (Singh *et al.*, 2019). Other proteins which have generated avid interest are *Pfs*47 required in mosquito stage and is associated with immune evasion (Molina-Cruz *et al.*, 2013) and a gametocyte membrane protein *Pf*HAP2 which is essential for fertilization (Angrisano *et al.*, 2017).

*Pfs*25 formulated with Montanide ISA51 underwent phase 1 clinical trials but were found to be highly reactogenic as a result was not pursued further development (Wu *et al.*, 2008). The focus was shifted to other delivery platforms, Pfs25 conjugated with exprotein A (EPA) (Shimp *et al.*, 2013) and another variant *Pfs*25-EPA/Alhydrogel was subjected to clinical trials (Talaat *et al.*, 2016). Identification of novel antigenic regions in *Pfs*25 aided by crystal structures of antigen-antibody complex (Scally *et al.*, 2017) might further improve our understanding of aid in structure-based vaccine design.

1.5 *P. berghei* as a model for malaria

Establishing continuous *in vitro* culture for *P. falciparum* has been a landmark in malaria research (Jensen & Trager, 1978). It has enabled the extensive dissection of the molecular pathways governing the basic parasite biology and studies with direct clinical relevance. However, due to the absence of any non-primate animal models compatible with *P. falciparum* (Zapata et al., 2002), its use becomes restricted to biochemical studies. This has led to the employment of other *Plasmodium* species as a model organism in pathogenesis and transmission studies. They have especially

enhanced the understanding of mosquito and liver-stage basic biology by increasing tractability. The ease in handling and breeding has led to the establishing of the rodent malaria system as the dominant model of use. There are several naturally occurring rodent malaria species. Amongst these most well-characterized are *Plasmodium berghei, Plasmodium chabaudi, Plasmodium yoelii, Plasmodium vinckei* which have been adapted for laboratory (Carlton & Carucci, 2002). Studies indicate a great deal of conservation in housekeeping genes and stage-specific proteomes amongst mammalian malaria parasites (Lasonder *et al.*, 2002) thereby validating the use of rodent malaria parasites as disease models.

In our lab, *P. berghei* is used as a model organism. It makes for an excellent malaria transmission model system, which provides efficient and reliable murine and mosquito infections. The investigators can work on malaria-infected mosquitos with no risk of infection (Butcher *et al.*, 1996). Another advantage of using *P. berghei* is that unlike *P. falciparum*, its vector stages such as zygote and ookinete can be cultured *in vitro* (Janse & Waters, 1995). *Anopheles stephensi* provides the most efficient transmission of *P. berghei* and is used for experiments.

1.6 Objectives:

The present study proposes to examine the role of Pb*S*14 and Pb*Scot*1 in *Plasmodium* life cycle stages. The proposed study will aim at the following:

1. Preparation of DNA constructs to carry out transfections for the generation of and HA-mCherry transgenic of Pb*S*14 and Pb*Scot*1.
2. Preparation of targeting DNA constructs to carry out transfections for the generation of Pb*S*14 and Pb*Scot*1 *KO* by Double Cross Over recombination.
3. Transfections in *Plasmodium berghei* to generate transgenics and knockouts.
4. Selection of stable transfectants by subjecting the parasites to antimalarial drug-pyrimethamine under *in vivo* conditions.
5. Limiting dilutions of the resistance transfected parasite to get a single clone.
6. Observation of HA-mCherry expression of *S*14 and SCOT1 and confirming respectively the localization and functional role in pre-erythrocytic stages.
7. Initiation of KO cycle in mosquitoes to study parameters associated with the sporozoite gliding pattern, ability to invade cells, development, and progression through liver stages by maintaining parasite in both mosquito and rodent (mice) hosts.
8. Analyzing the *in vivo* infection dynamics of the KO line to implicate its role in pre-erythrocytic stages.
9. Analyzing the protective immunity of the KO, if gene depletion leads to parasite attenuation leading to failure to progress through liver stages.

Chapter 2

Plasmodium berghei S14 regulates sporozoite gliding motility and infectivity

2.1. Introduction

As described in the previous chapter *Plasmodium* parasite in complying with the changing host microenvironment and its own pre-determined needs, alters its morphological forms. Facilitating these changes requires a paradigm shift in the gene expression for the parasite from one stage to another. The invasive forms of the *Plasmodium* parasite especially the sporozoite along with merozoite and the ookinete need to penetrate non-permissive biological membranes are deemed to have common features i.e. similar organellar protein expression profiles. Though the zooite forms of *Plasmodium* share the characteristic feature of invasion, they differ in the unique adaptations permitting them to interact and sustain in different host cell environments. Merozoites target either erythrocytes or reticulocytes and remain in mammalian hosts. In contrast, sporozoites are unique as they invade cells in both mosquito vector and hosts hence it becomes necessary for sporozoites to express a greater repertoire of cell surface invasins and adhesins to facilitate the recognition and invasion of the diaspora of cells it encounters in its long journey from oocyst to the hepatocyte. Another distinguishing feature between these invasive forms is that merozoites do not glide (Frénal *et al.,* 2017) whereas sporozoites glide for the majority of their lifespan. Along with invasion, gliding motility is the most important feature of the sporozoite stages and both processes have been studied in detail.

For the identification of the stage-specific pre-erythrocytic transcripts, suppression subtractive hybridization (SSH) of *Plasmodium yoelii* salivary gland sporozoite versus merozoites was performed (Kaiser *et al.,* 2004). This led to the identification of 25 genes unique to the *Plasmodium yoelii* salivary gland sporozoite stage and 12 of these genes contained the signal peptide indicative of their involvement in the secretory pathway. These genes together were termed as S genes and include the major sporozoite surface protein circumsporozoite protein (CSP) and thrombospondin related anonymous protein (TRAP). CSP and TRAP are amongst the well-characterized *Plasmodium* proteins (Menard *et al.,* 1997, Sultan *et al.,* 1997) and over the period of time, a substantial amount of work has been done in an attempt to characterize several other genes from this category. Post identification of these transcripts the initial interest was directed towards the ones that consisted of a signal sequence as previously identified secretory proteins had been found to play an

essential role in sporozoite motility of which TRAP is an example. Also, the genes with high levels of expression were also investigated and found to have an essential role in parasite development either in the mosquito or pre-erythrocytic stages. S4 localizes in the micronemes and was found to be essential in the process of cell traversal and was termed as CelTOS (cell traversal for ookinete and sporozoite), (Kariu *et al.*, 2006, Risco-Castillo *et al.*, 2015b), S13 also known as PLP1 is another micronemal protein involved in disruption of host cell membranes (Ishino *et al.*, 2005a) and successfully invading hepatocytes (Ishino *et al.*, 2004). SIAP-1/ S5 which expresses in oocyst sporozoites facilitates egress from oocyst and salivary gland invasion by the sporozoites (Engelmann *et al.*, 2009). S6 is a TRAP family adhesion and by disruption, its role has been implicated in parasite adhesion and gliding motility (Steinbuechel & Matuschewski, 2009). Another TRAP family protein S21/ thrombospondin related sporozoite protein (TRSP) containing a thrombospondin type 1 repeat region at its N-terminal is involved in cellular interaction and chaperoning sporozoites to the host cells (Tucker, 2004). SSP3/S23 localizes to the sporozoite surface and has been implicated in sporozoite gliding motility (Harupa *et al.*, 2014). Some of these genes such as S10 and S23 have been found to be essential beyond the sporozoite stage and play a role in the development of exo-erythrocytic form (EEF) (Togiri *et al.*, 2019). The fact that these genes are specific for the sporozoites also indicates their importance for the sporozoite and the pre-erythrocytic stages of the *Plasmodium* parasite. The work done in this thesis focuses on one of the S genes *S*14 which was in the microarray analysis showed one of the highest fold upregulation amongst the S genes (Kaiser *et al.*, 2004).

2.2 Material and methods

Materials and methods

Reagent	**Manufacturer**	**Catalogue No**
iProof DNA Polymerase	Bio-Rad	172/5301
Taq DNA Polymerase	Sigma Aldrich	B6677
X-Gal	Genetix	0428
IPTG	Fermentas	R0392
Luria Agar	Himedia	CR301
Luria Broth	Himedia	M1245
SeaKem® Agarose	Lonza	50004
Ampicillin sodium salt	Sigma Aldrich	A9518
Chloramphenicol	Sigma Aldrich	C0378
GenAmp® 10X PCR buffer	Applied Biosystems	4376212
Trizol	Invitrogen	15596-026
Random hexamer	Applied Biosystems	N8080127
RNase inhibitor	Applied Biosystems	N8080119
MULV Reverse transcriptase	Applied Biosystems	N8080018
SYBR® green mix	BioRad	1708882AP
Primers	IDT and GCC Biotech	NA
dNTPs mix	Promega	U1515
Nuclease-Free Water	Sigma Aldrich	95284
Trizma®	Sigma Aldrich	P6066
Boric Acid	Sigma Aldrich	B6768
EDTA	Sigma Aldrich	03690
Basic Parasite Nucleofactor™ Kit II	Lonza	VMI-1021
MiniPrep Plasmid Isolation Kit	Genetix Biotech Asia	27106
PureYield™ MidiPrep Plasmid Isolation Kit	Promega	A1222
Glucose	Sigma Aldrich	G8270
T4 DNA Ligase	Promega	M180A
Competent Cell Preparation Kit	Genei	1660200011730
SDS	Sigma Aldrich	L3771

Reagent	**Manufacturer**	**Catalogue No**
2-Propanol	Sigma Aldrich	19516
Chloroform:IAA	Sigma Aldrich	C0549
Ethanol	Merck	1.00983.0511
CutSmart® buffer	New England Biolabs	B7204S
XhoI	New England Biolabs	R0146S
NotI-HF®	New England Biolabs	R3189S
EcoRV-HF®	New England Biolabs	R0558S
SphI-HF®	New England Biolabs	R3182S
HindIII	New England Biolabs	R0104S
SacII	New England Biolabs	R0157S
PstI-HF®	New England Biolabs	R3140S
PvuII	New England Biolabs	R3151S
KpnI-HF®	New England Biolabs	R3142S
Hoechst 33342	Invitrogen	H13199
Prolong® Antifade diamond mounting medium	Invitrogen	P36970
Prolong® Antifade gold mounting medium with DAPI	Invitrogen	P36935
PCR purification/Gel Extraction kit	Genetix Biotech Asia	NP- 36105
ReliaPrep™ Blood gDNA extraction Kit	Promega	A5081
Sodium Hydroxide	Sigma Aldrich	S8045
Sodium Phosphate monobasic	Sigma Aldrich	S0751
Sodium Phosphate dibasic	Sigma Aldrich	S9390
Potassium Phosphate dibasic	Sigma Aldrich	P2222
Sodium acetate	Sigma Aldrich	71196
Glacial Acetic acid	Fisher Scientific	11005
Hydrochloric Acid	Rankem	H0100
Ketamine	Themis Medicare	NA
Xylazine	Indian Immunologicals Limited	NA

Reagent	Manufacturer	Catalogue No
Alsever's solution	Sigma Aldrich	A3551
Glycerol	Sigma Aldrich	G5516
Potassium acetate	Sigma Aldrich	791733
RNase	VWR	0675
Giemsa stain modified	Sigma Aldrich	48900
Collagen type 1	Sigma Aldrich	C3867
DMEM	Invitrogen	11995965
RPMI 1640	Invitrogen	22400071
Antibiotic Antimycotic	Invitrogen	15240062
FBS	Sigma Aldrich	F2442
Heparin	Sigma Aldrich	H3393
Pyrimethamine	Sigma Aldrich	46706
Trypsin-EDTA	Invitrogen	25200056
Amphotericin B	Sigma Aldrich	82942
Penicillin Streptomycin	Invitrogen	15140122
Histodenz™	Sigma Aldrich	D2158
LigaFast Rapid DNA ligation System	Promega	M8225
Paraformaldehyde	Sigma Aldrich	P6148
DMSO	Sigma Aldrich	D8418
Triton X-100	Sigma Aldrich	T8787
Methanol	Fisher Scientific	34457

2.2.1 Experimental animals

All animals used in this study were obtained from the laboratory animal facility (LAF) CSIR- Central Drug Research Institute, Lucknow, India. All animal experiments performed were approved by the Institutional animal ethics committee at Central Drug Research Institute (Approval no: IAEC/2013/83 and IAEC/2018/3)). For pre-patent studies 4-6 weeks old C57BL/6 mice were used. For blood-stage parasite maintenance, 4-6 week old Swiss albino mice were used. Swiss albino mice, 8-10 week old were used as a source of blood meal for *Anopheles stephensi* colony maintenance.

2.2.2 Parasite lines

To elucidate the role of *S*14 (PlasmoDB ID: PBANKA_060590), *Plasmodium berghei* ANKA, a species that infects rodents was used. For comparison with the GFP mutant, wild type GFP parasites were used. Wild type *Plasmodium berghei* ANKA (MRA 311) and *Plasmodium berghei* ANKA GFP (MRA 867 507 m6cl1) were obtained from BEI resources, USA.

2.2.3 Bacterial Strains

The E.coli DH5α strain: dlacZ Delta M15 Delta (lacZYA-argF) U169 recA1 endA1 hsdR17 (rK-mK+) supE44 thi-1 gyrA96 relA1 were used for all cloning reported in this study.

2.2.4 Cloning Vectors

Following vectors were used for cloning, expression and genetic manipulation (Annexure1) of the parasite:

1. pBluescript SK (+) vector
2. pBC-GFP-hDHFR
3. pBC-3XHA-mCherry-hDHFR

2.2.5 The nucleotide sequence of *S*14

The nucleotide sequence of *P. berghei S14* (PBANKA_060590) was downloaded from PlasmoDB.

2.2.6 Primers

Primers used in the *S*14 KO and study are given in Table 1.

Table 1. List of primers used to analyse expression of *S*14 gene, generation, and confirmation of *S*14 KO, and to check the excision of the gene.

Primer	Primers used in *S*14 real time, general knockout and transgenic (Primer sequence 5'-3')	Restriction site
1001	CCCTAAATAATGTGTCAACTGT	NA
1002	TGTCTCGATGTTGTTTCGG	NA
1003	AAGCTTCTCGAGTTACGAGAAATTAAAATTACACAAA	Xho1
1004	CTGCAAATCGATACTCAAATGATAAAGAAACAAAAAA	Cla1
1005	AAGCTTGCGGCCGCTGAGAGAATTTACCTTATCCTTA	Not1
1006	CTGCAGGGCGCGCCGTGCTCCCGCATTCTTTGTT	Asc1
1007	CTTTTAGCAACGTTTTATTTCCC	NA
1009	GGATCCATGTCTGATTATTTTACCATTTT	NA
1010	GCTTCTCGAGGAATCAATTACTGAACATGAAGA	NA
1011	GCCAGATCTTTTTTTGGCCGCCAAATTTATG	Bgl2
1215	GTTGTCTCTTCAATGATTCATAAATAG	NA
1225	TTCCGCAATTTGTTGTACATA	NA

2.2.7 *S*14 expression analysis by quantitative real-time PCR

For the absolute quantification of *S*14 transcripts, a standard was generated by amplifying a 120 bp fragment from *S*14 ORF using primers 1001/1002. Amplified product was cloned in pCR 2.1-TOPO vector. For the normalization of transcripts Hsp70 was used (Choudhary et al., 2019). Total RNA was isolated from blood stage schizont (Sc), liver stages (LS), midgut oocyst (MG) and salivary glands (SG) using Trizol reagent and RNA isolation kit (Genetix) following manufacturers instructions. cDNA was prepared by reverse transcription using Super script cDNA synthesis kit. Real-time PCR was carried out using SYBR green (Bio-Rad) and the ratio of transcript numbers of *S*14 and *Hsp70* was used to determine the copy number

2.2.8 Generation of knockout parasite line

2.2.8.1 Preparation of *S*14 knockout targeting construct

*S*14 KO parasite lines were generated by double-crossover homologous recombination. For the generation of the targeting construct, two fragments of length

0.730 Kb (F1) and 0.637 Kb (F2) were amplified from *P. berghei* ANKA genomic DNA using primer sets 1003/1004 and 1005/1006 respectively. High fidelity iProof DNA polymerase (BioRad) was used to set up the PCR (Table 1) to avoid any mutations in the targeted DNA fragments using temperature conditions given in Table 3. The amplified products were verified for correct size by running on 1% agarose gel followed by purification using PCR purification kit.

Table 2. Reaction Mix for insert amplification

Component	Working concentration
iProof® 5X PCR buffer	1X
$MgCl_2$	1.5 mM
dNTPs	200 µM
Forward primer	0.5 µM
Reverse primer	0.5 µM
WT gDNA	100 ng
iProof® DNA polymerase	0.4 U

Table 3. Reaction conditions for insert amplification with iProof DNA polymerase

Temperature	Time	Cycles
98°C	30s	1
98°C	10s	5
54°C	20s	
72°C	60s	
98°C	10s	30
62°C	20s	
72°C	60s	
72°C	600s	1

2.2.8.2 Agarose gel electrophoresis

For the separation of plasmids and purification of digested fragments, DNA samples were run on agarose gel. Agarose was dissolved in 1X TAE buffer by boiling and

EtBr was added and gel was poured in a casting tray. A comb was placed in the slots available in the casting tray for formation of wells and allowed to polymerize. The DNA was mixed with loading dye and run on a horizontal agrose gel electrophoresis apparatus containing 1XTAE buffer. The gels were documented using Alpha infotech gel documentation system.

The products amplified using iProof DNA polymerase are blunt-ended hence these can be directly cloned into pBluescript SK+ vector restriction digested by the enzyme EcoRV. The ligation was set up according to the setup given in Table 4.

Table 4 Ligation reaction mixture

Contents	**Concentration**
Vector	60ng
Insert	32ng
Ligation buffer	1X
T4 DNA ligase	3U

The vector and insert were used in a molar ratio of 1:3 to set up the ligation reaction with a final volume of 10 µl and incubated at 20^0C for 4hrs in a water bath. Post incubation the reaction mixture was transformed into competent DH5α cells.

The transformed cells plated on Luria agar plates spread with isopropyl thio galactoside (IPTG) which is a gratuitous inducer of the lac operon and X-Gal required for blue-white selection and ampicillin as selection antibiotic, the plate was incubated at 37^0C in an incubator overnight. The white colonies were inoculated in 5 ml Luria broth containing ampicillin and incubated overnight. Plasmids from these cultures were isolated using the alkaline lysis method procedure for which is described later and screened by setting up restriction digestion with a reaction volume of 30µl with PvuII (Table 5) which was run on a 1% agarose gel.

Table 5 Reaction for plasmid digestion

Contents	**Concentration**
Plasmid	1000ng
Cutsmart buffer	1X
Pvu II	2U

The plasmid positive for the insert was digested with *Xho*1 and *Cla* l, for F1 and *Not*1 and *Asc*1 for F2, run on 1% agarose gel and the band for the insert was excised using a scalpel. Restriction digestions were performed in conditions suggested by the manufacturer. Enzyme units added were as recommended by NEB: https://www.neb.com/tools-andresources/selection-charts/cleavage-of-supercoiled-dna. The insert was purified using a gel extraction kit according to the manufacturer's instructions and quantified on a nanodrop. The purified insert was used to set up ligation with vector digested with the same enzyme. The ligation mixture was transformed into DH5α competent cells and plated on chloramphenicol plates to select the resistant clones.

2.2.8.3 Preparation of competent cell

For the preparation of competent cells, DH5α cells were streaked on an LB agar plate using sterile inoculation loop incubated overnight on 37^0C. A single colony was inoculated in 5ml LB and grown overnight at 180 rpm at 37^0C. The overnight grown culture was diluted 1:100 in 100ml culture and grown till O.D 0.6 at 600nm. Culture was kept on ice for 10min and pelleted at 6000 rpm for 10min at 4^0C the supernatant was discarded and the pellet was resuspended in 80mM $MgCl_2$ + 20mM $CaCl_2$ and incubated on ice for 45min to 1hr. After incubation it was again centrifuged to pellet cells and finally resuspended in 2ml 15% glycerol + 100mM $CaCl_2$ aliquoted and flash frozen and stored at -80^0C.

Plates were incubated at 37°C overnight. Single colonies were inoculated in 5 ml Luria broth with chloramphenicol. Cultures were allowed to grow overnight at 37°C with shacking at 200 rpm. Plasmids were extracted from overnight grown cultures using the alkaline lysis method. Correct cloning was checked by specific restriction digestions.

2.2.8.4 Plasmid isolation

2.2.8.4.1 Alkaline lysis method

Innoculated colonies were cultured overnight in an incubator shaker at 180 rpm and temperature of 37^0C were pelleted at 12,000 rpm for 1min. These were resuspended in 150 µl ice cold resuspension buffer and 200 µl lysis buffer was added, mixed gently by inverting the tubes 4-5 times. The contents when mixed well turn into a transparent viscous solution to which 150 µl neutralization buffer is added and gently mixed. They were then centrifuged for 5min at maximum speed. The supernatant was collected and equal volume of chloroform isoamyl alchohol was added to it, mixed well by vortexing for 15 seconds and centrifuged for 5 min at maximum speed. The upper aqueous phase was carefully removed and transferred to a new vial. To this aqueous phase equal volume of chilled isopropanol was added vortexed and centrifuged for 20 min maximum speed at room temperature. DNA pellet was washed by 70% ethanol and dried for 10 min. These pellets were resuspended in 50 µl nuclease free water. Promega pureyeild miniprep kit was used for isolation of plasmid for sequencing purposes.

Plasmid isolation solutions:

- **Resuspension solution**: 50mM Glucose, 25mM Tris- Cl buffer, 10mM EDTA (pH 8.0). Filter through 20µm filter. Add 100 µg/ml RNase A and store at 4°C.
- **Lysis buffer**: 0.2 N NaOH (diluted from 10 N stock) and 1% SDS. It should be prepared freshly before use.
- **Neutralization buffer** (for 100ml): 5M Potassium acetate (60 ml), Glacial acetic acid (11.5 ml). Make up the volume with distilled water. Filter through 20 µm filter and store at 4°C.

2.2.8.4.2 Midiprep

For transfection purposes large scale cultures of upto 100ml were harvested and plasmids were isolated using Promega pureyeid midiprep kit following instructions by the manufacturer.

2.2.8.5 In vitro culture of *P. berghei* blood stages

Transfection of a purified DNA construct in the *P. berghei* parasite line was performed as previously described (Janse et al., 2006). Frozen stock of *P. berghei* ANKA was intraperitoneally injected into Swiss mice and parasitemia was monitored by making Giemsa-stained blood smears. When the parasitemia reached around 2-5% the blood was collected by cardiac puncture using a heparinized syringe and decanted into a 50 ml Conical tube containing 10 ml of schizont culture medium composition [RPMI (Roswell Park Memorial Institute) 10% Fetal calf bovine serum and 350µg gentamycin]. The collected blood was spun at 200g for 8 min without break in a swinging bucket rotor. After centrifugation, the supernatant was discarded and the pellet was resuspended in 35 ml of schizont culture medium and transferred into a non-vented T75 culture flask (BD Falcon™). The culture was gassed with 5% CO_2, 5% O_2 and 90% N_2 mixture for 1 min and incubated at 37°C with gentle shaking for 20-24 hours. After incubation, the presence of mature schizonts was checked by Giemsa staining of thin blood smear.

2.2.8.6 Giemsa staining of blood smears

The blood stages of the parasite can be monitored by Giemsa staining. To prepare smear, a drop of blood collected from culture or tail vein of the mouse was punctured and a drop of blood was put on a glass slide. Smear was made and air-dried. After drying, the smear was fixed with methanol. Modified Giemsa solution (Sigma) was diluted in Giemsa buffer (10X Giemsa buffer is composed of 6 mM Na_2HPO_4 and 8 mM KH_2PO_4). The staining solution was poured onto the smear and incubated for 15-20 min. After incubation, the slide was washed with water and air-dried. The smear was then observed under a light microscope at 100X magnification.

2.2.8.7 Purification of schizonts and electroporation

Histodenz stock composition:

- 5 mM Tris-Hcl
- 3 mM KCl
- 0.3 MM EDTA
- 27.6 g Histodenz (for 100 ml stock)
- Sterilized by autoclaving and stored at 4°C.

For the purification of schizonts density gradient centrifugation was performed using 50% histodenz gradient. Working solution made by diluting histodenz with PBS. Prewarmed 10ml of 1:1 mixture of histodenz and PBS was slowly pipetted into the 35ml schizont culture this was spun at 200g for 20min at room temperature without breaks. After centrifugation schizonts get setteled at the interphase and were collected using a pasture pipette. These schizonts were washed with 10ml schizont culture medium and centrifuged at 200g for 8min at room temperature. The pelleted shizonts were washed in 1ml schizont medium and re-pelleted by spinning at 13,000 rpm for 15 sec. Transfection mixture was prepared by mixing 82µl nucleofactor solution with 18µl supplement and 5-10µg of DNA. The shizont were mixed with the transfection solution, added to a cuvette and transfected using U-033 program on the lonza nucleofactor device. 100µl RPMI was added to the cuvette and mixed well injected promptly in a Swiss albino mice (Figure 8).

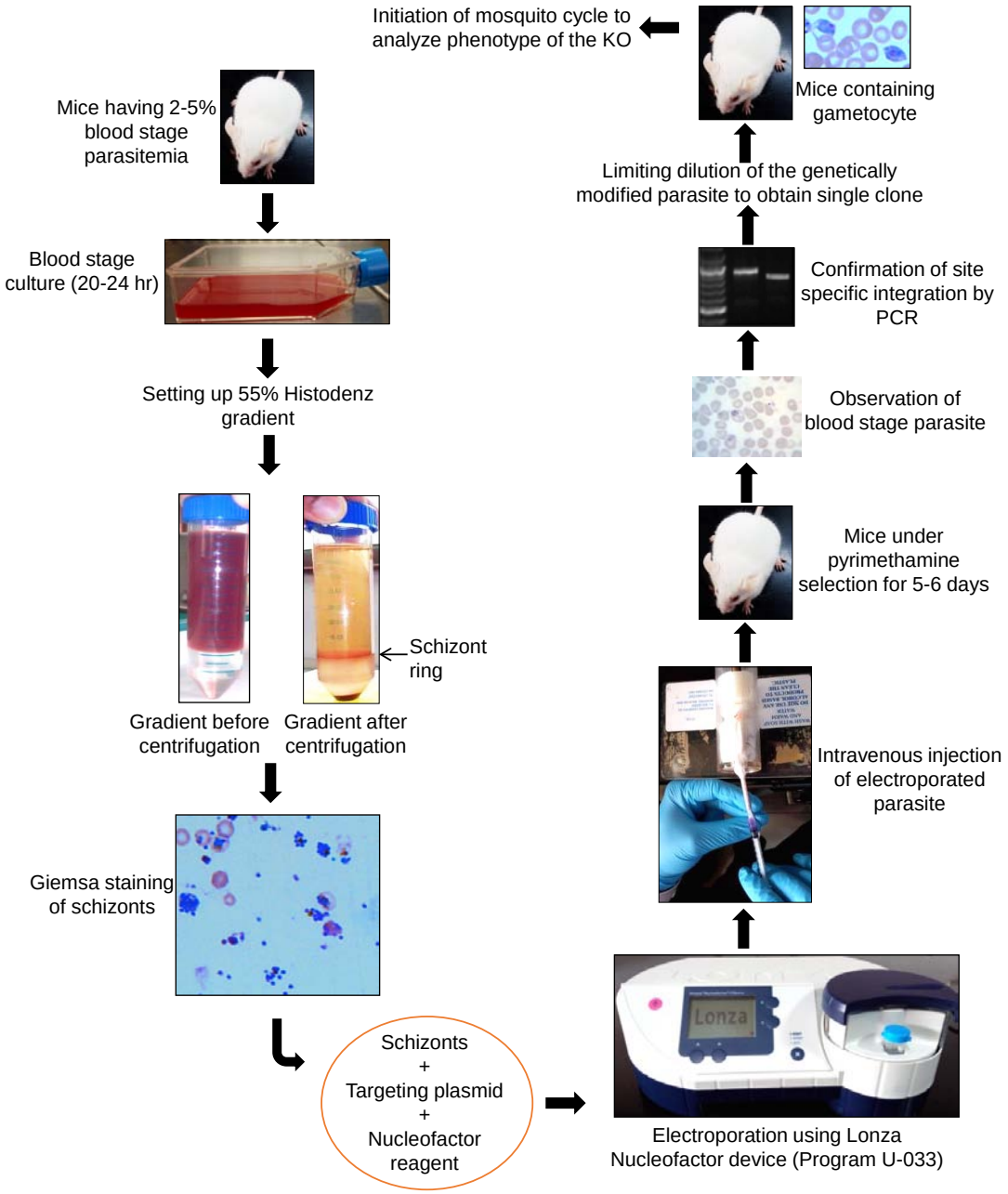


Figure 8. Schematic diagram showing the procedure for transfection, drug selection, confirmation of site-specific integration and phenotypic characterization of knockout parasites. Blood was collected from a mouse having 2-5% parasitemia and an overnight culture was set up. The next day, the schizonts were enriched on a density gradient. The purified schizonts were collected and electroporated with the targeting construct and immediately injected intravenously into a mouse. The mouse was kept on pyrimethamine, an antimalarial drug that facilitates the selection of the transfectants. The success of stable site-specific integration was confirmed by PCR. Following successful integration, mutant parasites were single cloned by limiting dilution. Mosquito cycle was initiated from clonal parasite lines to determine the phenotype.

2.2.8.8 Positive drug selection of recombinant parasites

A day post transfection giemsa stained blood smear were made to check for the presence of blood stage parasites. The transfected parasites contain the mutated version of parasite dihydrofolate reductase DHFR-TS in its cassette which confers resistance to pyramithamine. For the selection of transfected parasites pyramithamine was added to the drinking water of the mice injected with the transfected parasite. The clearance of wild type parasite was observed by making smears. Drug resistant parasites appear on day 5-7 post transfection blood is frozen and genomic DNA is isolated for confirmation of knockout generation by site specific integration PCR.

2.2.8.9 Limiting dilution to obtain clonal lines of the parasite

Once integration of cassette at the desired locus is confirmed parasite frozen stock was injected intraperitonealy into swiss mice. When parasitemia of 0.2-1 was achieved the blood was isolated from the infected mice and the number of infected RBC and total RBC were determined by counting. Serial dilutions were made in RPMI and injected in mice ensuring each mice received a single parasite. Smears were made one week post injection to check for the appearance of blood stage parasites. Blood from mice that were positive was frozen and genomic DNA was isolated and checked for contamination with wild type population.

2.2.8.10 Genomic DNA extraction from blood

The blood from infected parasites was extracted by performing cardiac puncture using a heparinized syringe. The infected blood was diluted with PBS to make a final volume of 5ml. RBC lysis was performed by adding 0.05% saponin and mixing gently. The mixture was incubated for 5min at room temperature and centrifuged at 6000 rpm for 20min. The supernatant was discarded and the pellet was washed twice with PBS, centrifuged for 5min at 12,000 rpm at room temperature. This pellet was resuspended in 200µl PBS, the genomic DNA was isolated using Promega pure yield blood gDNA miniprep kit following manufactures instructions. Nuclease free water was used to elute genomic DNA from the binding column, and stored at -20^0C. To confirm integration of the targeting cassette one of the primers was taken from

unmodified part of the genomic DNA flanking the targeted gene, the other primer was from inside the integration cassette.

2.2.8.11 Blood growth analysis

To analyse the asexual blood-stage propagation, 200 µl of infected blood having 0.2% parasitemia of *S*14 KO or WT GFP was intravenously injected into a group of four mice. The parasitemia was monitored daily in mice by Giemsa-staining of blood smears.

2.2.8.12 Analysis of parasite development in mosquito stages

Anopheles stephansi mosquitos' rearing was carried out at 28°C and 80% relative humidity. Following procedures were employed to maintain mosquito colony. Mice were anesthetized by intraperitoneal injection of Ketamine/Xylazine mixture. Mosquitoes were allowed to take blood meal for 20 min from anesthetized mice. Two days post blood meal, a beaker containing water with wet filter paper funnel at the top was put in mosquito cage. Eggs were collected and washed with 10% bleach solution and released into water trays. After hatching, larvae were fed on powdered food made of yeast extract and dog biscuit (pedigree). Larvae undergo a series of development (instars) during the next 10-14 days before differentiating into pupae. Early instar to pupae stages was fed on optimum fish food pellets along with the powdered food. Pupae were manually collected and placed inside cages where they mature and emerge as male and female mosquitoes. Adult mosquitoes were fed on 10% Sucrose. The mosquito rearing and sporozoites generation are described in Figure 9.

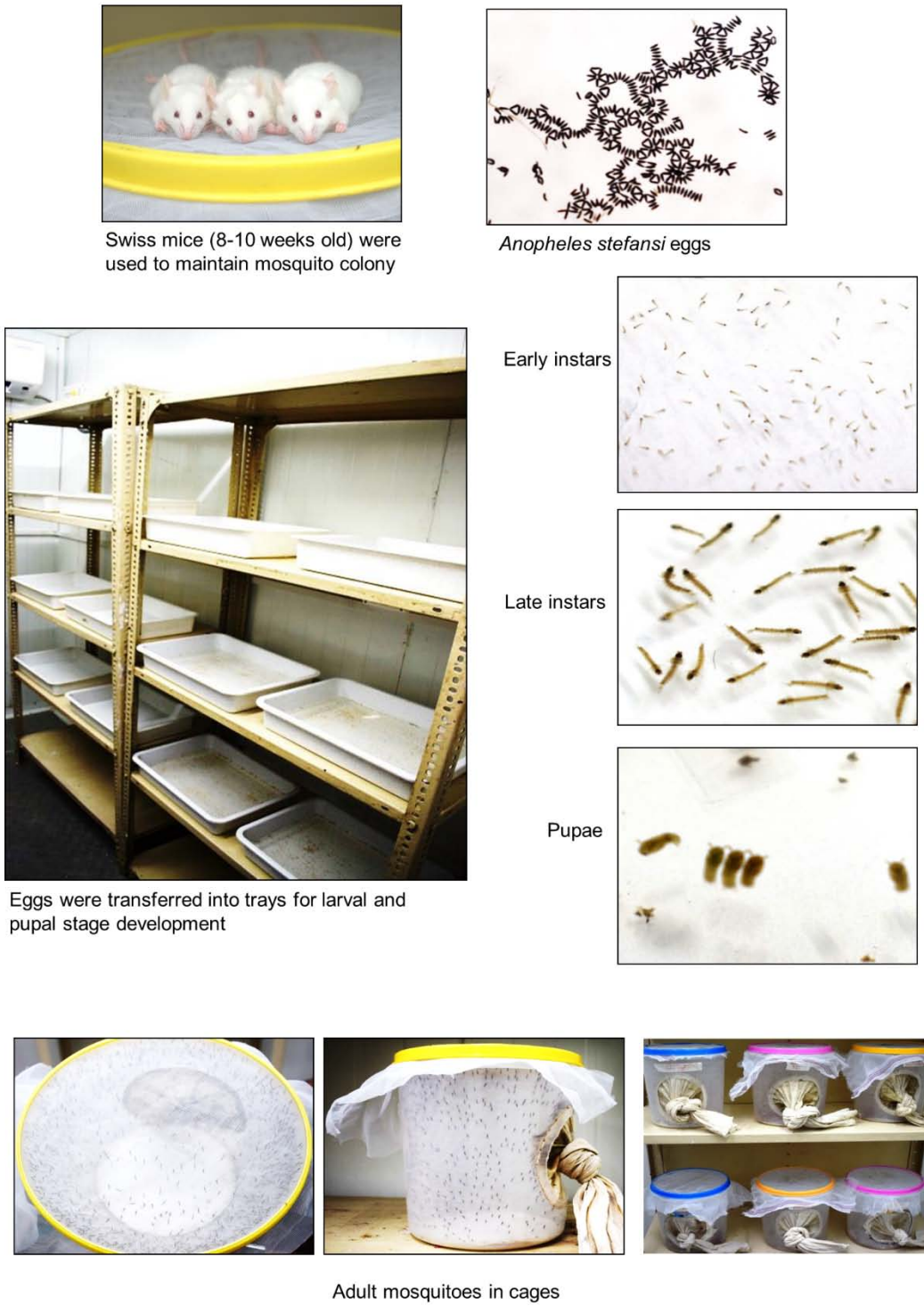


Figure 9. Rearing of *A. stephensi* and maintenance of *P. berghei* infected cages. (A) Blood-feeding to obtain mosquito eggs. (B) Collected eggs. Under these conditions, the eggs hatch and transforms into a series of instars and finally into pupae. (C) Different stages of mosquito development maintained in trays. (D) Different stages showing early instars, late instars, and pupae. (E) Cages containing adult mosquitoes.

For initiation of mosquito cycles, mosquitoes were allowed to feed on *S*14 KO or WT GFP infected mice to obtain sporozoites. Infected mosquitoes were kept in an environmental chamber maintained at 19°C and 80% relative humidity. On day 14 post blood meal, mosquitoes were dissected and midguts were isolated. Midguts were observed and oocysts numbers in individual midguts were enumerated. Oocyst sporozoite numbers were determined by crushing infected mosquito midguts.

2.2.8.13 Determination of pre-patent period

For the determination of the pre-patent period of *S*14 KO parasites, isolated hemocoel sporozoites were inoculated i.v. in C57BL/6 mice. As a control, equal number of WT hemocoel sporozoites was injected into another group of mice. Emergence of parasitemia in blood was checked by Giemsa-staining of blood smears.

2.2.8.14 In vivo infection through mosquito bite

To determine if *S*14 KO sporozoites are infective in vivo, groups of 10 mosquitos infected with *S*14 KO or WT parasites were allowed to feed on C57BL/6 mice on day 19 post-infective blood meal. Parasite in blood was observed by Giemsa-staining of blood smears.

2.2.8.14 Sporozoite gliding motility assay

To quantify sporozoite gliding motility, glass eight-well chamber slide was coated with 10 µg/ml anti-CSP antibody in PBS overnight and the assay was performed as described previously (Stewart & Vanderberg, 1988). Hemocoel sporozoites in 3% BSA/DMEM were added 5,000/well and incubated for 1 h at 37°C. After fixation with 4% PFA, trails were detected by staining with a biotinylated anti-CSP antibody, followed by streptavidin-FITC. Trails associated with sporozoites were counted using a Nikon 80i fluorescent microscope.

2.2.8.15 Sporozoite invasion assay

Cultured HepG2 cells were tyrpsinized and seeded 8 chamber LabTek slides were precoated with rat tail collagen type I 4hr prior to trypsiniztion of the HepG2 cells. HepG2 cells (60,000/well) were seeded to the collagen coated slides and maintained

in complete medium at 37^0C in a CO_2 incubator overnight. Next day isolated hemocoel sporozoites were added to the HepG2 cells (10,000 sporozoites/ well) and centrifuged at 310g for 4 min. The slide was incubated for 1hr at 37^0C in a CO_2 incubator and fixed using 4% PFA.

For invasion assay the wells were blocked in 1% BSA/PBS followed by staining with anti-CSP mouse monoclonal antibody (Yoshida *et al.*, 1980) for 1 h at room temperature. Wells were washed three times with PBS and bound CSP signal was revealed by incubation with Alexa-Flour 594 conjugated with anti-mouse IgG. After completing staining of extracellular sporozoites, cells were permeabilized with chilled methanol for 20 min at 4°C. Blocking and CSP antibody incubation was performed as described above and the signal was revealed by incubation with Alexa-Fluor 488 conjugated anti-mouse IgG. Finally, wells were incubated with Hoechst 33342 to stain nuclei and mounted in Diamond antifade reagent and visualized under a fluorescent microscope.

2.2.8.16 *In vitro* sporozoite infectivity

HepG2 cells (50,000/well) were seeded in 48 well plates containing sterilized cover slips pre-treated with collagen incubated overnight in a CO_2 incubator at 37^0C. Isolated hemocoel sporozoites were added to the HepG2 monolayers (5,000 sporozoites/well) in complete medium. After sporozoite addition plate was centrifuged at 310 g for 4min and incubated at 37^0C in a CO_2 incubator.

2.2.8.17 Mosquito wash procedure for in vitro culture

Mosquitoes were collected and anesthetized and washed twice in 70% ethanol. Next mosquitoes were washed thrice in mosquito wash medium (1X DMEM, 1X antimycotic-antibiotic, 5 µg/ml amphotericin B, 10 µg/ml gentamicin). Next, the mosquitoes were washed twice in DMEM with 2 min incubation for each wash. Incubations were performed on ice. After washing, mosquitoes were kept in DMEM and dissected.

2.2.8.18 Cell washing and maintenance of infected HepG2 cultures

After 1 hour of sporozoite addition, complete medium was removed and cells were washed once with cell wash medium (1X DMEM, 2X antimycotic-antibiotic, 5µg/ml amphotericin B) for 5 min. Then, cells were rinsed with DMEM and incubated in cell incubation medium (1X DMEM, 10%FBS, 1X Penicillin-Streptomycin and 1X antimycotic-antibiotic). The cells were maintained in incubation medium which was changed after every 12 hour. After 36 hr of infection antimycotic-antibiotic was removed from incubation medium being used for cell culture. Coverslips were removed at different stages of EEF development like 24 hr, 36 hr and 62 hr and fixed with 4% paraformaldehyde for 20 minutes at room temperature and rinsed with PBS.

2.2.8.19 Bulb transformation experiment

WT and *S*14 KO hemocoel sporozoites were suspended in DMEM supplemented with 10% FBS, 1X antibiotic/antimycotic, 5µg/ml amphotericin B. $5X10^3$ sporozoites were added per well in an 8-well chamber slide (Nunc) and incubated at 37^0C in 5% CO_2 for 4 hrs. These were fixed using 4% PFA and stained with anti HSP70 monoclonal antibodies. Signal was revealed by Alexa flour 488 anti-mouse IgG. The percentage sporozoite transformation into bulbs was quantified by examination of the stained slides under a fluorescent microscope.

2.2.8.20 Triton-x-100 membrane extraction

S14-mcherry sporozoites ($3X10^4$) were purified and treated with 0.1% TX-100 diluted in PBS and incubated on ice for 30 min. After incubation sporozoites were pelleted at 13,000 rpm for 5min at 4^0C. Both treated and untreated sporozoites were washed thrice with PBS and fixed with 2% paraformaldehyde diluted in PBS. Treated and untreated sporozoites were stained with anti-CSP and anti-mCherry antibodies.

2.2.8.21 Immunofluorescence assay

2.2.8.21.1 Staining of sporozoites

Purified salivary sporozoites were coated on 12 well slides. The spots were fixed using 4% PFA and permeablized by 0.1% TritonX-100 for 20 min at room

temperature. Sporozoites were washed thrice with PBS and blocked with 1% BSA/PBS for 1hr at room temperature. Further staining was done using anti-mCherry rabbit and anti-CSP mouse monoclonal antibodies. The signals for anti-mcherry and anti-CSP antibodies were revealed using Alexa fluor 594 anti-rabbit IgG and Alexa fluor anti-mouse IgG respectively. Nuclei were stained with Hoechst 33342 and mounted by diamond antifade. Imaging of the immunostained sporozoites was done using a confocal laser scanning microscope.

2.2.8.21.2 Staining of HepG2 cells

The infected HepG2 coverslips were fixed at time points 12hr, 24hr, 36hr and 48hr by treatment with 4% PFA. The fixed cover slips were washed with PBS twice for 5min. Permeablization was done using chilled methanol at 4^0C for 20 min. Coverslips were washed twice by PBS and blocked with 1% BSA/PBS for 1hr at room temperature. For the staining of cells anti-HSP70 (Tsuji et al., 1994) and anti-UIS4 (Mueller et al., 2005a) were used. The signals were revealed using alexa 594 anti-rabbit IgG and alexa 488 anti-mouse IgG respectively. The nuclei were stained by Hoechst 33342. Imaging was done using a confocal laser scanning microscope.

2.3 Results

2.3.1 Determination of *S*14 stage-specific expression

To perform the quantitative estimation of *S*14 transcript levels at different stages of the *Plasmodium* life cycle quantitative real-time PCR was performed. On analyzing the normalized expression, we observed *S*14 expression at the schizont and mosquito stages whereas liver-stage was devoid of S14 transcripts. Maximal *S*14 expression was seen in midgut sporozoites which was approximately six-fold higher than the schizont stages followed by salivary gland sporozoites. As mentioned earlier *S*14 belongs to a category of genes that have been recognized as unique sporozoite transcripts (Kaiser et al., 2004). Our results are consistent with the previous findings. These results indicate a possible role for *S*14 in the sporozoite stages. (Figure 10)

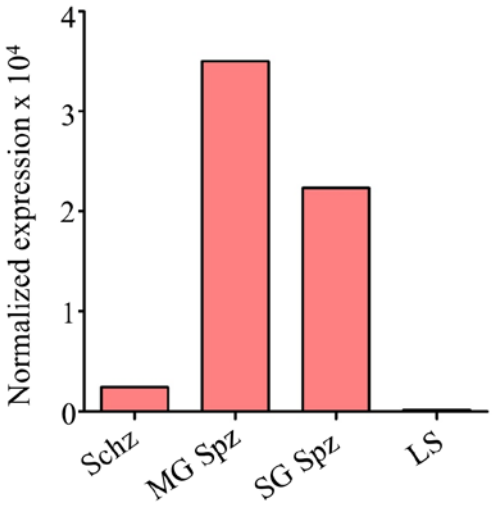


Figure 10. *S*14 expression analysis. The gene expression of *S*14 was analysed by quantitative PCR which reveals highest expression in sporozoite stages. The expression was normalized with *Pb Hsp70* rRNA. BS; blood stages, Schz; schizonts, MG Spz, midgut sporozoites, SG Spz; salivary gland sporozoites, LS; liver stages.

2.3.2 Generation of *S*14-3XHA mCherry transgenic parasites and expression of reporter

To study expression and localization of S14 protein in the *P. berghei* life cycle we generated a *S*14-3XHA-mcherry parasite line. In order to construct the targeting construct, two fragments from the 5' and 3'UTR regions of size 0.741 kb and 0.647 kb were amplified and cloned at *Xho*I-*Bgl*II and *Not*I-*Asc*I respectively in pBC-3XHA-mCherry-hDHFR vector (Figure 11A). For transfection, the targeting construct was digested with *Xho*I-*Asc*I for separating it from the vector backbone. Purified targeting fragment was transfected in *P. berghei*. After drug selection, genomic DNA was isolated from drug-resistant parasites. Site-specific integration PCR was performed using primer sets 1218/1007 and 1215/1008 for 5' and 3' integrations respectively (Figure 11B). Limiting dilutions of the KO parasite was performed to obtain pure clonal population which was used for all further experiments. The S14-3XHA-mCherry expression was not observed in the asexual blood stages. Next, parasite cycle was initiated to check if S14-mCherry expresses in the mosquito stages. For this S14-mCherry infected mice were used to transmit the infection to the vector stages then the infected mosquitos were dissected on day 14 and day 18 post-infection to check for the expression of the reporter gene in the midgut oocyst and salivary gland sporozoites respectively. We observed mCherry expression on both the oocyst and salivary gland sporozoites (Figure 11 C and D). The expression on sporozoites was uniform and the result indicates that S14 expresses throughout the mosquito stages which is consistent with real-time expression data.

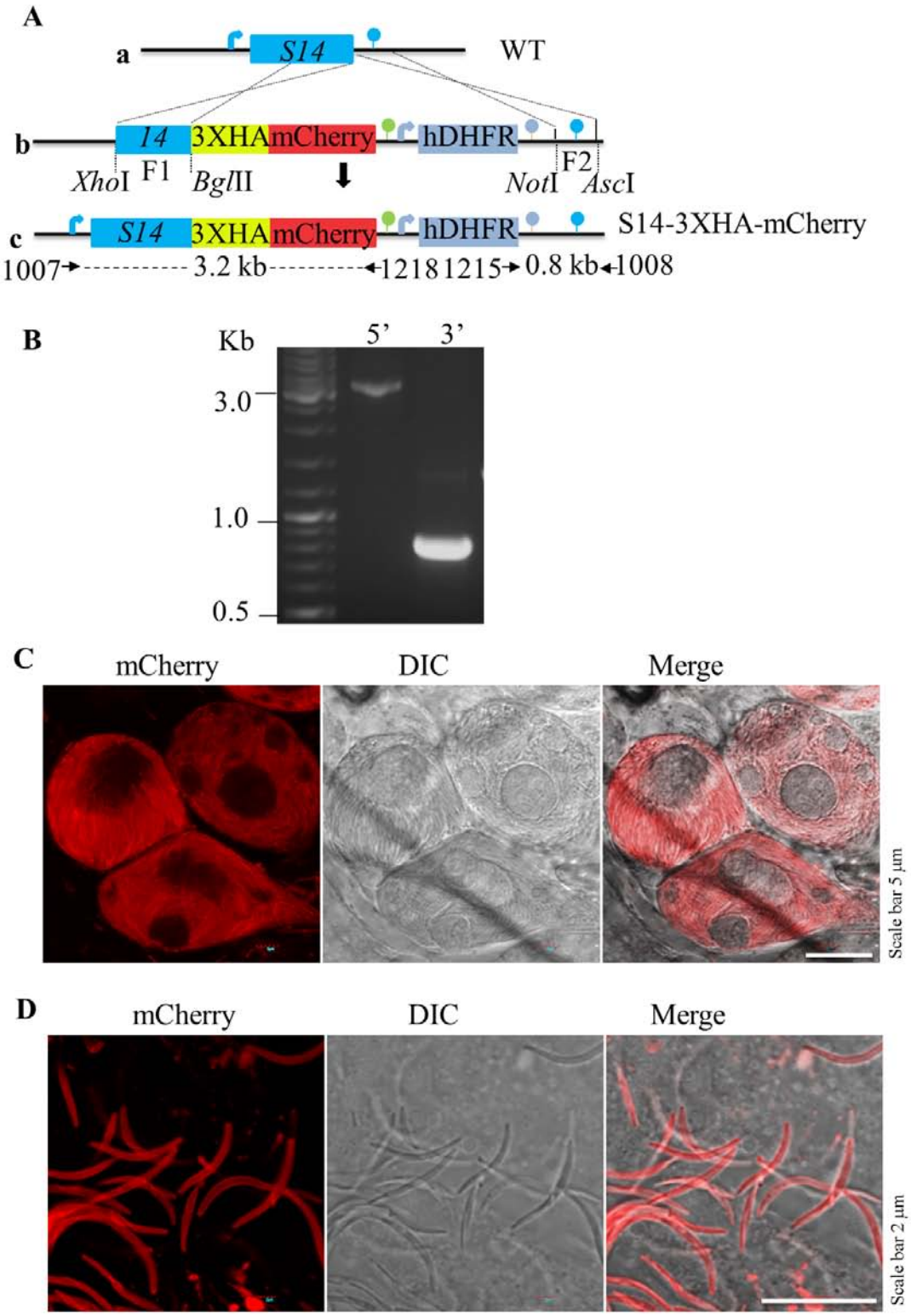


Figure 11. Generation of *S*14-HA-mcherry transgenic parasites and expression. (A) (a) Genomic locus of *S*14 (b) Targeting plasmid pBC-3XHA-mCherry-hDHFR. (c) *S*14 locus after integration of targeting plasmid. (**B**) Diagnostic PCR with a set of primers 1007/1218 and 1215/1008 confirming correct integration of 5' and 3' fragments respectively in the parasite genome. **(C)** Live microscopy image of midgut oocyst expressing mcherry reporter **(D)** Image of salivary gland sporozoites.

2.3.3 *S*14 localizes on the sporozoite inner membrane

The *S*14-3XHA-mCherry salivary gland sporozoites were isolated by dissecting infected mosquitos on day 18 post-infection. Directly observing salivary gland sporozoites under fluorescent microscope revealed its presence on the surface (Figure 12A). Sporozoites were stained with anti-CSP and anti-mCherry antibodies. We observed that mCherry colocalizes with CSP (Figure 12B). This result indicates that *S*14 expresses on the parasite membrane. To further confirm a more precise localization we extracted the outer membrane of the sporozoite by using Triton-X-100 as described previously (Steinbuchel et al., 2004). After membrane extraction, the sporozoites were fixed on a glass slide and immunostained anti-CSP and anti-mCherry antibodies. The abolition of the CSP signal indicated the successful extraction of the outer membrane. Triton-X-100 treated sporozoites stained positively for mcherry indicating *S*14 is present on the inner membrane (Figure 12C).

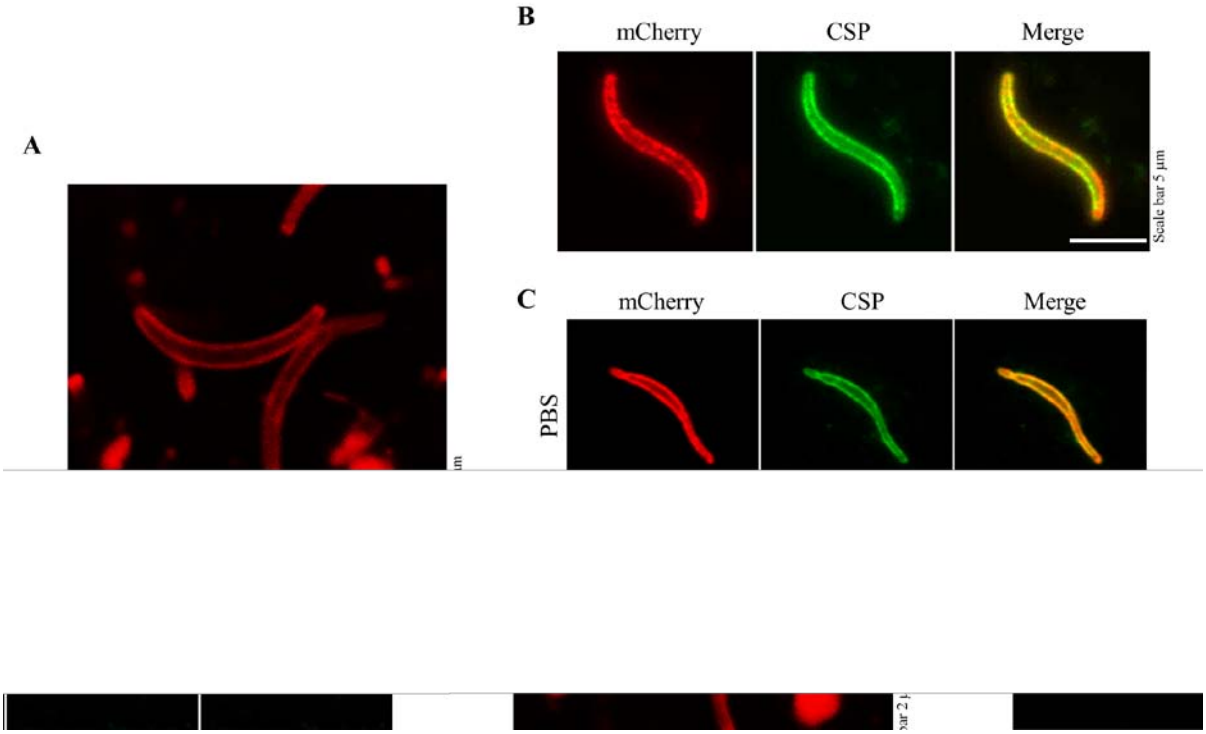


Figure 12. *S*14-HA-mcherry sporozoite CSP staining (A) Image of salivary gland sporozoites exressing mCherry on the surface **(B)** *S*14-HA-mcherry salivary gland sporozoites stained with anti-CSP monoclonal antibody. **(C)** Triton-X membrane extraction reveals the presence of S14-mCherry on the inner membrane complex of sporozoites.

2.3.4 Generation of *S*14 knockout parasites

With the purpose of investigating the role of *S*14 in the *P.berghei* life cycle, we used gene replacement strategy to generate a stable KO parasite line. In order to achieve this two fragments from the 5' and 3'UTR regions were amplified using primers 1003/1004 and 1005/1006 and cloned at *Xho*I-*Cla*I and *Not*I-*Asc*I of the pBC-GFP-hDHFR vector respectively. The cloning was confirmed using restriction digestion. Targeting cassette was seperated from vector backbone by digestion with *Xho*I-*Asc*I and transfected into purified *P.berghei* schizonts. The parasite population which incorporates the targeting construct and undergoes the double homologous recombination (Figure 13A) event was selected under the drug pyrimethamine. Further confirmation was made by observing GFP expression under a fluorescent microscope (Figure 13B) and by diagnostic PCR using primers 1225/1007 and 1215/1008 to check correct 5' and 3' site-specific integrations respectively (Figure 13C). A PCR for the amplification of whole cassette containing GFP and hDHFR was set up using primers 1007/1008 which amplified the correct size (Figure 13D). Limiting dilutions of the KO parasite was performed to obtain pure clonal population which was used further for all the experiments.

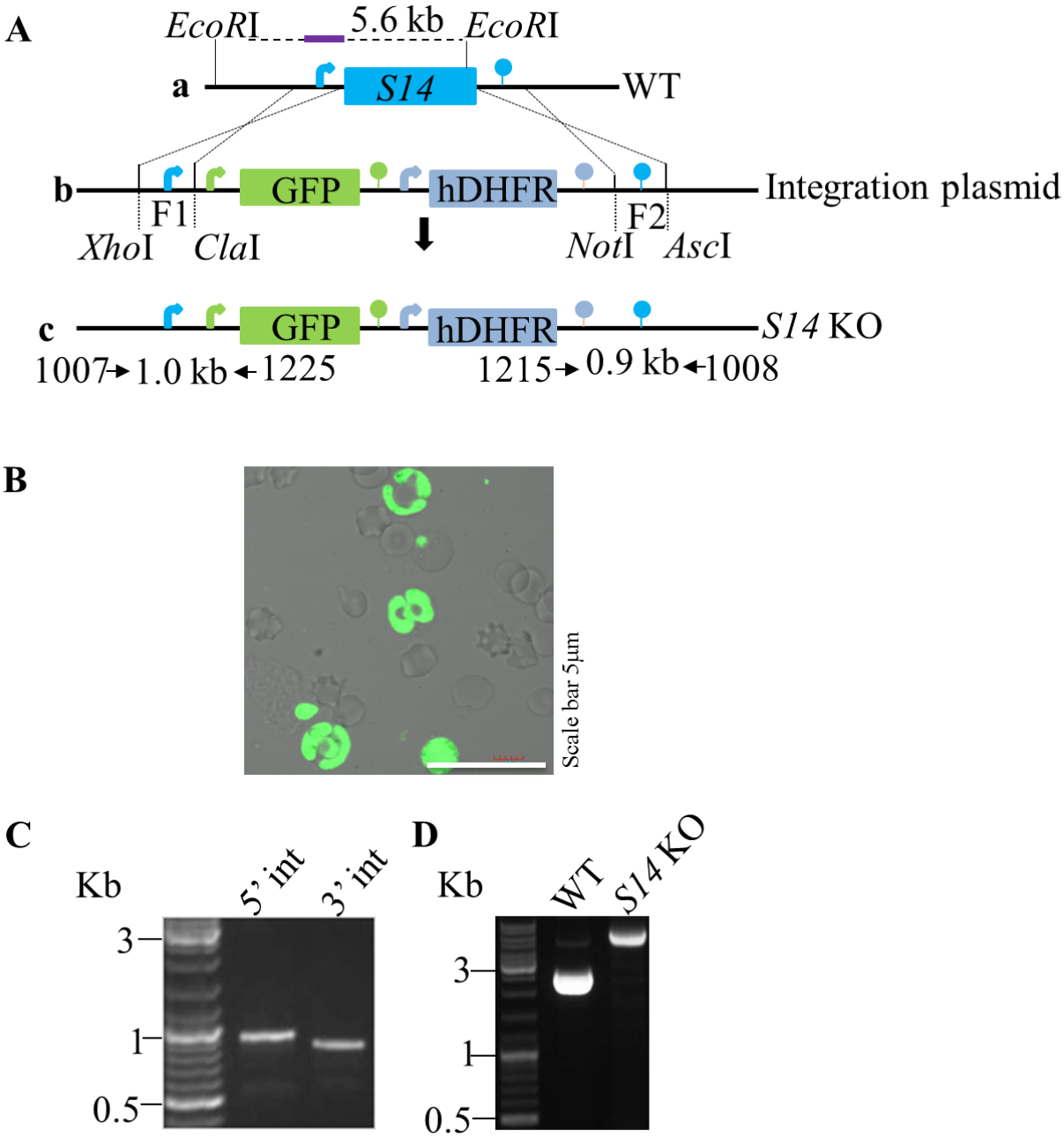


Figure 13. Generation of *S14* KO parasites. (A) (a) WT locus showing ORF, 5' and 3' UTR. (b) Targeting vector pBC-GFP-hDHFR consists of GFP and hDHFR cassette regulated by 5' (arrow) and 3' (lollipop) regulatory sequences. (c) Replacement of *S*14 locus by GFP and hDHFR cassette after double crossover (DCO) homologous recombination **(B)** *S*14 KO parasites showing GFP expression in blood stages. (**C**) Diagnostic PCR to confirm ′5and 3′ site-specific integration in the genome using primer sets 1007/1225 and 1215/1008 respectively. **(D)**Whole cassette amplification PCR using primer sets 1007/1008.

2.3.5 *S*14 is not required for the asexual blood stages

The dispensability of *S*14 indicates the non-essentiality of *S*14 in the asexual blood stages. To confirm whether the deletion of *S*14 had any effect on the parasite propagation in asexual blood stages, growth of the *S*14 KO parasite was monitored along with the WT type parasites (Figure 14). For this Swiss mice were intravenously injected with the equivalent number of *S*14 KO and WT parasites and the parasitemia was monitored consecutively for three days post-infection by counting Giemsa stained blood smears. We found similar propagation of WT and KO parasites suggesting that deletion of *S*14 does not affect the asexual blood stage growth.

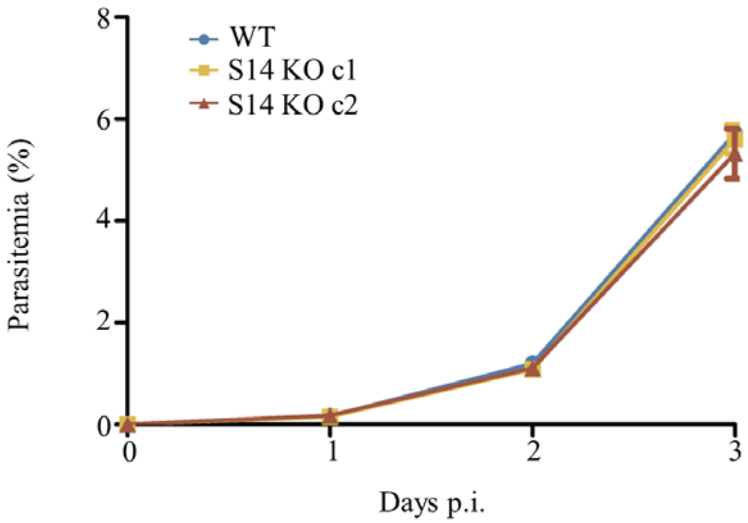


Figure14. Blood stage propagation of S14 KO parasites. Blood stage growth was measured by making Giemsa stained blood smears after equal number of *S*14 KO or WT parasites were injected intravenously in Swiss mice (n=4). Parasitemia was monitored and no difference was observed between *S*14 KO and WT parasites (P=0.998).

2.3.6 *S*14 is not required for the oocyst development and sporulation

The natural progression of the *P. berghei* parasite is the transmission of the parasite from rodent to mosquito stages. To investigate this, uninfected mosquitoes were allowed to feed on mice infected with either *S*14 KO or WT parasites. Post feeding, the mosquitos were kept in an environmental chamber maintained at 19^0C with a relative humidity of 80%. To check the successful transmission, mosquitos were dissected on day 14 post-feeding and midguts were isolated and checked for the presence of oocysts. We found that oocyst development, numbers and sporulation

inside the oocyst was normal (Figure 15A, B and C). The isolated midguts were also subjected to mechanical sheering to determine the number of midgut sporozoites which was also normal in KO parasite line (Figure 15D).

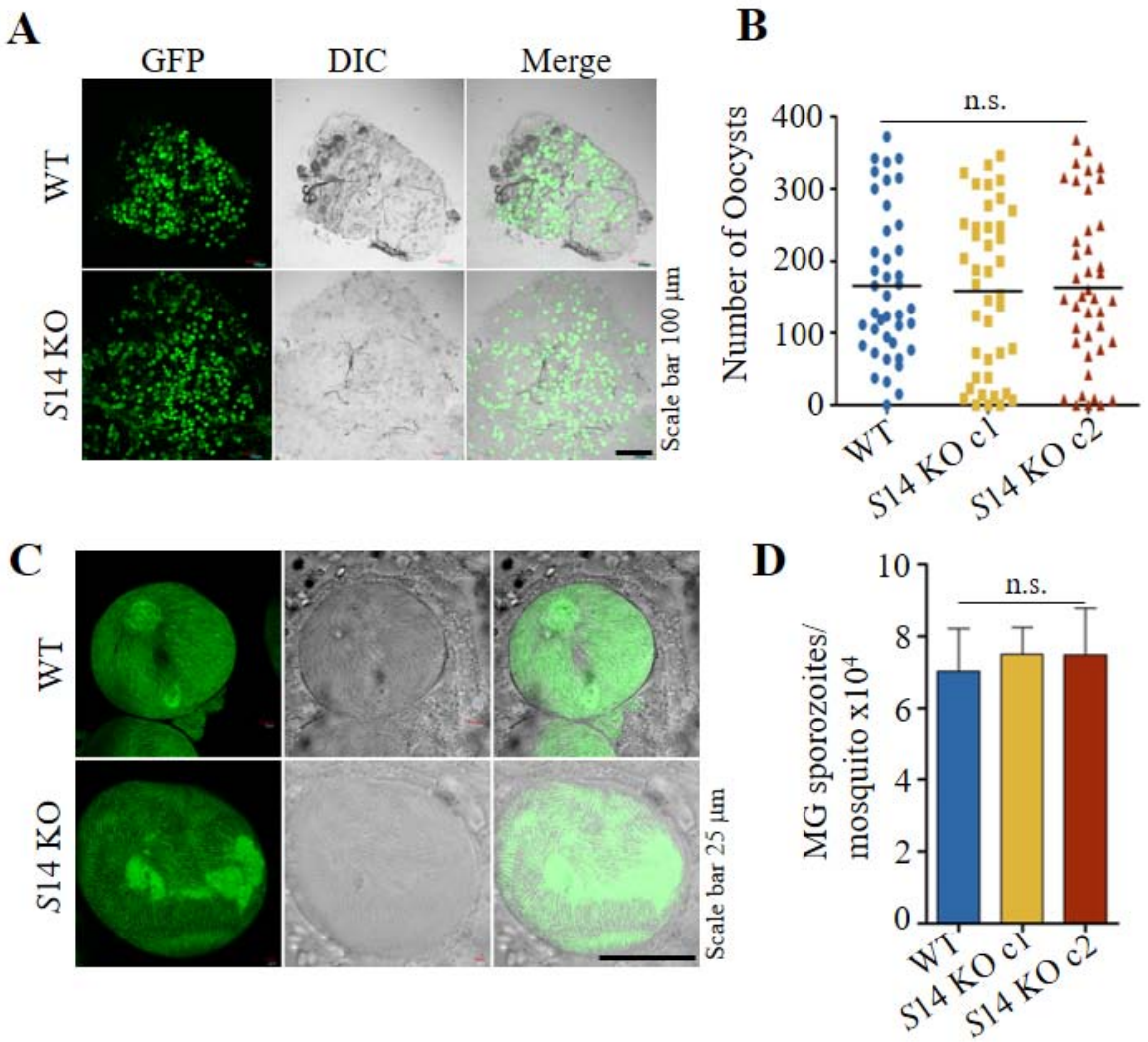


Figure 15. *S*14 KO parasites development in mosquito stages (A) Live microscopy images of mosquito midgut. **(B)** Number of midgut oocyst for WT and *S*14 KO parasites (P= 0.952). **(C)** Live microscopy images of sporulating oocysts of WT and *S*14 KO parasites. **(D)** Midgut oocyst sporozoite number (P= 0.942**)**

2.3.7 *S*14 KO sporozoites egress oocyst but failed to invade salivary glands

On day18 post-infection the mosquitoes were dissected and salivary glands were isolated and observed under a fluorescent microscope. We found flouresecent salivary glands in WT but all the KO glands were non-fluorescent (Figure 16A). For the numerical determination of sporozoite number, the salivary glands were crushed to release sporozoites and the numbers were counted using a hemocytometer. We observed that the *S*14 KO infected mosquito salivary glands were completely devoid of any sporozoites when compared to WT infected mosquito salivary glands (Figure 16B). Next, we determined whether sporozoites were defective in egressing oocyst or invading salivary gland. The oocyst sporozoites complete development on day14 post-infective blood meal and start egressing from oocyst into the hemolymph. In order to determine if the midgut sporozoites egress oocysts normally, hemocoel sporozoite number was determined by injecting medium into the mosquito thorax and collecting it from the posterior end of the mosquito and counting the hemocoel sporozoite numbers. This was done on alternate days starting from day 15 to day 23 for WT and *S*14 KO. The data indicates that the hemocoel sporozoite numbers are comparable on day 15 but there is a significant difference in the haemocoel sporozoite numbers on day 17 post-infection indicating an accumulation of sporozoites in the hemocoel as they are unable to invade the salivary glands (Figure 16C). On later time points (days 19 to 23) we observed a significantly higher number of sporozoites in KO hemolymph compared to WT. Our data clearly indicates that the *S*14 KO oocyst sporozoites egress the midgut normally but failed to invade salivary glands.

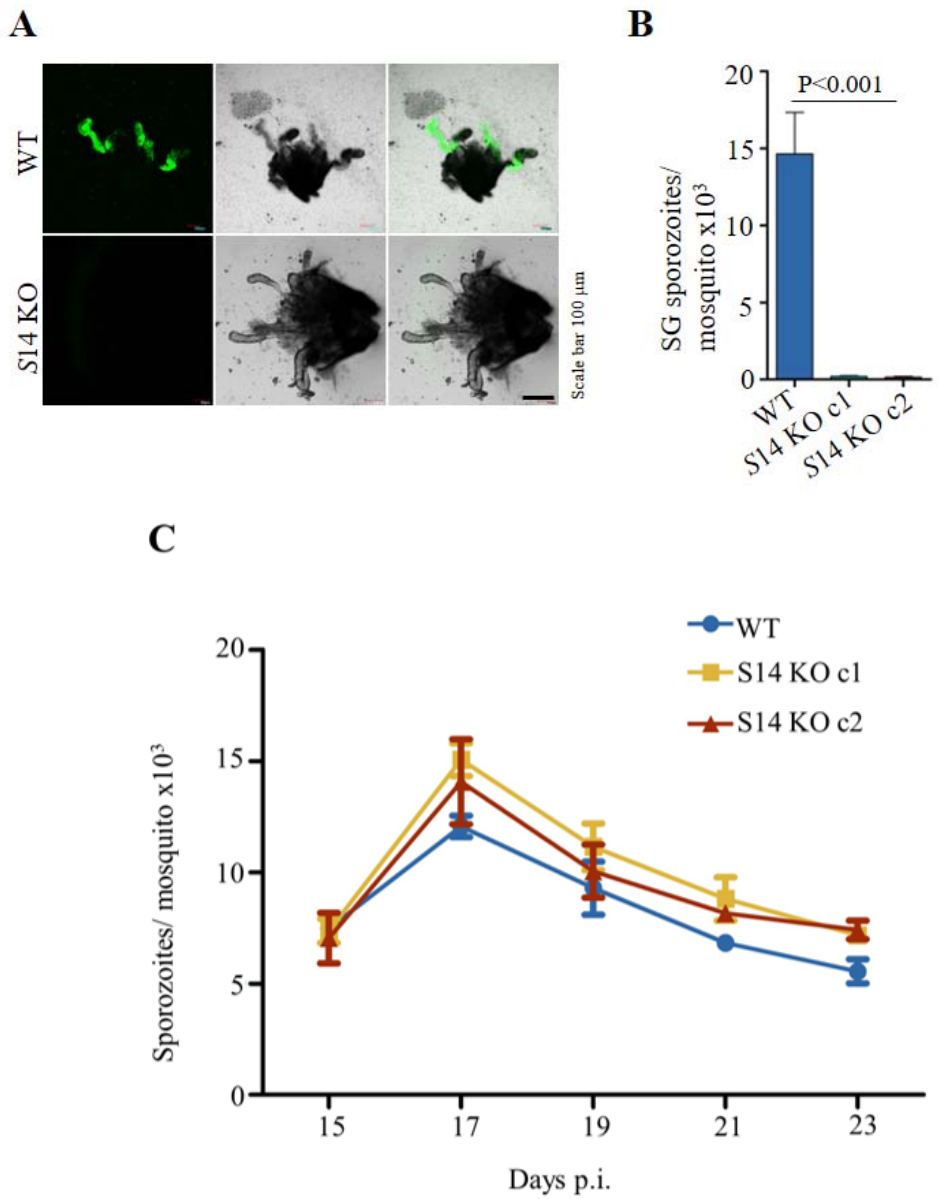


Figure 16. *S*14 KO hemolymph sporozoite count. **(A)** Dissected salivary glands. In contrast to WT, no S14 KO sporozoites could be detected in salivary glands of infected mosquitoes. **(B)** Salivary gland sporozoites number. (P<0.0001) **(C)** *S*14 KO and WT hemocoel sporozoite were collected on alternate days starting from day 15 till day 23 post-infective blood meal. Hemolymph sporozoite numbers were quantified.

2.3.8 *S*14 KO hemolymph sporozoites failed to infect mice

After it was experimentally determined that *S*14 KO sporozoites are unable to invade the salivary glands we proceeded to investigate its role in pre-erythrocytic stages of malaria. For this C57/BL6 mice were inoculated with WT and S14 KO sporozoites by mosquito bite. Groups of 10 *S*14 KO and WT infected mosquitos were used to feed

per mice (Table 6). Alternatively, C57/BL6 mice were also intravenously injected with *S*14 KO and WT hemolymph sporozoites. Giemsa-stained thin blood smear was made to check the appearance of parasite in blood stage (Table 6). We observed that in both set of experiments *S*14 KO infected mice did not exhibit any blood-stage parasitemia compared to WT which exhibited a normal 3-day prepatent period.

Table 6 Infectivity of *S14 KO* sporozoites in C57BL/6 mice. *S*14 KO parasites showed no pre-patency. C57BL/6 mice were inoculated with WT or *S*14 KO sporozoites by mosquito bite or intravenous injection into tail vain.

Experiments	**Parasites**	**Route**	**Number of sporozoites injected**	**Mice infected**	**Pre-patent period (day positive)**
1	WT	Mosquito bite	From 10 mosquitos	4/4	3
	*S*14 KO c1	Mosquito bite	From 10 mosquitos	4/0	NA
	*S*14 KO c2	Mosquito bite	From 10 mosquitos	4/0	NA
2	WT	intravenous	5,000	5/5	3
	*S*14 KO c1	intravenous	5,000	5/0	NA
	*S*14 KO c2	intravenous	5,000	5/0	NA
3	WT	intravenous	5,000	4/4	3
	*S*14 KO c1	intravenous	5,000	4/0	NA
	*S*14 KO c2	intravenous	5,000	4/0	NA

2.3.9 *S*14 KO sporozoites failed to infect hepatocytes

The *S*14 KO parasites did not show any prepatent period which could be due to impaired liver stage development. To investigate if the *S*14 gene had a role in the development of hepatocyte stages, HepG2 monolayers were infected with *S*14 KO and WT hemolymph sporozoites. Cells were stained with anti UIS4 (Muller et al., 2005) which recognizes the PV membrane. Hoechst 33342 was used to stain the nuclei of both host and parasite. The EEF's was observed only in WT infected

hepatocytes (Figure 17 A and B). Results indicate WT hemocoel sporozoites developed normally into EEF's whereas no EEF's were observed in the case of *S*14 KO infected hepatocytes.

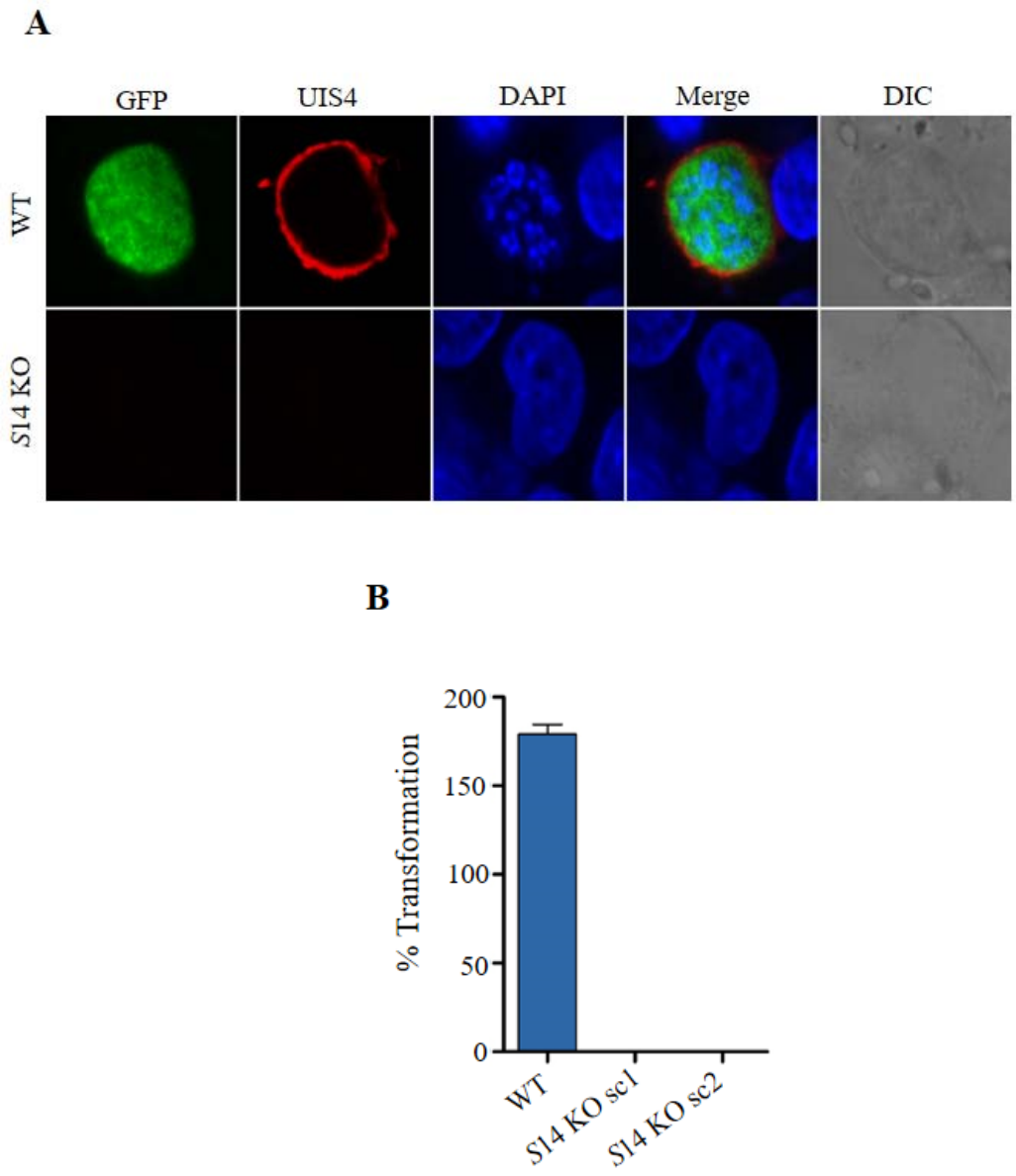


Figure 17. S14 KO sporozoites liver stage development (A) HepG2 cells infected with *S*14 KO and WT hemolymph sporozoites were stained with UIS4 antibody and host and parasite nuclei was stained with Hoechst. **(B)** Quantitation of EEF numbers. ($P<0.0001$).

2.3.10 *S*14 KO sporozoites were developmentally normal

The sporozoites after productively invading hepatocyte form PV membrane, undergo morphogenesis and maturing into EEF. This intracellular development can be

phenocopied in vitro. In an attempt to investigate if *S*14 plays a role in the EEF development bulb transformation assay was performed (Kaiser et al., 2003). *S*14 KO and WT hemolymph sporozoites were incubated at 37^0C in DMEM supplemented with 10% FBS and incubated for 4 hr. The percentage transformation into bulbs was determined by counting sporozoites that transformed into spherical structures (Figure 18 A). There was no significant difference between *S*14 KO and WT sporozoites transformation into bulbs (Figure 18 B).

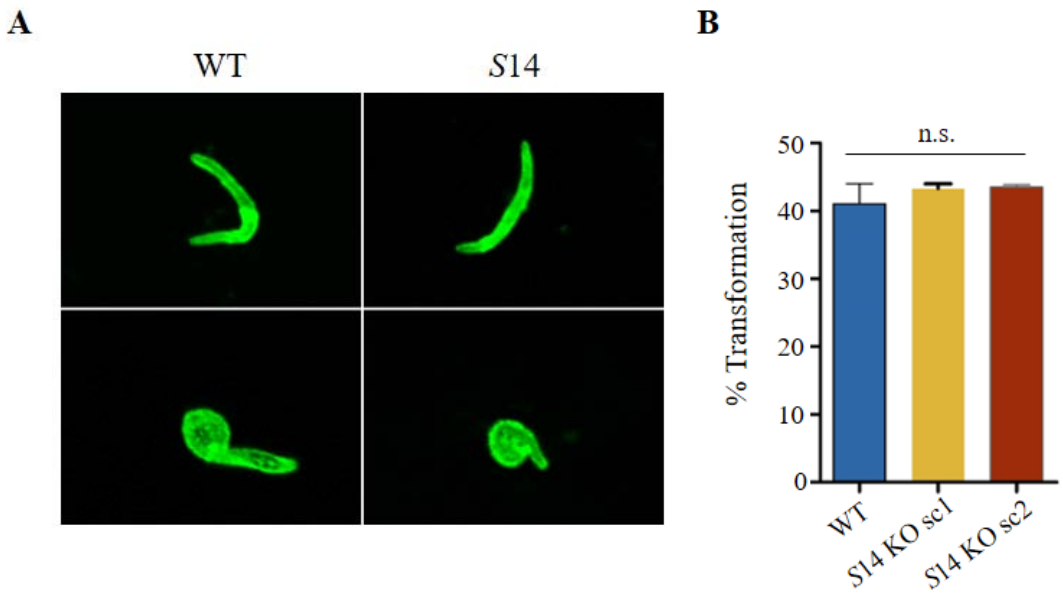


Figure 18. *S*14 KO sporozoites developmentally normal. (A) *S*14 KO and WT hemocoel sporozoites transformation into bulbs **(B)** Quantification of sporozoites transformation into bulbs (P=0.418)

2.3.11 *S*14 KO sporozoites were non-invasive

To check whether *S*14 KO sporozoites invade hepatocytes normally, HepG2 monolayers were infected with *S*14 KO and WT hemolymph sporozoites and fixed after 1 hr. Dual-color immunostaining was performed as previously described (Reina et al., 1988) and sporozoite inside and outside of the cells were counted (Figure 19 A). Counting revealed that *S*14 KO sporozoites were completely incapable of invading hepatocytes (Figure 19 B).

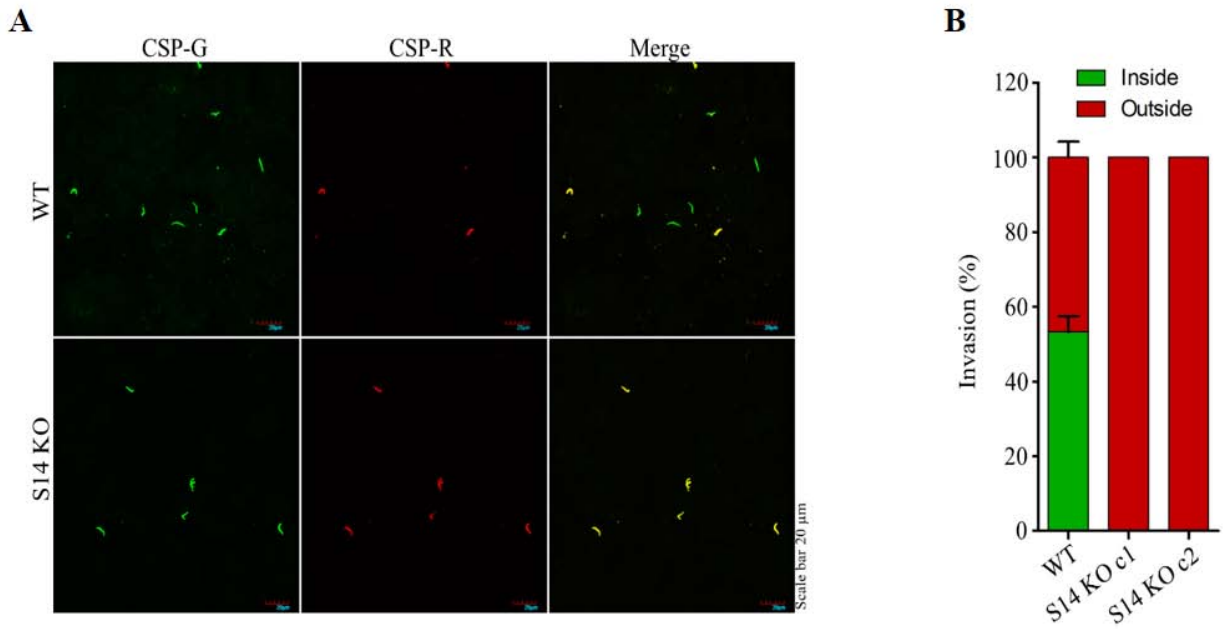


Figure 19. Hepatocyte invasion of *S*14 *KO* sporozoites. (A) Immunofluorescent detection of sporozoite invasion by sequential CSP staining pre and post permeabilization revealed by anti-mouse 594 and anti-mouse 488 fluorescent antibodies before and after permeabilization respectively. **(B)** Quantification of sporozoites inside vs outside (P<0.001).

2.3.12 *S*14 KO sporozoites were non-motile

Gliding and invasion are correlated as they share molecular machinery. To examine whether the gliding motility was affected by *S*14 deletion, *S*14 KO and WT hemolymph sporozoites were added to Nunc glass slide precoated with anti-CSP antibodies and visualized the trails using Biotin-labeled CSP antibody followed by streptavidin-FITC (Stewart & Vanderberg, 1988). The CSP trails were counted for both KO and WT sporozoites, (Figure 20 A and B) the counting revealed that *S*14 KO sporozoites were completely non-motile which indicates that *S*14 is essential for sporozoite gliding motility.

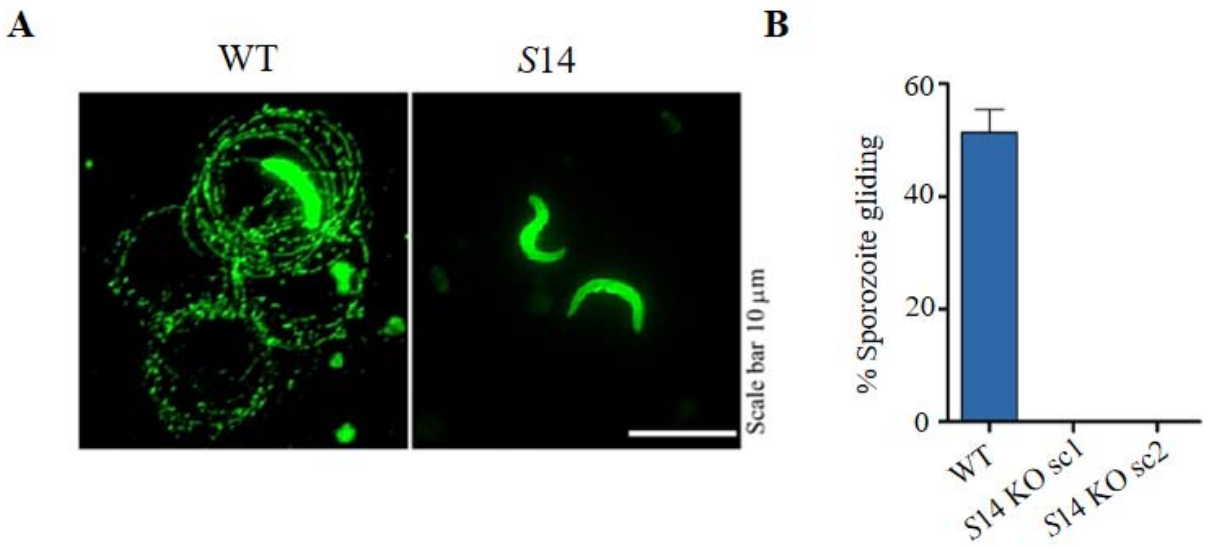


Figure 20. *S*14 KO hemocoel sporozoites are deficient in gliding motility (A) The WT and *S*14 KO hemocoel sporozoites IFA revealing CSP trails **(B)** Graph showing percentage of motile sporozoites (P<0.0003)

2.4 Discussion

The S genes which were identified as sporozoite specific transcripts (Kaiser *et al.*, 2003) have been found to play crucial role in sporozoite biology mostly affecting processes specific to the zoite stages such as motility, adhesion, invasion and hepatocyte development (Kariu *et al.*, 2006, Risco-Castillo *et al.*, 2015b, Ejigiri & Sinnis, 2009). Similar to the other S genes *P. berghei S*14 play an essential role in *Plasmodium* sporozoite stages. Expression analysis revealed *S*14 expression in sporozoites confirming previously conducted SSH study (Kaiser et al., 2004). Tagging of S14 with 3XHA-mCherry expressed reporter in oocyst and salivary gland sporozoites. Immunostaining colocalized mcherry with MTIP which localizes on the IMC (Bergman et al., 2003). As *S*14 does not possess any transmembrane domains, it probably interacts with the membrane peripherally. In silico analysis (data not shown) predicts the presence of palmitoylation motif on *S*14 is consistent with several other glideosome associated proteins that localize on the IMC or parasite plasma membrane through post-translational modifications such as dual N-terminal acylation in GAP45 (Frenal et al., 2010). The presence of the palmitoylation motif is a common feature in most *Plasmodium* peripheral membrane proteins. We have established through our studies that *S*14 is essential for gliding and invasion of *Plasmodium* sporozoite. Several other gene depletions studies exhibit similar phenotype such as the deletion of

TRAP (Sultan *et al.*, 1997) and *S6* which also belongs to the TRAP family adhesins (Steinbuechel & Matuschewski, 2009) and are also members of S genes. TRAP and similar adhesins like CSP interact with extracellular substrates anchoring sporozoite to the substratum providing tracks for the sporozoite to propel forward.

From a mechanistic stand point gliding motility is vastly different from previously observed modes of unicellular locomotion namely amoeboid, cilliary and fllagelar motion. The studies in *Plasmodium spp* sporozoite contributed vastly in the understanding of the concept of gliding motility especially the circumsporozoite protein precipitation reaction (Vanderberg, 1974). The parasite gliding and invasion requires a dedicated multiprotein complex, it consists of essential elements that are indispensable and proteins that are not essential but facilitate the process. This complex localizes on the parasite IMC and was termed as glideosome in context to *Toxoplasma* (Opitz & Soldati, 2002) which can be extended to other Apicomplexans. The central motor component that drives the process is MYOA which in Apicomplexa is uncharacteristically devoid of the tail domain which in other organisms is responsible for interaction with cargo molecules and localization (Schliwa & Woehlke, 2003). The velocity of gliding sporozoites (3µm/s) (Green *et al.*, 2006, Meissner *et al.*, 2002) indicates that Apicomplexan myosin is a fast motor drawing similarity to skeletal muscle myosin in its mechanical properties. The MYOA head domain contains the ATP binding domain which is responsible for mechanochemical coupling of ATP hydrolysis and molecular movement which is further transduced into locomotion by Myosin light chain 1 (MLC1) also known as Myosin A tail interacting protein (MTIP) which functions as a lever (Bookwalter *et al.*, 2014, Bosch *et al.*, 2007) for the glidosome machinery. Several other proteins that associate with the central MYOA motor directly or to the glidosome peripherally have been termed as gliding associated proteins (GAP). GAP45 is responsible for the maintenance of pellicular integrity and links the glideosome to plasma membrane during motility (Frénal *et al.*, 2010) also acts as a central anchor to the whole complex. GAP45 is targeted to plasma membrane by an N-terminal acylation and its C-terminal domain interacts with the IMC (Frénal *et al.*, 2010, Ridzuan *et al.*, 2012). GAP40 and GAP50 interact with MYOA and ensure its strong adherence to the cytoskeleton (Frénal *et al.*, 2010). The glideosome interacts with the alveolins through gliding associated proteins with multiple membrane spans (GAPM) which does not interact directly with MYOA

but with GAP50 (Bullen *et al.*, 2009). GAPM along with GAP50 and GAP40 plays pivotal role in the synthesis of the inner membrane complex in *Toxoplasma* (Harding *et al.*, 2016).

The IMC localized MYOA motor drives the anterioposterior translocation of the F-actin linked adhesion complexes along the sporozoite axis. The forward locomotion of the sporozoite necessitates synchronized cleavage of cytoplasmic tails of these adhesion complexes by intramembrane ROM4 (Ejigiri *et al.*, 2012) which leads to shedding of these adhesins during gliding. Unlike S6 and TRAP, *S*14 is not an adhesin or a cell surface protein. This enumerates the complexity of gliding motility which is driven by multiple proteins working in concert. The deletion of *Pb S*14 abolishes gliding completely but does not affect the CSP shedding, this suggests that rhomboid protease activity is unaffected in absence of *S*14.

We have also established that *S*14 localizes on the sporozoite IMC. The co-localization of *S*14 with the gliding associated proteins along with the loss of function phenotype strongly suggests its potential mechanistic role in the working glideosome complex. In a separate study yeast two-hybrid study confirmed the interaction of *S*14 with MTIP and GAP45 (data not shown). This result unequivocally establishes *S*14 as a part of the glidosome complex. As discussed above the shedding of CSP by proteolytic cleavage remains unaffected in *S*14 KO, a possibility could be that *S*14 similar to GAP45 is responsible for the tethering of the complex to the membrane. The forward propulsion of the sporozoite aided by posterior movement of host attached complexes requires the generation of traction for which a strong attachment of the molecular motor to the pellicle membrane is essential. The absence of *S*14 seems to destabilize the glideosome complex which leads to the inability of the parasite to glide. Our investigations establish *S*14 as sporozoite specific component of the glidosome which is essential for gliding and invasion.

Chapter 3

Plasmodium berghei SCOT1 is essential for liver stage Development

3.1 Introduction

Plasmodium parasite has a complex life cycle consisting of multiple stages. The blood stages of the *Plasmodium* parasite which have been studied most extensively, though the staging point in the invertebrate host are the liver stages also termed as the exo-erythrocytic forms (EEF's). The liver stages lead to the formation of merozoites which further propagate in the blood-stage by infecting the erythrocyte. The biology of liver-stage parasite is still unexplored. The use of high throughput methods in analysing stage specific gene expression has led to the enumeration of several putative genes that could have crucial roles in parasite virulence (Le Roch *et al.*, 2003, Le Roch *et al.*, 2004, Zhou *et al.*, 2008). Transcriptomic analysis of sporozoite stages revealed changes in transcript profiles responsible for their infectivity (Matuschewski *et al.*, 2002). Important insights to *Plasmodium* gene regulation was aided by a comprehensive study of the sporozoite transcriptome (Kappe *et al.*, 2001) and paved way to identification of unique transcripts specific to sporozoites (Kaiser *et al.*, 2004). The group of genes discovered through these studies includes the UIS genes (Kappe *et al.*, 2001) and S genes (Kaiser *et al.*, 2004). Characterization of these genes has led to the annotation of genes crucial in liver stage development. Which include genes such as UIS4 and UIS3 both localize on the PV membrane (Mueller *et al.*, 2005a) UIS4 mutants arrest early in liver stage development (Mueller *et al.*, 2005a) and UIS3 has been shown to interact with LC3 playing a crucial role in avoiding parasite clearance through autophagy (Yao & Klionsky, 2018, Real *et al.*, 2018). The role of S genes has been discussed in detail in the previous chapter. Similarly expressed sequence tag (EST) data analysis of salivary gland sporozoites identified several proteins essential for diverse sporozoite associated functions. These include micronemal protein SPECT and membrane attack complex/perforin (MACPF) both are essential for cell traversal (Ishino *et al.*, 2004, Ishino *et al.*, 2005a). Though SPECT depleted parasites have the ability to invade hepatocytes *in vitro*, whereas MACPF deletion renders sporozoites non infective (Ishino *et al.*, 2005a).

The pursuit of putative genes identified through the high throughput techniques has provided in depth insights into sporozoite and liver stage biology. In a previous study, samples from infected *P. vivax* patients and mosquitos was used to examine stage specific transcriptional changes in an attempt to annotate gene function and regulatory

sequences (Westenberger *et al.*, 2010). They found that mRNA modulation plays a key role in developmental regulation in different life cycle stages of the parasite. They were able to identify DNA sequence motifs that are conserved across *Plasmodium* species present upstream of co-expressed genes which could serve as binding sites for stage specific transcription regulatory proteins. Through this study they also ranked transcripts exhibiting maximal expression in different stages which also led to identification of novel transcripts (Table 7). As discussed above several transcripts upregulated in or specific to sporozoite stages have been found to play crucial role in liver stage development (Mueller *et al.*, 2005a, Ejigiri & Sinnis, 2009, Ishino *et al.*, 2005b). The highly ranked transcripts in sporozoite stages are genes known to be upregulated in sporozoites which include UIS1 which is eukaryotic initiation factor kinase (Turque *et al.*, 2016), and UIS2 a serine threonine phosphatase (Zhang *et al.*, 2016), the list also has candidates that have been used previously for pre-erythrocytic subunit vaccine development including the apical membrane antigen 1 (AMA1) gene and the circumsporozoite protein (CSP). In addition there are genes whose disruption has led to genetically attenuated sporozoites including the ortholog of *P. falciparum* UIS4 in *P. yoelii* (Matuschewski *et al.*, 2002, Mueller *et al.*, 2005a), UIS3 (Mueller *et al.*, 2005b) and P52 (van Dijk *et al.*, 2005) that have been used in genetically attenuated parasite vaccine development. This study also identified a set of transcripts upregulated throughout the *Plasmodium* species which have not been annotated previously and could possibly yield genetically attenuated sporozoites on disruption. These transcripts have been termed as Sporozoite conserved orthologous transcripts (SCOT). *P. berghei* orthologue for one of these genes SCOT2 has been characterized by gene disruption, the targeted deletion of SPELD (sporozoite surface protein essential for liver stage development) led to abortion of EEF development (Al-Nihmi *et al.*, 2017). We chose SCOT1 which was ranked at the top of the list for characterization using reverse genetic approaches.

Table 7 list of highly expressed sporozoite transcripts ranked in descending order of maximum expression value. Sporozoite Conserved Orhtologus Transcript (SCOT) are either putative genes or genes which have not been annotated previously upregulated in sporozoites of all species. (Westenberger *et al.*, 2010)

Alias	Description	*P. vivax* Gene	Exp (E)	Rank
SCOT1	hypothetical protein, conserved in *Plasmodium*	PVX_122458*	17726	1
UIS4	UIS4, ETRAMP10.3	PVX_001715	16095	2
SCOT2	hypothetical protein, conserved	PVX_092505	13536	3
CSP1	circumsporozoite protein precursor, putative	PVX_119355	13189	4
PvSpz1	hypothetical protein	PVX_092255	8649	5
H3	histone H3, putative	PVX_114020	6864	6
SUI1	translation initiation factor SUI1, putative	PVX_101080	6656	7
PvSpz2	hypothetical protein, conserved	PVX_113796	6201	8
UVS1	hypothetical protein, conserved	PVX_089045	5424	9
AMA1	apical merozoite antigen 1	PVX_092275	4856	10
ECP1	cysteine protease serine-repeat antigen (SERA)	PVX_003790	4799	11
	Falstatin	PVX_099035	4358	13
UVS	hypothetical protein, conserved	PVX_122910	3975	14
PvROM1	rhomboid-like protease 1, putative	PVX_091350	3501	15
DIM1	DIM1 rRNA dimethylase, putative	PVX_100520	3383	17
	hypothetical protein, conserved	PVX_087095	3236	18
PvSpz3	hypothetical protein	PVX_114765	3074	19
S11	S11 *P. yoelii* conserved hypothetical protein	PVX_086200	3056	20
SCOT3	hypothetical protein, conserved	PVX_085040	2794	23
SCOT4	hypothetical protein, conserved	PVX_123360	2793	24
DHFR-TS	dihydrofolate reductase-thymidylate synthase	PVX_089950	2729	25
UVS2	protein tyrosine phosphatase, putative	PVX_091305	2713	26
UVS3	hypothetical protein, conserved	PVX_115405	2697	27
PvSpz4	hypothetical protein	PVX_118250	2688	28
SPECT	sporozoite microneme protein, putative	PVX_083025	2671	29
G10	G10 protein, putative	PVX_080110	2499	32
UIS2	UIS2, Ser/Thr protein phosphatase	PVX_117230	2332	34
D13	CCCH zinc finger domain D13 protein	PVX_089510	2282	35
UVS4	hypothetical protein, conserved	PVX_091250	2273	36
SCOT5	hypothetical protein, conserved	PVX_094795	2063	43
S14	S14 *P. yoelii* Sporozoite-specific gene	PVX_084410	2037	46
UVS5	hypothetical protein, conserved	PVX_122540	2022	47
PvSpz5	hypothetical protein, conserved in *Plasmodium*	PVX_091306*	1978	50

3.2 Material and methods

3.2.1 Cloning vectors

Following vectors were used for cloning, expression and genetic manipulation (Annexure 1) of the parasite:

1. pBluescript SK (+) vector
2. pBC-GFP-hDHFR-yFCU
3. pBC-3XHA-hDHFR

Table 8 List of primers used for the generation, and confirmation of *Scot1* KO and transgenic parasite lines.

Primers	**Primers for *Scot1* general knock out and transgenic (primer sequence 5'-3')**	**Restriction sites**
1165	CTCGAGGTTCTCTTTAATTAATGAAAGCTTG	Xho1
1166	ATCGATAAATATATCTCATATGTTACAAC	Cla1
1167	GCGGCCGCCAAGCCACGGAGAAAATGC	Not1
1168	GGCGCGCCCCAAGATTAATATATATTTCC	Asc1
1171	CAGATCTTATTATTACTGTTACGGGAGA	Bgl2
1169	AGCAATATCAAAAATACAAGTTT	NA
1170	TAAAATTGGCTATTTAGACATAA	NA
1215	GTTGTCTCTTCAATGATTCATAAATAG	NA
1225	TTCCGCAATTTGTTGTACATA	NA

3.2.2 Preparation of *Scot*1 knockout targeting construct

The PCR amplification, confirmation, agarose gel electrophoresis protocols have been described in the previous chapter. The fragments F1 (630 bp) and F2 (519 bp) from the 5' and 3' UTR sequences flanking the *Scot1* gene were amplified using primer sets 1165/1166 and 1167/1168 respectively and cloned at *Xho*1/*Cla*1 and *Not*1/*Asc*1 in pBC-GFP-hDHFR-yFCU vector. Final digestion was setup using *Xho*1/*Asc*1 to separate the vector backbone from the targeting construct.

3.2.3 Preparation of SCOT1-3XHA-mCherry targeting construct

For the tagging of SCOT1 with 3XHA-mCherry, fragment F2 (519) was taken from KO targeting vector and a new fragment F3 (842) from the c-terminus of the gene was amplified using primers 1165/1171. Fragments F3 and F2 were cloned at *Xho*1/*Bgl*2 and *Not*1/*Asc*1 respectively in pBC-3XHA-mCherry-hDHFR vector. Final digestion was setup using *Xho*1/*Asc*1 to separate the vector backbone from the targeting construct.

3.2.4 Determination of the pre-patent period

To determine the the infectivity of *Scot*1 KO sporozoites in vivo, C57BL/6 mice were inoculated intravenously. As a control, equal number of WT sporozoites was injected into another group of mice. Parasite in blood was observed by Giemsa-staining of blood smears.

3.2.5 *In vitro* sporozoite infectivity

To study the EEF development *in vitro*, salivary gland sporozoites were added on the monolayer of HepG2 culture in above chapter.

3.2.6 Immunofluorescence assay

Immunofluorescence staining of *Scot*1 KO and WT EEF harvested till 48h was performed using UIS4 antibody. Late EEFs harvested 62h p.i. stained with MSP1 antibody as described in above chapter.

3.2.7 Immunization experiment

Female C57BL/6, 6-8 week old mice were used for the study and were divided into 4 groups. All the animals were of the same age group. One group was used for pre-erythrocytic immunity and one for cross-stage immunity with respective control groups. All the groups were injected with three doses of sporozoites with an interval of 14 days. The DMEM was injected as vehicle control. The control and immunized groups were challenged with WT sporozoites 10 days after the last immunization. For cross-stage immunity, mice were challenged with 50 iRBCs. The parasitemia was monitored every day by making Giemsa-stained blood smears.

3.2.8 Fluorescence associated cell sorting (FACS) of *Plasmodium* infected HepG2 cells

HepG2 cells (55,000/ well) were seeded in 24 well cell culture plates and infected with *Scot*1 KO or WT salivary gland sporozoites using similar protocol as described above. The infected cells were allowed to grow for 55 hrs after which the cells were washed with PBS and harvested by trypsinization. Harvested cells were pelleted by

centrifuging at 500g for 3 min at 4^0C and resuspended in MACS buffer (1X PBS, 2mM EDTA, 0.5% BSA). For sorting of cells gating was done on the basis fluorescence and granularity.

3.2.9 RNA isolation for sequencing

Trizol (200µl) was added to the sorted cell and thoroughly mixed with equal volume of 95% ethanol. The mixture was loaded on Zymo IC column and centrifuged for 30 sec at 10,000 g. Column was washed with 400µl RNA wash buffer. Remaining steps were followed as per manufacturer's guidelines including on column DNase treatment. RNA was eluted using 15µl nuclease free water (Ambion. Cat#AM9938)

3.2.10 Library prepration and sequencing

Total RNA was used for preparation of RNA sequencing library (SMART-seq v4 ultra low input RNA kit clonetech, SA) used for cDNA synthesis followed by Nextera XT DNA library prep kit (Illumina, USA) at Genotypic Pvt Ltd. Bangalore, India. The input RNA primed by 3' SMART-seq CDS primer II A was subjected to first strand cDNA synthesis and MARTseq v4 oligonucleotide was used for template switching at the 5' end of the transcript. First strand synthesis was followed by cDNA amplification using LD PCR for 10 cycles. For purification of double stranded cDNA AMPure XP magnetic beads (Agentcourt, MA, USA) and quantification was done using Qubit fluorometer (Thermo Fisher Scientific, MA, USA). Amplicon tagment mix (Nextera XT kit) was used to fragment and tag 1ng of Qubit quantified cDNA and was subjected to 10 cycles of indexing-PCR (72^0C for 3 mins followed by denaturation at 95^0C for 30 sec, cycling 95^0C for 10sec, 55^0C for 30 sec, 72^0C for 30 sec and 72^0C for 5 mins) to enrich adapter tagged fragments.

Final PCR product (sequencing library) was purified followed by library quality control assessment. Quantifications of the Illumina-compatible sequencing library were made using Qubit fluorometer (Thermo Fisher Scientific, MA, USA) followed by analysis of fragment size distribution on Agilent tape station. Sequencing of the libraries was performed on Illumina HiSeq 4000 sequencer (Illumina, San Diego, USA) for 150 bp paired-end chemistry following the manufacturer's protocol.

3.3 Results

3.3.1 Generation of *Scot*1-3XHA-mCherry transgenic parasite line

For the generation of a stable *Scot*1-3XHA-mCherry transgenic parasite line, two homology fragments F2 and F3 from the 3' UTR (512bp) and c-terminus (753bp) of the gene was amplified. These fragments were cloned into pBluescriptSK+ vector and the transformants were screened by blue-white screening and restriction digestion. Purified fragments were sequentially cloned at the *Xho*1/*Bgl*2 and *Not*1/*Asc*1 sites in the pBC-3XHA-mCherry vector and cloning was confirmed by restriction digestion (Figure 21A). Post confirmation of the final targeting construct the vector backbone was separated by double digestion with *Xho*1/*Asc*1 (Figure 21B). The targeting construct was transfected into the *P.berghei* schizont. The transfectants were selected using the drug pyrimethamine and further single cloning of the Knock-out parasite was done by limiting dilution. Diagnostic PCR with primer sets 1225/1169 and 1215/1170 was performed and successful integration of the targeting construct at the appropriate loci was confirmed (Figure 21 C).

3.3.2 *Scot*1-3XHA-mCherry expression in mosquito and liver stages

To investigate the expression of SCOT1 protein, the *Scot*1-3XHA-mcherry transgenic parasite was progressed through the mosquito stages by feeding mosquitos on transgenic parasite-infected Swiss mice. Mosquitos were dissected on day 14 and day18 post-infection to isolate oocyst and salivary gland sporozoites. We found that mCherry reporter expressed throughout the mosquito stages. The mCherry expression in the sporozoite stage showed a non-uniform punctate distribution in the sporozoite (Figure 22 A). To observe the SCOT1-3XHA-mCherry expression in EEFs, cultured HepG2 monolayers were infected with Scot1-3XHA-mCherry sporozoites and fixed at different time points. EEFs were stained with UIS4 antibody (Muller et al., 2005) as described earlier. The expression and localization of mCherry was revealed by fluorescent microscopy (Figure 22 B). We observed that SCOT1 expresses till the

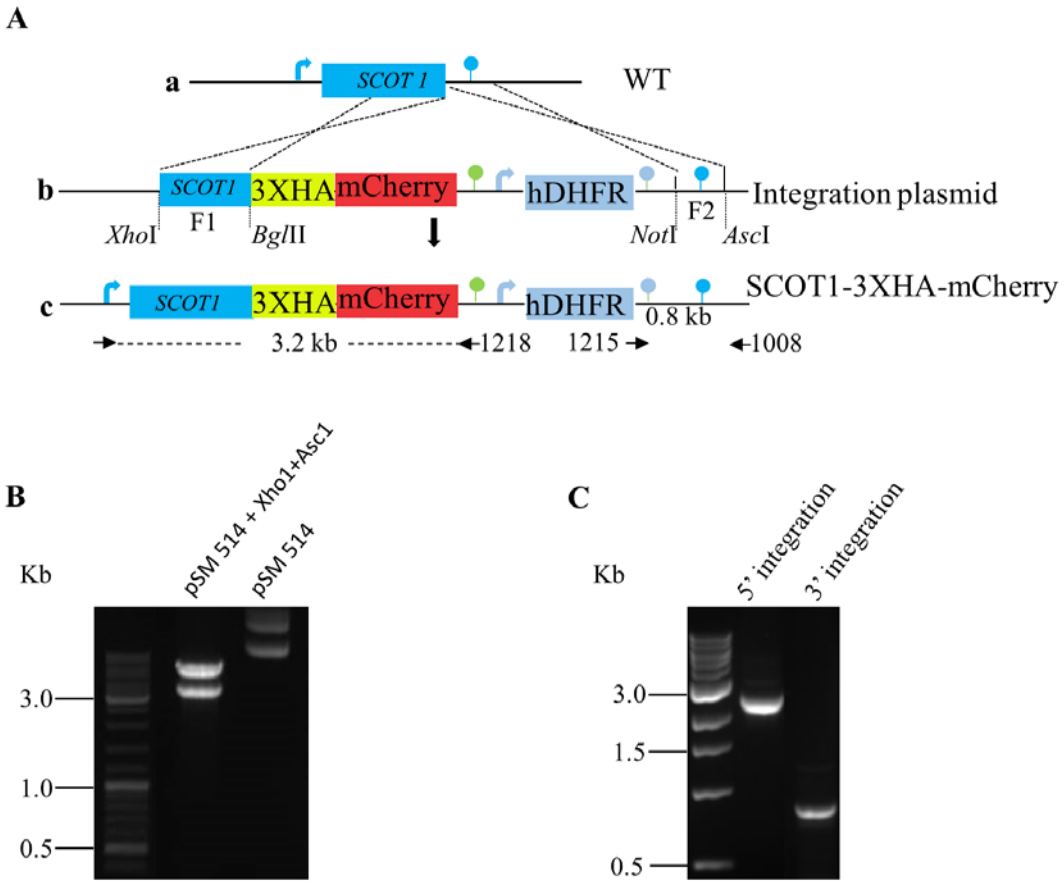


Figure 21. Generation of *Scot*1-3XHA transgenic parasites (**A**) **(a)** Genomic locus of *Scot*1. **(b)** Targeting plasmid pBC-3XHA-mCherry-hDHFR. (c) *Scot*1 locus after integration of targeting plasmid. (**B**) Targeting construct was separated from the vector backbone by restriction digestion with *Xho*I/*Asc*I. **(C)** Diagnostic PCR with a set of primers 1169/1171 and 1170/1167 confirming correct integration of 5' and 3' fragments respectively in the parasite genome.

3.3.2 *Scot*1-3XHA-mCherry expression in mosquito and liver stages

To investigate the expression of SCOT1 protein, the *Scot*1-3XHA-mcherry transgenic parasite was progressed through the mosquito stages by feeding mosquitos on transgenic parasite-infected Swiss mice. Mosquitos were dissected on day 14 and day18 post-infection to isolate oocyst and salivary gland sporozoites. We found that mCherry reporter expressed throughout the mosquito stages. The mCherry expression in the sporozoite stage showed a non-uniform punctate distribution in the sporozoite (Figure 22 A). To observe the SCOT1-3XHA-mCherry expression in EEFs, cultured HepG2 monolayers were infected with *Scot*1-3XHA-mCherry sporozoites and fixed at different time points. EEFs were stained with UIS4 antibody (Muller et al., 2005) as described earlier. The expression and localization of mCherry was revealed by fluorescent microscopy (Figure 22 B). We observed that SCOT1 expresses till the

24hr time point in the liver stages and it shows a uniform distribution throughout the early stage of EEF cytoplasm.

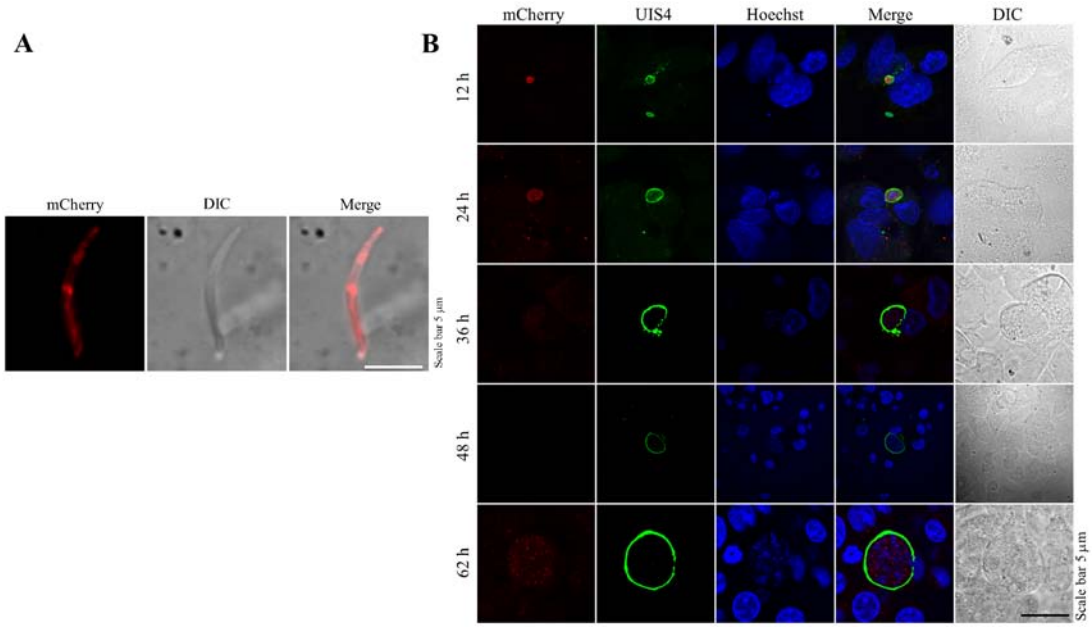
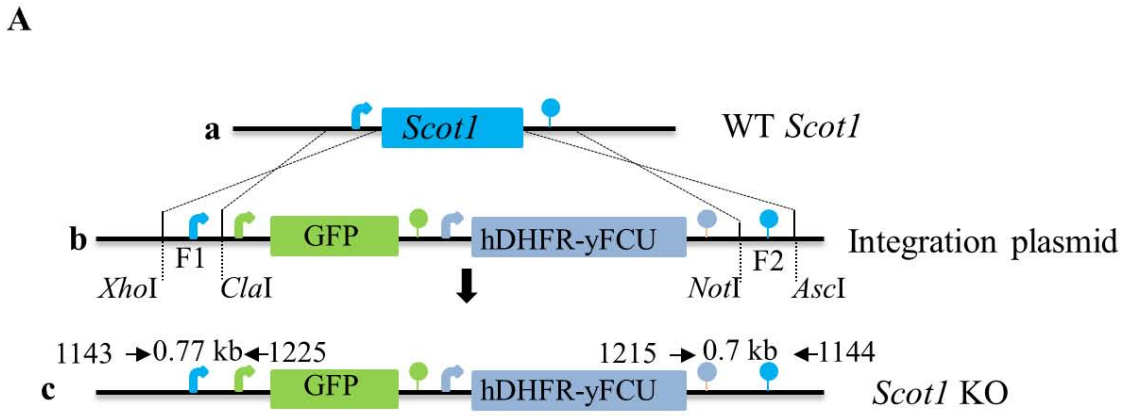
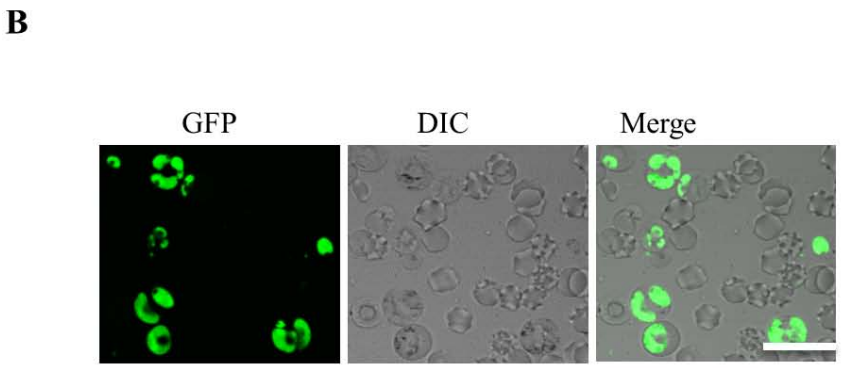


Figure 22. *Scot*1-3XHA-mCherry transgenic parasites expression in mosquito and liver stages *(*A) Salivary gland sporozoites showing SCOT1-mCherry expression. **(B)** HepG2 cells infected with *Scot*1-mcherry sporozoites were harvested at different time points. Cultures harvested at 12, 24, 36, 48 and 62 h p.i. were stained with UIS4 antibody and host and parasites nuclei was stained with Hoechst. SCOT1-mcherry localizes in the cytosol and expression is seen till 24 h.p.i.

3.3.3 Generation of *Scot*1 knockout parasites in *P. berghei*

For the generation of a stable *Scot*1 knockout parasite line through double crossover homologous recombination the pBC-GFP-hDHFR-yFCU plasmid was used. Two homology fragments F1 and F2 from the 5' and 3' UTR sequences flanking the *Scot*1 gene with lengths of 630bp and 519bp respectively, were amplified using primers 1165/1166 and 1167/1168. These fragments were sequentially cloned at the *Xho*1/*Cla*1 and *Not*1/*Asc*1 sites in the above mentioned vector and cloning was confirmed by restriction digestion. Post confirmation of the final targeting construct the vector backbone was separated by double digestion with *Xho*1/*Asc*1. The targeting construct was transfected into the schizont stages of *P. berghei* (Figure 23 A). Blood of the transfected parasites was observed under a fluorescent microscope to confirm the GFP expression (Figure 23 B). Diagnostic PCR was performed to confirm the successful integration of the targeting construct using primer sets 1225/1169 and 1215/1170 for the 5' and 3' integrations respectively (Figure 23 C). The single cloned parasite was used for further studies.





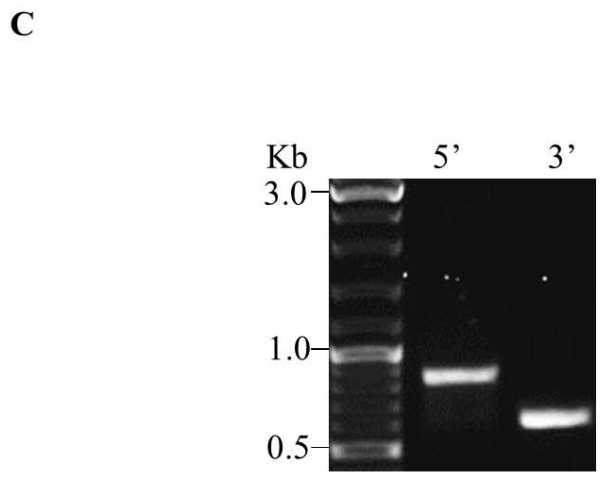


Figure 23. Generation of *Scot*1 KO parasites. (A) (a) WT locus showing ORF, 5' and 3' UTR. (b) Targeting vector pBC-GFP-hDHFR consists of GFP and hDHFR cassette regulated by 5' (arrow) and 3' (lollipop) regulatory sequences. (c) Replacement of *Scot*1 locus by GFP and hDHFR cassette after double crossover (DCO) homologous recombination (**B**) *Scot*1 KO parasites showing GFP expression in blood stages. (**C**) Diagnostic PCR to confirm 5′ and 3′ site-specific integration in the genome using primer sets 1225/1169 and 1215/1170 respectively.

3.3.4 *Scot*1 KO parasites propagate normally in asexual blood stages

To determine whether the deletion of *Scot*1 had any effect on the dynamics of blood growth, two groups of mice were intravenously inoculated with WT and *Scot*1 KO parasites. The parasite growth was monitored by making Giemsa-stained blood smears. No difference was observed between WT and *Scot*1 KO parasites (Figure 24).

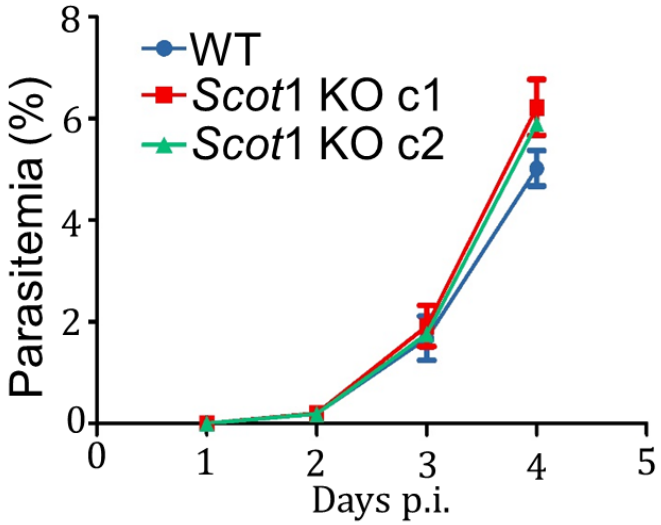


Figure 24. Blood stage growth for *Scot*1 and WT parasites. (no difference, p=0.9980)

3.3.5 Deletion of *Scot*1 does not affect the *P. berghei* mosquito stage development

For the phenotypic characterisation of the *Scot*1 KO parasite, the mosquito cycle was initiated by infecting *A. Stephensi* mosquitos with *Scot*1 KO or WT parasites. The infected mosquitos were kept in an isolated environmental chamber maintained at a temperature of 19^0C and a relative humidity (RH) of 80%. On day 14 post-infection mosquito midguts were dissected to check for the presence of oocysts which was comparable in both WT and KO parasites (Figure 25 A and B). The sporulation in oocyst and sporozoite numbers were also normal (Figure 25 C and D). Further on day 18 p.i. intact salivary glands were observed under a fluorescent microscope and quantifications of the sporozoite numbers were made by mechanical disruption of the isolated salivary glands and counting the number under haemocytometer. GFP fluorescence and salivary gland sporozoite numbers for *Scot*1 and WT on comparison showed no significant difference (Figure 25 E and F). These results demonstrate that the deletion of *Scot*1 does not affect mosquito stage development.

3.3.6 *Scot*1 deficient sporozoites were unable to initiate blood-stage infection

In order to investigate the infectivity of *Scot*1 KO sporozoites, two sets of C57/BL6 mice were intravenously injected with equal number of salivary gland *Scot*1 KO and WT sporozoites. Giemsa stained blood smears were made to check for the appearance of parasites in blood-stage. The WT injected mice were found to be positive after day 3 post-injection whereas in the case of *Scot*1 KO no blood-stage parasites were observed (Table 9).

Table 9. Infectivity of *Scot*1 KO sporozoites in C57BL/6 mice. *Scot*1 KO parasites showed no PPP. C57BL/6 mice were inoculated with WT GFP or *Scot*1 KO sporozoites by intravenous injection into tail vain. These experiments were repeated with higher doses of sporozoite inoculation with similar results.

Experiment	Parasite	Sporozoite injected/mouse	Mice+/Mice injected	Average PPP (Days)
1	WT	5,000	10/10	3
	*Scot*1 *KO*	5,000	0/15	N/A
2	WT	10,000	10/10	3
	*Scot*1 *KO*	10,000	0/20	N/A
3	WT	40,000	3/3	3
	*Scot*1 *KO*	40,000	0/5	N/A
4	WT	50,000	5/5	3
	*Scot*1 *KO*	50,000	0/10	N/A

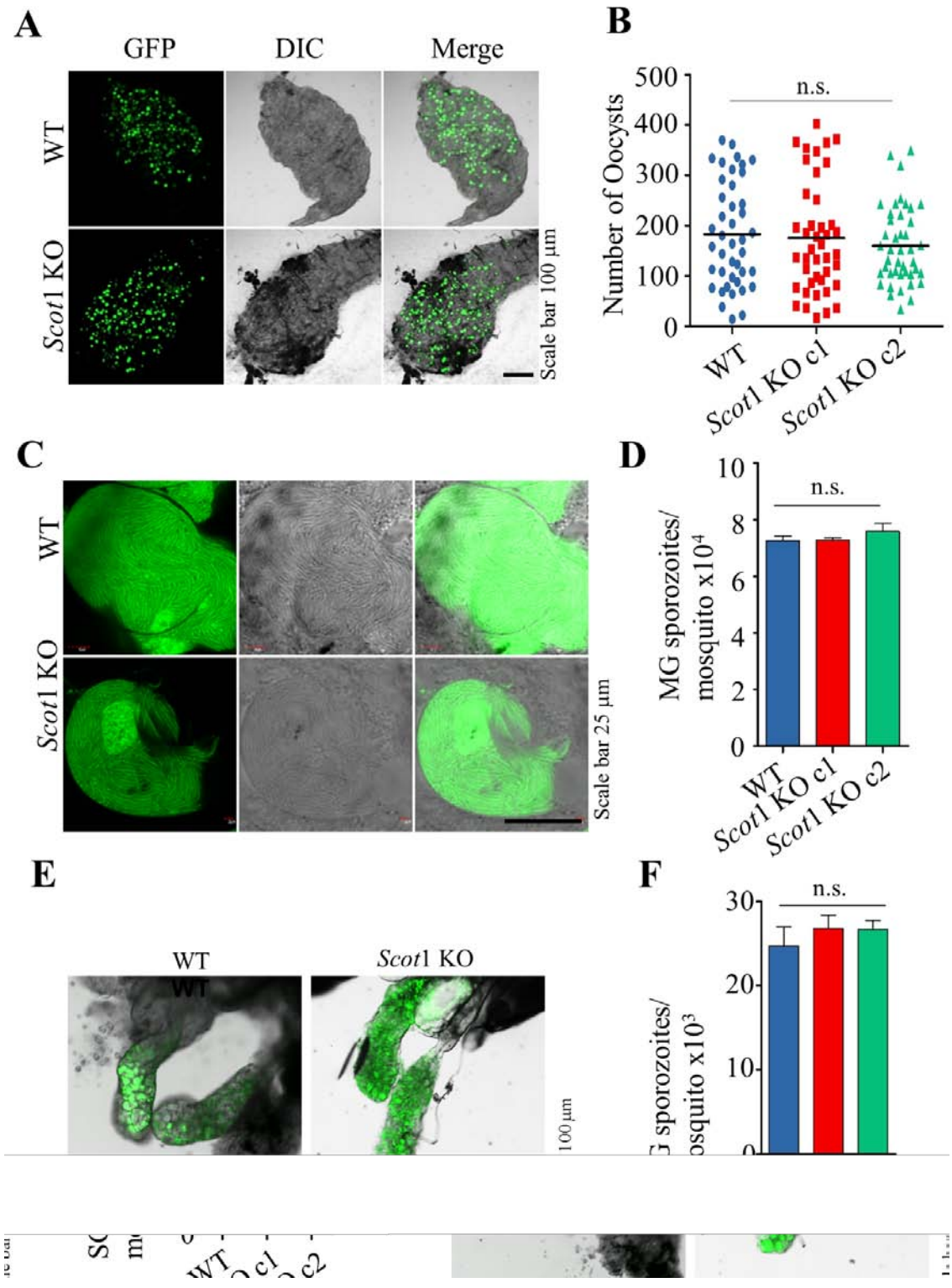


Figure 25. *Scot*1-KO mosquito stages (A) Midgut showing oocysts. (**B**) Oocyst numbers.(no difference, p=0.5482) **(C)** Live microscopy images of sporulating oocysts of WT and *Scot*1 KO. **(D)** Midgut sporozoite numbers. (no difference, p=0.4734) **(E)** Dissected salivary glands. **(F)** Salivary gland sporozoites number. (no difference, p=0.6606)

3.3.7 *Scot*1 KO parasites develop normally in the early exo-erythrocytic forms

The failure of *Scot*1 KO sporozoites to initiate blood stage infection suggests that either they failed to develop into EEFs or egress from hepatocytes. To further investigate liver stage development of KO parasite, HepG2 monolayers were infected with KO and WT sporozoites and fixed at different time points i.e., 24hr, 36hr, 48hr and 62hr. EEFs from 24-48h were stained with UIS4 antibodies and 62h EEFs were stained with MSP1 antibodies to observe the development of merozoites. Various qualitative and quantitative observations were made using these samples such as the presence of Exo-Erythrocytic Forms (EEF's) along with the determination of EEF number and area using NIS elements basic research software on a Nikon fluorescent microscope using 40X magnification for all time points. The results demonstrate completely normal growth of *Scot*1 KO parasites when compared to WT parasites (Figure 26). The EEF numbers were comparable and the evaluation EEF size indicates robust development up until 48hr of liver stage development (Figure 27 A-D).

3.3.8 *Scot*1 KO parasites showed impaired merozoite development

Similar quantifications for the late liver stage time point 62hr EEF's were made which suggests that though the area and size were comparable to the WT but the development of merozoite was impaired (Figure 28 A-C). When compared to the 62hr WT EEF's, *Scot*1 KO EEF's exhibited diminished nuclear division revealed by staining with DAPI (Figure 28 A). Also, a clear abnormality in the MSP1 staining pattern in *Scot*1 KO 62hr EEF's was observed when compared to WT EEF's. To make quantitative estimations from the qualitative observations made by the immunofluorescent slides the nuclei were counted for both *Scot*1 KO and WT 62hr EEF's using the ImageJ software (Figure 28 D). We found a significant reduction in the nuclear division for *Scot*1 KO late liver stage EEF's.

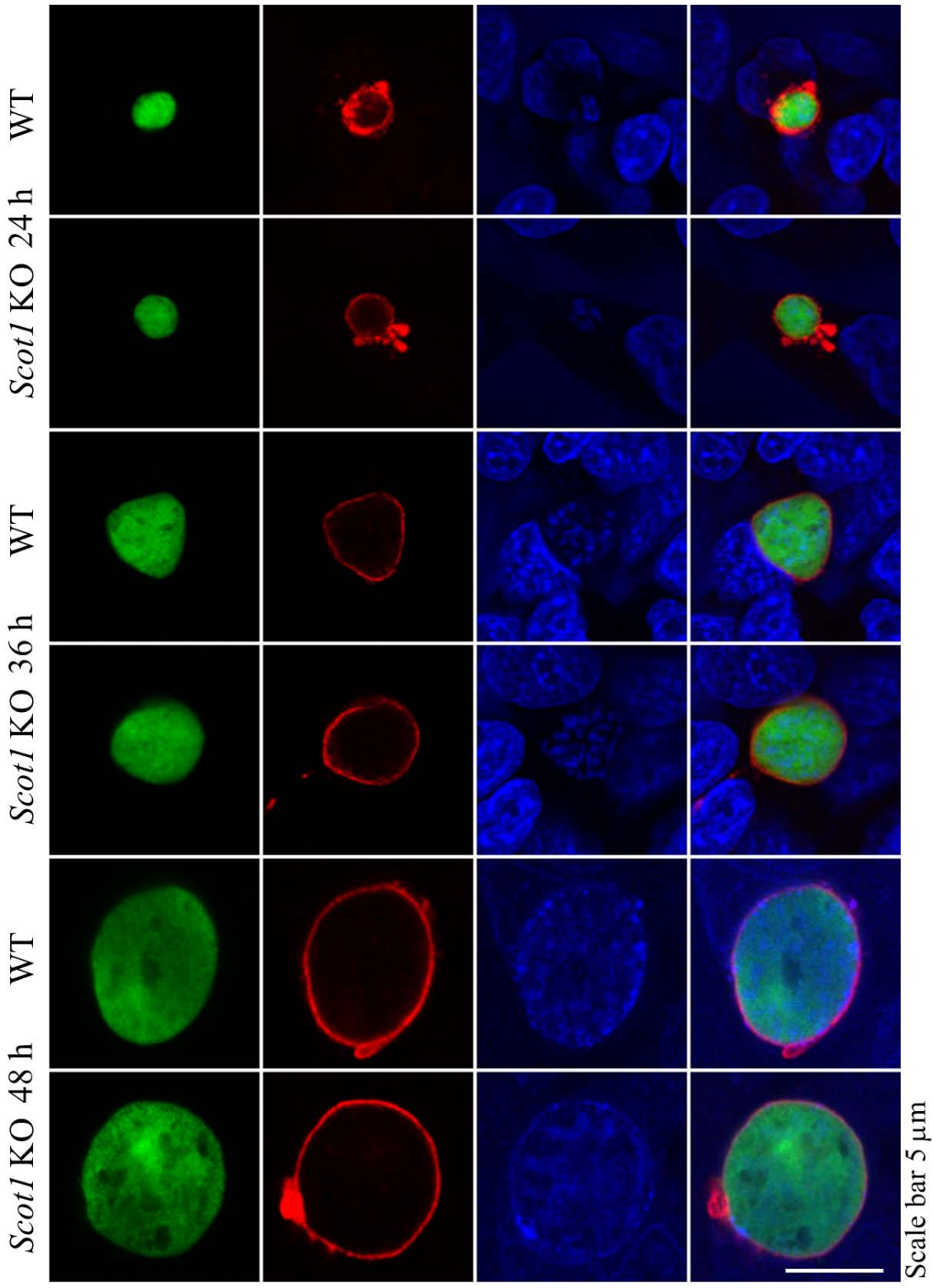


Figure 26. *Scot*1-KO parasites showing normal EEF development. HepG2 cells infected with *Scot*1 KO and WT SG sporozoites were harvested at different time points. Cultures harvested at 24, 36, 48 h p.i. were stained with UIS4 antibody and host and parasite nuclei was stained with Hoechst.

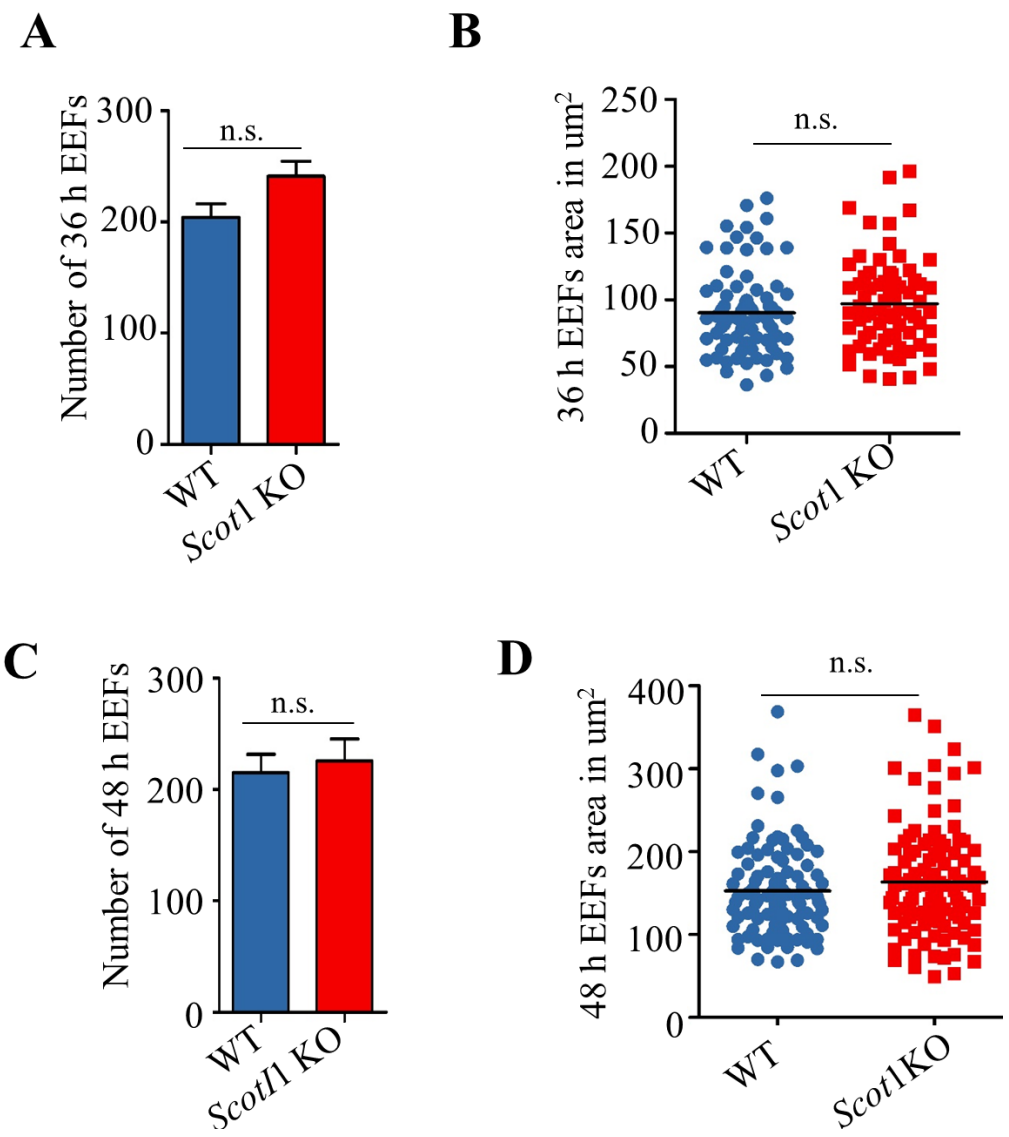


Figure 27. Quantification of EEF numbers and area (A) Number of EEFs at 36 h.p.i (no difference p=0.1167) **(B)** EEF areas at 36 h.p.i (no difference p=0.2064) **(C)** Number of EEFs at 48 h.p.i (no difference p=0.7023) **(D)** EEF areas at 48 h.p.i (no difference p=0.2084)

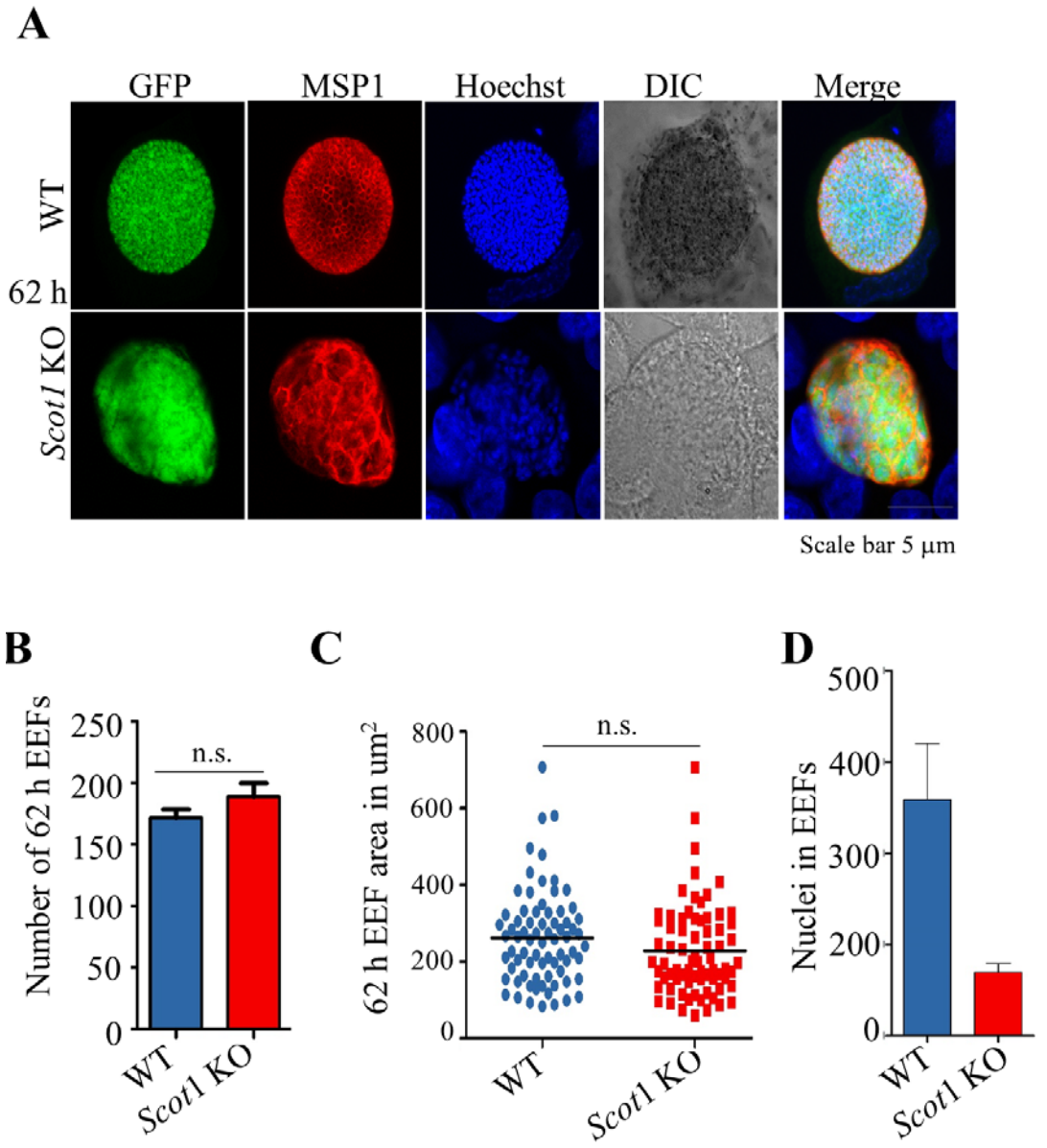


Figure 28. Scot1KO parasites fail to mature into hepatic merozoites. (A) Infected cultures harvested at 62 h p.i. were stained with MSP1 antibody to visualize the development of hepatic merozoites. **(B)** EEF numbers 62h p.i. (no difference, p=0.2506) **(C)** EEF area 62h p.i.(no difference, p=0.0908) **(D)** Nuclei number 62 h p.i. showing a significant decrease in nuclei in *Scot*1 KO parasites (p<0.0001).

3.3.9 Immunization with *Scot*1 KO parasites protected against the WT challenge and also provided cross-stage immunity.

The use of genetically attenuated parasites (GAP) as vaccines has been well established and discussed in detail in chapter1. The above mentioned results establish *Scot*1 KO as a candidate GAP vaccine which attenuates at the late liver stage. UIS gene deletion mutants that have been scrutinized as GAP vaccines, arrested early in

liver stage development they were found to demonstrate protection against WT sporozoite challenge (Mueller *et al.*, 2005a). However late liver stage arresting GAP's could greatly increase and diversify antigen repertoire thereby serving a more robust vaccine candidate. To investigate if the immunization with *Scot*1 KO sporozoites provides sterile protection against WT challenge C57/BL6 mice were i.v. immunized, thrice with varying doses of *Scot*1 KO sporozoites (Table 10) at intervals of 14 days and 28 days post-infection and challenged with WT sporozoites on day 38 post-infection, Giemsa stained thin smears were monitored to check the emergence of blood-stage parasites. As several immunization regimens were followed we found in one of the experiments mice demonstrated breakthrough before even being challenged with WT sporozoites (Exp 1 Table 10) others exhibited complete protection from WT challenge. The data (Table 10) demonstrates that immunization with *Scot*1 KO provides pre-erythrocytic stage protection preventing the onset of blood-stage parasitemia.

Late liver-stage attenuated GAP parasites express proteins that are essential for blood-stage infection and propagation. Hence the late liver stage arresting parasites introduce the prospect of immune priming of blood-stage antigens before the appearance of asexual blood stages. To further check if the immunization with KO sporozoites could protect against blood-stage parasite challenge.

C57/BL6 mice were i.v. immunized thrice with 10,000 *Scot*1 KO sporozoites following a similar regimen as described earlier. These mice were challenged with 50 WT infected RBCs on day 10 following the last immunization. The parasitemia was monitored by Giemsa stained blood smears from day 5 to day 8 post-challenge. The results indicate a reduction of 53% in parasite burden in comparison to WT control mice (Figure 29). The results indicate that immunization with *Scot*1 KO sporozoites provides protection from both pre-erythrocytic and blood-stage challenge.

Table 10. *P.berghei Scot*1 KO immunization regimen and protection from WT challenge. The immunization doses indicate the number of salivary gland sporozoites injected intravenously in respective groups of C57/BL6 mice. Number of WT sporozoites used to challenge. Number of mice monitored in each group and the number of days in which patent.

Exp	Group	Immunization dose	Challenge Dose (Day 10)	No. of patent/no. of challenged	Day patent
1	Control	(3x) Mock	10^4	5/5	3
	*Scot*1 KO	(3x) 10^4 *Scot*1 KO	10^4	1/5	5.5
2	Control	(3x) Mock	$5X10^3$	5/5	3
	*Scot*1 KO	(3x) 10^4 *Scot*1 KO	$5X10^3$	0/5	NA
3	Control	(3x) Mock	10^3	5/5	3
	*Scot*1 KO	(3x) 10^4 *Scot*1 KO	10^3	0/5	NA
4	Control	(3x) Mock	$5X10^3$	5/5	3
	*Scot*1 KO	(3x) $2X10^4$ *Scot*1 KO	$5X10^3$	0/5	NA

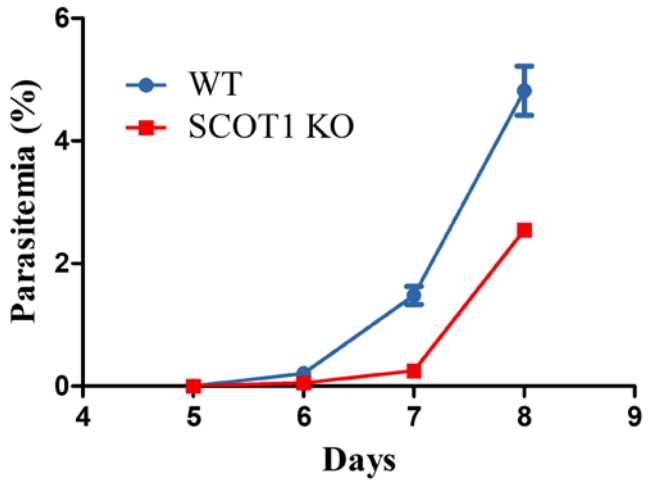


Figure 29. Immunization of C57BL/6 with *Scot*1 KO sporozoites elicit cross-stage immunity. After immunization with *Scot*1 KO sporozoites, mice were challenged with 50 iRBCs and parasitemia was monitored by Giemsa stained blood smears. Approximately 53% reduction in parasitemia was observed ($P<0.05$) compared to naive mice.

3.3.10 RNA-Sequencing revealed changes in global gene expression in *Scot*1 KO parasites

Immunofluorescence assay of exoerythrocytic forms (EEFs) revealed that in vitro development of *Scot*1 KO were similar to WT GFP up to 48 h p.i. However, merozoite formation in *Scot*1 KO parasites was severely compromised both in vitro and in vivo. In order, to determine any changes in global gene expression in both the host and the parasites, HepG2 cells were infected with WT GFP or *Scot*1 KO sporozoites and 55 h p.i. culture was harvested and GFP expressing infected cells were sorted by FACS (Figure 30). RNA was extracted from both the samples and subjected to RNA sequencing.

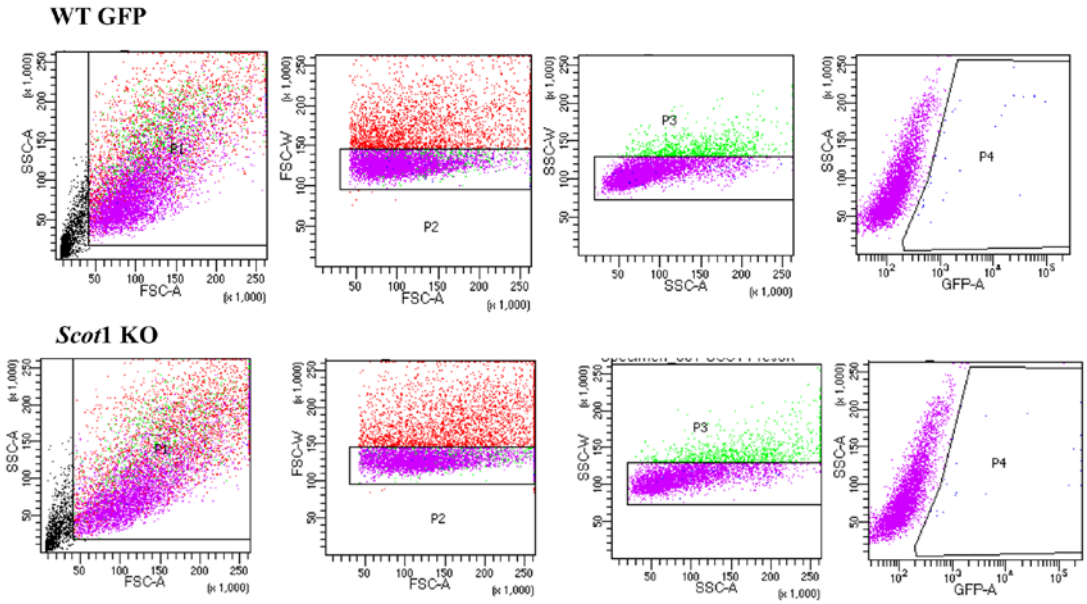
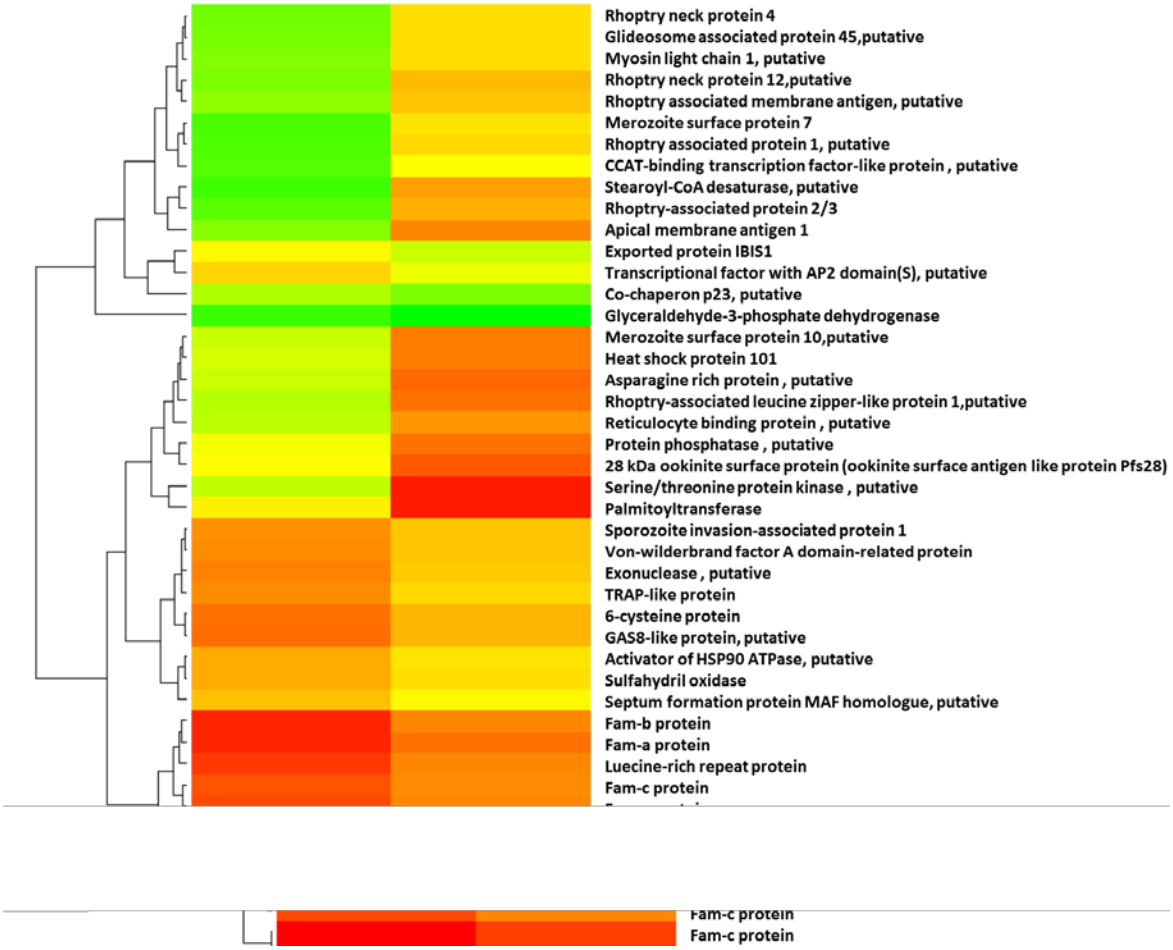


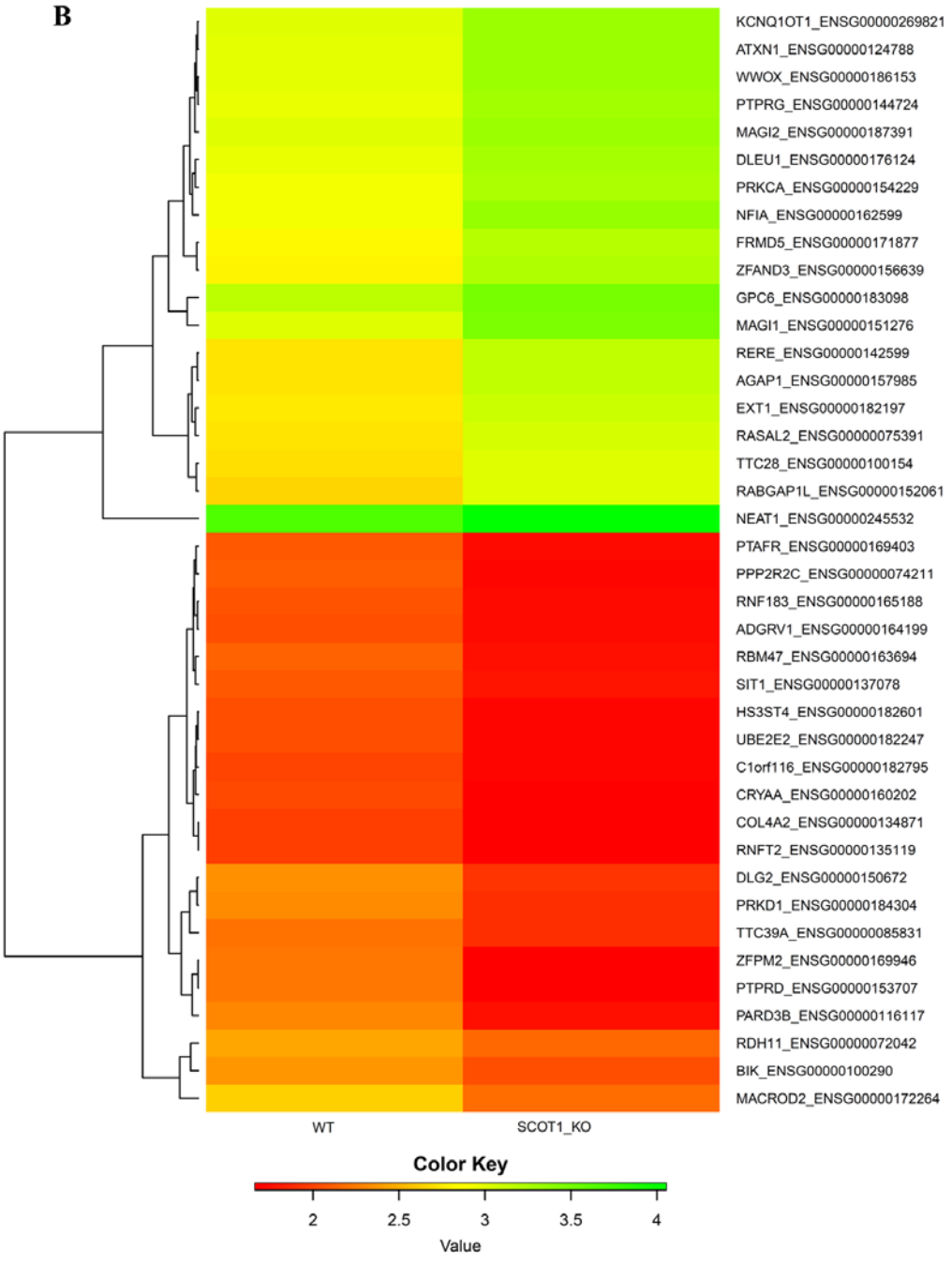
Figure 30. FACS analysis of GFP expressing parasites

The total reads obtained were aligned to the *P. berghei* reference genome and the human reference genome. Total 4,773 parasite genes and 21,512 host genes were identified out of which 209 parasite genes were found to be upregulated, 684 downregulated, 3,880 neutrals, 82 genes found only in WT GFP and 10 genes were found only in *Scot*1 KO parasites. In the case of host 1186 genes were upregulated, 3,247 downregulated, 17,079 neutrals, 7467 found only in WT GFP and 1588 were found only in *Scot*1 KO parasites infected cells. The heat maps of top 20 upregulated and downregulated genes along with major pathways affected of parasite and host are

shown in Figure 31 A, B, C and D. Amongst downregulated genes several have roles merozoite maturation and invasion (MSP7, AMA1, GAP45 etc), though diverse pathways are affected in parasite including orgenellar biogenesis and maintainance, metabolism, cellular interaction, adhesion trafficking etc. Similarly several host pathways also exhibit altered expression which include signaling, cell cycle, immune response, transcription etc.

A




B
KCNQ1OT1_ENSG00000269821
ATXN1_ENSG00000124788
WWOX_ENSG00000186153
PTPRG_ENSG00000144724
MAGI2_ENSG00000187391
DLEU1_ENSG00000176124
PRKCA_ENSG00000154229
NFIA_ENSG00000162599
FRMD5_ENSG00000171877
ZFAND3_ENSG00000156639
GPC6_ENSG00000183098
MAGI1_ENSG00000151276
RERE_ENSG00000142599
AGAP1_ENSG00000157985
EXT1_ENSG00000182197
RASAL2_ENSG00000075391
TTC28_ENSG00000100154
RABGAP1L_ENSG00000152061
NEAT1_ENSG00000245532
PTAFR_ENSG00000169403
PPP2R2C_ENSG00000074211
RNF183_ENSG00000165188
ADGRV1_ENSG00000164199
RBM47_ENSG00000163694
SIT1_ENSG00000137078
HS3ST4_ENSG00000182601
UBE2E2_ENSG00000182247
C1orf116_ENSG00000182795
CRYAA_ENSG00000160202
COL4A2_ENSG00000134871
RNFT2_ENSG00000135119
DLG2_ENSG00000150672
PRKD1_ENSG00000184304
TTC39A_ENSG00000085831
ZFPM2_ENSG00000169946
PTPRD_ENSG00000153707
PARD3B_ENSG00000116117
RDH11_ENSG00000072042
BIK_ENSG00000100290
MACROD2_ENSG00000172264
WT
SCOT1_KO
Color Key
2
2.5
3
3.5
4
Value

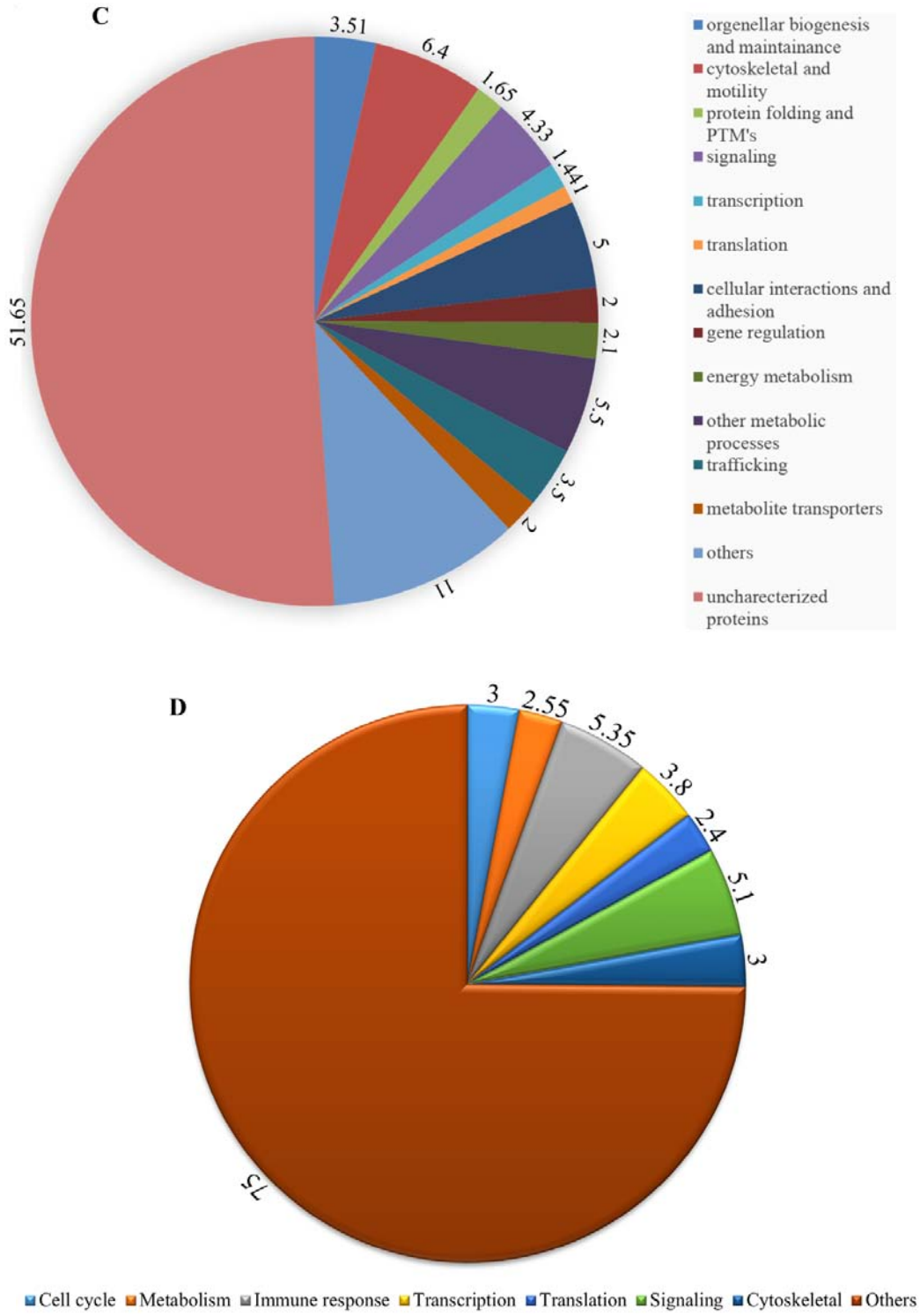


Figure 31 RNA seq analysis of EEFs 55 h.p.i (A) Heat maps of parasite genes showing altered expression globally in *Scot*1 KO compared to WT **(B)** Heat map of host genes with altered expression **(C)** Pie diagram showing the pathways (%) affected in parasite due to *Scot*1 deletion **(D)** Pie diagram showing the host pathways (%) affected due to *Scot*1 deletion

3.4 Discussion

Sporozoite active invasion of the hepatocyte forming a PV membrane marks the beginning of the parasite development in mammalian hosts; the PV membrane provides a niche for sporozoites metamorphosis into EEF. Though the pre-erythrocytic stages are acyclic and pathologically silent they act as a staging point for the initiation of the asexual blood stages additionally the pre-erythrocytic stages also present a bottleneck in parasite life cycle. Hence, therapeutic targeting of the pre-erythrocytic stages would ensure the abolition of blood stage onset preventing any clinical manifestations of the disease. Existing high throughput data such as transcriptomic and differential expression studies have laid the foundation for in depth investigation of putative gene candidates identified previously by employing reverse genetic approaches and have led to the characterization of several essential pre-erythrocytic genes and biological pathways. In this study we investigate the role of PBANKA_0141100, an orthologue of a *P. vivax* gene which was amongst the highest scored transcripts in a systems biology based transcriptomic study (Westenberger *et al.*, 2010).

The targeted deletion of *Scot*1 had no manifestations in the mosquito stages of the parasite life cycle. We observed robust infection in the mosquito midgut and oocyst formation was normal indicating that *Scot*1 has no role in ookinete stage and oocyst formation which is consistent with the previous study which indicate *Scot*1 upregulation in the sporozoite stage (Westenberger *et al.*, 2010). Sporozoite invasion of the mosquito salivary glands and infection of the hepatocytes was normal. Though *Scot*1-3XHA-mCherry transgenic parasite demonstrated mCherry reporter expression in the salivary gland sporozoite as previously reported, KO data suggests lack of *Scot*1 expression does not affect sporozoite gliding or hepatocyte infectivity. Early liver stage development of *Scot*1 KO parasites was completely normal and EEF numbers and size were comparable to WT EEFs. SCOT1 mCherry expression was seen in early liver stages (24 hrs) and localized in the EEF cytoplasm. Contrastingly *Scot*1 deletion exerted its effects in the late liver stage development. The late liver stage parasite exhibited aberrant schizogony as *Scot*1 KO parasites failed to initiate blood stage infection. Genes form diverse pathways have been shown to exhibit similar phenotype such as the enzymes of the FAS II pathway for fatty acid

biosynthesis are essential for the late liver stages but not in the blood stages (Vaughan *et al.*, 2009, Stanway *et al.*, 2019). A major difference is FAS II pathway mutants display complete abrogation of MSP1 expression and cytokinesis (Annoura *et al.*, 2012, Yu *et al.*, 2008) which is not the case with *Scot*1 KO.

*Scot*1 KO parasites demonstrated a severe defect in nuclear division similar phenotype was observed in *PlasMei2* knock out parasites which is an RNA binding protein. *PlasMei2* shows granular cytoplasmic localization similar to *Scot*1 but only expresses in the late stage shizogony and *PlasMei2* knock out liver stage schizonts had atypical DNA segregation and abolition of merozoite formation (Dankwa *et al.*, 2016). Though *Scot*1 and *PlasMei2* deletions have similar effects on *Plasmodium* liver stages they vary in there temporal expression in liver stages. *P. falciparum* gene LSA-1 knockouts also exhibited very similar phenotype, with severe defects in late liver stage development and compromised exo-erythrocytic merozoite formation (Mikolajczak *et al.*, 2011). Several, studies in the last decade have been helpful in understanding *Plasmodium* liver stage development other than the genes mentioned above there are several others from diverse metabolic pathways which lead to late liver stage developmental arrest of EEF's which include apicoplast localised *Lip-B* (lipoic acid protein ligase) (Falkard *et al.*, 2013) and *P. yoelii* glycerol 3-phosphate dehydrogenase and glycerol 3-phosphate acyltransferase (Lindner *et al.*, 2014) deletion of both affects merozoite formation. *LISP2* an exported protein involved in interaction with host proteins is also essential for merozoite development (Orito *et al.*, 2013). Enumeration of these pathways all of which affect development in late liver stages emphasizes on the drastic metabolic requirements for the process of merozoite development to occur successfully.

As *Scot*1 KO parasite exhibited no breakthrough to blood stages even at high doses we attempted to evaluate their potential as genetically attenuated parasite vaccine (GAP) using C57BL/6 mice which are a susceptible rodent model for *P. berghei*. We found that *Scot*1 KO conferred complete protection form WT sporozoite challenge. Immunization with sporozoites has been found to elicit CD8 T-cell IFN-gamma response and sporozoite specific antibodies during pre-erythrocytic stage development (Schofield *et al.*, 1987, Nardin *et al.*, 1999, Purcell *et al.*, 2008, Kumar *et al.*, 2009). The late liver stage arresting parasites express several blood stage proteins and could

potentially elicit immune response against both pre-erythrocytic and erythrocytic stages of infection (Nahrendorf *et al.*, 2015). Immunization with GAP parasites provide both B and T cell responses essential for stage transcending protection also the anti-erythrocytic response is found to be dominated by F_c- mediated response such as complement fixation (Sack *et al.*, 2015). In the same study they also found that the antibody response toward the erythrocytic stages were not dominated towards MSP-1 which is the major merozoite surface protein elucidating that the response could be targeted against shared antigens for both stages. The challenge of immunized mice with infected RBC showed 53% reduction in parasite growth indicating *Scot*1 KO parasites ability to elicit cross stage immunity. To generate whole parasite vaccines, double and triple knockouts have been generated to prevent any possible break-through infection (Mikolajczak *et al.*, 2014, Labaied *et al.*, 2007). Recent studies have also demonstrated that immunization with double knockout parasites generates immune responses against pre-erythrocytic and erythrocytic stages in inbred and outbred mice (Vaughan *et al.*, 2018).

In order to evaluate possible transcriptional changes which could have led to parasite attenuation, *Scot*1 KO and WT liver stage late liver stages (55 h.p.i) were used for RNA sequencing. Analysis of the generated data suggests major differences in gene expression in *Scot*1 KO compared to WT. SCOT1 protein is devoid of any nucleic acid binding domains, hence it is improbable for SCOT1 to act as a transcriptional factor or RNA binding protein similar to *PlasMei2* and DOZI or AP2. Deletion for these genes leads to the downregulation of several transcripts (Mair *et al.*, 2006, Balaji *et al.*, 2005). But we observed significant changes in several crucial pathways in both host and parasite. In parasite these include transcripts involve in motility and merozoite maturation, other prominently affected pathways included those responsible for organellar biogenesis, parasite metabolism related genes and cell signaling etc. Similarly host biological pathways such as cell signaling, transcription, metabolism and immune response related expression profiles were also severely affected. Even though *Scot*1 expresses early in liver stage, the effect of gene deletion results in late stage attenuation. Our investigations demonstrate *Scot*1 is essential for liver stage development and could be used as a potential GAP vaccine.

Chapter 4
Summary

4 Summary

Malaria remains a major threat to huge population in tropical regions of the world. Resistance to available drugs could lead to increase in mortality and morbidity. The parasite asexual blood stages have been studied in detail over time because these stages elicit disease symptoms. Hence initial research was completely focused in unravelling the mysteries of asexual blood stage associated biological pathways aimed at designing methods for chemotherapeutic interventions and vaccine to counter disease pathology. The mosquito and the pre-erythrocytic stages however remained largely neglected. The pre-erythrocytic stages specifically which are responsible for the establishment of infection in host provides a bottleneck in the parasite life cycle. Targeting these stages would also provide the advantage of prohibiting the blood stage cycle automatically preventing onset of disease symptoms. Over the recent decade considerable research effort has been dedicated to the mosquito and pre-erythrocytic stages. The advent of high throughput techniques has aided in the discovery of novel genes and comparative analysis using bio-informatic tools helped immensely in annotating these genes. While transcriptomic experiments generate considerable amount of data, the validation of this data requires biochemical and reverse genetic approaches preceded by careful curation of the existing data for characterization of the predicted genes. The work done in this thesis focuses on investigating the role of genes *S*14 and *Scot*1 in *Plasmodium* life cycle using reverse genetic approaches in order to develop novel therapeutics and uncovering there role in parasite biology.

The malaria parasite life cycle alternates between sessile replicative and motile invasive stages the sessile stages undergo rapid cell division giving rise to thousands of motile progeny which invade and infect target cells. The mosquito midguts harbor parasite oocysts which are responsible for the production of sporozoites. The fate of sporozoites is to infect mammalian liver and transform into EEF. In order to achieve this target the sporozoite undergoes a long and arduous journey from oocyst to hepatocyte encountering numerous cells on the way. The sporozoite use a specific type of substrate dependent motility known as gliding motility. We characterized *S*14 and found that it maximally expressed in the mosquito stages of the parasite life cycle. Tagging of *S*14 with mCherry revealed its expression in sporozoites which localizes

on the inner-membrane of the *Plasmodium* sporozoite. We generated *S*14 KO using double cross over (DCO) homologous recombination. The *S*14 KO parasites exhibited proper development in blood stage and mosquito midgut oocyst but did not invade salivary glands. We enumerated hemolymph sporozoite numbers and found that sporozoites egress from oocysts normally. *S*14 KO hemocoel sporozoites failed to initiate blood stage infection though they were developmentally normal, it was due to inability of *S*14 KO sporozoites to invade hepatocytes. Deletion of *S*14 led to the abolition of sporozoite gliding motility.

We next investigated the role of *Scot*1 in the parasite life cycle. *Scot*1-3XHA-mCherry was generated similarly to check the localization in the parasite. We found that *Scot*1 expressed in sporozoite and early liver stages. We generated *Scot*1 KO parasite line using double crossover homologus recombination. The *Scot*1 KO parasites exhibited no defects in the mosquito stages. On intravenous administration of *Scot*1 KO and WT sporozoites to C57BL/6 mice *Scot*1 KO parasites failed to initiate blood stage infection even at high doses. To investigate this further we infected HepG2 cells with *Scot*1 KO and WT sporozoites. *Scot*1 KO parasites were normal in early lever stage development but nuclear division and merozoite formation was impared. We observed immunization with *Scot*1 KO provided sterile protection against WT sporozoite challenge. We found immunization with *Scot*1 KO sporozoites could provide stage transcending protection, challenging immunized mice with infected RBC there was 53% reduction in parasitemia compared to WT. In an attempt to understand the underlying mechanisms of liver stage attenuation RNA seq for HepG2 cells infected with *Scot*1 KO and WT sporozoites was performed. 55 h.p.i infected HepG2 cells were sorted using fluorescent associated cell sorting of and RNA sequencing was performed. The RNA seq data revealed significant changes in expression of several genes in both host and parasite compared to WT. Pathways crucial to cellular homeostasis were affected such as signaling, various metabolic pathways, most importantly several genes essential for merozoite formation were affected which include GAP45, AMA1 which are essential for merozoite invasion machinery. Hence in our study we have established *Scot*1 gene is essential for merozoite formation and maturation and can serve as robust GAP vaccine target.

References

References

Adini, A., and Warburg, A. (1999) Interaction of Plasmodium gallinaceum ookinetes and oocysts with extracellular matrix proteins. *Parasitology* **119 (Pt 4)**: 331-336.

Aikawa, M., Torii, M., Sjolander, A., Berzins, K., Perlmann, P., and Miller, L.H. (1990) Pf155/RESA antigen is localized in dense granules of Plasmodium falciparum merozoites. *Exp Parasitol* **71**: 326-329.

Al-Nihmi, F.M., Kolli, S.K., Reddy, S.R., Mastan, B.S., Togiri, J., Maruthi, M., Gupta, R., Sijwali, P.S., Mishra, S., and Kumar, K.A. (2017) A Novel and Conserved Plasmodium Sporozoite Membrane Protein SPELD is Required for Maturation of Exo-erythrocytic Forms. *Sci Rep* **7**: 40407.

Al-Olayan, E.M., Beetsma, A.L., Butcher, G.A., Sinden, R.E., and Hurd, H. (2002) Complete development of mosquito phases of the malaria parasite in vitro. *Science* **295**: 677-679.

Aly, A.S., and Matuschewski, K. (2005) A malarial cysteine protease is necessary for Plasmodium sporozoite egress from oocysts. *J Exp Med* **202**: 225-230.

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

Angrisano, F., Sala, K.A., Da, D.F., Liu, Y., Pei, J., Grishin, N.V., Snell, W.J., and Blagborough, A.M. (2017) Targeting the Conserved Fusion Loop of HAP2 Inhibits the Transmission of Plasmodium berghei and falciparum. *Cell Rep* **21**: 2868-2878.

Annoura, T., Ploemen, I.H., van Schaijk, B.C., Sajid, M., Vos, M.W., van Gemert, G.J., Chevalley-Maurel, S., Franke-Fayard, B.M., Hermsen, C.C., Gego, A., Franetich, J.F., Mazier, D., Hoffman, S.L., Janse, C.J., Sauerwein, R.W., and Khan, S.M. (2012) Assessing the adequacy of attenuation of genetically modified malaria parasite vaccine candidates. *Vaccine* **30**: 2662-2670.

Aravind, L., Iyer, L.M., Wellems, T.E., and Miller, L.H. (2003) Plasmodium biology: genomic gleanings. *Cell* **115**: 771-785.

Arnot, D.E., and Gull, K. (1998) The Plasmodium cell-cycle: facts and questions. *Ann Trop Med Parasitol* **92**: 361-365.

Arredondo, S.A., Cai, M., Takayama, Y., MacDonald, N.J., Anderson, D.E., Aravind, L., Clore, G.M., and Miller, L.H. (2012) Structure of the Plasmodium 6-cysteine s48/45 domain. *Proc Natl Acad Sci U S A* **109**: 6692-6697.

Baer, K., Klotz, C., Kappe, S.H., Schnieder, T., and Frevert, U. (2007) Release of hepatic Plasmodium yoelii merozoites into the pulmonary microvasculature. *PLoS Pathog* **3**: e171.

Balaji, S., Babu, M.M., Iyer, L.M., and Aravind, L. (2005) Discovery of the principal specific transcription factors of Apicomplexa and their implication for the evolution of the AP2-integrase DNA binding domains. *Nucleic Acids Res* **33**: 3994-4006.

Bannister, L., and Mitchell, G. (2003) The ins, outs and roundabouts of malaria. *Trends Parasitol* **19**: 209-213.

Bannister, L.H., Butcher, G.A., Dennis, E.D., and Mitchell, G.H. (1975) Structure and invasive behaviour of Plasmodium knowlesi merozoites in vitro. *Parasitology* **71**: 483-491.

Bannister, L.H., Hopkins, J.M., Fowler, R.E., Krishna, S., and Mitchell, G.H. (2000) A brief illustrated guide to the ultrastructure of Plasmodium falciparum asexual blood stages. *Parasitol Today* **16**: 427-433.

Bano, N., Romano, J.D., Jayabalasingham, B., and Coppens, I. (2007) Cellular interactions of Plasmodium liver stage with its host mammalian cell. *Int J Parasitol* **37**: 1329-1341.

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

Beier, J.C. (1998) Malaria parasite development in mosquitoes. *Annu Rev Entomol* **43**: 519-543.

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

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

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

Bongfen, S.E., Torgler, R., Romero, J.F., Renia, L., and Corradin, G. (2007) Plasmodium berghei-infected primary hepatocytes process and present the

circumsporozoite protein to specific CD8+ T cells in vitro. *J Immunol* **178**: 7054-7063.

Bookwalter, C.S., Kelsen, A., Leung, J.M., Ward, G.E., and Trybus, K.M. (2014) A Toxoplasma gondii class XIV myosin, expressed in Sf9 cells with a parasite co-chaperone, requires two light chains for fast motility. *J Biol Chem* **289**: 30832-30841.

Bosch, J., Turley, S., Roach, C.M., Daly, T.M., Bergman, L.W., and Hol, W.G. (2007) The closed MTIP-myosin A-tail complex from the malaria parasite invasion machinery. *J Mol Biol* **372**: 77-88.

Brier, B. (2004) Infectious diseases in ancient Egypt. *Infect Dis Clin North Am* **18**: 17-27.

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

Burda, P.C., Caldelari, R., and Heussler, V.T. (2017) Manipulation of the Host Cell Membrane during Plasmodium Liver Stage Egress. *mBio* **8(2). pii**: mBio.

Butcher, G.A., Sinden, R.E., and Billker, O. (1996) Plasmodium berghei: infectivity of mice to Anopheles stephensi mosquitoes. *Exp Parasitol* **84**: 371-379.

Carlton, J.M., and Carucci, D.J., (2002) Rodent models of malaria in the genomics era. In: Trends Parasitol. England, pp. 100-102.

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

Carter, V., Shimizu, S., Arai, M., and Dessens, J.T. (2008) PbSR is synthesized in macrogametocytes and involved in formation of the malaria crystalloids. *Mol Microbiol* **68**: 1560-1569.

Cerami, C., Frevert, U., Sinnis, P., Takacs, B., Clavijo, P., Santos, M.J., and Nussenzweig, V. (1992) The basolateral domain of the hepatocyte plasma membrane bears receptors for the circumsporozoite protein of Plasmodium falciparum sporozoites. *Cell* **70**: 1021-1033.

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

Claudianos, C., Dessens, J.T., Trueman, H.E., Arai, M., Mendoza, J., Butcher, G.A., Crompton, T., and Sinden, R.E. (2002) A malaria scavenger receptor-like protein essential for parasite development. *Mol Microbiol* **45**: 1473-1484.

Coatney, G.R. (1968) Simian malarias in man: facts, implications, and predictions. *Am J Trop Med Hyg* **17**: 147-155.

Cogswell, F.B. (1992) The hypnozoite and relapse in primate malaria. *Clin Microbiol Rev* **5**: 26-35.

Collins, K.A., Snaith, R., Cottingham, M.G., Gilbert, S.C., and Hill, A.V.S. (2017) Enhancing protective immunity to malaria with a highly immunogenic virus-like particle vaccine. *Sci Rep* **7**: 46621.

Combe, A., Moreira, C., Ackerman, S., Thiberge, S., Templeton, T.J., and Menard, R. (2009) TREP, a novel protein necessary for gliding motility of the malaria sporozoite. *Int J Parasitol* **39**: 489-496.

Coppens, I. (2011) Metamorphoses of malaria: the role of autophagy in parasite differentiation. *Essays Biochem* **51**: 127-136.

Coppi, A., Natarajan, R., Pradel, G., Bennett, B.L., James, E.R., Roggero, M.A., Corradin, G., Persson, C., Tewari, R., and Sinnis, P. (2011) The malaria circumsporozoite protein has two functional domains, each with distinct roles as sporozoites journey from mosquito to mammalian host. *J Exp Med* **208**: 341-356.

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

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

Cowman, A.F., Berry, D., and Baum, J. (2012) The cellular and molecular basis for malaria parasite invasion of the human red blood cell. *J Cell Biol* **198**: 961-971.

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

Cox-Singh, J., Davis, T.M., Lee, K.S., Shamsul, S.S., Matusop, A., Ratnam, S., Rahman, H.A., Conway, D.J., and Singh, B. (2008) Plasmodium knowlesi malaria in humans is widely distributed and potentially life threatening. *Clin Infect Dis* **46**: 165-171.

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

Dadimoghaddam, Y., Daryani, A., Sharif, M., Ahmadpour, E., and Hossienikhah, Z. (2014) Tissue tropism and parasite burden of Toxoplasma gondii RH strain in experimentally infected mice. *Asian Pac J Trop Med* **7**: 521-524.

Dankwa, D.A., Davis, M.J., Kappe, S.H.I., and Vaughan, A.M. (2016) A Plasmodium yoelii Mei2-Like RNA Binding Protein Is Essential for Completion of Liver Stage Schizogony. *Infect Immun* **84**: 1336-1345.

Dawn, A., Singh, S., More, K.R., Siddiqui, F.A., Pachikara, N., Ramdani, G., Langsley, G., and Chitnis, C.E. (2014) The central role of cAMP in regulating Plasmodium falciparum merozoite invasion of human erythrocytes. *PLoS Pathog* **10**: e1004520.

De Niz, M., Burda, P.C., Kaiser, G., Del Portillo, H.A., Spielmann, T., Frischknecht, F., and Heussler, V.T. (2017) Progress in imaging methods: insights gained into Plasmodium biology. *Nat Rev Microbiol* **15**: 37-54.

De Silva, E.K., Gehrke, A.R., Olszewski, K., Leon, I., Chahal, J.S., Bulyk, M.L., and Llinas, M. (2008) Specific DNA-binding by apicomplexan AP2 transcription factors. *Proc Natl Acad Sci U S A* **105**: 8393-8398.

Dessens, J.T., Beetsma, A.L., Dimopoulos, G., Wengelnik, K., Crisanti, A., Kafatos, F.C., and Sinden, R.E. (1999) CTRP is essential for mosquito infection by malaria ookinetes. *EMBO J* **18**: 6221-6227.

Dessens, J.T., Mendoza, J., Claudianos, C., Vinetz, J.M., Khater, E., Hassard, S., Ranawaka, G.R., and Sinden, R.E. (2001) Knockout of the rodent malaria parasite chitinase pbCHT1 reduces infectivity to mosquitoes. *Infect Immun* **69**: 4041-4047.

Dessens, J.T., Siden-Kiamos, I., Mendoza, J., Mahairaki, V., Khater, E., Vlachou, D., Xu, X.J., Kafatos, F.C., Louis, C., Dimopoulos, G., and Sinden, R.E. (2003) SOAP, a novel malaria ookinete protein involved in mosquito midgut invasion and oocyst development. *Mol Microbiol* **49**: 319-329.

Doi, Y., Shinzawa, N., Fukumoto, S., Okano, H., and Kanuka, H. (2011) Calcium signal regulates temperature-dependent transformation of sporozoites in malaria parasite development. *Exp Parasitol* **128**: 176-180.

Dorin-Semblat, D., Quashie, N., Halbert, J., Sicard, A., Doerig, C., Peat, E., and Ranford-Cartwright, L. (2007) Functional characterization of both MAP kinases of the human malaria parasite Plasmodium falciparum by reverse genetics. *Mol Microbiol* **65**: 1170-1180.

Douglas, A.D., Baldeviano, G.C., Lucas, C.M., Lugo-Roman, L.A., Crosnier, C., Bartholdson, S.J., Diouf, A., Miura, K., Lambert, L.E., Ventocilla, J.A., Leiva, K.P., Milne, K.H., Illingworth, J.J., Spencer, A.J., Hjerrild, K.A., Alanine, D.G., Turner, A.V., Moorhead, J.T., Edgel, K.A., Wu, Y., Long, C.A., Wright, G.J., Lescano, A.G., and Draper, S.J. (2015) A PfRH5-based vaccine is efficacious against heterologous strain blood-stage Plasmodium falciparum infection in aotus monkeys. *Cell Host Microbe* **17**: 130-139.

Draper, S.J., Angov, E., Horii, T., Miller, L.H., Srinivasan, P., Theisen, M., and Biswas, S. (2015) Recent advances in recombinant protein-based malaria vaccines. *Vaccine* **33**: 7433-7443.

Dundas, K., Shears, M.J., Sun, Y., Hopp, C.S., Crosnier, C., Metcalf, T., Girling, G., Sinnis, P., Billker, O., and Wright, G.J. (2018) Alpha-v-containing integrins are host receptors for the Plasmodium falciparum sporozoite surface protein, TRAP. *Proc Natl Acad Sci U S A* **115**: 4477-4482.

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

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

Ejigiri, I., and Sinnis, P. (2009) Plasmodium sporozoite-host interactions from the dermis to the hepatocyte. *Curr Opin Microbiol* **12**: 401-407.

Eksi, S., Czesny, B., van Gemert, G.J., Sauerwein, R.W., Eling, W., and Williamson, K.C. (2006) Malaria transmission-blocking antigen, Pfs230, mediates human red blood cell binding to exflagellating male parasites and oocyst production. *Mol Microbiol* **61**: 991-998.

Engelmann, S., Silvie, O., and Matuschewski, K. (2009) Disruption of Plasmodium sporozoite transmission by depletion of sporozoite invasion-associated protein 1. *Eukaryot Cell* **8**: 640-648.

Epstein, J.E., Tewari, K., Lyke, K.E., Sim, B.K., Billingsley, P.F., Laurens, M.B., Gunasekera, A., Chakravarty, S., James, E.R., Sedegah, M., Richman, A., Velmurugan, S., Reyes, S., Li, M., Tucker, K., Ahumada, A., Ruben, A.J., Li, T., Stafford, R., Eappen, A.G., Tamminga, C., Bennett, J.W., Ockenhouse, C.F., Murphy, J.R., Komisar, J., Thomas, N., Loyevsky, M., Birkett, A., Plowe, C.V., Loucq, C., Edelman, R., Richie, T.L., Seder, R.A., and Hoffman, S.L. (2011) Live attenuated malaria vaccine designed to protect through hepatic CD8(+) T cell immunity. *Science* **334**: 475-480.

Espinosa, D.A., Gutierrez, G.M., Rojas-López, M., Noe, A.R., Shi, L., Tse, S.W., Sinnis, P., and Zavala, F. (2015) Proteolytic Cleavage of the Plasmodium falciparum Circumsporozoite Protein Is a Target of Protective Antibodies. *J Infect Dis* **212**: 1111-1119.

Falkard, B., Kumar, T.R., Hecht, L.S., Matthews, K.A., Henrich, P.P., Gulati, S., Lewis, R.E., Manary, M.J., Winzeler, E.A., Sinnis, P., Prigge, S.T., Heussler, V., Deschermeier, C., and Fidock, D. (2013) A key role for lipoic acid synthesis during Plasmodium liver stage development. *Cell Microbiol* **15**: 1585-1604.

Favretto, F., Assfalg, M., Molinari, H., and D'Onofrio, M. (2013) Evidence from NMR interaction studies challenges the hypothesis of direct lipid transfer from L-FABP to malaria sporozoite protein UIS3. *Protein Sci* **22**: 133-138.

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

Foquet, L., Schafer, C., Minkah, N.K., Alanine, D.G.W., Flannery, E.L., Steel, R.W.J., Sack, B.K., Camargo, N., Fishbaugher, M., Betz, W., Nguyen, T., Billman, Z.P., Wilson, E.M., Bial, J., Murphy, S.C., Draper, S.J., Mikolajczak, S.A., and Kappe, S.H.I. (2018) Plasmodium falciparum Liver Stage Infection and Transition to Stable Blood Stage Infection in Liver-Humanized and Blood-Humanized FRGN KO Mice Enables Testing of Blood Stage Inhibitory Antibodies (Reticulocyte-Binding Protein Homolog 5) In Vivo. *Front Immunol* **9**: 524.

Frevert, U., Sinnis, P., Cerami, C., Shreffler, W., Takacs, B., and Nussenzweig, V. (1993) Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes. *J Exp Med* **177**: 1287-1298.

Frénal, K., Dubremetz, J.F., Lebrun, M., and Soldati-Favre, D. (2017) Gliding motility powers invasion and egress in Apicomplexa. *Nat Rev Microbiol* **15**: 645-660.

Frénal, K., Polonais, V., Marq, J.B., Stratmann, R., Limenitakis, J., and Soldati-Favre, D. (2010) Functional dissection of the apicomplexan glideosome molecular architecture. *Cell Host Microbe* **8**: 343-357.

Galaway, F., Drought, L.G., Fala, M., Cross, N., Kemp, A.C., Rayner, J.C., and Wright, G.J. (2017) P113 is a merozoite surface protein that binds the N terminus of Plasmodium falciparum RH5. *Nat Commun* **8**: 14333.

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

Garnham, P.C. (1966) Comments on biology of human malaria. *Mil Med* **131**: Suppl:961-962.

Ghosh, A.K., Devenport, M., Jethwaney, D., Kalume, D.E., Pandey, A., Anderson, V.E., Sultan, A.A., Kumar, N., and Jacobs-Lorena, M. (2009) Malaria parasite invasion of the mosquito salivary gland requires interaction between the Plasmodium TRAP and the Anopheles saglin proteins. *PLoS Pathog* **5**: e1000265.

Giovannini, D., Spath, S., Lacroix, C., Perazzi, A., Bargieri, D., Lagal, V., Lebugle, C., Combe, A., Thiberge, S., Baldacci, P., Tardieux, I., and Menard, R. (2011) Independent roles of apical membrane antigen 1 and rhoptry neck proteins during host cell invasion by apicomplexa. *Cell Host Microbe* **10**: 591-602.

Gissot, M., Ting, L.M., Daly, T.M., Bergman, L.W., Sinnis, P., and Kim, K. (2008) High mobility group protein HMGB2 is a critical regulator of plasmodium oocyst development. *J Biol Chem* **283**: 17030-17038.

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

Gonzalez, V., Combe, A., David, V., Malmquist, N.A., Delorme, V., Leroy, C., Blazquez, S., Ménard, R., and Tardieux, I. (2009) Host cell entry by apicomplexa parasites requires actin polymerization in the host cell. *Cell Host Microbe* **5**: 259-272.

Grassi, B., and Noe, G. (1900) The Propagation of the Filariae of the Blood Exclusively by means of the Puncture of Peculiar Mosquitos. *Br Med J* **2**: 1306-1307.

Green, J.L., Martin, S.R., Fielden, J., Ksagoni, A., Grainger, M., Yim Lim, B.Y., Molloy, J.E., and Holder, A.A. (2006) The MTIP-myosin A complex in blood stage malaria parasites. *J Mol Biol* **355**: 933-941.

Grützke, J., Rindte, K., Goosmann, C., Silvie, O., Rauch, C., Heuer, D., Lehmann, M.J., Mueller, A.K., Brinkmann, V., Matuschewski, K., and Ingmundson, A. (2014) The spatiotemporal dynamics and membranous features of the Plasmodium liver stage tubovesicular network. *Traffic* **15**: 362-382.

Harding, C.R., Egarter, S., Gow, M., Jiménez-Ruiz, E., Ferguson, D.J., and Meissner, M. (2016) Gliding Associated Proteins Play Essential Roles during the Formation of the Inner Membrane Complex of Toxoplasma gondii. *PLoS Pathog* **12**: e1005403.

Harupa, A., Sack, B.K., Lakshmanan, V., Arang, N., Douglass, A.N., Oliver, B.G., Stuart, A.B., Sather, D.N., Lindner, S.E., Hybiske, K., Torii, M., and Kappe, S.H. (2014) SSP3 is a novel Plasmodium yoelii sporozoite surface protein with a role in gliding motility. *Infect Immun* **82**: 4643-4653.

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

Hegge, S., Kudryashev, M., Barniol, L., and Frischknecht, F. (2010) Key factors regulating Plasmodium berghei sporozoite survival and transformation revealed by an automated visual assay. *FASEB J* **24**: 5003-5012.

Inselburg, J. (1983) Gametocyte formation by the progeny of single Plasmodium falciparum schizonts. *J Parasitol* **69**: 584-591.

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

Ishino, T., Chinzei, Y., and Yuda, M. (2005b) Two proteins with 6-cys motifs are required for malarial parasites to commit to infection of the hepatocyte. *Mol Microbiol* **58**: 1264-1275.

Ishino, T., Orito, Y., Chinzei, Y., and Yuda, M. (2006) A calcium-dependent protein kinase regulates Plasmodium ookinete access to the midgut epithelial cell. *Mol Microbiol* **59**: 1175-1184.

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

Itoe, M.A., Sampaio, J.L., Cabal, G.G., Real, E., Zuzarte-Luis, V., March, S., Bhatia, S.N., Frischknecht, F., Thiele, C., Shevchenko, A., and Mota, M.M. (2014) Host cell phosphatidylcholine is a key mediator of malaria parasite survival during liver stage infection. *Cell Host Microbe* **16**: 778-786.

Janse, C.J., and Waters, A.P. (1995) Plasmodium berghei: the application of cultivation and purification techniques to molecular studies of malaria parasites. *Parasitol Today* **11**: 138-143.

Jensen, J.B., and Trager, W. (1978) Plasmodium falciparum in culture: establishment of additional strains. *Am J Trop Med Hyg* **27**: 743-746.

Jewett, T.J., and Sibley, L.D. (2003) Aldolase forms a bridge between cell surface adhesins and the actin cytoskeleton in apicomplexan parasites. *Mol Cell* **11**: 885-894.

Kaiser, K., Camargo, N., and Kappe, S.H. (2003) Transformation of sporozoites into early exoerythrocytic malaria parasites does not require host cells. *J Exp Med* **197**: 1045-1050.

Kaiser, K., Matuschewski, K., Camargo, N., Ross, J., and Kappe, S.H. (2004) Differential transcriptome profiling identifies Plasmodium genes encoding pre-erythrocytic stage-specific proteins. *Mol Microbiol* **51**: 1221-1232.

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

Kappe, S.H., Gardner, M.J., Brown, S.M., Ross, J., Matuschewski, K., Ribeiro, J.M., Adams, J.H., Quackenbush, J., Cho, J., Carucci, D.J., Hoffman, S.L., and Nussenzweig, V. (2001) Exploring the transcriptome of the malaria sporozoite stage. *Proc Natl Acad Sci U S A* **98**: 9895-9900.

Kappe, S.H., Noe, A.R., Fraser, T.S., Blair, P.L., and Adams, J.H. (1998) A family of chimeric erythrocyte binding proteins of malaria parasites. *Proc Natl Acad Sci U S A* **95**: 1230-1235.

Kapulu, M.C., Da, D.F., Miura, K., Li, Y., Blagborough, A.M., Churcher, T.S., Nikolaeva, D., Williams, A.R., Goodman, A.L., Sangare, I., Turner, A.V., Cottingham, M.G., Nicosia, A., Straschil, U., Tsuboi, T., Gilbert, S.C., Long, C.A., Sinden, R.E., Draper, S.J., Hill, A.V., Cohuet, A., and Biswas, S. (2015) Comparative assessment of transmission-blocking vaccine candidates against Plasmodium falciparum. *Sci Rep* **5**: 11193.

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

Kariu, T., Yuda, M., Yano, K., and Chinzei, Y. (2002) MAEBL is essential for malarial sporozoite infection of the mosquito salivary gland. *J Exp Med* **195**: 1317-1323.

Kaushansky, A., Douglass, A.N., Arang, N., Vigdorovich, V., Dambrauskas, N., Kain, H.S., Austin, L.S., Sather, D.N., and Kappe, S.H. (2015) Malaria

parasites target the hepatocyte receptor EphA2 for successful host infection. *Science* **350**: 1089-1092.

Kaushansky, A., and Kappe, S.H. (2011) The crucial role of hepatocyte growth factor receptor during liver-stage infection is not conserved among Plasmodium species. *Nat Med* **17**: 1180-1181; author reply 1181.

Kaushansky, A., Ye, A.S., Austin, L.S., Mikolajczak, S.A., Vaughan, A.M., Camargo, N., Metzger, P.G., Douglass, A.N., MacBeath, G., and Kappe, S.H. (2013) Suppression of host p53 is critical for Plasmodium liver-stage infection. *Cell Rep* **3**: 630-637.

Kauth, C.W., Epp, C., Bujard, H., and Lutz, R. (2003) The merozoite surface protein 1 complex of human malaria parasite Plasmodium falciparum: interactions and arrangements of subunits. *J Biol Chem* **278**: 22257-22264.

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

Kazmin, D., Nakaya, H.I., Lee, E.K., Johnson, M.J., van der Most, R., van den Berg, R.A., Ballou, W.R., Jongert, E., Wille-Reece, U., Ockenhouse, C., Aderem, A., Zak, D.E., Sadoff, J., Hendriks, J., Wrammert, J., Ahmed, R., and Pulendran, B. (2017) Systems analysis of protective immune responses to RTS,S malaria vaccination in humans. *Proc Natl Acad Sci U S A* **114**: 2425-2430.

Kester, K.E., Cummings, J.F., Ofori-Anyinam, O., Ockenhouse, C.F., Krzych, U., Moris, P., Schwenk, R., Nielsen, R.A., Debebe, Z., Pinelis, E., Juompan, L., Williams, J., Dowler, M., Stewart, V.A., Wirtz, R.A., Dubois, M.C., Lievens, M., Cohen, J., Ballou, W.R., and Heppner, D.G., Jr. (2009) Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. *J Infect Dis* **200**: 337-346.

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

Kisalu, N.K., Idris, A.H., Weidle, C., Flores-Garcia, Y., Flynn, B.J., Sack, B.K., Murphy, S., Schon, A., Freire, E., Francica, J.R., Miller, A.B., Gregory, J., March, S., Liao, H.X., Haynes, B.F., Wiehe, K., Trama, A.M., Saunders, K.O., Gladden, M.A., Monroe, A., Bonsignori, M., Kanekiyo, M., Wheatley, A.K., McDermott, A.B., Farney, S.K., Chuang, G.Y., Zhang, B., Kc, N., Chakravarty, S., Kwong, P.D., Sinnis, P., Bhatia, S.N., Kappe, S.H.I., Sim, B.K.L., Hoffman, S.L., Zavala, F., Pancera, M., and Seder, R.A. (2018) A

human monoclonal antibody prevents malaria infection by targeting a new site of vulnerability on the parasite. *Nat Med* **24**: 408-416.

Kooij, T.W., and Matuschewski, K. (2007) Triggers and tricks of Plasmodium sexual development. *Curr Opin Microbiol* **10**: 547-553.

Krotoski, W.A., Collins, W.E., Bray, R.S., Garnham, P.C., Cogswell, F.B., Gwadz, R.W., Killick-Kendrick, R., Wolf, R., Sinden, R., Koontz, L.C., and Stanfill, P.S. (1982) Demonstration of hypnozoites in sporozoite-transmitted Plasmodium vivax infection. *Am J Trop Med Hyg* **31**: 1291-1293.

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

Kwong, P.D. (2017) What Are the Most Powerful Immunogen Design Vaccine Strategies? A Structural Biologist's Perspective. *Cold Spring Harb Perspect Biol* **9**.

Labaied, M., Camargo, N., and Kappe, S.H. (2007) Depletion of the Plasmodium berghei thrombospondin-related sporozoite protein reveals a role in host cell entry by sporozoites. *Mol Biochem Parasitol* **153**: 158-166.

Lasonder, E., Ishihama, Y., Andersen, J.S., Vermunt, A.M., Pain, A., Sauerwein, R.W., Eling, W.M., Hall, N., Waters, A.P., Stunnenberg, H.G., and Mann, M. (2002) Analysis of the Plasmodium falciparum proteome by high-accuracy mass spectrometry. *Nature* **419**: 537-542.

Lasonder, E., Janse, C.J., van Gemert, G.J., Mair, G.R., Vermunt, A.M., Douradinha, B.G., van Noort, V., Huynen, M.A., Luty, A.J., Kroeze, H., Khan, S.M., Sauerwein, R.W., Waters, A.P., Mann, M., and Stunnenberg, H.G. (2008) Proteomic profiling of Plasmodium sporozoite maturation identifies new proteins essential for parasite development and infectivity. *PLoS Pathog* **4**: e1000195.

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

Le Roch, K.G., Zhou, Y., Blair, P.L., Grainger, M., Moch, J.K., Haynes, J.D., De La Vega, P., Holder, A.A., Batalov, S., Carucci, D.J., and Winzeler, E.A. (2003) Discovery of gene function by expression profiling of the malaria parasite life cycle. *Science* **301**: 1503-1508.

Leiriao, P., Mota, M.M., and Rodriguez, A. (2005) Apoptotic Plasmodium-infected hepatocytes provide antigens to liver dendritic cells. *J Infect Dis* **191**: 1576-1581.

Levy, S., Todd, S.C., and Maecker, H.T. (1998) CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. *Annu Rev Immunol* **16**: 89-109.

Limviroj, W., Yano, K., Yuda, M., Ando, K., and Chinzei, Y. (2002) Immuno-electron microscopic observation of Plasmodium berghei CTRP localization in the midgut of the vector mosquito Anopheles stephensi. *J Parasitol* **88**: 664-672.

Lindner, S.E., Sartain, M.J., Hayes, K., Harupa, A., Moritz, R.L., Kappe, S.H., and Vaughan, A.M. (2014) Enzymes involved in plastid-targeted phosphatidic acid synthesis are essential for Plasmodium yoelii liver-stage development. *Mol Microbiol* **91**: 679-693.

Liu, Y., Tewari, R., Ning, J., Blagborough, A.M., Garbom, S., Pei, J., Grishin, N.V., Steele, R.E., Sinden, R.E., Snell, W.J., and Billker, O. (2008) The conserved plant sterility gene HAP2 functions after attachment of fusogenic membranes in Chlamydomonas and Plasmodium gametes. *Genes Dev* **22**: 1051-1068.

Lobo, C.A., Rodriguez, M., Reid, M., and Lustigman, S. (2003) Glycophorin C is the receptor for the Plasmodium falciparum erythrocyte binding ligand PfEBP-2 (baebl). *Blood* **101**: 4628-4631.

Longley, R.J., Halbroth, B.R., Salman, A.M., Ewer, K.J., Hodgson, S.H., Janse, C.J., Khan, S.M., Hill, A.V., and Spencer, A.J. (2017) Assessment of the Plasmodium falciparum Preerythrocytic Antigen UIS3 as a Potential Candidate for a Malaria Vaccine. *Infect Immun* **85**.

Lopes da Silva, M., Thieleke-Matos, C., Cabrita-Santos, L., Ramalho, J.S., Wavre-Shapton, S.T., Futter, C.E., Barral, D.C., and Seabra, M.C. (2012) The host endocytic pathway is essential for Plasmodium berghei late liver stage development. *Traffic* **13**: 1351-1363.

Mahairaki, V., Voyatzi, T., Sidén-Kiamos, I., and Louis, C. (2005) The Anopheles gambiae gamma1 laminin directly binds the Plasmodium berghei circumsporozoite- and TRAP-related protein (CTRP). *Mol Biochem Parasitol* **140**: 119-121.

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

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

Manson, P. (1900) Experimental Proof of the Mosquitomalaria Theory. *Br Med J* **2**: 949-951.

March, S., Ng, S., Velmurugan, S., Galstian, A., Shan, J., Logan, D.J., Carpenter, A.E., Thomas, D., Sim, B.K., Mota, M.M., Hoffman, S.L., and Bhatia, S.N. (2013) A microscale human liver platform that supports the hepatic stages of Plasmodium falciparum and vivax. *Cell Host Microbe* **14**: 104-115.

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

Meis, J.F., Verhave, J.P., Jap, P.H., Sinden, R.E., and Meuwissen, J.H. (1983) Ultrastructural observations on the infection of rat liver by Plasmodium berghei sporozoites in vivo. *J Protozool* **30**: 361-366.

Meissner, M., Schlüter, D., and Soldati, D. (2002) Role of Toxoplasma gondii myosin A in powering parasite gliding and host cell invasion. *Science* **298**: 837-840.

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

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

Mikolajczak, S.A., Lakshmanan, V., Fishbaugher, M., Camargo, N., Harupa, A., Kaushansky, A., Douglass, A.N., Baldwin, M., Healer, J., O'Neill, M., Phuong, T., Cowman, A., and Kappe, S.H. (2014) A next-generation genetically attenuated Plasmodium falciparum parasite created by triple gene deletion. *Mol Ther* **22**: 1707-1715.

Mikolajczak, S.A., Sacci, J.B., Jr., De La Vega, P., Camargo, N., VanBuskirk, K., Krzych, U., Cao, J., Jacobs-Lorena, M., Cowman, A.F., and Kappe, S.H. (2011) Disruption of the Plasmodium falciparum liver-stage antigen-1 locus causes a differentiation defect in late liver-stage parasites. *Cell Microbiol* **13**: 1250-1260.

Mikolajczak, S.A., Silva-Rivera, H., Peng, X., Tarun, A.S., Camargo, N., Jacobs-Lorena, V., Daly, T.M., Bergman, L.W., de la Vega, P., Williams, J., Aly, A.S., and Kappe, S.H. (2008) Distinct malaria parasite sporozoites reveal transcriptional changes that cause differential tissue infection competence in the mosquito vector and mammalian host. *Mol Cell Biol* **28**: 6196-6207.

Mitchell, G.H., Thomas, A.W., Margos, G., Dluzewski, A.R., and Bannister, L.H. (2004) Apical membrane antigen 1, a major malaria vaccine candidate,

mediates the close attachment of invasive merozoites to host red blood cells. *Infect Immun* **72**: 154-158.

Molina-Cruz, A., Garver, L.S., Alabaster, A., Bangiolo, L., Haile, A., Winikor, J., Ortega, C., van Schaijk, B.C., Sauerwein, R.W., Taylor-Salmon, E., and Barillas-Mury, C. (2013) The human malaria parasite Pfs47 gene mediates evasion of the mosquito immune system. *Science* **340**: 984-987.

Montagna, G.N., Beigier-Bompadre, M., Becker, M., Kroczek, R.A., Kaufmann, S.H., and Matuschewski, K. (2014) Antigen export during liver infection of the malaria parasite augments protective immunity. *MBio* **5**: e01321-01314.

Moorthy, V.S., Newman, R.D., and Okwo-Bele, J.M. (2013) Malaria vaccine technology roadmap. *Lancet* **382**: 1700-1701.

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

Mota, M.M., and Rodriguez, A. (2002) Invasion of mammalian host cells by Plasmodium sporozoites. *Bioessays* **24**: 149-156.

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

Mueller, A.K., Camargo, N., Kaiser, K., Andorfer, C., Frevert, U., Matuschewski, K., and Kappe, S.H. (2005a) Plasmodium liver stage developmental arrest by depletion of a protein at the parasite-host interface. *Proc Natl Acad Sci U S A* **102**: 3022-3027.

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

Mueller, I., Shakri, A.R., and Chitnis, C.E. (2015) Development of vaccines for Plasmodium vivax malaria. *Vaccine* **33**: 7489-7495.

Myung, J.M., Marshall, P., and Sinnis, P. (2004) The Plasmodium circumsporozoite protein is involved in mosquito salivary gland invasion by sporozoites. *Mol Biochem Parasitol* **133**: 53-59.

Müller, H.M., Reckmann, I., Hollingdale, M.R., Bujard, H., Robson, K.J., and Crisanti, A. (1993) Thrombospondin related anonymous protein (TRAP) of Plasmodium falciparum binds specifically to sulfated glycoconjugates and to HepG2 hepatoma cells suggesting a role for this molecule in sporozoite invasion of hepatocytes. *EMBO J* **12**: 2881-2889.

Müller, K., Matuschewski, K., and Silvie, O. (2011) The Puf-family RNA-binding protein Puf2 controls sporozoite conversion to liver stages in the malaria parasite. *PLoS One* **6**: e19860.

Nahrendorf, W., Scholzen, A., Sauerwein, R.W., and Langhorne, J. (2015) Cross-stage immunity for malaria vaccine development. *Vaccine* **33**: 7513-7517.

Nardin, E., Zavala, F., Nussenzweig, V., and Nussenzweig, R.S. (1999) Pre-erythrocytic malaria vaccine: mechanisms of protective immunity and human vaccine trials. *Parassitologia* **41**: 397-402.

Narum, D.L., and Thomas, A.W. (1994) Differential localization of full-length and processed forms of PF83/AMA-1 an apical membrane antigen of Plasmodium falciparum merozoites. *Mol Biochem Parasitol* **67**: 59-68.

Nussenzweig, R.S., Vanderberg, J., Most, H., and Orton, C. (1967) Protective immunity produced by the injection of x-irradiated sporozoites of plasmodium berghei. *Nature* **216**: 160-162.

Olotu, A., Fegan, G., Wambua, J., Nyangweso, G., Leach, A., Lievens, M., Kaslow, D.C., Njuguna, P., Marsh, K., and Bejon, P. (2016) Seven-Year Efficacy of RTS,S/AS01 Malaria Vaccine among Young African Children. *N Engl J Med* **374**: 2519-2529.

Opitz, C., and Soldati, D. (2002) 'The glideosome': a dynamic complex powering gliding motion and host cell invasion by Toxoplasma gondii. *Mol Microbiol* **45**: 597-604.

Orito, Y., Ishino, T., Iwanaga, S., Kaneko, I., Kato, T., Menard, R., Chinzei, Y., and Yuda, M. (2013) Liver-specific protein 2: a Plasmodium protein exported to the hepatocyte cytoplasm and required for merozoite formation. *Mol Microbiol* **87**: 66-79.

Oyen, D., Torres, J.L., Wille-Reece, U., Ockenhouse, C.F., Emerling, D., Glanville, J., Volkmuth, W., Flores-Garcia, Y., Zavala, F., Ward, A.B., King, C.R., and Wilson, I.A. (2017) Structural basis for antibody recognition of the NANP repeats in Plasmodium falciparum circumsporozoite protein. *Proc Natl Acad Sci U S A* **114**: E10438-e10445.

Pei, Y., Tarun, A.S., Vaughan, A.M., Herman, R.W., Soliman, J.M., Erickson-Wayman, A., and Kappe, S.H. (2010) Plasmodium pyruvate dehydrogenase activity is only essential for the parasite's progression from liver infection to blood infection. *Mol Microbiol* **75**: 957-971.

Pimenta, P.F., Touray, M., and Miller, L. (1994) The journey of malaria sporozoites in the mosquito salivary gland. *J Eukaryot Microbiol* **41**: 608-624.

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

Pinzon-Ortiz, C., Friedman, J., Esko, J., and Sinnis, P. (2001) The binding of the circumsporozoite protein to cell surface heparan sulfate proteoglycans is required for plasmodium sporozoite attachment to target cells. *J Biol Chem* **276**: 26784-26791.

Pradel, G., Garapaty, S., and Frevert, U. (2004) Kupffer and stellate cell proteoglycans mediate malaria sporozoite targeting to the liver. *Comp Hepatol* **3 Suppl 1**: S47.

Prado, M., Eickel, N., De Niz, M., Heitmann, A., Agop-Nersesian, C., Wacker, R., Schmuckli-Maurer, J., Caldelari, R., Janse, C.J., Khan, S.M., May, J., Meyer, C.G., and Heussler, V.T. (2015) Long-term live imaging reveals cytosolic immune responses of host hepatocytes against Plasmodium infection and parasite escape mechanisms. *Autophagy* **11**: 1561-1579.

Preiser, P., Renia, L., Singh, N., Balu, B., Jarra, W., Voza, T., Kaneko, O., Blair, P., Torii, M., Landau, I., and Adams, J.H. (2004) Antibodies against MAEBL ligand domains M1 and M2 inhibit sporozoite development in vitro. *Infect Immun* **72**: 3604-3608.

Purcell, L.A., Yanow, S.K., Lee, M., Spithill, T.W., and Rodriguez, A. (2008) Chemical attenuation of Plasmodium berghei sporozoites induces sterile immunity in mice. *Infect Immun* **76**: 1193-1199.

Ralph, S.A., van Dooren, G.G., Waller, R.F., Crawford, M.J., Fraunholz, M.J., Foth, B.J., Tonkin, C.J., Roos, D.S., and McFadden, G.I. (2004) Tropical infectious diseases: metabolic maps and functions of the Plasmodium falciparum apicoplast. *Nat Rev Microbiol* **2**: 203-216.

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

Rankin, K.E., Graewe, S., Heussler, V.T., and Stanway, R.R. (2010) Imaging liver-stage malaria parasites. *Cell Microbiol* **12**: 569-579.

Rayner, J.C., Galinski, M.R., Ingravallo, P., and Barnwell, J.W. (2000) Two Plasmodium falciparum genes express merozoite proteins that are related to Plasmodium vivax and Plasmodium yoelii adhesive proteins involved in host cell selection and invasion. *Proc Natl Acad Sci U S A* **97**: 9648-9653.

Real, E., Rodrigues, L., Cabal, G.G., Enguita, F.J., Mancio-Silva, L., Mello-Vieira, J., Beatty, W., Vera, I.M., Zuzarte-Luis, V., Figueira, T.N., Mair, G.R., and Mota, M.M. (2018) Plasmodium UIS3 sequesters host LC3 to avoid elimination by autophagy in hepatocytes. *Nat Microbiol* **3**: 17-25.

Regules, J.A., Cicatelli, S.B., Bennett, J.W., Paolino, K.M., Twomey, P.S., Moon, J.E., Kathcart, A.K., Hauns, K.D., Komisar, J.L., Qabar, A.N., Davidson, S.A., Dutta, S., Griffith, M.E., Magee, C.D., Wojnarski, M., Livezey, J.R., Kress, A.T., Waterman, P.E., Jongert, E., Wille-Reece, U., Volkmuth, W., Emerling, D., Robinson, W.H., Lievens, M., Morelle, D., Lee, C.K., Yassin-Rajkumar, B., Weltzin, R., Cohen, J., Paris, R.M., Waters, N.C., Birkett, A.J., Kaslow, D.C., Ballou, W.R., Ockenhouse, C.F., and Vekemans, J. (2016) Fractional Third and Fourth Dose of RTS,S/AS01 Malaria Candidate Vaccine: A Phase 2a Controlled Human Malaria Parasite Infection and Immunogenicity Study. *J Infect Dis* **214**: 762-771.

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

Ridzuan, M.A., Moon, R.W., Knuepfer, E., Black, S., Holder, A.A., and Green, J.L. (2012) Subcellular location, phosphorylation and assembly into the motor complex of GAP45 during Plasmodium falciparum schizont development. *PLoS One* **7**: e33845.

Risco-Castillo, V., Topcu, S., Marinach, C., Manzoni, G., Bigorgne, A.E., Briquet, S., Baudin, X., Lebrun, M., Dubremetz, J.F., and Silvie, O. (2015a) Malaria Sporozoites Traverse Host Cells within Transient Vacuoles. *Cell Host Microbe* **18**: 593-603.

Risco-Castillo, V., Topcu, S., Son, O., Briquet, S., Manzoni, G., and Silvie, O. (2014) CD81 is required for rhoptry discharge during host cell invasion by Plasmodium yoelii sporozoites. *Cell Microbiol* **16**: 1533-1548.

Risco-Castillo, V., Topçu, S., Marinach, C., Manzoni, G., Bigorgne, A.E., Briquet, S., Baudin, X., Lebrun, M., Dubremetz, J.F., and Silvie, O. (2015b) Malaria Sporozoites Traverse Host Cells within Transient Vacuoles. *Cell Host Microbe* **18**: 593-603.

Ross, R., and Smyth, J. (1897) On some peculiar pigmented cells found in two mosquitos fed on malarial blood. *Wilderness Environ Med* **10**: 29-33.

Sack, B.K., Keitany, G.J., Vaughan, A.M., Miller, J.L., Wang, R., and Kappe, S.H. (2015) Mechanisms of stage-transcending protection following immunization

of mice with late liver stage-arresting genetically attenuated malaria parasites. *PLoS Pathog* **11**: e1004855.

Sanders, P.R., Gilson, P.R., Cantin, G.T., Greenbaum, D.C., Nebl, T., Carucci, D.J., McConville, M.J., Schofield, L., Hodder, A.N., Yates, J.R., 3rd, and Crabb, B.S. (2005) Distinct protein classes including novel merozoite surface antigens in Raft-like membranes of Plasmodium falciparum. *J Biol Chem* **280**: 40169-40176.

Scally, S.W., McLeod, B., Bosch, A., Miura, K., Liang, Q., Carroll, S., Reponen, S., Nguyen, N., Giladi, E., Ramisch, S., Yusibov, V., Bradley, A., Lemiale, F., Schief, W.R., Emerling, D., Kellam, P., King, C.R., and Julien, J.P. (2017) Molecular definition of multiple sites of antibody inhibition of malaria transmission-blocking vaccine antigen Pfs25. *Nat Commun* **8**: 1568.

Scally, S.W., Murugan, R., Bosch, A., Triller, G., Costa, G., Mordmuller, B., Kremsner, P.G., Sim, B.K.L., Hoffman, S.L., Levashina, E.A., Wardemann, H., and Julien, J.P. (2018) Rare PfCSP C-terminal antibodies induced by live sporozoite vaccination are ineffective against malaria infection. *J Exp Med* **215**: 63-75.

Schliwa, M., and Woehlke, G. (2003) Molecular motors. *Nature* **422**: 759-765.

Schofield, L., Villaquiran, J., Ferreira, A., Schellekens, H., Nussenzweig, R., and Nussenzweig, V. (1987) Gamma interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites. *Nature* **330**: 664-666.

Schussek, S., Trieu, A., Apte, S.H., Sidney, J., Sette, A., and Doolan, D.L. (2013) Immunization with apical membrane antigen 1 confers sterile infection-blocking immunity against Plasmodium sporozoite challenge in a rodent model. *Infect Immun* **81**: 3586-3599.

Shahabuddin, M. (1998) Plasmodium ookinete development in the mosquito midgut: a case of reciprocal manipulation. *Parasitology* **116 Suppl**: S83-93.

Shimp, R.L., Jr., Rowe, C., Reiter, K., Chen, B., Nguyen, V., Aebig, J., Rausch, K.M., Kumar, K., Wu, Y., Jin, A.J., Jones, D.S., and Narum, D.L. (2013) Development of a Pfs25-EPA malaria transmission blocking vaccine as a chemically conjugated nanoparticle. *Vaccine* **31**: 2954-2962.

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

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

Silvie, O., Franetich, J.F., Boucheix, C., Rubinstein, E., and Mazier, D. (2007) Alternative invasion pathways for Plasmodium berghei sporozoites. *Int J Parasitol* **37**: 173-182.

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

Silvie, O., Greco, C., Franetich, J.F., Dubart-Kupperschmitt, A., Hannoun, L., van Gemert, G.J., Sauerwein, R.W., Levy, S., Boucheix, C., Rubinstein, E., and Mazier, D. (2006) Expression of human CD81 differently affects host cell susceptibility to malaria sporozoites depending on the Plasmodium species. *Cell Microbiol* **8**: 1134-1146.

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

Sim, B.K., Chitnis, C.E., Wasniowska, K., Hadley, T.J., and Miller, L.H. (1994) Receptor and ligand domains for invasion of erythrocytes by Plasmodium falciparum. *Science* **264**: 1941-1944.

Sinden, R., and Matuschewski, K., (2005) The sporozoite. In: Molecular Approaches to Malaria. S. I (ed). Washington, DC: American Society for Microbiology Press, pp. 169–190.

Sinden, R.E. (1974) Excystment by sporozoites of malaria parasites. *Nature* **252**: 314.

Sinden, R.E., and Garnham, P.C. (1973) A comparative study on the ultrastructure of Plasmodium sporozoites within the oöcyst and salivary glands, with particular reference to the incidence of the micropore. *Trans R Soc Trop Med Hyg* **67**: 631-637.

Singh, A.P., Buscaglia, C.A., Wang, Q., Levay, A., Nussenzweig, D.R., Walker, J.R., Winzeler, E.A., Fujii, H., Fontoura, B.M., and Nussenzweig, V. (2007) Plasmodium circumsporozoite protein promotes the development of the liver stages of the parasite. *Cell* **131**: 492-504.

Singh, S.K., Thrane, S., Chourasia, B.K., Teelen, K., Graumans, W., Stoter, R., van Gemert, G.J., van de Vegte-Bolmer, M.G., Nielsen, M.A., Salanti, A., Sander, A.F., Sauerwein, R.W., Jore, M.M., and Theisen, M. (2019) Pfs230 and Pfs48/45 Fusion Proteins Elicit Strong Transmission-Blocking Antibody Responses Against Plasmodium falciparum. *Front Immunol* **10**: 1256.

Sinnis, P., Clavijo, P., Fenyö, D., Chait, B.T., Cerami, C., and Nussenzweig, V. (1994) Structural and functional properties of region II-plus of the malaria circumsporozoite protein. *J Exp Med* **180**: 297-306.

Sirima, S.B., Durier, C., Kara, L., Houard, S., Gansane, A., Loulergue, P., Bahuaud, M., Benhamouda, N., Nebie, I., Faber, B., Remarque, E., and Launay, O. (2017) Safety and immunogenicity of a recombinant Plasmodium falciparum AMA1-DiCo malaria vaccine adjuvanted with GLA-SE or Alhydrogel(R) in European and African adults: A phase 1a/1b, randomized, double-blind multi-centre trial. *Vaccine* **35**: 6218-6227.

Spring, M., Murphy, J., Nielsen, R., Dowler, M., Bennett, J.W., Zarling, S., Williams, J., de la Vega, P., Ware, L., Komisar, J., Polhemus, M., Richie, T.L., Epstein, J., Tamminga, C., Chuang, I., Richie, N., O'Neil, M., Heppner, D.G., Healer, J., O'Neill, M., Smithers, H., Finney, O.C., Mikolajczak, S.A., Wang, R., Cowman, A., Ockenhouse, C., Krzych, U., and Kappe, S.H. (2013) First-in-human evaluation of genetically attenuated Plasmodium falciparum sporozoites administered by bite of Anopheles mosquitoes to adult volunteers. *Vaccine* **31**: 4975-4983.

Srinivasan, P., Baldeviano, G.C., Miura, K., Diouf, A., Ventocilla, J.A., Leiva, K.P., Lugo-Roman, L., Lucas, C., Orr-Gonzalez, S., Zhu, D., Villasante, E., Soisson, L., Narum, D.L., Pierce, S.K., Long, C.A., Diggs, C., Duffy, P.E., Lescano, A.G., and Miller, L.H. (2017) A malaria vaccine protects Aotus monkeys against virulent Plasmodium falciparum infection. *NPJ Vaccines* **2**.

Srinivasan, P., Beatty, W.L., Diouf, A., Herrera, R., Ambroggio, X., Moch, J.K., Tyler, J.S., Narum, D.L., Pierce, S.K., Boothroyd, J.C., Haynes, J.D., and Miller, L.H. (2011) Binding of Plasmodium merozoite proteins RON2 and AMA1 triggers commitment to invasion. *Proc Natl Acad Sci U S A* **108**: 13275-13280.

Srinivasan, P., Fujioka, H., and Jacobs-Lorena, M. (2008) PbCap380, a novel oocyst capsule protein, is essential for malaria parasite survival in the mosquito. *Cell Microbiol* **10**: 1304-1312.

Stanway, R.R., Bushell, E., Chiappino-Pepe, A., Roques, M., Sanderson, T., Franke-Fayard, B., Caldelari, R., Golomingi, M., Nyonda, M., Pandey, V., Schwach, F., Chevalley, S., Ramesar, J., Metcalf, T., Herd, C., Burda, P.C., Rayner, J.C., Soldati-Favre, D., Janse, C.J., Hatzimanikatis, V., Billker, O., and Heussler, V.T. (2019) Genome-Scale Identification of Essential Metabolic Processes for Targeting the Plasmodium Liver Stage. *Cell* **179**: 1112-1128.e1126.

Steinbuechel, M., and Matuschewski, K. (2009) Role for the Plasmodium sporozoite-specific transmembrane protein S6 in parasite motility and efficient malaria transmission. *Cell Microbiol* **11**: 279-288.

Stewart, M.J., and Vanderberg, J.P. (1988) Malaria sporozoites leave behind trails of circumsporozoite protein during gliding motility. *J Protozool* **35**: 389-393.

Stowers, A.W., Keister, D.B., Muratova, O., and Kaslow, D.C. (2000) A region of Plasmodium falciparum antigen Pfs25 that is the target of highly potent transmission-blocking antibodies. *Infect Immun* **68**: 5530-5538.

Sturm, A., Amino, R., van de Sand, C., Regen, T., Retzlaff, S., Rennenberg, A., Krueger, A., Pollok, J.M., Menard, R., and Heussler, V.T. (2006) Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science* **313**: 1287-1290.

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

Talaat, K.R., Ellis, R.D., Hurd, J., Hentrich, A., Gabriel, E., Hynes, N.A., Rausch, K.M., Zhu, D., Muratova, O., Herrera, R., Anderson, C., Jones, D., Aebig, J., Brockley, S., MacDonald, N.J., Wang, X., Fay, M.P., Healy, S.A., Durbin, A.P., Narum, D.L., Wu, Y., and Duffy, P.E. (2016) Safety and Immunogenicity of Pfs25-EPA/Alhydrogel(R), a Transmission Blocking Vaccine against Plasmodium falciparum: An Open Label Study in Malaria Naive Adults. *PLoS One* **11**: e0163144.

Tan, J., Sack, B.K., Oyen, D., Zenklusen, I., Piccoli, L., Barbieri, S., Foglierini, M., Fregni, C.S., Marcandalli, J., Jongo, S., Abdulla, S., Perez, L., Corradin, G., Varani, L., Sallusto, F., Sim, B.K.L., Hoffman, S.L., Kappe, S.H.I., Daubenberger, C., Wilson, I.A., and Lanzavecchia, A. (2018) A public antibody lineage that potently inhibits malaria infection through dual binding to the circumsporozoite protein. *Nat Med* **24**: 401-407.

Tavares, J., Formaglio, P., Medvinsky, A., Ménard, R., and Amino, R. (2013) Imaging sporozoite cell traversal in the liver of mice. *Methods Mol Biol* **923**: 401-410.

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

Thieleke-Matos, C., da Silva, M.L., Cabrita-Santos, L., Pires, C.F., Ramalho, J.S., Ikonomov, O., Seixas, E., Shisheva, A., Seabra, M.C., and Barral, D.C. (2014) Host PI(3,5)P2 activity is required for Plasmodium berghei growth during liver stage infection. *Traffic* **15**: 1066-1082.

Thieleke-Matos, C., Lopes da Silva, M., Cabrita-Santos, L., Portal, M.D., Rodrigues, I.P., Zuzarte-Luis, V., Ramalho, J.S., Futter, C.E., Mota, M.M., Barral, D.C.,

and Seabra, M.C. (2016) Host cell autophagy contributes to Plasmodium liver development. *Cell Microbiol* **18**: 437-450.

Tiegs, G., and Lohse, A.W. (2010) Immune tolerance: what is unique about the liver. *J Autoimmun* **34**: 1-6.

Timmann, C., and Meyer, C.G. (2010) Malaria, mummies, mutations: Tutankhamun's archaeological autopsy. *Trop Med Int Health* **15**: 1278-1280.

Togiri, J., Segireddy, R.R., Mastan, B.S., Singh, D., Kolli, S.K., Ghosh, A., Al-Nihmi, F.M.A., Maruthi, M., Choudhary, H.H., Dey, S., Mishra, S., and Kumar, K.A. (2019) Plasmodium berghei sporozoite specific genes- PbS10 and PbS23/SSP3 are required for the development of exo-erythrocytic forms. *Mol Biochem Parasitol* **232**: 111198.

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

Triglia, T., Thompson, J., Caruana, S.R., Delorenzi, M., Speed, T., and Cowman, A.F. (2001) Identification of proteins from Plasmodium falciparum that are homologous to reticulocyte binding proteins in Plasmodium vivax. *Infect Immun* **69**: 1084-1092.

Trueman, H.E., Raine, J.D., Florens, L., Dessens, J.T., Mendoza, J., Johnson, J., Waller, C.C., Delrieu, I., Holders, A.A., Langhorne, J., Carucci, D.J., Yates, J.R., and Sinden, R.E. (2004) Functional characterization of an LCCL-lectin domain containing protein family in Plasmodium berghei. *J Parasitol* **90**: 1062-1071.

Tucker, R.P. (2004) The thrombospondin type 1 repeat superfamily. *Int J Biochem Cell Biol* **36**: 969-974.

Turque, O., Tsao, T., Li, T., and Zhang, M. (2016) Translational Repression in Malaria Sporozoites. *Microb Cell* **3**: 227-229.

Usynin, I., Klotz, C., and Frevert, U. (2007) Malaria circumsporozoite protein inhibits the respiratory burst in Kupffer cells. *Cell Microbiol* **9**: 2610-2628.

van de Sand, C., Horstmann, S., Schmidt, A., Sturm, A., Bolte, S., Krueger, A., Lutgehetmann, M., Pollok, J.M., Libert, C., and Heussler, V.T. (2005) The liver stage of Plasmodium berghei inhibits host cell apoptosis. *Mol Microbiol* **58**: 731-742.

van Dijk, M.R., Douradinha, B., Franke-Fayard, B., Heussler, V., van Dooren, M.W., van Schaijk, B., van Gemert, G.J., Sauerwein, R.W., Mota, M.M., Waters,

A.P., and Janse, C.J. (2005) Genetically attenuated, P36p-deficient malarial sporozoites induce protective immunity and apoptosis of infected liver cells. *Proc Natl Acad Sci U S A* **102**: 12194-12199.

van Dijk, M.R., Janse, C.J., Thompson, J., Waters, A.P., Braks, J.A., Dodemont, H.J., Stunnenberg, H.G., van Gemert, G.J., Sauerwein, R.W., and Eling, W. (2001) A central role for P48/45 in malaria parasite male gamete fertility. *Cell* **104**: 153-164.

van Schaijk, B.C., van Dijk, M.R., van de Vegte-Bolmer, M., van Gemert, G.J., van Dooren, M.W., Eksi, S., Roeffen, W.F., Janse, C.J., Waters, A.P., and Sauerwein, R.W. (2006) Pfs47, paralog of the male fertility factor Pfs48/45, is a female specific surface protein in Plasmodium falciparum. *Mol Biochem Parasitol* **149**: 216-222.

VanBuskirk, K.M., O'Neill, M.T., De La Vega, P., Maier, A.G., Krzych, U., Williams, J., Dowler, M.G., Sacci, J.B., Jr., Kangwanrangsan, N., Tsuboi, T., Kneteman, N.M., Heppner, D.G., Jr., Murdock, B.A., Mikolajczak, S.A., Aly, A.S., Cowman, A.F., and Kappe, S.H. (2009) Preerythrocytic, live-attenuated Plasmodium falciparum vaccine candidates by design. *Proc Natl Acad Sci U S A* **106**: 13004-13009.

Vanderberg, J., and Rhodin, J. (1967) Differentiation of nuclear and cytoplasmic fine structure during sporogonic development of Plasmodium berghei. *J Cell Biol* **32**: C7-10.

Vanderberg, J.P. (1974) Studies on the motility of Plasmodium sporozoites. *J Protozool* **21**: 527-537.

Vanderberg, J.P. (1975) Development of infectivity by the Plasmodium berghei sporozoite. *J Parasitol* **61**: 43-50.

Vanderberg, J.P. (2014) Imaging mosquito transmission of Plasmodium sporozoites into the mammalian host: immunological implications. *Parasitol Int* **63**: 150-164.

Vanderberg, J.P., Chew, S., and Stewart, M.J. (1990) Plasmodium sporozoite interactions with macrophages in vitro: a videomicroscopic analysis. *J Protozool* **37**: 528-536.

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

Vaughan, A.M., Mikolajczak, S.A., Wilson, E.M., Grompe, M., Kaushansky, A., Camargo, N., Bial, J., Ploss, A., and Kappe, S.H. (2012) Complete Plasmodium falciparum liver-stage development in liver-chimeric mice. *J Clin Invest* **122**: 3618-3628.

Vaughan, A.M., O'Neill, M.T., Tarun, A.S., Camargo, N., Phuong, T.M., Aly, A.S., Cowman, A.F., and Kappe, S.H. (2009) Type II fatty acid synthesis is essential only for malaria parasite late liver stage development. *Cell Microbiol* **11**: 506-520.

Vaughan, A.M., Sack, B.K., Dankwa, D., Minkah, N., Nguyen, T., Cardamone, H., and Kappe, S.H.I. (2018) A Plasmodium Parasite with Complete Late Liver Stage Arrest Protects against Preerythrocytic and Erythrocytic Stage Infection in Mice. *Infect Immun* **86**.

Vial, H.J., and Ancelin, M.L. (1992) Malarial lipids. An overview. *Subcell Biochem* **18**: 259-306.

Vinetz, J.M., Dave, S.K., Specht, C.A., Brameld, K.A., Xu, B., Hayward, R., and Fidock, D.A. (1999) The chitinase PfCHT1 from the human malaria parasite Plasmodium falciparum lacks proenzyme and chitin-binding domains and displays unique substrate preferences. *Proc Natl Acad Sci U S A* **96**: 14061-14066.

Vinetz, J.M., Valenzuela, J.G., Specht, C.A., Aravind, L., Langer, R.C., Ribeiro, J.M., and Kaslow, D.C. (2000) Chitinases of the avian malaria parasite Plasmodium gallinaceum, a class of enzymes necessary for parasite invasion of the mosquito midgut. *J Biol Chem* **275**: 10331-10341.

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

W.H.O, (2019) World Malaria Report. In., pp.

Wang, Q., Fujioka, H., and Nussenzweig, V. (2005) Exit of Plasmodium sporozoites from oocysts is an active process that involves the circumsporozoite protein. *PLoS Pathog* **1**: e9.

Weiner, H.L. (1997) Oral tolerance: immune mechanisms and treatment of autoimmune diseases. *Immunol Today* **18**: 335-343.

Westenberger, S.J., McClean, C.M., Chattopadhyay, R., Dharia, N.V., Carlton, J.M., Barnwell, J.W., Collins, W.E., Hoffman, S.L., Zhou, Y., Vinetz, J.M., and Winzeler, E.A. (2010) A systems-based analysis of Plasmodium vivax lifecycle transcription from human to mosquito. *PLoS Negl Trop Dis* **4**: e653.

White, M.T., Shirreff, G., Karl, S., Ghani, A.C., and Mueller, I. (2016) Variation in relapse frequency and the transmission potential of Plasmodium vivax malaria. *Proc Biol Sci* **283**: 20160048.

White, M.T., Verity, R., Griffin, J.T., Asante, K.P., Owusu-Agyei, S., Greenwood, B., Drakeley, C., Gesase, S., Lusingu, J., Ansong, D., Adjei, S., Agbenyega, T., Ogutu, B., Otieno, L., Otieno, W., Agnandji, S.T., Lell, B., Kremsner, P., Hoffman, I., Martinson, F., Kamthunzu, P., Tinto, H., Valea, I., Sorgho, H., Oneko, M., Otieno, K., Hamel, M.J., Salim, N., Mtoro, A., Abdulla, S., Aide, P., Sacarlal, J., Aponte, J.J., Njuguna, P., Marsh, K., Bejon, P., Riley, E.M., and Ghani, A.C. (2015) Immunogenicity of the RTS,S/AS01 malaria vaccine and implications for duration of vaccine efficacy: secondary analysis of data from a phase 3 randomised controlled trial. *Lancet Infect Dis* **15**: 1450-1458.

Wiegard, C., Wolint, P., Frenzel, C., Cheruti, U., Schmitt, E., Oxenius, A., Lohse, A.W., and Herkel, J. (2007) Defective T helper response of hepatocyte-stimulated CD4 T cells impairs antiviral CD8 response and viral clearance. *Gastroenterology* **133**: 2010-2018.

Wu, Y., Ellis, R.D., Shaffer, D., Fontes, E., Malkin, E.M., Mahanty, S., Fay, M.P., Narum, D., Rausch, K., Miles, A.P., Aebig, J., Orcutt, A., Muratova, O., Song, G., Lambert, L., Zhu, D., Miura, K., Long, C., Saul, A., Miller, L.H., and Durbin, A.P. (2008) Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51. *PLoS One* **3**: e2636.

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

Yao, Z., and Klionsky, D.J. (2018) Plasmodium protein UIS3 protects the parasite from autophagy clearance. *Autophagy* **14**: 1291-1292.

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

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

Yu, M., Kumar, T.R., Nkrumah, L.J., Coppi, A., Retzlaff, S., Li, C.D., Kelly, B.J., Moura, P.A., Lakshmanan, V., Freundlich, J.S., Valderramos, J.C., Vilcheze, C., Siedner, M., Tsai, J.H., Falkard, B., Sidhu, A.B., Purcell, L.A., Gratraud, P., Kremer, L., Waters, A.P., Schiehser, G., Jacobus, D.P., Janse, C.J., Ager, A., Jacobs, W.R., Jr., Sacchettini, J.C., Heussler, V., Sinnis, P., and Fidock,

D.A. (2008) The fatty acid biosynthesis enzyme FabI plays a key role in the development of liver-stage malarial parasites. *Cell Host Microbe* **4**: 567-578.

Yuda, M., Iwanaga, S., Shigenobu, S., Mair, G.R., Janse, C.J., Waters, A.P., Kato, T., and Kaneko, I. (2009) Identification of a transcription factor in the mosquito-invasive stage of malaria parasites. *Mol Microbiol* **71**: 1402-1414.

Zapata, J.C., Perlaza, B.L., Hurtado, S., Quintero, G.E., Jurado, D., Gonzalez, I., Druilhe, P., Arevalo-Herrera, M., and Herrera, S. (2002) Reproducible infection of intact Aotus lemurinus griseimembra monkeys by Plasmodium falciparum sporozoite inoculation. *J Parasitol* **88**: 723-729.

Zenklusen, I., Jongo, S., Abdulla, S., Ramadhani, K., Lee Sim, B.K., Cardamone, H., Flannery, E.L., Nguyen, T., Fishbaugher, M., Steel, R.W.J., Betz, W., Carmago, N., Mikolajczak, S., Kappe, S.H.I., Hoffman, S.L., Sack, B.K., and Daubenberger, C. (2018) Immunization of Malaria-Preexposed Volunteers With PfSPZ Vaccine Elicits Long-Lived IgM Invasion-Inhibitory and Complement-Fixing Antibodies. *J Infect Dis* **217**: 1569-1578.

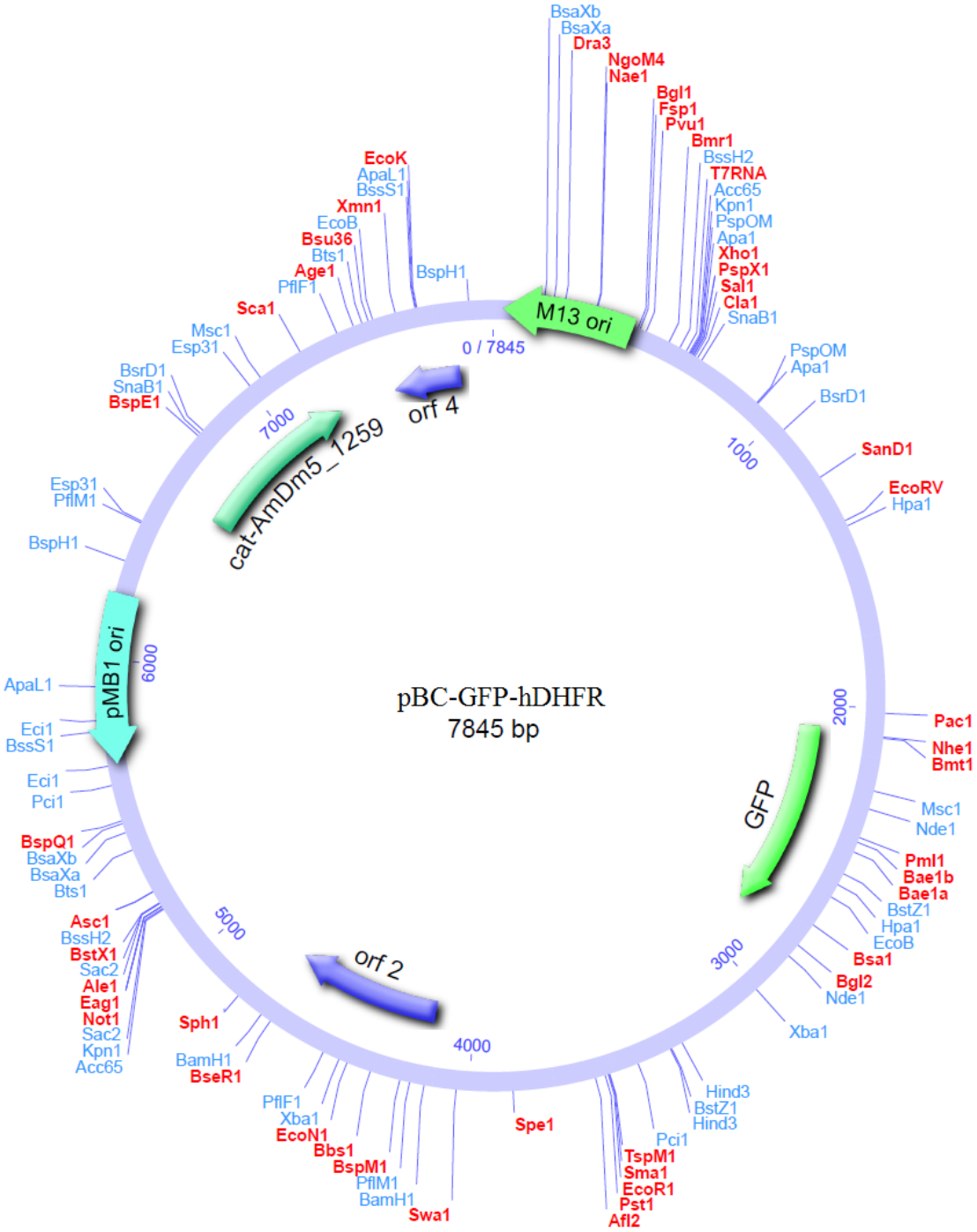
Zhang, M., Mishra, S., Sakthivel, R., Fontoura, B.M., and Nussenzweig, V. (2016) UIS2: A Unique Phosphatase Required for the Development of Plasmodium Liver Stages. *PLoS Pathog* **12**: e1005370.

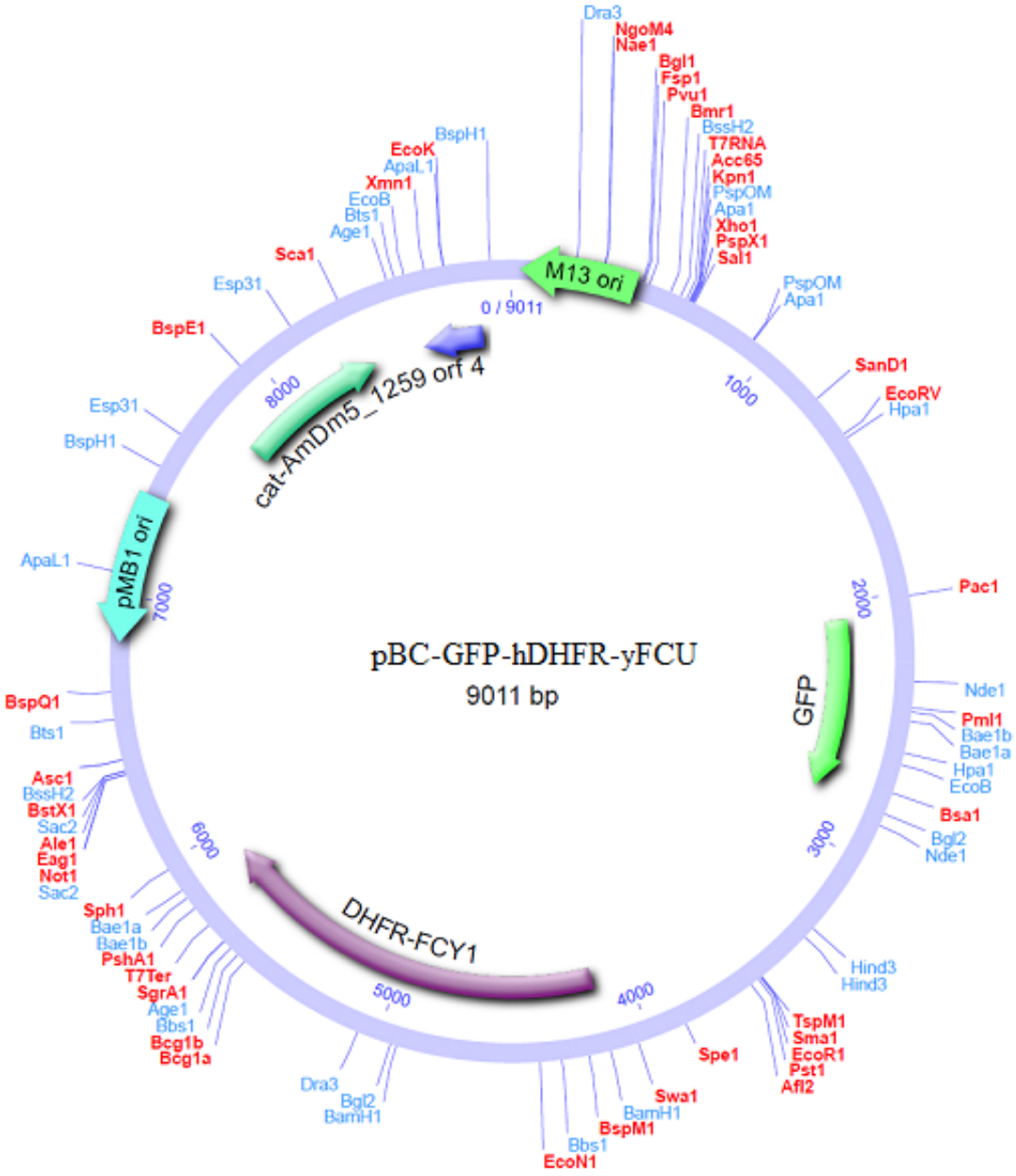
Zhou, Y., Ramachandran, V., Kumar, K.A., Westenberger, S., Refour, P., Zhou, B., Li, F., Young, J.A., Chen, K., Plouffe, D., Henson, K., Nussenzweig, V., Carlton, J., Vinetz, J.M., Duraisingh, M.T., and Winzeler, E.A. (2008) Evidence-based annotation of the malaria parasite's genome using comparative expression profiling. *PLoS One* **3**: e1570.

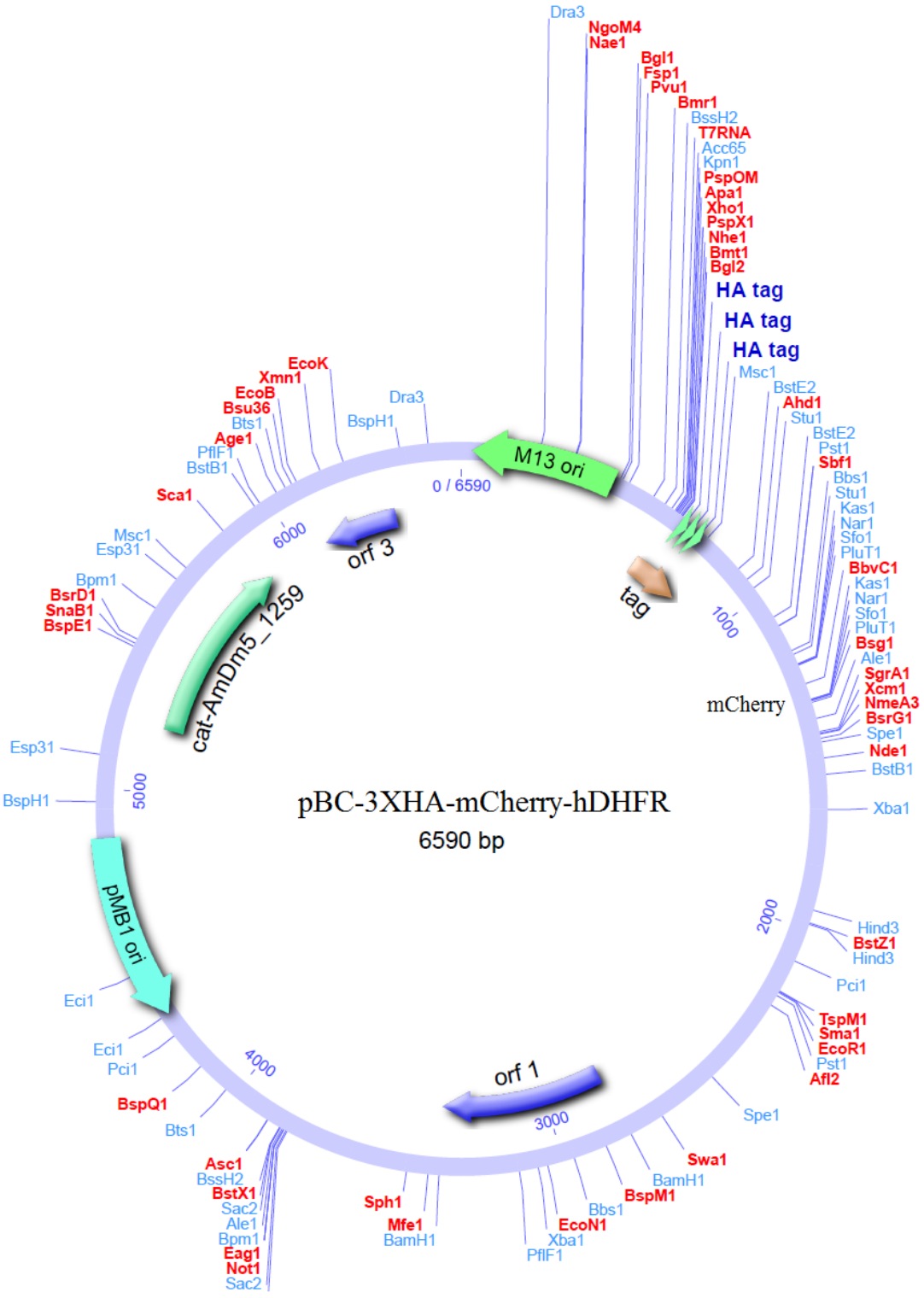
Zöller, B., Koethe, M., Ludewig, M., Pott, S., Daugschies, A., Straubinger, R.K., Fehlhaber, K., and Bangoura, B. (2013) Tissue tropism of Toxoplasma gondii in turkeys (Meleagris gallopavo) after parenteral infection. *Parasitol Res* **112**: 1841-1847.

Appendix

Appendix


pBC-GFP-hDHFR
7845 bp
M13 ori
orf 4
cat-AmDm5_1259
pMB1 ori
GFP
orf 2
0 / 7845
1000
2000
3000
4000
5000
6000
7000
BsaXb
BsaXa
Dra3
NgoM4
Nae1
Bgl1
Fsp1
Pvu1
Bmr1
BssH2
T7RNA
Acc65
Kpn1
PspOM
Apa1
Xho1
PspX1
Sal1
Cla1
SnaB1
PspOM
Apa1
BsrD1
SanD1
EcoRV
Hpa1
Pac1
Nhe1
Bmt1
Msc1
Nde1
Pml1
Bae1b
Bae1a
BstZ1
Hpa1
EcoB
Bsa1
Bgl2
Nde1
Xba1
Hind3
BstZ1
Hind3
Pci1
TspM1
Sma1
EcoR1
Pst1
Afl2
Spe1
Swa1
BamH1
PflM1
BspM1
Bbs1
EcoN1
Xba1
PflF1
BamH1
BseR1
Sph1
Kpn1
Sac2
Not1
Eag1
Ale1
BstX1
Sac2
BssH2
Asc1
Bts1
BsaXa
BsaXb
BspQ1
Pci1
Eci1
BssS1
Eci1
ApaL1
BspH1
PflM1
Esp31
BspE1
SnaB1
BsrD1
Esp31
Msc1
Sca1
PflF1
Age1
Bts1
Bsu36
EcoB
Xmn1
BssS1
ApaL1
EcoK
BspH1


pBC-GFP-hDHFR-yFCU
9011 bp
M13 ori
cat-AmDm5_1259 orf 4
pMB1 ori
GFP
DHFR-FCY1
0 / 9011
1000
2000
3000
4000
5000
6000
7000
8000
Dra3
NgoM4
Nae1
Bgl1
Fsp1
Pvu1
Bmr1
BssH2
T7RNA
Acc65
Kpn1
PspOM
Apa1
Xho1
PspX1
Sal1
PspOM
Apa1
SanD1
EcoRV
Hpa1
Pac1
Nde1
Pml1
Bae1b
Bae1a
Hpa1
EcoB
Bsa1
Bgl2
Nde1
Hind3
Hind3
TspM1
Sma1
EcoR1
Pst1
Afl2
Spe1
Swa1
BamH1
BspM1
Bbs1
EcoN1
Dra3
Bgl2
BamH1
Sph1
Bae1a
Bae1b
PshA1
T7Ter
SgrA1
Age1
Bbs1
Bcg1b
Bcg1a
Asc1
BssH2
BstX1
Sac2
Ale1
Eag1
Not1
Sac2
BspQ1
Bts1
ApaL1
Esp31
BspH1
BspE1
Esp31
Sca1
Age1
Bts1
EcoB
Xmn1
ApaL1
EcoK
BspH1


pBC-3XHA-mCherry-hDHFR
6590 bp
M13 ori
tag
mCherry
orf 3
cat-AmDm5_1259
pMB1 ori
orf 1
0 / 6590
1000
2000
3000
4000
5000
6000
Dra3
NgoM4
Nae1
Bgl1
Fsp1
Pvu1
Bmr1
BssH2
T7RNA
Acc65
Kpn1
PspOM
Apa1
Xho1
PspX1
Nhe1
Bmt1
Bgl2
HA tag
HA tag
HA tag
Msc1
BstE2
Ahd1
Stu1
BstE2
Pst1
Sbf1
Bbs1
Stu1
Kas1
Nar1
Sfo1
PluT1
BbvC1
Kas1
Nar1
Sfo1
PluT1
Bsg1
Ale1
SgrA1
Xcm1
NmeA3
BsrG1
Spe1
Nde1
BstB1
Xba1
Hind3
BstZ1
Hind3
Pci1
TspM1
Sma1
EcoR1
Pst1
Afl2
Spe1
Swa1
BamH1
BspM1
Bbs1
EcoN1
Xba1
PflF1
Sph1
Mfe1
BamH1
Asc1
BssH2
BstX1
Sac2
Ale1
Bpm1
Eag1
Not1
Sac2
BspQ1
Bts1
Eci1
Eci1
Pci1
Esp31
BspH1
Esp31
Bpm1
BsrD1
SnaB1
BspE1
Msc1
Esp31
Sca1
BstB1
PflF1
Age1
Bts1
Bsu36
EcoB
Xmn1
EcoK
Dra3
BspH1

Publications

1) Togiri J, Reddy SR, Mastan BS, Singh D, Kolli SK, **Ghosh A**, Abdul Al-Nihmi FM, Maruthi M, Choudhary HH**,** Dey S, Mishra S, Kumar KA. Plasmodium berghei sporozoite specific genes- PbS10 and PbS23/SSP3 are required for the development of exo-erythrocytic forms. ***Mol Biochem Parasitol***. 2019; 232:111198.

Scientific Abstracts, Posters and Presentations

1. Narwal SK, Choudhary HH, **Ghosh A**, Mishra S. Parasite mediated desaturation of fatty acid is essential for completion of liver stage schizogony. 7th International symposium on current trends in drug discovery research, CSIR-CDRI, Lucknow, India. February 20-23, 2019.

2. **Ghosh A**, Narwal SK, Gupta R, Gaurav S, Choudhary HH, Ahmed S, Mishra S. Plasmod A novel glideosome-associated protein coordinates motility and invasion of Plasmodium sporozoites. 7th International symposium on current trends in drug discovery research, CSIR-CDRI, Lucknow, India. February 20-23, 2019.

3. **Ghosh A**, Narwal SK, Gupta R, Gaurav S, Choudhary HH, Ahmed S, Mishra S. A novel glideosome-associated protein coordinates motility and invasion of *Plasmodium* sporozoites. International symposium on malaria biology and 29th National congress of parasitology on basic and applied aspects. University of Hyderabad, Hyderabad, India, November 1-3, 2018.

4. Abdul Al-Nihmi FM, Togiri J, Reddy SR, Jalapali R, Kolli SK, Mastan BS, Mulaka M, Singh D, Dey S, Gupta R, **Ghosh A**, Choudhary HH, Narwal SK, Sijwali PS, Mishra S, Kota KA. Design of Plasmodium liver arresting mutants by targeting sporozoite specific genes: Implications for developing a whole organism vaccine. International symposium on malaria biology and 29th National congress of parasitology on basic and applied aspects. University of Hyderabad, Hyderabad, India, November 1-3, 2018.

5. Narwal SK, Choudhary HH, **Ghosh A**, Mishra S. *Plasmodium berghei* Stearoyl-CoA delta 9 desaturase is essential for liver stage maturation. International Congress of Cell Biology, Hyderabad, India, January 27-31, 2018.

6. **Ghosh A**, Narwal SK, Gupta R, Choudhary HH, Mishra S**.** *Plasmodium berghei S*14 protein regulates sporozoites gliding motility and infectivity. International Vaccine Conference, ICGEB, New Delhi, India, November 27-29, 2017.
7. Narwal SK, Choudhary HH, **Ghosh A**, Mishra S. *Plasmodium berghei* Stearoyl-CoA delta 9 desaturase is essential for liver stage maturation. International Vaccine Conference, ICGEB, New Delhi, India, November 27-29, 2017.
8. Narwal SK, Togiri J, Kolli SK, **Ghosh A**, Choudhary HH, Mastan BS, Reddy SR, Kumar KA, Mishra S. Protein Kinase 9 regulates sexual reproduction in Plasmodium: A novel malaria transmission-blocking drug target. 40th Annual Conference of Indian Association of Medical Microbiologists, PGIMER, Chandigarh, India, November 25-27, 2016.
9. **Ghosh A**, Narwal SK, Gupta R, Choudhary HH, Kolli SK, Kumar KA, Mishra S. Plasmodium berghei S14 is Essential for Gliding Motility and Infectivity of Sporozoites. International Conference on Cell Biology of Infections. National Centre for Biological Sciences, Bengaluru, India, October 13-14, 2016.
10. **Ghosh A**, Narwal SK, Gupta R, Choudhary HH, Kolli SK, Kumar KA, Mishra S. A Novel Protein-PbS14 is Essential for Gliding Motility and Infectivity of Plasmodium Sporozoites. Malaria Parasite Biology: Drug Designing & Vaccine Development. Nirma University, Ahmedabad, India, September 9-10, 2016.
11. **Ghosh A**, Narwal SK, Gupta R, Choudhary HH, Kolli SK, Kumar KA, Mishra S. A sporozoite transmembrane protein-PbS14 produced by oocyst sporozoites facilitates commitment of sporozoites to invade *Anopheles stephensi* salivary glands. 6th International Symposium on "Current trends in drug discovery & research, CSIR-CDRI, Lucknow, India, February 25-28, 2016.
12. Narwal SK, Choudhary HH, Gupta R, **Ghosh A**, Kumar KA, Mishra S. *Plasmodium* Stearoyl-CoA Δ9-desaturase is important for the late liver stage development or initiation of blood stages. 6th International Symposium on "Current trends in drug discovery & research, CSIR-CDRI, Lucknow, India, February 25-28, 2016.