Elucidating the effects of *Plasmodium berghei* sporozoite and *Toxoplasma gondii* infection on host cell SUMOylation

&

Functional characterization of *PI31* (proteasome inhibitor-31) in *Plasmodium berghei* by reverse genetics approach

Thesis Submitted for the Award of Degree of

DOCTOR OF PHILOSOPHY

By

Mulaka Maruthi

08LAPH14

DEPARTMENT OF ANIMAL BIOLOGY

SCHOOL OF LIFE SCIENCES

UNIVERSITY OF HYDERABAD

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HYDERABAD- 500 046, INDIA

April 2016

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

By

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

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DECLARATION

I, **Mulaka Maruthi**, hereby declare that the thesis entitled **"Elucidating the effects of *Plasmodium berghei* sporozoite and *Toxoplasma gondii* infection on host cell SUMOylation & Functional characterization of *PI31* (proteasome inhibitor-31) in *Plasmodium berghei* by reverse genetics approach"** submitted by me under the guidance and supervision of Dr. Kota Arun Kumar is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Name : **Mulaka Maruthi**

Reg. No : **08LAPH14**

Signature :

Date :

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

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CERTIFICATE

This is to certify that the thesis **"Elucidating the effects of *Plasmodium berghei* sporozoite and *Toxoplasma gondii* infection on host cell SUMOylation & Functional characterization of *PI31* (proteasome inhibitor-31) in *Plasmodium berghei* by reverse genetics approach"** is a record of bonafide work done by Mr. Mulaka Maruthi, for the Ph.D. programme in the Department of Animal Biology, University of Hyderabad, under my guidance and supervision. The thesis has not been submitted previously in part or full to this or any other University or Institution for the award of any degree or diploma.

Dr. Kota Arun Kumar
(Supervisor)

Head of the Department

Dean of the School

Acknowledgements

I express my gratitude to my supervisor Dr. Kota Arun Kumar for his constant guidance, encouragement and support throughout my doctoral research. He taught me to do good research and helped me to shape my character.

I thank, Prof. Jagan Pongubala, Head, Department of Animal Biology, and former heads Prof. B. Senthil Kumaran, Prof. Manjula Sritharan and Prof. S. Dayananda for extending departmental facilities.

I thank Prof. P. Reddanna, Dean, School of Life Sciences, and former Deans, Prof. M. Ramanadham, Prof. R.P. Sharma, Prof. Aparna Dutta Gupta and Prof. A.S. Raghavendra for allowing me to use the school facilities.

I thank my Doctoral Committee members Dr. K. Gopinath and Dr. Sharsmista Banerjee for their valuable suggestions.

I thank our lab collaborators Dr. Satish Mishra, CSIR-CDRI-Lucknow, Dr. Dhanasekharan Shanmugam, NCL-Pune and Dr. Agam Singh Prasad, NII-New Delhi for sharing valuable reagents and parasites.

I am grateful to all my teachers, who taught me and are instrumental in shaping up my life.

I would like to express my sincere gratitude to Rameswar, Mastan, Dipti and Sandeep for providing me mosquitoes for my experiments.

I extend my thanks to my lab mates and very good friends Jyothi, Ravi, Surendra, Faisal, Mastan, Rameswar, Dipti and Sandeep for their cooperation in the lab throughout my research.

I thank master's dissertation students of the lab Abhishek, Shravani, Dipti and Deepak for their help.

I would like to thank my friends Anand, Prasad, Praveen, Madhavi, Khamushavalli, Chaithanya, Suresh, Sagar, Ashok, Papa Rao, Malathi, Divya and all JNVL friends for being there for me always and supporting me.

I thank Uma, Pasha, Deepthi, Mahesh, Mano, Rameswar and Prathibha for their help during difficult times. I would like to acknowledge the support of all my friends.

I would like to thank all the lab members of Prof. Aparna Dutta Gupta, Prof. Prakash Babu, Dr. Suresh Yenugu, Dr. Naresh Babu and Dr. Radheshyam Mourya for their timely help when required.

I thank Surendra and Ravi for their good companionship and Dr. Abhijit for his valuable suggestions about research and future.

I thank Narasimha and Yadagiri for their assistance in the lab.

The help and cooperation of the non-teaching staff of the Dept. of Animal Biology and School of Life Sciences is highly acknowledged.

I acknowledge CSIR for providing me financial assistance through CSIR-JRF and CSIR-SRF fellowship.

I thank DST, CSIR, DBT, ICMR, UGC and UPE-II for funding the lab.

I acknowledge the financial support of DBT, CSIR, ICMR, UGC, PURSE and DBT-CREEB to the department and school.

I am extremely grateful to my parents and family members for their unwavering love, support and patience.

To everyone who made this journey possible but I forgot to mention, please know that I am deeply grateful and would have never made it without you.

Above the all, I owe my thanks to Almighty, for everything.

Maruthi...

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Abbreviations

AMA	Apical membrane antigen
AOS	Activator of SUMO
APC	Antigen presenting cells
ATP	Adenosine triphosphate
CD	Cluster differentiation
CP	Core particle
CSP	Circumsporozoite protein
DAPI	4', 6', diamino-2 phenyl indole
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxy ribonucleic acid
DUB	De ubiquitinase
ECM	Extra cellular matrix
EEFs	Exo-erythrocytic forms
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immune sorbent assay
ER	Endoplasmic reticulum
FCM	Flow cytometry
GPI	Glycophosphatidylinositol
HDAC	Histone deacetylase
HSP	Heat shock protein
HSPG	Heparan sulphate proteoglycan
HT	Host targeting motif
IBIS1	Inta erythrocyte *P.berghei* induced structure
IFN	Interferon
IL	Interleukin
iRBC	*Plasmodium* infected red blood cell
IRF	Interferon regulatory factor
ISG	Interferon stimulate gene

KO	Knockout
LAMP	Loop mediated isothermal amplification
LB broth	Luria-Bertani broth
LISP	Liver stage specific protein
LSECs	Liver sinusoidal endothelial cells
LSTVN	Liver stage tubo vesicular network
MAPK	Mitogen activated protein kinase
MAT	Modified agglutinin test
MHC	Major histo compatibility complex
MIC	Micronemal protein
MJ	Moving junction
MOI	Multiplicity of infection
MS	Mass spectrometry
MSP	Merozoite specific protein
mRNA	Messenger RNA
NER	Nucleotide excision repair
NFκB	Nuclear factor kappa light chain enhancer of activated B cells
NO	Nitric oxide
OD	Optical density
PA	Proteasome activator
PCR	Polymerase chain reaction
PBS	Phosphate buffer saline
P. berghei	*Plasmodium berghei*
Pb	*Plasmodium berghei*
PEXEL	*Plasmodium* export element
P. falciparum	*Plasmodium falciparum*
PfEMP	*P. falciparum* erythrocyte membrane protein
PI31	Proteasome inhibitor 31 kDa protein
PNEP	PEXEL negative exported proteins
PSMF	Proteasome inhibitor PI31 subunit

PTM	Post translational modification
PVM	Parasitophorous vacuolar membrane
RAPD	Rapid amplification polymorphic DNA
RBC	Red blood cell
RFLP	Rapid fragmented length polymorphism
RH	Relative humidity
RIFIN	Repetitive inter spread family
RNA	Ribonucleic acid
RON	Rhoptry neck protein
RPMI	Roswell park memorial institute medium
SAE	SUMO activating enzyme
SDS-PAGE	Sodium dodecyl sulphate poly acrylamide gel electrophoresis
SERA	Serine repeat antigen
STEVOR	Sub telomeric variable open reading frames
SUMO	Small ubiquitin like modifier
TBS	Tris buffer saline
TBV	Transmission blocking vaccine
TGF	Transforming growth factor
Tg	*Toxoplasma gondii*
T. gondii	*Toxoplasma gondii*
TNF	Tumor necrosis factor
UBC	Ubiquitin conjugating enzyme
Ubl	Ubiquitin like protein
UIS	Up regulated in infective sporozoites
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system
UTR	Untranslated region
VCP	Valosin containing protein
WT	Wild type
mg	Milligram

ng	Nanogram
µl	Microlitre
µg	Microgram
µm	Micrometer

Chapter 1

Review of Literature

1.1 Introduction

Malaria word comes from the Italian origin Mal'aria meaning spoiled air. Malaria, a scourge of humanity since antiquity, is caused by the parasites of the genus *Plasmodium*, with an incidence of 300-500 million people and a worldwide death toll of 800,000 per year (1, 2).

1.1.1 Prevalence of malaria

By using a combination of epidemiological, demographic and geographical data, it was estimated that there were 300-600 million clinical episodes of *Plasmodium falciparum* (*P. falciparum*) worldwide (1). At regional level, most of the clinical cases attributable to *P. falciparum* were concentrated in the African continent with around 70% (with high prevalence in the sub Saharan region); the other 25% of world's clinical attacks are contributed from the highly populated South East Asia region (Fig 1). The risk of death after clinical attack is much higher in the African region than the Western Pacific and South East Asia regions.

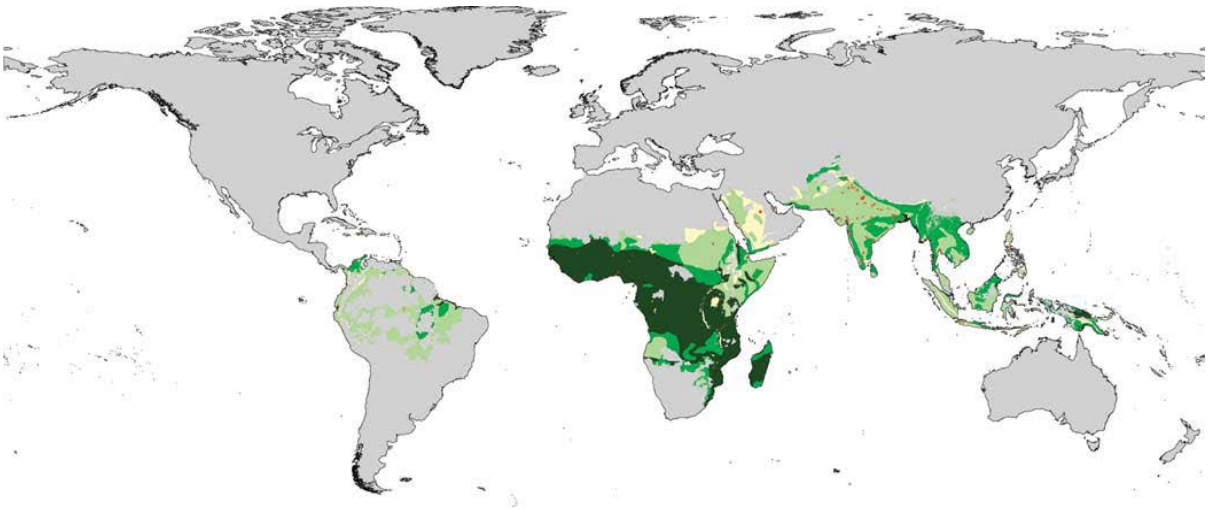


Fig. 1: *P. falciparum* endemicity distribution within the global limits of risk. Endemicity classes: light green, hypoendemic (areas in which prevalence is less than 10% in childhood infection); medium green, mesoendemic (areas with infection prevalence is between 11% and 50%); dark green, hyperendemic and holoendemic (areas with an infection prevalence of 50% or more). Unclassified areas (yellow). Grey areas are a combined mask of areas outside of the transmission limits and areas with population density of less than 1 person /km (3).

1.1.2 History and discovery of the parasite

Malaria is an ancient disease and the occurrence was documented in the historical texts that dates back as 2700 BC (Chinese document), 2000 BC (Mesopotamia clay tablets) and Sixth century BC (Hindu texts). The early Greeks were well aware of the poor health characteristics, malarial fevers and spleen enlargement in people living in marshy areas. The discovery of bacteria, the incrimination of microorganisms as infectious agents and the germ theory of infection intensified the search for the cause of malaria (4). Scientific studies became possible only after Charles Alphonse Laveran (1880) glimpsed the parasite through his microscope and the discovery of the mosquitoes as vectors for avian malaria by Ronald Ross (1897). Human malaria was identified by the Italian scientists Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, Camillo Golgi, Angelo Celli and Ettore Marchiafava between 1898 and 1900 (5).

1.1.3 *Plasmodium*: the causative agent of malaria disease

Plasmodium belongs to the phylum Apicomplexa which includes many parasitic genera of medical importance. The apicomplexan parasites are characterized by the possession of a unique, essential organelle, chloroplast- like juxta-nuclear apicoplast which have evolved by secondary endosymbiosis (6). *Plasmodium* genera include different species which causes the disease malaria in specific hosts (Fig 2) (7).

1.2 *Plasmodium* life cycle

The Apicomplexan parasites have complex life cycles, with some passing directly between vertebrate hosts (*Toxoplasma, Eimeria and Cryptosporidium*). In contrast, others including *Plasmodium* have a life cycle including an arthropod vector transmitting the parasite to the vertebrate host during blood feeding.

Plasmodium life cycle initiates with bite of female *Anopheles* mosquitoes injecting sporozoites into the vertebrate host during a blood meal. These extracellular sporozoites migrate rapidly to the liver via the blood stream by passing through a number host cells before actively invading hepatocytes. Inside the hepatocytes each sporozoite divides mitotically and differentiates into thousands of liver merozoites resulting in a multi-nucleated schizont structure (Exoerythrocytic schizogony).

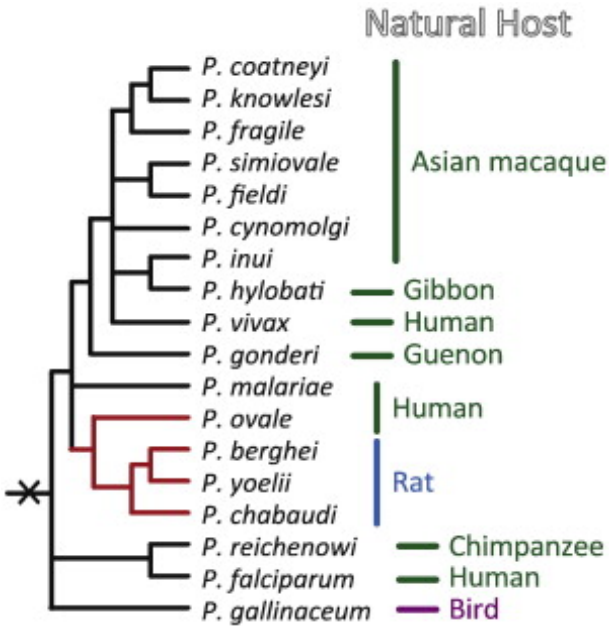


Fig. 2: Schematic tree of the genus *Plasmodium*. Ambiguous relationships are shown by multi-furcation. Close relationships confirmed by recent analysis using apicoplast genome-encoded protein genes were shown by red (7).

Merozoites when released from the schizont enter into the blood stream and invade the erythrocytes initiating the asexual blood-stage life cycle of the parasite. Free merozoites in the blood, rapidly invade erythrocytes, develop through ring, trophozoite and schizonts stages where they replicate and generate further infectious merozoites (Erythrocytic schizogony) (8). Some of the intra-erthrocytic forms develop into the sexual forms of the parasite- the male and female gametocytes. The gametocytes are taken up by the mosquito during a blood feeding and within the lumen of mosquito gut, male and female gametes emerge from respective gemetocytes. The gametes fuse to form zygote that tarnsforms into an ookinete and acquires motitlity to across the gut epithelium. Motile ookinete transforms into a multi nucleated oocyst on the basal lamina of the mosquito mid gut. The oocyst partitions into sporoblasts, where sporozoite formation or sporogenesis takes place. Sporozoites bud from the oocysts and are released into the hemolymph. Sporozoites migrate to the salivary glands and wait for transmission to the vertebrate host when they happen to obtain another round of blood meal (Fig 3).

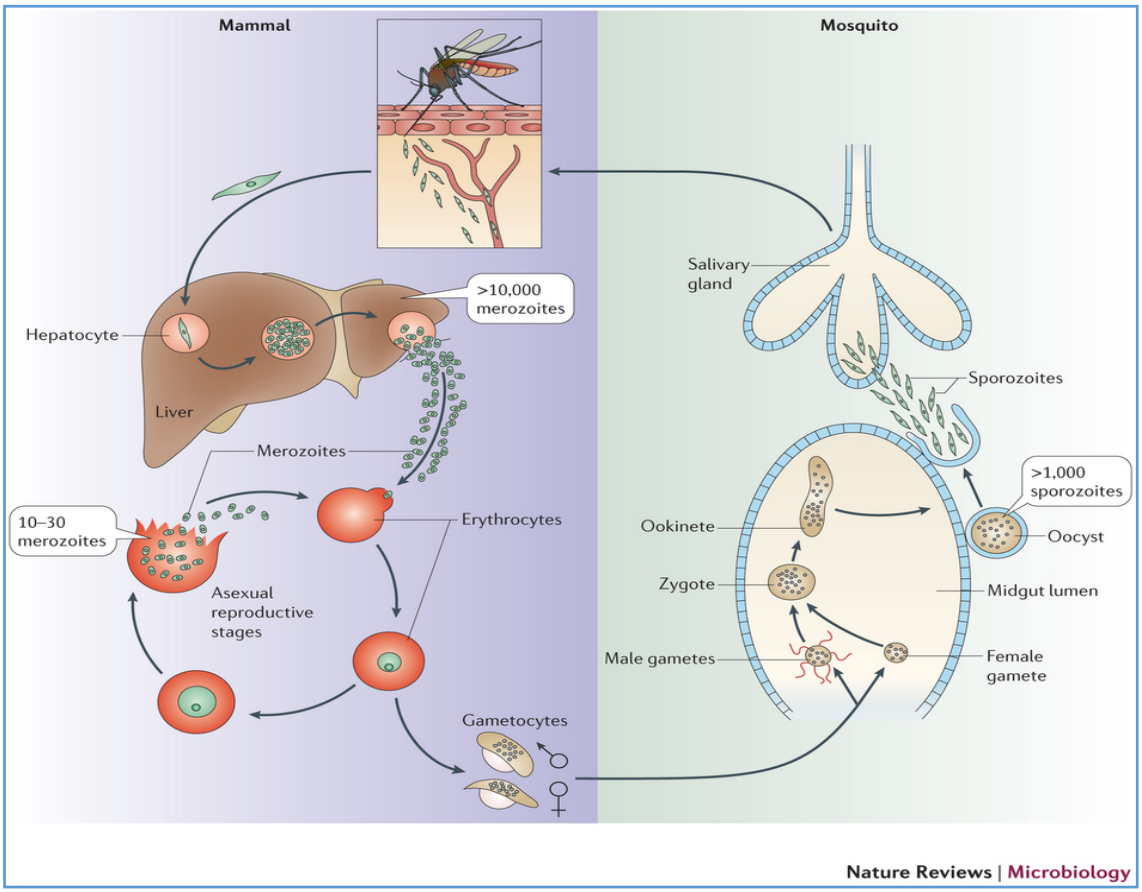


Fig. 3: The life cycle of *Plasmodium* species. *Plasmodium* cycle is initiated by injection of sporozoites by a female *Anopheles* mosquito during blood feeding; motile sporozoites enters the blood stream and reach the liver. Prior to hepatocyte invasion, the sporozoites breach several cellular barriers-notably the cells of the dermal layer of skin, the endothelial cells of blood vessels, the endothelial cells lining of the sinusoidal lumen, the Kupffer cells and several hepatocytes before they successfully establish infection. An intra-hepatocytic sporozoite generates thousands of daughter merozoites. These merozoites enter the blood stream and invade erythrocytes. Merozoites inside RBC divide and form new merozoites. Some intra-erythrocytic parasites transform into male or female gametocytes, which are taken up by a mosquito. Sexually dimorphic gametocytes give rise to male and female gametes respectively that fuse in the mosquito midgut. The motile zygote, called an ookinete, crosses the gut epithelium to transform into an oocyst and attaches on the hemocoel side, in which thousands of sporozoites develop. Sporozoites are released into the mosquito body cavity and later passes through salivary gland cells to enter the salivary ducts (9).

1.2.1 The sporozoite: journey from the mosquito gut to the liver hepatocytes

Sporozoites are the invasive stages of the *Plasmodium* life cycle. *Plasmodium* sporozoite is slender sickle shaped cell and is characterized by the presence of apicoplast, the apical ring, and unique secretory compartments called micronemes and rophtries that define the apical complex (Fig 4) (10). Sporozoites display complex behaviors, during their passage through the vertebrate host and within the mosquito vector. The behaviors include, gliding motility, invasion of target

cells, migration through and egress from target cells. The sporozoite plasma membrane is supported beneath by a unique actin-myosin motor, which allows the sporozoite for extracellular migration, cell traversal and invasion (10).

The sporozoites reach the salivary glands, attach to and invade the salivary gland cells where they exit into the secretory cavity and become dormant and stay viable for weeks' time, awaiting transmission to the vertebrate host during the process of obtaining a blood meal. Mosquitoes release the salivary gland sporozoites into the skin from where they are rapidly and selectively transported to the liver. At liver the sporozoites exit from the blood vessels and invade hepatocytes- the final destination of the sporozoites. Inside the hepatocyte sporozoite develop into Exoerythrocytic forms (EEFs), which replicate and forms thousands of liver merozoites (11).

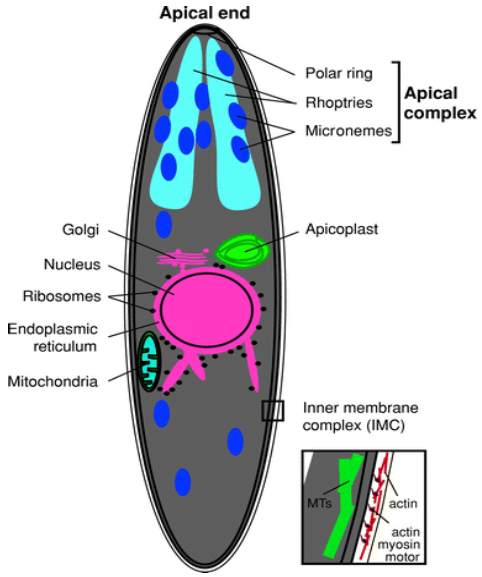


Fig. 4: Schematic representation of a *Plasmodium* sporozoite showing organization of apical complex at the apical end, cell organelles and subcellular structures (10).

1.2.2 Liver stage infection: Pre-erythrocytic stage – gliding and hiding

Plasmodium species the causative agents of malaria in mammals are obligatory intracellular parasites. The first site of infection is hepatocytes, where they replicate in few infected cells without showing any clinical manifestations (12). The pre-erythrocytic stages of *Plasmodium* are initiated by deposition of sporozoites in the skin by an infectious *Anopheles* mosquito. The sporozoites are highly mobile, though they do not have any cilia or flagella; they are propelled by a substrate dependent locomotion termed as gliding motility. Gliding motility maintains a fixed shape of the parasite and is essential for sporozoites to infect hepatocytes. For gliding motility and cell invasion, sporozoites requires ordered and coordinated release of proteins and other molecules, from sporozoites apical end that harbour the secretory organelles called micronemes and rhoptries (13).

Sporozoites traverse and move between the dermal cells until they find a blood capillary, which they penetrate and enter into blood circulation, finding their way to the liver. The sporozoite can disrupt membranes of host cells to move through and out of the cell. The cell traversal behavior of sporozoites was described in macrophages, epithelial cells and fibroblasts (14,15). Once they reach the liver sinusoids (liver blood capillaries with fenestrated endothelium), sporozoites traverse through liver resident macrophages (Kupffer cells) (16) or liver sinusoidal endothelial cells (LSECs) (17) to escape from the blood stream, passing through parenchyma and infect the hepatocytes (Fig 5). The sporozoites are highly selective for infecting host hepatocytes. The choice of host cell is an evolutionary event to support the prolific replication of parasite and ensures progression of further life cycle with the release of hepatic merozoites to infect RBC.

The commitment of sporozoites switching from migration through cells to infection of hepatocytes is still unknown. The tissue tropism of sporozoite towards hepatocytes is facilitated by specific interactions between secreted proteins of *Plasmodium* and host molecules. Once the cell traversal activity of the *Plasmodium* sporozoites is switched off, they productively invade hepatocytes by attaching to hepatocytes and form a moving junction (18). The interactions between hepatocyte and *Plasmodium* sporozoite are necessary for establishment and success of infection. The highly sulfated heparan sulfate proteoglycans (HSPGs) present on the surface of hepatocytes play a critical role by interacting with Circumsporozoite protein (CSP), a major surface protein of sporozoite (19). This binding is only required for sporozoite attachment to hepatocytes and not necessary for invasion under *in vitro* conditions (20). HSPGs belong to glycoprotein family and are majorly present on surfaces of mammalian cells and in the extra cellular matrix (ECM), and act as co-receptors for growth factors.

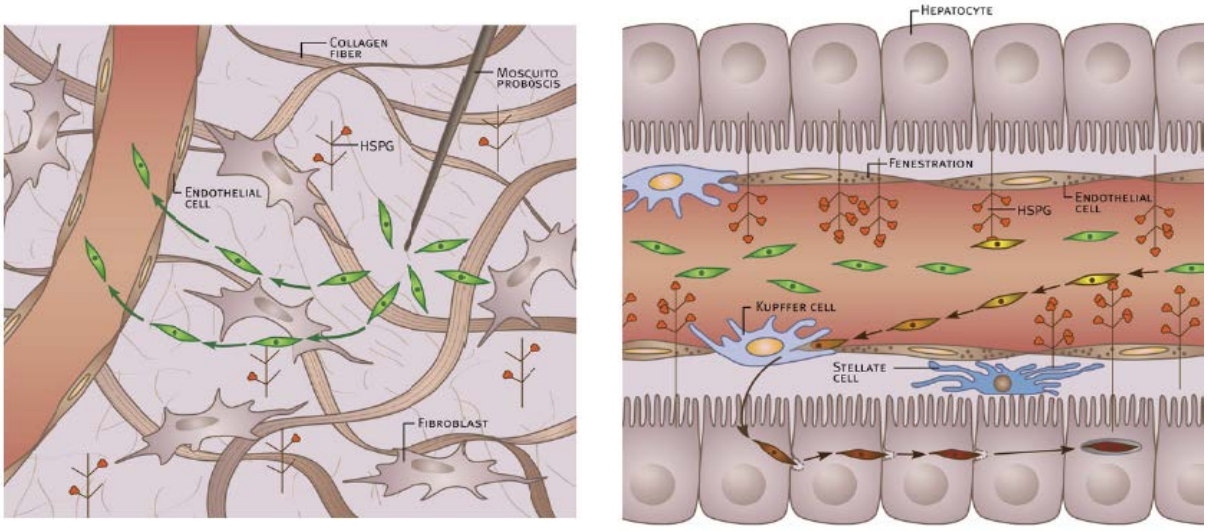


Fig. 5: The journey of sporozoites from the site of injection (skin) to the site of infection (liver). The *Plasmodium* sporozoites are deposited under the skin by the bite of a female infected mosquito. The sporozoites move through dermis, a low HSPGs expressing tissue, then contact with a blood vessel, enter into circulatory system and reach the liver. In the liver sinusoids, sporozoites glide over endothelium that contain high content of HSPGs, cross Kupffer cells by cell traversal activity. In liver parenchyma sporozoites migrate through several hepatocytes before it gets activated for productive invasion, a process that involves invagination of the hepatocyte plasma membrane mediated by invading sporozoite. The invagination subsumes resulting in the formation of the vacuolar structure called parasitophorous vacuole (PV) within which the sporozoites transforms into an exo-erythrocytic form (EEF's) (21).

The attachment of sporozoite to the host surface forms a ring-shaped intimate junction referred to as moving junction (MJ) formed when micronemes discharge their contents on host cell surface. The formation of MJ is the hallmark of host cell entry of the parasite. Host cell penetration by sporozoite is extremely rapid and completes within 20-30 secs. The moving junction exerts force and is pulled towards the posterior end of the sporozoite inside the nascent vacuole (Fig 6) and the sporozoite invades the cell and forms a Parasitophorous vacuole (PV), surrounded by invagination of the host cell plasma membrane resulting in a parasitophorous vacuolar membrane (22, 23 and 24).

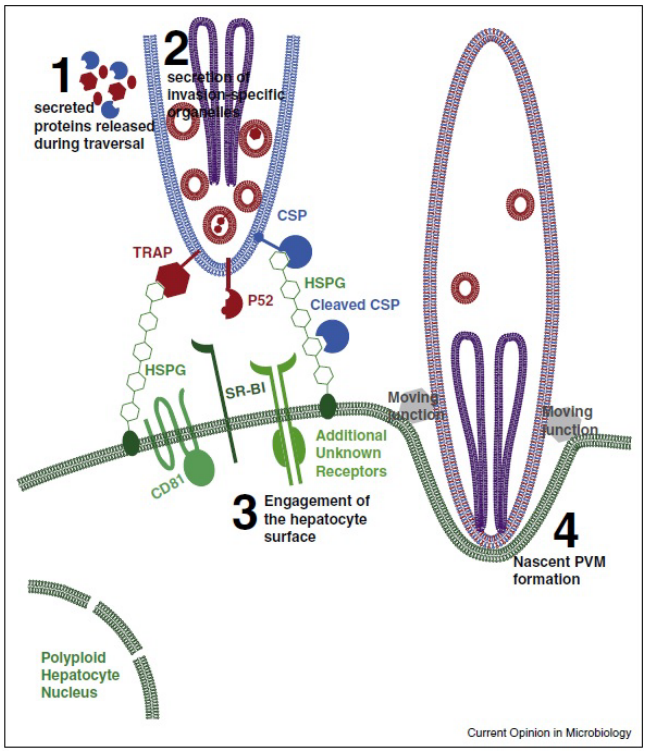


Fig. 6: Attachment and invasion of *Plasmodium* sporozoite. Gliding and traversal of sporozoites though skin and blood involves secretion of micronemal proteins (events 1, 2). After reaching the liver sinusoids, they traverse through LESCs, and engage a hepatocyte for invasion (event 3). The binding of sporozoite involves the interaction of CSP and HSPGs, upon attachment a moving junction is initiated by the sporozoite forming a nascent PVM (event 4) (25).

PVM shelters the parasite and prevents acidification of the compartment. PVM constitutes host cell-parasite interface, and is most likely central to nutrient acquisition, environmental sensing, host cell remodeling, waste disposal, and protection from host innate defenses. The presence of parasite proteins on the PVM was shown to be indispensable for the parasite to escape from elimination by the host cell (26). *Plasmodium* exports specific protein repertoire into the host cytosol using specialized machinery and the exported parasite proteins are speculated to coordinate the process of infection which involves several changes in host cells. Many of these exported proteins contain a canonical export motif called *Plasmodium* export element (PEXEL) or host targeting motif (HT) with a sequence RxLxE (x-any amino acid) (27,28). This signature motif is found in protein families in *P. falciparum* which belong to repetitive inter spread family (RIFINs) or sub-telomeric variable open reading frames (STEVORs) (29,30). Of approximately 100 single proteins, nearly 33 have orthologues in all sequences of *Plasmodium* species including *P. vivax* and rodent malaria species suggesting that protein export is not restricted to *P. falciparum* (31). However, not all exported proteins require a PEXEL/HT motif as export of PEXEL-negative exported proteins (PNEPs) have also been reported (32).

The presence of *Plasmodium* derived material was first observed in 1983 when anti sporozoite antibodies were used to stain the cells containing liver stage parasites (33). CSP, the immuno dominant antigen of sporozoites (34), and a major surface protein of the sporozoites harbours two putative PEXEL motifs one at N-terminus and other at the cleavable GPI anchor at C-terminus. CSP is consistently found and distributed in a punctate fashion throughout the host cell in the early hours after sporozoite invasion and upto 24 hr of liver stage development. Two inhibitory roles of exported CSP in host cells has been proposed based on CSP's association with host cell ribosomes and the nucleus (35,36), (i) inhibition of translation through binding of CSP to ribosomes (37) and (ii) rewiring of host cell gene expression by inhibiting the nuclear translocation of NFκB (36), though evidences for both the roles are still conflicting. Another PEXEL containing protein IBIS1 has also been localized during liver stage development; IBIS1 is retained at the PVM and also localized throughout liver stage tubo vesicular network (LSTVN), which includes membranous connecting structures from the PVM and autonomous vesicles in the host cells (38). However, depletion of IBIS1 had no apparent defects in the liver stage development. It is speculated that, as PEXEL/HT containing liver stage proteins could be present in the cytoplasm of the infected hepatocytes, they constitute potential candidates for antigen presentation on class I MHC molecules to CD8+ effector cells. An additional exported protein during liver stage infection is *P.berghei* liver specific protein -2 (LISP2), which can be detected in cytoplasm and nucleus of the host cell in early schizogony of *P.berghei* at 36 hr post infection (39). LISP2 lacks a PEXEL/HT motif at N-terminal, but possess a pentameric amino acid sequence that matches the PEXEL/HT motif suggesting the possibility of considerably more number of secreted proteins contributing to the parasite secretome during the liver stage infection than predicted.

During liver stage, sporozoite transforms into a trophozoite, which grows into a multinucleated schizont, with multiplication of its genome by $10^4 – 10^5$ times over a time course of 2-10 days. Parasite spends one third of the intra-hepatocytic phase by undergoing a process called de-differentiation, involving the transformation of a spindle, elongated shaped sporozoite into a rounded, trophic form. It involves the disassembly of molecular and cellular components that are essential for motility and invasion and discharge some entire structures (40). Parasite modifies the PVM during de-differentiation thereby enhances the parasites' ability to complete LS development. Following one round of asexual replication, the EEF's develop into a multinucleated schizont, which upon rupture release thousands of hepatic merozoites into the blood circulation (41). The merozoites initiate erythrocytic cycle by infecting red blood cells.

The escape from the host cell is crucial for the *Plasmodium* to progress through its life cycle and to promote its propagation. The parasite egress from the host cell follows a fixed program and involves the sequential breakdown of two membranes, the rupture of PVM preceding the breakdown of the host cell membrane (42-45). The actual egress of the merozoites from the host cells is initiated by the detachment of the infected hepatocyte. Facilitated by a process called budding, parasites are released into the blood stream enveloped in host cell membrane derived vesicles- the merosomes that contain hundreds to thousands of merozoites (44,46,47). Cysteine proteases of the serine repeat antigen (SERA) family are being studied for their potential role in membrane disruption during liver stage egress (48). An up-regulation of SERA proteins has been reported in the late liver stages; *Pb*SERA3 is detected in PVM and is released into hepatic cytoplasm following the rupture of the PVM (49,50). Another plasmodial protein reported to localize to the PVM of the liver stages termed liver- specific protein 1 (LISP1) is involved in host cell egress by the liver stage parasites, as LISP1 deficient parasites fail to rupture the PVM and remain entrapped inside the host cell (51).

1.2.3 Blood stages

Once into the blood, multiplication of *Plasmodium* inside erythrocytes is responsible for all related morbidity and mortality. Intense morphological and structural changes occur in erythrocytes after parasite invasion, impairing their circulation and altering physical properties (52). Parasitized RBC are more rigid and adhere to vascular endothelium and to other cell types (53). The increased rigidity and cytoadherance of infected RBC result in increased haemodynamic resistance in the microvasculature and play crucial role in the pathogenesis of malaria (54). After invasion, *Plasmodium* forms a membranous envelope surrounding it designated as PV whose organisation is similar to that described for liver stages. *Plasmodium* export many proteins beyond the PVM to the erythrocyte (55,56). The exported proteins modify the host erythrocytes in many ways, making the erythrocyte environment suitable for parasite proliferation. Few of these exported proteins involved in the active formation of knob like structures on the erythrocyte membrane (57), some are associated with membranous structures called Maurer's clefts (58), which are primarily required for the trafficking of parasite proteins to the host cell surface through cytoplasm. The exported proteins are also thought to have remarkable roles in the acquisition of nutrients and supplements from the host cells, and conferring rigidity to the host erythrocyte cytoskeleton (59).

1.3 Immune evasion by *Plasmodium* and antigenic polymorphism

Host immune responses to *Plasmodium* involves the antibody responses elicited against sporozoites, arresting sporozoite entry of hepatocytes and cytotoxicity of CD8+ T cells on hepatocytes harboring EEF's (60). CD4+ T cells are crucial for protection against blood stage parasites which provide assistance for antibody production and secretion of IFN-γ to activate macrophages thereby promoting the clearance of infected RBCs. Pro inflammatory cytokines IFN-γ, IL-12 and TNF-α are essential mediators for protective immunity against blood stage parasites (61,62). IFN-γ and TNF-α act synergistically to optimize the production of NO which is involved in killing of the parasite (63,64). IFN-γ confers resistance to reinfection and protects from clinical attacks of malaria (65,66).

Immune evasion of malaria parasites includes adoption of intracellular parasitism, as once the sporozoites or merozoites enter the host cells, antibodies can no longer access them. Furthermore, RBC do not express MHC on their surface that facilitates merozoites to escape from CD8+ T cells recognition. The adherence of infected RBC to endothelial cells protect the parasites from entering into liver and spleen where mature parasites are trapped and digested. T- cells and antibodies recognize the antigens in a specific manner and will not recognize different epitopes. Therefore, changing antigenicity termed as antigenic diversity/polymorphism is a strategy for parasites, to have expression of different alleles of a gene in different parasite populations resulting under the immune pressures of the host. MSP-1 a leading vaccine candidate has many alleles and antibodies to MSP-1 specific to one allele does not recognize the MSP-1 of other alleles (67,68). There is a high occurrence of tandem repeats in malaria antigens, which have a pivotal function in immune evasion by affecting the affinity maturation of antibodies and function as a molecular smokescreen for the critical epitopes. PfEMP1 an infected RBC surface associated antigen which is highly accessible to antibodies, the "var" gene encoding PfEMP1 is present in approximately 50 multiple loci on different chromosomes and parasites are capable of switching expression between different loci in blood stages showing "antigenic distinct wave" of parasitemia (69).

PfEMP1 binds to CD36 expressing on antigen presenting cells (APC), inhibits the maturation of APC resulting in impaired recognition of parasite antigens by T cells (70) and alters the secretion pattern of cytokines. It induces the secretion of suppressive IL-10 instead of protective IFN-γ and IL-12 (71,72). The parasites can activate APCs or T cells to activate TGF-β (71), a suppressive cytokine and parasites can produce prostaglandins which can act as immune suppressive biological compounds (73). Sexual stages of *Plasmodium* recruit the soluble human

complement regulator factor H, an evasion strategy to avoid complement mediated parasite clearance within the mosquito host (74).

1.4 Pathogenesis, diagnosis and treatment for malaria

Malaria is a multifactorial disease, the clinical outcome depends on many factors, such as host and parasite genetics, age, previous exposure to infection, nutritional status, geographic and socio-economic status (75). The first symptoms of malaria in normal individuals include fever, chills, headache, muscle pain, vomiting and lethargy. Malarial paroxysms associated with high levels of circulating cytokines appear between 7-15 days post infection. If left untreated, the symptomatic uncomplicated malaria caused by *P. falciparum* can rapidly emerge into severe illness and lethality.

1.4.1 Diagnosis of malaria

The prompt and accurate diagnosis of malaria is critical to the effective treatment, which alleviates suffering and decreases community transmission effectively. The nonspecific nature of symptoms and clinical signs of malaria may result in non-treatment of other diseases or over-treatment of malaria in endemic areas, and misdiagnosis in non-endemic areas (76). Malaria is diagnosed using different techniques. Conventional microscopic examination of thin and thick smears remains gold standard for the malaria diagnosis, smears are stained using Giemsa, Wright's or Field's stain (77), a technique that is both inexpensive and reliable. Other concentration techniques like Quantitative buffy coat method (QBC) (76) enhance the microscopic examination. Quick and convenient rapid diagnostic tests like Paracheck (78), OptiMAL (79), and ICT (80) are currently implemented, but are costly. Recent molecular diagnostic methods like PCR (81), mass spectrometry (MS) and flow cytometry (FCM) (82) are appropriate for research laboratories to identify drug resistance, species identification and for quantifying parasite density in low parasitemia conditions. Factors like the urgency of diagnosis, level of endemecity, budget and effectiveness of technicians are influencing the choice of the malaria diagnostic method.

1.4.2 Treatment of malaria

Early malaria detection and proper treatment with safe and effective drugs still remains the important measure for the malaria case management. If not properly treated, due to missed or delayed diagnosis, the disease may progress from a mild condition through complicated to severe form of malaria (83). Treatment depends on severity of the disease, patient's age, and therapeutic efficacy and availability of the antimalarial drug. There is a limited armoury of anti-malarial drugs and a lack of affordable drugs which can be used to treat or prevent malaria. The widely used drugs

are (i) quinine and its derivatives which include chloroquine, primaquine, amodiaquine and mefloquine, (ii) antifolate drugs including pyrimethamine, proguanil and trimethoprim, (iii) sulfa drugs including salfalene, sulfamethoxazole and sulfadoxine, (iv) artemisinin, its derivatives and other combination drugs include arteether, artelinate, artemether, and artesunate (84). Tetracycline derivatives like doxycycline are also used in combination with quinine for treatment and prophylaxis.

1.5 Vaccines against malaria

Vaccines against malaria are considered amongst the most important modalities for potential prevention and transmission reduction of malaria disease. There is an intense effort for research and development in this field by many groups over the last few decades. Despite these, there are no vaccines against malaria currently. The complex life cycle of *Plasmodium* provides many targets for vaccine design (85). Normally the immunity is naturally acquired which is short lived and allows intermittent infections to occur. In contrast to naturally acquired protective immunity, a long lasting protective immunity is achieved through γ-irradiated sporozoite inoculation which prevents the development of blood stage infection in response to infective sporozoites challenge (86-88). At the blood stage level, highly accessible surface antigens such as *Pf*EMP1 and MSP offer potential targets for vaccine development (85). Extensive antigenic variation and complex interactions with the immune system are the major hurdles for identification of conserved and optimal epitopes as targets for malaria vaccine development (89).

There are a significant number of vaccines being evaluated in clinical trials (90). RTS, S/AS01 a pre-erythrocytic stage targeting protein particle vaccine with a powerful adjuvant is currently under phase III multicenter trials (91-93), and VAR2CSA-PAM vaccine is in pre-clinical development and manufacturing phase. Vaccines targeting asexual blood stages are expected to prevent disease but not infection, while on other hand, the use of sporozoites and liver stage parasites offer highly potential targets for vaccines that can completely block malaria transmission (94). Transmission blocking vaccines (TBVs) target the sexual stages of the parasites which infect mosquitoes and induce immune responses against them. TBVs protect the immunized individuals from becoming as a source of transmission (95) and will work in conjunction with other vaccines (96).

Despite great progress in the field of malaria vaccine development, the achieved protective efficacies are rather very low (eg. RTS,S has only 30-50% efficiency) (91,97), which urges for a thorough understanding of immunological and epidemiological factors that might influence the protective efficacy of the vaccines. Hitherto, out of many *Plasmodium* genes, a very

small number have been pipelined into sound pre-clinical development (90) due to inadequate protection, poor immunogenicity and other efficacy related issues.

1.6 Toxoplasmosis

Toxoplasmosis is the pathological and clinical consequence of acute infection with the apicomplexan obligate intracellular parasite *Toxoplasma gondii*. *Toxoplasma* can infect humans as well as almost all warm blooded animals. Though there is tremendous variation in the local rates, at least one third of the human population are infected by *Toxoplasma*, making it one of the most successful parasitic infections. It is generally assumed that an estimate of 25 to 30% of world population is infected by *Toxoplasma* (98). The disease is generally benign and goes unnoticed often, and has health-threatening and fatal implications in immunocompromised individuals and congenitally infected foetuses. *Toxoplasma* infection has very diverse clinical manifestations ranging from encephalitis in immune deficiency conditions, myocarditis, ocular-toxoplasmosis, and hydrocephalus to mental diseases.

1.6.1 Life cycle of *Toxoplasma*

In nature the parasite exists in 3 forms: (i) the sporozoite harbouring oocyst which are exclusively produced in the intestine of the felid family animals and are released in their faeces contaminating soil and water resources. (ii) The tissue cyst, which contain and release bradyzoites, and (iii) the tachyzoites, the rapidly dividing proliferative form of the parasite which can infect virtually all nucleated cells. They reside and multiply within the parasitophorous vacuole inside host cells. The tachyzoites dissemination into different tissues represents acute infection.

Toxoplasma gondii has 2 phases of life cycle and is completed in two hosts: (1) sexual cycle occurs in the small intestine of the feline family (definitive host) and (2) asexual cycle takes place in infected animals, including humans (intermediate hosts). It can be transmitted not only between the definitive and intermediary hosts, but also between definitive hosts and or even between intermediary hosts via carnivorism. Sexual reproduction of *Toxoplasma* occurs only in domestic and wild cats, after the ingestion of tissue containing cysts from an intermediary host, the cyst wall is destroyed by the gastric enzymes releasing the bradyzoites. Bradyzoites settle within enterocytes and undergo self-limiting number of asexual multiplications resulting in the development of merozoites within the schizonts (99). Few merozoites undergo sexual development forming male and female gametes (gametogony) (100). Oocysts are formed within enterocytes after fertilization, and are liberated by the disruption of the enterocytes and excreted as unsporulated forms in cat faeces. The shedding of oocysts starts after 3 to 7 days after the ingestion of tissue cysts, and the number ranges up to 100 million oocysts in their faeces (101).

In the external environment, the process of sporogony occurs with a meiotic reduction and associated morphological changes resulting in the formation of a sporulated oocyst, with two sporocysts, each containing 4 haploid sporozoites. Within the intermediary hosts, oocysts upon ingestion along with food or water undergoes asexual development. After oocysts ingestion, sporozoites are liberated, and penetrate the intestinal epithelium and differentiate into tachyzoites. Tachyzoites replicate rapidly by endodyogeny inside any kind of nucleated cell and disseminate throughout the organism. After 7 to 10 days post infection tissue cysts arise predominantly in brain or musculature resulting in the conversion of tachyzoites to bradyzoites. Through uncooked or raw meat, tissue cysts are ingested by the intermediary hosts, and they pass through the digestive tract, cysts are ruptured causing the release of bradyzoites. The bradyzoites will infect the new host's intestinal epithelium and differentiate back into tachyzoite stage for dissemination throughout the body.

1.6.2 Infection

Following egress, tachyzoites glide in the extracellular environment for relatively short duration which provides the parasite an opportunity to disseminate to new tissues. The first initial contact of the parasite with the new host cell is through the GPI anchored surface antigens known as SAGs, SAG1 recognizes the sulfated proteoglycans on the host cell membrane (102). Reduced amount of SAG1on the surface (103) or addition of exogenous glycans (104) inhibits the host cell attachment by the parasite. Following initial recognition, parasite engages into a tighter interaction mediated by the secretion of MIC (micronemal) proteins resulting in the formation of a MJ (105). The MJ is a cooperative structure formed collectively by AMA1 and rhoptry neck proteins (RONs) (106). AMA1 anchors towards the parasite plasma membrane and interacts with the RON4/5 protein complex which is anchored in the host cell plasma membrane mediated by RON8 for proper interaction (107,108).

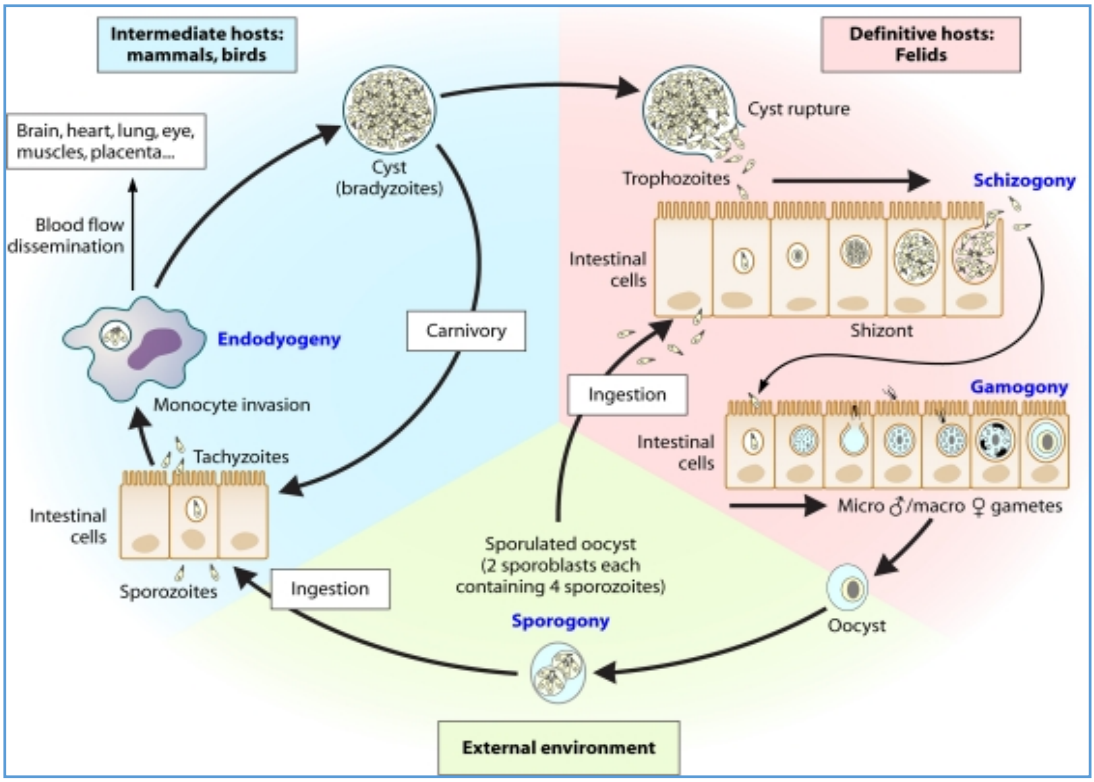


Fig.7: Life cycle of *Toxoplasma gondii.* Infection, biology and replication of trophozoite, sporozoite and bradyzoite stages of the parasite in their respective hosts (109).

1.6.3 Immune evasion by *Toxoplasma*

Host cell resistance to *Toxoplasma* requires both innate and adaptive immune responses. IFNγ is critical for both responses, which upregulate the expression of anti-parasitic genes through a variety of mechanisms to kill the parasite. IFNγ activates degradation of the PVM by IFNγ-regulated GTPases and degradation of essential nutrients such as tryptophan by indoleamine dioxygenase (110). *Toxoplasma* evades the IFNγ effectors by interfering with the expression of IFNγ regulated genes (111). CD40 a member of TNF family can kill *Toxoplasma* by inducing mobilization of autophagy related proteins on to the PVM, followed by degradation of PV and the parasite (112). *Toxoplasma* can evade CD40 induced autophagy by activating EGFR signaling, thereby escaping killing by autophagy (113). *Toxoplasma* inhibits host cell apoptosis via inhibiting caspase 3 activation (114), activation of cell survival signaling pathways (115), increased expression of anti-apoptotic proteins (116,117) and inhibition of pro-apoptotic proteins (118).

1.6.4 Diagnosis, treatment and vaccines for Toxoplasmosis

Traditional non DNA based techniques to detect *T. gondii* include microscopic examination of tissue cysts following staining with H&E, Giemsa and PAS stains (119,120). Bioassays using laboratory animals (cats and mice) is a gold standard for detecting *T.gondii* infection, but not applicable to large scale screening (121). A variety of serological tests such as modified agglutinin test (MAT) (122), dye test (DT) (123), ELISA (124), and indirect fluorescent antibody test (IFAT) (125) have been developed to detect different antigens. Imaging techniques such as magnetic resonance imaging (MRI) and computerized tomography can facilitate the toxoplasmosis diagnosis and to monitor the therapeutic effect (126). In addition to conventional serological methods, molecular diagnostic techniques are used to diagnose the infection in prenatal and immunocompromised patients. Molecular techniques include conventional PCR (127), real-time PCR (128), Loop-mediated isothermal amplification (LAMP) (129), PCR-RFLP (130) and Rapid Amplification Polymorphic DNA (RAPD) PCR (131).

For the treatment of toxoplosmosis, the most recommended drugs are those that primarily act against the tachyzoite form of the *T. gondii*. Among all, pyrimethamine is the effective agent. Folinic acid should be administered coupled with the drug to prevent bone marrow suppression. Under certain circumstances a second drug such as sulfadiazine or clindamycin should be added (132). The most effective therapeutic combination available is pyrimethamine with sulfadiazine (133) or trisulfapyrimidines (134) which are active against tachyzoites and function synergistically when used in combination.

Currently there is no licensed vaccine to human toxoplasmosis. Only live attenuated S48 parasite strain "Toxovax" has been licensed for use in ewes and not suitable for human use (135). Current research is focused on vaccine candidates which can induce protective and systemic responses to mimic the lifelong immunity conferred by natural infection. Vaccine developing approaches has included the use of purified *T. gondii* surface antigens such as p30 and SAG1 (136,137), mutant or live attenuated strains of the parasite (138), or plasmids encoding colony stimulating factors (139).

1.7 Post translational modifications

The reversible post translational modifications (PTMs) of proteins provide cells with a rapid and dynamic mechanism to modulate the functionality of protein pathways in response to many stimuli, including pathogen invasion and development. Certain type of PTMs, such as acetylation, phosphorylation, glycosylation and proteolytic cleavage (irreversible) of substrates are highly conserved among prokaryotes and eukaryotes, while other modifications such as ubiquitin and ubiquitin like molecules are found mostly in eukaryotic organisms (140).

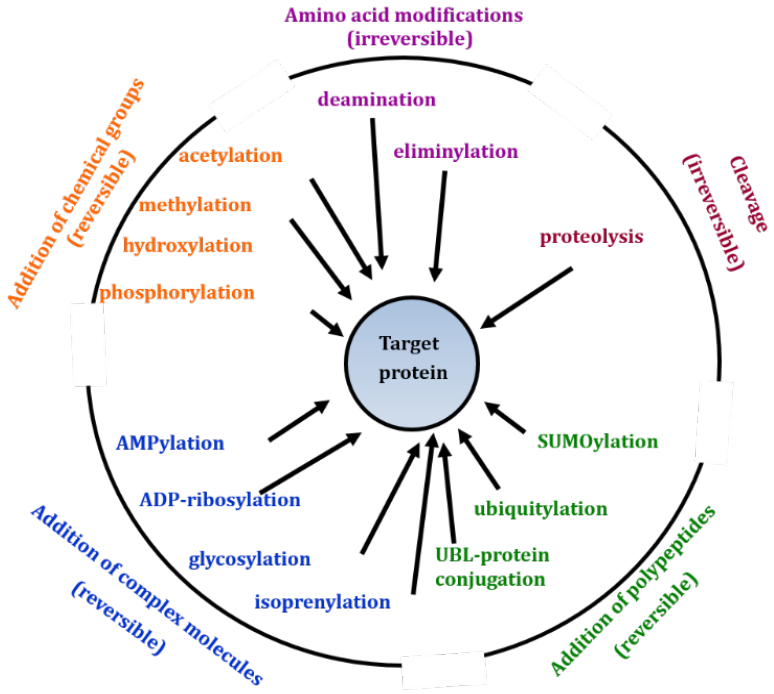


Fig. 8: Diversity of post translational modifications. Schematic representing the reversible and irreversible modifications of proteins (141).

Recently ubiquitin and ubiquitin like modifiers/proteins (Ubls) have emerged as central regulators in mediating host immune response to infection. They are diverse and coordinated by specialized enzymatic cascades, and have a range of linkage topologies that contribute to an incredibly rich regulatory potential. Ubls include Atg8 (142), Nedd8 (143), ISG15 (144) and SUMO (145) as shown in Fig 8. Among all Ubls, the small ubiquitin like modifiers (SUMOs) are predominantly located in the cell nucleus, and are reversibly attached to lysine residues of target proteins only by a small set of known enzymes (146). Nonetheless, SUMOs can conjugate to a large repertoire of proteins (147) and functionally implicated in regulation of distinct cellular processes, including nuclear functions such as chromatin remodeling, RNA processing, DNA

repair, transcriptional regulation, cell cycle progression, nucleocytoplasmic transport, differentiation and development, and apoptosis (148). More recently, a significant number of non-nuclear functions of SUMOylation have been identified, such as regulation of metabolic pathways (149,150) and ion channel activity (151,152). Due to this pleiotropic ability to modify many proteins and influence a wide range of cellular processes, SUMOylation is an attractive target for pathogenic organisms to modulate the host cellular milieu to favor pathogen survival, replication and/or maintenance.

The SUMO conjugating machinery is highly dynamic and responsive to stress stimuli, with global changes to SUMOylation happening rapidly after cells have been exposed to DNA damage (153,154), heat shock (155,156) or proteasome inhibition (157,158). Thus, stimuli triggered SUMOylation modulates nuclear functions to protect cell integrity. Recent investigations also have implicated SUMOylation as a critical function in activating host responses against intracellular pathogens, particularly against bacteria (159,160), DNA viruses that enter the nucleus (161,162), HIV (163) and other RNA viruses (164). As such, some bacteria and many viruses encode proteins that actively reduce the global content of SUMO conjugates or suppress host SUMOylation in infected cells (160,165).

While ubiquitinylation is predominantly involved in quality and turnover of proteins, SUMOylation is functionally less well understood and appears to have major pleiotropic effects on activity and/or intracellular localization of proteins. The relationship between SUMOylation and phosphorylation is complex, although for some proteins phosphorylation is important for SUMOylation (166), and there are examples where both these PTMs antagonize with each other (167). There is an extensive cross talk between ubiquitin and SUMO modifications, as they are seen in coordination in a mutually exclusive manner in some cases (168).

1.7.1 Host cell PTMs and exploitation by pathogens

Post translational modifications are growingly considered as key strategies used by pathogens to modulate host factors which are critical for infection. PTMs represent efficient targeting strategies to modify host proteins by altering their half-lives, activities or intracellular localization. The first ever report that a pathogen could modulate a host PTM occurred with the discovery that *Corynebacterium diphtheriae* produces diptheria toxin which mediates ADP-ribosylation and thus inhibits the activity of host Elongation-Factor-2 (EF-2) (169). YopH, an effector secreted by *Yersinia* species with its potent phosphatase activity dephosphorylates several host proteins leading to the inhibition of host cell phagocytosis (170,171). OspF, produced by *Shigella flexneri* is secreted into host cell cytoplasm and dampens the host immune responses by dephosphorylating

host MAP kinases (172). Virulence factors produced by *Vibrio parahaemolyticus* (VopS) and *Histophilus somni* (IbpA) are able to mediate AMPylation (addition of AMP) of host Rho-GTPases, which regulates the cell cytoskeleton dynamics (173,174).

The papain like proteases of SARS virus and NS1 protein of influenza B interfere with the ISGylation (addition of ISG15 (interferon stimulated gene 15) machinery of the host cell (175). BPLF1 protein of the Epstein- Barr virus (176) and Cif effector protein secreted by *E.coli* interfere with the neddylation (addition of Nedd8) of host proteins leading to a deregulation of host cell cycle (177). To interfere with host ubiquitination process, pathogens encode their own E3 ligases, deubiquitinase (DUB) like proteins or adapter proteins that bind to host E3 ligases. The E6 onco-proteins of HPV16/18 recruit a host E3 ligase and induce the degradation of host p53 facilitating the transformation of the infected cells (178). SseL, secreted by *S. typhimurium* displays DUB activity and inhibits the degradation of host IκBα thereby deregulating the NFκB pathway (179).

As similar to the ubiquitin system, several bacterial effector and viral encoded proteins target or mimic the components of the SUMOylation machinery, thus increasing or decreasing SUMOylated protein content of the host cell (141). K-bZip, encoded by KSHV displays E3 SUMO ligase activity, and participates in the SUMOylation of host cell proteins like p53 and Rb, thereby modulating the host gene expression to facilitate viral infection (180). VP35, encoded by Ebola virus binds to host E3 ligase –PIAS1 and increases the SUMOylation of IRF7, which downregulates the interferon expression that contributes to the dampening of antiviral response (181). Gam1, encoded by adenovirus targets the host SUMO E1 enzyme to degradation thereby inhibiting the SUMOylation machinery and further affecting the host cell transcription (182).

Listeria monocytogenes, a well-studied food borne bacterial pathogen secretes a toxin "Listeriolysin", which degrades the E2 enzyme (UBC9) of host SUMOylation machinery, leading to a global decrease in the level of SUMOylated proteins in the infected cells, thus altering the activities of host factors critical for infection (160). XopD, an effector protein secreted by a plant pathogen *Xanthomonas campestris* into the host cell cytoplasm induce deSUMOylation of several host factors by acting as an SUMO- specific protease. XpoD is known to alter host gene expression, promote pathogen multiplication, and delays the onset of leaf necrosis and chlorosis (183).

1.8 Protein homeostasis

All proteins must accomplish their native confirmation to ensure optimal cellular functioning. This is achieved by homeostasis of proteins termed "proteostasis", comprised of several conserved stress response pathways that regulate protein synthesis, trafficking, folding and degradation, to collectively attain the stability and functionality of the proteome (184). The proteostasis phenomenon integrates the physiological milieu of the cell through stress signaling pathways including the endoplasmic reticulum (ER) and heat shock response (HSR), and mitochondrial unfolded protein responses (UPR) (185,186) that sense an imbalanced compartmental distribution of proteins in the face of extrinsic and intrinsic challenges. Proteostasis constitutes other essential mechanisms including the ubiquitin-proteasome and the autophagy system providing the major pathways for degradation and clearance; the inflammatory and oxidative stress defense pathways; pathways regulating Ca^{2+} gradient between the endoplasmic reticulum and cytoplasm; and histone deacetylases (HDACs) that regulate the chromatin structure (187).

The ubiquitin-proteasome system (UPS) is the major proteolysis complex responsible for the degradation and recycling of target proteins, thus playing an important role in intracellular protein quality control. It mediates the degradation of several short lived proteins that are involved in signal transduction, cell cycle regulation and apoptosis, and is responsible for the recycling of damaged or abnormal proteins, which would otherwise accumulate and turn harmful to the cell (188). The proteasome manages the proteostasis in the cell via an UPS-specific enzymatic cascade, in which proteins become labelled with a small ubiquitin (Ub) tag. Selectively based on the type of ubiquitin tag on the protein, it is determined and designated for further roles in cellular processes like signal transduction, trafficking or DNA repair, or it will be degraded by the proteasome (189,190). Deficiencies in the proteasome pathway leads to metabolic, neurodegenerative, cardiovascular and oncogenic disorders (184). Ubiquitin conjugated proteins are degraded by 26S proteasome, a 2000 kDa ATP dependent proteolytic complex (191). The 26S proteasome is a dynamic structure built in a modular manner, comprising a barrel shaped catalytic core complex (20S) capped at one or both ends by a regulatory complex (19S). 19S regulatory complex is also called the proteasome activator (PA) 700 and confers substrate specificity and regulation. Another regulatory complex the 11S (or PA28) can replace the 19S and activate the proteolysis of short peptides.

1.8.1 Regulation of proteasome activity

Protein quality control and turnover by the proteasome is of paramount significance for cell homeostasis. The regulation involving activation and inhibition of proteasome is complex and still widely unexplored. Under physiological conditions activation of the 20S core particle (CP) requires the binding of 19S (PA700) regulatory particle to one of the outer rings of the CP leading to the formation of the 26S proteasome which is functionally active. On the other hand, CP can be activated by binding of other components such as PA200 (PSME4) monomeric and PA28α/β/γ (11S) hetero-multimeric complexes (192-195). The physiological inhibition of proteasome is attained by binding of PSMF1 (proteasome inhibitor PI31 subunit) (196). PSMF1/PI31 is a 31 kDa protein, proline rich and highly conserved among metazoans. PI31 inhibits the proteasome activity by directly binding to the outer rings of CP or by competing with the activating partners of CP (197-199).

PI31, initially identified based on its ability to inhibit activity of 20S proteasome *in vitro* (196) can nevertheless, also activate the 26S proteasome *in vitro* and serves as a crucial physiological regulator (200). PI31 possesses a functionally important HbYX (Hydro phobic amino acid- Tyrosine- any amino acid) motif at the C terminus, which cause inhibition by hindering substrate access to the CP (201,202). Previous work indicated that PI31 function is regulated to increase proteasome activity under conditions where the proteolytic activity is maximal (200). ADP-ribosylation controls the activity of PI31 by blocking its binding to the CP (197). However, the exact mechanism by which PI31 regulates proteasome activity remains unknown.

1.8.2 Proteasome system of *Plasmodium*

While completing the different stages of its complex life cycle, *Plasmodium*, experiences different host environments and undergoes phenomenal variation in shape, size and motility. Protein regulation is an important mechanism for rapid transformations of *Plasmodium*, during its life cycle progression in target organs of the vertebrate host and the mosquito vector, specifically in the stages having high replication rates (203). The rise and fall of protein subsets expression during specific stage transitions in life cycle suggest a coordinated control of protein turnover during parasite development. *Plasmodium* while rapidly adapting from human to mosquito and vice versa is exposed to shifts in temperature that might additionally induce a stress response requiring regulation by the UPS-proteasome. *In silico* predictions implicate that nearly half of the *Plasmodium* represents targets for ubiquitination (204). Inhibitor studies in *Plasmodium* sp. reveal an essential

role of proteasome for liver, blood and transmission stages of the parasite, suggesting proteasome as possible promising target in malaria therapy (205-207).

Plasmodium, appears to possess a functional eukaryotic proteasome and a bacterial ClpQ/hsIV threonine peptidase like complex, but the specific roles of each of them within the parasite are not known (208,209). Although a complete intact proteasome complex has not been isolated from *Plasmodium,* the sequencing of *P. falciparum* revealed a complete set of homologs for subunits of the eukaryotic proteasome (210-212). The expression of the subunits of the 20S particle (7α and 6β) and 19S regulatory particle of the putative *P. falciparum* proteome suggest an important role of this multi-catalytic complex in parasite intra-erythrocytic cycle (213). The expression of these subunits peaks during stage specific transition of parasite that accompany structural, developmental and metabolic changes and also during generation of new daughter parasites enabling to complete the cycle and invade new host cells (214).

Chapter 2

Elucidating the effects of *P. berghei* (*Pb*) sporozoite and *T. gondii* (*Tg*) infection on host cell SUMOylation

2.1 Introduction

The development of *Plasmodium* sporozoites inside the liver hepatocytes is the first obligatory step before the onset of the disease. Very little is known about the interactions between the parasite liver stage and the host cell. Understanding the parasite's prerequisites for establishing infection during this period may be crucial for developing any form of early intervention and may have implications for developing effective drug or vaccines strategies. Further it provides a timely intervention to interrupt the parasites life cycle at an early stage before the onset of clinical symptoms associated with emergence of the blood stage infections. Hepatocytes are responsible for multitudinous metabolic processes, including metabolism of carbohydrates, lipids, synthesis of proteins and uptake of nutrients. Liver also has clearance functions that involve uptake of waste products and pathogens from blood (215). As an obligate intracellular parasite, *Plasmodium* depends on host cell resources for nurturing and supporting its development. This dependency is important for the liver stage parasites, as the replication rate in the liver is higher than that in the blood.

Unlike erythrocytes, hepatocytes are capable of processing and presenting *Plasmodium* derived peptides on infected hepatocytes that forms the basis for activation of antigen specific T cells (216-218). These antigen specific T cells secrete effector cytokines like IFN-γ that eliminate the infected hepatocytes in a cytolytic manner (219). While inhibitory effects of cytokines like TNF-α has also been reported on the pre-erythrocytic stages of *Plasmodium* (220), several lines of evidence also reveal that liver stages inhibit the host cell apoptosis (221), implicating the parasite's strategic mechanism to overcome immune clearance and initiate a successful break through infection. Nevertheless, the range of mechanisms used by the parasite to manipulate and exploit the host cell environment for their successful survival, and the gene pool responsible for this process remain largely unknown.

Intracellular pathogens such as bacteria and viruses have evolved multiple times to adopt highly sophisticated mechanisms to accommodate their specific developmental needs and survive in the harsh host cell environment. Recent evidences has shown that protozoan pathogens from the phylum Apicomplexa, also subvert their host cell functions to access essential nutrients and to escape from defense mechanisms and immune responses. They profoundly reorganize the host cell components for their needs and ensure optimal growth and

persistence (222). Targeted host processes include modulation of gene expression, protein synthesis, membrane trafficking, antigen presentation and apoptosis (215).

In addition to the orchestration of the above host cellular mechanisms, parasites have evolved to manipulate specialized host cellular processes viz. post translational modifications (PTMs). PTMs include the conjugation of simple chemical groups, such as methyl, acetyl, phosphate, or hydroxyl groups; more complex molecules, such as lipids, sugars, ADP ribose or AMP; modification of specific amino acid side chains, such as deamidation of glutamine residues; proteolysis by cleavage of peptide bond; and addition of small peptides, such as ubiquitin or ubiquitin-like proteins (UBLs). PTMs proved to be highly versatile tools and stratagem used by both eukaryotic and prokaryotic cells to regulate the function of key proteins. PTMs constitute systematic strategies to direct the target proteins to modify half-lives, activities or intracellular localization that are crucial for infection. The first study has been reported four decades ago providing evidence that pathogens could use PTMs for efficient infection (169). Since then, significant number of host PTMs induced, mediated or counteracted by different pathogens have been reported (223) (141).

Besides ubiquitin, a family of substrates called ubiquitin like proteins are covalently linked to the target proteins. One such member of UBLs is SUMO (Small Ubiquitin-like Modifier), a 10kDa polypeptide found ubiquitously in the eukaryotic kingdom. SUMOylation, the covalent linkage of SUMO moiety on lysine residue of the target protein is mediated by the sequential action of three enzymes- viz. E1- SAE1/SAE2 heterodimer (Activating enzyme), E2-UBC9 (Conjugating enzyme) and E3 enzymes (Ligating enzymes) (Fig 9). SUMOylation is essential for many cellular functions as its targets are involved in transcription regulation, intracellular transport, maintenance of genome integrity, protein stability, stress responses and many other biological functions (224,225). Consistent with the indispensable role of SUMOylation in the host cell, it has been shown that parasites can interfere with this PTM. Effector proteins from pathogens can alter the SUMOylation of host proteins, modify host SUMOylation machinery or use SUMOylation to modify parasite proteins (226). The exploitation of host SUMOylation by bacterial pathogens such as *Listeria* (160)*, Yersinia (227), Xanthomonas (228), Salmonella* (229)*, Anaplasma* (230) and many viral pathogens (182,226,231) is well documented. However, there are no reports till date showing the exploitation of host SUMOylation by Apicomplexan parasites.

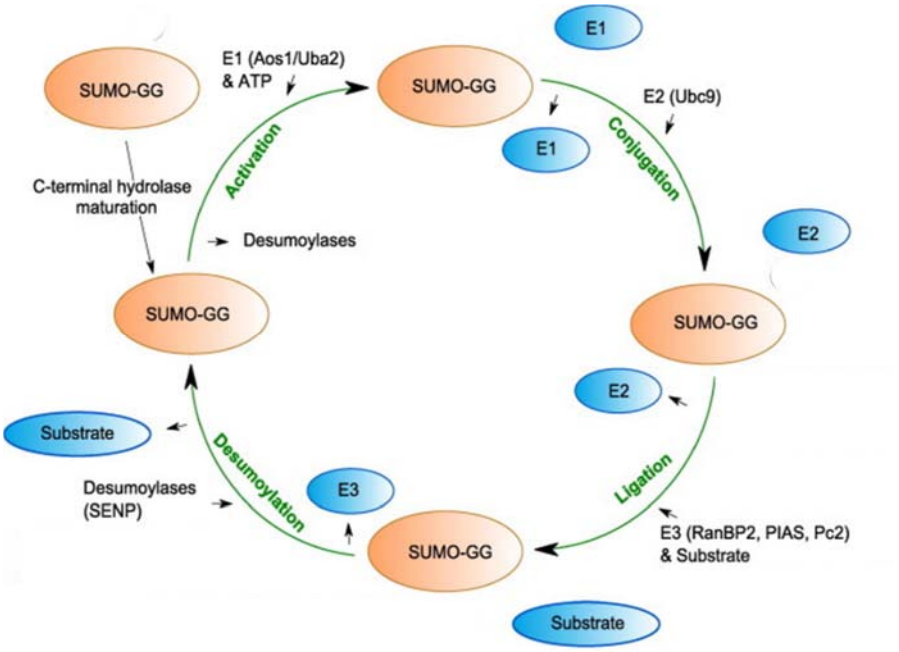


Fig.9: SUMOylation cycle. Initially SENPs proteolytically modify SUMO by removing C-terminal from the diglycine motif. E1 heterodimer AOS1-UBA2 activates the processed SUMO in an ATP dependent manner forming a thioester bond between C173 in UBA2 and C-terminal glycine residue. Activated SUMO is shifted to the catalytic cysteine residue of Ubc9 (E2 enzyme). Subsequently, SUMO is transferred to its target protein by an E3 ligase through an isopeptide bond between the C-terminal glycine residue of SUMO and a lysine residue of the target protein (232).

The host cells that *Plasmodium* sporozoites invades and establishes infection in vertebrates are hepatocytes. After entering the hepatocytes, sporozoite resides in a membranous structure called parasitophorous vacuolar membrane (PVM), where it multiplies and transforms into EEF's. *Plasmodium* exports specific proteins into the host cytosol using specialized machinery (233). The exported parasite proteins are speculated to coordinate the process of infection which involves several changes in host cells such as suppression of host p53 signaling (234), alteration of ER stress (235), interference in host NFκB pathway (36) and inhibition of host cell apoptosis (236). How *Plasmodium* is able to modulate these changes in the host has been a longstanding unanswered question. Further if host SUMOylation machinery is affected during *Plasmodium* and *Toxoplasma* infections is unknown and investigation in these lines may help us understand the range of host processes manipulated by the Apicomplexan parasites.

Our studies on host SUMOylation process during *Plasmodium* and *Toxoplasma* infection reflected several parallels that included down regulation of the SUMO transcriptional machinery together with an overall decrease in the efficacy of the cellular effector proteins to get SUMOylated. The over expression of SUMO1 in host hepatocytes showed a detrimental effect on the intracellular development of *Plasmodium* EEF's and *Toxoplasma*. Contrary to this observation, shRNA mediated silencing of the only conjugation enzyme of the host SUMO-machinery-the E2/Ubc9 favored the intracellular growth of *Plasmodium* EEF. Analysis of global gene expression of the HepG2 cells by microarrays under 4 different conditions viz., control/no infection (Spz-), sporozoite infection (Spz+), over expression of SUMO1 (SUMO1+) and infection during SUMO1 over expression (SUMO1+Spz+) yielded piercing insights as how host cell modulation occurs during infection and how over expression of SUMO1 counteracts the of anti-apoptotic and anti-proliferative effects induced *Plasmodium* and *Toxoplasma* infections. Our observations provide evidence for the first time that apicomplexan parasites like *Plasmodium* and *Toxoplasma* hijack SUMO1 signaling pathways that can globally alter the gene expression of the host. By altering the levels of SUMOylation of host cellular proteins, by yet to be discovered mechanism, the parasites reduce the SUMO conjugation of the targets proteins that likely induces a favorable environment for its growth and dissemination.

2.2 Materials and Methods

2.2.1 Maintenance of rabbits, BALB/c, C57BL/6 and Swiss albino mice

Rabbits, BALB/c, C57BL/6, and Swiss albino mice were procured from National Institute of Nutrition, Hyderabad, India and house at the animal house facility of University of Hyderabad. All animal experiments performed at the School of Life Sciences, University of Hyderabad were in compliance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) national guidelines. The animal protocols were approved by Institutional Animal Ethics Committee (IAEC).

2.2.2 Maintenance of *Anopheles stephensi* (*A. stephensi*) mosquitoes

A. stephensi mosquito strain colony was obtained from National Institute of Malaria Research (NIMR), New Delhi, India. The mosquito colony was maintained in a 3 level safety insect breeding and maintenance facility at the School of Life Sciences, University of Hyderabad.

To generate the mosquito stages of *P. berghei*, a colony of *A. stephensi* was continuously maintained. Following is the brief description of the activity associated with breeding and maintenance of the colony. Adult male and female mosquitoes were allowed to mate in captivity within 24-36 hours following their emergence from pupae. Following mating the mosquitoes received a blood meal from an anesthetized rabbit that was sedated using a combination of ketamine and xylazine [0.8 ml ketamine [50mg/ml] + 0.3 ml of xylazine (20mg/ml) + 3.9 ml of 1X PBS). Administration of 0.5 ml of this anesthesia intramuscularly to the rabbit maintained it in a sedative state for 30-45 minutes. The sedated rabbit was placed on the top of the mosquito cages to facilitate the uptake of blood meal by the female mosquitoes. The blood meal was given two times within the interval of 24 h. After 36 h of second blood meal, a blow of water was placed inside the mosquito cage to allow egg laying by female mosquitoes. The eggs were collected over a period of 4 days. The eggs were transferred into water trays and shifted into a chamber that was maintained at 27°C and 80% RH [relative humidity]. The eggs hatched under these conditions and transformed through a series of instars resulting in the pupae. The pupae were manually collected and placed in a new cage to facilitate the emergence of adult mosquitoes.

2.2.3 Transmission of *P. berghei* mCherry to *Anopheles* mosquitoes

To allow malaria transmission into mosquitoes, a separate cage containing only female *Anopheles* mosquito was prepared. The separation of the female mosquitoes was achieved by placing the hand on the outer side of the adult mosquito cage that attracted the female mosquitoes by sensing the body temperature (37°C). All the female mosquitoes (on inside of the cage) gathered in the region of the palm were collected into a tube attached to a vacuum pump. The female mosquitoes were immediately transferred into a new plastic cage and were maintained in another chamber maintained at 20-21°C and 80% RH. The mosquitoes received a 10% sugar solution soaked in cotton as food. Immediately before obtaining an infective blood meal, the mosquitoes were starved for 2 h by removing the sugar soaked cotton pads. Four to five BALB/c mice that were positive for gametocytes were sedated by injecting 200µl of anesthesia (prepared by mixing 0.8 ml ketamine [50mg/ml] + 0.3 ml of xylazine (20mg/ml) + 3.9 ml of 1X PBS) into each mouse. The mice were placed on top of the female mosquito cage to transmit malaria. The mice were allowed to take a blood meal for a total time of 18 minutes, with three changes in the position of the mice to ensure that majority of the mosquitoes received blood meal from all the mice. The feeding was done for two times within 24h interval. After second feeding the mosquito cage was placed back in same chamber maintained at 20°C and

80% RH for 18-22 days to facilitate the completion of sexual development and for the formation of salivary gland sporozoites. During this entire period, the mosquitoes were fed with sugar soaked cotton pads that were replaced on a daily basis.

2.2.4 Observing the midgut oocyst

Successful transmission of malaria in the mosquitoes was monitored by observing the oocysts on mosquito midguts. Since the P. berghei parasites constitutively expressing mCherry, all the mosquito stage were readily monitored under a fluorescent microscope. On day 14 post infection, 10-15 mosquitoes were dissected to isolate the midguts that provided an idea about the parasite burden in the mosquito. Similarly on day 18-21 post infection, the salivary glands were dissected to obtain sporozoites (Fig 10).

2.2.5 Dissection and purification of salivary gland sporozoites

To isolate the salivary glands, day 18 post infection mosquitoes were collected and were washed in 70% ethanol for three times. Followed by this, the mosquitoes were additionally washed in DMEM containing 1X antibiotic and antimycotic (Life Technologies, Cat# 152400-062, 100X) for three times. The salivary glands were dissected and collected into a 1.5ml of eppendorf tube in small volume of 80-100µl. The glands were crushed using a plastic pestle and disrupted to release the sporozoites. The crushed samples were spun at 800rpm for 3 min at 4°C in Eppendorf centrifuge (Model 5415R). The supernatants were collected in a 1.5ml eppendorf tube. A small volume [2-3µl] was diluted in a 1:10 ratio and 10µl of this dilution was placed on a haemocytometer and the number of sporozoites was counted. Sporozoite count from all the four quandrants was averaged and the actual sporozoite numbers were calculated using the following formula:

No. of sporozoites = Average number of sporozoites from 4 quadrants X 10 (dilution fold) $X10^4$ (Hemocytomter correction factor)/ml

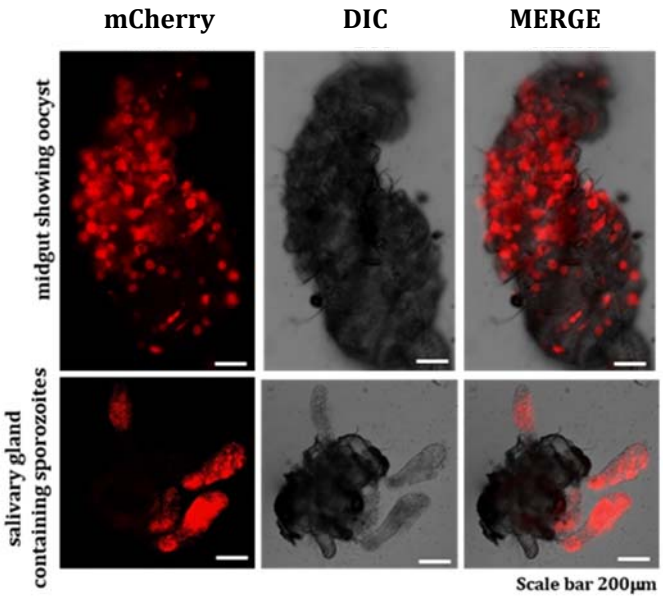


Fig. 10: Generation of *P. berghei* mCherry sporozoites. On day 14 post infection, oocyst burden in the *Pb* mCherry infected mosquito midguts (upper panel). On day 18 post infection, infected mosquito salivary glands loaded with *Pb* mCherry sporozoites (lower panel), images were acquired using Nikon fluorescence microscope at 10X magnification. Scale bar 200µm.

2.2.6 Plasmids

pcDNA3.1- SUMO1-FLAG and pTRE3G-SUMO1-GFP encode mature forms of SUMO1 with C-terminal FLAG and GFP tags. PCMV-Tet3G (Clonetech) regulator plasmid was used to generate stable clones of HepG2 expressing Trans-activator protein. Cignal SMAD4 reporter plasmid encodes the monster GFP gene under minimal CMV promoter and tandem repeats of the SMAD4 transcriptional response elements (SA Biosciences).

2.2.7 Doxycycline regulated expression of SUMO1 in HepG2 cells

HepG2 cells that were used as a cell model in this study were first transfected by the pCMV-Tet3G plasmid to express the trans-activator that is required to bind to the Tet promoter in the response plasmid pTRE3G. The transfected cells are kept on drug selection with Geneticin (Invitrogen) at a concentration of 700µg/ml. The drug resistant colonies were sub cultured separately and analyzed for trans-activator protein expression by western blotting. The clonal population with maximal expression of trans-activator protein was passaged and used for

further experiments. A second round of transfection was done on trans-activator cell line with a response plasmid pTRE3G-SUMO1-GFP where the SUMO1-GFP protein was under the control of tetracycline responsive elements. The responsiveness of the pTRE3G transfected cells to doxycyline was tested at different concentration (10ng to 100ng/ml) by analyzing the expression of SUMO-1 by western blotting. After 24 hours of transfection with response plasmid, cells were infected with *P. berghei* sporozoites and *T. gondii* tachyzoites as mentioned below. After 2 hours of infection, cells were replenished with fresh culture medium with 10ng/ml of doxycycline and incubated for the indicated times.

2.2.8 Infection of HepG2 cells with salivary gland sporozoites

Human liver carcinoma cells (HepG2) were obtained from National Centre for Cell Sciences, Pune, India. Cells were maintained in DMEM (Invitrogen) containing 10% fetal bovine serum, 100 U/ml penicillin, 2mM L-glutamine and 100 µg/ml streptomycin (all Invitrogen) at 37^0C and 5 % CO_2. Nearly $2x10^5$ cells were seeded per well on coverslip in a 24 well plate. *P.berghei* mCherry sporozoites were isolated from infected *A. stephensi* mosquitoes, and infected to HepG2 cells in DMEM. After 3h, cells were washed, replenished with fresh culture medium and incubated for the indicated time points of 16h, 25h, 42h and 52h for real-time PCR analysis. In a different experiment, the infected HepG2 cells were maintained for 48h and processed for RNA microarray and fixed after 48h post infection for immunofluorescence assay (IFA).

2.2.9 Culturing of *T. gondii* tachyzoites and infection to HepG2 cells

HFF cells were grown to 90% confluency and cryopreserved *T. gondii* RH tachyzoites (Parasites form NCL Pune) were added to the cultures. The culture were maintained for 4 days to allow the parasites to multiply and infected cells were harvested by scraping. The tachyzoites were purified by passing the infected cells through 24G syringes and subsequently by filtering through 5 µm filter. The parasites were pelleted down by centrifuging at 5000 rpm for 15 min. The parasites were counted using hemocytometer chamber and the required number of parasites were added to HepG2 cells at MOI of 0.5. Infected samples were collected at 24h post infection. Infection mediated changes in SUMOylation pattern in cells were analyzed by immuno-fluorescence assay and real time PCR.

2.2.10 Immunofluorescence assay

HepG2 cells were infected with *P. berghei* and *T. gondii* as described above. Cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. The cells were permeabilized with pre cooled acetone/methanol in a ratio of 1:3 for 15 minutes. The non-specific blocking was done with 3% BSA in PBS for 1 hour. The cells were incubated for one hour with anti-SUMO1 rabbit primary antibody (Abcam) at 1:250 dilution or anti-Tg TP3 mouse primary antibody (Abcam) at 1:300 dilution made in 3% BSA.

Cells are washed sequentially with TBS, TBST and TBS for 15 minutes each. To visualize immunoreactivity by fluorescent microscope, secondary antibodies, goat anti-rabbit IgG-Alexa flour 488 (Invitrogen) at 1:200 dilution or goat anti-mouse IgG-Alexa flour 594 (Invitrogen) at 1:250 dilution and DAPI (1μg/ml) (sigma) was added to the cells in 3% BSA and incubated for 1 hour. Cells are washed sequentially with TBS, TBST and TBS for 15 minutes each. Following completion of staining procedure, coverslips were mounted on a glass slide using Prolong Gold (Invitrogen) anti-fade reagent and stored at 4^0C before and after microscopy. Stained cells were analyzed using Nikon AR fluorescence microscope.

2.2.11 Western blot analyses

Protein samples were prepared by lysing the cultured cells in RIPA buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO4 and 1 μg/ml leupeptin). Samples were resolved by SDS-PAGE, transferred to nitrocellulose membrane, blocked in 3% skimmed milk in TBS, and probed with antibodies. Primary antibodies were mouse anti-TetR (Clonetech) used at 1:2000 dilution, rabbit anti- ubc9 (Abcam) used at 1:3000 dilution, rabbit anti-SUMO1 (Abcam) used at 1:3000 dilution, mouse anti-actin (Abcam) used at 1:5000 dilution, and mouse anti-β tubulin (Millipore) used at 1:5000 dilution. Secondary antibodies were horse radish peroxidase (HRP) - linked goat anti-rabbit IgG and goat anti-mouse IgG (both from Biorad) used at a dilution of 1:5000. Blots were developed using ECL prime western blotting detection kit (Amersham), and images were scanned using Versadoc (Biorad).

2.2.12 Isolation of RNA from infected samples

Total RNA was isolated from the cultured cells using PureLink micro-to-midi total RNA purification system **(**Ambion). Briefly, cells were washed with 1X sterile PBS, lysed in lysis buffer and passed through 35G insulin syringe for efficient cell lysis. The RNA was precipitated with 70% ultra-pure ethanol and applied to the silicon membrane column. Following two washes with wash buffer and one additional dry spin to remove residual ethanol, RNA was eluted in DEPC treated water and assessed for concentration and purity using Nanodrop® ND-1000 spectrophotometer. RNA was stored at -80^{0}C until further use.

2.2.13 Generation of cDNA

cDNA was generated using the total RNA as template. The reaction mixture for cDNA synthesis was consisted of MulV reverse transcriptase, dNTPs, $MgCl_2$, RNase inhibitor, random hexamer primer (for short length cDNA fragments) or Oligo dT (for full length cDNA). Typically 1-2µg of RNA was used to perform reverse transcription in a volume of 30µl reaction mixture. cDNA was generated in a single cycle using following thermal cycling conditions: 25^{0}C for 10 min, 42^{0}C for 20 min, 98^{0}C for 5 min and 5^{0}C for 5 min.

2.2.14 TA cloning to generate standards for real time PCR quantification

For quantification of target gene expression, real time PCR analysis was done by absolute quantification method. Briefly, primers were designed for genes as indicated in Table 1. Primers spanning the exon-exon junctions were selected that amplified a product in the range of 100-150bp. The PCR products were ligated into TA vector (pTZ57 R/T, Thermo) using T4 DNA ligase. The ligations were further confirmed by double digestion with 2 enzyme specific for the vector.

S.No	Primer name	Sequence of the primer 5'- 3'
1	Pb18SrRNA FP	GGGGATTGGTTTTGACGTTTTTTG
2	Pb18SrRNA RP	AAGCATTAAATAAAGCGAATACATCCTTA
3	Tg18SrRNA FP	GGCATTCCTCGTTGAAGATT
4	Tg18SrRNA RP	CCTTGGCCGATAGGTCTAGG
5	hGAPDH FP	CCTCAACTACATGGTTTACAT

6	hGAPDH RP	GCTCCTGGAAGATGGTGATG
7	hSUMO1 FP	GGGAAGGGAGAAGGATTTGT
8	hSUMO1 RP	CCTCAGTTGAAGGTTTTGCC
9	hUBC9 FP	GGCACGATGAACCTCATGAA
10	hUBC9 RP	CCCCGAAGGGTACACATT
11	hAOS1 FP	GTTGCCAAAGTTAGCCAAGGA
12	hAOS1 RP	TTTCTCACTGCTCCAGTCCACC
13	hPIAS1 FP	ACAACGAGAAGCTTCAGGAGA
14	hPIAS1 RP	CAGCTGGTGTCTGAGTAACAG
15	hCBX4 FP	TGCAAGAAGCGGCTGACTGCG
16	hCBX4 RP	ATCCAGGTCTGAGTCTAGCAG
17	hKIT FP	CGTTCTGCTCCTACTGCTTCG
18	hKIT RP	CCCACGCGGACTATTAAGTCT
19	hKLF5 FP	CCTGGTCCAGACAAGATGTGA
20	hKLF5 RP	GAACTGGTCTACGACTGAGGC
21	hKLF6 FP	GGCAACAGACCTGCCTAGAG
22	hKLF6 RP	CTCCCGAGCCAGAATGATTTT
23	hHERC5 FP	GGTGAGCTTTTTGCCTGGG
24	hHERC5 RP	TTCTCCGGCAGAAATCTGAGC
25	hIFIT2 FP	AAGCACCTCAAAGGGCAAAAC
26	hIFIT2 RP	TCGGCCCATGTGATAGTAGAC
27	hOASL FP	CTGATGCAGGAACTGTATAGCAC
28	hOASL RP	CACAGCGTCTAGCACCTCTT
29	hSKIL FP	GTTAAGCGAACCTGTACTTCTGT
30	hSKIL RP	GTAGGCGACATGCTTTCTTGG
31	hCDKN1A FP	TGTCCGTCAGAACCCATGC
32	hCDKN1A RP	AAAGTCGAAGTTCCATCGCTC
33	hIRF2BP2 FP	CCCATGACTCCTACATCCTCTT
34	hIRF2BP2 RP	GAGGGCGGACTGTTGCTATTC

Table. 1: Primer sequences used for Real Time PCR analysis

2.2.15 Real Time PCR quantification

Real-time PCR was performed with Mastercycler ep Realplex (Eppendorf) using IQ SYBR green super mix (Biorad). Real-time PCR was performed using following Thermal cycling conditions: 1 cycle at 95^0C for 2 min, followed by 40 cycles at 95^0C for 15 s, 60^0C for 1 min followed by a melt curve detection with 1 cycle at 95°C for 15 s, 60^0C for 15 s, 95^0C for 15 s. Copy numbers were determined by absolute quantification method.

2.2.16 Normalization of the mRNA expression values

Normalization of gene expression was done by dividing the copy numbers of specific target gene with the copy numbers of the internal control (*GAPDH).* The normalized value of samples were used to plot the graphs indicating the fold change among the samples. Microsoft excel was used to plot the graphs and for statistical analysis.

2.2.17 Preparation of RNA samples for human whole genome microarray

To generate RNA for microarray studies, $1X10^5$ HepG2 cells were grown in 8 well Labtek chambered slides. Samples were generated for four experimental conditions: 1.uninfected cells (Spz-), 2. Infected with 20,000 *P. berghei* sporozoites (Spz+), 3. Transfected with 0.5 microgram of *SUMO1* encoding plasmid (SUMO1+) and 4. Infection with *P. berghei* sporozoites after transfection with SUMO1 encoding plasmid (SUMO1+, Spz+). RNA was isolated from all the four samples using Purelink total RNA mini kit (Ambion).

2.2.18 Microarray analysis of HepG2 cells

For microarray analysis**,** an Agilent Custom human gene expression microarray slide having 8X60k format was used. The microarray slide contains 900X10 replicates of biological probes and 96X10 positive control probes. The hybridization, scanning, data extraction and data analysis was performed under project number SO_3724 at Genotypic Technology Private Limited, Bangalore, India.

2.2.18.1 RNA Quality Control

The concentration and purity of the RNA extracted were evaluated using the Nanodrop Spectrophotometer (Thermo Scientific; ND 1000). The integrity of the extracted RNA were analysed on the Bioanalyzer (Agilent; 2100). The RNA was considered to be of good quality based on the 260/280 values (Nanodrop), rRNA 28S/18S ratios and RNA integrity number (RIN) (Bioanalyzer)

2.2.18.2 Labeling and microarray hybridization

The samples for Gene expression were labeled using Agilent Quick-Amp labeling Kit (p/n5190-0442). Five hundred nanogram each of total RNA were reverse transcribed at 40°C using oligo dT primer tagged to a T7 polymerase promoter and RNA was converted to double stranded cDNA. Synthesized double stranded cDNA were used as template for cRNA samples generation. cRNA was generated by in vitro transcription and the dye Cy3 CTP(Agilent) was incorporated during this critical step. The cDNA synthesis and in vitro transcription steps were carried out at 40°C. Labeled cRNA was purified using Qiagen RNeasy columns (Qiagen, Cat No: 74106) and quality assessed for yields and specific activity using the Nanodrop ND-1000.

2.2.18.3 Hybridization and scanning

Six hundred nanogram of labeled cRNA sample were fragmented at 60ºC and hybridized on to a Agilent Human Gene Expression Microarray 8x60K (AMADID No: 39494) arrays. Fragmentation of labeled cRNA and hybridization were performed using the Gene Expression Hybridization kit from Agilent Technologies (In situ Hybridization kit, Part Number 5190-0404). Hybridization was carried out in Agilent's Surehyb Chambers at 65º C for 16 hours. The hybridized slides were washed using the Agilent Gene Expression wash buffers (Agilent Technologies, Part Number 5188-5327) and slides were scanned using the Agilent Microarray Scanner (Agilent Technologies, Part Number G2600D).

2.2.18.4 Feature Extraction: Image Analysis

Data extraction from Images was done using Agilent Feature Extraction software.

2.2.18.5 Microarray Data Analysis

Feature extracted raw data was analyzed using Agilent GeneSpring GX software. Normalization of the data was done with GeneSpring GX using the 75th percentile shift method. Percentile shift normalization is considered as a global normalization, where the locations of all spot intensities in the array are adjusted. This normalization takes each column in an experiment independently, and calculates the n^{th} percentile of the expression values for this array, across all the spots (where n has a range from 0-100 and n=75 is the median). It subtracts this value from the expression value of each entity and fold change values were obtained by comparing test samples with respect to specific control samples. Significant genes up regulated fold> 0.6 (logbase2) and down regulated <-0.6 (logbase2) in the test samples with

respect to the control sample were identified. Statistical student T-test p-value among the replicates was calculated based on volcano Plot Algorithm. Differentially regulated genes were clustered using hierarchical clustering based on the Pearson coefficient correlation algorithm to identify significant gene expression patterns**.** Genes were classified based on functional category and pathways using Biological Analysis tool DAVID (http://david.abcc.ncifcrf.gov/).

2.2.19 shRNA mediated silencing of Ubc-9 expression

shRNA was used to silence the expression of the SUMOylation E2 enzyme *Ubc9* in HepG2 cells. Approximately $2x10^5$ cells per well were seeded in 24 well plate with or without coverslips. Cells were transfected with p-GFP-VRS- UBE2I human shRNA plasmid variants encoding 4 unique 29mer shRNAs targeting human *Ubc9* mRNA and one non-targeting scrambled shRNA (all obtained from Origene TG300697). Transfection with transfection reagent alone served as negative control. Forty eight hours post transfection, cells were infected with $3x10^3$ *P. berghei* sporozoites per well and incubated for additional 48 hours prior to processing. Cells were collected and lysed in RIPA buffer for analysis by western blotting. Alternatively, the cells were processed for real-time PCR as described above. Cells fixed on cover slips and analyzed by fluorescent microscopy by Nikon AR.

2.2.20 TGF-β reporter assay

HepG2 cells were transfected with SMAD4 reporter plasmid (SA Biosciences). Twenty four hours after transfection, the cells were infected with *P.berghei* sporozoites ($3x10^3$/well) or *T. gondii* tachyzoites (MOI 0.5). Two hours after infection, cells were incubated with recombinant human TGFβ1 (20ng/ml) for additional 20 hours. Cells were harvested and transferred to a black 96 well plate. Intensity of GFP fluorescence was measured using Tecan Infinite 200 pro microplate reader. The monster GFP intensity was measured using 480nm excitation and 515nm emission filters. Intensities were normalized with un-transfected HepG2 cells as negative control.

2.2.21 Inside/out assay to quantify *P. berghei* invasion

HepG2 cells were grown in Lab-tek chambered slides and infected with $2X10^4$ *P. berghei* mCherry sporozoites and incubated at 37^0C for 2 hrs. Medium was gently removed and the sporozoites were fixed with 4% PFA for 30 minutes followed by two washes in with PBS. Nonspecific blocking was done using 1% BSA for 1 h, followed by incubation for 1 h with 1μg/ml of 3D11 monoclonal antibody (specific for *P. berghei* CSP repeats) prepared in 1% BSA and incubated at 37^0C. The cells were washed sequentially with PBS, PBST and PBS for 15 min each. To reveal the immunoreactivity, the cells were further incubated with anti-mouse Alexa-flour 488 antibody (Invitrogen) and 1μg/ml of DAPI (Invitrogen) in 1% BSA for 45 min at 37^0C. The cells were washed sequentially with PBS, PBST and PBS for 15 min each. The slide was observed under fluorescent microscope to score for red (total) and green (extracellular) sporozoites in 25-30 random fields. The number of sporozoites that invaded per field was calculated using the formula: total number of sporozoites (outside and inside) - extracellular sporozoites (only outside).

2.3 Results

2.3.1 Cell lines for growing *Plasmodium* exo-erythrocytic forms and *Toxoplasma* tachyzoites

The sporozoite stages of rodent malaria species *P. berghei* invade and undergo successful transformation into EEF's within the HepG2 cells and is one of the most widely used *in vitro* model to study the *Plasmodium* liver stage biology and host-parasite interactions. *T. gondii* infects all nucleated cells where they transform into tachyzoites. We used human foreskin fibroblasts (HFF) cells initially to propagate and obtain tachyzoites from the culture supernatants, following which the HepG2 cells were infected with tachyzoites at an MOI of 0.5.

2.3.2 Fluorescent microscopic analysis for studying the cellular distribution of SUMO1 during intracellular development of *Plasmodium* liver stages and *Toxoplasma* tachyzoites

While addressing the sporozoite infection induced changes in the HepG2 cell SUMOylation, we investigated SUMO1 immunoreactivity in the nuclei of the cells harbouring *Plasmodium* EEF's. We found significantly less SUMO-1 immunoreactivity in nuclei of infected cells as compared non-infected cells, suggesting that the developing EEF's precluded the nuclear translocation of the SUMOylated proteins into the host nucleus (Fig 11). Such observations were consistent, in some instances with the preferential accumulation of the SUMOylated proteins towards the nuclear periphery of the infected host nuclei. We also noted a similar phenomenon in *Toxoplasma gondii* where cells harbouring the parasites revealed a decreased SUMO-1 immunoreactivity in the host nucleus (Fig 12).

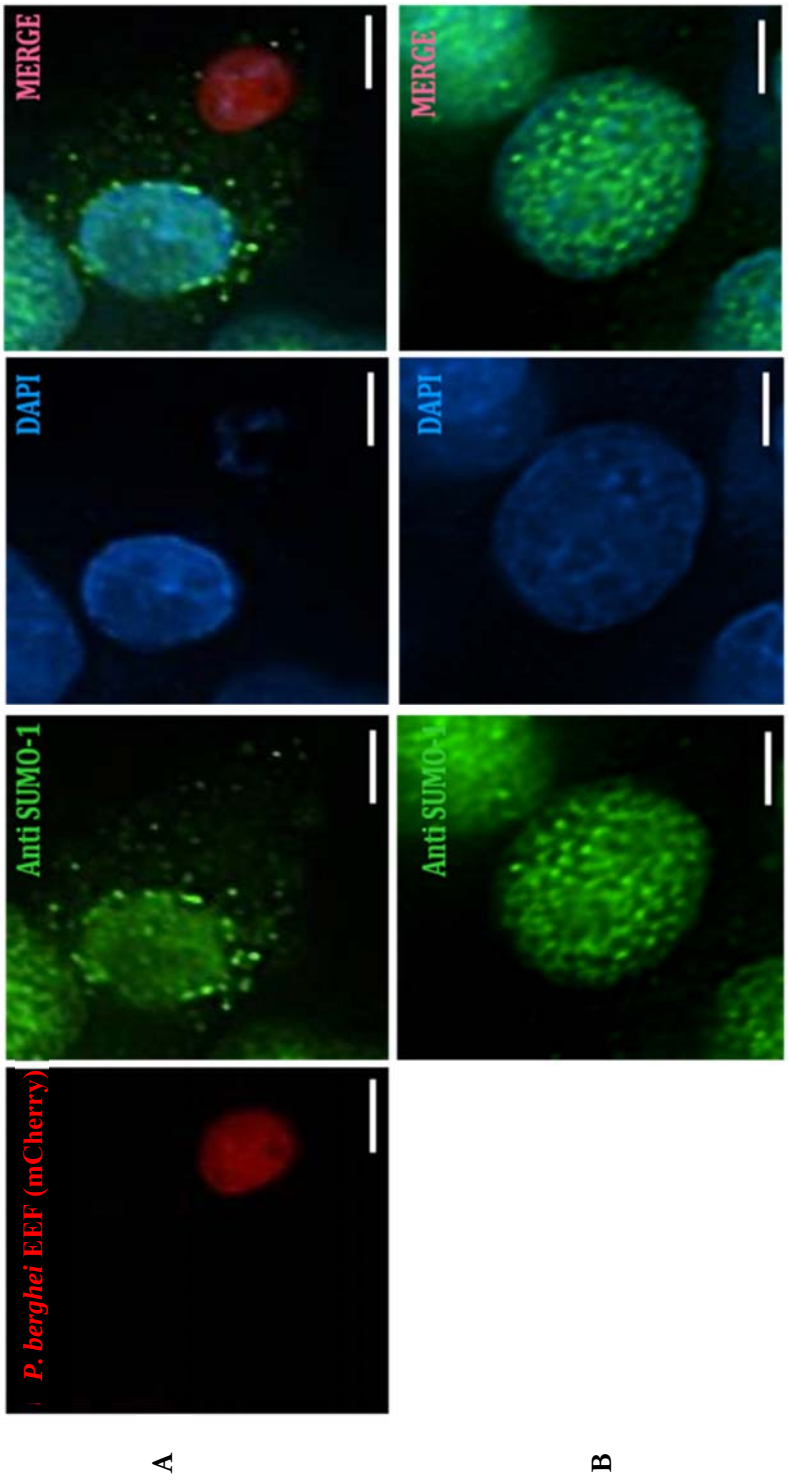


Fig. 11: Monitoring the cellular SUMOylation levels in *Plasmodium* infected and non-infected HepG2 cells. *P. berghei* mCherry sporozoites were added to monolayers of HepG2 cells and cultures were maintained for 48 hours. The cells were fixed with 4% PFA and IFA was performed. The cultures were stained with anti-SUMO1 antibody and revealed with anti-mouse secondary antibody conjugated to Alexa flour 488. The host cell nuclei were stained with DAPI. Top panel (A) shows HepG2 cells harbouring EEF precludes nuclear translocation of SUMO-1. Lower panel (B) shows the nuclear translocation of SUMO-1 is uninhibited in non-infected cells. (Scale bar- 10µm)

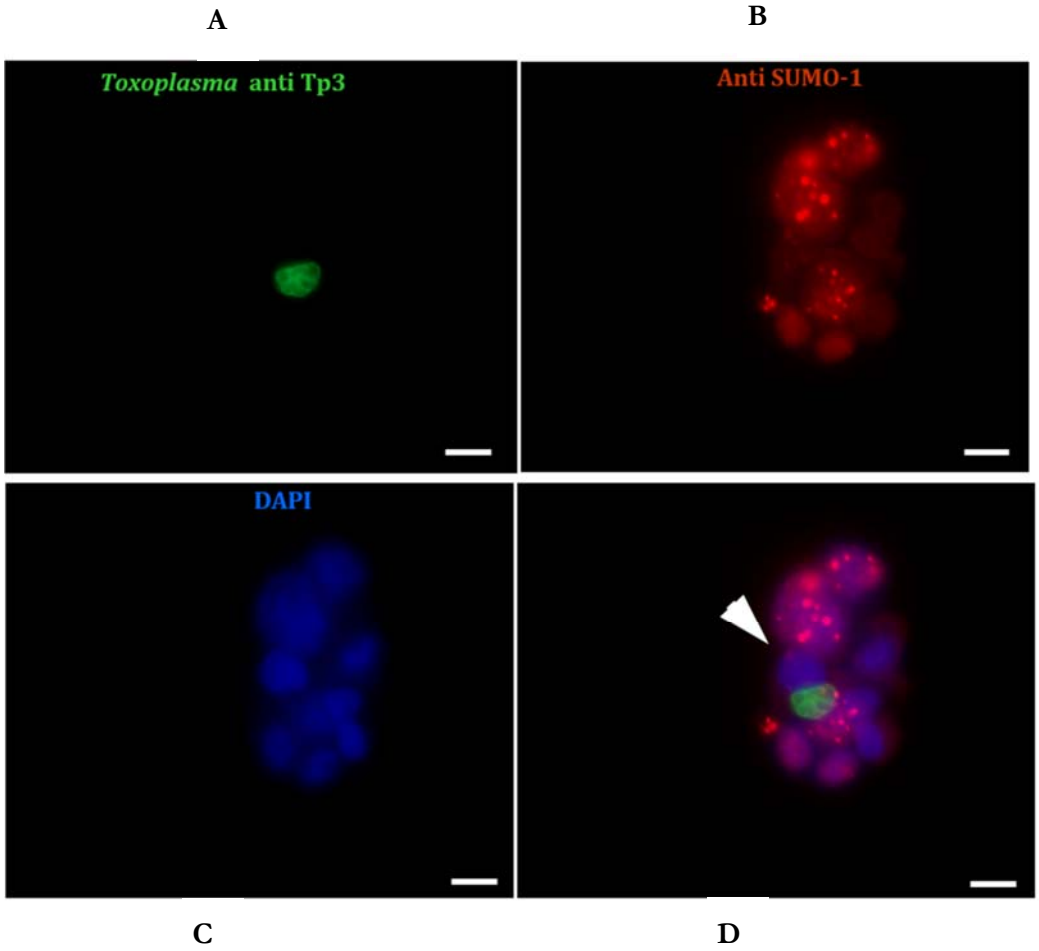


Fig12. Monitoring the cellular SUMOylation levels in *Toxoplasma* infected and non-infected HepG2 cells. *Toxoplasma* tachyzoites were added to a monolayer of HepG2 cells and cultures were maintained for 48 hours. The cells were fixed with 4% PFA and IFA was performed. (A) For visualizing *Toxoplasma*, the cultures were stained with TgTp3 antibody and revealed with anti-mouse secondary antibody conjugated to Alexa flour 594. B) For visualizing SUMO-1 immunoreactivity the cultures were stained with SUMO-1 antibody and revealed with anti-rabbit secondary antibody conjugated to Alexa flour 488. (C) DAPI staining of host cell nuclei. (D) Merge of A, B and C. (Scale bar -10µm).

Fig. 11: Monitoring the cellular SUMOylation levels in *Plasmodium* infected and non-inf

2.3.3 Transient over expression of SUMO1 in HepG2 cells does not compromise *Plasmodium* sporozoite or *Toxoplasma* tachyzoite entry into the host cells, but attenuates the growth of the intracellular parasites

In order to investigate whether the observed decrease was due to a compromised invasion of sporozoites into the SUMO-1 over expressing cells or due to block in the sporozoite development into EEF's following invasion, we performed sporozoite invasion assay and quantified the infectivity of HepG2 cells that were transiently expressing SUMO1. We observed that the sporozoites were not compromised in their ability to invade the SUMO1 over expressing cells as invasion efficiency was almost same in transfected and non-transfected cultures (Fig 13). We next investigated the development of EEF within the HepG2 over expressing SUMO1. We observed that following 48 hours of EEF development within HepG2 cells, the transfected SUMO1 over expressing cells had EEF's that were drastically attenuated in their growth as compared to EEF's growing in the non-transfected cultures. We conclude that transient over expression of SUMO1 has detrimental effect on the *Plasmodium* EEF development. We next measured the burden of *Plasmodium* and *Toxoplasma* at 48 and 24 hours respectively in SUMO1 transfected and non-transfected cells following addition of equal number of sporozoites or tachyzoites. We observed a 7 and 4 fold decrease in the 18S rRNA copy numbers of *Plasmodium berghei* and *Toxoplasma* in cultures over expressing SUMO1 (Fig 14). We conclude that transient over expression of SUMO1 leads to significant decrease in both *Plasmodium* and *Toxoplasma* parasite burden.

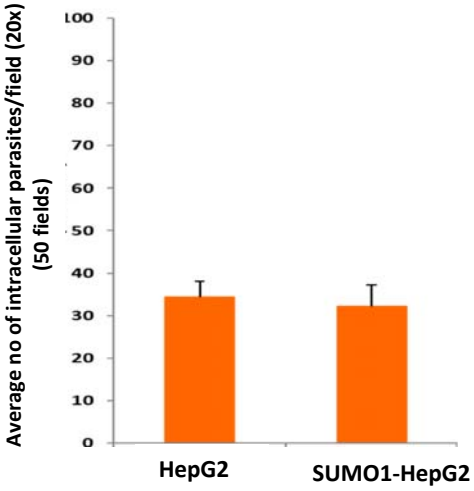


Fig. 13: Bar graph showing the average number of intracellular parasites per field. The number of intracellular parasites were calculated by performing inside /out assay as described in methodology. Error bars-s.e.m (n=3) A similar invasion capacity of *Plasmodium berghei* sporozoites was noted in control and SUMO1 over expressing HepG2 cells.

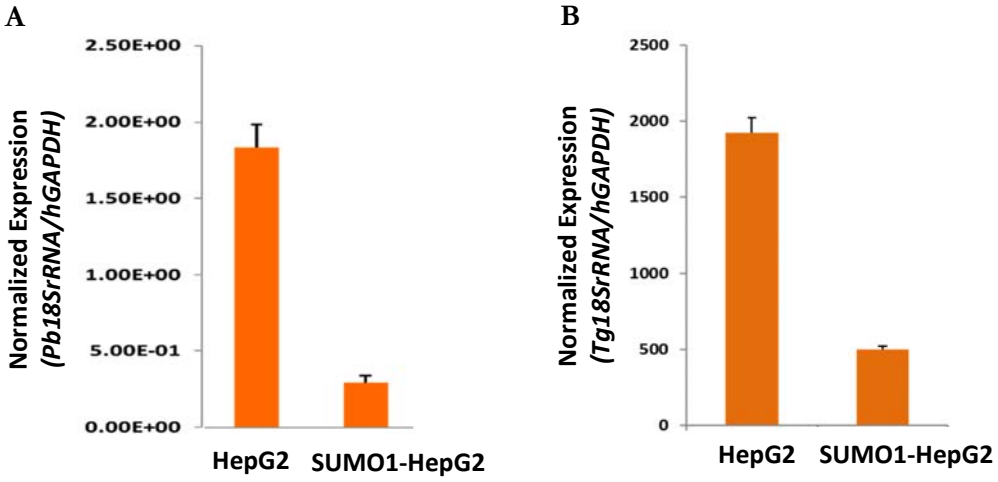


Fig. 14: Bar graphs showing the decrease in the parasite load of *Plasmodium berghei (*A) and *Toxoplasma gondii* (B) in SUMO-1 over expressing HepG2 cells. The parasite loads were quantified by analyzing the expression of *Pb* and *Tg 18SrRNA* and its absolute copy numbers were normalized to *GAPDH*, error bars-s.e.m. A 7 fold decrease in *Plasmodium berghei* and 4 fold decrease in *Toxoplasma* parasite loads were observed in SUMO1+HepG2 cells.

2.3.4 A minimal induction of SUMO1 using a tet- regulated expression system attenuates the growth of the *Plasmodium* EEFs and *Toxoplasma* tachyzoites

To reiterate our findings of the detrimental effect of SUMO1 over expression on EEF growth in HepG2 cells, we next generated a tet-regulated SUMO1 expression system in HepG2 cells, so as the look at the fate of EEF development following minimal induction of SUMO1. For generation of a stable Tet-transactivator cell line, we transfected the HepG2 cultures with pCMV-tet3G vector and selected for clonal population with G418 drug (500 μg/ml) (Fig 15). Following establishment of the clonal line, the cultures were transfected with tet responsive plasmid pTRE-3G-SUMO1-GFP and analyzed for the expression of SUMO1 at different concentrations of doxycycline. We observed the sensitivity of these lines to tetracycline at a minimum concentration 10 ng/ml that induced SUMO1 (Fig 16). Under these conditions, we infected these cells with sporozoites and monitored their growth. We arrived at the identical observation of EEF growth attenuation as seen previously using the non-reporter transient over expression of SUMO1 (Fig 17). The growth of *Toxoplasma* was also analyzed using the tet-regulated SUMO1 expression system and we observed that in transfected cells, the ability to form tachyzoites were highly compromised (Fig 18). We conclude that a minimal or subtle expression of SUMO1 likely activates the cellular effectors mechanisms of host cells that negatively regulate the growth of *Plasmodium* EEF's and *Toxoplasma* thus preventing its maturation and replication respectively.

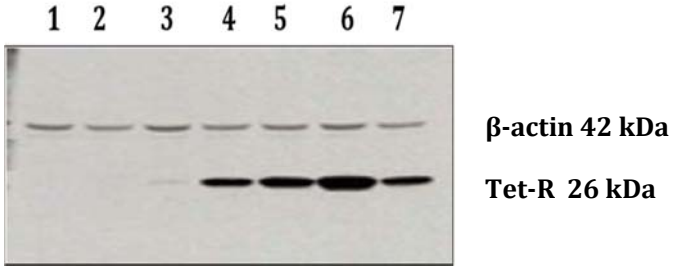


Fig. 15: Generation of a transactivator cell line in HepG2 cells. Western blot showing the expression of trans-activator protein in clones 4, 5, 6 and 7. Anti-beta actin was used for loading control.

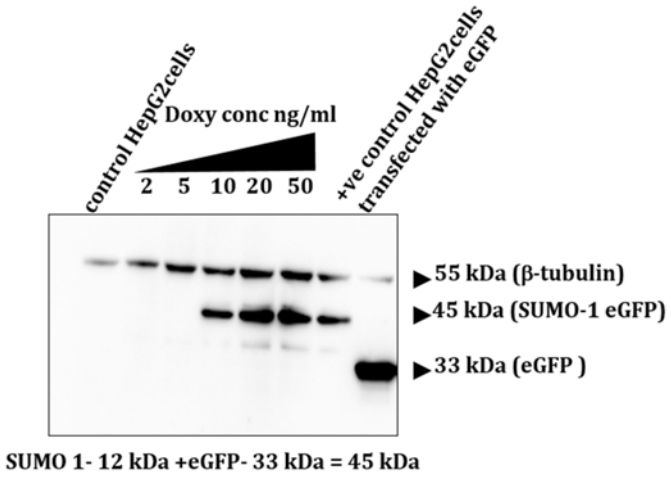


Fig. 16: Tet- regulated expression of SUMO1-GFP in trans-activator cell line by doxycycline treatment. Western blot showing the expression of SUMO1-GFP induced in the presence of varying concentrations of doxycycline (0 [control], 2, 5, 10, 20 and 50ng/ml). The minimum concentration of doxycycline that induced the expression SUMO1 GFP was noted at 10ng/ml. Anti-β-tubulin was used for loading control.

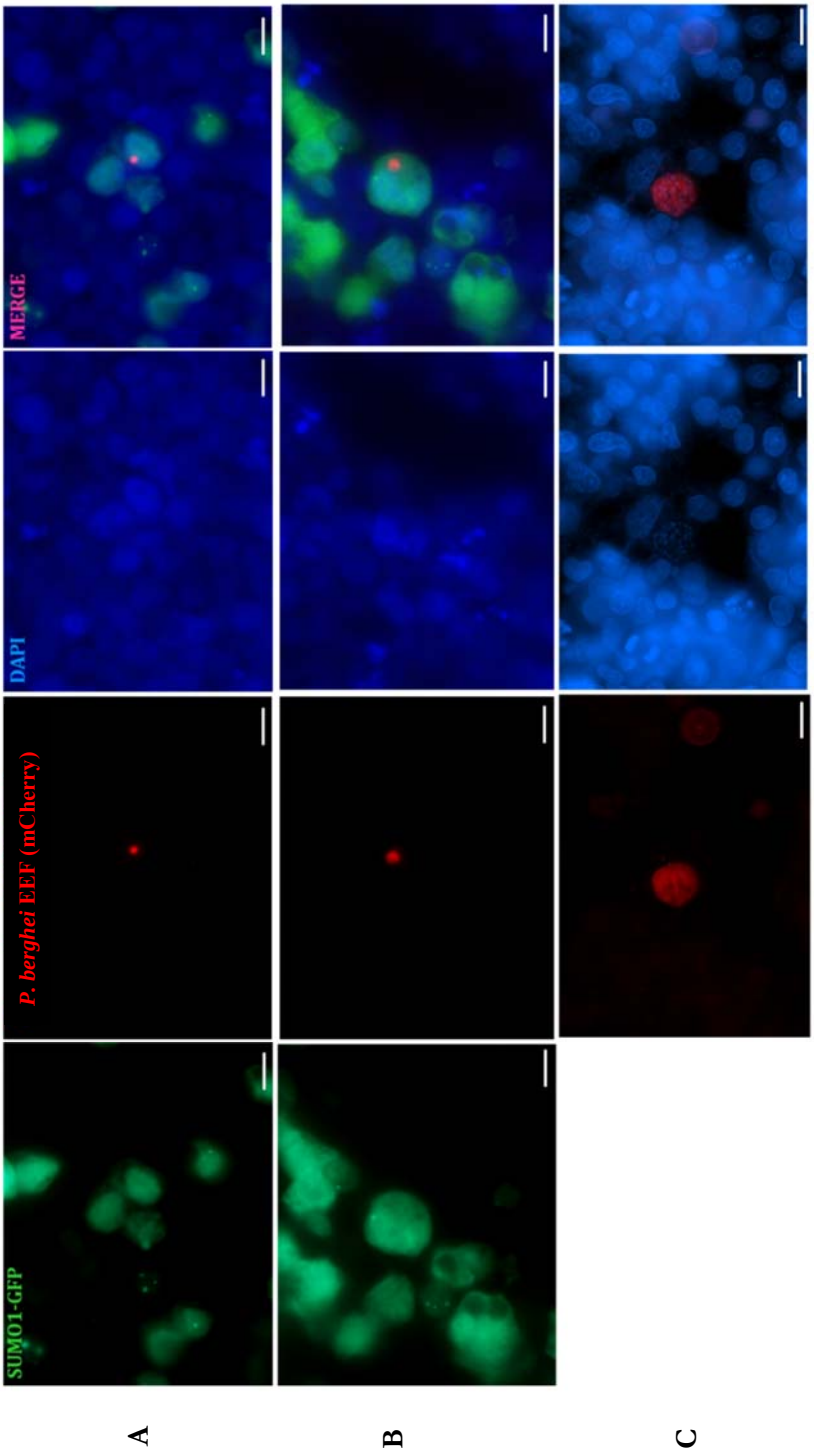


Fig. 17: The minimal induction of SUMO1 GFP in HepG2 cells by tet-regulated system inhibits the development of *P. berghei* EEF's. HepG2 cells were transfected with pTRE3G plasmid containing the SUMO1 GFP cassette. *P. berghei* mCherry sporozoites were added to cultures. Following 2h post infection, the SUMO1 GFP expression was induced with 10ng/ml of doxycycline. The growth of developing EEF's was monitored at 48 hours. Panel (A) and (B) attenuated EEF's growing in SUMO-1 GFP transfected cells. Panel (C) EEF development in non-transfected cultures. Scale bar 10µm.

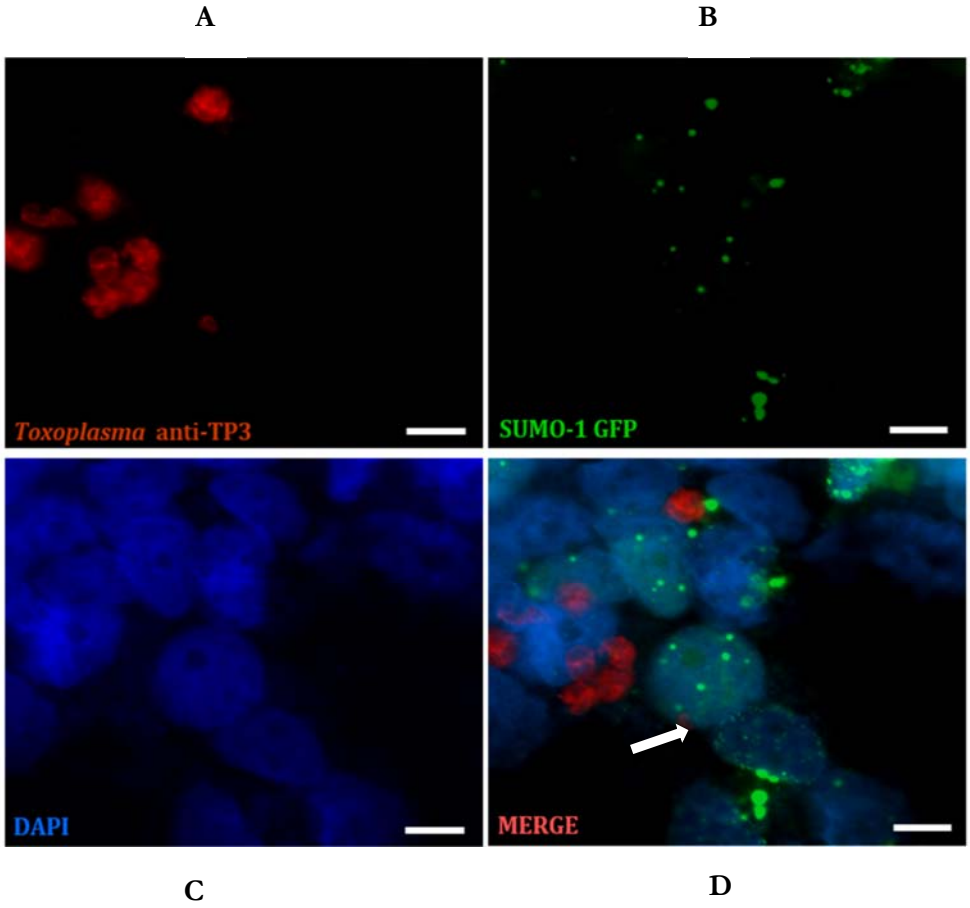


Fig 18. The minimal induction of SUMO-1 GFP in HepG2 cells by tet-regulated system inhibits the multiplication of *Toxoplasma*. HepG2 cells were transfected with pTRE3G containing SUMO1 GFP cassette. The cultures were infected with *Toxoplasma* tachyzoites. Following 2h post infection, the SUMO-1 GFP expression was induced with 10ng/ml of doxycycline. The intracellular multiplication of Toxoplasma was monitored at 48 hr. The cells were fixed and IFA was performed. A) For visualizing *Toxoplasma*, the cultures were stained with TgTp3 antibody and revealed with anti-mouse secondary antibody conjugated to Alexa flour 594. B) Transfected cells expressing SUMO1 GFP expression. C) DAPI staining of host cell nuclei. (D) Merge of A, B and C. Scale bar 10µm.

2.3.5 shRNA mediated down regulation of the E2/Ubc9- the only SUMO conjugation enzyme facilitates better development *P. berghei* EEF's

shRNA silencing of host signaling molecules are valuable tools to study host-parasite interactions at molecular level. Since over expression of SUMO1 was unfavorable for EEF development, we next analyzed if down regulation of any components of SUMOylation enzyme machinery would favor better growth of EEF. We transfected HepG2 cells with shRNA GFP reporter constructs specific for host Ubc9 (SUMO conjugating enzyme) followed by exposure of these culture to sporozoites. Following transfection with 4 variants (shRNA81, 82, 83, 84) including one scrambled (Origene TG300697), we monitored the growth of the EEF's at 50h by measuring the size of the parasite. We found a significant increase in the size of the parasites maturing within the GFP positive HepG2 cells transfected with shRNA 83 and 84 (Fig 20 & Fig 21). The efficacy of each of the 4 shRNA mediated down regulation was confirmed independently by analyzing the levels of E2 in cellular lysates following transfection (Fig 19). We conclude that perturbing the SUMO machinery of the host cells has a favorable impact on the maturation of *Plasmodium* EEF's there by promoting its growth.

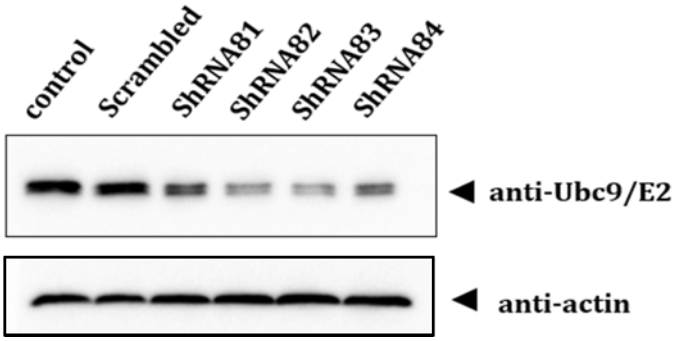


Fig. 19: Western blot showing the decreased expression of Ubc9 in cells transfected with shRNA against *UBC-9*. Anti-actin was used for loading control.

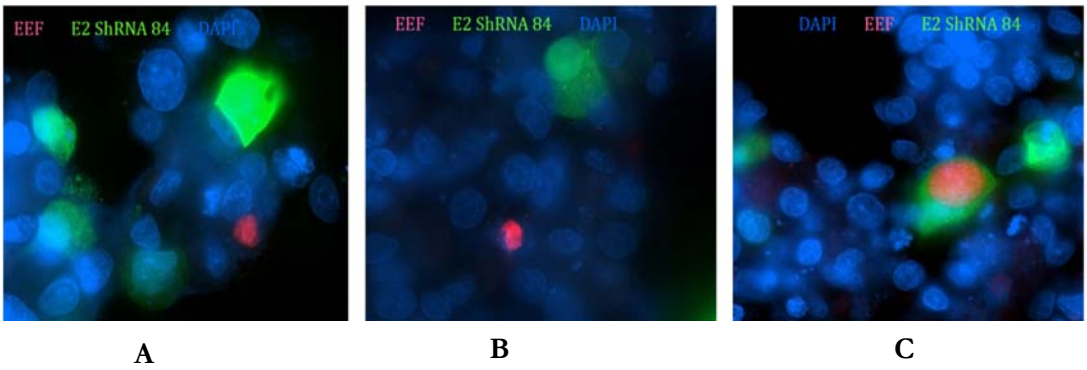


Fig. 20: shRNA mediated silencing of Ubc9 promotes *Plasmodium berghei* EEF development. Microscopy images of *Pb* mCherry EEFs in un-transfected cells (A&B) and EEFs in *UBC-9* shRNA transfected cells (GFP expressing cells) (C) 48hr post infection.

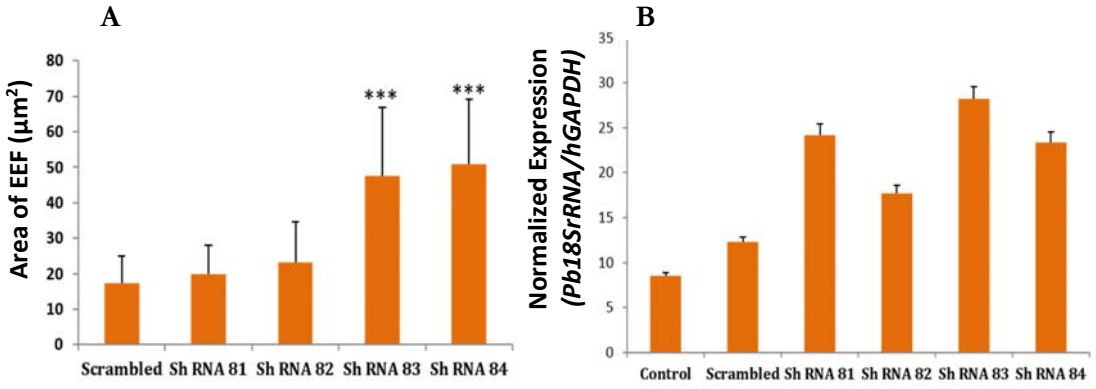


Fig. 21: Average area of *Plasmodium berghei* EEF's growing in HepG2 cells transfected with different shRNAs against *UBC-9* (A). Error bars s.e.m, (***= p value <0.05). The burden of *Plasmodium berghei* EEF's growing in HepG2 cells transfected with different shRNAs against *UBC-9* measured by quantifying *Pb 18SrRNA* (B), values were normalized with host *GAPDH.*

2.3.6 HepG2 cultures harboring developing *Plasmodium* EEF's or *Toxoplasma* species down regulate the expression of SUMO1 and SUMOylation machinery enzymes

Having shown that during EEF development, host SUMOylation machinery is altered, we next analyzed if infection caused a global change in the expression of transcripts that encode the enzymes of the SUMOylation. We analyzed the expression of *SUMO1*, *E1, E2* and *E3* during the progression of *Plasmodium* EEF development by quantitative real time PCR. We observed the down regulated expression of all the three enzymes viz., E1, E2 and E3 with a range of 2 to 20 fold during 12h to 50h post infection (Fig 22). Similar down regulation of

SUMOylation enzymes was noted in case of 36hr cultures infected with *Toxoplasma* (Fig 23). These observations suggest an interference of SUMOylation machinery at transcription level by developing intracellular *Plasmodium* and *Toxoplasma* parasites.

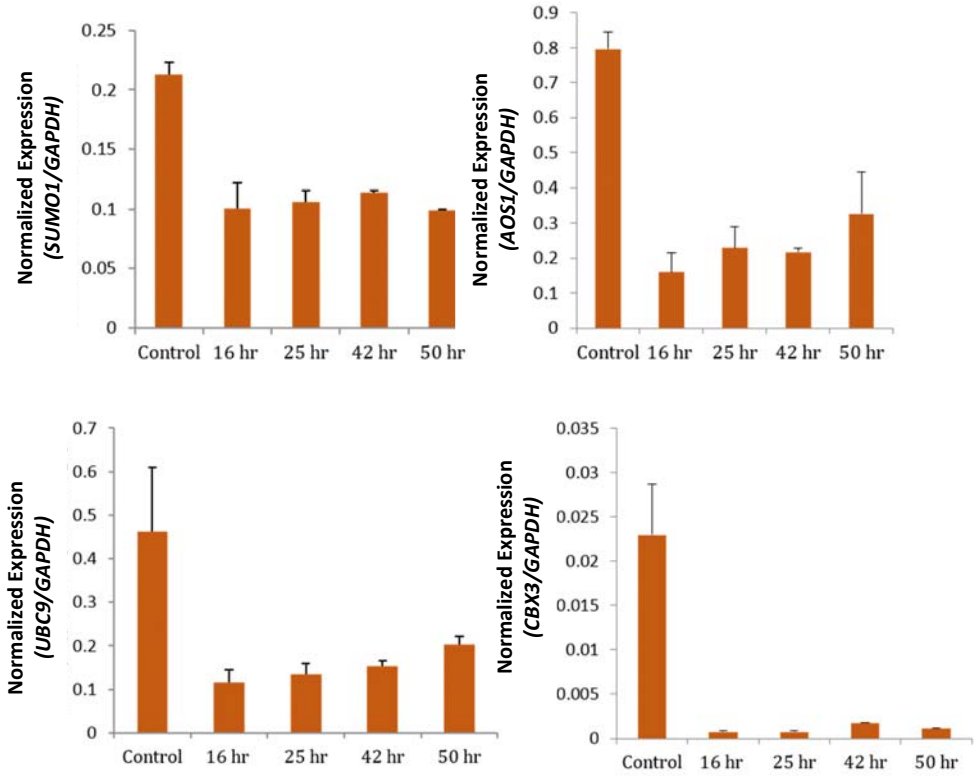


Fig. 22: *P. berghei* infection induced down regulation of *SUMO1*, *AOS1 (E1)*, *UBC9 (E2)* and *CBX3 (E3)* genes expression. Experiments were performed in duplicates and values were normalized with *GAPDH.* Error bars-s.e.m.

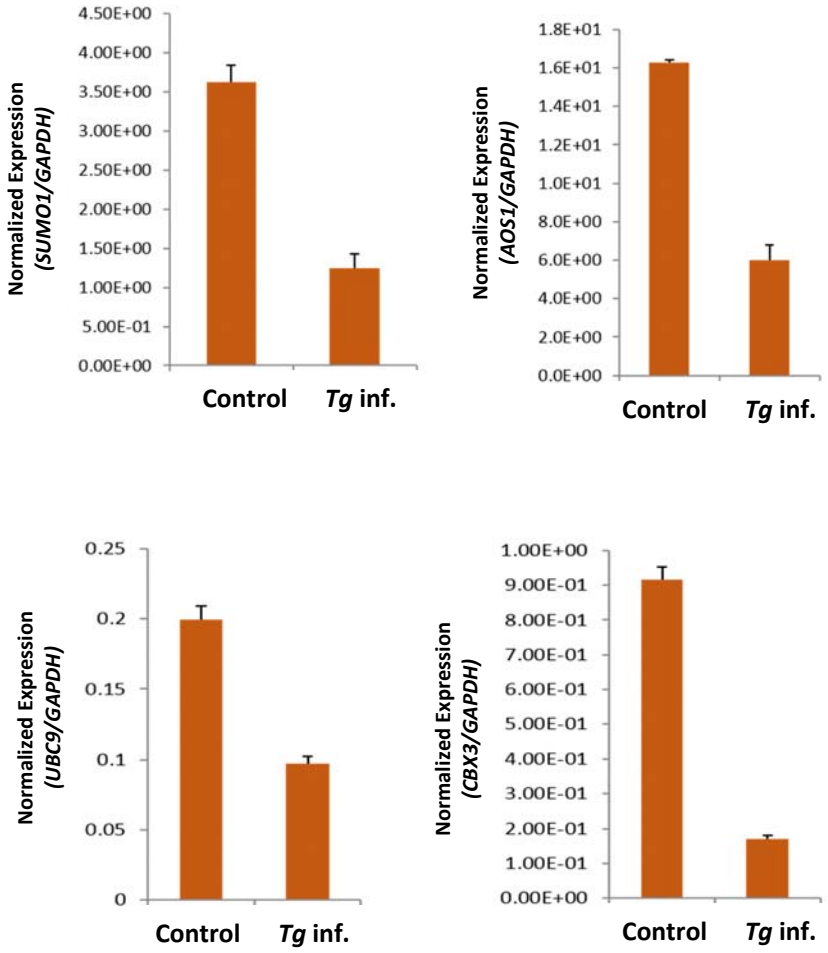


Fig. 23: *T. gondii* infection induced down regulation of *SUMO1*, *AOS1 (E1)*, *UBC9 (E2)* and *CBX3 (E3)* genes expression. The RNA samples were generated from HepG2 cultures infected with *T. gondii*, 24h post infection. Experiments were performed in duplicates and values were normalized with *GAPDP.* Error bars-s.e.m.

2.3.7 Translocation of a stabilized SUMOylated SMAD4 complex to nucleus is precluded in HepG2 cells harboring *Plasmodium* EEFs and *Toxoplasma* tachyzoites.

SUMOylation leads to the change in the sub cellular localization of the target protein leading to their preferential accumulation in the nucleus (237). If the effector target proteins are transcription factors (238), they are likely to affect the host cell gene expression, by inducing a plethora of host transcripts involved in defense response or anti-apoptosis. Towards this end we analyzed the endogenous activity of SMAD4 using a TGF-beta SMAD-4 reporter activity. The assay is based on the translocation of a stabilized SUMOylated SMAD4 complex to nucleus following TFG-β treatment of the cells. Since the cells are transfected prior to TGF-β treatment with expression plasmids having SMAD4 responsive elements upstream to GFP coding sequence, the expression of GFP fluorescence provides is an indirect measure of the SUMOylated SMAD4 complex translocation into nucleus (Fig 24). Using this reporter assay, we observed a 50% decrease in the SMAD4 translocation activity in the presence of both *Plasmodium* EEF and during *Toxoplasma* infections when compared to SUMOylated SMAD4 translocation under no infection condition (Fig 25). We infer that intracellular parasites like *Plasmodium* and *Toxoplasma* reduce the nuclear trafficking of SUMOylated SMAD4 during their development that may likely alter the expression of genes that are regulated by SMAD-4. In order to investigate this hypothesis we analyzed the expression of genes that are induced following nuclear translocation of SUMOylated SMAD4 following TGF beta treatment. We observed that IRF2BP2, CDKN1A and SKIL which are the targets of SMAD4 were all down regulated during infection in both *Plasmodium* and *Toxoplasma* (Fig 26). We conclude that cytoplasmic SMAD4 is indeed a target for both the parasites, whose SUMOylation levels are reduced thus preventing its nuclear translocation. While SMAD4 is one of the definitive target for altered SUMOylation during infections, we cannot rule out the possibility of other cellular effectors which may similarly alter the host gene expression.

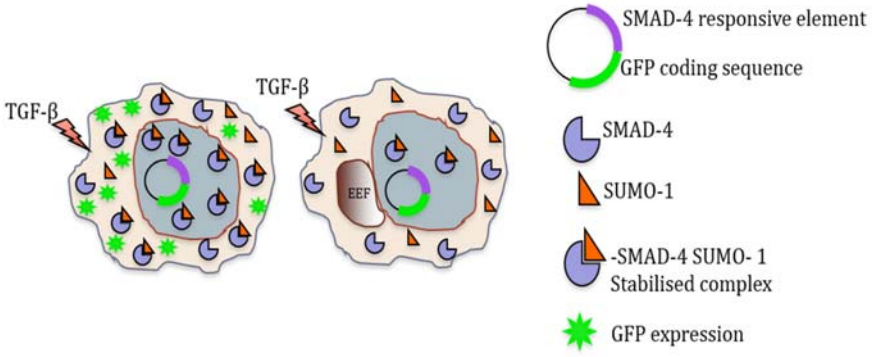


Fig. 24: Schematic showing the method of SMAD4 reporter assay during parasite development. HepG2 cells are initially transfected with Cignal-SMAD4-GFP reporter plasmid. The cells were treated with TGF-β that induces the SUMOylation of SMAD4 resulting its nuclear translocation. Activated SMAD4 (SUMOylated) binds to the SMAD4 response elements of the plasmid and drives the expression of GFP. Transfected cells were infected with *Pb* sporozoites or *Tg* tachyzoites. After 24 hours incubation, the cultures were lysed and GFP intensity was measured. The change in SMAD4 activity was expressed as difference in the GFP intensities between control and infected cells.

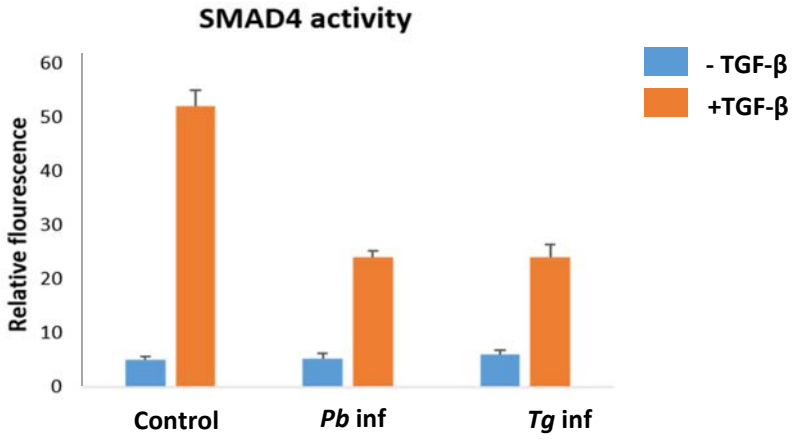


Fig. 25: Infection with *P.berghei* (*Pb*) and *T.gondii* (*Tg*) decreases the transcriptional activity of SMAD4 in HepG2 cells. GFP fluorescence quantification in HepG2 cells transfected with SMAD4 reporter plasmid followed by with or without TGF-β treatment in the presence of *P.berghei (Pb* inf) or *T. gondii* (*Tg* inf). (n=4, error bars s.e.m).

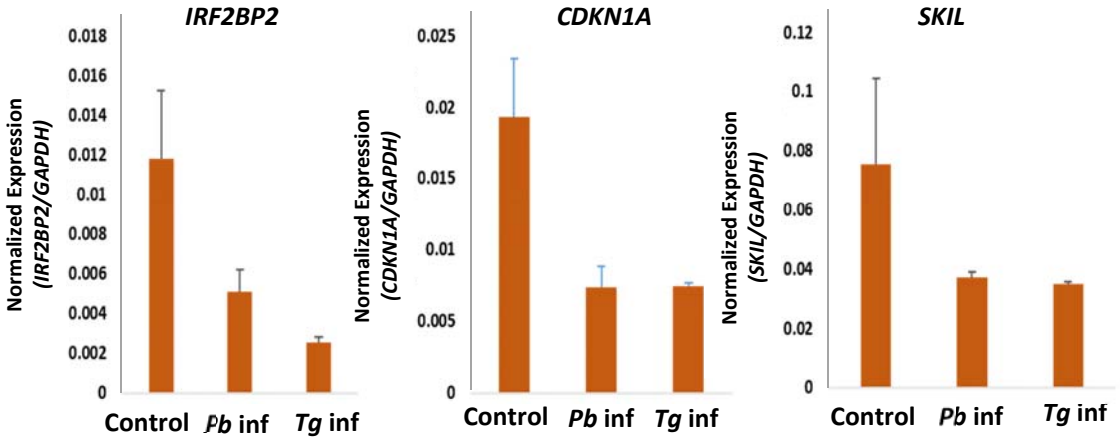


Fig. 26: Quantification of expression levels of SMAD4 target genes *IRF2BP2, CDKN1A, SKIL* in non-infected HepG2 cells (Control) and HepG2 cells infected with *P. berghei (Pb* inf) or *T. gondii* (*Tg* inf). Error bars s.e.m.

2.3.8 Microarray reveal induction of defense related genes associated with SUMO1 over expression that may mitigate the growth of *Plasmodium* EEF's

As the role of SUMO1 tagged transcription factors in activation and repression of global gene expression is well drafted in literature, we wanted to investigate the differences in the global gene expression profiles of HepG2 cells infected with *Pb* sporozoites and HepG2 cells over expressing SUMO1 infected with *Pb* sporozoites. By using human HepG2 microarrays, we investigated the differential gene expression in HepG2 cells under four different conditions: 1) Control or no sporozoite infection, 2) sporozoite infection, 3) SUMO-1 over expression and 4) SUMO1 over expression and sporozoite infection. From the raw data and heat maps (Fig 27) obtained from gene expression analysis, we pooled certain genes with their transcription factor having SUMO marks (likely to get conjugated by SUMO) and generated separate heat maps with relevant to their functions and associated pathways (Fig 28). Overall analysis of the effects of infection versus infection under conditions of SUMO1 over expression gave an indication that, *P. berghei* infection usurps the host SUMOylation process to bring out changes to create an anti-apoptotic, proliferative environment that favors the parasite development inside the host cell (Fig 29). This was evident by the altered expression of *PTGS2, KIT, SOCS3, IL1B, PLA2G, HERC5* and *NOS2* following infection with sporozoites. However, contrary to this scenario, SUMO-1 over expression upregulated several defense responsive genes like *IFNG, IL10, NOS2, IL4, CCL5* and *IFIT2* whose products may successfully revert the parasite induced effects resulting in parasite growth arrest. Validation of the expression values obtained from microarray data was done by real-time PCR analysis of 6 genes randomly picked from the expression data. There is a clear correlation between the expression values from microarray and quantification from real-time PCR (Fig 30).

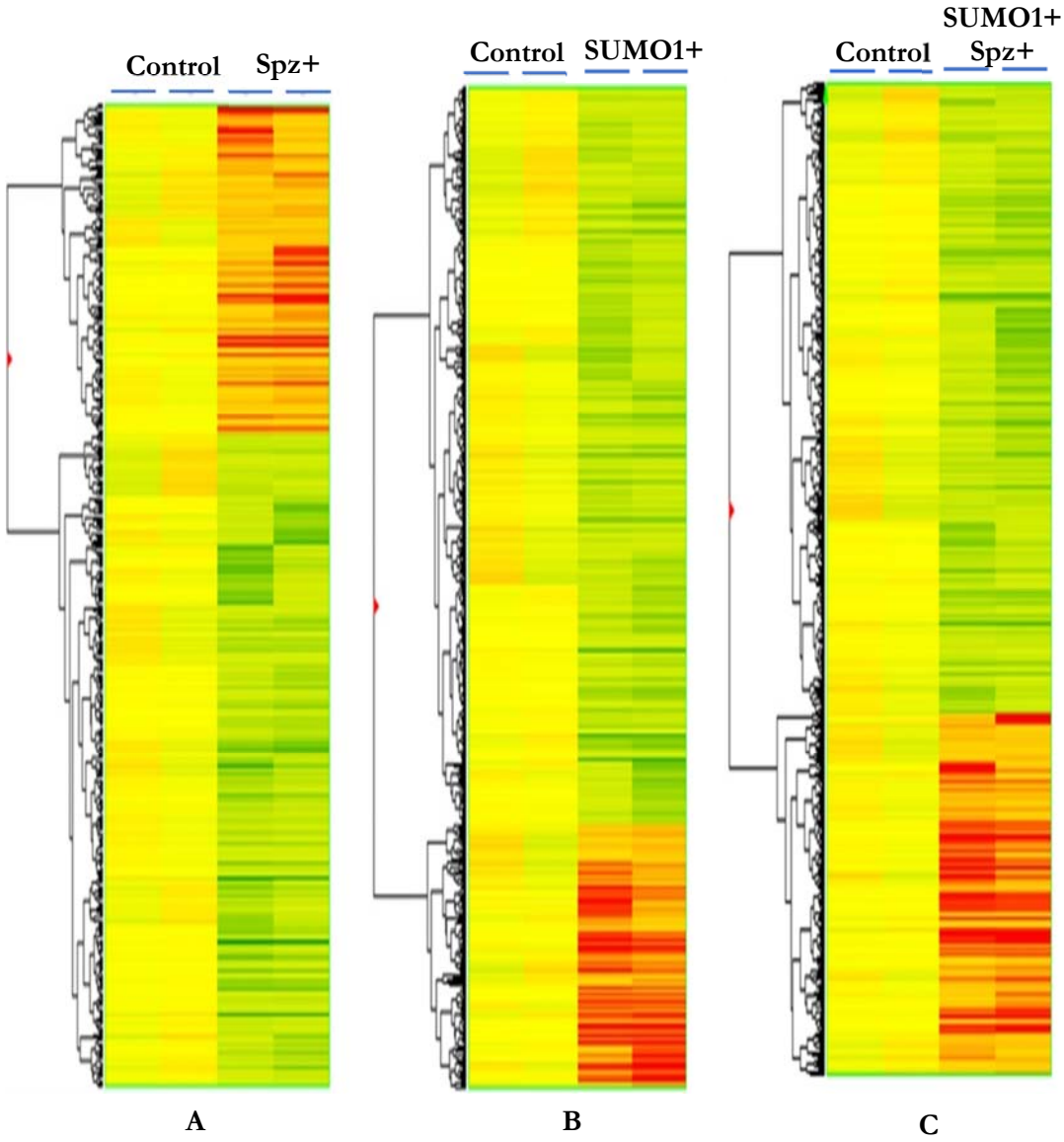


Fig. 27: Heat maps of differentially expressed genes under conditions of (A) *Plasmodium berghei* sporozoite infection (Spz+), (B) SUMO1 over expression (SUMO1+) and (C) SUMO1 over expression and *P. berghei* sporozoite infection (SUMO1+Spz+) as compared to control (Spz-).

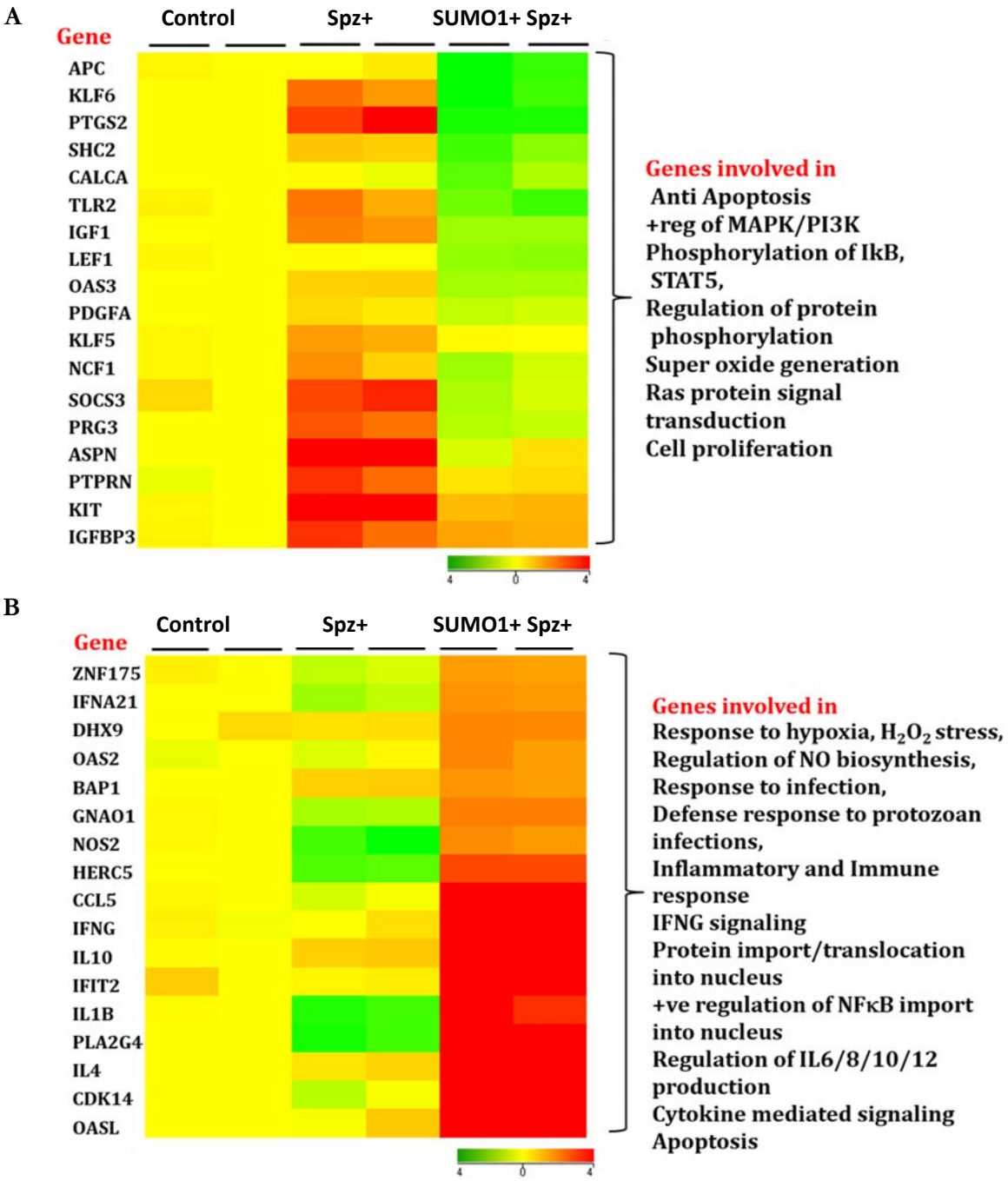


Fig. 28: A) Heat maps showing differentially expressed genes in pathways as specified in figure are upregulated during sporozoite infection (Spz+) as compared to control (Spz-) and down regulated during SUMO1 over expression and sporozoite infection (SUMO1+Spz+). B) Heat maps showing differentially expressed genes in pathways as specified in figure are downregulated during sporozoite infection (Spz+) as compared to control (Spz-) and upregulated during SUMO1 over expression and sporozoite infection (SUMO1+Spz+).

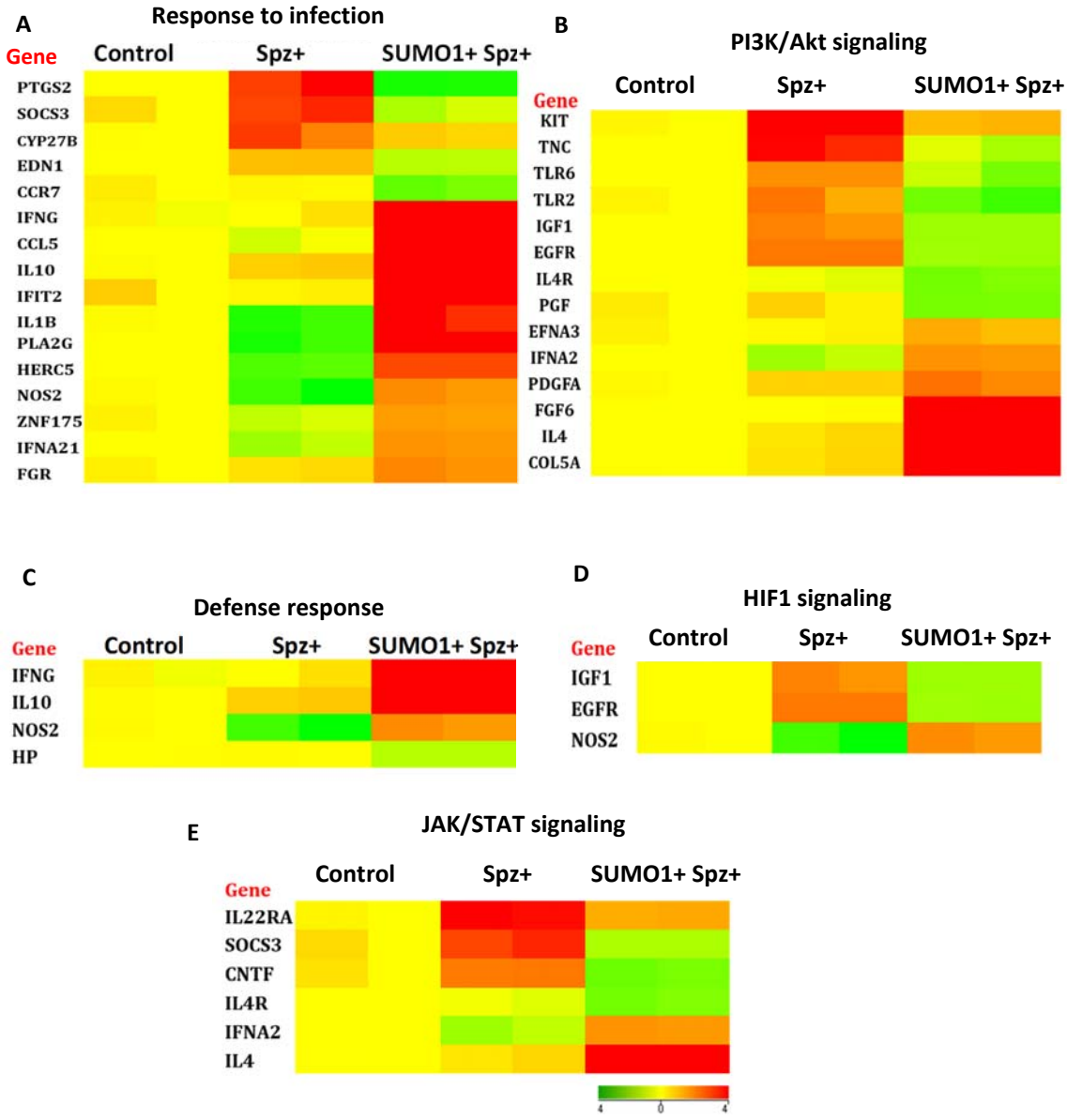


Fig. 29: Heat maps showing differentially expressed genes in pathways like (A) response to infection, (B) PI3K/Akt signaling, (C) defense response, (D) HIF1 signaling pathway and (E) Jak/STAT signaling pathway under conditions of no infection i.e. control (Spz-), sporozoite infection (Spz+) and SUMO1 over expression and sporozoite infection (SUMO1+ Spz+).

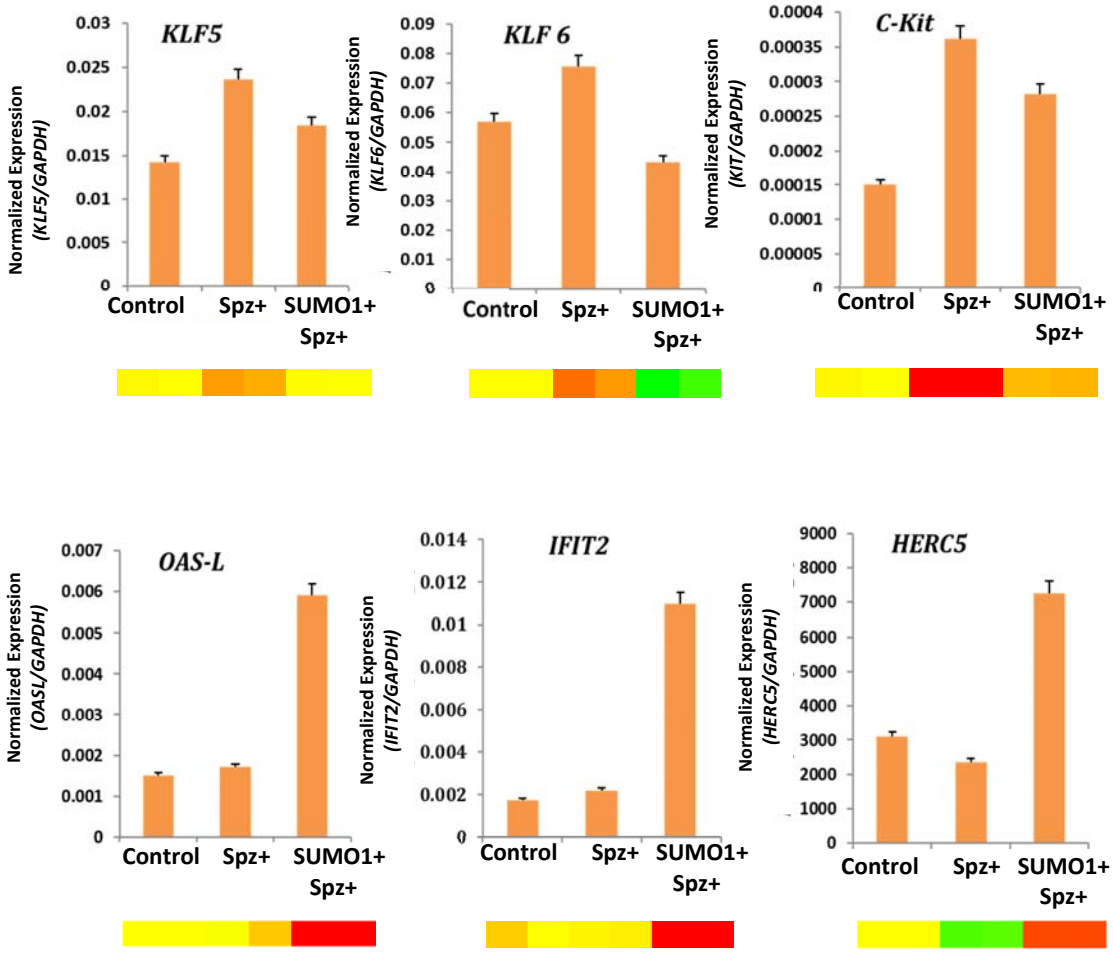


Fig. 30: Validation of micro array results by analyzing the expression of selected genes by Real-Time PCR. Gene expression analysis of 6 transcripts as indicated in figure was done by absolute real time-PCR. The cDNA was prepared from same RNA samples as used for microarray analysis. Bar graphs are normalized expression values obtained by taking ratio of target genes/ *GAPDH*. Control (Spz-), Sporozoite infected (Spz+), SUMO1 over expression and Sporozoite infected (SUMO1+Spz+).

2.4 Discussions

Several lines of evidence suggests that intracellular viruses and pathogens modify the host SUMOylation machinery for their successful establishment, propagation and dissemination of infection. The spectrum of activities altered by modulating SUMO conjugation include signal transduction, gene expression, chromatin structure and maintenance of genome (239). In the current study we have investigated how *Plasmodium* EEF's and *Toxoplasma* tachyzoites alter the host cell SUMOylation following establishment of infection and during their intracellular growth. Since over expression of SUMO1 inhibited the intracellular growth of the parasite we investigated the mechanism that leads to the parasite growth arrest. While SUMO1 over expression may facilitate its conjugation to several effector proteins within the cytoplasm, we focused to investigate a signaling pathway mediated by transforming growth factor β family which is regulated by SUMOylation and affect several biological processes central to cell growth, differentiation, cell death/apoptosis, motility and cellular transformation and production of extra cellular matrix (240). The ligands of the TGF-β family transduce signals using two distinct downstream adaptors-the heteromeric complexes of type-I and type-II serine threonine kinase receptors and intracellular SMAD proteins (240,241).

Previous studies (242) have shown that SMAD-4 mediates the transcriptional response for TGF-β mediated signaling pathway. These studies have demonstrated that, in cells transfected with SUMO1, SMAD-4 transcriptional activity is regulated by SUMOylation levels. SUMO1 expression strongly enhances the SMAD-4 levels while inhibition of SUMO1 over expression by small interfering RNA (SiRNA) mediated silencing of the E2 enzyme Ubc9 reduced endogenous SMAD 4 levels. Further, SUMO1 conjugation of SMAD-4 rescues it from ubiquitin dependent degradation and consequently enhances the transcriptional response of SMAD-4. We integrated this known information of SMAD-4 regulation by SUMOylation to analyze if developing *Plasmodium* EEF's and *T. gondii* precluded the nuclear translocation of SMAD4 by preventing its SUMOylation.

Using a TGF-β reporter based assay to investigate the modulation of SMAD-4, our results showed that both developing *Plasmodium* EEF's and *Toxoplasma* tachyzoites significantly reduced the nuclear translocation suggesting that SMAD-4 was indeed the target for SUMOylation that was hijacked by both parasites. We further provided evidence that such

reduced nuclear translocation of SMAD-4 led to down regulation of several SMAD-4 targets involved in diverse cellular functions. For example CDKN1A/p21 is a key cell cycle regulator and is involved in cellular repair process like DNA damage repair. P21 is involved in apoptosis, cell cycle arrest and antioxidant activity (243). SKIL/SNON is a component of SMAD pathway and regulates cell growth and differentiation through TGFβ. SKIL is a direct activator of p53 to accelerate aging and inhibit tumorigenesis (244). IRF2BP2 is a direct target gene of p53 and promotes cell cycle arrest and interfere with p53 mediated apoptosis (245). In fact resistance to host cell apoptosis during EEF development has already been documented (221) and suppression of host p53 is critical for *Plasmodium* liver stage infection (234). Thus infection induced conditions that lead to failure of SMAD-4 to translocate to host nucleus may likely be envisaged to inhibit P21 induced apoptosis and also prevent SKIL mediated activation of p53. Taken together, these observations collectively refer to the ability of infected cells to resist apoptosis for the successful propagation of the both *Plasmodium* and *Toxoplasma.*

Other than SMAD4, it has been shown that STAT-1 is a target for modification by SUMO1. STAT-1 is a signal transducer of the IFN-induced gene responses. A distinct SUMOylation consensus site (ψKXE) 702IKTE705 is localized in the C-terminal region of STAT-1 where Lys 703 is a target for PIAS (Protein inhibitor of activated STATs) induced SUMOylation. In light of these observations, it is tempting to speculate if STAT-1 could also serve as one of the potential transcription factors to be SUMOylated during over expression of SUMO1. Our assumption is based on independent investigations in *Plasmodium* (246) and *Toxoplasma* (111) that have shown that IFN-gamma activates several immune responsive genes that likely confer resistance for the completion of intracellular growth of *Plasmodium* EEF or *Toxoplasma* tachyzoites. IFN-gamma response is mediated by STAT-1 transcription factor, however, if the activation of such cellular defense response is under the influence of SUMOylated STAT-1 trafficking into the nucleus is not known.

For example the activity of recombinant interferon (IFN)-gamma was assessed against *Plasmodium berghei* exo-erythrocytic forms (EEF's) grown in HepG2 where complete inhibition of parasite growth was noted when IFN-gamma was added 6 hr prior to sporozoite invasion at 10^2 U/ml (247). These data strongly suggest that IFN-gamma exerts its antimalarial activity by binding to the surface receptors on hepatocytes and inducing intracellular changes unfavorable for parasite development. The natural source of IFN-gamma under *in vivo* conditions are T cells and it could be envisaged this lymphokine released from antigen specific T cells in response to the pre-erythrocytic antigen exposure, may be an important effector mechanism that contributes

to the sterile immunity following challenge of immunized mice with infectious sporozoites. Such assumptions are in agreement with a recent study where using RNA sequencing, it was shown that a robust innate immune response including type-I interferon (IFN) and IFNγ pathways are activated during rodent malaria liver-stage infection (248). Further the liver-stage infection was suppressed by the infection-engendered innate responses whose effect was abolished in mice that were deficient in IFNγ, the type I IFN α/β receptor (IFNAR), and interferon regulatory factor 3. Thus an intact signaling loop of IFNAR is critical for recruiting NK-T cell numbers in the liver, thus linking type-I IFN signaling with cell recruitment and subsequent parasite elimination.

Similarly, *Toxoplasma's* ability to block the potent IFN-gamma response might be one of the key mechanisms that allows intracellular persistence of the parasites (111). A complete dysregulation of IFN-γ inducible gene expression was demonstrated in human fibroblasts infected with *Toxoplasma* by using a genome-wide microarray analysis. When *Toxoplasma* infected cultures were exposed to IFN-gamma, none of the 127 IFN- γ responsive genes could be significantly induced in infected cells while uninfected cells from IFN-γ treated cultures expressed IFN regulatory factor 1 normally. Interestingly, in all uninfected and infected cells treated with IFN-gamma, STAT1 trafficked to the host nuclei indistinguishably. These observations likely point to the possibility that the inhibitory effects of *Toxoplasma* infection likely occurred via blocking STAT1 transcriptional activity in the nucleus. Analysis of our gene expression data set during over expression of SUMO1 indeed revealed the induction of IFN gamma and other genes involved in response to infection, apoptosis, protein translocation into nucleus and defense response to protozoan infection. Nonetheless, a definitive proof of SUMOylation of STAT-1 in our studies awaits further investigation.

Our studies have also documented the better growth of EEF's when Ubc9, the only E2 enzyme for performing conjugation activity within the members of the SUMO family was silenced by shRNA. This suggested that an overall decrease in the SUMOylation process benefits the growth of *Plasmodium* and *Toxoplasma*. Interestingly, a similar kind of observation were also made in *Listeria monocytogenes*, where a significant decrease in the level of SUMOylated cellular proteins was noted, a phenomenon likely attributed to the degradation of the conjugating enzyme UBC9. Thus, usurping the host cellular machinery may utilize a common theme irrespective of the nature of the pathogen that causes the disease. While these studies highlighted the role of Listeriolysin as an effector in modulating the host cell SUMOylation, it is currently unknown how *Plasmodium* and *Toxoplasma* achieve overall reduction of host SUMOylation. The

scenario may not be as simple as *Listeria* that reside in the cytoplasm and have access to directly modulate the host machinery. *Plasmodium* and *Toxoplasma* that reside in membrane bound PVM compartment may have more elaborate mechanisms of protein export that may directly modulate the components of SUMO machinery or indirectly regulate through altering the expression of the SUMO machinery enzymes as documented in this study. We did not observe any changes in the expression of the de-SUMOylase (SENP4) suggesting that this pathway was not affected by both *Plasmodium* and *Toxoplasma* (data not shown).

To unravel the global changes in the host gene expression during (1) Spz-, (2) Spz+, (3) SUMO1+ and (4) SUMO1+Spz+, we performed global microarray analysis of these samples at 40hr post infection. Comparison of differentially expressed (DE) genes between Spz+ and Spz+SUMO1+ revealed that during intra hepatic development, *P. berghei* usurps the host SUMOylation to create an anti-apoptotic, proliferative environment that is beneficial for *Plasmodium*. We observed the levels of PTGS2, KIT, and SOCS3 were up regulated in Spz+ compared to Spz+SUMO1+ condition. Although no gene ontology based pathways can be clearly identified for these differentially regulated gene sets, some predictions were made based on the existing literature evidence. For example, PTGS2/COX-2 is responsible for production of the inflammatory prostaglandins which plays important roles in modulating cell motility, resistance to apoptosis and proliferation (249).

In *Trypanosoma cruzi,* down regulation of COX-2 by celecoxib impairs the parasite entry into cardiac cells and modulates the inflammatory response. This inhibition was in association with increase in NO and IL-1β (250). In *Leishmania infantum*, production of the Prostagalandin E2 mediated by COX2, negatively regulates the production of inflammatory cytokines and IL-17 benefiting the parasite survival (251). Thus, *Plasmodium* hepatic stages may follow a similar strategy for modulation of host COX2 pathway to establish successful infection. SOCS3 is induced by cytokines including IL-6, IL-10 and IFN-G. SOCS3 inhibit STAT3 activation by binding to both JAK kinase and the cytokine receptor (252). SOCS3 play a role in regulation of STAT3 signaling in the outcome of infection with *Mycobacterium tuberculosis (253)*. C-KIT (KIT) plays an important role in regulation of cell proliferation and survival, stem cell maintenance, migration and function. KIT can activate several signaling pathways including activation of Akt/PI3K, STAT1, STAT3, STAT5 and PLCG1. PI3K pathway is required for successful infection of *Yersinia*, *Listeria* and *Salmonella* (254-256). In *Plasmodium* HGF/MET signaling protects infected host cells via activation of PI3-kinase pathway (257).

However, contrary to this scenario during SUMO1+Spz+ condition, several defense responsive genes like *NOS-2*, *IFN-G, IL1-B, IL10, IL4, CCL5*, *IFIT2* and *HERC5* were upregulated whose products may be recruited to revert the parasite induced effects and promote parasite growth arrest. NOS2 (iNOS) activity produces nitric oxide which is a messenger molecule with diverse functions. NO mediates tumoricidal and bactericidal actions. NO has been proposed to have a relevant role in malaria pathogenesis, but its mechanisms of action in different stages of infection remains unknown. NOS2 promoter polymorphisms are associated with different malaria clinical outcomes (258). Further, NOS2 acts downstream to HIF1 signaling. HIF signaling reduces the susceptibility to mycobacterial infection via a nitric oxide dependent mechanism (268). IGF1, a component of HIF1 signaling is upregulated during Spz+ condition. IGF1 is likely associated with promoting cell growth activity which is reversed during SUMO1+Spz+ condition.

IFNG activates JAK/STAT pathway, leading to synthesis of IRF1 and stimulation of NOS2 expression. NOS2 expression in phagocytes results in efficient killing of *Leishmania* parasites *in vitro* and is critical for controlling the infection *in vivo* (259). IL1β is an important mediator of inflammatory response and is involved in apoptosis, cell proliferation and differentiation. IL1β is critical for host resistance during *Mycobacterium tuberculosis* infection (260).

HERC5 protein localizes in the cytoplasm and on perinuclear region and functions as an interferon-induced E3 protein ligase that mediates ISGylation of protein targets. ISGylation is another PTM involving addition of ISG15 to target proteins. HERC5 broadly targets the newly synthesized proteins for ISG15 conjugation, and many endogenous targets of HERC5 have been identified that function in diverse cellular pathways including RNA splicing, chromatin remodeling/polII transcription, cytoskeleton organization and regulation, translation, glycolysis, stress responses, interferon signaling and antiviral responses. HERC5 inhibits HIV-1 replication by targeting a unique step of HIV-1 particle assembly at the plasma membrane (261).

IFIT2 a protein involved in Interferon signaling and RNA binding. RNA-binding is required for antiviral activity and can promote apoptosis. IFIT2 can interact and modulate the microtubule network that is implicated in cell reorganization during *A. algerae* infection as a host response to clear the infection (262). The upregulation of IFIT2 in SUMO1+Spz+ condition may direct the cells to undergo apoptosis. CCL5 (also termed as RANTES) is a β chemokine and induces leukocyte migration by binding to CCR1, CCR3 or CCR5. An elevated level of

CCL5 has been associated with a variety of inflammatory disorders. CCL5 is also induced by multiple inflammatory stimuli and is also constitutively expressed in T cells. Decreased CCL5 levels in $CCR5^{-/-}$ mice also reflects a decreased inflammatory response in the $CCR5^{-/-}$ mice during *Toxoplasma* infection (263). CCL5 is part of the cascade of events leading to efficient parasite growth control in *Leishmania major* infection and blockade of CCL5 rendered mice more susceptible to infection (264). IL4 is a pleiotropic cytokine produced by activated T cells. IL4 regulates the functions including B-cell activation, induces the expression of class II MHC and enhances the expression of IgE and IgG1. The sequence variations in IL4 leads to host resistance in disease like Chagas disease and malaria (265,266). IL4 induction in listeriosis contributes to host defense without impairing the development of acquired T cell response (267).

Infection with the promastigote form of *Leishmania major*, *Leishmania pifanoi* and *Leishmania amazonensis* activates signaling through p38 mitogen-activated protein kinase (MAPK), NFκB and PI3K/Akt (269). Inhibition of PI3K/Akt pathway with LY294002 and Akt IV inhibitor reversed resistance of infected RAW 264.7 macrophages and bone marrow-derived macrophages to potent inducers of apoptosis. Moreover, reduction of Akt levels with siRNA resulted in the inability of infected macrophages to resist apoptosis. Further evidence of the role of PI3K/Akt signaling in the promotion of cell survival by infected cells was obtained with the finding that Bad, a substrate of Akt, becomes phosphorylated during the course of infection. Thus *Leishmania* promastigotes engage PI3K/Akt signaling, which confers to the infected cell, the capacity to resist death from activators of apoptosis. Our observations of Akt pathway being upregulated during (SUMO1+Spz+) over expression may also hint to the fact that a similar host cell resistance may be offered during SUMO over expression that counter acts the effects of *Plasmodium* and *Toxoplasma* EEFs.

In an independent study, transcriptional profiling during malaria liver stage infection revealed 3 distinct phases during which time the host profiles are modified (270). These include an initial stage of stress response, the engagement of host metabolic processes and finally maintenance of cell viability throughout infection (270). Our data of the transcriptional analysis of HepG2 cell response in Spz+ conditions, when compared to this study where transcriptional landscape of murine hepatoma (Hepa1-6) cells was done following infection with *P. berghei* sporozoites (270) revealed a similarity in the differential expression of several host genes and pathways involved in apoptosis, signaling, transport and transcription/translation. The expression of stress response genes to infection were similarly modified. The interacting partners

of JAK/STAT1 signaling pathway are significantly changed in HepG2 cells but not in infection to Hepa1-6 cells. Our experimental results shows that *Plasmodium* modulates the JAK/STAT1 pathway as happens in *Toxoplasma* infection (111) and the changes are reversed in SUMO1+Spz+ group.

We further observed up regulation of *TLR2* and *TLR6* in Spz+ condition. During *Brucella abortus* infection, the MAP kinase signaling is impaired in *TLR2* and *TLR6* KO dendritic cells, leading to a decrease in the production of proinflammatory cytokines (271) likely hinting that TLR2 and TLR6 act as positive regulators of MAPK pathway. Previous studies have shown that during sporozoite infection to HepG2 cells both PI3-kinase and MAPK pathways are activated though only the PI3-kinase/Akt pathway plays a critical role in protecting host cell from apoptosis (61). While our studies do not demonstrate a direct role of MAPK pathway in Spz+ condition, we nonetheless hypothesize that the TLR2 and TLR6 upregulation observed in our study may involve MAPK loop and that both TLR2 and TLR6 expression is reversed in SUMO1+ Spz+ condition.

The differentially expressed genes reported in Hepa1-6 cells such as *AARS, CHAC1, DPP7, GPR137, KIF5C, KLF4, MAF, NR4A2, NRP, PROC, PTPN14 and SLC16A4* which are involved in diverse cellular functions are also showed similar differential expression in infected HepG2 cells. The differential regulation of similar functional clusters in two independent transcriptomic profiling studies, suggests that the observed changes in the transcriptional patterns are indeed true. The pro- and anti-apoptotic gene expressions are modified in both Hepa1-6 and HepG2 cells infected with *P. berghei* suggesting a tendency of infected cells to undergo apoptosis and the modulation of host cell by the parasite for host cell survival. In contrast, in SUMO1+Spz+ condition, the host cell responses are clearly biased towards the suppression of cell proliferation and promoting apoptosis which may be responsible for the parasites growth arrest and clearance.

2.5 Conclusions

The overall investigations from this part of work revealed that apicomplexan parasites like *Plasmodium* and *Toxoplasma* hijack the host SUMOylation machinery to the benefit of their intracellular growth and dissemination. We show convincingly that *Plasmodium* and *Toxoplasma* alter the host SUMO machinery at the transcriptional level, as well as decrease in the overall conjugation of the SUMO to cellular proteins. Of the several host proteins that may be altered by these intracellular parasites, we provide a definitive evidence for the role of SMAD-4 of TGF-β pathway as a target for altered SUMOylation. Over expression of SUMO1 mitigates the intracellular development of *Plasmodium* EEF and replication of *Toxoplasma* hinting to regulation of the global gene expression in favor of host such that the cellular environment is rendered hostile for the parasite development. Contrary to this observation, shRNA mediated silencing of the only conjugation enzyme of the host SUMO machinery-the E2/Ubc9 favored the intracellular growth of *Plasmodium* EEF's. SUMO1 over expression promotes the expression of several genes that play a role in the defense responses and overcomes the parasite mediated effects. This was evident by counteracting/down regulating the expression of genes central to anti-apoptosis and cell proliferation induced by the parasite when SUMO1 was over expressed. Our findings provide a mechanism of how *Plasmodium* and *Toxoplasma* usurp the host post translational pathway for their benefit and to the best of our knowledge this is a novel report in apicomplexan parasites that dissects the host parasite interactions central to SUMO modification of cellular proteins.

Chapter 3

Functional characterization of Proteasome inhibitor 31 (PI31) in *Plasmodium berghei* by reverse genetics approach

3.1 Introduction

The life cycle of *Plasmodium* involves transitions at various stages which vary in terms of cellular physiology. This requires a tight control over protein turnover, a feature regulated by proteasome that maintains protein homeostasis by carefully degrading redundant and mis-folded proteins through ubiquitin mediated pathway. In fact, *in silico* predictions indicates that, half of the parasite proteins represent targets for ubiquitination (204). Catabolism of proteins occurs chiefly through ubiquitin mediated proteasome pathway. Proteasome is a multi-subunit catalytic protein complex involved in maintenance of protein homeostasis. The proteasome is a major proteolytic complex, involved in the degradation and recycling of proteins and in maintaining intracellular protein quality control. The proteasome carries out the degradation of many ephemeral proteins that are involved in signal transduction, cell cycle regulation, stress response and apoptosis. The proteasome is responsible for the recycling of damaged or abnormal proteins, which would otherwise accumulate and become lethal to the cell (188). Proteostasis is central to cell development, abnormalities can lead to metabolic disorders that may affect the cell survival.

The proteasome is a member of the Ubiquitin-proteasome system (UPS), by which most intracellular proteins are kept in quality check, degraded and recycled. Proteins become labelled with ubiquitin via an UPS specific enzymatic cascade. The type of ubiquitination linkage on the target protein determines its destiny for their role in cellular processes like degradation by the proteasome, signal transduction, DNA repair and trafficking (189,190). The proteasome of eukaryotes is a modular protease system with multiple holoenzyme complexes formed from multiple interchangeable protease and regulatory components (272). The catalytic heart of these complexes, called the core particle or 20S proteasome is a 700 kDa barrel shaped structure composed of four axially stacked hetero- heptametrical rings (α1-7, β1-7, β1-7, α1-7) (273). The two outer rings are composed of seven different α subunits which serves as entry sites, the two inner rings consists of seven different β subunits that exert trypsin, chymotrypsin and caspase like protease activities with unique specificities (274). Substrates reach this catalytic core chamber via the pores formed by α subunit rings at either end of the cylinder (275).

20S proteasome possesses three distinct and specific proteolytic active sites present in the two central rings of β subunits (Fig 31). The active sites hydrolyze almost all peptide bonds with an exception of those bonds which follow glycine and proline. The elimination of

potentially catastrophic nonspecific substrates is averted by sequestration of active sites within the 20S proteasome hollow structure (274). The access for substrates to the central catalytic chamber is through axial ports at the end of α subunit rings (276). In the absence of activators the channels remain closed and the proteasome activity is suppressed. Regulation of proteasome is a complex and slightly unresolved issue.

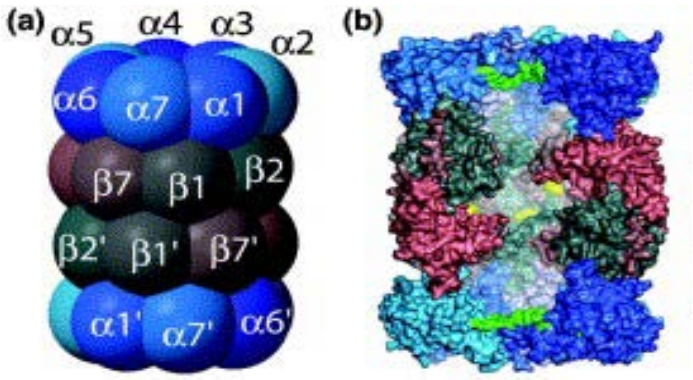


Fig. 31: Architecture of the 20S proteasome. (a) Side view of the proteasome. N termini of β1, β2 and β5 forms the active sites, with substrate preferences: β1- PGPH (peptidylglutamyl peptide hydrolase) substrates; β2- trypsin like substrates and β5- chymotrypsin like substrates. (b) cutaway stereo view showing the active sites (yellow) within the central catalytic chamber and α –annulus, the opening for substrates through the middle of the α subunits ring (green) (274).

Proteasomes are activated by binding of proteasome regulators to α subunits at the apical face which induces the opening of the 20S cylinder allowing the proteolytic activity of the β subunits. Various regulators have distinct mechanisms for 20S proteasome binding and activation. Under physiological conditions the activation of the 20S core requires the binding of 19S regulatory particle (PA700 a 20 subunit complex) to both or either ends of the outer α subunit rings, positioning as a gatekeeper and leading to the formation of a functionally active 26S proteasome (194). PA700 is the only activator of proteasome that is known to stimulate the degradation of proteins and recognizes poly-ubiquitin modification of target proteins (277). PA700 employs a single structural element, the HBYX motif for binding to the proteasome and activating functions. The interaction of C-terminal HBYX motif with cognate sites between adjacent α subunits induces conformational changes which results in the gate opening (201). Substrate entry is a complex process that is catalyzed by the PA700 subunit, and the fine architecture of PA700 is highly evolved to confine proteolysis to a nano-sized compartment and

prevents the nonspecific destruction of cellular proteins. The functioning of PA700 ensures the proteolysis as an exquisitely selective process (278). PA700 possess chaperone like activity to destabilize the substrates and deliver them to the proteolytic chamber. The overall process of proteolysis by 26S proteasome is energy consuming and depends on ATP hydrolysis (188).

In addition to PA700, two other protein complexes, PA28 (11S or REG) and PA200 bind specifically to the 20S proteasome and activate it against peptide substrates (279,280). PA28 utilizes separate C-terminal and internal structural elements for binding to the proteasome and activation, respectively (281), whereas PA200 contain HEAT (Huntingtin, Elongation factor 3, protein phosphatase 2A and yeast kinase TOR1) repeats that activate proteasome by relieving the occlusions at the proteasome's outer rings (282). PA28 does not change the proteolytic specificity of 20S, but it can influence the quality and quantity of the protein repertoire that undergo cleavage (283). Though the biochemical basis for activation of proteasome by PA28 is well characterized, the role and biological relevance of PA28 induced peptidase activity is poorly understood and remain obscure (194). The small molecule synthetic proteasome inhibitor, Bortezomib (Velcade) has proved to be effective against multiple myeloma and has raised considerable clinical interest in proteasomes (284). Several viral and cellular proteins have also been known to inhibit 20S proteasome activity *in vitro*. The most abundant chaperone hsp90 inhibits the constitutive 20S proteasomes, and inhibition is abrogated by low levels of PA28 (285). Physiological inhibition of 20S proteasome is achieved by PSMF1 (Proteasome inhibitor PI31 subunit, commonly called PI31) and Pr39 by directly blocking proteasome activity and attenuating the binding of proteasome activators (194).

PI31 is a proline rich 31 kDa protein, highly conserved throughout metazoan evolution, that inhibits the activity of proteasome by directly binding to the entry points (outer α rings) of the 20S proteasome or by competing with the proteasome activators i.e PA700 and PA28 for binding to the 20S proteasome (286). PI31 preferentially inhibits the post-glutamylpeptidyl-hydrolysing and chymotrypsin (CT) active sites of the proteasome rather than the trypsin (T) site (286). Contrastingly, PI31 acts as a selective modulator for the proteasome mediated steps in antigen processing rather than inhibiting the proteasome (198). PI31 undergoes a post translational modification through ADP-ribosylation, which drastically reduces the affinity of PI31 towards 20S proteasome and relieve 20S repression by PI31. ADP-ribosylation of PI31 increases its binding and sequestration of 19S assembly chaperones, promoting 26S proteasome assembly and activity (197).

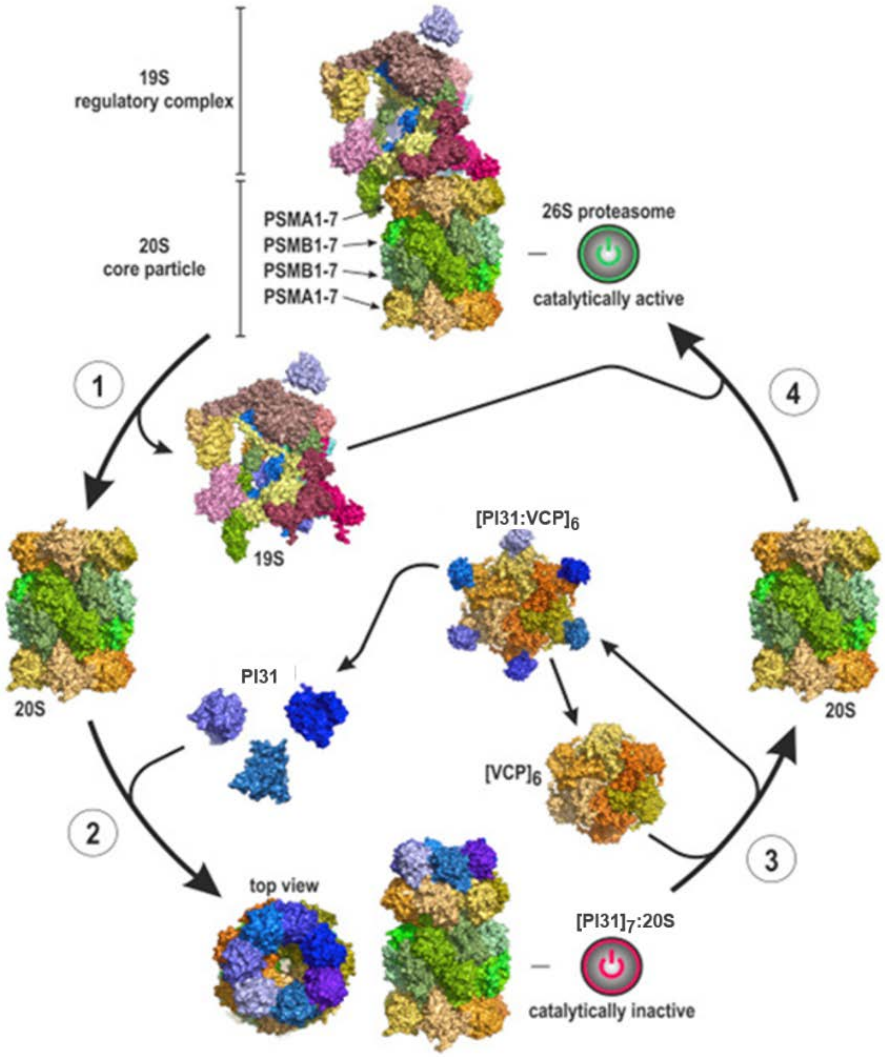


Fig. 32: Regulation of proteasome activity. Architecture of functionally active 26S proteasome with 20S core particle and 19S regulatory particles. The dissociation of 19S particle from 26S proteasome generates free 20S core particle. PI31 binds to the 20S particle and inhibit the catalytic activity. VCP (Valosin containing protein) sequesters PI31 form the inhibitory complex and generates free 20S core particles. Free 20S can be further activated by binding of 19S (287).

It is speculated that the N-terminal part of PI31 is responsible for binding to the functional 20S proteasome and mediates its inhibition; but, the exact function of the proline rich C-terminal domain remains unknown (198). A recent study showed that PI31 was able to suppress the assembly of 26S proteasome from 20S and 19S particles without showing any effect on the preformed 26S proteasome *in vitro*, suggesting that only free 20S proteasome seems to interact with PI31 (288). PI31 molecules indeed acts as caps for the 26S proteasome with C-terminal ends of PI31 monomers blocking the entry of substrate proteins to the active sites

inside the 20S core particle. This PI31 mediated inhibition of the proteasome can be counteracted by a regulatory protein - Valosin containing protein (VCP). VCP is an AAA-ATPase which is involved in diverse cellular functions interact directly with PI31 and regulate the activity of proteasome in an antagonistic fashion (287) (Fig 32). VCP sequesters PI31 from PI31:20S complex enabling the reassembly of 26S proteasome from free 20S core and 19S regulatory complexes.

Spo0M a sporulation controlling factor in *Bacillus subtilis* which has significant structural similarity with FP domain of human PI31 plays an essential role in the early stage of endospore formation (289). The proteasome components aside from their protease activity, also functions non-proteolytically in a variety of cellular processes including chromatin remodeling, DNA repair and transcription. Studies in yeast demonstrated the direct activities of PA700 in transcription initiation and elongation supporting a role of co-trans-activator (290). PA700 is also involved in nucleotide excision repair (NER), inhibition of ATPase activity of PA700 inhibits the NER activity in a Rad23 (poly-ubiquitin chain binding protein) dependent manner (291).

Given the fact that precise regulation of protein turnover and proper folding is critical for the rapid transformation of malaria parasite (to different morphological forms) and for their adaptation to environmental stress (temperature and oxidative stress) and during progression of life cycle in mammals and vectors, it is perceptive to speculate that the 26S proteasome is essential for the survival of malaria parasites (203). Existing literature indicates that the 26S proteasome is essential for parasite development throughout all life stages (292-295), emphasizing the proteasome as a promising target for malaria therapy. Considering the critical functions of proteasome in maintaining cellular proteostasis, we wanted to address how the regulation of 26S proteasome in *Plasmodium* species is achieved and if some of the known cellular proteins that act as activators or inhibitors of proteasome in other eukaryotes function in a similar way in *Plasmodium*.

It is known that *Plasmodium* possess a functional eukaryotic 26S proteasome and an additional bacterial peptidase like protein complex (ClpQ/HsIV) (209), but the specific functions of these complexes within the parasite are unknown. Inhibitor studies in *Plasmodium* reveals an essential role of the proteasome in the liver, blood and transmission stages, suggesting the proteasome as a multi-stage target in malaria therapy (203). However, all the inhibitors studied so far that inhibit the *Plasmodium* proteasome also inhibit the host proteasome, which

limits their tolerated dose and usage as antimalarial drugs (292). Focusing on exploitation of proteasome activity regulators may provide new clues in the field of malaria treatment. Recent research evidences unravel the integrity of *Plasmodium*'s 26S proteasome with all known proteasome subunits and provided hints for the occurrence of hybrid proteasomes (19S-20S-PA28), highlighting the possible existence of proteasome regulating function by PA28 in *Plasmodium* (296).

The presence of PA28 (proteasome activator) in *Plasmodium* prompted us to look for the annotation of its antagonistic protein i.e PI31 (proteasome inhibitor). We did extensive database mining and found orthologues to PI31 in *Plasmodium* species. In a recent study, the predicted *PI31* mRNA expression was shown exclusively in liver stages of *Plasmodium yoelii* (297). While the function of the proteasome is well characterized, the influence of the regulatory protein complexes on proteasome activity is not yet clear. Studying the role of PI31 in regulating the proteasome function, at different stages of life cycle may shed light on stage specific transitions. Loss of function studies using reverse genetics approach proved to be successful tool to dissect the role of a specific gene, and this method is extensively used in *Plasmodium* research.

3.2 Materials and methods

3.2.1 Animals and parasite lines used for the experiments

Rabbits, BALB/c mice, Swiss albino mice and C57BL/6 mice were procured from National Institute of Nutrition, Hyderabad, India and house at the animal house facility of University of Hyderabad. All animal experiments performed at the School of Life Sciences, University of Hyderabad were in compliance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) national guidelines. The animal protocols were approved by Institutional Animal Ethics Committee (IAEC). Transgenic parasite lines were generated by transfecting respective targeting plasmid in wild type *P. berghei* ANKA parasite line. WT *P.berghei* ANKA GFP parasite line (Surendra KK and Kumar KA, personal communications) was used for comparison of phenotype of transgenic parasites throughout the parasite life cycle. Life cycle of *P. berghei PI31* KO parasites was studied by cycling the parasites through BALB/c and female *A. stephensii* mosquitoes. In experiments involving analysis of pre-patent period by intravenous injection of sporozoites or mosquito bite, C57BL/6 mice were used.

3.2.2 Bioinformatics analysis

Plasmodium genome database PlasmoDB (www.plasmodb.org) was scanned to generate a list that included predicted protein sequences with PI31 like motifs. *P. berghei PI31* (Gene Id PBANKA_120850) gene and protein sequences were retrieved from PalsmoDB. Primers were designed based on the sequence information.

Conserved Domain Search (CDS) was performed using PbPI31 amino acid sequence. MULTALIN (http//multalin.toulouse.inra.fr/multalin) was used to check the homology of PI31 protein among different *Plasmodium* species. The gene IDs for the sequences of PI31 used for multiple sequence alignment are: PBANKA_120850, PY17X_1211700, PCHAS_120920, PF3D7_1010100, PRCDC_1009500, PKH_080960 and PVX_094725 (obtained from PlasmoDB).

For phylogenetic analysis, multiple sequence alignment was done using ClustalX 2.0 and phylogenetic analysis was performed using MEGA.4. The evolutionary history was interpreted using neighbor-joining method. The bootstrap scores for the tree are inferred using 1000 replicates taken to represent the evolutionary history of the taxa analyzed. The phylogenetic tree is drawn to scale, with branch lengths representing the evolutionary distances. The GenBank accession numbers of the PI31 orthologues are: *Plasmodium falciparum* 3D7 XP_001347383, *Plasmodium yoelii yoelii* 17XNL XP_724250, *Plasmodium berghei* ANKA CDS49095, *Plasmodium knowlesi strain H* CAQ39511*, Plasmodium vivax* Sal-1 XP_001614412, *Homo sapiens* CAC10383.1, *Chlamydomonas reinhardtii* EDP07961, *Selaginella moellendorffii* XP_002970386, *Physcomitrella patens* EDQ62410, *Micromonas pusilla* XP_003055701, *Micromonas sp.* RCC299 XP_002499999, *Neospora caninum* XP_002371034, *Toxoplasma gondii* KFH10377, *Leishmania infantum* XP_001462933, *Leishmania major* CAJ08078, *Gallus gallus* NP_001026753.1, *Rattus norvegicus* NP_001094475.1, *Mus musculus* EDL05928.1, *Drosophila melanogaster* NP_610715.1, *Nematostella vectensis* EDO37152, *Paramecium tetraurelia* CAK84047, *Tetrahymena thermophile* XP_001033021, *Naegleria gruberi* EFC38623, *Volvox carteri f. nagariensis* EFJ48166, *Danio rerio* XP_699466.

3.2.3 *PbPI31* gene expression analysis by quantitative real time PCR (qRT PCR)

To quantify the gene expression of the *PbPI31,* cDNA from rings, mixed blood stages, midgut sporozoites, salivary gland sporozoites and liver stages at 16 h, 25 h, 42 h, 50 h and 65 h were used. Gene specific standards were generated by cloning 150 bp coding sequence of *Pb PI31* and *Pb18S rRNA* into TA vector followed by serial dilution in a log range from 10^2 to 10^7

copies per µl. RNA from the above mentioned stages was isolated using using Purelink Total RNA isolation kit (Ambion). Subsequently 1.5 µg of RNA from each sample was used to generate cDNA using 10X PCR buffer, $MgCl_2$, dNTPs, RNase inhibitor, random hexamer primers and MulV reverse transcriptase (all from Applied Biosystems). The cDNA samples and gene specific standards generated were used for absolute quantification of *PbPI31* expression in different stages of *P. berghei* life cycle. Reaction for real-time analysis was set up in a 96 well format plate with the specified primers, cDNA samples and 2X SYBR Green mix (Biorad), the PCR run and detection was done in Eppendorf Real-Plex machine. The expression values of *PbPI31* were normalized with the values of *Pb18SrRNA* for each specific sample. The primer sequences of *PbPI31* and *Pb18SrRNA* were listed in table1.

3.2.4 Primers

S.No	Primer name	Primer Sequence (5'-3')
1	PbPI31 5' FP (FP1)	GTA**CTCGAG**CTTTGCTTGTATGCTATCT
2	PbPI31 5' RP (RP1)	GTA**ATCGAT**AGTAAAAAAAGAGGAAATAC
3	PbPI31 3' FP (FP2)	ATA**GCGGCCGC**TATCTCCTTCAATAATACTA
4	PbPI31 3' RP (RP2)	AAT**GGCGCGCC**TTTAGAGTGGATAATAGCT3
5	PbPI31 cnf FP (FP3)	ACTATATTTTCTTTTTATTCCAA
6	PbHSP70 5' UTR RP (RP3)	TTCCGCAATTTGTTGTACATA
7	hDHFR FP (FP4)	GTTGTCTCTTCAATGATTCATAAATAG
8	PbPI31 cnf RP (RP4)	ACATTAGTTACACGCATTAATTTGA
9	PbPI31 ORF FP	CCAGGATCCATGGAAAAGTGTGAAACCTT
10	PbPI31 ORF RP	CCACTCGAGTTAGAAAGGAAAATTGTTCTG
11	PbPI31 RT FP	TTCATCAGAACAATTACGTGTTT
12	PbPI31 RT RP	ATAGGGTCATAACGCAAGTTTTG
13	Pb18SrRNA FP	GGGGATTGGTTTTGACGTTTTTTGCG
14	Pb18SrRNA RP	AAGCATTAAATAAAGCGAATACATCCTTAT

Table 1: Information of primer sequences used for construction of transfection plasmids, to confirm site specific integration, to check WT contamination from cloned *PbPI31* KO lines and real-time PCR analysis.

3.2.5 Generation of *PbPI31* (PBANKA_120850) knockout construct

3.2.5.1 PCR

To generate *PbPI31* knockout construct, we used double cross over recombination strategy for target gene replacement. To achieve this, *P. berghei ANKA* genomic DNA was used as a template to amplify 554bp of the 5'flanking region of *PbPI31* using primers *PbPI31* 5' forward (FP1) and *PbPI31* 5' reverse primers (RP1). A 507 bp of the 3' flanking region of *PI31* was amplified using *PbPI31* 3' forward primer (FP2) and *PbPI31* 3' reverse primers (RP2) (primer sequences provided in table 1). To amplify 5' and 3' fragments of *PbPI31*, 100 µl PCR mix was prepared by adding 1X PCR buffer, 1 mM dNTP mix, 5 mM MgCl2, 0.5 µM forward primer, 0.5 µM reverse primer, 2.5 units Taq polymerase and 50 ng genomic DNA. Volume was made up to 50 µl with nuclease free water. The reaction mix was kept in PCR machine with thermal cycling conditions, 94°C for 2 min for initial denaturation, 94°C for 30 sec followed by annealing at 54°C 30 sec and extension at 72°C for 1 min. The PCR cycles were repeated for 35 times with a final extension at 74°C for 10 min. After completion of PCR, 5 µl of the product was analyzed on 1% agarose gel that revealed an expected size of the amplicon. PCR products were purified using PurelinkTM quick gel extraction and PCR purification combo kit (Invitrogen, Cat No.K2200-01).

3.2.5.2 Preparation of *Escherichia coli (E.coli)* XL1 Blue MRF' competent cells and transformation

Escherichia coli XL1 blue MRF' cells were cultured overnight in LB medium with tetracycline (15µg/ml). After diluting the overnight culture a 1:100 diluion in LB medium with tetracycline, it was incubated at 37°C in orbital shaker maintained at 200 rpm until it reached an OD_{600} of 0.5. The culture was incubated on ice for 30 min followed by centrifugation at 6000 rpm at 4°C for 10 min. Bacterial cell pellet was resuspended in 3-5 ml of ice-cold 0.1M $CaCl_2$ solution and incubated for 1 h on ice followed by centrifugation at 4°C for 10 min at 6000 rpm. Finally, the cell pellet was resuspended in 13% glycerol prepared in 0.1M $CaCl_2$ solution and 50 µl of competent cells were aliquoted in 1.5 ml micro centrifuge tubes and instantly frozen in liquid nitrogen which were further stored at -80°C.

XL-Blue MRF' competent cells were used for transformation of plasmids or ligation reaction samples in molecular cloning. Ligation mixture of 10 µl was added to 50 µl of competent cells and incubated on ice for 20 min, followed by a heat shock at 42°C for 90 sec and further

incubation for another 5 minutes in ice. Pre-warmed LB medium of 1 ml was added to the cells and incubated for 1 h at 37°C under constant shaking at 200 rpm. Cells were briefly centrifuged at 5000 rpm for 5 min and supernatant was removed by pipetting. The cells were resuspended in 100 µl of fresh LB medium and spread on LB-Agar plates supplemented with specific antibiotic tetracyline (5 µg/ml) or ampicillin (100 µg/ml) and incubated overnight at 37°C.

3.2.5.3 Cloning of 5'UTR

Concentration of purified 5' PCR product was quantified using Nanodrop 2000. Sample was prepared for digestion by mixing 3 µL of restriction enzyme compatible buffer, 1U of XhoI, 1U of ClaI, 1-2 µg PCR product and nuclease free water made up to 30 µL in volume. The sample was incubated for 3-4 h at 37°C. Transfection vector, pBC-GFP-DHFR was also digested with XhoI and ClaI and incubated at 37°C for 3-4 h. After digestion, samples were purified using Purelink quick gel extraction and PCR purification combo kit (Invitrogen, Cat No.K2200-01). After purification, concentration of digested vector and PCR product was quantified using Nanodrop 2000. Ligation was set up by mixing 1 µL of 10X ligation buffer, 1U of ligase, purified vector and insert in 1: 4 ratio in concentration and made up to 10 µL with nuclease free water. The ligation mix was incubated at 22°C for 3 h and transformed into XL-blue MRF' competent cells. After transformation, cells were plated on LB culture plates containing ampicillin. Colony PCR was performed to identify the clones having the insert. Following this preliminary confirmations, plasmid was isolated from positive colony by Sure spin plasmid mini kit (Genetix, Cat No. NP-37105). Restriction digestion by XhoI and ClaI confirmed the presence of 5' fragment resulting in pBC-GFP-DHFR-PI31 KO 5' plasmid.

3.2.5.4 Cloning of 3'UTR

Double digestion was performed for purified 3'PCR product and pBC-GFP-hDHFR *PbPI31* 5' KO plasmid with NotI and AscI and purified using PurelinkTM quick gel extraction and PCR purification combo kit (Invitrogen, Cat No.K2200-01). After the purification of digested samples, ligation was set up and the ligation mix was transformed into XL-blue MRF' competent cells. Colony PCR was used to select positive colonies. Plasmid was isolated from a single positive colony. The plasmid was digested with XhoI/ClaI and NotI/AscI to release 5' and 3' fragments. Sequencing was performed for the plasmid with 5' and 3' fragments with sequence specific primers. After the sequence confirmation, *PbPI31* knockout plasmid was isolated in large scale by Sureprep plasmid MAXI kit (Genetix, Cat No-NP-15162) and digested

with XhoI and AscI enzymes. The digested fragment containing the targeting construct (5'UTR fragment+GFP-hDHFR cassette+ 3' UTR fragment) was gel excised and purified using PurelinkTM quick gel extraction and PCR purification combo kit (Invitrogen, Cat No.K2200-01). The concentration of the targeting plasmid was quantified on Nanodrop 2000. The digested and purified product was used as *PI31* KO construct for transfection.

3.2.6 *In vitro* culture and purification of schizonts

Blood was collected from *P. berghei* infected mice by cardiac puncture when the parasitemia was 2-3% and place in a 50ml tube containing 0.25ml of heparin (conc 10U/ml). To the collected blood, 10 ml of schizont culture medium (RPMI with 20% FBS and 0.5 mg/ml gentamicin) was added and centrifuged at 200 g without brake for 8 min in a swinging bucket rotor. The pellet was resuspended in 5 ml culture medium and 1ml of this is added to 20 ml culture medium in T75 flask. Gently culture was aerated with gas (5% CO_2, 5% O_2, 90% N_2) for 2-3 minutes and incubated at 36.5°C with 42 rpm for 16-23hrs. A 60% Nycodenz was prepared in PBS and used for purification of schizonts. Twenty five milliliter parasite culture was aliquoted into a 50 ml tube and 10 ml of 60% nycodenz was added slowly under the culture suspension. To separate schizonts from the mixture of uninfected RBC the mixture was centrifuged at 370 g for 20 min without brake in swinging bucket rotor. Purified schizonts were collected from the interface. Schizonts were washed with culture medium and centrifuged (Fig 33).

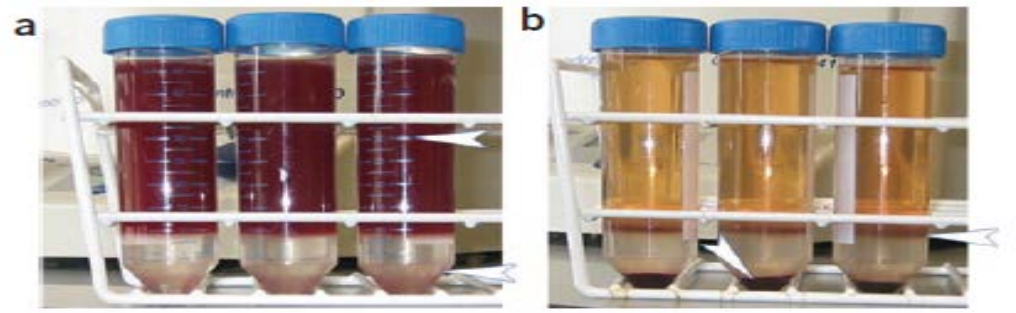


Fig. 33: Nycodenz density gradient purification of schizont containing erythrocytes from uninfected erythrocytes. (a) Nycodenz solution layered under the culture suspension (**b**). The separated density gradients after centrifugation. The schizont containing erythrocytes collect at the interface between Nycodenz solution and the culture suspension, whereas the uninfected erythrocytes settle at the bottom of the tubes (298).

3.2.7 Transfection of *PbPI31* knockout construct into purified schizonts

PbPI31 KO gene disruption plasmid was digested with XhoI and AscI and gel purified. Nucleofector solution was prepared by mixing 90 µl mouse T cell nucleofector solution and 20 µl supplement. 10µg of DNA was added to the nucleofector solution. Schizont pellet was resuspended with DNA/nucleofector solution and transferred into the cuvette. Cuvette was placed in Amaxa nucleofector device and electroporated using program U-033 (Fig 34). One hundred microliters of cold culture medium was added to the transfected schizonts and injected immediately into the mice by intravenous route. The following day, blood smear was made and stained with Giemsa stain and monitored for parasites under light microscope. After checking the parasitemia, pyrimethamine was prepared by dissolving 7mg/1ml DMSO. The stock solution was diluted with 100ml tap water and pH was adjusted to 4.0. The mouse was kept under drug pressure to select recombinant parasites which will have GFP and hDHFR cassette at target gene locus following successful integration. Following drug selection, the recombinant parasites were collected from the mouse by ocular puncture and genomic DNA was isolated. Gene disruption was confirmed by integration specific PCR.

3.2.8 Isolation of genomic DNA from *PbPI31* KO

Parasite infected blood was collected in heparin (10 U/ml) from mice through ocular puncture and centrifuged at 10,000 rpm for 10 min at 24°C. Supernatant was discarded and pellet was resuspended in equal volume of 0.5% saponin. This was followed by centrifugation at 10,000 rpm for 5 min and saponin treatment repeated. Pellet was further processed for genomic DNA isolation. Two hundred microliters of lysis buffer, 200 µl BT3 buffer and 25 µl of Proteinase K was added to parasite pellet. The sample was resuspended and incubated at 70°C for 15 min. This was followed by addition of 200 µl of absolute ethanol and mix loaded onto the column. After 1 min centrifugation at 13200 rpm, washing was performed twice using 700 µl of wash buffer each time. Finally, genomic DNA was eluted in 25 µl elution buffer.

3.2.9 Analysis of asexual blood stage propagation of *PbPI31* KO parasites

To determine the effect of *PbPI31* knockout on intra-erythrocytic development, $1X10^3$ infected RBC of either WT or *PbPI31* KO were intravenously injected into a group of 5 BALB/c mice and parasitemia was monitored daily by microscopic examination of Giemsa stained blood smears for seven days.

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

Adult *A. stephansii* mosquitoes were maintained by feeding on 10% sucrose solution in a humidified chamber at 28°C and relative humidity 80%. The breeding and continuous maintenance of *A. stephansii* mosquitoes was done by using the following protocol. A healthy rabbit (New Zealand White) was sedated by injecting anesthesia (ketamine-50 mg/kg and xylazine 10 mg/kg body weight) intramuscularly, and used as a source of blood meal. The blood feeding each of 30 min duration was done for two consecutive days with an interval of 24 h. After two days following second blood meal, a bowl of water was placed inside the mosquito cage to facilitate the egg laying. The eggs were collected for 3 to 4 consecutive days. The eggs were transferred into environmental chambers maintained at 27°C and 85% RH. During a period of 10-14 days the eggs undergo a series of transformations- early instars, late instars and pupae. The pupae were collected manually into a petri dish placed inside a fresh cage. Adult male and female mosquitoes emerge from pupae and mate within 24-36 hours of emergence. For preparing infection cages, the female mosquitoes were collected by vacuum suction and placed in a new cage (Fig 35). The female mosquitoes were allowed to obtain a blood meal from mice harboring gametocytes from WT or *PbPI31* KO line. Infected mosquitoes were maintained at 20-22°C with 80% RH.

3.2.11 Transmission of *PbPI31* KO parasites to female mosquitoes

BALB/c mice were injected either with WT or *PbPI31* knockout parasites and the parasitemia was allowed to progress upto 2-3%. Infected mice were screened for occurrence of gametocytes in blood smears. The gametocyte positive mice were anesthetized (200µl of anesthesia mix containing 0.8 ml ketamine (50mg/ml) + 0.3 ml of xylazine (20mg/ml) + 3.9 ml of 1X PBS) and used for blood feeding the mosquitoes. Infective blood meal was given for 15 min and repeated on the consecutive day and the cage was maintained at 20-22°C with 80% relative humidity. On day 14 post infection, mosquitoes infected with WT or *PbPI31* KO were dissected and midguts were observed for oocyst development under fluorescence microscope (Nikon Eclipse NiE AR). On day 18 post infection, mosquitoes were dissected to obtain salivary gland sporozoites of either WT or *PbPI31* KO lines.

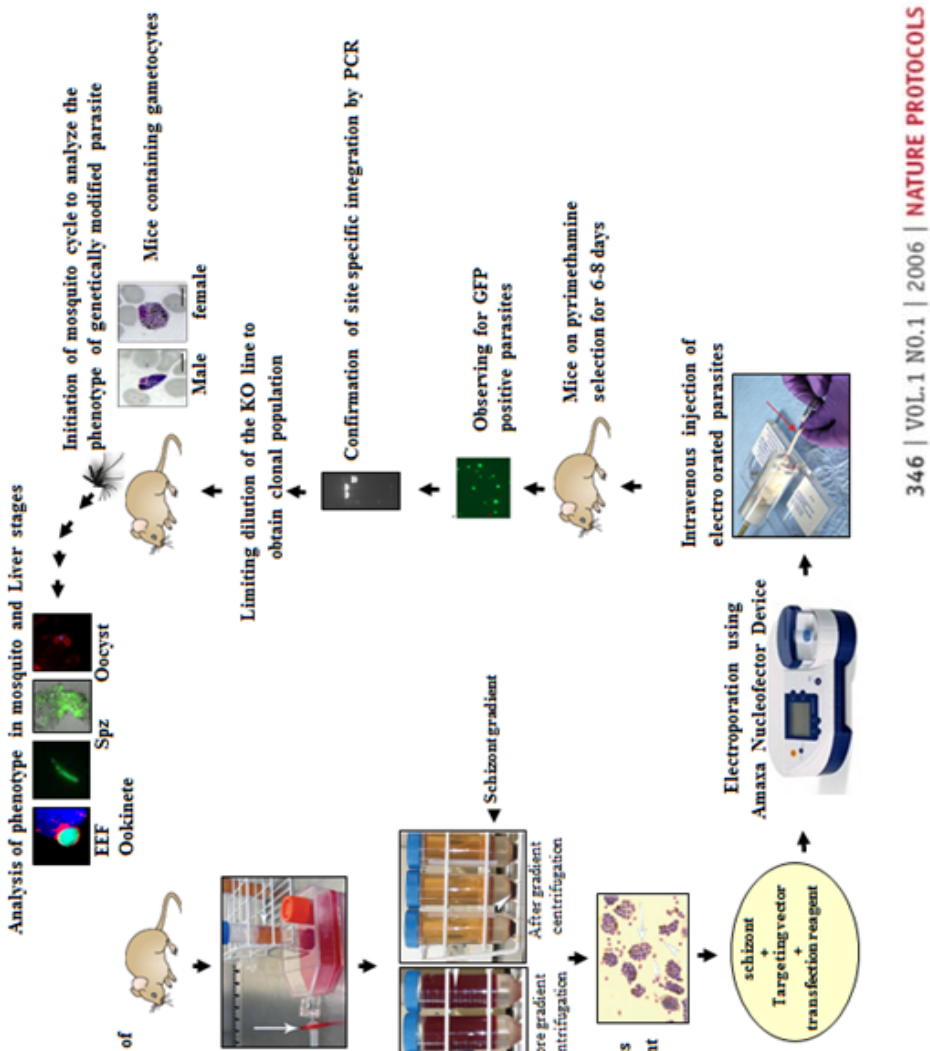


Fig. 34: Schematic representation of *Plasmodium berghei* transfection, drug selection, confirmation of the site specific integration and phenotypic characterization of the genetically modified knockout/reporter line. Infected blood is collected from mouse having 2-3% parasitemia and an overnight culture is set up. Next day, the schizonts were enriched on a Nycodenz density gradient. The purified schizonts were collected and electroporated with the targeting construct and immediately injected intravenously into mouse. The mice are given pyramethimine orally, an antimalarial drug that facilitates selection of the transfected parasites. The success of stable site specific integration is confirmed by PCR or Southern blot. The transfected parasites were cloned out and passed through mosquito stages to analyze the phenotype in other life cycle stages like oocysts, sporozoites and liver stages (EEFs) (3).

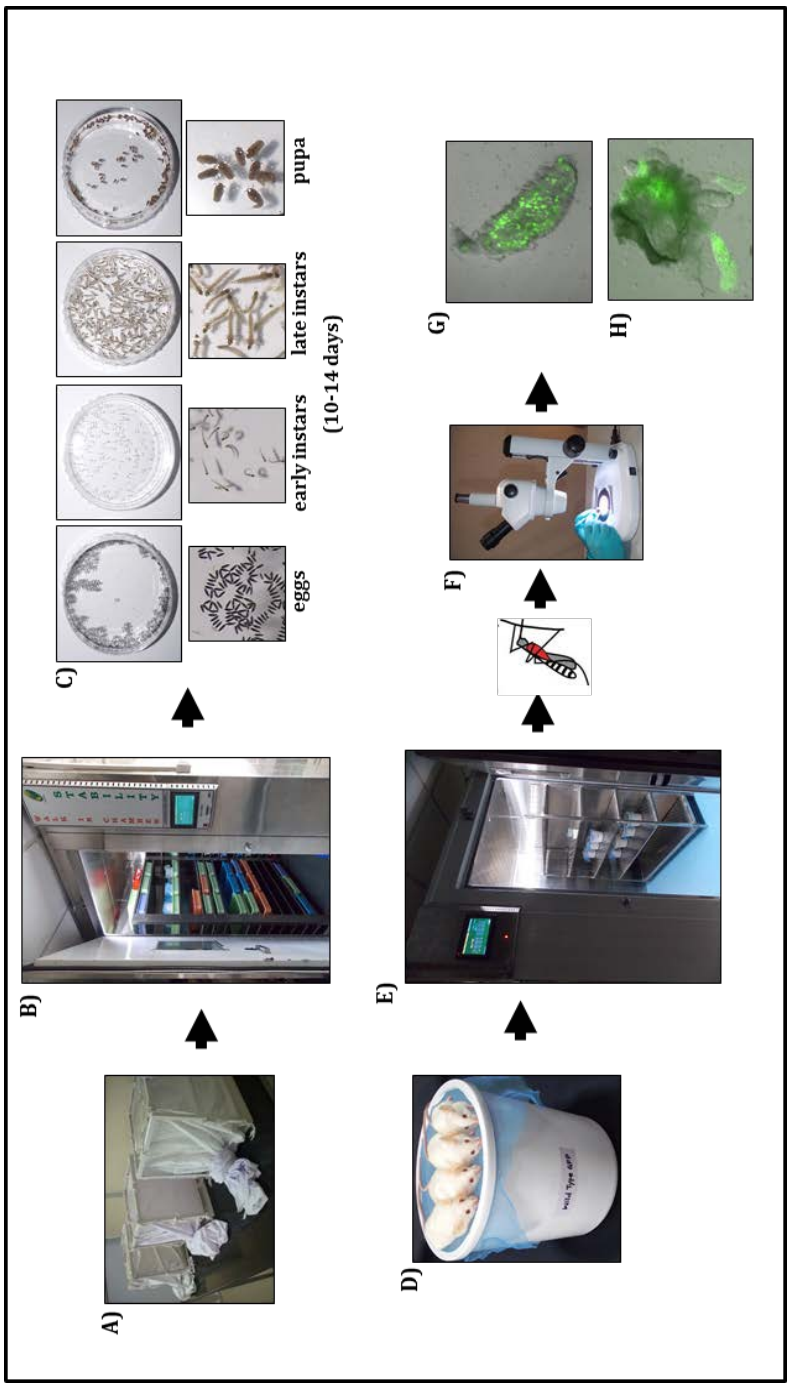


Fig. 35: Maintenance of *Anopheles stephensi* colony. The process includes activities like preparation of breeding cages, where pupa are placed in water bowls that emerge into adults and mate within 24-36 hours (A). Two successive blood meals from anesthetized rabbit are given to the mated female mosquitoes at an interval of 24 hours. Thirty six hours after second blood meal, a bowl of water is placed inside the breeding cage and eggs are collected for four consecutive days. The eggs are then transferred into an environmental chamber maintained at 27°C and RH 80% (B). Eggs hatch and transforms into a series of instars and develop finally into pupae (C). The pupae are collected and placed inside the breeding cage for emergence of adult mosquitoes. For preparation of infection cages, female mosquitoes are separated using a vacuum pump. The cage containing female mosquitoes were allowed to obtain an infective blood meal from mice which are positive for *Plasmodium berghei* gametocytes (D). The infection cage was maintained in an environmental chamber at 22°C and RH 80% to facilitate the completion of sexual reproduction of *P. berghei* (E). Mosquitoes were dissected under dissection microscope (F) on D14 (G) and D18 (H) post infection to observe the oocysts and sporozoites in the salivary glands respectively under a fluorescent microscope.

3.2.12 Isolation of *PbPI31* KO sporozoites from mosquito salivary glands

The infected salivary glands containing *PbPI31* KO sporozoites were dissected on D18 post infection in DMEM. The glands were crushed and centrifuged at 800 rpm for 3 min at 4^0C. Supernatant containing sporozoites were collected and used for *in vitro* and *in vivo* experiments to study the *Plasmodium* liver stage development.

3.2.13 *In vitro* EEF development

HepG2 cells were seeded two days prior to the experiment. Approximately $1X10^5$ cells were seeded per each well of Labtek chamber slide that contained 500 µl of DMEM supplemented with 2 mM L-glutamine, 25 mM HEPES and 4.5 g/L glucose, 10% fetal bovine serum (FBS) and 1X antibioc-antimycotic. The *PbPI31* KO sporozoites isolated from the disrupted salivary glands were counted using hemocytometer and $2X10^4$ sporozoites were added to each well. Labtek chamber slides were centrifuged after addition of sporozoites to facilitate adhesion to HepG2 cells. Complete DMEM with 1X antibiotic was replaced with 4X antibiotic-antimycotic containing DMEM throughout the experiment. At 12 h, 36 h and 62 h time points, the HepG2 cells were fixed in 4% paraformaldehyde and taken for immunofluorescence assay.

3.2.14 Immunofluorescence assay

HepG2 cultures harbouring EEF's at different developmental stages were fixed in 4% para formaldehyde for 20 min and washed for 1 min with PBS (pH 7.2). Permeabilization was done by using acetone:methanol (1:3) solution for 15 min at room temperature . Nonspecific blocking was performed in 3% BSA prepared in PBS (pH 7.2) and was incubated for 1 h at room temperature. This was followed by incubation with rabbit polyclonal antibody-UIS4 specific for parasitophorous vacuolar membrane (PVM). The primary antibody was used at a dilution of 1:1000 and was incubated for 90 min. Following primary antibody incubation, the cells were washed for three consecutive times, with PBS, PBS-tween (0.1% tween in PBS) and PBS for 20 min each. Alexaflour 594 (Molecular probes) conjugated anti-rabbit secondary antibody was diluted at 1:300 dilution and incubated for 1 h at 37°C followed by washes with PBS, PBS-tween (0.1% tween in PBS) and PBS for 30 min each. Nuclear staining was done with 4', 6' diamidino-2 phenyl indole (DAPI) used at 1µg/ml dilution which was mixed with secondary antibody solution. Slides were air dried and mounted with an antifade reagent covered

with coverslips. The border of the coverslip was coated with nail polish for sealing. Slides were observed under fluorescence microscope (Nikon Eclipse NiE AR).

3.2.15 Transmission dynamics of *PbPI31* KO sporozoites

To unravel the role of *PbPI31* in liver stage development, sporozoites were inoculated by mosquito bite or by intravenous injection with either WT or *PI31* KO sporozoites. The mice were monitored for blood stage infection daily by Giemsa staining of blood smears.

3.3 Results

3.3.1 PI31 is conserved among *Plasmodium* species

Alignment of *P. berghei* PI31 protein with other *Plasmodium* orthologue sequences like *P. yoelii, P. chabaudi, P. falciparum, P. reichenowi, P. knowlesi and P. vivax* showed 76- 85% homology (Fig 36). The proline rich region is present at the C-terminus of all species. To show the evolutionary conservedness of PI31 amongst other organisms, multiple sequence alignments was performed using ClustalX 2.0 and phylogenetic analysis and drawing of phylogenetic tree was performed using MEGA.4 with bootstrap percentages indicated at each branch. Phylogenetic analysis showed that PI31 is well conserved among *Plasmodium* species with high bootstrap percentages (Fig 37).

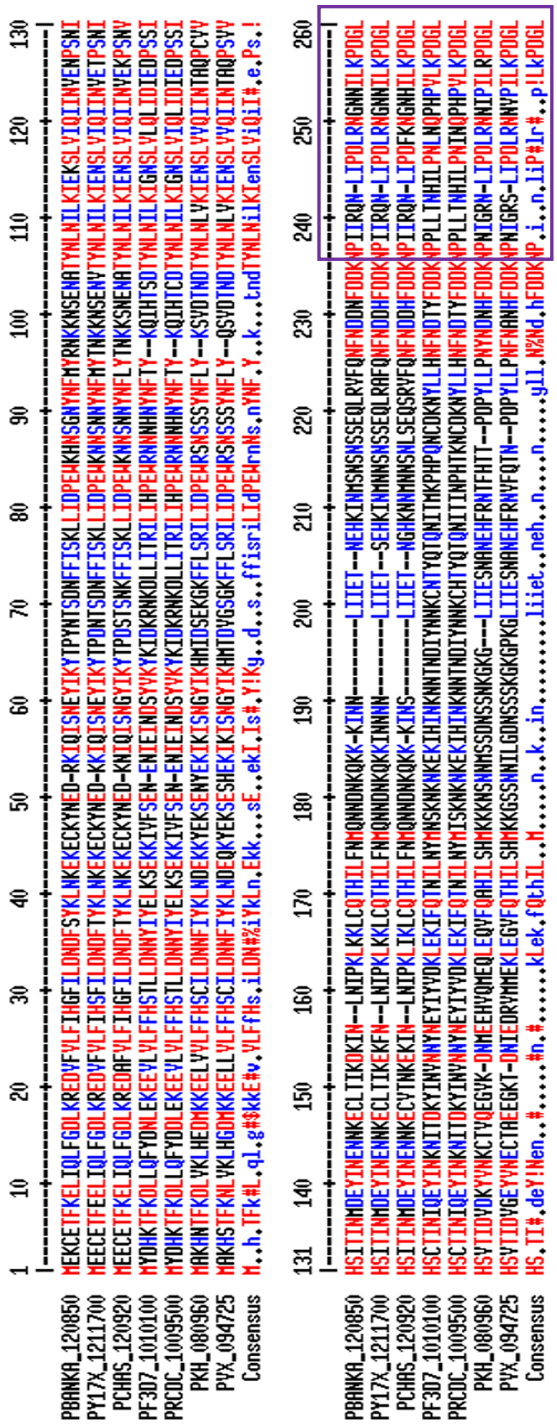


Fig. 36: Multiple sequence alignment of PI31 sequences across *Plasmodium* species reveals a high degree of conservation. The blue box indicates proline rich C-terminal of PI31.

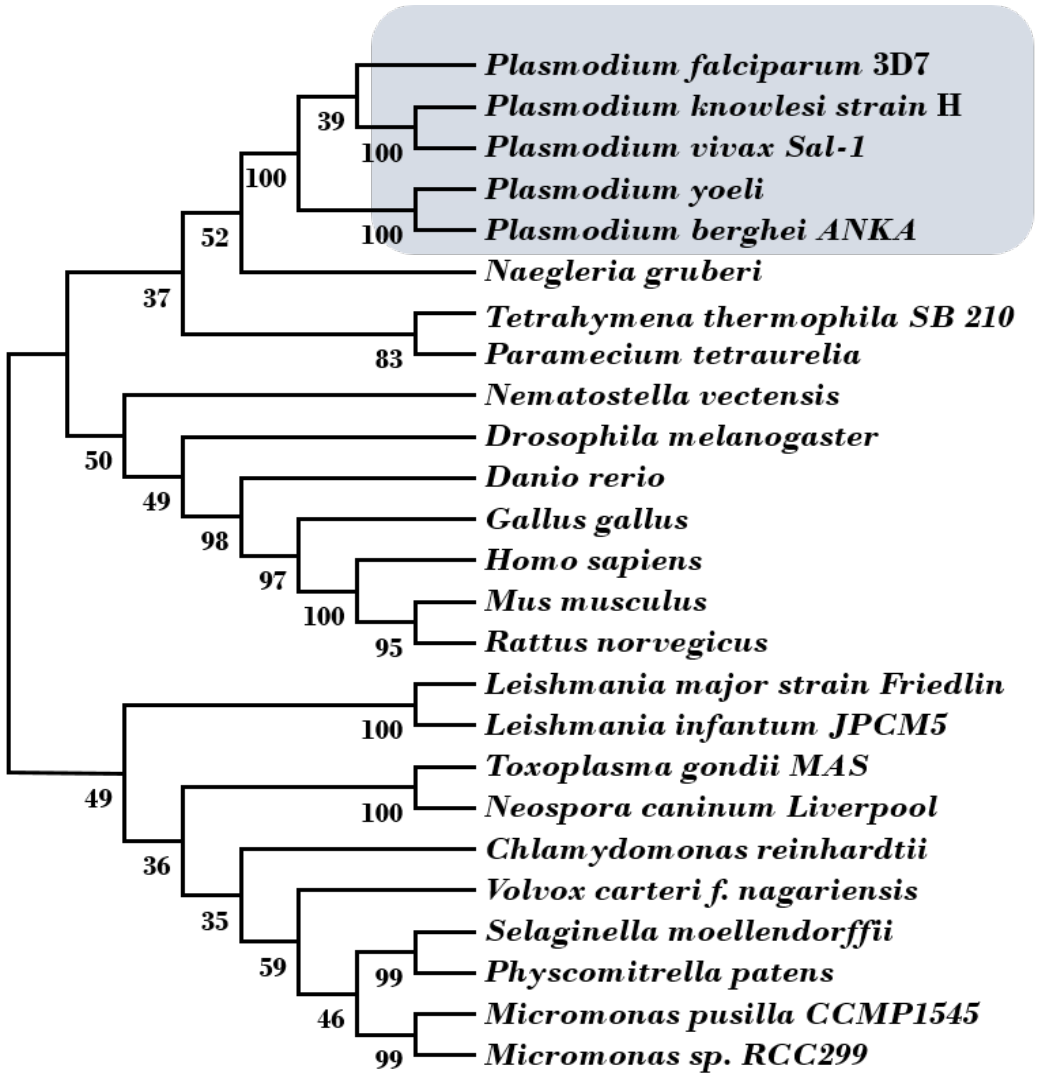


Fig. 37: Phylogenetic tree of PI31 proteins. Phylogenetic tree showing the evolutionary distances related to PI31 protein between different organisms.

3.3.2 *PbPI31* is expressed in all life cycle stages of *P. berghei*

To analyze the expression of *PbPI31* in various life cycle stages of *P. berghei,* RNA was isolated from mixed blood stages, insect stages (oocyst and salivary gland sporozoites) and infected HepG2 cells at different time points. Primers were used that specifically amplified *PbPI31* and *Pb18SrRNA* (internal control gene) from cDNA samples by absolute quantification using real-time PCR. Our expression analysis revealed that *PbPI31* is transcribed at all life cycle stages with maximum expression at LS 17 followed by salivary gland sporozoite stage (Fig 38).

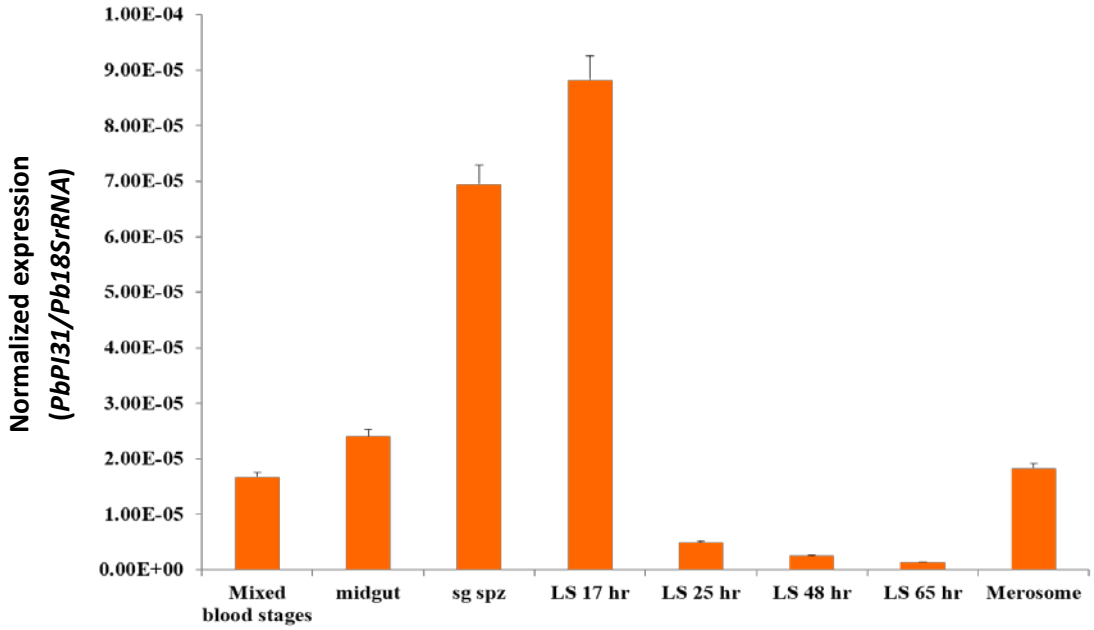


Fig. 38: Expression of *PbPI31* in *P. berghei* life cycle stages. Bar graph showing the expression profile of *PbPI31* mRNA. The expression values were normalized with *Pb18SrRNA* mRNA expression values for respective samples. The expression of *PbPI31* was observed throughout the life cycle with a maximal expression 17hr liver stages followed by at salivary gland sporozoite stage.

3.3.3 Homologous recombination using 5'UTR and 3'UTR fragments of *PbPI31* results in replacement of the target gene with a GFP-hDHFR cassette

The strategy for *PbPI31 gene* knockout is shown in Fig 39. Genomic organization of *PbPI31* gene locus was shown in Fig 39A. Two regions selected for homologous recombination are shown in the *PbPI31* KO plasmid in Fig 39B. *PbPI31* locus was replaced with GFP-hDHFR cassette as shown in Fig 39C. PCR of *PbPI31* 5' and 3' fragments was shown in Fig 39D. Double digestion of *PbPI31* KO plasmid with XhoI/ClaI and NotI/AscI relased *PI31* 5' and 3' UTRs respectively as shown in Fig 39E. Digestion of *PbPI31* knockout plasmid with XhoI and AscI released the vector backbone and GFP-hDHFR cassette bearing two homologous DNA fragments on either side of the cassette, is shown in Fig 39F. Integration PCR with indicated primers designed at sites beyond the recombination event confirmed the 5' and 3' UTR site specific integration (Fig 39G). It also confirmed that *PbPI31* gene locus was replaced with GFP-hDHFR cassette in *PbPI31* knockout parasites. After limiting dilution genomic DNA was isolated from *PI31* KO clonal population (4 clones) and PCR with gene specific primers confirmed the deletion of *PbPI31* ORF (Fig 39H). *PbPI31* KO parasites constitutively express GFP under the HSP70 promoter (Fig 39I).

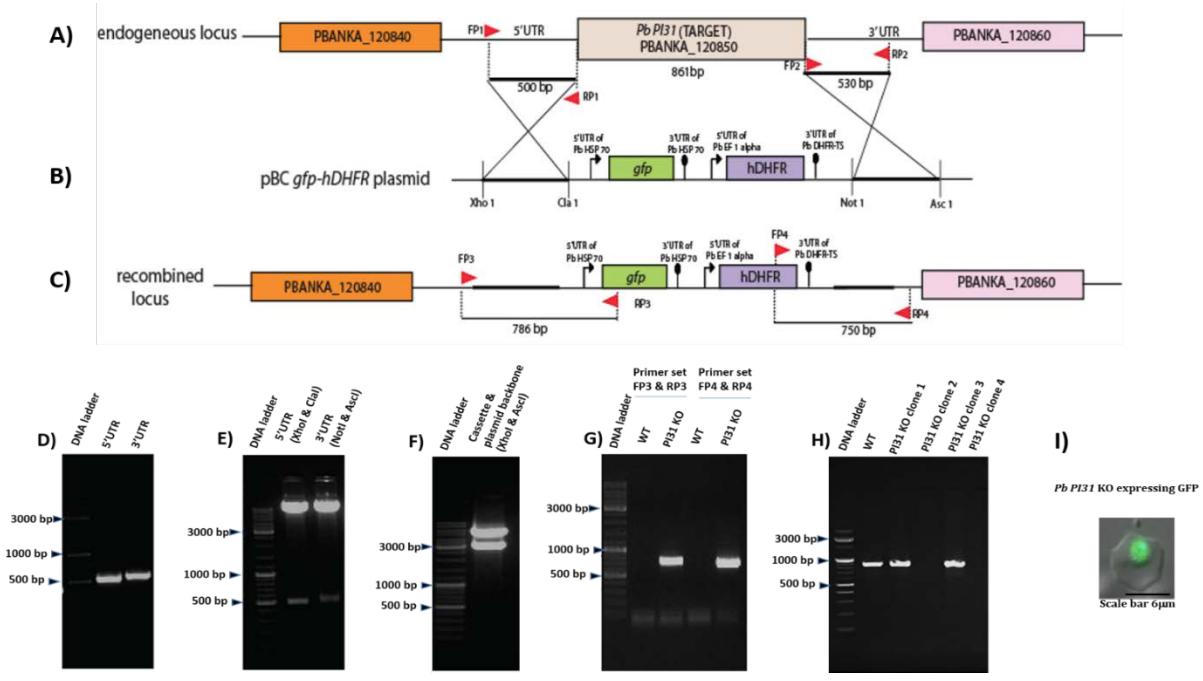


Fig. 39: Generation of *PI31* KO parasite line. A) Genomic locus of *PbPI31* (PBANKA_120850) showing 5' and 3' UTRs. **B)** Targeting vector showing pBC-GFP-hDHFR. A 500 bp 5' UTR of *PbPI31* was cloned in XhoI/ClaI site of the targeting vector. A 530 bp 3' UTR was cloned into NotI/AscI site of the targeting vector. **C)** Recombined locus following successful double cross over recombination resulting in replacement of target gene, *PbPI31* by GFP-DHFR cassette. **D)** Agarose gel showing PCR products of 5' and 3' UTRs. The 5' UTR part was amplified with primers *PbPI31* 5' FP (FP1) and *PbPI31* 5' RP (RP1) and 3' UTR part was amplified with *PbPI31* 3' FP (FP2) and *PbPI31* 3' RP (RP2). **E)** Release of *PbPI31* 5' UTR part (using restriction enzymes XhoI/ClaI) and release of *PbPI31* 3'UTR part (using restriction enzymes NotI/AscI) from transfection vector. **F)** Release of targeting cassette (5' UTR part+GFP-DHFR cassette+3' UTR part) and vector backbone using restriction enzymes XhoI/AscI. **G)** Diagnostic PCR using primers within the targeting cassette and beyond sites of recombination revealing the correct site specific integration. A PCR product with primers *PbPI31* 5' confirmation FP (FP3) and *PbHSP70* 5' UTR (RP3) indicated a correct 5' UTR integration and a PCR product with primers *hDHFR* FP (FP4) and *PbPI31* 3' confirmation RP (RP4) indicated a correct 3' UTR integration. **H)** Genomic DNA isolated from *PbPI31* KO parasite clones 2 and 4 does not amplify a PCR product from the *PbPI31* ORF indicating the deletion of *PbPI31* gene, whereas WT parasites and *PbPI31* KO parasite clones 1 and 3 amplify a product of 861bp with primers for full ORF of *PbPI31* indicating presence of WT parasites. Further studies were carried out using *PbPI31* KO clonal populations 2 and 4 **I)** A merged DIC image showing a GFP expressing *PbPI31* KO parasite inside RBC.

3.3.4 *PbPI31* KO exhibits no defect in the asexual blood stage propagation

To monitor, if *PbPI31* depletion affected asexual blood stage propagation, two groups of BALB/c mice (5 mice per group) were intravenously injected with $1X10^3$ iRBC (infected RBC) of either WT or *PbPI31* KO and the asexual blood stage replication was monitored for 7 days by making Giemsa stained blood smears. There is no difference between *PI31* KO and WT parasites in the onset of infection, active multiplication and propagation. Presence of all stages of asexual forms in *PI31* KO revealed its non-essential role in asexual blood stages (Fig 40).

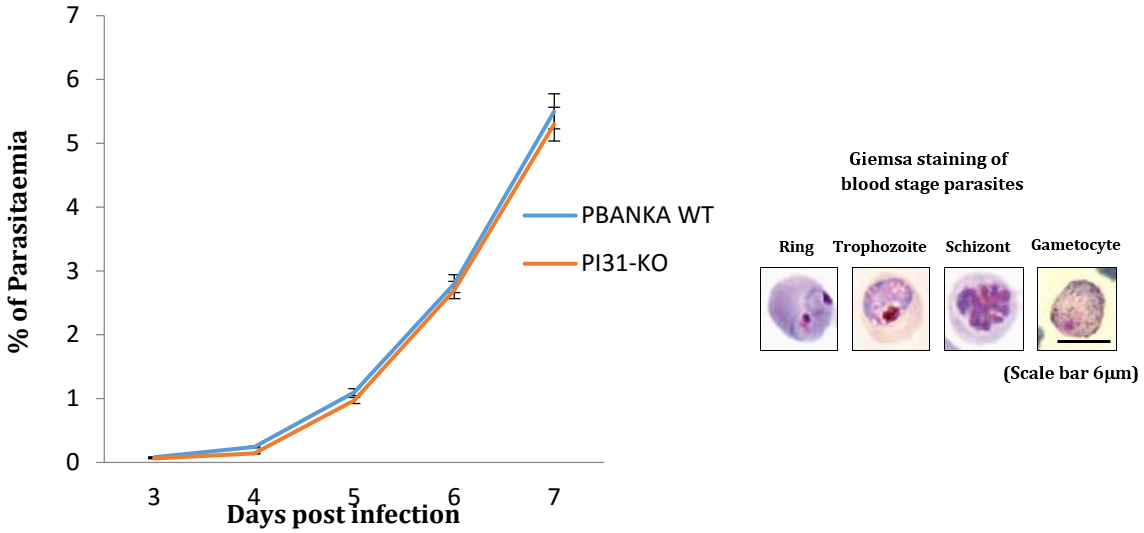


Fig. 40: *Pb PI31* KO asexual parasites propagate at similar rates as WT parasites. A) $1X10^3$ infected RBC of either WT or *PI31* KO were intravenously injected in two groups of mice (5 mice/group) and monitored for propagation of the parasites daily for 7 days by making Giemsa stained smears. B) Representative pictures showing asexual blood stages (299).

3.3.5 *PbPI31* KO does not exhibit any defect in the production of midgut oocysts and salivary gland sporozoites

Transmission of *PbPI31* KO parasites to mosquitoes leads to formation of oocysts, whose numbers were comparable to the oocysts derived from the WT parasites (Fig 41A). The sporulation pattern inside oocyst (Fig 41B) and the ability of the egressed sporozoites to reach salivary gland (Fig 41C) also were comparable to that of WT parasites suggesting that *PbPI31* KO manifested no defect in the mosquito stages of *P. berghei*.

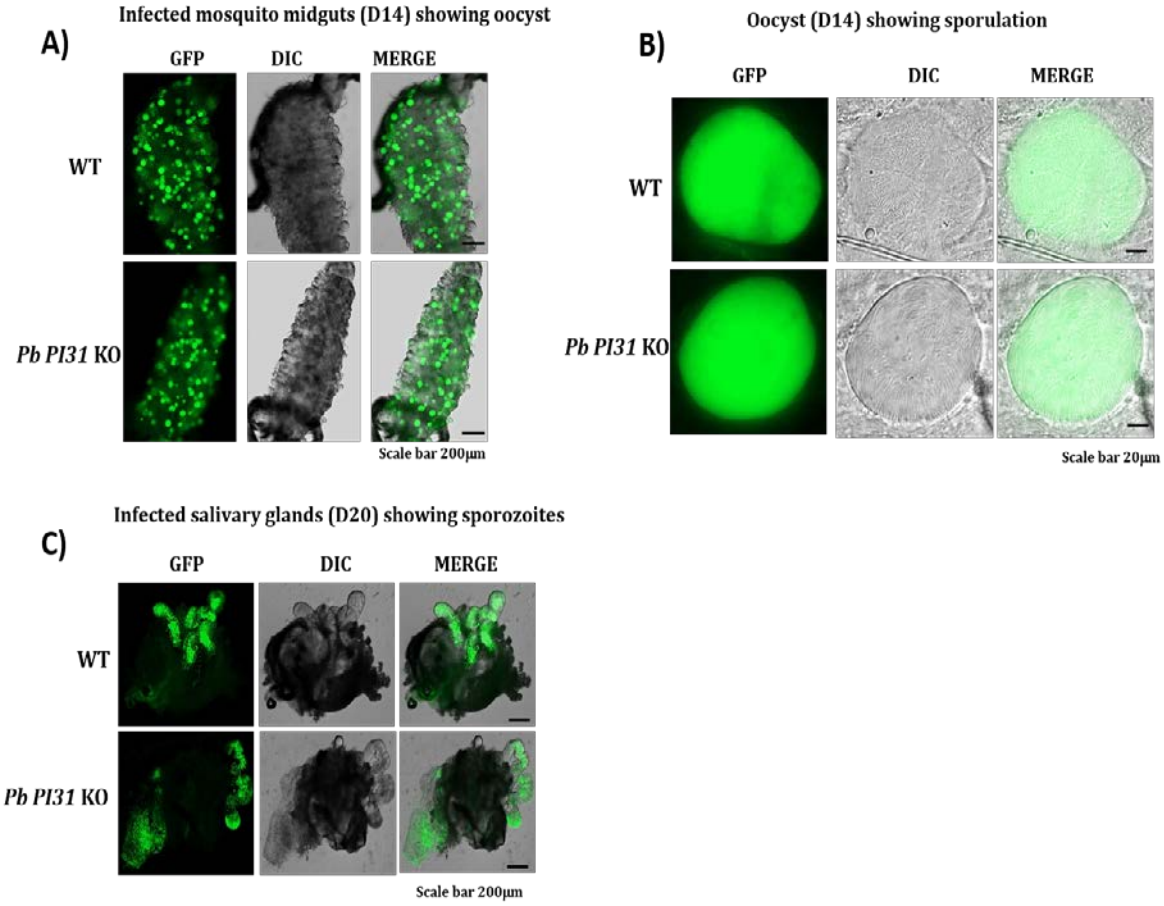


Fig. 41: Mosquito stages of *PbPI31* KO do not show any defect in sexual development or sporulation. Malaria was transmitted to female *Anopheles* mosquitoes from mouse harboring gametocyte stages of either WT or *PbPI31* KO. (A) Midguts showing oocyst derived from WT and *PbPI31* KO parasites. (B) A single magnified oocyst of WT and *PbPI31* KO showing similar pattern of sporulation (C). Dissected salivary glands showing WT GFP expressing sporozoites and *PbPI31* KO sporozoites.

3.3.6 *PbPI31* is not essential for sporozoites invasion of hepatocytes and completion of EEF development

Mosquito salivary glands harbouring *PbPI31* KO sporozoites were dissected. The *PbPI31* KO sporozoites were added to HepG2 cultures to study the transformation of sporozoites into EEF's. *PbPI31* KO sporozoites developed into fully grown EEF's in HepG2 cells. EEF development at 12hr, 36hr and 62hr were similar to that of WT EEF development (Fig 42).

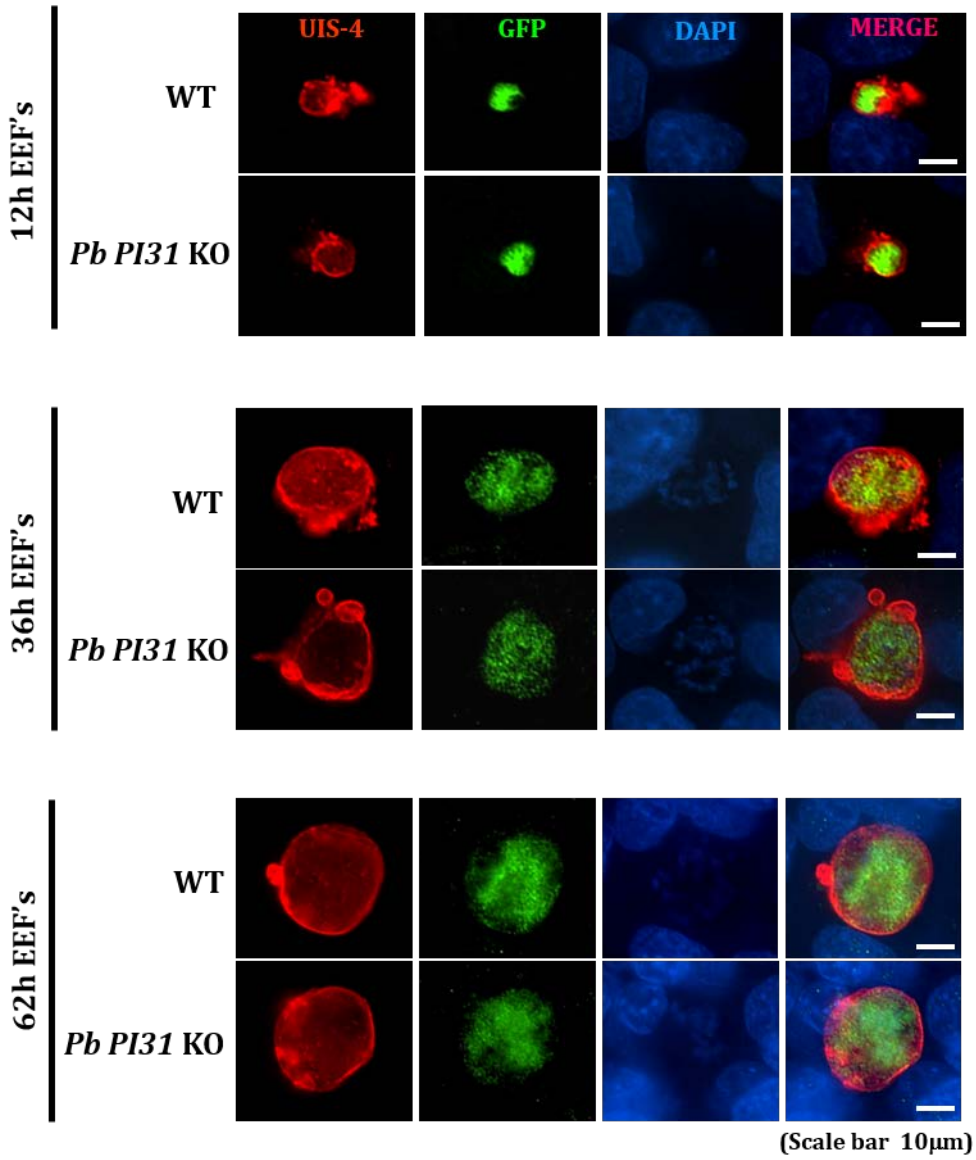
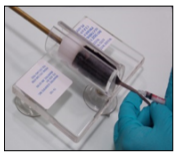


Fig. 42: The EEF's of *PbPI31* KO reveals no growth defect in liver stages development *in vitro*. Salivary glands sporozoites were isolated by dissection and $2X10^4$ sporozoites of either WT or *PbPI31* KO were added to HepG2 cultures, that supported the complete development of the *P. berghei* EEF's. The cultures were fixed at different time points: 12hr, 36hr and 62hr. The cultures were stained with anti-UIS4 antibody that reacts with the parasitophorous vacuolar membrane (PVM) of EEF and DAPI for visualization of HepG2 and parasite nuclei. EEFs derived from *PbPI31* KO sporozoites at 12hr, 36hr and 62hr were comparable to that of the WT EEF's.

3.3.7 *PbPI31* depletion has no effect in initiating blood stage infection after exo-erythrocytic schizogony

Inoculation of *PbPI31* KO sporozoites through mosquito bite or by intravenous injection (i.v.,) of $2X10^4$ sporozoites successfully initiated blood stage infection on D4 post infection. WT sporozoite infection through mosquito bite or i.v., route were used as control. (Table 2).

Parasite Strain	Mode of sporozoite infection	Number of animals used for bite	Number of animals positive for blood stage infection	*Pre-patent period (in days)
WT	by mosquito bite	3	3	4
Pb PI31 KO	by mosquito bite	3	3	4



Parasite Strain	Mode of sporozoite infection	Number of animals used for i.v.,	Number of animals positive for blood stage infection	*Pre-patent period (in days)
WT	intravenous route (i.v.,) ($2X10^4$)	3	3	4
Pb PI31 KO	intravenous route (i.v.,) ($2X10^4$)	3	3	4

* Pre-patent period: defined as time required for the appearance of blood stages following infection with sporozoites.

Table 2. Transmission dynamics of WT and *PbPI31* KO sporozoites. All blood meal positive mosquitoes following bite experiment were dissected and the salivary glands were screened to confirm the presence of GFP expressing WT or *PbPI31* KO sporozoites under fluorescence microscope. Table showing the kinetics of mosquito bite experiment and infection by i.v., route. The details of number of animals used for experiment, the number of animals positive for blood stage infection and the pre patent period are shown in the table.

3.4 Discussion

Plasmodium has several genes whose functions are conserved across different species. This might mean that certain biological processes may be common irrespective of the complexity of the organism or the ecological niche the organisms occupy. One of the themes central to progression of *Plasmodium* life cycle is the coordinated regulation of stage specific gene expression that encodes proteins unique to a stage. These stage specific proteins influence infectivity and tropism of parasites to certain cell types both in *Anopheles* species and vertebrate host. Central to the regulation of stage specific functions is the proteasome, a multi-subunit catalytic protein complex primarily involved in the maintenance of protein homeostasis through degradation and recycling of proteins and maintaining intracellular protein quality control.

Several lines of evidence indicate that the activity of proteasome is regulated by activators and inhibitors. The functional role of regulatory proteins of proteasome in *Plasmodium* life cycle, particularly in high replicative stages is not well studied. PI31 is a component of proteasome complex with dual functionality of activation and inhibition of proteasome function. The current work has investigated the similarity of PbPI31 sequence with its orthologues in other *Plasmodium* species, mRNA expression profiling at different stages of *P. berghei* life cycle and functional characterization of *PbPI31* through gene replacement strategy using double homologous recombination method. These investigations provide the first report revealing a dispensable role of PI31 in *P. berghei* parasites.

We initially assumed that upregulation of *PI31* in infective salivary gland sporozoites may be required for reprogramming of sporozoites in preparation for establishment of infection in the vertebrate host similar to the role of UIS genes that are implicated to govern several aspects of sporozoite and liver stage biology (300). Modulation of sporozoite infectivity through regulation of gene expression is in fact controlled at multiple levels by transcription factors and regulators like PUF2 (301,302), SLARP (303), AP2-sp (304) and IK2/UIS1 (305). Consistent with such observations, the repertoire of proteins unique to sporozoite stages requires a tight regulation of the proteasome function that is mediated by the activators and inhibitors. The surprisingly high expression pattern of *PbPI31* in the pre-erythrocytic stages of *P. berghei* inclined us to explore its function in these stages and to address whether *PbPI31* is required for maintaining the proteasome integrity.

For functional investigation of *PbPI31*, we generated a gene replacement plasmid that was designed to replace the *PbPI31* by double homologous recombination following transfection with the *PbPI31* KO vector containing an h*DHFR-GFP* cassette to enable the selection of recombinant *P.berghei* parasites using pyrimethamine drug. The recombinant parasites expresses GFP protein under a constitutive HSP70 promoter that facilitated monitoring of the parasite's development through different stages of life cycle. The recombinant parasites were selected using antifolate drug pyrimethamine. Subsequent clonal dilution resulted in generation of parasite lines form a single clone *PbPI31* KO. Genotyping by diagnostic PCR confirmed the *PI31* gene replacement in two parasite lines i.e clone 2 and clone 4. To monitor the population expansion during blood stage infection *in vivo*, we passaged 10^3 *Pb* WT or *PbPI31* KO iRBC into naïve Swiss albino mice and monitored parasitemia by daily microscopic examination of Giemsa stained peripheral blood smears. Parasite growth was similar when compared between the *Pb* WT and *PbPI31* KO parasites, suggesting non-essential role for *PbPI31* during blood stage propagation *in vivo*.

We next analyzed the colonization of the *Anopheles* mosquitoes by *PbPI31* KO parasites. Laboratory bred female *A. stephensi* mosquitoes were allowed to take a blood meal from Swiss albino mice harbouring WT or *PbPI31* KO gametocytes. Dissection of mosquito midguts on day 14 post infection revealed that *PbPI31* KO parasites were capable to invade the midguts and subsequently develop into oocysts similar to WT parasites. The number of oocysts derived from *PbPI31* KO parasite were comparable to that of WT parasites. The sporulation pattern when observed microscopically under high magnification revealed no difference between *PbPI31* KO and WT parasites. On day 18 post infection, mosquitoes were dissected to monitor the sporozoite load in the salivary glands. An almost similar load of sporozoites was noted in salivary glands infected with WT and *PbPI31* KO. We conclude that depletion of *PbPI31* did not affect the progression of *P. berghei* through the *Anopheles* vector or that the product encoded by *PbPI31* may not be necessary at least in the mosquito stages of *Plasmodium*.

We next analyzed the ability of *PbPI31* KO sporozoites to initiate a blood stage infection in vertebrate host. Towards this end we allowed mosquitoes to obtain a blood meal from anesthetized C57BL/6 mouse, a process during which the sporozoites are inoculated into the skin of the mouse. As evident by analysis of pre-patent period, we observed no defect in the initiation of blood stage infection when *PbPI31* KO parasites were introduced through natural mosquito bite (table 2). In a parallel experiment, we injected $2X10^3$ salivary gland sporozoites

of WT or *PbPI31* KO into C57BL/6 mice through i.v., route. The prepatent period in this mode of sporozoite delivery was also similar to that of WT suggesting that *PbPI31* KO were not compromised to invade liver and complete exo-erythrocytic development. These conclusion were supported by *in vitro* analysis that revealed growth of EEF's as monitored at 12h, 36h and 62h to be identical in both WT and *PbPI31* KO. The frequency of EEFs in HepG2 cultures remained similar for both WT and *PbPI31* KO parasites suggesting that PbPI31 has no role in the invasion of sporozoites to the host cell. The maintenance of the PVM appeared to be normal in *PbPI31* KO EEF as visualized by staining a PVM signature protein UIS4.

Despite the fairly good expression of *PbPI31* in some specific stages of *P.berghei*, we show that PI31 deficient parasites are viable throughout the life cycle. In one of the significant studies that was aimed at investigating the cellular roles of PI31 on regulation of proteasome function, both the peptide corresponding to the PI31 C- terminus and the intact PI31 protein were tested to evaluate its effect on modulating the activity of 20S proteosome (25). The PI31 C terminus peptide binds and activates the 20 S proteasome in an HbYX-dependent manner, whereas the intact PI31 protein inhibits *in vitro* 20 S activity. These studies ruled out the absolute requirement of HbYX motif though binding and inhibition of the proteasome by PI31 are conferred by the HbYX-containing proline-rich C-terminal domain. These studies further showed that while PI31 blocks the ATP-dependent *in vitro* assembly of the 26S proteasome from 20S proteasome and PA700 sub-complexes, it apparently has no effect on *in vitro* activity of the intact 26S proteasome. The physiologic significance of these *in vitro* findings was further explored by assessing the cellular proteasome content and function following alteration of PI31 levels. These observations revealed no change in overall cellular proteasome content or function when PI31 levels were either increased by ectopic overexpression or down regulated by RNA interference (RNAi). Though multiple regions of PI31 bind independently to the proteasome and collectively determine its effects on activity *in vitro*, mechanism of PI31 regulation of proteasome function still remains un-elucidated. While such paradigms are conserved in *Plasmodium* is speculative, further investigation is required to prove if any component of proteasome complex is compensating the loss of function of *PbPI31*.

3.5 Conclusions

Using data base search engines, we found the existence of *PI31* like genes in all *Plasmodium* species, whose function in higher eukaryotes is shown to regulate proteasome activity. Gene expression analysis of *PbPI31* was performed across all life cycle stages and maximal expression was found to be associated with 17h liver stage followed by sporozoite stage. A loss of function mutant of *PbPI31* was generated in a rodent species-*Plasmodium berghei* to investigate the phenotype across all life cycle stages. The *PbPI31* KO did not manifest any defect in asexual blood stage propagation, in mosquito stages (oocyst and sporozoites formation) and in the ability of the sporozoites to invade and complete development in HepG2 cells. The role of *Pb PI31* KO in onset of blood stage infection following delivery of sporozoites through mosquito bite and by intravenous route proved the dispensable role of *PbPI31*. There are two possible explanations to account for the dispensable role of *PI31* in *P. berghei*. Firstly that PI31 may not be a key player in regulation of proteasome activity unlike in other metazoans and secondly, that there could be other yet to be identified unique regulators of the proteasome in *Plasmodium* that needs to be further investigated. Our studies also point to the fact that several of the conserved mechanism of proteasome regulation may not be applicable in *Plasmodium* and that a mere occurrence of the orthologues may not indicate a parallels in mechanism of proteasome functions. From the perspective of *Plasmodium*, the obvious advantages of retaining a similar machinery as host may confer the advantage of safe guarding its survival against target based chemotherapy. However, if the regulation of proteasome activity is mediated by other proteins that act hierarchically, then identifying these unique regulators may help devise drugs specific to parasite and having minimal effects on host.

Summary

Summary

We provide evidence for the ability of the apicomplexan parasites to hijack the host SUMOylation machinery for benefit of their intracellular growth and dissemination. By designing several elegant experiments, we show convincingly that *Plasmodium* and *Toxoplasma* alter the host SUMOylation machinery at the transcriptional level as well as decrease the overall conjugation of the SUMO1 to the cellular proteins. The over expression of SUMO1 mitigates intracellular development of *Plasmodium* EEF and replication of *Toxoplasma*. These observations likely hint to SUMO1 mediated global regulation of host gene expression in its favor so that the cellular environment is rendered hostile for the developing intracellular parasites. Contrary to this observation, shRNA mediated silencing of the only conjugation enzyme of the host SUMO machinery-the E2/Ubc9 favoured the intracellular growth of *Plasmodium* EEF's. SUMO1 over expression counteracts/downregulates the expression of genes central to anti-apoptosis and cell proliferation induced by the parasite. Our findings provide a mechanism that explains how *Plasmodium* and *Toxoplasma* usurp the host post translational pathway for their benefit.

The role of proteasome complex in the stage specific transitions of *Plasmodium* has started gaining interest. The functional role of regulatory proteins of proteasome in *Plasmodium* life cycle, particularly in high replicative stages is poorly studied. PI31 is a component of proteasome complex with dual functionality of activation and inhibition of the proteasome. We investigated its functional role in *Plasmodium berghei* life cycle by reverse genetics approach. Surprisingly, we observed a dispensable role of *PbPI31* in parasite life cycle. Further investigations are needed to understand the compensatory mechanisms that operate in *PbPI31* KO mutants that enable these parasites to complete their life cycle normally in vertebrate host and *Anopheles* vector.

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by Maruthi Mulaka

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