Investigation of stress responses as determinants of artemisinin resistance and cellular heterogeneity in *Plasmodium falciparum*

A thesis

Submitted in partial fulfillment of the requirements

Of the degree of

Doctor of Philosophy

By

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ID: 20122024

Under the guidance of

Dr. Krishanpal Karmodiya

At

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

2019

This thesis is dedicated to my parents

for their love and support

CERTIFICATE

Certified that the work incorporated in the thesis entitled "**Investigation of stress responses as determinants of artemisinin resistance and cellular heterogeneity in *Plasmodium falciparum***" Submitted by Mukul Rawat was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution.

Dr. Krishanpal Karmodiya

(Supervisor)

Date: 19/12/2019

DECLARATION

I declare that this written submission represents my ideas in my own words and where others' ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

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Mukul Rawat

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Date: 19/12/2019

ACKNOWLEDGEMENTS

On my concluding note for my thesis, I would like to express my utmost gratitude towards everyone who has made my PhD journey extremely enjoyable and certainly a memorable one. I am so grateful that I decided to join IISER Pune to pursue my Integrated MS-PhD. I got an opportunity to work with amazing people who have always helped me to become a better researcher. This work would not have been possible without the support and guidance of so many wonderful individuals.

To begin with, I would like to express my sincere thanks to my supervisor Dr. Krishanpal Karmodiya who has supported me throughout my Ph.D. journey as well as challenged my way of thinking. I cannot thank him enough for sharing his ideas and knowledge. Without his constant support, I would not be here today. Thank you for all the scientific liberty and intellectually stimulating discussions that I received from you. I am blessed to have a supervisor like you. Your patience and mentoring during my Ph.D. gave me the strength to keep moving forward.

Besides my supervisor, I would like to thank my Research Advisory Committee members, Prof. Sanjeev Galande and Dr. Vasudevan Seshadri, for their insightful comments and encouragement. I would particularly like to acknowledge Prof. Galande for always encouraging me to have enthusiastic scientific discussions.

Many thanks to my collaborator Dr. Juan Jose Lopez Rubio, University of Montpellier, for allowing me to work in his lab as a part of EMBO short term fellowship program. I am also grateful to Dr. Shruthi Vembar for providing PfAlba3 antibodies and her valuable comments and suggestions on the manuscript, which immensely helped us, improve the quality of the article. Special thanks to Dr. Ishaan, Indian Institute of Technology, Delhi for all the help with data analysis of single cell RNA sequencing.

I would also like to express my heartfelt thanks to IISER Pune biology faculty Dr. Thomas Pucadyil (Chair, Biology Department), Dr. Sudha Rajamani, Prof. L. S. Shashidhara, Dr. Siddhesh Kamat, and Dr. Mayurika Lahiri for their support.
Thanks a bunch, Dr. Anamika Krishnapal, for the lovely lab parties and interesting discussions that accompanied them.

I am greatly thankful to all the staff at the IISER Pune Academic section, Accounts section, Purchase department, Library stores, Microscopy facility, NGS facility, Animal house

facility, and all other staff. Special thanks to Tushar, Sayali, Kalpesh, Piyush, Shabnam, Mrinalini, Santosh, and Mahesh for making timely documentation, orders and lab related chores so convenient.

I gratefully acknowledge the principal source of my PhD funding received from the Department of Biotechnology. I would also like to acknowledge DST-SERB for providing a travel grant for attending the MPM 2019 conference. Special thanks to EMBO for providing short term fellowship to carry out a small part of my work at the University of Montpellier. I am also grateful to DST INSPIRE, DBT IYBA and IISER Pune for project funding.

I would like to take this opportunity to thank my undergraduate teachers Dr. Sundip Malik, Dr. Sandeep Arora, Dr. Sonu Ambwani, Dr. Dinesh Pandey, for motivating and inspiring me to pursue the field of science.

An exceptional thanks to Krish lab - current and past lab members, for enthusiastic discussions, an ever-friendly lab environment and all the lab-get-togethers and trips that we had in the last five years. Special thanks to one and only – Abhishek, for his great talks, for proof-reading all my documents, for supporting me constantly and being such a great friend. I will certainly miss a lab mate like you. Thank you, Deepak for all the love and support. I also want to thank current and past lab members of Krish lab members Anjani (CRISPR girl), Rashim (Antisense RNA girl), Bhagyashree (Bhaggu), Mamatha (Queen), Mohit, Shayantan, Sharvani, Dilsha, Samarendra, Saakshi, Pratima, Madhvi, Shweta, Vandana, Aishwarya, Ratnal, Astha, Kalpshree, Sameer, Jaimin, Vijay, Sunil, Nilam, Prachi, Punita, Saikat, Feba, Deepa, Yajush, Haripriya, Achala, Bodas, Shivani and Yuva who were always so helpful in several ways.

I would like to take this opportunity to lovingly thank - Ashish. I don't think I have ever seen a person more sorted, caring, and dedicated to science. I will always cherish the short time spent with him. Excellent work done with you for single-cell RNA seq project has made a valuable contribution towards my Ph.D.

Special thanks to Sanjeev lab members Ankitha, Saurabh, Rini, Manu, Suyog, Ankita, Ayush, Jasmine, Sneha, Satya, Mouli, Akhila, Mrinmoy, Ameya, Indu, Prateek, Keshav, Rahul's, Ashwin, Sudipta, Anirudh, Amit, Greg, Anjali and everyone else. I owe special thanks to Saurabh for helping me during all the NGS library preparation, other experiments, and career advice.

I am also grateful to JJ's lab members, who were always so helpful and supportive. I am especially indebted to Pratima for everything. Thanks, Pratima, for being my guardian and taking care of each and everything during my stay in Montpellier. Special thanks to Ana and Elodie for all the guidance and support during my stay. Special thanks to Rohan (brother from another mother) and Sheena for being wonderful friends.

To all my friends - thank you for listening, offering me advice, and supporting me through this entire process. Thanks to Shruti, Intikhab, Himanshu, Mayank, Swati, Deepika, Rishi, Romain, Pant, Ekta, Ankit, Shake, CP d dude, Ruchika, Vineet, Dabba who were more confident than me that I should pursue a career in research. I consider myself fortunate to have great batchmates like Ankitha, Ashish, Neha, Sukrut, Aditi, Nandi, Jerrin, Tomin, Chetan, and Rahi at IISER.

Special thanks to my best friends Ankitha and Ashish. This journey would not have been possible without their love, support, video calls, and constant push. They were always there to help me in every possible way. 'Thank you' doesn't seem sufficient, but it is said with appreciation and respect to both of them for their support, encouragement, care, understanding, and precious friendship. I convey special acknowledgment to Ankitha. She was always beside me during the happy and hard moments to push me and motivate me. I doubt that I will ever be able to convey my appreciation fully, but I owe her my eternal gratitude.

I wish to thank my juniors: Simran (My Cross Fit partner), Madhumita, Neel, Sandip, Bharat, Anish, Ron, Akhila, Amar, Swati, Shivani, Dhriti, Kashyap, and Nilam for creating a friendly and enjoyable environment.

Finally, I would like to thank my parents for their love and support. Without them, this day would not have been possible. I would also like to thank my entire family (Harish, Arti, Ishan, Nayansi, Aadrika, and Tin-Tin) for good times in my life and never allowing me to give up.

Finally, I would like to extend my thanks to everyone who has helped me in one way or another during my Ph.D.

Sincerely,
Mukul

CONTENTS

ABSTRACT

Plasmodium falciparum has evolved resistance to almost all front-line drugs including artemisinins, which threatens malaria control and elimination strategies. Oxidative stress and protein damage responses have emerged as key players in the generation of artemisinin resistance. In this study, we show that PfGCN5, a histone acetyltransferase, binds to the stress responsive and multi-variant family genes in poised state and regulates their expression under stress conditions. We have also provided biochemical and cellular evidences that PfGCN5 regulates stress responsive genes by acetylation of PfAlba3. Furthermore, we show that upon artemisinin exposure, genome-wide binding sites for PfGCN5 are increased and it is directly associated with the genes implicated in artemisinin resistance generation. Moreover, inhibition of PfGCN5 in artemisinin resistant parasites reverses the sensitivity of the parasites to artemisinin treatment indicating its role in drug resistance generation. Together, these findings elucidate the role of PfGCN5 as a global chromatin regulator of stress-responses with potential role in modulating artemisinin drug resistance. Moreover, stress responses and drug resistance can show significant variation in cellular adaptation due to phenotypic cell-to-cell variability. To investigate this, we performed single cell RNA-sequencing (scRNA-seq) to quantify the cellular heterogeneity under temperature stress condition. High-resolution clustering of scRNA-seq datasets and a combination of gene signatures allow identification of cellular heterogeneity and stage transition during stress adaptation. Interestingly, we identified a rare population of cells, which is only emerged during the stress condition, showing the reactive state of the pathogen against the temperature stress condition. Thus, in this study, we have identified PfGCN5 as a global modulator of artemisinin resistance and revealed magnitude of gene expression heterogeneity under temperature stress condition within populations of *P. falciparum*.

SYNOPSIS

Malaria is a mosquito borne infectious disease known to affect millions of people worldwide every year. One of the major roadblocks in the elimination of malaria is the robustness of its causative agent *Plasmodium*, to generate resistance against different antimalarial. Artemisinin based combination therapies (ACTs) against *Plasmodium falciparum* have significantly reduced death due to malaria infections. Unfortunately, over the last decade there has been an increase in number of reports of artemisinin resistance. Emergence and spread of artemisinin resistance poses serious threat to malaria eradication goals, hence it becomes necessary to understand the molecular mechanism and key players involved in artemisinin resistance.

Recent studies have shown that artemisinin resistant parasites have elevated adaptive response against protein damage and oxidative stress linked with artemisinin treatment. Understanding the mechanism of this elevated response can help researchers in designing better drugs to fight against artemisinin resistant parasites. This thesis focuses on the study conducted to understand the transcriptional regulation during unfavorable conditions in *P. falciparum*. Histone acetyltransferase GCN5 is known to regulate stress responses in higher eukaryotes hence we investigated the role of PfGCN5 in mediating stress response in *Plasmodium falciparum*. A part of work also focuses on understanding cellular heterogeneity present within parasite population during stress condition. The scientific rationale behind this work is to appreciate and explore the importance of stress induced heterogeneity in emergence of artemisinin resistant parasites.

Chapter 1 covers general introduction about the malaria and its causative agent *Plasmodium*. It discusses status of malaria burden, symptoms, *Plasmodium* life cycle in two different hosts along with vaccines and antimalarial drugs currently available in market. It also discusses transcriptional regulation in *Plasmodium* through different mechanisms like epigenetics, transcription factors etc.

In **chapter 2**, we show that PfGCN5 binds to the stress responsive and multi-variant family genes in poised state and regulates their expression under various stress conditions. With the help of chromatin immunoprecipitation coupled high-throughput sequencing (ChIP-seq) we identified PfGCN5 binding sites during control and stress conditions like elevated temperature and artemisinin exposure. Interestingly, we found that PfGCN5 is associated

with the genes which are upregulated during stress conditions. We have also provided biochemical and cellular evidences that PfGCN5 regulates stress responsive genes by acetylation of PfAlba3.

Chapter 3 provides the evidence that PfGCN5 plays an important role in modulating artemisinin resistance in *Plasmodium falciparum*. We show that upon artemisinin exposure, genome-wide binding sites for PfGCN5 are increased and it is directly associated with the genes implicated in artemisinin resistance generation like BiP and TRiC chaperone. Moreover, inhibition of PfGCN5 in artemisinin resistant parasites, Kelch13 mutant, K13I543T and K13C580Y (RSA~ 25% and 6%, respectively) reverses the sensitivity of the parasites to artemisinin treatment indicating its role in drug resistance generation. Together, these findings elucidate the role of PfGCN5 as a global chromatin regulator of stress-responses with potential role in modulating artemisinin drug resistance, and identify PfGCN5 as an important target against artemisinin resistant parasites.

Study carried out in **chapter 4** investigated the role of PfGCN5 in transcription regulation during nutrient stress condition. With the help of ChIP-seq and transcriptomic (RNA-sequencing) analyses, we show that PfGCN5 is associated with the genes that are important for the maintenance of parasite cellular homeostasis upon nutrient stress condition. Furthermore, we identified various metabolic enzymes as interacting partners of PfGCN5 by immunoprecipitation coupled with mass spectroscopy, possibly acting as a sensor of nutrient conditions in the environment. We also demonstrated that PfGCN5 interacts and acetylates PfGAPDH *in vitro*. Collectively, our data provides important insights into transcriptional deregulation upon nutrient stress condition and elucidate the role of PfGCN5 during nutrient stress condition.

Chapter 5 discuses heterogeneity within the *Plasmodium* population under stress conditions using single cell RNA sequencing (scRNA-seq). The malaria parasite has a complex life cycle exhibiting phenotypic and morphogenic variations in two different hosts. Phenotypic cell-to-cell variability can be an important determinant of cellular adaptation, stress tolerance and immune evasion in the host. To investigate cellular heterogeneity, we performed single cell RNA-sequencing of 4949 and 6873 synchronized *Plasmodium* cells in control and under temperature stress condition (phenocopying the cyclic bouts of fever experienced during

malarial infection). High-resolution clustering of scRNA-seq datasets and a combination of gene signatures allow identification of cellular heterogeneity and stage transition during stress adaptation. We identified a subset of parasites primed for gametogenesis and another subset primed for stress adaptation. Interestingly, temperature stress inducted the process of gametogenesis by upregulation of master regulator (AP2-G) of sexual conversion. Moreover, pseudotime analysis indicated bifurcation for cell-fate decision to gametogenesis at two different stages of intra-erythrocytic cycle. Furthermore, we identified a rare population of cells, which is only emerged during the stress condition, showing the reactive state of the pathogen against the temperature stress condition. Interestingly, genes associated with the gametogenesis, chaperon activity and maintenance of cellular homeostasis showed maximum variation under temperature stress condition. Thus, our study suggests that the variability and versatility of the maintenance of cellular homeostasis should enable cells to survive under different stress conditions, and may act as an important stimulator of development of drug-resistance in *Plasmodium falciparum*.

Chapter 6 discusses the conclusions and future perspective of the work carried out as the part of this thesis.

ABBREVIATIONS

GCN5	General Control Non-derepressible 5
RBCs	Red Blood Cells
EMP-1	Erythrocyte Membrane Protein 1
ICAM-1	Intercellular adhesion molecule 1
NKT	Natural Killer T
NK	Natural Killer
KAHRP	Knob-associated histidine-rich protein
WHO	World Health Organization
NVBDCP	National Vector Borne Disease Control Programme
NSP	National Strategic Plan
HAT	Histone Acetyltransferase
HDAC	Histone deacetylase
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
GNAT	Gcn5-related N-acetyltransferases
HSFA3	Heat stress transcription factor A-3
UVH6	Ultraviolet hypersensitive 6
AREB1	Abscisic acid responsive element binding protein 1
SAGA	Spt-Ada-Gcn5 acetyltransferase
ROS	Reactive oxygen species
PMSF	Phenylmethylsulfonyl fluoride
PIC	Protease inhibitor cocktail
NP-40	4-Nonylphenyl-polyethylene glycol
PVDF	Polyvinylidene fluoride
TBST	Tris-buffered saline (TBS) and Tween 20
PFA	Para formaldehyde
BSA	Bovine serum albumin
RPM	Revolutions per minutes

NFW	Nuclease free water
PBS	Phosphate buffer saline
ChIP	Chromatin immunoprecipitation
HP1	Heterochromatin protein 1
Alba	Acetylation lowers binding affinity
GST	Glutathione S transferse
ACT	Artemisinin based combination therapy
DHA	Dihydroartemisinin
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
RSA	Ring stage survival assay
BIP	Binding immunoglobulin protein
TCP1	T-Complex Protein 1
PI3K	Phosphoinositide 3-kinase
PERK	Protein kinase R (PKR)-like endoplasmic reticulum kinase
GEMS	Gel Bead-In emulsions
AP2	Apetala 2
ScRNA-seq	Single cell RNA Coefficient of variation
SURFIN	Surface associated interspersed protein family
CPSF	Cleavage and polyadenylation specificity factor
PEXEL	*Plasmodium* export element
FIKK	Phenylalanine, isoleucine, lysine, lysine
Sec	Seconds

Chapter 1: Introduction to Malaria

1.1 Malaria overview

1.1.1 History of malaria

Malaria, an infectious disease is caused by parasitic protozoa belonging to genus *Plasmodium*. It is one of the oldest infectious diseases with recorded history dating back to ~4000 years [1]. First written records of malaria description can be found in Chinese medical writings, Nei Ching which means 'The Canon of Medicine' in 2700 B.C.E [2]. It was referred as the "Mother of fevers" by Chinese. Symptoms of malaria infections are also documented in Susruta, a Sanskrit medical treatise. Hippocrates had also documented the symptoms of malaria as it was responsible for high toll of deaths in Greek city-states [1]. Qinghao plant was mentioned in a Chinese medical treatise called 52 Remedies [3]. Later Ge Hong first described the anti-fever properties of this plant [4].

Malaria got its name from Italian word 'mala aria' meaning bad air [5]. Before the term was coined it was referred as intermittent fever, Roman fever, death fever, swamp fever [5]. Refuting the earlier belief that malaria is caused by bacterial infection, in 1880 Charles Louis Alphonse Laveran identified parasite in the blood of humans which were suffering from malaria [6]. He discovered that malaria is caused due to a protozoan, after observing the blood smear prepared from the blood of patient who just died of malaria. He named it *Oscillaria malariae* but later changed it to *Plasmodium* [6]. He was awarded Nobel Prize in 1907 for his contribution. This was the first time any protozoon was identified as responsible for any disease. Later in 1886, Camillo Golgi identified that there are different species of the malaria parasites and symptoms caused due to malaria infection is species specific [6]. *Plasmodium vivax* and *Plasmodium malariae* were named by Giovanni Batista Grassi and Raimondo Filetti [6]. *P. falciparum* was named by William H. Welch in 1897 [7]. Following this vector for the avian malaria was identified by Ronald Ross in 1897 in India [8]. Later in 1898, Giovanni Batista Grassi and his team of researchers observed the full life cycle of *Plasmodium* [6]. Other scientist like Amico Bignami, Giuseppe Bastianelli, Angelo Celli and Ettore Marchiafava also contributed in identification of vectors of *Plasmodium* [6, 9]. Mosquitoes have transmitted malaria in different organism likes reptiles, birds, humans,

rodents, apes and monkey [10]. Animals which are affected by the malaria infection are considered as host whereas mosquito acts as parasite vector.

1.1.2 Malaria burden

According to the World Health Organization (WHO) 2018 report around 219 million cases of malaria infections were reported worldwide (Figure 1.1) [11]. Around 435,000 deaths were reported due to malaria globally. Most of the malaria cases were reported from the Sub-Saharan Africa and south east Asia. Pregnant women and children under the age of 5 are the most vulnerable group affected by malaria infection [11]. According to the WHO 2018 report fifteen countries in Sub-Saharan Africa and India contributes to 60% of the global malaria burden. Nigeria (25%), Democratic Republic of the Congo (11%), Mozambique (5%), India (4%) and Uganda (4%) contributes nearly half of the malaria cases reported in 2017 [11]. On the other hand, eight countries including Paraguay, Algeria, Argentina, Uzbekistan and Sri Lanka have been declared malaria free since year 2000. Many other countries have significantly reduced the number of malaria cases [12]. China and El Salvador have reported zero cases of malaria in 2017 [13, 14].

Malaria is considered as the disease of poverty, primarily affecting developing countries. There are several funding agencies all over the world which provides support in the form of policy making, technical, operational and financial assistance. The Roll Back Malaria (RBM) partnership, US President's Malaria Initiative (PMI), Asia Pacific Malaria Elimination Network (APMEN), World Health Organization (WHO), Malaria No More (MNM), End Malaria Council (EMC), Asia Pacific Leaders Malaria Alliance (APLMA) are some of the organizations which supports malaria elimination at global level [15]. Many malaria control interventions like long lasting insecticides treated nets (LLINs), indoor residual spraying (IRS) and artemisinin based combination therapies (ACTs) have significantly reduced the cases of malaria infections globally [16].

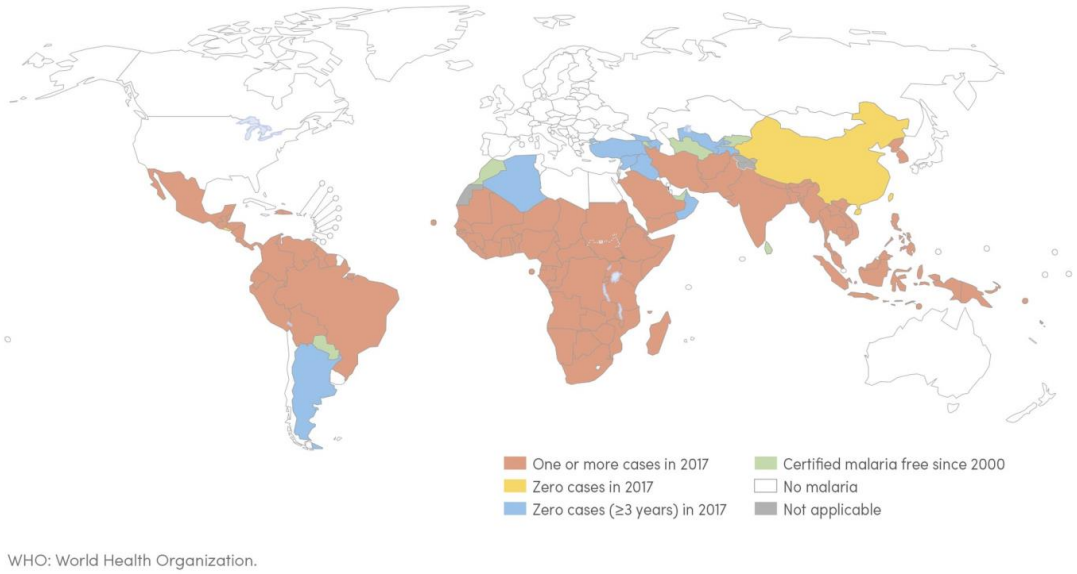


Figure 1. 1: Global distribution of malaria. Malaria is confined to tropical and subtropical regions of the world. *Different color represents the numbers of malaria cases reported in 2017 according to the WHO Malaria Report 2018 [11].*

1.1.3 Malaria in India

According to the 2017 report, India accounts for the 4% of the global malaria burden and 90% of the malaria cases in the WHO south east Asia region [11]. Malaria is one of the common health problems in India. The states which are majorly affected by malaria are Odisha, Chhattisgarh, Jharkhand, Tamil Nadu, Madhya Pradesh, Maharashtra, Tripura, Meghalaya and Mizoram [15, 17, 18]. With the help of various Malaria control strategies number of cases in India have consistently declined from 2.08 million in 2001 to 0.4 million in 2018 and 24% reduction in malaria cases from last year [11]. Out of five species of *Plasmodium*, *P. falciparum* and *P. vivax* are most common in India. *P. malariae* is reported in the Odisha whereas *P. ovale* is extremely rare. *P. falciparum* and *P. vivax* vary in their distribution across India. *P. vivax* infections are mostly common in southern states like Tamil Nadu whereas *P. falciparum* infections are found in Orissa. Mixed infections of both the species are prevalent in the west states like Gujarat. Earlier *P. vivax* infection cases were more common than *P. falciparum* infections. However the ratio of *P. falciparum* to *P. vivax* infection increased from 0.41 in 1985 to 1.01 in 2010 [19].

1.1.4 Symptoms of Malaria

Malaria is classified into two categories on the basis of severity; complicated and uncomplicated malaria. Most of the clinical manifestations observed during malaria infections are during intraerythrocytic stage of the *Plasmodium* [20]. Malaria is characterized by symptoms like fever, vomiting, diarrhoea, headache, body ache, stomach ache. It also results in hypoglycemia, hyperlactatemia and anaemia [21]. Uncomplicated malaria has three stages; hot stage which is characterized by fever, headache and vomiting followed by sweat stage consisting of sweating and tiredness. Finally cold stage of infection results in shivering. These cycles last for 6-10 hours [22]. *P. falciparum*, *P. ovale* and *P. vivax* have tertian attacks which means symptomatic stage occurs every second day [23]. *P. malariae* results in quartan attacks resulting in symptomatic stage every third day [23]. If the malaria is left untreated it can result in complicated malaria resulting in organ failure, severe anaemia, cerebral malaria, acute respiratory distress syndrome etc. If not treated on time, it can eventually result into the death of the patient [22].

1.1.5 Human Cerebral malaria

Plasmodium infection can result in severe infection resulting in cerebral malaria. It is responsible for the 50,000 deaths per year. Patients who survive cerebral malaria usually have cognitive and physical dysfunction [21, 24, 25]. Cerebral malaria can happen within two week of mosquito bite and usually results in 2-7 days of fever. Some of the most common clinical features of cerebral malaria are brain swelling, retinopathy, seizures and elevated intracranial pressure [21, 24, 25]. There are very limited studies done to understand the pathogenesis of cerebral malaria. One of the first attempts to study the cerebral malaria was in 1900 when a patient died due to cerebral malaria [26]. Histopathological analysis of the cerebral malaria patient revealed blockage of brain vessels due to infected RBCs. This blockage results in the hemorrhage of different parts of the brain like corpus callosum, cerebella and cerebral cortices. Blockage of the brain vessels results from the parasite sequestration in cerebral microvasculature. Parasite sequestration within brain vessels results from the binding of parasite protein exported on the surface of RBCs, Erythrocyte membrane protein-1 (PfEMP-1) with host receptor like intercellular adhesion molecule -1 (ICAM-1). Expression of ICAM-1 was high at the areas where parasite sequestration was found in patient sample with cerebral malaria [21, 24, 25]. The size of the sequestered parasites keeps

increasing due to the other uninfected RBCs keep aggregating with infected RBCs to form rosettes [27] .

1.2 Prevention and control

1.2.1 Vector and vector control

Around 30-40 species of *Anopheles* are known to act as the vector for human malaria. Malaria is transmitted by mosquitoes belonging to the genus *Anopheles* [28]. The *Anopheles* have characteristic feature with abdomen pointing upwards, in contrast to parallel position, when in a resting position [29]. Different species of Anopheles have different capacity to transmit *Plasmodium*. *Anopheles gambiae* and *A. funestus* are considered efficient transmitters of *Plasmodium*. Female *Anopheles* bites human in order to get proteins from the blood meal which is required for their egg production [30]. The parasite life cycle takes 10-21 days within the mosquito [31].

Several studies have shown that mosquitoes produce immune response against the *Plasmodium* infection [32, 33]. They are known to secrete several molecules which are secreted by mosquito organ, fat bodies, and haemocytes and midgut cells [33]. Once the gametocytes are taken up by the mosquitoes from infected human, they enter into the harsh environment of the digestive tract. Parasites can positively or negatively regulate the expression of mosquitos' digestive enzymes. Mosquitoes are known to produce nitric oxide synthase (NOS) which further results in the production of several antimicrobial peptides which can result in death of large number of ookinete [34]. NOS also activate complement system in mosquitoes through thioester containing protein 1 (TEP1) protein [35]. Very few numbers of ookinete are able to survive the immune response which results in formation of sporozoites. Haemocytes in mosquito can perform phagocytosis of sporozoites as reported in *Ae. aegypti* and *An. Gambiae* [36]. Fat body in mosquito is known to produce antimicrobial peptides which help in controlling the infection [37].

One of the most effective tools to prevent the transmission of malaria is through the use of insecticide treated bed nets and spraying of insecticides, which prevents mosquitoes to feed on human blood. However mosquito has a great ability to evolve and generate resistance against the insecticide use for prevention [38]. Recently studies suggested the role of male *Anopheles* mosquito in malaria transmission through deregulation of female Anopheles mosquito midgut transcriptome. The change in transcriptome is mediated by the steroid

hormone 20-hydroxyecdysone [39]. This hormone is contained within mating plug and was found to increase female susceptibility to *P. falciparum* infection [39].

Six species of *Anopheles* are known to be vector of malaria infection in India although *Anopheles culicifacies* is responsible for 60-65% of the malaria burden. The launch of National Malaria Control Programme (NMCP) in 1953 resulted in significant decrease in number of malaria cases with the widespread use of DDT indoor residual spray (IRS) [40]. Later in 1958, NMCP was converted into National Malaria Eradication Programme (NMEP). Unfortunately after the huge success in 1976, 6.4 million cases of malaria were reported [15, 17, 40]. Resistance against antimalarial in *Plasmodium* and insecticide resistance in vector were responsible for resurgence. Later in 2002, the NMCP became part of the National Vector Borne Disease Control Programme (NVBDCP) which is currently responsible for the control and prevention of the various vector borne diseases like malaria in India [15]. The National Strategic Plan (NSP) has been proposed by NVBDCP in collaboration with World Health Organization to make India free of malaria infections by 2027 and elimination by 2030 [15].

1.2.2 Host immunity against *Plasmodium*

Individuals are known to acquire adaptive immunity against malaria infection after repeated rounds of infections. The adaptive response generated due to repeated infection is largely mediated by Immunoglobulin G (IgG), which is short lived and becomes weaker in the absence of parasite exposure. Immune response at the pre-erythrocytic stage targets sporozoites and infected hepatocytes [41]. Antibodies against the circumsporozoite protein of sporozoites are known to prevent invasion of hepatocytes. It is known to activate complement system, phagocytosis by cytotoxic Natural Killer (NK) and NKT cells. Infected hepatocytes are also identified by parasite neoantigens expressed at the surface of hepatocytes and killed through antibody dependent cell mediated mechanism by Kupffer cells and NK cells [41]. Interferon- γ secreted by $CD8^+$T cells, NK, NKT, and γδT cells results in killing of parasites present inside hepatocytes [41]. Antibodies are also generated against merozoites which can block the invasion of RBCs. These antibodies are also known to mark merozoites for lysis by complement system. $CD4^+$T helper cells are known to produce proinflammatory cytokines for activation of macrophages. NK cells are known to secrete IFN- γ, perforin and granzymes which can kill infected RBCs [41, 42]. Recent studies have shown that NK cells play an important role during the early phase of *Plasmodium* infection by inhibiting parasite growth

through antibody dependent cellular cytotoxicity mechanism (ADCC) [41]. Antibodies can be also generated against the gametocytes by human immune system, result in killing of gametocytes. Macrophages are also known to kill gametocytes through release of nitric oxide [41].

1.2.3 Immune evasion through cytoadherence

Infected RBCs are known to bind several host cell endothelial receptors to prevent splenic clearance. Parasites are known to express and export different proteins on the surface of infected RBCs 12-14 hours post invasion [43]. Various knobs like structures appear on the surface of infected RBCs which are mainly consist of knob-associated histidine rich proteins (KAHRP) [44]. Parasites are also known to express *P. falciparum* erythrocyte membrane protein (PfEMP) 1 on the surface of iRBCs [45, 46]. Variants of PfEMP1 are encoded by 60 members of the var gene family. PfEMP1 proteins are known to play an important role in cytoadherence [46, 47]. The extracellular domain of PfEMP1 is known to bind host endothelial receptors like "Cluster of Differentiation 36" (CD36) and "Intercellular adhesion molecule 1" (ICAM-1), which results in their sequestration and avoidance by splenic clearance (Figure 1.2) [46, 48]. Infected RBCs are also known to form rosettes by binding to several uninfected RBCs [27, 49, 50]. PfEMP1 are also known to undergo antigenic variation [45]. It is a process by which pathogen continuously change the antigenic epitopes exposed to the immune system. *Plasmodium* performs antigenic variation through transcription regulation and switching of different var genes [45, 51-53]. Thus parasite is able to evade the immune response generated through switching of var gene on the surface of iRBCs. Other variant proteins like rifins and stevors are also known to mediate cytoadherence and antigenic variation [54, 55].

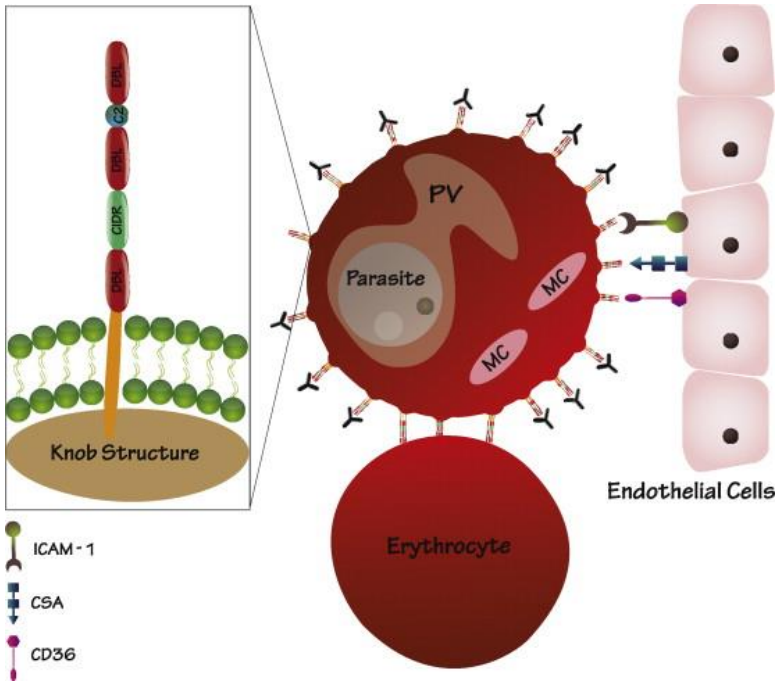


Fig. 1.2: Schematic representation of the interaction between PfEMP1 and endothelial receptors. *Parasitised RBC express PfEMP1 proteins on the knob like structure present on their surfaces. These proteins bind to the various endothelial receptors like ICAM1, CD36 etc. This helps in sequestration of Plasmodium parasite by avoiding splenic clearance of infected RBC. PV is parasitophorous vacuole; DBL is Duffy binding like domain; MC is Maurer's cleft; ICAM-1 is intracellular adhesion molecule; CSA is chondroitin sulphate A; CD36 is cluster determinant 36 and CIDR is cysteine-rich interdomain regions. Adapted from Pasternak and Dzikowski, 2009 [56].*

1.2.4 Vaccines available for the treatment of malaria or malaria vaccines

One of the initial studies related to the development of malaria vaccines started in 1960s with the mice infected using irradiated sporozoites. One study suggested that high level of protection can be obtained in volunteer but this requires multiple numbers of bites by irradiated sporozoite infected mosquitos [57]. Later various studies have assessed sporozoite coat protein circumsporozoite as well as various blood stage antigens as vaccine candidates (Table 1.1). Later peptide based candidate vaccine was developed in Colombia, called SPf66 [58]. But the vaccines failed to provide protection during filed trial in Africa and Asia. One of the most promising vaccines is the RTS, S/AS01E which targets pre erythrocytic stages of the *Plasmodium* parasite in the human host [59-61]. It is a hybrid protein formulated in a multi-component adjuvant named AS01. RTS vaccine is the result from collaboration between Walter Reed Army Institute of Research in the USA and GSK Biologicals. RTS, S is

scientific names which represent its composition. The 'R' stands for the central repeat region of the *P. falciparum* circumsporozoite protein (CSP); the 'T' stands for T cell epitopes of CSP protein: the 'S' stands for hepatitis B surface antigen [59]. This fusion protein (RTS) is expressed in yeast cells. To make it more immunogenic it is assembled further with the unfused S protein to yield RTS, S. Since this vaccine also contains the AS01 adjuvant system, it is referred as 'RTS, S/AS01'. In 2001, along with Bill and Melinda Gates Foundation vaccines was developed for infants and young children living in endemic region. Phase 3 trial was concluded in 2014 [62]. It received positive scientific opinion from the European Medicines Agency in 2015 and is currently recommended by World Health Organization for a pilot implementation programme. This vaccine is provided to young children in three sub Saharan African countries from 2019 beginning [59].

1.2.5 Antimalarial drugs

Antimalarial drugs are considered as the powerful tool to treat malaria infection. Several drugs are currently available in the market which target different stages of the parasite life cycle. According to the World Health Organization list of medicine, around 14 antimalarial drugs are listed for treatment of malaria whereas 4 drugs are mentioned for prophylactic treatment [63, 64]. Various combinations of drugs are recommended by the WHO for the treatment of malarial infection. Artemisinin based combination therapy (ACT) is considered as the first line of defense in the areas where chloroquine resistance is present. Other drugs which are currently used for the treatment of malaria include lumefantrine, piperaquine and atovaquone etc. In 1999, Medicine for Malaria Venture (MMV) was formed to boost research related to antimalarial product development [65]. The potential drug candidate is judged on the basis of several factors like the stage targeted by the drug, novel mode of action, cross resistance potential, property to kill hypnozoites etc. MMV also provide aid for research to fight against malaria infection. One way to identify new drugs is to repurpose the existing drugs by demonstrating their antimalarial properties [65]. Currently several drugs are in the pipeline identified by such approach worldwide. Methylene blue in combination with Primaquine has completed its Phase II trials in 2017. Fosmidomycin in combination with piperaquine is currently in phase II trial [66]. Rosiglitazone (antidiabetic drug), Imatinib (cancer therapy drug), sevuparin (sickle cell disease drug) are currently in the clinical trial [67]. Some of the new drugs under preclinical/clinical stages are NPC1161B (chiral 8-

aminoquiniline), MK4815, CDRI 97/78, GSK369796, artemisone, SAR97276, AQ-13 etc [67]. Different antimalarial drugs and their targets are discussed in detail in section 3.1.

Vaccine candidate	Clinical trial registration number	Clinical trial stage
PRE-ERYTHROCYTIC		
RTS,S/AS01	NCT01345240	Phase 3
R21/AS01B	NCT02600975	Phase 1
R21/ME-TRAP	NCT02905019	Phase 2
ChAd63/MVA ME-TRAP	NCT01635647	Phase 2
R21/Matrix-M1	NCT02572388/NCT02925403	Phase 1/2
PfSPZ Vaccine	NCT03510481	Phase 1
PfSPZ-CVac (PfSPZ Challenge + chloroquine or + chloroquine/pyrimethamine	NCT03083847	Phase 1
GAP 3KO (52-/36-/sap1-)	NCT02313376	Phase 1
BLOOD-STAGE		
pfAMA1-DiCo	NCT02014727	Phase 1
P27A	NCT01949909	Phase 2
PAMVAC	NCT02647489	Phase 1
PRIMVAC	NCT02658253	Phase 1
SEXUAL-STAGE		
ChAd63 Pfs25-IMX313/MVA Pfs25-IMX313	NCT02532049	Phase 1
Pfs25-EPA/Alhydrogel	NCT01867463, 51	Phase 1
Pfs230D1M-EPA/Alhydrogel and/or Pfs25-EPA/Alhydrogel	NCT02334462	Phase 1
Pfs25M-EPA/AS01 and/or Pfs230D1M-EPA/ASOI	NCT02942277	Phase 1
Pfs25 VLP-FhCMB	NCT02013687	Phase 1
Pfs25-Pfs25	NCT00977899	Phase 1
Pfs25 & Pvs/Monatide ISA 51	NCT00295581	Phase 1

Table 1.1 Status of various vaccines under clinical trial against malaria. *Around 20 candidate vaccines are currently under clinical trials. These vaccines target different stages of the malaria parasite. Currently RTS, S is licensed and undergoing pilot administration programmes before being approved for immunization. Adapted from Frimpong et al, 2018 [68].*

1.3 *Plasmodium*

Malaria is caused by unicellular eukaryotic protozoa of *Plasmodium* species. *Plasmodium* belongs to the Apicomplexan phylum that includes various other parasites like *Toxoplasma, Cryptosporidium* etc, which are known to cause infection in warm blooded organisms [69]. The phylum is known as Apicomplexa since most of these organisms' possess a unique organelle that comprise of a plastid called apicoplast and an apical complex structure [70]. There are more than 250 species of *Plasmodium* which can infect different organisms like humans, birds, monkeys etc. There are five species of *Plasmodium* which can infect human beings namely *P. falciparum, P. malariae, P. vivax, P. ovale, and P. knowlesi* [71]. Out of these five species, *P. falciparum* is the most lethal and causes highest mortality.

P. falciparum infections are most common in the tropical Africa, Asia and Latin America. It causes most dangerous form of the disease resulting in high level of parasites in the blood which can result in cerebral malaria or even death of the host. *P. vivax* is predominant in tropical as well as some temperate areas like Latin America., Indian subcontinent etc and it is absent in West Africa [72]. On the other hand *P. ovale* is found mostly in West Africa. *P. malariae* is less common than *P. vivax* and *P. falciparum* infections. *P. vivax* and *P. ovale* are known to have dormant stage in liver resulting in relapse of malaria. Interestingly, *P. knowlesi* naturally occurs in macaques. However, recently it has been shown to cause infection in humans. First case of *P. knowlesi* infection in human was reported from Sarawak, a state in Malaysia [72, 73]. Thereafter number of *P. knowlesi* cases has kept on increasing and now it is found in other countries too in south east Asia. *P. knowlesi* is known to complete its asexual life cycle in 24 hours and can result in high parasitemia resulting in death of human [72, 73].

Plasmodium like other members of Apicomplexa phylum is known to possess several organelles. It has apical complex localized at the apical pole of the organism. It houses machinery like granules, micronemes and rhoptries, necessary for the invasion of the parasite into the host cells [70]. It carries apicoplast which harbours several essential metabolic pathways like mevalonate independent 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway to synthesise isoprenoids and type II fatty acid synthesis (FAS II) [74]. It also carries food vacuole where the digestion of the host haemoglobin is carried out. Detoxification of haem into non-toxic haemozoin is carried out in food vacuole [75]. *Plasmodium* also contains other organelles like Golgi apparatus, mitochondria, nucleus and endoplasmic reticulum [76].

1.3.1 Life cycle of *Plasmodium*

Plasmodium life cycle is consists of two phases; sexual and asexual in mosquito and human, respectively (Figure 1.3) [77]. It uses female *Anopheles* mosquitoes as a vector for transmission of disease from one human to another. The sexual life cycle is harbored in the *Anopheles* mid gut where fertilization of male and female gametocytes takes place whereas the asexual cycle occurs in humans [77]. Incidence of malarial infection begins when a female *Anopheles* feeds on human blood, during the process of which it injects sporozoites into the skin. Sporozoites are deposited in the skin along with vasodilators in order to

increase their chance of finding blood vessel [78, 79]. Interestingly, when mosquito reach blood vessel, it no longer releases sporozoites. The sporozoites present in the skin now traverse to reach hepatocytes [78, 79]. *In vitro* studies have shown that sporozoites can travel through an epithelial layer which might be the mechanism through which sporozoites travels through vessels to reach hepatocytes. Sporozoites are harbored in the hepatocytes for 5-16 days and undergo rapid division cycles to release thousands of merozoites from each liver cell [77]. Some of them form hypnozoites which remain dormant in the liver cell for long time and are responsible for relapse of malaria [80]. The merozoites released on account of hepatocytes rupture enter into circulation where they now infect red blood cells. Merozoites are held within parasitophorous vacuole within red blood cells. In red blood cells (RBCs) they undergo different morphological stages like ring, trophozoite and schizont. During early stages in RBCs, parasites are referred as ring due to its ring shape appearance. During trophozoite stage parasites is metabolically active. Moreover, hemoglobin from the RBC is transported into parasite food vacuole where it is degraded to release amino acids required for the growth of the parasites [81]. Finally during schizont stage, parasites undergo multiple rounds of nuclear division without cytokinesis. It results in formation of 16-32 merozoites at the end of 1-3 days depending on the species of parasite. This is followed by rupture of the infected RBCs and release of the merozoites which can now potentially re-invade new RBCs. Some of the merozoites infected RBCs provide conducive conditions for the parasite to exit from the asexual stage and form gametocytes [77]. The infected RBCs harboring the sexual form of the parasite is then re-ingested by the female *Anopheles* while feeding on a malaria-infected human. Within the *Anopheles* midgut, gametocytes are released and developed into mature male (microgamete) and female (macrogamete) gametes. One female gametocyte develops into one macrogamete whereas one male gametocyte develops into eight flagellar motile microgametes. They fuse to form a zygote which further develops to form ookinete that later travels to salivary gland to form oocysts. Each oocyst then divides to form thousands of sporozoites [82]. These sporozoites travel through haemolymph to finally reach salivary glands where they are ready to be released into the next human host during time of feeding [82].

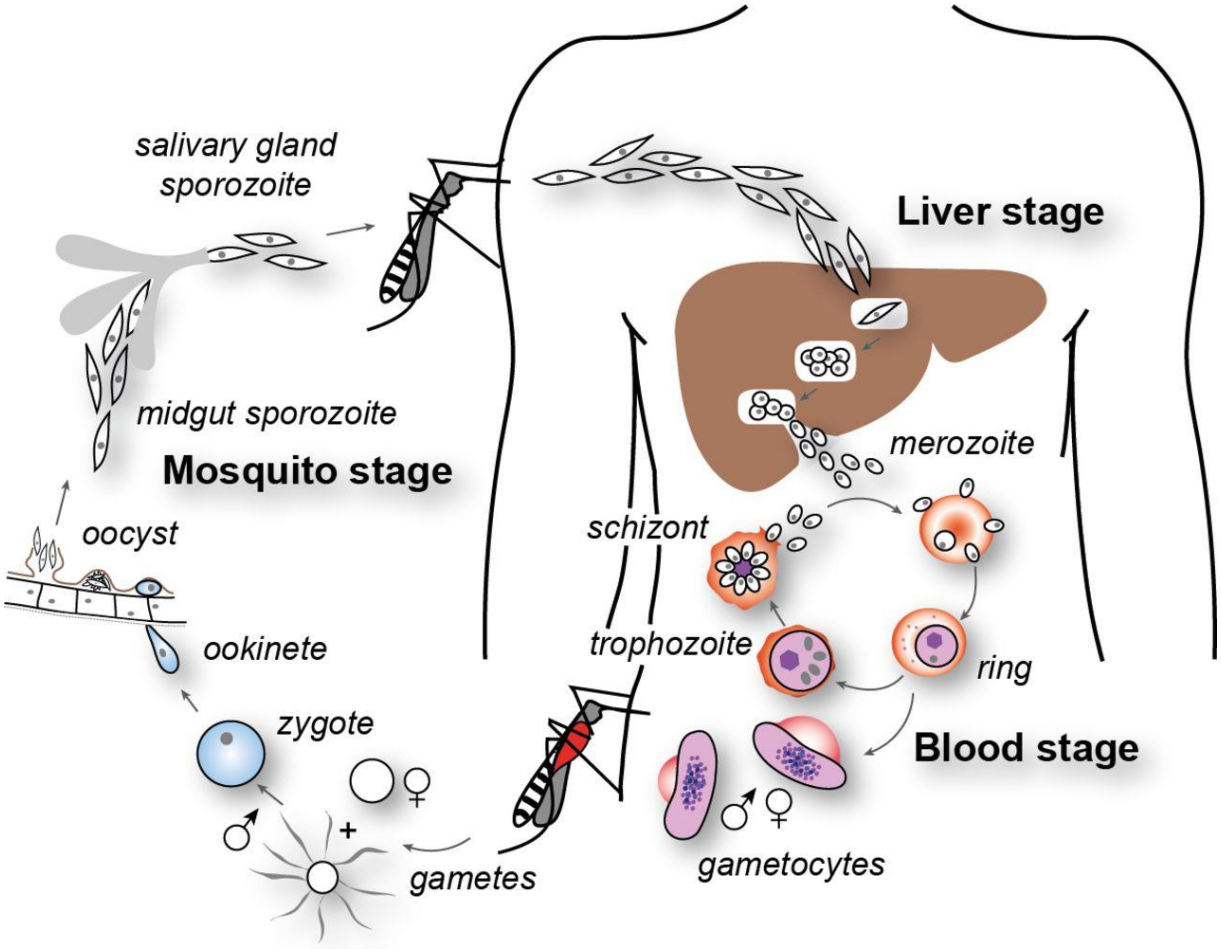


Fig. 1.3: Schematic representation of Plasmodium life cycle. *Plasmodium completes its life cycle in two different hosts. Sexual phase take place in female Anopheles and asexual reproduction take place in humans. Reproduced from Cowman et al. 2012[83].*

1.3.2 *Plasmodium* genome

Plasmodium is the first eukaryotic parasite whose genome was sequenced in the year 2002 [84]. Six species of *Plasmodium* genome sequence have been published till now which includes *P. falciparum* strain 3D7, rodent malaria *P. y. yoelii* 17XNL clone, *P. berghei* ANKA clone, *P. chabaudi*, *P. vivax* Salvador 1 strain and the human/simian malaria *P. knowlesi* H strain [85]. Genome sequence revealed that all *Plasmodium* species are haploid with genomic size approximately 23.3 Mb, which is organized in 14 chromosomes [84]. Genomic sequence revealed high A + T content (protein coding region 80.6% and non-coding region 90%) in *P. falciparum* and rodent *Plasmodium* species [84]. However *Plasmodium vivax* and *P. knowlesi* have higher GC content in comparison to *P. falciparum* (45% and 40% respectively). *Plasmodium falciparum* contains approximately 5800 genes present on 14 chromosomes. 51% of the genes contain at least one intron. Mean length of the genes in

Plasmodium is around 2.25 kb which is longer than in higher organism. More than 60% of the genes *of P. falciparum* have no sequence similarity with any other organism [84]. Hence, most of the genes in *Plasmodium* are still not annotated [84].

Apart from nuclear genome, *Plasmodium* also has a linear mitochondrial genome which is 6 kb in size in *P. falciparum* [84]. It also harbors ~35 kb circular apicoplast genome. *Plasmodium* genome encodes around 43 tRNA which binds to all codons except TGT and TGC which codes for cysteine [84]. In *Plasmodium*, mitochondrial genome do not encode for any tRNA hence it imports tRNAs from the cytoplasm [84]. On the other hand apicoplast encodes sufficient tRNAs for carrying out protein synthesis within the apicoplast [84]. Unlike higher eukaryotes, *Plasmodium* contains individual rRNA unit on 7 different chromosomes. There is difference in the sequence in the rRNA gene present on different chromosome. Interestingly expression of these rRNA is known to be dependent on the stage of the parasite. It is believed that parasite changes their translation properties by regulating the expression of different ribosome RNA. Ribosome expression is also specific to the host. S type (sexual type) ribosomes are expressed in mosquitoes whereas A type (asexual type) ribosomes are expressed during stages in humans [86].

1.3.3 Transcription regulation in *Plasmodium*

Several transcriptomics studies have been conducted which are fundamental to understand gene expression variation during *Plasmodium* life cycle in two different hosts. These studies have also helped in deciphering the role of different transcription factors vital for the parasite pathogenesis. Bozdech *et al.* in 2003 published a comprehensive transcriptome data of highly synchronized HB3 parasites at 1 hour interval [87]. This study showed the unique mode of transcriptional regulation in *Plasmodium* where most of the genes are transcribed once per cycle. Moreover, the expression of genes is correlated with the developmental cycle of the parasites. Most of the genes required for the general cellular process like protein synthesis are expressed initially followed by genes required for DNA replication [87]. Most of the genes required for invasion are expressed at the end of the intraerythrocytic developmental (IDC) cycle [87].

Le Roch *et al.* published the transcriptome of nine different stages of the parasites including salivary gland sporozoites and sexual stage gametocytes [88]. This study showed that genes with similar function have similar expression profiles. Furthermore, they identified the

possible function of more than 1000 hypothetical proteins [88]. Eventually transcriptome of other parasite strains like Dd2 and 3D7 were published [89]. Since *Plasmodium* completes its life cycle in two different hosts, it requires mechanisms for coordinated modulation of gene expression. Transcriptional and post transcriptional regulation of gene expression enables it to be causative for chronic infection in humans [90, 91]. Morphological changes observed during the development of the malaria parasite in erythrocytes are also governed by the fine tuning of gene expression [92].

1.3.3.1 Basic transcription apparatus

Previous studies have shown that gene expression regulation can take place at different levels like transcriptional, post transcriptional and translational as well as via epigenetic mechanisms like histone modifications etc. Studies focusing on understanding gene regulation on the level of transcription initiation revealed that *Plasmodium* codes for the key eukaryotic transcription regulation proteins. Similar to higher eukaryotic system, general transcription factors (GTFs) and RNA polymerase II (RNAPII) assemble to form pre-initiation complex at core promoter region [93]. Sequence specific transcription factors can regulate the transcriptional activity of RNAPII though regulation of pre initiation complex (PIC) assembly. Largest subunits of RNAPII as well as TATA-binding protein (TBP) were identified before the whole genome sequencing of *P. falciparum* [93]. Bioinformatics studies later identified common component of eukaryotic PIC likes TATA binding protein associated factors (transcription factor IID), transcription factor A, B, B-like, E, F, H and TBP-like (TLP) [93].

Also nucleosome mapping studies have identified that genic regions have higher levels of nucleosome occupancy as compared to the intergenic region. Studies have found the presence of nucleosome just downstream of the TSSs. Interestingly, homopolymeric poly (dA: dT) sequence is found to be enriched next to start codon, stop codon and splice sites. Homopolymeric nucleotide sequence is known to be resistant to nucleosome formation. These homopolymeric tracts are known to have impact on organization of DNA and intrachromosomal recombination [94].

1.3.3.2 DNA methylation in *Plasmodium*

One of the epigenetic mechanisms of gene regulation is through DNA methylation. DNA methylation is the process during which a methyl group is added to the 5- carbon of the

cytosine ring. It is commonly known as the fifth base of DNA [95, 96]. DNA methylation is known to involve in several functions likes regulation of gene expression, genomic imprinting and maintenance of chromosomal stability [95, 96]. Presence of DNA methylation in *Plasmodium* is still debatable. Earlier studies have indicated the absence of any DNA methylation in *Plasmodium* genome. It is believed that *Plasmodium* due to its high A-T rich genome might have lost the machinery of DNA methylation [97]. However, later technological advances led detection of low level of DNA methylation in *Plasmodium* [98]. Putative C5 methyltransferase was identified but was later found to methylate's tRNA aspartic acid at the C38 position [98, 99]. Hence, the role of DNA methylation in *Plasmodium* is still not clear.

1.3.3.3 Long non-coding RNA

Long non-coding RNA (lncRNA) is known to play an important role in gene regulation in *Plasmodium*. One of the most important phenomena regulated by lncRNAs is the monoallelic var gene expression [100, 101]. Var gene contains two exons and the intron in between act a bidirectional promoter resulting in transcription of two lncRNAs. These lncRNAs are known to play an important role in var gene expression. Antisense long noncoding RNA are known to activate their corresponding var gene [100, 101]. Any interference with antisense RNA results in the repression of var gene. Recent studies have shown that gdv1 antisense RNA regulates the process of sexual development by regulating sense transcription of gdv1 expression [102].

1.3.3.4 3D structure of chromatin organization regulates gene expression

Understanding transcriptional regulation in *Plasmodium* is important to dissect the pathogenesis of the parasite and its development in two different hosts under varied environmental conditions. Among the several ways of transcription regulation, the chromatin organization is believed to play an important role, though not properly understood in *Plasmodium* [103]. Studies have shown that during developmental transition of the parasite from one stage to another and/or in response to environmental cues there is a significant change in the chromatin organization [104]. This reorganization of the chromatin can allow discrete regulatory regions of the genome to make or break contacts leading to formation of transient regulatory hubs where supramolecular complexes can assemble and regulate gene expression paradigms. Enhancer and promoter are few such genetic elements which are

guided by chromatin conformations and are crucial for gene regulation across vast distances [105]. Chromatin accessibility control via heterochromatization and de-condensation can also allow genomic regions to be actively involved in gene expression or suppression [106]. During the sexual development parasite genome undergoes extensive heterochromatization [104]. As a result, genes that are not required may be repressed whereas genes which are important for sexual development are reorganized from repressive cluster to active (euchromatic) clusters [104]. An example of such a regulation can be observed during the var gene switching which mediates antigenic variation [107]. Chromatin looping plays an integral role in mediating the switch from repressive to active cluster and vice versa. Several methods which help in the investigation of 3D organization of the genome includes chromosome conformation capture (3C), Chromosome conformation capture on chip (4C), chromosome conformation capture carbon copy (5C) and Hi-C [108]. Several microscopic observations including the presence of repressive region in the subtelomeric and internal region was validated with the help of Hi-C. Hi-C experiments showed that *P. falciparum* does not contain well defined topologically associating domains like in higher eukaryotes. Interestingly, var genes which are repressed in *Plasmodium* are known to colocalize to the periphery of the nucleus within a heterochromatin compartment [104]. Hi-C studies have also observed the changes in the 3D organization of the genome throughout the parasite lifecycle. Trophozoite stage of the parasite is known to display more interchromosomal contacts which correlate with the high transcriptional activity [104]. During the development of gametocytes heterochromatin region remodels to transcribe genes which are important for sexual development. Hi-C data have shown that AP2-G, a master regulator of sexual development, leaves the heterochromatin cluster to express gametogenesis specific transcriptional cascade [104]. Similar rearrangements of the chromatin are also observed during the sporozoites stage of the parasites [104].

1.3.4 Transcription regulators in *Plasmodium*

1.3.4.1 Transcription factors

Plasmodium genome encodes very few transcription factors in comparison to higher eukaryotes. Apicomplexan specific transcription factors, ApiAP2 have been identified to play an important role in transcriptional regulation in a stage specific manner [109, 110]. Other transcription factors characterized in *Plasmodium* are PfMyb1 and PRE binding protein (PREBP) which are known to regulate cell cycle progression [111, 112]. It has been difficult

to decipher core promoter elements in *Plasmodium* due to extremely high A/T rich genome. Moreover, *in silico* studies have identified various cis-regulatory elements crucial for *Plasmodium* gene regulation. Recently substantial work has been done using CAGE (cap analysis of gene expression) to characterize the *Plasmodium* promoter structure and transcription initiation sites [113].

In higher eukaryotes, a wide range of transcription factors are known to regulate the expression of different subsets of genes in a cell-type specific manner. However, the repertoire of the transcription factors in *Plasmodium* is limited. Apicomplexan AP2 (Api-AP2) transcription factors help in development of gametocyte [114]. Several studies using next generation sequencing technologies have identified different transcription factors that play crucial role in the stage specific gene expression [109]. Transcriptomics studies have identified 27 ApiAP2s, 12 C2H2-type zinc finger (ZnF-C2H2), 8 helix-turn-helix [HTH including high mobility group box 3 protein (HMGB3)], 1 β-scaffold factor, K homology domain containing PREBP and a homeodomain –like TF [109, 115, 116].

Zinc finger C2H2 domain containing protein PfTRZ (terlomere repeat vinding ZnF protein) binds to the telomeric repeats and helps in maintenance of telomere length and cell cycle progression [117]. *Plasmodium* genomes encode a family of transcription factor which is homologous to the plant apetala 2/ethylene response factor TF family [110]. Different Ap2 proteins show different DNA binding preferences. Translational modifications of these proteins can also modulate their DNA binding. Recent studies have deciphered the function of these transcription factors through domain deletion and knockout studies and assigned names according to their functions [109] (Figure 2.1). For example PfAP2-I play an important role in regulating invasion related genes, whereas AP2tel is known to bind to the telomeric repeats hence play crucial role in maintenance of telomeric ends [109, 118]. Various AP2 transcription factors are also known to play important roles in the gametocyte conversion. AP2-G which is known to be under the regulation of heterochromatin protein 1 (HP1) is known as the master regulator of gametocyte induction [102]. AP2-G2 plays an important role in the repression of the asexual genes and promoting gametocyte conversion [109]. Similarly AP2-G3 also play crucial role in gametocyte commitment. AP2 transcription factors are also known to play important roles in the successful mosquito infection. AP2-O play critical role in the ookinete gene expression. AP2 transcription factors also regulate sporozoites development within mosquito [109, 119].

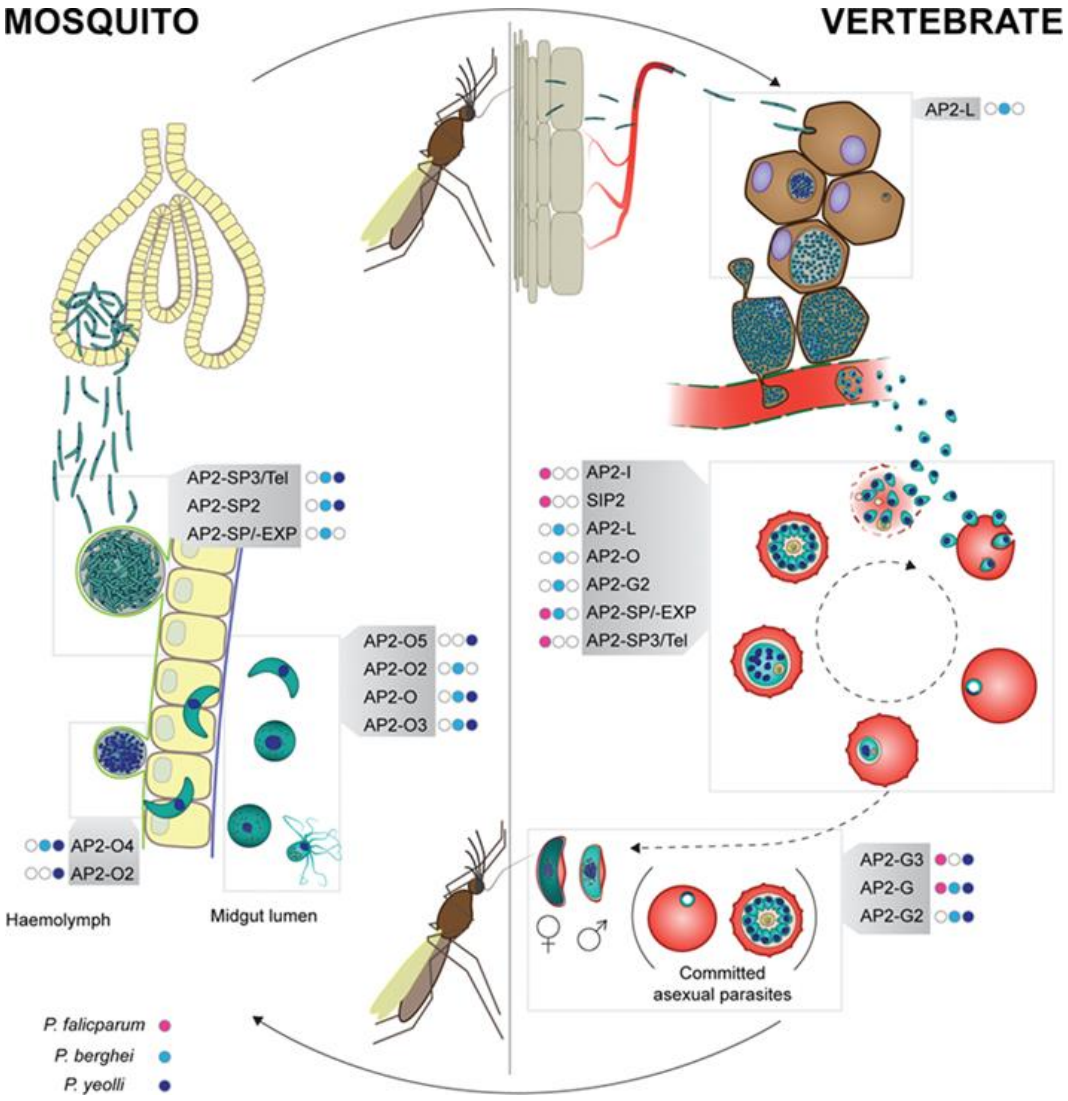


Fig. 1.4 Schematic showing role of different AP2 transcription factors during different stages of Plasmodium life cycle. *Adapted from Toenhake et al. 2019 [120].*

1.3.4.2 Histone modifiers

Large number of *Plasmodium* genes are known to encode proteins involved in chromatin structure maintenance, mRNA decay etc. This suggests that epigenetic and post transcriptional machinery might be playing a lead role in gene regulation in the parasite. Heritable changes in the transcription without altering the primary sequence of DNA are called epigenetic regulation of gene expression [121, 122]. Epigenetic modifications include DNA modification like methylation and changes in the chromatin organization through post translation modification of the histones [123]. In *Plasmodium*, epigenetic mechanisms have been shown to play an important role in regulation of difference processes like invasion,

gametogenesis, antigenic variation etc [124-126]. Epigenetic modification also regulate the way in which genome is packaged which in turn dictate the gene expression and suppression.

In eukaryotic systems genetic material, DNA is wrapped around the histone proteins H2A, H2B, H3 and H4 to form nucleosomes; the fundamental unit of the chromatin. During this organization of DNA into chromatin, 150 bp of DNA is wrapped around histone octamer in 1.75 super helical turn like "bead on a string" [127]. Linker histone (H1) is responsible for tight packaging of DNA [127]. Interestingly, histone H1 is absent in *Plasmodium* which leads to loose packaging of the parasite genome [128]. Most of the modifications which determine the genome organization occur on histones. Structurally histones have C terminal tail, a central domain and N terminal tail. Certain residues of both core domain and N terminal tail undergo different post translational modifications like acetylation, methylation, sumoylation, ubiquitination and ADP ribosylation [96]. Both location and type of modification is tightly regulated and has a crucial effect on transcription. These modifications can change the affinity of the DNA to histones. Those modifications which enhance the affinity of histones to DNA results in tight packaging of DNA forming heterochromatin and lead to gene silencing [96]. On the other hand modifications which result in decreasing affinity of histones to DNA leads to open chromatin also called euchromatin and promotes gene activation by making DNA accessible to transcription machinery. There are different enzymes which have been characterized to mark different histone modifications. Together these enzymes determine the "histone code" of the gene. Histone code is defined as the set of histone modifications which together facilitate the downstream events [129].

The DNA of *Plasmodium* like all eukaryotes is compacted into condensed structures of increasing order of chromatin organization. It is however made conspicuous by the absence of histone H1. The N-terminal tails of these histone proteins serve as substrates for a variety of post-translational modifications (PTMs) such as acetylation, methylation, phosphorylation, and ubiquitination [123, 126] (Figure 2.2). The modifications are catalyzed by a number of enzymes which often act in an opposing fashion to balance out the dynamic modification on the histones to regulate gene expression. *Plasmodium* has a significant pool of histone acetyltransferases (HATs)/deacetylases, histone methyltransferase/demethylases, and proteins that specifically recognize and target these PTMs along with a number of ATP dependent chromatin remodelers [130, 131]. Dynamic distributions of these post translation

modifications decide the chromatin structure which in turn regulates gene expression. Histone modifications are known to play an important role in the regulation of gene expression in *Plasmodium.* Most of the genes in *Plasmodium* are reported to be having both active and repressive marks (poised), which favours the plasticity of its gene expression status. H3K27me3, a global repressive mark in humans is reported to be absent in *Plasmodium*. On the other hand, H3K36me2 acts as a global repressive mark in the parasite. Moreover, virulence (var) genes that govern pathogenicity of the parasite are marked by a unique set of activation (H4ac) and repression (H3K9me3) marks, which are mutually exclusive from the ones occurring on the *Plasmodium* housekeeping genes [126]. Various histone modifiers are known to play an important role in mediating heterochromatization. Histone deacetylase HDA2 is known to mediate repression of var gene and sexual stage master regulator PfAP2-G [132]. Heterochromatin protein 1 (HP1) also play a crucial role in mediating repression through heterochromatization [133]. Other histone modifiers playing important role in var gene regulation includes PfSET2, which is known to mark H2K36me3 (mark for repression) [134]. Sirtuin family proteins, PfSIR2A and PfSIR2B are known to mediate var gene repression through removal of acetylation mark present on histones N terminal tails [135]. Histone modification which governs the expression of single var gene includes H3K9ac, H3K27ac, H3K4me3 and H4K8ac [126]. There are around 10 lysine methyltransferases known in *Plasmodium*. They contain characteristic catalytic methyltransferase SET domain. During intraerythrocytic life cycle of *Plasmodium* PfSET1, PfSET2, PfSET3 and PfSET8 enzymes are responsible to methylate at H3K4, H3K36, H3K9 and H4K20 respectively [131]. During sporozoite stage in salivary gland of mosquito and liver stage of parasite PfSET7 methylate H3K4 and H3K9 residues [136, 137]. Various other modifications of histones like H2A and H3B ubiquitination and H4 sumoylation are also identified in *P. falciparum* [138].

Along with the canonical histones, there are several unusual histone variants present in the *Plasmodium*. Its genome encodes 8 histone proteins H2A, H2B, H3, H4, H2A.z, H2Bv, H3.3 and cenH3 [103]. Mass spectrometry studies have identified several post translational modifications present on the histone variants like acetylation at H2A.ZK25, H2A.ZK30, H2BvK13, H2BvK18, H3K9, H3K14, H3K18, H3K27, H3.3K9 and methylations at H3K14, H4K8 and H3K12 [139]. These unique modifications help *Plasmodium* to organize and regulate expression of genes during its life cycle in human and mosquito.

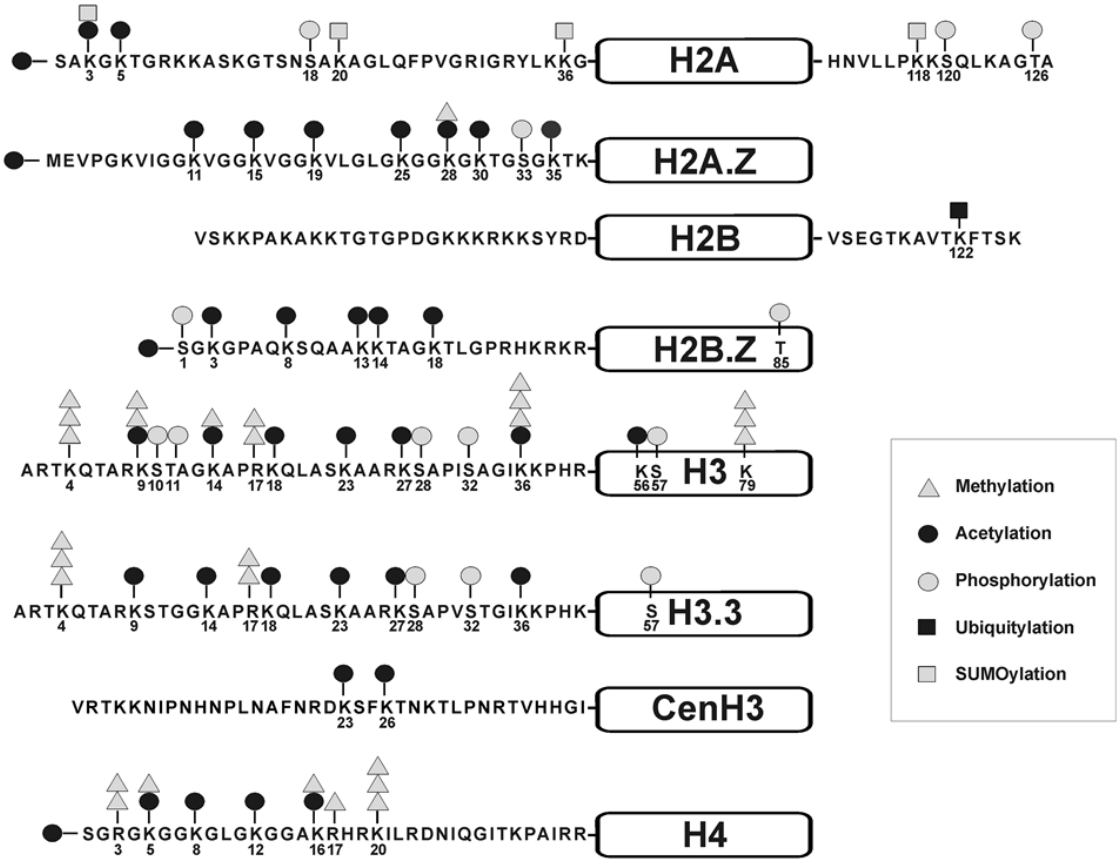


Fig. 1.5: Schematic showing the different histone modifications identified on the Plasmodium histones. *Adapted from Duffy et al., 2012[122].*

1.3.5 Histone acetyltransferase/Histone deacetylase

Reversible acetylation of histones by HATs and histone deacetylases (HDACs) is among the well-studied covalent histone modifications. These enzymes serve as the 'writers' and 'erasers' of acetyl marks on histone tails, and hence manipulate the histone code [140]. Histone acetylation is shown to play an important role in a plethora of processes in *P. falciparum*. Furthermore, in the light of recent advances in molecular medicine; HATs and HDACs have emerged as very potent therapeutic targets with a range of inhibitors being tested for their efficacy as anti-malarial agents [141].

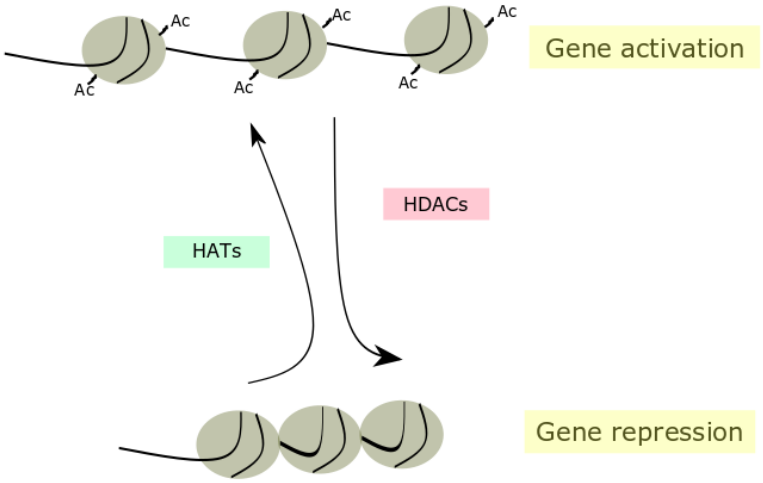


Fig. 1.6: Transcription regulation in eukaryotes through post transcriptional modifications of N terminal tail of histones. *These modifications play an important role in gene expression regulation. Acetylation of lysine residues present on N terminal tail of histone is mediated by histone acetyltransferases (HATs). It results in gene activation. Acetyl marks are erased by histone deacetylase (HDACs) resulting in gene repression.*

1.3.5.1 Histone acetyltransferases

Histone modification, acetylation occurs primarily at multiple lysine residues and is catalyzed by a set of enzymes called histone acetyltransferases (Figure 2.3). The yeast GCN5 (General Control Non-derepressible 5) was the first histone acetyltransferase (HAT) enzyme to link histone acetylation and transcriptional activation. Characterization of HATs on the basis of protein sequence and domain organization recognizes five major families of HATs; Gcn5-related N-acetyltransferases (GNATs), MYST, p300/CBP, nuclear receptor coactivator and other transcription associated families [130, 142]. Our genome-wide survey of HATs in *P. falciparum* identified ten putative HATs of which only PF08_0034 (GCN5) and PF11_0192 (MYST) have been experimentally demonstrated to have HAT activity [130].

GCN5 related N-acetyltransferase (GNAT) family is a superfamily of acetyltransferases that is known to acetylate a diverse set of proteins including histones and non-histone substrates [143]. This family includes a set of proteins which have been clustered together on the basis of acetylation related motifs. In higher organisms, this group includes GCN55, HAT1, ELP3, and HPA2 [143]. The GNAT proteins share a domain composed of four conserved sequence

motifs A-D. Among these motifs, motif A is involved in the recognition and binding of the Acetyl Coenzyme A substrate. Motif A is highly conserved and it is present in GNAT, MYST and p300/CBP family of HATs [143]. Motif C on the other hand is shared uniquely among the GCN5 family of HATs [143]. The GNAT members play a critical role in the regulation of transcription, DNA repair, cell growth and development [143].

Gene	Family	Gene ID	Maximum sequence similarity with	Percent identity
A. HATs				
PfMYST	MYST	PF3D7_1118600	*Homo sapiens* MYST	56.65
PfGcn5	GNAT	PF3D7_0823300	*Homo sapiens* GCN5	64.29
MAL8P1.200	GNAT	PF3D7_0805400	*Erythrobacter* pro_acteyltransferase	25.64
PF10_0036	GNAT	PF3D7_1003300	*Crocosphaera watsonii* proHAT	25.97
PF13_0131	GNAT	PF3D7_1323300	*Cytophaga hutchinsonii* proHAT	21.52
PF14_0350	GNAT	PF3D7_1437000	*Crocosphaera watsonii* proHAT	23.38
PFA0465c	GNAT	PF3D7_0109500	*Cytophaga hutchinsonii* proHAT	26.56
PFF1405c	GNAT	PF3D7_0629000	*Crocosphaera watsonii* proHAT	20.25
PFL1345c	GNAT	PF3D7_1227800	*Schwanella* pro HAT	25.76
PF10_0200	GNAT	PF3D7_1020700	*Homo sapiens* PCAF	14.29

Table 1.2: Histone acetyltransferases identified in P. falciparum and their closet homolog in other eukaryotic organisms. *Table reproduced from Kanyal et al. 2017 [130].*

GCN5 is a well-studied histone acetyltransferase (HAT) in higher eukaryotic systems. It is known to acetylate various histone lysine residues like H3K9, H3K14, H3K18, H3K27 and lysine residues of histone H4 and H2A. GCN5 belongs to GCN5- related N acetyltransferase (GNAT) superfamily [144]. The major functional domains of GCN5 include a highly conserved catalytic or HAT domain followed by a carboxy terminal bromodomain, which helps GCN5 binding on acetyl lysine residues [144]. Previous studies have demonstrated that yeast recombinant GCN5 acetylates free histones but fails to acetylate histones contained in nucleosomes. This emphasized the need for identification of other components required for acetylation of chromosome histones. Workman (1997) reported that the yeast GCN5 functions as a component of two major complexes, SAGA (Spt-Ada-Gcn5-Acetyltransferase) and ADA complex [145]. PfGCN5 was identified in *Plasmodium* by Fan *et al.* 2004 [146]. Furthermore, PfGCN5 was found to acetylate histone H3 at K9 and K14 residues [146]. PfADA2 was found to be interacting partner of PfGCN5 in further studies [147]. PfGCN5 contain bromodomain along with HAT domain at the C terminal end of the protein. Therefore it functions as both writer and reader in *Plasmodium* [130]. Other putative members of the GNAT family of acetyltransferases are mentioned in Table 1.2.

PfMYST is the only gene in the MYST family gene in *P. falciparum*. It contain highly conserved MYST domain, chromo domain as well as C2H2- type zing finger domain [130].

Plasmodium is known to have two isoforms of PfMYST, long (~ 72 kDa) and short (~ 37 kDa) [148]. PfMYST is known to acetylate histone H4K5, H4K8, H4K12 [148]. It play important role in cell cycle progression, transcriptional regulation and DNA damage repair [148].

1.3.5.2 Histone deacetylase

Histone deacetylases are broadly classified into four classes on the basis of their homology with the yeast HDACs [130]. Class I HDACs are known to have nuclear localisation. They utilizes Zn^{2+} ion as cofactor for their HDAC activity. *Plasmodium* HDAC1 belongs to class I HDACs. They are known to play an important role in schizogony, hepatocyte invasion and gametocytogenesis [149]. Class II HDACs are known to shuttle between nucleus and cytoplasm. Hence they are also involved in deacetylation of non-histone proteins. *Plasmodium* HDA1 and HDA2 belong to this class. HDA2 is known to play an important role in antigenic variation and gametogenesis [132]. Class III HDACs are homologous with the yeast Sir2 family of proteins. Sirtuins require NAD+ as cofactor for their activation. They also have nucleo-cytoplasmic distribution. PfSir2A and PfSir2B belong to this class. PfSir2A is known to localize at telomeres and regulate their length. They are involved in maintenance and heterochromatization in *P falciparum*. Both Sir2A and Sir2B are also known to regulate var gene expression [135]. Class IV HDACs have homology with both class I and class II HDACs. *Plasmodium* doesn't have any HDAC belonging to this category [130].

Summary: Malaria is one of the most infectious parasitic diseases responsible for several thousand deaths world-wide each year. *Plasmodium* has exhibited excessive ability to generate resistances against wide range of antimalarials. Currently, artemisinin based combination therapy is considered as the last line of defense available against *P. falciparum*. However, several reports of reduced sensitivity against artemisinin drug are reported from different parts of the world. Unfortunately, the global transcriptional regulators of the key players involved in drug-resistance remain unexplored. Understanding how *Plasmodium* regulates gene expression during drug-resistance generation is fundamental for understanding the mechanism of drug-resistance which can be targeted for future interventions. *Plasmodium,* like other eukaryotes regulate transcription at several levels through different mechanisms. One of the mechanisms of gene activation for transcription is through acetylation of histone tails through epigenetic writer histone acetyltransferases. This thesis

focuses in investigating transcriptional regulation in *Plasmodium* via histone acetyltransferase PfGCN5.

Chapter 2: Investigating the stress mediated transcriptional regulation by PfGCN5 during different stress conditions

2.1 Introduction

The gene PF08_0034 (PfGCN5) was identified as a homolog of yeast GCN5 in *P. falciparum* [146]. PfGCN5 has a catalytic GNAT and a bromo-domain. The N terminal region of PfGCN5 is longer than GCN5 in any other organism and bears little similarity with them [146]. It is postulated to be involved in protein-protein interaction and recognition of nucleosomal substrates [150]. PfGCN5 is constitutively expressed at the asexual stages and transcription can be triggered from multiple transcription start sites [146]. Recombinant PfGCN5 possess conserved *in vitro* HAT activity with preference to H3 histone at K9 and K14 and to certain extent towards histone H4 [146]. Presence of PfADA, homologs of which are also present in other organisms, indicates minimal functional SAGA complex in *P. falciparum* [147]. In yeast, GCN5 and ADA proteins are part of the SAGA complex, which plays an important role in regulation of stress-induced genes [151].

ChIP-on-chip analysis indicates a weak yet positive correlation between PfGCN5, H3K9ac mark and gene expression [152]. PfGCN5 is found to be enriched at all the chromosomes and it is associated with processes such as catalytic activity, transcription/translation, enzyme regulation, signaling and transport. Interestingly, the largest subset of genes targeted by PfGCN5 still does not have any documented functions. PfGCN5 has been also implicated to be expressed in response to DNA damage and repair and is also significantly upregulated in chloroquine drug-resistant Pfcrt mutants [152].

2.1.1 Role of GCN5 in stress response in other eukaryotic system

2.1.1.1 Yeast

Previous studies in higher eukaryotes demonstrates that GCN5 plays an important role during stress conditions where it has been associated with high level of transcriptional reprogramming required for stress adaptation [151, 153]. Studies have shown that GCN5 is required for the adaptation of *S. pombe* to KCl and CaCl2 mediated stress [151]. GCN5 was found to be associated and involved in the regulation of a subset of stress response genes

activated during KCl stress [151]. Further studies showed that despite of regulating common group of stress responsive genes in both *S. cerevisiae* and *S. pompe*, GCN5 also regulates few genes unique to both strain of yeast [151]. Genome wide localization studies suggested the relocalisation of GCN5 from short genes to the transcribed region of long genes in *S. cerevisiae*. Such redistribution of GCN5 is absent in *S. pombe* during KCl stress conditions [154]. Wright et al (2013) have shown two functionally distinct roles of GCN5 in yeast. Firstly, GCN5 re-localizes to the ORF of long genes during stress conditions. Secondly, it interacts with histones close to the transcriptional start site of various stress-induced genes [154].

2.1.1.2 *Toxoplasma*

Stress induced developmental changes plays an important role in the pathogenesis of the parasite *Toxoplasma*. Alkaline stress is known to induce the conversion of rapidly growing form (tachyzoite) to dormant cyst (bradyzoite) stage of the parasite. *Toxoplasma* is known to possess two GCN5 HATs, TgGCN5-A and B [155]. Knockdown of TgGCN5-A was not lethal under normal culture conditions [156]. However, during alkaline stress condition, TgGCN5-A knockout parasites failed to upregulate bradyzoite specific genes and these parasites are not successful in recovering from the alkaline stress [156]. This observation led to further investigation of TgGCN5-A role in developmental reprogramming during alkaline stress. Transcriptomic studies during alkaline stress suggested that stress response genes are not upregulated in TgGCN5-A knockout condition. Furthermore, chromatin immunoprecipitation studies showed that TgGCN5-A is enriched at the promoter of the genes which were found to be upregulated during the alkaline stress suggesting the role of TgGCN5-A in mediating activation of the genes crucial for the survival and inducing the bradyzoite stage development [156].

2.1.1.3 Plants

Plants are regularly exposed to different types of environmental conditions. One of the most common stresses which plants are exposed to is heat stress. Studies have shown that exposure of plants to temperature above optimum levels can drastically affect the growth and development of the plant. Studies have shown that GCN5 plays an important role in mediating thermo tolerance by inducing heat responsive genes [157]. Chromatin immunoprecipitation studies have shown that GCN5 is enriched over the promoter of heat

shock (HS) transcription factors like HSFA3 and UVH6 [157]. Another study has investigated the role of GCN5 in maintaining cell wall integrity and salt stress tolerance. Mutation in the GCN5 resulted in the cell wall defects and growth inhibition during salt stress [158]. AREB1-ADA2b-GCN5 complex is known to regulate expression of genes important for overcoming unfavourable condition like draught stress [159]. In *Brachypodium distachyon*, GCN5 is known to regulate acetylate temperature stress responsive genes [160]. Interestingly, *B. distachyon* has two splice variants of GCN5 which are known to be induced transcriptionally during stress conditions [160].

2.1.1.4 Humans

Gaupel et al showed that deletion of GCN5 or any of the SAGA complex components makes mammalian cells lies sensitive to the inhibitor responsible for inducing oxidative stress in human cell lines [161]. These *in vitro* experiments assayed the role of GCN5 and SAGA complex in inducing oxidative stress response in cells [161]. Since GCN5 has been known to be involved in dealing with stress conditions from higher eukaryotes like humans to lower eukaryotes like *Toxoplasma*, it is possible that it also helps *Plasmodium* in adapting to stress environments during its intra-erythrocytic cycle.

2.1.2 Different stress conditions during intraerythrocytic like cycle of *Plasmodium*

Plasmodium completes its asexual life cycle in erythrocytes. While being harbored within the RBCs, it is exposed to different kinds of environmental and physiological stresses i.e. oxidative, temperature and nutrient stress as discussed below.

2.1.2.1 Oxidative stress

The parasite employs very slow metabolism during the initial ring stage of infection. Twenty four hours post invasion, *Plasmodium* enters into the trophozoite stage where it begins uptake and digestion of haemoglobin from the erythrocyte cytoplasm. The conversion of hemoglobin to hemozoin accumulates reactive oxygen species (ROS) in parasites which results in oxidative stress [162-164]. Various drugs like artemisinin, arteether etc. used for antimalarial treatment also result in similar ROS build up [165]. High level of reactive oxygen species results in parasite death. ROS are also released by different innate immune cells like neutrophil and macrophages against infected RBCs. Recent study demonstrated the role of Vitamin E as antioxidant in *Plasmodium* [166]. Vitamin E is considered as a potent

antioxidant which prevents lipid peroxidation of polyunsaturated fatty acid. Use of Vitamin E biosynthesis inhibitor usnic acid leads to higher ROS level in the parasites whereas adding exogenous Vitamin E to the parasite results in decreased ROS level [166].

2.1.2.2 Temperature stress

Another characteristic feature of malarial infection is the acute cyclical episode of fever which is triggered by release of merozoites from RBCs. Fever occurs every 48hrs in *Plasmodium falciparum* infection which coincides with the intra-erythrocytic cycle of *Plasmodium* and the temperatures increases to 41 degree for about 2-6 hours [167]. It has been reported that growth of *Plasmodium* is inhibited when grown at 40^0C for 6 hrs *in vitro* [168]. Late stages of parasites like trophozoite and schizont are more susceptible to heat stress than early stages [167-169]. Since *Plasmodium* faces the same set of stress conditions during each of its infectious cycles, it is possible that it has evolved mechanisms to resist the metabolic perturbations caused on account of stress.

2.1.2.3 Nutrient stress

Plasmodium obtains most of the nutrients from the host for their growth and development like glucose, amino acids, purines etc. Any change in the level of these nutrients can result in the stress condition within the parasites. Malarial infection results in hypoglycaemia in patients where the glucose level decreases to 50 mg/dl. *Plasmodium* lacks TCA cycle during its intra-erythrocytic growth phase, thus the parasite entirely depends on glycolysis for energy [170]. The parasite largely relies on the host for its high glucose demand, owing to which it undergoes nutrient stress when the glucose levels in the infected host drops [171]. Factors which results in nutrient stress have been discussed in section 4.1.3 in detail.

2.2 Material and Methods

2.2.1 *Plasmodium in vitro* culture

P. falciparum strain 3D7 was cultured as previously described. Briefly, parasites were cultured in RPMI1640 medium supplemented with 25 mM HEPES, 0.5 % AlbuMAX I, 1.77 mM sodium bicarbonate, 100 μM hypoxanthine and 12.5 μg ml^{-1} gentamicin sulfate at 37 °C. Parasites were sub-cultured after every two days. Subculturing was done by splitting the flask into multiple flasks in order to maintain parasitemia around 5%. Hematocrit was maintained to 1 -1.5% by adding freshly washed O +ve human RBC isolated from healthy human donor.

Synchronization was done with the help of 5% sorbitol at ring stage. Late stage synchronization was performed using the Percoll density gradient method (63%) [172]. Parasitemia was monitored using Giemsa staining of thin blood smear.

2.2.2 Freezing of parasites

Parasites were frozen according to the protocol mentioned in Radfar et al., 2009 [172]. This protocol was used to freeze early ring stage parasites in liquid nitrogen. *Plasmodium* culture was centrifuged followed by washing with 5 ml of incomplete media. Freezing solution (0.0065% of NaCl, 0.0302% of sorbitol and 0.28% of glycerine) was added to the equal volume of the culture [172]. Freezing solution was added drop wise to the pellet and mixed properly before aliquoting 500 µl of the mix in each cryovial. Cryovial were immediately stored in liquid nitrogen [172].

2.2.3 Thawing of the *Plasmodium* culture

Parasites were thawed using two reviving solution, A and B. Frozen pellet was thawed for 1 minute in water bath at 37^0C. Solution A (12% NaCl solution) was added drop wise to the *Plasmodium* pellet [172]. Solution A was added $1/10^{th}$ volume of frozen pellet. It was kept for 5 minutes at room temperature. After the incubation was over Solution B (10 volumes of parasite pellet) was added to the mix and then centrifuged again [172]. Parasites were washed using incomplete media and kept back in culture after adding fresh RBCs to maintain hematocrit [172].

2.2.4 Transfection

Plasmodium culture was synchronized in the earlier cycle of infection and grown in fresh RBCs. Culture was washed once with incomplete media followed by washing with complete cytomix (1X). Culture was then washed with cytomix and supernatant was discarded. Infected RBC (100 µl, ~8% parasitemia) were mixed with 270 µl cytomix and 60 µg plasmid dissolved in 30 µl cytomix. 400 µl of the mix was added to the cuvettes (Biorad, 0.2 cm). Infected RBC's were then electroporated using Biorad Gene Pulser. After electroporation 1 ml of warm RPMI was added to the cuvettes. Whole content of cuvette was then removed and transferred into 25 cm^2 flask containing 7 ml pre-warmed media. Culture was kept at the normal growth conditions in the incubator. Parasites were washed after 24 hours in order to get rid of the dead RBCs followed with drug treatment for the selection of positive parasites.

Full length and truncated (containing HAT and bromodomain) PfGCN5 were cloned in pARL1a overexpression vector using Kpn1 and AvrII restriction sites under PfCRT promoter. Transfection was performed as mentioned above in the protocol followed by stress experiment after 48 hours. Images of PfGCN5_GFP overexpression were captured using Zeiss confocal microscope LSM710.

2.2.5 Antibodies

Anti-actin (Sigma A2066) and Anti-Rabbit IgG (OSB PM035) were used for Western blotting and immunoprecipitation, respectively. Goat Anti-Rabbit Alexa Fluor 647 (A21245), Goat anti-Rat Alexa Fluor 488 (A 11006), Goat Anti-Rabbit Alexa Fluor 488 (A11034) were used for immunofluorescence. Rabbit polyclonal antibodies against PfAlba3 resulting from immunizations of rabbits with the KLH-conjugate peptide IGKRMFTGNEEKNP were obtained from GenScript Corporation. Rat polyclonal antibodies against full-length recombinant GST-tagged PfAlba3 were from GenScript Corporation. For generating PfGCN5 peptide antibody, online software LBtope was used for selecting the antigenic peptide. PfGCN5 peptide (CEYCNVLYDGNELLRKRK) used for raising antibody was obtained from Apeptide Co., Ltd., China. PfGCN5 peptide was conjugated to Keyhole limpet hemocyanin (KLH) carrier protein for the immunization purpose. Both Anti-GCN5 peptide and protein antibodies were raised at The National Facility for Gene Function in Health and Disease, IISER Pune. The New Zealand White rabbits (3-4 months old) were used for antibody generation. Antibodies were further purified using affinity chromatography on the sulfolink resin.

2.2.6 Western blotting

Parasites were harvested using 0.15% saponin. Parasites pellets were washed using phosphate buffer saline (PBS). Parasites were lysed using ice cold parasite lysis buffer (Tris-Cl pH 8.0, 150 mM sodium chloride (NaCl), 0.5% nonyl phenoxypolyethoxylethanol (NP-40), 0.5% sodium deoxycholate, 0.1 mM ethylenediaminetetraacetic acid, 1.5 mM magnesium chloride ($MgCl_2$), 1X protease inhibitor cocktail (PIC) , 1 mM phenylmethylsulfonyl fluoride (PMSF). Three freeze thaw cycles were performed using liquid nitrogen to achieve proper lysis of the parasites. To get rid of debris, parasites were spun at 17949 x g for 30 minutes. Supernatant was transferred to another tube. The lysate proteins were separated on 7.5% - 12% polyacrylamide gels and transferred to PVDF membrane. The membrane was blocked using

5% skimmed milk and probed using primary antibody overnight at 4^0C. After overnight incubation membrane were washed using 1X Tris-buffered saline, 0.1% Tween 20 (TBST) followed by 1hr incubation with secondary antibody in TBST (1:5000, Biorad). Three washes were given for 10 minutes each after the secondary antibody incubation. Blots were developed using Clarity Western ECL substrate (Biorad).

2.2.7 Immunofluorescence assay

Parasites were fixed using 4% PFA and 0.00075% glutaraldehyde for 30 minutes at 37^0C. Permeabilisation was carried out using 0.1% Triton X-100 in PBS. Washing was performed using 1X PBS after every step. Blocking was done using 3% BSA for 1hr at room temperature followed by incubation with primary antibody in BSA for 3 hours. Three PBS washes were given to remove the unbound primary antibody. Secondary antibody incubation was done for 1 hour at room temperature. Parasites were washed before mounting on glass slides using ProLong Gold Antifade with DAPI (Invitrogen).

2.2.8 RNA isolation

Parasite pellets were homogenized in 1 ml TRIzol (Biorad) reagent properly. Add 0.2 volume of chloroform to the TRIzol. Tubes were properly mixed and kept at room temperature for 10 minutes. Samples were centrifuged at 13,000 RPM for 12 minutes at 4^0C. Upper aqueous phase was collected in a different tube. Isopropanol (0.8 volumes) was added to the tubes and properly mixed. Samples were incubated for 15-20 minutes at room temperature for precipitation followed by centrifugation at 13,000 RPM for 12 minutes at 4^0C. Supernatant was removed and pellet was washed once with 75% ethanol. Pellet was air dried before resuspending in nuclease free water. RNA quantity and quality was estimated using Nanodrop or Qubit reading.

2.2.9 Quantitative RT-PCR

RNA isolation was carried out using TRIzol reagent (Biorad). 2 µg of DNAse free RNA was used for cDNA synthesis using ImProm-II Reverse transcription system (Promega), as per the manufacture's recommendation. Random primers were used for the cDNA synthesis. Real time PCR was carried out using CFX96 Real Time PCR detection system (Biorad). 18S rRNA and tRNA synthetase were used as internal controls to normalize for variability across different samples. Quantification of the expression was done with the help of fluorescence

readout of SYBR green dye incorporation into the amplifying targets (Biorad). Each experiment included technical triplicates and was performed over three independent biological replicates.

2.2.10 Chromatin immunoprecipitation

Infected RBCs were crosslinked using 1% formaldehyde (Thermo Scientific, 28908) for 10 mins at RT. 150 mM glycine was added for quenching the cross-linking reaction. The samples were washed using 1X PBS (chilled) before proceeding with lysis. Sample homogenization was performed using swelling buffer (25 mM Tris-Cl pH 7.9, 1.5 mM MgCl2, 10 mM KCL, 0.1% NP40, 1 mM DTT, 0.5 mM PMSF, 1x PIC) followed by cell lysis in sonication buffer (10 mM Tris-Cl pH 7.5, 200 mM NaCl, 1 % SDS, 4 % NP-40,1mM PMSF, 1X PIC). Sonication was performed using Covaris S220 to obtain the chromatin size of 200-400 bp. Pre-clearing was performed for 1 hour at 4^0C using recombinant protein G conjugated sepharose beads with continuous gentle inverting. 30 µg purified chromatin was used per antibody (both α-HAT and α-peptide antibodies) and incubated for 12 h at 4^0C. Samples were then incubated with saturated Protein G Sepharose beads for 4 hours at 4^0C. Bound chromatin was finally washed and eluted using ChIP elution buffer (1 % SDS, 0.1 M sodium bicarbonate). Both IP sample and input were reverse crosslinked using 0.3 M NaCl overnight at 65^0C along with RNAse. Proteinase K treatment was performed at 42^0C for 1 hour. Finally DNA was purified using phenol chloroform precipitation. Target sites identified from ChIP sequencing analysis were further validated by ChIP-qPCR using the Biorad SYBR Green Master Mix (Biorad). Gene ontology was performed using PlasmoDB (www. plasmodb.org) [173].

2.2.11 ChIP-sequencing library preparation and sequencing

ChIP-sequencing libraries for all the samples were prepared from 5-10 ng of DNA using the NEB Next Ultra II DNA Library Prep kit. Chromatin immunoprecipitated, fragmented DNA samples were end repaired and adapters ligated. Size selection was performed using Agencourt XP beads (Beckman Coulter). Adapter ligated fragments were PCR amplified using indexing primers followed by purification using the Agencourt XP beads (Beckman Coulter). The library electropherograms were assessed using Agilent Bioanalyzer 2100 and Agilent DNA 1000 kit. The libraries were pooled in equimolar concentration and 50 bp reads

were sequenced using Illumina HiSeq2500 (BENCOS Research Solutions Pvt. Ltd., Maharashtra).

2.2.12 Data pre-processing and peak calling

ChIP-seq data were mapped to *Plasmodium falciparum 3D7* genome version 37 (http://plasmodb.org/plasmo/) using Bowtie2 with default parameters. The mapped reads were used for peak calling against an input control data, using the MACS2 peak calling software (default parameters) [174]. Peaks were annotated using Bedtools [175]. ChIP-seq signals were background subtracted using MACS2 bdgcmp tool and the significantly enriched peaks were visualized using Integrative Genomics Viewer (IGV) [176].

2.2.13 Average profile calculations

We extracted the tag density in a 5 kb window surrounding the gene body using the seqMINER tool which generates heatmap as well as the enrichment profiles of factors over gene bodies [177]. For average gene profiles, genes (+/-5000 bp from binding site) were divided in 100 bins relative to the gene length. Moreover 10 equally sized (50 bp) bins were created on the 5' and 3' of the gene and ChIP-seq densities were collected for each dataset in each bin.

2.2.14 Data source and analysis

ChIP seq data for Heterochromatin protein 1 (HP1) trophozoite stage was downloaded from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/gds) with accession number: GSM2743113. Histone modification ChIP-seq data were downloaded from database under the accession number GSE63369. SeqMINER was used for generating scatter plots and average gene occupancy profiles. Correlation analysis and box plot were generated using 'R' software (http://r-project.org/).

2.2.15 Stress induction

Parasites were subjected to heat and therapeutic (artemisinin treatment) stresses for 6 hours from late ring (~17 hrs) to early trophozoite (~23 hrs) stage. Double synchronization was carried out to achieve tight synchronization of parasite stages. Parasites were exposed to a) Heat stress (40^0C for 6 hours) and b) Therapeutic stress (30 nM artemisinin for 6 hours).

2.2.16 RNA sequencing and data analysis

Parasites were harvested for RNA isolation after 6 hours of stress induction. Total RNA was isolated using TRIzol reagent according to the protocol. DNAse treated RNA was used for cDNA synthesis. Quality of the RNA was verified using Agilent Bioanalyzer 2100. Three biological replicates were pooled together for performing RNA sequencing. The cDNA libraries were prepared for samples using Illumina TruSeq RNA library preparation kit. Transcriptome sequencing was performed using Illumina NextSeq 500 system (1x150 bp read length) at BioServe Biotechnologies (India) Pvt. Ltd. Hyderabad in replicate. Quality control of the RNA-sequencing reads was performed using FASTQC and reads were trimmed based on the quality estimates. The quality verified reads were then mapped onto the reference genome (PlasmoDB_v37) using the HISAT2 software (New Tuxedo Suite) [178]. After verification of the mapping percentage, the alignment data (SAM format) was converted into its binary counterpart (BAM format) using samtools. The same step also sorts the aligned reads positionally according to their genomic coordinates, making them easier to process further. In order to quantify the reads mapped onto the genomic features (genes, exons, etc.), the htseq-count feature was used. The count data was then used to perform differential gene expression (DGE) analysis and statistical validation using the Deseq2 package in the R computational environment [179]. MA plot is generated using 'R' software (http://r-project.org/).

2.2.17 Saponin lysis of the infected RBCs

Parasites were harvested from the infected RBC using saponin lysis. *Plasmodium* culture (10 ml) was centrifuged followed by PBS washes. Saponin (4ml, 0.15%) was added to the *Plasmodium* culture along with 3 ml of 1x PBS. Parasite pellet was vortexed to mix the pellet followed by incubation at 37^0C for 15 minutes. After the incubation parasites were centrifuged at 6000 RPM for 15 minutes at 4^0C. This was followed by 1X PBS washes to get rid of the lysed RBC. Parasites pellets were stored at -80^0C immediately after the washes.

2.2.18 Immunoprecipitation

In order to harvest the parasites, infected RBCs were lysed using 0.15% saponin at 37^0C. Harvested parasites were then lysed using ice cold parasite lysis buffer (20 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 0.1 mM EDTA, 1.5 mM

$MgCl_2$, 1X PIC, 1 mM PMSF). Lysed parasites were then centrifuged at 20817 x g for 30min at 4^0C. Pre-clearing was performed using recombinant protein G conjugated sepharose beads for 1 hour at 4^0C. Precleared lysate was then used for overnight incubation with antibody at 4^0C. After the overnight incubation of lysate with antibody, sepharose Protein G beads were added to the lysate for 4 hours incubation. Washes were done using immunoprecipitation buffer (25 mM TRIS pH 7.9, 5 mM $MgCl_2$, 10% glycerol, 100 mM KCl, 0.1% NP-40, 0.3 mM DTT) followed by elution of the proteins using glycine (pH – 2.5). Eluted proteins were neutralized using 1 M Tris pH 8.8. For mass spectrometry analysis samples were digested with trypsin for 16 hrs at 37^0C. The digested samples were cleared using C18 silica cartridge. Peptides were then analyzed using EASY-nLC 1000 system (Thermo Fisher Scientific) coupled to QExactive mass spectrometer (Thermo Fisher Scientific) equipped with nanoelectrospray ion source (Valerian Chem Private Limited, New Delhi). Immunoprecipitation followed by mass spectrometry was performed in three biological replicates. Samples were processed and RAW files were generated. Files were then analyzed with Proteome Discoverer against the Uniprot *P. falciparum* reference proteome database. For Sequest search, the precursor and fragment mass tolerances were set at 10 ppm and 0.5 Da, respectively. The protease used to generate peptides, i.e. enzyme specificity was set for trypsin/P (cleavage at the C terminus of "K/R: unless followed by "P") along with maximum missed cleavages value of two. 0.001 FDR was set for both peptide spectrum match and protein false discovery rate.

2.2.19 Protein expression and purification

PfGCN5 (HAT and bromodomain) DNA sequence was amplified from parasite genomic DNA using gene specific primers. The PCR-amplified fragment was cloned in frame with glutathione S-transferase (GST) fusion protein in pGEX-4T1 plasmid vector using XhoI and BamHI restriction enzymes. For expression in *E.coli*, pGEX-4T1 (GCN5) plasmid was transformed in BL21 (DE3) star competent cells. Expression was induced at an optical density of 0.6 at 600 nm, with 0.5 mM isopropyl-1-thio-β-d-galactopyranoside (IPTG) for 5 hrs at 25^0C. Protein was purified using glutathione sepharose 4B beads (GE healthcare life science). 20 mM concentration of reduced glutathione was used for protein elution. PfAlba3 was cloned in $pET28a^+$ vector using NdeI and XhoI. Histidine-tagged PfAlba3 was expressed in the *E. coli* BL21 (DE3) competent cells. Expression was induced at an optical density of 0.6 at 600nm, with 0.5 mM IPTG for 5 hours at 25^0C. Bacterial pellet was resuspended in

sonication buffer (10 mM Tris-Cl pH 8.0, 150 mM NaCl, 10 % Glycerol, 1X PIC and 1X PMSF) followed by sonication using Probe Sonicator (Thomas Scientific, 70% Amplitude, 10 minutes, 02 Sec On/ 06 Sec off). After the sonication lysate was centrifuged at high speed 14,000 RPM for 30 minutes at 4^0C. Supernatant was stored at -80^0C and pellet was resuspended in 8 M Urea prepared in sonication buffer. Pellet was kept in shaking condition for 1 hour at room temperature followed by centrifugation. Earlier supernatant as well as pellet supernatant were run on SDS PAGE gel along with uninduced bacterial lysate to check the induction of protein expression. Protein expression was also confirmed using Western blotting (Anti-His antibody). Once the expression was confirmed protein purification was performed using Ni-NTA beads. Protein lysate was kept in incubation with Ni-NTA beads for 4 hours at 4^0C. This was followed by washing of the protein bound to beads using low concentration of Imidazole (20 mM) in sonication buffer. Protein was eluted using different concentration of Imidazole (50 mM, 150 mM and 300 mM). Purification of protein was checked using SDS PAGE. Purified proteins were dialyzed and stored at -20^0C.

2.2.20 *In vitro* interaction assay

In vitro interaction study was carried out using GST-tagged PfGCN5 and His tagged PfAlba3 proteins. Recombinant proteins (2 μg) were incubated together overnight at 4^0C. GST protein was used as the negative control. Glutathione beads were added to the protein mix for 4 hours at 4^0C. The beads were washed and the bound proteins were eluted from the beads using 20 mM reduced glutathione. Western blotting was performed using Anti-His antibody to verify presence of PfAlba3 in the elutions.

2.2.21 Acetyltransferase assay

Acetyltransferase assay was performed using PfGCN5 complex pulled down using PfGCN5 specific antibodies. Eluted PfGCN5 complex was incubated with recombinant PfAlba3 along in acetyltransferases buffer (250 mM Tris-Cl pH 8, 50% glycerol, 0.5 mM EDTA, 5mM DTT, 250 mM KCl, 100 mM Na-Butyrate, 1X PIC) freshly supplemented with 1mM acetyl CoA. It was incubated at 30^0C for 1 hour. Proper controls were kept to check the basal level of recombinant PfAlba3 acetylation which included only acetyl-coA and PfAlba3 without PfGCN5 complex, PfGCN5 complex with acetyl-coA, PfAlba3 with acetyl-coA. Western blotting was performed to check the level of acetylation using pan-acetyl antibody (ab22550).

2.3 Results

2.3.1 Generation of polyclonal antibodies against PfGCN5

While paralogs of GCN5 are well studied in multiple systems, little is known about the function of GCN5 in *P. falciparum*. PfGCN5, encoded by *PF3D7_0823300,* contains histone acetyltransferase (HAT) and bromo (for binding to acetylated histones) domains at its C-terminal end (Figure 2.1).

Fig. 2.1: Schematic diagram showing the domain organization of PfGCN5. *Histone acetyltransferase (HAT) domain and bromodomain (represented in blue and orange color, respectively) are present at C terminal end. PfGCN5 peptide from N-terminal region of the protein was commercially synthesized for raising antibody. The HAT and bromodomain was cloned in pGEX 4T1 vector for expression of PfGCN5 protein tagged with GST. Protein expression was induced using 0.5mM IPTG in BL21 (DE3) star competent cells.*

To gain further insight into the function of PfGCN5 during asexual growth, we generated polyclonal antibodies against recombinant C-terminal HAT and bromo domains (amino acid 1183-1448) of PfGCN5 (also called α-HAT antibody, Figure 2.1 and Figure 2.2A). We also raised polyclonal antibodies against a peptide from the N-terminal region of PfGCN5 (amino acids 9-25; called α-peptide antibody; Figure 2.1). Antiserum raised against protein was tested against bacterially expressed PfGCN5 protein to check specificity (Figure 2.2B). Specificity of the generated antibodies was determined by Western blotting using parasite lysate (Figure 2.2C), and by immunoprecipitation coupled with mass spectroscopy.

Interestingly, we detected multiple forms of PfGCN5 in parasite lysate by western blotting with the α-HAT antibody: a band migrating at 190 kDa and two lower molecular weight (LMW) bands at approximately 72 and 65 kDa (Figure 2.2C). A slightly different pattern was observed with the α-peptide antibody: in addition to a higher molecular weight 124 kDa band and a 80 kDa band, a 43 kDa band was also observed (Figure 2.2D). These multiple bands observed in the Western blotting are the proteolytic cleaved bands as discussed in Bhowmick et al [180]. Further, we performed immunoprecipitation using α-HAT PfGCN5 antibody and subjected the eluted product to Western blotting with α-peptide PfGCN5 antibody. We could clearly detect the fain band of full length PfGCN5 with α-peptide PfGCN5 antibody (Figure 2.2D). Possibly, we need much larger amount of PfGCN5 to be detected with anti-peptide antibody as the numbers of antigens present in a peptide are expected to be much smaller than the α-HAT PfGCN5 protein. Furthermore, we confirmed the specificity of peptide antibody by Western blotting of PfGCN5 tagged with GFP overexpression (Figure 2.2D).

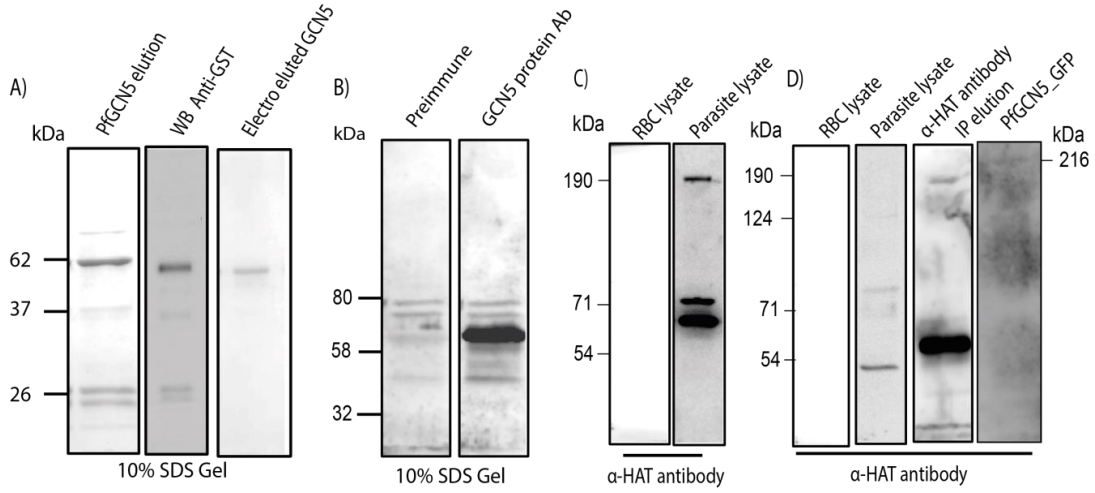


Fig. 2.2: PfGCN5 specific antibody generation. *A) Protein purification of GST tagged PfGCN5_HAT protein. Protein was purified using glutathione beads and eluted using reduced glutathione (20mM). Protein expression was confirmed using anti-GST Western blotting. Protein was further purified using electro elution before injected in rabbit for Anti-PfGCN5 antibody generation. Single band protein (PfGCN5-GST) was observed after electro elution in SDS-PAGE. B) Specificity of α-HAT antibody against bacterial lysate expressing PfGCN5 HAT and bromodomain. Antiserum raised against PfGCN5 protein (HAT and bromodomain) was checked for specificity using bacterial lysate expressing PfGCN5.Western blotting assessing the specificity of raised antibodies against parasite lysate. Specificity of the antibody C) α-HAT and D) α-peptide was further checked using parasite protein lysate from*

asynchronous culture. Western blotting result indicates presence of more than one forms of PfGCN5 in Plasmodium. Western blotting was performed on proteins which are pulled down by α-HAT antibody and probed with α-peptide antibody. The presence of full length band indicates that both antibodies detect full length PfGCN5. α-peptide antibody specificity was also assessed using overexpression of PfGCN5-GFP in parasites.

Differential profile observed between two different PfGCN5 antibodies can be explained through schematic shown in Figure 2.3A. In order to determine whether the multiple forms of PfGCN5 detected by Western blotting are degradation products, post-translationally modified forms, or transcriptional isoforms, we treated the parasites with proteosomal inhibitor MG132 and analyzed PfGCN5 profiles in parasite lysate at various stages of asexual growth. Interestingly, two forms of PfGCN5 were consistently observed with the α-HAT antibodies upon proteosomal inhibition, the 190 kDa and 70 kDa bands (Figure 2.3B).

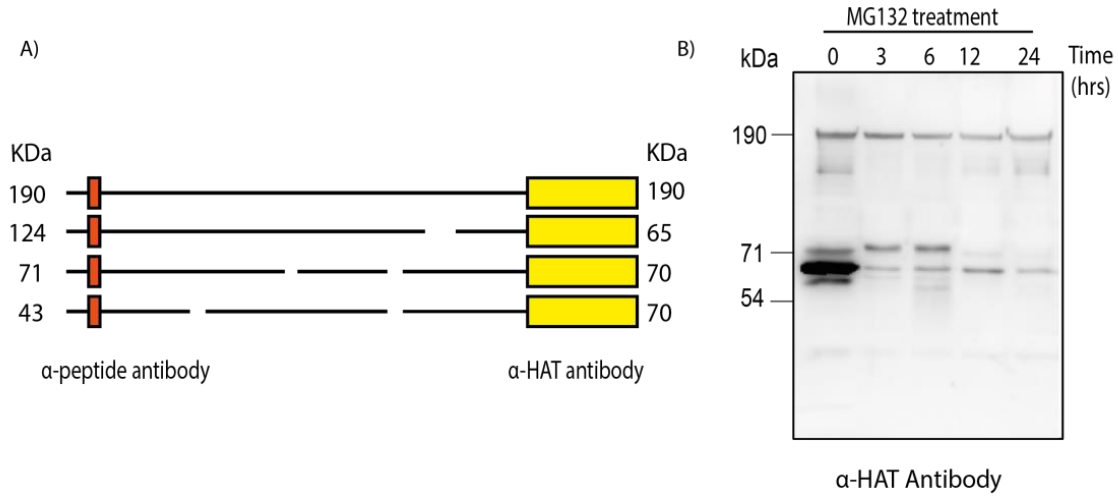


Fig. 2.3: Proteosomal degradation of PfGCN5 protein *A) Schematic to possibly explain the bands which are observed during Western blotting for both the antibodies generated against PfGCN5. B) In order to check whether the extra bands observed in Western blotting are the result of possible proteosomal degradation, parasites were treated with MG132 inhibitor for different duration of time. MG132 treatment resulted in significant decrease in number of bands observed. Only two bands were observed after 24 hours treatment of MG132 which might indicate two isoforms of PfGCN5.*

2.3.2 PfGCN5 is associated with virulence and stress responsive genes

Next, to comprehend the transcriptional regulation mechanisms of PfGCN5, we performed chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) using the α-HAT and α-peptide PfGCN5 antibodies. ChIP-seq was performed at early trophozoite stage (24 hpi) of parasite growth as PfGCN5 exhibits high mRNA expression at this stage (Figure 2.4A). Peaks of local enrichment of PfGCN5 were determined after sequence alignment and normalization to input sequences using the MACS2 peak calling software. In total, we identified 754 high confidence common binding sites (fold enrichment >= 2; q value < 0.1) with α-HAT and α-peptide PfGCN5 antibodies, which corresponds to 403 genes (Figure 2.4BA). When we averaged PfGCN5 binding density for the α-HAT and α-peptide antibodies across the average gene structure of *P. falciparum*, we observed identical profiles for the two (Figure 2.4C). This confirmed that the antibodies are well-correlated and specifically recognize PfGCN5.

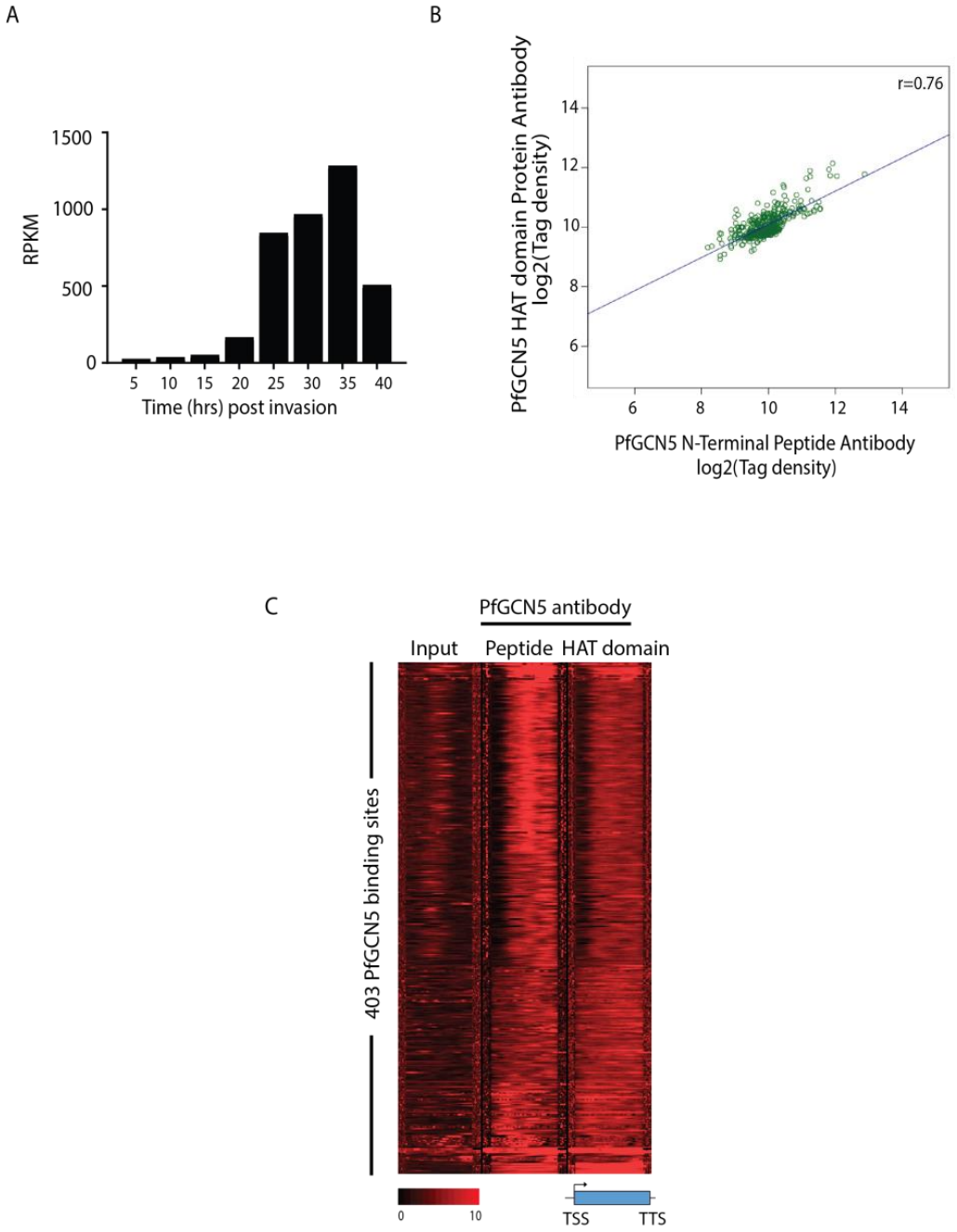


Fig. 2.4: PfGCN5 α-HAT and α-peptide antibody detects similar binding sites. *A) Dynamics of PfGCN5 transcript expression during different stages of intraerythrocytic life cycle of P. falciparum. Expression profile suggests the low expression of PfGCN5 during the ring stages and a sudden burst of PfGCN5 mRNA expression during early trophozoite stage. B) Scatter plot depicting a linear correlation of the target tag densities in ChIP pull down performed using the scores of overlapping peaks from the PfGCN5 HAT domain and peptide antibodies. This is indicative of the fact that both antibodies have similar pull down profile in ChIP*

sequencing reads. C) Heat map showing PfGCN5 occupancy over 403 genes identified as the targets using PfGCN5 (HAT) antibody and peptide antibody.

Since different gene sets in *P. falciparum* have distinct histone modification distribution profiles, we measured the signal density of H3K9ac, a histone modification that is known to be mediated by PfGCN5, and compared it to PfGCN5 density distribution (as measured by α-peptide antibody) across all 5712 *P. falciparum* genes. Interestingly, PfGCN5 was enriched at the 3' end and center of the gene body of the 403 target genes identified by MACS2 analysis. In contrast, these genes have H3K9ac marks distributed along the entire gene body (Figure 2.5A).

When compared with the heterochromatin protein (PfHP1) occupancy, which uniformly coats chromosome ends that contain a majority of the multi-copy variant genes (var, rifin and stevor), we found that PfGCN5 exhibits specific binding to antigenic variation genes, as shown in the representative example for Chromosome 1 from the trophozoite stage (Figure 2.5B). These results corroborate our earlier findings where we have shown that stress and stimuli dependent genes show enrichment of histone modifications at the center and towards the 3'-end of the genes, while genes belonging to other housekeeping functions demonstrate uniform distribution of histone modifications.

Further, to validate the peaks obtained in ChIP-seq, we performed ChIP-qPCR on randomly selected genomic loci enriched for PfGCN5 and confirmed its binding (Figure 2.5C). Lastly, gene ontology (GO) analysis of PfGCN5 bound genes indicated enrichment of terms such as antigenic variation, stress response to heat, and response to unfolded proteins (Figure 2.5D), suggesting that PfGCN5 may play a role in the regulation of stress responsive and stimuli-dependent genes in *P. falciparum*. The presence of an expanded repertoire of GCN5-related N-acetyltransferase (GNAT) family of histone acetyltransferases clearly indicates the possibility of uncharacterized HATs as writers of H3K9ac mark in *P. falciparum* [130].

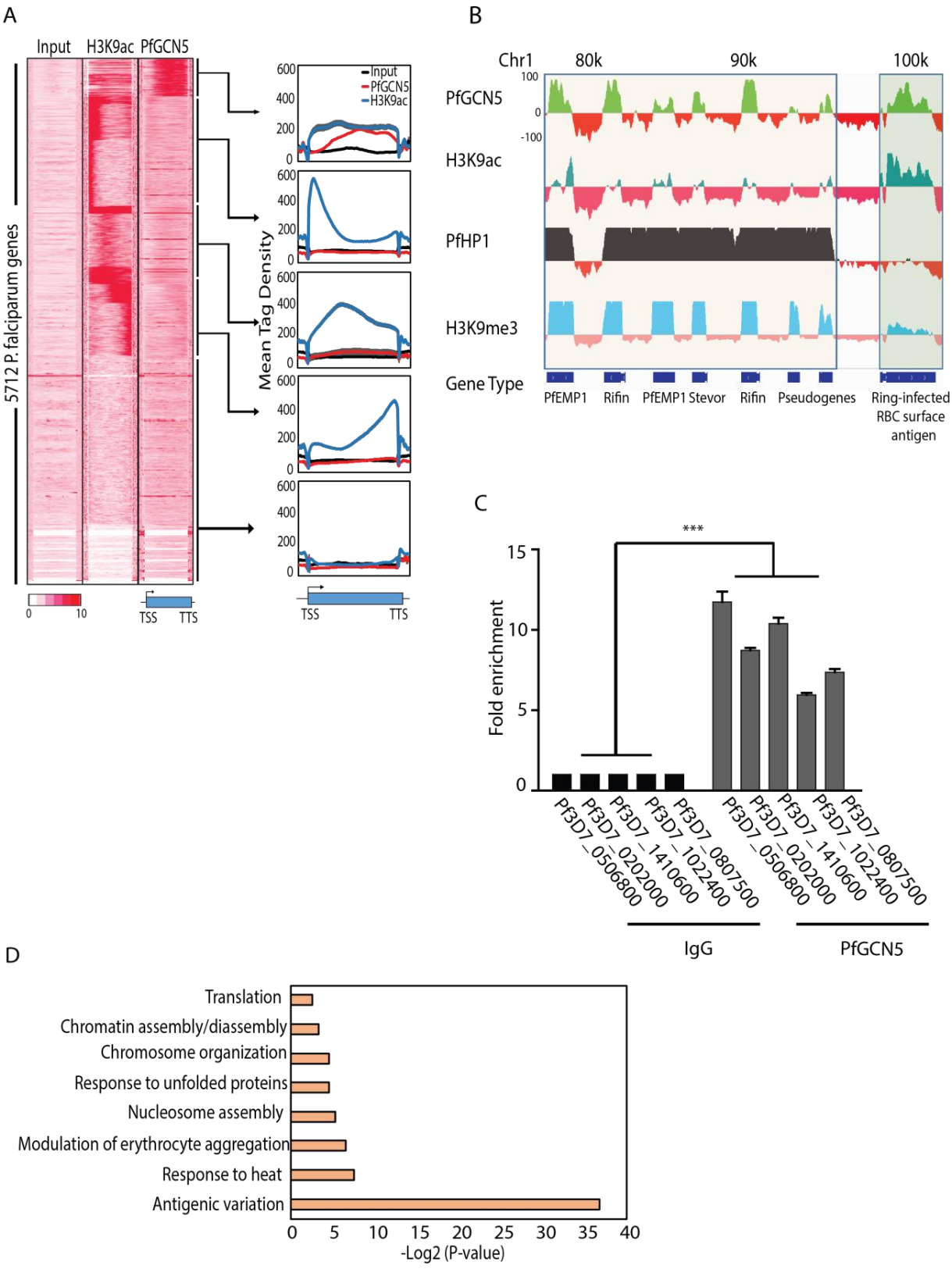

A
Input
H3K9ac
PfGCN5
5712 P. falciparum genes
Mean Tag Density
TSS
TTS
B
Chr1
80k
90k
100k
PfGCN5
H3K9ac
PfHP1
H3K9me3
Gene Type
PfEMP1
Rifin
PfEMP1 Stevor
Rifin
Pseudogenes
Ring-infected RBC surface antigen
C
Fold enrichment
***
IgG
PfGCN5
D
Translation
Chromatin assembly/diassembly
Chromosome organization
Response to unfolded proteins
Nucleosome assembly
Modulation of erythrocyte aggregation
Response to heat
Antigenic variation
-Log2 (P-value)

Fig. 2.5: Density distribution of PfGCN5 binding over average gene structure of P. falciparum. *A) Heat map showing the ChIP-seq tag counts at 5712 P. falciparum genes for H3K9ac and PfGCN5. PfGCN5 was enriched only over a subset of genes having H3K9ac enrichment indicating that it is not a general transcription co-activator. PfGCN5 was found to be enriched mostly at the 3' end of the genes and towards the center of the genes. B) IGV browser snapshot of representative genes having PfGCN5 binding. Binding of H3K9ac, Heterochromatin protein 1 (HP1) and H3K9me3 are also represented for comparison on the same genes. PfGCN5 was present over both active genes and repressed genes. C) ChIP-qPCR of selected genes confirms PfGCN5 binding to ChIP-seq targets. The results are shown as fold enrichment of ChIP performed with PfGCN5 α-peptide antibody versus non-immune IgG. D) Gene ontology for the genes which were found to be bound by PfGCN5 using ChIP sequencing. Antigenic variation and other genes required during stress conditions are overrepresented in gene ontology.*

2.3.3 PfGCN5 is not a general transcriptional co-activator; it is specifically associated with stress/stimuli associated genes

Next, we investigated how PfGCN5 binding relates to transcriptional activity of a gene at the trophozoite stage. We systematically calculated the enrichment levels of PfGCN5 and H3K9ac, a general activation mark, at the gene body of all *P. falciparum* genes and compared it to the relative expression levels of genes as evaluated by RNA-seq-based transcriptomic analysis. As expected, we observed a positive correlation between H3K9ac enrichment and the expression status of the downstream gene (Figure 2.6A; left panel). On the other hand, we did not observe strong positive correlation between PfGCN5 gene-body occupancy and the expression of nearby genes (Figure 2.6A; right panel). Genes with either high or low gene expression levels (outlier points for log2 read density) showed high PfGCN5 occupancy (Figure 2.6B), suggesting that PfGCN5 binds to both active and suppressed/poised genes. In order to confirm this, we compared the expression levels of genes bound by PfGCN5 and contrasted them with the expression of all the *P. falciparum* genes. The expression level of PfGCN5 bound genes spreads from high expression to low expression values (Figure 2.6B) indicating its presence on expressed as well as suppressed genes. Thus, absence of global correlation with transcription and occupancy on suppressed/poised as well as active genes,

suggest that PfGCN5 is not a general transcriptional co-activator rather it may specifically regulate stress responsive genes in *P. falciparum*.

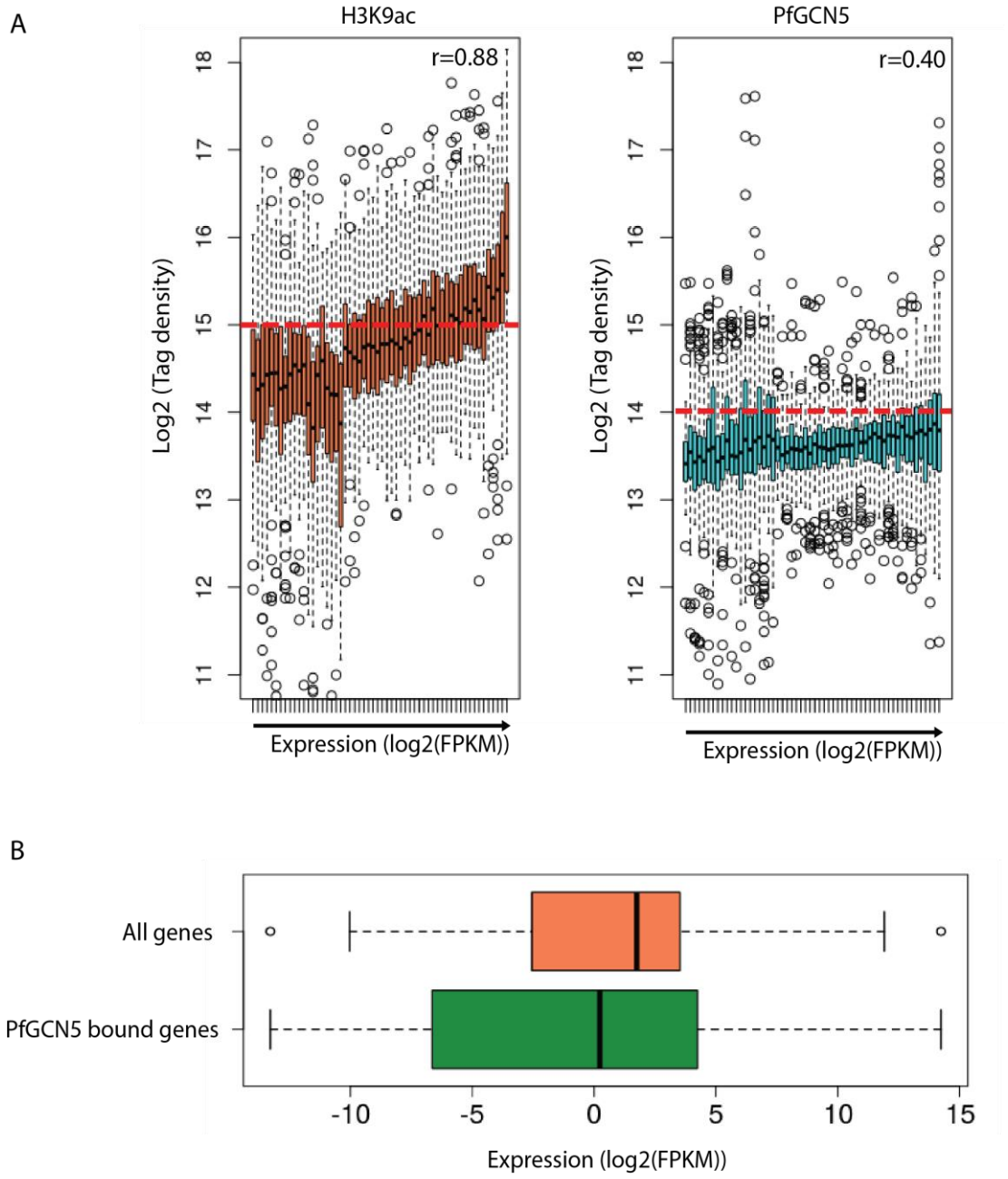


Fig. 2.6: PfGCN5 is not a general transcriptional co-activator; it is specifically associated with stress/stimuli associated genes. *A) Box and whisker plots representing the correlation of genome-wide H3K9ac prevalence and PfGCN5 occupancy with the global gene expression. Absence of global correlation was found for the recruitment of PfGCN5 and gene expression. This indicates that PfGCN5 is not a general transcription coactivator. PfGCN5 is associated*

with highly as well as least expressed genes. B) The expression level of the genes bound by PfGCN5 in comparison to all genes in P. falciparum is represented by the box plots.

2.3.4 PfGCN5 is a specific regulator of stress responsive genes

Next, we decided to look into the role of PfGCN5 during different stress conditions. Synchronized ring stage parasites were exposed to two different physiological stress conditions; temperature (40^0C) and drug (30 nM artemisinin) exposure for 6 hours. Temperature stress was performed to mimic the *in vitro* fever conditions during malaria infection and artemisinin treatment was performed to induce oxidative stress within the parasite. Artemisinin exposure will also help us in understanding the response of parasite to drug exposure. In order to confirm the stress response we looked at the expression level of marker genes, which are known to be upregulated during stress conditions in *Plasmodium*. For temperature stress we looked at the expression level of the heat shock protein, HSP70 (Figure 2.7). Since the artemisinin is known to induce oxidative stress through production of reactive oxygen species, we confirmed the oxidative stress by validating the expression levels of glutathione S-transferase and superoxide dismutase (Figure 2.7).

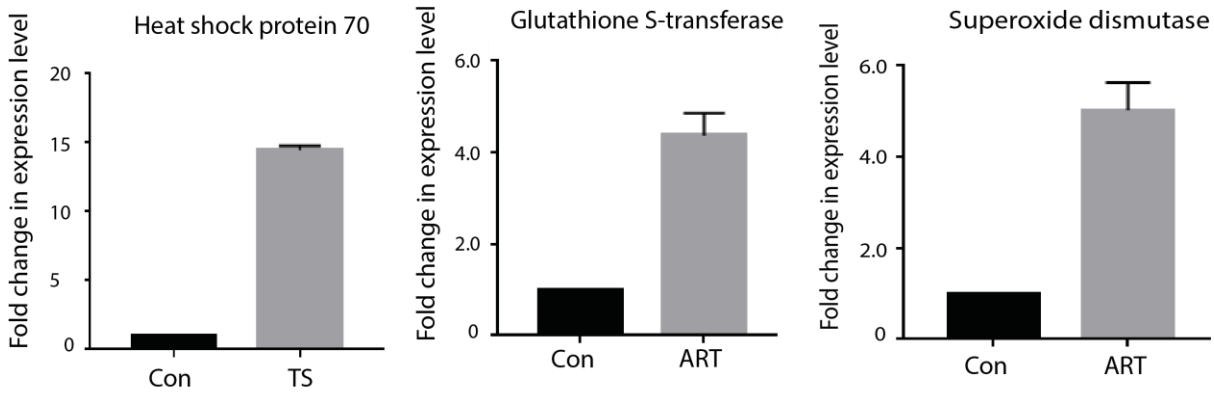


Fig. 2.7: Markers genes were found to be upregulated during stress conditions. *Temperature stress (TS) results in up regulation of HSP70. Similarly artemisinin (ART) treatment results in the increase in expression of Glutathione S-transferase and Superoxide dismutase which indicates the presence of ROS in parasites due to artemisinin treatment in comparison to untreated parasites (Con). Up regulation of these genes is indicative of the fact that stress is induced in the parasite upon artemisinin treatment and increase in temperature.*

Interestingly, PfGCN5 was several fold upregulated upon physiological stress conditions as shown by quantitative real-time PCR (qPCR) using gene specific primers (Figure 2.8).

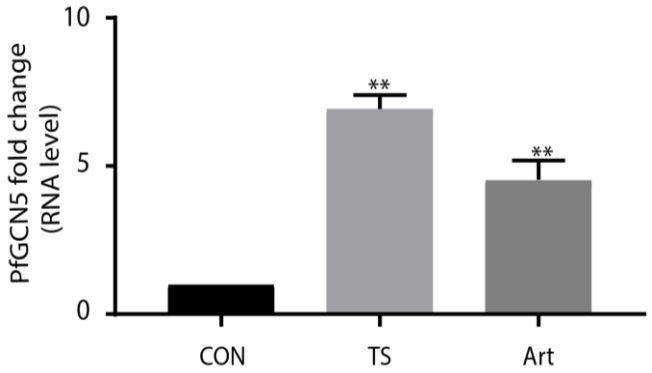


Fig. 2.8: Change in the expression level of PfGCN5 during various stress conditions (N=3). *PfGCN5 is found to be upregulated during heat stress and artemisinin treatment conditions. Data shows the mean ±SEM for three independent experiments. TS: Temperature stress (40^{0}C), Art: Artemisinin treatment (30nM).*

To identify the genes that are deregulated under these stress conditions, we performed transcriptomic analysis using RNA-sequencing and identified 727 and 942 genes (>2 fold change) deregulated upon artemisinin and high temperature exposure, respectively. Genes showing deregulation during stress conditions were also validated by qRT-PCR (Figure 2.9).

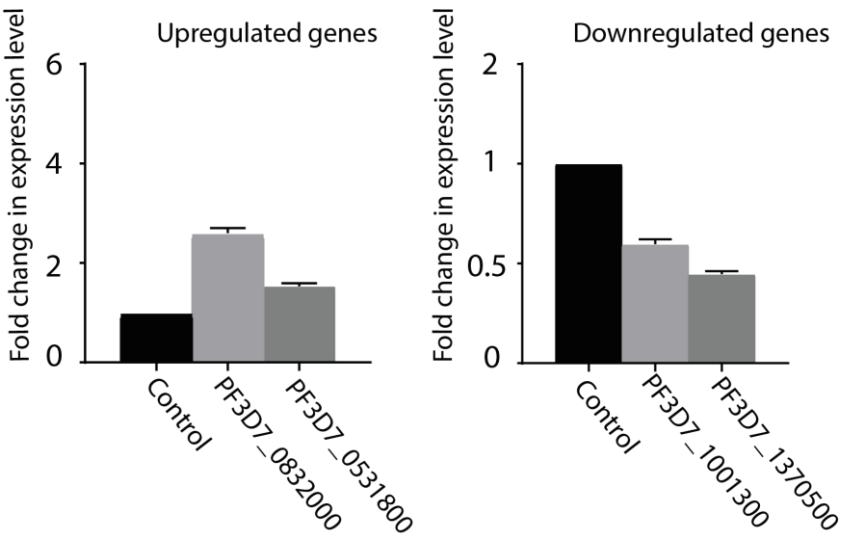


Fig. 2.9: RT-qPCR validation of the genes which are deregulated during artemisinin stress condition, identified through RNA sequencing in comparison to control condition.

Most of the genes that are upregulated during both artemisinin and temperature stress conditions are reported to maintain cellular homeostasis (Figure 2.10A and B). To further dissect the functional correlation between transcriptome deregulation and recruitment of PfGCN5 under different stress conditions, we performed ChIP-sequencing for PfGCN5 using α-peptide antibody during both temperature and artemisinin stress conditions. Notably, most of these genes which are bound by PfGCN5, are upregulated under artemisinin and temperature stress conditions (Figure 2.10C), indicating that PfGCN5 is associated with the activation of stress responsive genes.

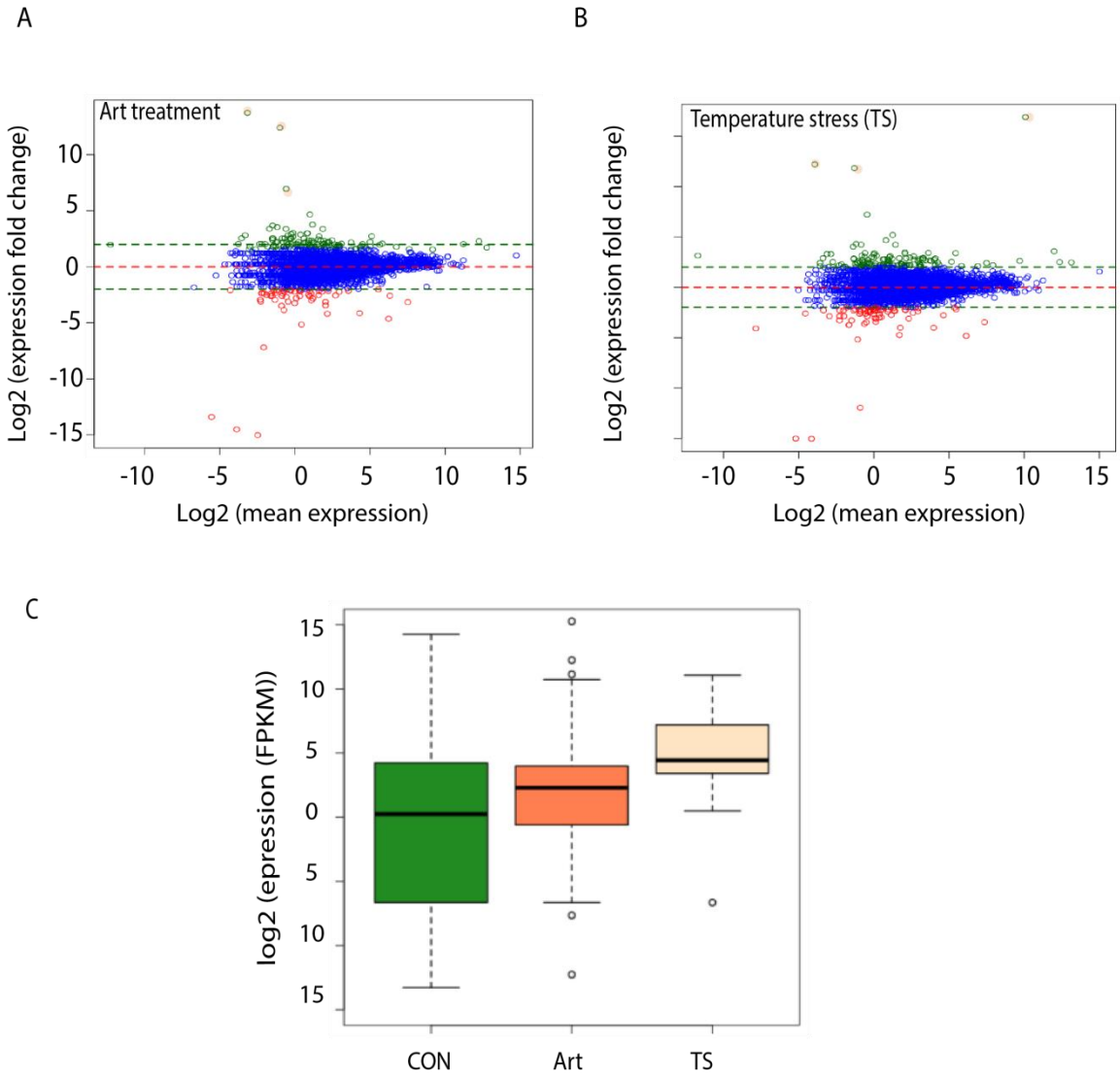


Fig. 2.10: Deregulation of gene expression during stress conditions. *MA plot showing the deregulation in the expression of protein coding genes during artemisinin treatment with A) 30 nM concentration for 6 hours and B) during temperature stress at 40^0C for 6 hours. C) Expression profiles of the genes bound to PfGCN5 during stress conditions. PfGCN5 bound genes are upregulated upon stress induction as compared to control condition. Untreated parasites (TS), Artemisinin treatment (ART) and temperature stress (TS).*

Further to investigate the role of PfGCN5 during stress conditions, we overexpressed HAT and bromo domains of PfGCN5 under normal and stress conditions as previous attempts failed to knockout GCN5 in *P. berghei* and *P. falciparum* indicating it is essential for parasite survival. Remarkably, we observed cell death upon overexpression of PfGCN5 HAT and bromo domains during stress conditions (Figure 2.11). This in turn suggests that overexpression of PfGCN5 possibly leads to hyperactivation of stress responses, eventually resulting in cell death.

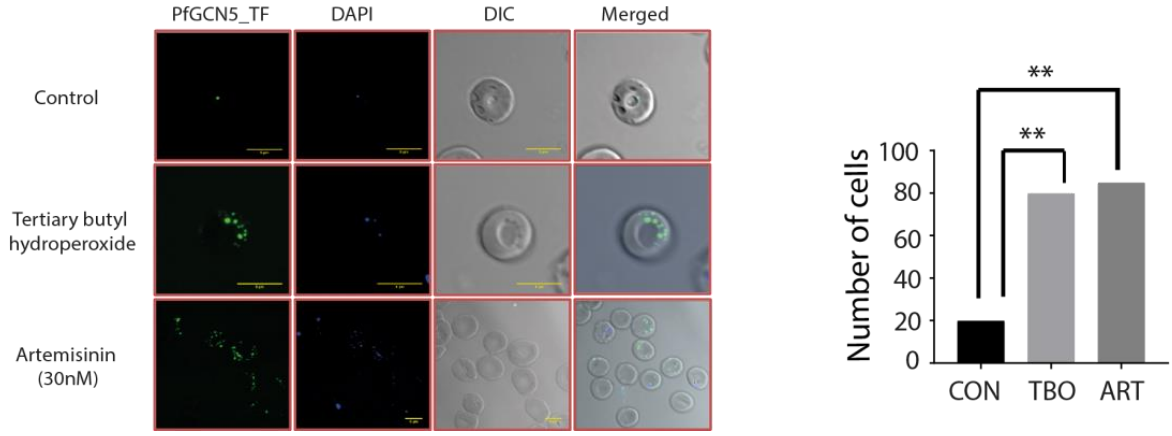


Fig. 2.11: Parasites with episomal overexpression of truncated PfGCN5 (HAT and bromodomain) exhibited cell death during stress conditions. *Truncated PfGCN5 tagged with GFP (green) was expressed in the parasites which were further exposed to Artemisinin (Art, 30 nM) and Tert-Butyl hydroperoxide (TBO, 10 mM) for 6 hours. TBO was used to induce ROS stress in parasites. Bar graph indicates the number of cells showing fragmented nucleus due to stress induction.*

2.3.5 PfGCN5 interacts with PfAlba3 and regulates its chromatin binding

PfGCN5 has an unusually large N-terminal tail as compared to GCN5 in higher eukaryotes as well as other members of the phylum Apicomplexa. This large N-terminal region of PfGCN5 might play an additional role in protein-protein interactions to regulate *Plasmodium*-specific pathways. Further to understand the PfGCN5 mediated transcriptional regulation and to identify its interacting partners, we performed immunoprecipitation-coupled mass spectrometry using the two anti-PfGCN5 antibodies, α-HAT and α-peptide. We identified approximately 125 proteins interacting specifically with PfGCN5, representing four major pathways namely chromatin assembly, response to stimuli, metabolic pathways and translation regulation (Figure 2.12).

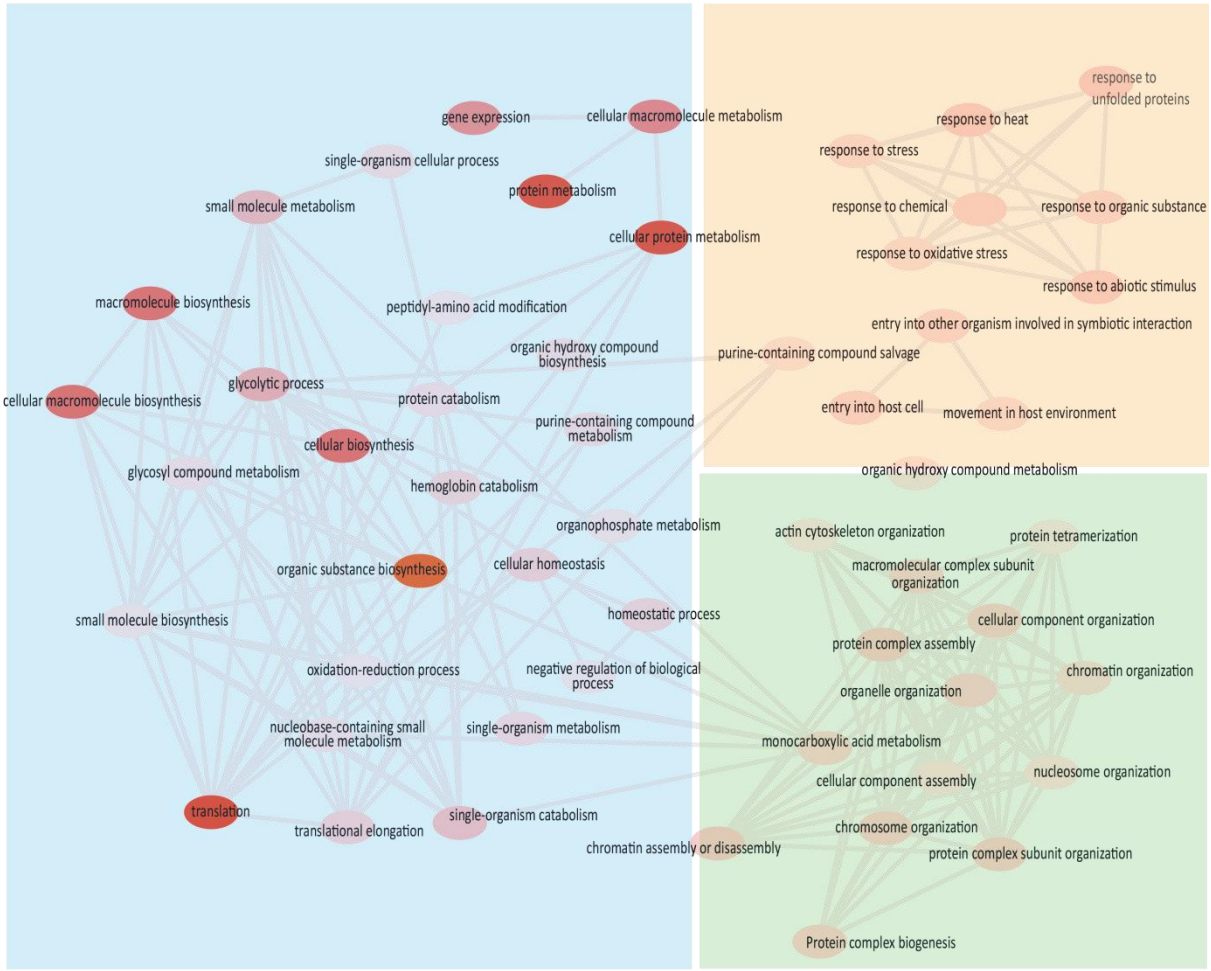


Fig. 2.12: Gene ontology of proteins interacting with PfGCN5. *Gene ontology analysis of PfGCN5 interacting proteins indicate the overrepresentation of four major biological pathways namely chromatin assembly, response to stimuli, metabolic pathways and translation regulation. Gene ontology was performed using PlasmoDB and the plot was generated using Revigo (http://revigo.irb.hr/) [181].*

Interestingly, one of the family of proteins identified as the interacting partners of PfGCN5 is PfAlba (Acetylation lowers binding affinity). PfAlbas are known to play diverse role during transcriptional and translational regulation [182-184]. Alba proteins are also known to play important roles in stress response pathways in higher eukaryotic system [185, 186]. As PfGCN5 was found to be majorly associated with stress responsive genes, we decided to further study PfGCN5 and PfAlba3 interaction.

First, to validate the interaction of PfGCN5 with PfAlba3, we cloned, expressed and purified recombinant His-tagged PfAlba3. As we were unable to express the full length PfGCN5 due to its large size, we cloned and overexpressed GST-tagged HAT and bromo domains of PfGCN5. Surprisingly, *in vitro* binding assay using recombinant His-tagged PfAlba3 and GST-tagged PfGCN5-HAT did not show any interaction (Figure 2.13A). Thus, it is possible that PfAlba3 either interacts with PfGCN5 outside of the HAT and bromodomain or it interacts indirectly with the PfGCN5 complex *in vivo*. Next, we performed immunoprecipitation using PfGCN5 peptide antibody and looked for PfAlba3 as its interacting partner in the pulled down fractions by Western blotting. As shown in Figure 2.13B, PfGCN5 co-elutes with PfAlba3 indicating an interaction with the PfGCN5 complex.

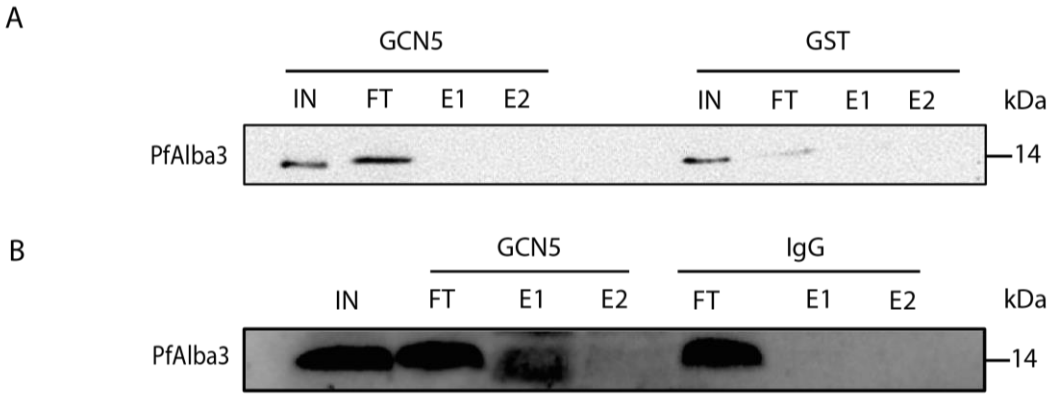


Fig. 2.13: PfGCN5 interacts with PfAlba3. *A) In vitro binding assay of recombinant PfGCN5 (HAT and bromodomain) and PfAlba3. Absence of binding was confirmed with Western blotting using Anti-His antibody. B) Immunoprecipitation was performed to confirm the interaction of PfGCN5 and PfAlba3. PfGCN5 interacting proteins were pull down using PfGCN5 peptide antibody and PfAlba3 interaction binding was confirmed with Western*

blotting using PfAlba3 antibody. Input (IN), flow through (FT), E1 and E2 stands for elutions using 1 M Glycine (pH 2.5).

Lastly, to understand the physiological role of PfGCN5 and PfAlba3 interaction, we performed *in vitro* acetyltransferase assays with the PfGCN5 complex and found that PfGCN5 indeed acetylates PfAlba3 (Figure 2.14). Together, these data suggest that PfGCN5 interacts with PfAlba3 and mediates its acetylation.

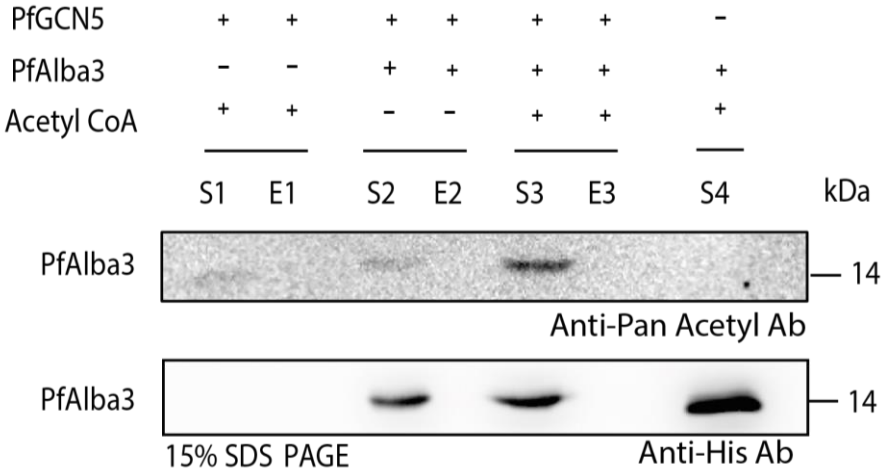


Fig. 2.14: Histone acetyltransferase (HAT) assay to verify PfGCN5 mediated acetylation of PfAlba3. *The assay was performed using recombinant PfAlba3 and the PfGCN5 complex (pulled down with the help of PfGCN5 antibody). PfGCN5 was found to acetylate PfAlba3. Acetylation assay was performed on bead with PfGCN5 bound using antibody. S1, S2 and S3 stands for the supernatant of the acetylation assay whereas E1, E2 and E3 stands for the elution obtained using 1M Glycine (pH 2.5).*

Further to comprehend PfGCN5-mediated regulation of PfAlba3, we performed chromatin-immunoprecipitation using anti-PfGCN5 and anti-PfAlba3 antibodies. Previous studies as well as our RNA sequencing data suggest that temperature stress results in deregulation of stress responsive and multicopy variant (var) genes. Importantly, virulence genes are known to be enriched for PfAlba occupancy and our data suggests enrichment of PfGCN5, hinting at involvement of the two factors in var gene regulation. Thus, to explore the possible crosstalk between PfGCN5 and PfAlba3 in regulation of virulence and stress responsive genes,

parasites were subjected to temperature stress at 16 hpi for a period of 6 hours. This was followed by ChIP and qPCR with anti-PfGCN5 (peptide antibody) and anti-PfAlba3 (protein antibody) antibodies. Interestingly, we observed an increased occupancy of PfGCN5 and a corresponding decreased occupancy of PfAlba3 at stress responsive and virulence genes under temperature stress condition (Figure 2.14). Thus, the interplay between PfGCN5 and PfAlba3 may play an important role in the regulation of various stress responsive and virulence genes depending on external cues.

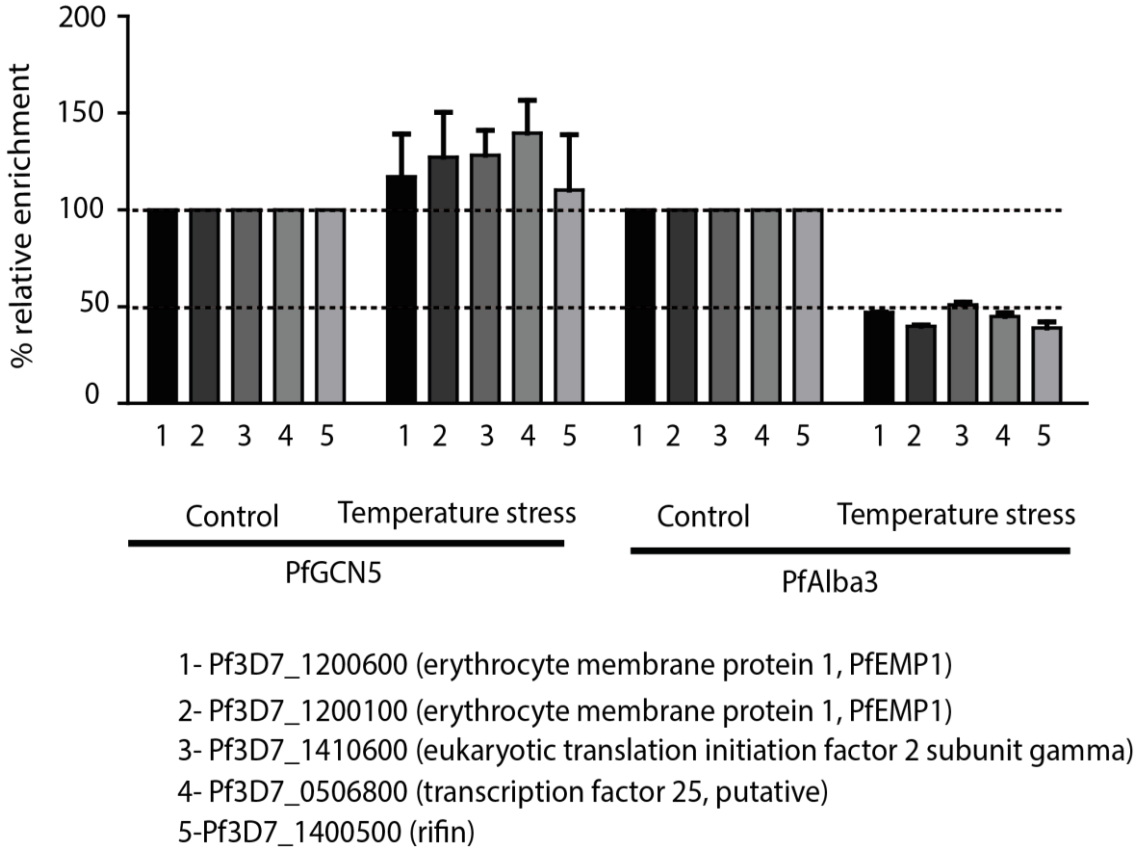


Fig. 2.15: ChIP-qPCR showing the switching in the PfGCN5 and PfAlba3 occupancy on multivariant/stress responsive genes under temperature stress. *ChIP was performed using PfGCN5 (peptide) and PfAlba3 antibodies. Significant decrease in the enrichment of PfAlba3 was observed during temperature stress condition at gene loci which are known to be upregulated during heat stress.*

2.4 Discussion

PfGCN5 is a stress/stimuli specific regulator and not a general transcriptional coactivator

Plasmodium must have evolved efficient machineries to overcome changes in environmental conditions experienced in two different hosts. The ability of *Plasmodium* to develop resistance against artemisinins is attributed to the competent stress responsive pathways and the unfolded protein response machinery, which are activated upon artemisinin exposure [187]. Here, we establish the role of the histone acetyltransferase PfGCN5 as a global regulator of stress responsive pathways in *P. falciparum*. Genome-wide analysis of PfGCN5 occupancy shows that it is associated with stress responsive and multivariant gene family (virulence genes). Interestingly, PfGCN5 occupancy at various genomic loci was found to establish a transcriptionally poised state, which may allow these genes to be switched on or off immediately in response to stimuli. Such regulation is crucial for the genes implicated in stress response and host immune evasion. We and others have previously shown that H3K14ac, another histone modification mediated by GCN5, is specifically present on poised stress responsive genes in higher eukaryotic systems indicating a conserved role of GCN5 in *P. falciparum*. Together, these results suggest that PfGCN5 is not a general transcription coactivator and it specifically regulates the stress responsive and multicopy variant (virulence) genes in *P. falciparum*.

Plasmodium falciparum has evolved an extensive machinery to evade the host immune system through changes in the expression of multicopy variant proteins (var, rifin and stevor), which are expressed on the surface of infected RBCs [45]. A switch in expression of these proteins also helps the parasite in evading splenic and immune clearance by a process called antigenic variation. Though the environmental cues responsible for virulence gene switching are not known, several factors have been identified to play regulatory roles in antigenic variation under physiological conditions. Various histone modifying enzymes like PfSir2, PfHda2, PfSET2 and PfSET10 are shown to repress expression of virulence genes [132, 135, 188]. *P. falciparum* heterochromatin protein 1 (HP1) is another key player known to repress the expression of virulence genes by binding to H3K9me2/3 and heterochromatization [133, 134, 189]. Here, we have identified PfGCN5 as a regulator of virulence gene expression under temperature stress condition.

Furthermore, we also found PfAlba3, a DNA/RNA binding protein, as an interacting partner of PfGCN5. PfAlba superfamily is known to play an important role in translation regulation in *P. falciparum* [182-184]. Moreover, acetylation of PfAlba3 is known to lower its binding

to DNA and results in gene activation [184]. Conversely, PfSir2a deacetylates PfAlba3 and makes it competent to bind DNA and leads to gene suppression [184]. Here we show that PfGCN5 binds to stress responsive and virulence genes and most probably regulates their expression by the acetylation of PfAlba3. Thus, the interplay between PfGCN5, PfSir2A and PfAlba3 possibly helps in regulation of stimuli dependent and virulence genes and contribute to stress responsive and virulence phenotype. Several studies in prokaryotes have investigated the link between virulence and resistance generation [190]. There are clear evidences that virulence modulates resistance level in microorganisms and *vice versa*. Geisinger *et al.* has showed the presence of a key stress response system in *Acinetobacter baumannii*, which enhances the virulence and resistance level in response to different physiological stresses [191]. Similarly, in *P. falciparum,* drug sensitivity of parasites is shown to be virulence dependent, where virulent parasites are shown to have higher likelihood to survive drug treatment. Thus, it is plausible that regulation of both virulence as well stress responsive genes, which are responsible for drug resistance generation is mediated by same machinery involving GCN5 in *P. falciparum.*

Summary: Emergence of drug resistance against artemisinin is one of the biggest hurdles in malaria control and eradication. Recent reports have implicated stress responsive pathways in drug resistance generation. Understanding the regulation of stress responses and virulence gene is crucial to fathom the pathogenesis of the parasites. Our study identifies PfGCN5 as a global transcriptional regulator of stress response and virulence genes in *P. falciparum*. Interestingly we found the PfGCN5 was associated with genes which were found to be upregulated during stress conditions like temperature stress or artemisinin stress. Furthermore, through immunoprepitation followed by mass spectrometry we were able to identify PfAlba3 as an interacting partner of PfGCN5 in regulating stress response genes.

Chapter 3: Role of PfGCN5 in modulating artemisinin resistance in *P. falciparum*

3.1 Introduction

Antimalarial are the drugs used to treat malaria infection. Currently there is no licensed malaria vaccine available in the market to fight against malarial infection hence antimalarial drugs are known to play critical role in the control of malarial infection. Despite of several drugs available in the market, elimination of malaria is still not possible due to inherent ability of *Plasmodium* to generate resistance against most of the drugs [64] (Figure 3.1).

Antimalarial drugs can be classified according to their a) chemical structure or b) the stage of the parasite growth they inhibit [192, 193].

a) Currently most of the antimalarial drugs broadly fall into three categories broadly according to their chemical structure:

1) **Aryl amino alcohol compounds:** quinine, chloroquine, amodiaquine, mefloquine, lumefantrine, piperaquine, tafenoquine
2) **Antifolate compounds:** pyrimethamine, proguanil
3) **Artemisinin compounds:** artemisinin, dihydroartemisinin, artemether, artesunate

Atovaquone is another antimalarial which does not lie in any of the above three categories. It is usually given along with the other drug such as proguanil. It is known to interfere with the electron transport chain in *P. falciparum* mitochondria.

b) Antimalarial drugs can also be classified according to the stage of the parasite life cycle they target.

1) **Blood schizonticides:** Anti-malarials which target blood stage of the parasites are known as blood schizonticides. Most of the drugs currently available in the market

belong to this class for example chloroquine, pyrimethamine, sulphadoxine, quinine, artemisinin etc [194].

2) **Tissue schizonticides:** These drugs act against the parasite growth within the liver which prevents the parasites to reach blood stages of the infection. They kill the parasites in the liver stage of the development. Pyrimethamine and Primaquine are known to have this activity. Primaquine also act on the hypnozoites of *P. vivax* and *P. ovale* in the liver [194].

3) **Gametocytocides:** These are the drugs which targets the sexual form of the parasites. Chloroquine, quinine and primaquine have gametocytocidal activity [194].

4) **Sporontocides:** These drugs are also known to prevent the development of oocysts within the mosquito midgut. Primaquine and chloroguanide have sporontocides activity [194].

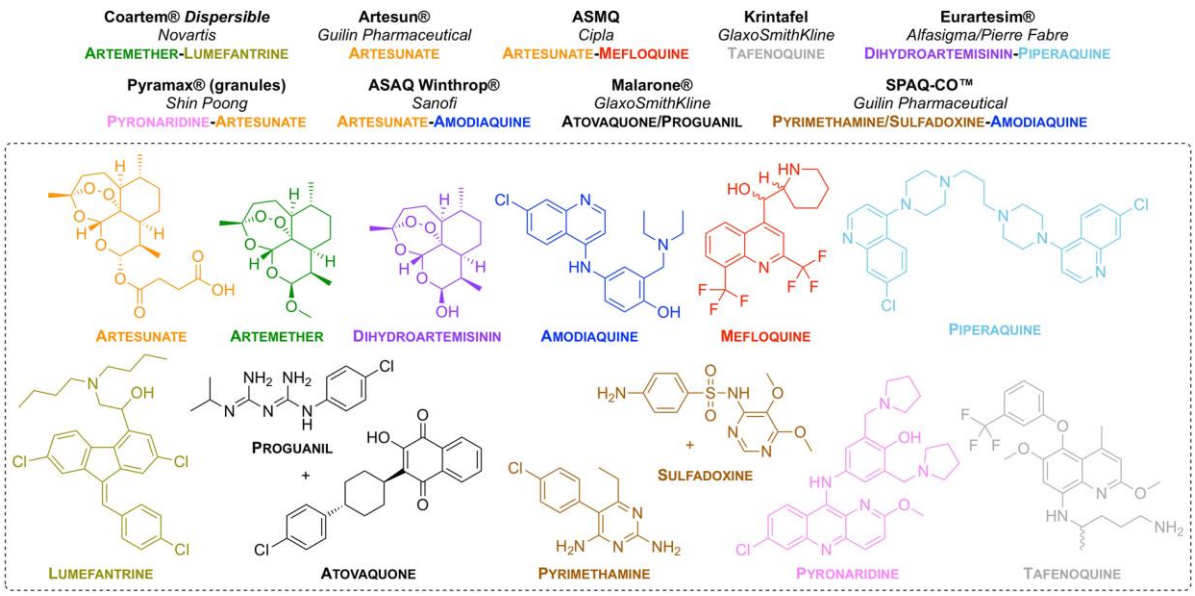


Fig. 3.1: Chemical structure of different antimalarial drugs used for the treatment of malaria. *Adapted from Tse et al. 2019 [67].*

3.1.2 Quinine

Quinine is one of the first antimalarial drugs used in the history. It is extracted from the bark of the *Cinchona* tree [195]. During early 1600 quinine was used in South America to treat fever associated with malaria. By late 1600 usage of this drug was spread to different part of the world including Europe. Chemically quinine is an aryl amino alcohol [195]. Commercial synthesis of quinine is complicated and not cost effective. Later chloroquine was discovered which proved to be more cost effective. Quinine is still considered as the most effective antimalarial where artemisinin based combination therapy (ACT) is not available. In 2010, WHO guidelines recommended the use of quinine along with doxycycline, tetracycline, and clindamycin as the second line of defense [195]. During the World War II there was a decrease in the supply of quinine as a result different countries gained interest in developing commercial antimalarial to replace quinine. Different modified compounds were synthesized by modifying methyl side groups keeping the quinolone core.

3.1.3 Chloroquine

One of the modified quinine is chloroquine [196, 197]. Chloroquine was considered as the gold standard for malaria treatment. Chloroquine is effective against different forms of *Plasmodium*. It is currently used as the first line of defense against *P. vivax* in countries where chloroquine resistance has not emerged. Chloroquine act by accumulating within the food vacuole and interferes with haemoglobin metabolism [196, 197]. In 1950 and early 1960 chloroquine was added to the table salt resulting in emergence of drug-resistance [198]. First report of chloroquine resistance was reported from Thailand Cambodia border [199, 200]. After 2 year, resistance was also reported from South America [201]. Resistance to chloroquine from Africa was reported after 17 years, in 1978 [202]. Through epidemiology studies it was found that the resistance in Africa was originated from the south east Asian resistant strain. There are several mutations which contribute to chloroquine resistance. Mutation (K76T) in chloroquine resistance transporter gene (PfCRT) is considered as the marker of chloroquine resistance [203]. Mutated transporter results in export of chloroquine outside of food vacuole. As a result there is no chloroquine accumulation in the food vacuole. Studies have shown that in south east Asia where the administration of chloroquine is ceased, parasites have started to become sensitive to the chloroquine with less prevalence of PfCRT K76T mutation [204].

Inorder to overcome the chloroquine resistance aromatic ring like structure was incorporated in one of the side chains of the chloroquine to increase its lipophilicity. This modified chloroquine was called amodiaquine [205]. It is usually given in places where there is low level of chloroquine resistance. Piperaquine is another drug which is synthesized by attaching two 4-aminoquinolone structures with various linkers which prevents the binding of this drug to the PfCRT [205].

3.1.4 Sulphadoxine-pyrimethamine

Pyrimethamine and suphadoxine inhibit folate biosynthesis and recycling which result in blockage of DNA synthesis. Suphadoxine targets dihydropteroate synthase and pyrimethamine inhibits dyhydrofolate reductase. Mutations in the genes of these two proteins are associated with resistance to suphadoxine-pyrimethamine.

Protein	Function	Location	Principal drugs affected†	Comments	Polymorphisms
ATP6 (SERCA), putative	Membrane-bound Ca^{2+}-transporting ATPase	Endoplasmic reticulum	ART	Putative determinant	L263E (not yet seen in field isolates)
CRT	Transporter	Membrane of food vacuole	CQ, ADQ	Major determinant	C72S, M74I, N75D/E, K76T‡, A220S/H, Q271E/V, N326S/D, I356T/L, I371R
			MQ, HF, LMF, ART, QN, PIP	Minor determinant	
CYT b	Subunit of complex III (cytochrome bc_1 complex) electron transport chain	Mitochondrion	ATV	Administered as synergistic combination of ATV and PG	Y268S/N/C
DHFR	Folate pathway enzyme	Cytoplasm (principally)	PYR, PG, chlorproguanil	DHPS and DHFR targeted simultaneously in synergistic combinations of antifolates	N51I, C59R, S108N‡, I164L or C50R, N51I, S108N‡, I164L for PYR; A16V, S108T‡ or N51I, C59R, S108N‡, I164L for PG
DHPS	Folate pathway enzyme	Cytoplasm (principally)	SDX, dapsone	DHPS and DHFR targeted simultaneously in synergistic combinations of antifolates	S436A/F, A437G‡, K540E, A581G, A613S/T
MDR1 or PGH1	Transporter	Membrane of food vacuole	MQ, HF, LMF, QN	Major determinant	N86Y‡, Y184F, S1034C, N1042D, D1246Y; also copy number important
			CQ, ADQ, ART	Minor determinant	
MRP1	Transporter	Membrane-bound vesicles on parasite surface membrane	CQ, QN	Possibly also LMF, ART and antifolates	Possibly H191Y, S436A, I876V, R1466K
NHE1	Transporter	Parasite surface membrane/membrane of food vacuole (putatively)	QN	Limited corroboration in field studies to date	Copy numbers of repeat motifs DNNND and NHNDNHNNDDD affect susceptibility levels
TCTP, putative	Ortholog of human histamine-releasing factor	Not yet established	ART	2.5-fold overexpression of TCTP in resistant rodent parasites; possible influence in host histamine synthesis	Not yet known

Table 3.1: Different mutations identified in resistance P. falciparum against different antimalarials. Adapted from Muller et al.,2010 [206].

3.1.5 Mefloquine

Mefloquine (MQ) is a methanol derivative of quinolone [64]. It is known to show high activity against chloroquine resistance isolates. It showed rapid clearance of parasites and reduced relapse. It is given in combination with artemisinin because resistance to mefloquine can generate quickly. It is fast acting drug and has long half-life. Mefloquine is also known to accumulate in the food vacuole and the resistance is associated with the overexpression of the *Plasmodium falciparum* multidrug resistant protein (pfmdr1) gene and its protein product P-glycoprotein homologue (Pgh) 1 [207, 208]. This drug transporter results in the efflux of the mefloquine resulting in the resistance against the drug.

3.1.6 Artemisinins and Artemisinin based combination therapy

Artemisinin based combination therapy (ACT) is considered as the last line of defense against *Plasmodium falciparum* infection [209]. Active component of artemisinin is derived from the natural herb, *Artemisia annua* [210]. This plant was known to have antipyretic properties for more than a millennium in China. Antimalarial properties of this herb were discovered by You You Tu along with fellow researchers Yagang Yu and Guoming Gu in 1972 [210]. Artemisinin is a sesquiterpene lactone and contains a unique peroxide bridge. Presence of free ferrous ion inside the parasites due to haemoglobin degradation is responsible for activation of the artemisinin drug [211]. Activation of artemisinin involves breaking of endoperoxide-bridge and generation of free radical intermediates [211]. Due to the low solubility of artemisinin, other semi synthetic derivatives like artemether (oil soluble) or artesunate (water soluble) are currently used for the treatment of *P. falciparum* infections [212]. Artemisinin and its derivatives have very short half-life. Due to the reduced efficacy of the artemisinin drug again *P. falciparum* infections, World Health Organization (WHO) recommended ACT for treatment of *P. falciparum* infections in 2010. Efficacy of the artemisinin drug is improved by giving it along with the other antimalarial drug which can help in killing remaining parasites. Presently there are five ACTs which are approved by the WHO namely, artemether-lumefantrine, and artesunate (AS)-amodiaquine, artesunate-mefloquine, artesunate-sulfadoxine pyrimethamine and dihydroartemisinin (DHA)-piperaquine [11].

3.1.6.1 Mechanism of action

Artemisinin is a sesquiterpene lactone which contains an endoperoxide bridge which is considered crucial for its antimalarial activity. This endoperoxide bridge is unstable in the presence of ferrous ion (Fe^{2+} ions) [210]. *Plasmodium* is known to uptake haemoglobin from the cytosol of RBCs into food vacuole. Here haemoglobin is degraded to release ferrous ion and globin. This ferrous ion is known to further activate artemisinin by breaking of endoperoxide-bridge, resulting in release of various highly reactive free radicals [210]. These free radicals are known to alkylate and modify various *Plasmodium* proteins and other molecules resulting in the damage and ultimately death of the parasite. The activation of artemisinin due to haemoglobin degradation also makes the activity of artemisinin specific to the parasite infected RBCs.

Another structurally similar compound commonly used as Ca2+ SERCA (sarco/endoplasmic reticulum membrane calcium ATP-ase) inhibitor is thapsigargin [213]. Structurally thapsigargin is also a sesquiterpene lactone. Hence it was believed that both artemisinin and thapsigargin work through similar mechanisms. Interestingly, artemisinin was also found to be interacting with PfATPase6 (Ca2+ ATPase). Furthermore, similarly to thapsigargin, artemisinin was also found to inhibit PfATPase. Inorder to test whether activity of artemisinin is iron dependent, infected RBCs were treated with artemisinin and thapsigargin in the presence of iron chelator desferrioxamine (DFO) [213]. Interestingly, artemisinin was not able to inhibit PfATPase6 in the presence of DFO however activity of thapsigargin was unaffected in the presence of DFO [213]. Moreover, artemisinin can also accumulate within neutral lipid and cause damage to parasite membrane [214].

Recently, clickable artemisinin probe has been used for identification of the protein targets. Around 124 artemisinin targets have been identified through this approach [215]. Targets identified using artemisinin probe are known to play important role in several biological processes. These targets were further validated using *in vitro* binding assays. *In vitro* binding assay also suggested that haem rather than free ferrous ion plays an important role in activation of the artemisinin. Some of the proteins which directly interact with artemisinin are plasmepsin I and II, merozoites surface protein 1 (MSP 1), actin, pyruvate kinase (PyrK), L-lactate dehydrogenase (LDH) etc [215].

3.1.6.2 Emergence of artemisinin resistance, molecular markers and mechanism

According to the WHO, antimalarial drug resistance is defined as the ability of a parasite species to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within limits of tolerance of the subject. Later, in order to have proper clarification definition was further modified by adding "form of the drug active against the parasite must be able to gain access to the parasite or the red blood cell for the duration of time needed for its normal action".

Artemisinin resistance is defined as the delay in the clearance of parasite from the blood following treatment with the artemisinin along with combination drug. Since artemisinin resistance developed mostly at the ring stage of *Plasmodium* life cycle it is considered as 'partial resistance' due to the stage specific nature of the resistance. It is believed that partial resistance can further evolve into full resistance resulting in resistance against all the stages of parasites. Artemisinin resistance emerged around 2001, almost one year after WHO recommended artemisinin based combination therapy against chloroquine resistant parasite in Thailand and Cambodia border areas. Currently, resistance has spread and established well in western Cambodia, eastern Thailand, southern Laos and southern Vietnam. DHA-PPQ is no more effective in these regions. AS-MQ is also becoming ineffective in various regions on Greater Mekong region.

Since artemisinin based combination therapy is the last line of defense available against *P. falciparum* infection, intense genome wide association studies (GWAS) have been conducted to identify the molecular makers associated with the artemisinin resistance. In 2012, Ariey *et al* generated artemisinin resistant line using the artemisinin sensitive F32 Tanzania clone through step wise exposure of increasing artemisinin drug over period of 5 years. Whole genome sequencing of the artemisinin resistant line identified M476I mutation present on the PfKelch13 gene as the molecular marker of artemisinin resistance. This was the first single nucleotide polymorphism (SNP) gained during the generation of artemisinin resistance and was directly linked with sharp increase in survival of the parasites. Later association between the artemisinin resistance and PfKelch13 mutation were confirmed by ring stage survival assays on culture adapted parasite isolates. Although these assays identified several SNPs (Y493H, R539T, I543T, C580Y) associated with artemisinin resistance, M476I mutation was

not found in the clinical studies. Furthermore, genome editing and several genome-wide association studies have confirmed mutations in PfKelch13 (Y493H, R539T, I543T, C580Y) as primary molecular markers of artemisinin resistance.

Interestingly, studies on clinical samples collected from 1984 to 2011from Amazon region of Brazil, Acre, Suriname reported no mutation in the propeller domain of kelch protein [216]. However, in clinical studies in Guyana reported 5 out of 98 samples collected in 2010 having C580Y mutation in the propeller domain of kelch protein [217]. Molecular investigation suggested that emergence of resistance is independent from Asian parasites. Moreover, whole genome sequencing studies have identified different mutations present in the patient samples collected from different region of Africa. However, these mutations present in the propeller domain of kelch protein were different from those identified in south east Asia like A578S. Another mutation A675V was also identified in Uganda, which has been associated with delayed sensitivity in Asia [218].

Currently, there is limited knowledge on the mechanism by which the parasites develop resistance against artemisinin. Only the earliest stage of rings (0-3 hours) shows artemisinin resistance. Moreover, artemisinin resistant parasites are characterized by slow growth and reduced drug susceptibility at the ring stage of asexual growth. Artemisinin resistant parasites are also shown to have extensive transcriptional deregulation, with transcriptional regulators emerging as important players in the evolution of drug resistance. Multiple transcriptomics studies have revealed dormancy, oxidative stress response and protein metabolism to be key players in mechanism of artemisinin drug resistance generation. Furthermore, ubiquitin/proteasome machinery is shown to play an important role in delayed sensitivity of artemisinin resistant parasites. Artemisinin resistant parasites are known to have lower level of ubiquitinated proteins. Interestingly, efficient stress response machinery is active in the resistant parasite which engages ubiquitin/proteasome system in maintaining the lower level of ubiquitinated proteins. Use of proteasome inhibitor like epoxomicin along with DHA resulted in enhanced clearance of the artemisinin resistant parasites.

In order to better understand the artemisinin resistance phenotype Rocamora *et al.* generated two artemisinin resistant lines through *in vitro* drug pressure cycles. Interestingly, transcriptomic analysis of these resistant lines showed that oxidative stress and protein

damage response play a crucial role in determining sensitivity of parasite against artemisinin drug. Genome wide analysis of these resistant parasite lines identified novel mutations in the parasite genome. However, there were no mutations present on the K13 loci of the resistant parasite which is considered as the main marker of artemisinin resistance. These results indicate that the emergence of artemisinin resistance can be due to different mutations in the genotype of the parasite but the pathways which play a crucial role in the artemisinin resistance are related to protein damage response and oxidative stress.

Another recent study has identified the importance of phosphatidylinositol-3-phosphate (PI3P) in mediating artemisinin resistance. Cryoimmunoelectron microscopy has shown the presence of P13P tubules/vesicles which houses various proteins like kelch13 and adhesion protein like PfEMP1. These vesicles also contain various proteins important for the protein quality control, folding and unfolded protein responses in *Plasmodium*. Overexpression of phosphatidylinositol 3-kinase VPS34 in order to induce PI3P vesicles resulted in artemisinin resistant phenotype. *In vitro* generation of resistance in sensitive parasites has played an important role in understanding the emergence of artemisinin resistance. These studies have identified several mutations which are crucial for delayed sensitivity against artemisinin treatment. One such study has identified mutations in the actin binding protein PfCoronin [219]. This protein is known to share homology with PfKelch protein in carrying beta-propeller domain. In order to validate the role of PfCoronin in mediating artemisinin resistance, CRISPR/Cas9 mediated gene editing was performed. Mutation in the PfCoronin genes was found sufficient in delaying *Plasmodium* sensitivity to artemisinin treatment [219].

3.1.6.3 Kelch protein

Kelch gene is shown to be associated with the artemisinin resistance in both *in vitro* as well as genome-wide association studies of clinical samples [220]. Kelch is located on chromosome number 13 and it is known to code for Kelch protein [220]. Domain organization of Kelch protein reveals that there are 6 kelch motifs present at the C terminal end of the protein (Figure 3.3) [221]. Each motif is composed of 50 amino acids that form four antiparallel beta sheet secondary structure. The propeller domain of Kelch protein is responsible for the protein-protein interaction. There are several mutations (Y493H, R539T, I543T and C580Y) which have been reported in the beta propeller domain of the Kelch protein [221-223].

One of the most common and studied mutation is C580Y which is present in the first beta sheet of the fourth motif and it is associated with the delayed clearance of parasites on artemisinin exposure [223]. C580Y mutation is predominantly found in artemisinin resistant parasites from Cambodia, Thailand, Laos, Myanmar and Vietnam. Interestingly most of these mutations are absent in the artemisinin resistant parasites collected from Africa [223]. This indicates that there are factors other than kelch mutations which determine artemisinin resistance in the *Plasmodium.* Few mutations were identified in the non-propeller domain of the kelch protein but their role in artemisinin resistance is still not clear. Another mutation, E252Q in Kelch protein is identified in patients along Thai-Myanmar border with reduced efficacy of artemisinin [224].

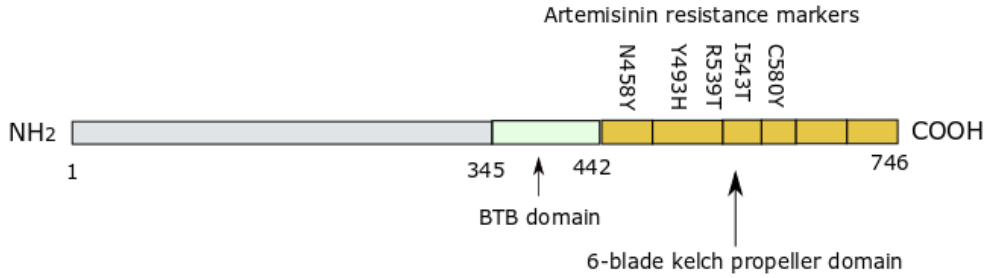


Fig. 3.2: Domain organization of PfKelch protein showing different single nucleotide polymorphism known to be linked with artemisinin resistance. *Most of the mutations identified in various clinical isolates are present on the β propeller domain of the kelch protein.*

3.1.6.4 Dormancy in Artemisinin resistant *P. falciparum*

Upon artemisinin treatment many parasites enter morphologically distinct quiescent stage with reduced metabolism. Once the drug pressure is removed parasites enter back into the ring stage with normal growth progression. This stage is called dormant stage of the parasites [225]. Artemisinin resistant parasites are known to recrudesce earlier from the dormant stage than the sensitive parasites. Dormancy is associated with the selection of the resistant parasites. Studies performed on five different strain of *P. falciparum* showed that all strains are capable of undergoing dormancy while the rate of recovery is varies between strains [225]. It is believed that dormancy is a mechanism employed by the parasite to fight against stress conditions during the ring stage of intra erythrocytic life cycle [225].

3.1.6.5 Ring stage survival assays

Ring stage survival assay (RSA) is the method to detect artemisinin resistance in *Plasmodium falciparum* [226]. In this assay the parasites during the early ring stage (0-3 hours) is exposed to high concentration of artemisinin (700 nM) for a period of 6 hours (Figure 3.4). Survival of parasites is assessed after 72 hours using both SyBr green detection method and manual calculation of the parasitemia using Giemsa staining [226]. When used for cultured adapted parasites, it is called *in vitro* ring stage survival assay whereas it is called *ex vivo* ring stage survival assay if performed on parasite sample freshly isolated from the patient [226].

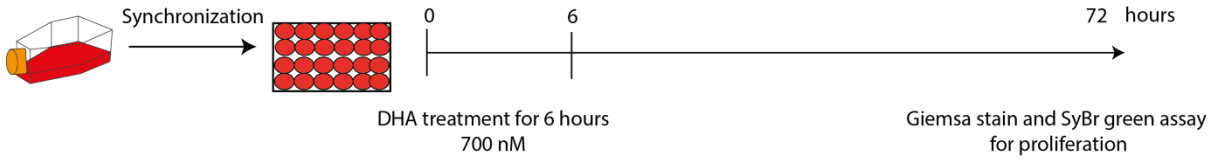


Fig. 3.3: Schematic representation of the ring stage survival assay performed to assess the resistance level of artemisinin drug. *Tightly synchronized parasites at early ring stage are treated with 700 nM concentration of artemisinin drug for 6 hours. Drug is washed and growth of the parasites is monitored after 72 hours.*

3.1.6.6 Artemisinin resistance in India

Artemisinin combination therapy is considered as the first line of defense against *Plasmodium* infection since 2004 [209]. Emergence of resistance in greater Mekong region has raised substantial concern regarding the status of artemisinin resistance in northern eastern region of India due to the cross border migration between India and Myanmar [227, 228]. Chakrabarti *et al.* analyzed the status of artemisinin sensitivity from patient samples from north east and southwestern region of India [229]. Interestingly, samples from northern east showed 50 percent reduced sensitivity against artemisinin. On the other hand only two samples out of 10 showed reduced sensitivity from south western region of India. Sequence analysis of the kelch gene showed no mutation present in the parasites showing reduced sensitivity to artemisinin [229]. In another study Indian patients were assessed for the K13 mutation using the PCR based screening and Sanger sequencing in large number of samples (384 samples). Four patients were identified in north eastern India to contain non

synonymous mutation in the K13 propeller region [227]. However despite these mutations there were no ACT treatment failures. This is one of the first reports which suggested the presence of K13 mutation in the parasites in eastern India. In order to check the presence of artemisinin resistance in India, Das *et al.* assayed parasites for clearance half-life in West Bengal. *Ex vivo* ring stage survival was evaluated for parasites obtained from patients from April 2013 to March 2014 [228]. Interestingly 14% of the patients had increased parasite clearance half-lives (more than 5 hours). Furthermore these patients carried polymorphisms in kelch13 locus. This study identified G625R as a potential novel mutation, along with R539T in Indian population [228].

3.2 Material and Methods

3.2.1 Parasite culture and transfection

P. falciparum strain 3D7 was cultured as previously described in section 2.2.1. Artemisinin resistant lines along with their sensitive counterpart (MRA-1236, MRA-1241, MRA-1254, and MRA-1253) were obtained from MR4. Parasites were cultured in RPMI1640 medium supplemented with 25 mM HEPES, 0.5% AlbuMAX I, 1.77 mM sodium bicarbonate, 100 μM hypoxanthine and 12.5 μg ml^{-1} gentamicin sulfate. Media was supplemented with A^{+ve} human serum.

3.2.2 Histone acetyltransferase activity of recombinant PfGCN5/ Inhibition assay

Histone acetyltransferase activity was analyzed using recombinant PfGCN5-HAT protein. Recombinant protein was incubated with the unique histone substrate which is stably captured on the strip well. Acetylation of the histone in the presence of recombinant PfGCN5-HAT protein was detected using ELISA based calorimetric assay (The EpiQuik™ HAT Activity/Inhibition Assay Kit, EpiGentek). Assay was also performed in the presence of PfGCN5 inhibitor garcinol. GST protein was used as a negative control for acetylation.

3.2.3 Drug response assay

Drug response assay were performed according to the protocol described in Johnson et al, 2007 [230]. Parasites were cultured at 1% parasitemia in 2% hematocrit in 100 μl total volume in 96 well plates. Assay was performed in a sterile 96-well cell culture plate. Serial

dilution of the garcinol containing well was done in triplicates with final concentration ranging from 0.1 µM to 20 µM. Final volume of the complete media in each well was 200 µl. Infected RBCs without any inhibitor were used as a control to check the growth of the parasites. The plate was then incubated at 37^0C for 72 hours. Growth of the parasites was assayed using the SYBR Green I dye intercalation assay.

3.2.4 Ring stage survival assay (RSA)

In vitro RSA was performed according to the protocol described in Witkowski *et al.* (2013) [226]. Parasites were synchronized at early ring stage. Tightly synchronized 0-3 hrs rings were treated with 700 nM of artemisinin for 6 hrs. Drug was washed after 6 hrs with RPMI. Culture was then cultivated for 66 hrs. Parasites were then lysed and the parasite growth was calculated with the help of SYBR Green I reagent which intercalates with the DNA and gives a fluorescent readout upon excitation. Parasite survival rate was calculated by comparing the growth rate between drug treated and untreated control parasites.

3.3 Results

3.3.1 PfGCN5 regulates the expression of genes important for artemisinin resistance in *Plasmodium*

Several studies have been carried out in order to understand the molecular mechanism of artemisinin action and emergence of resistance. Responses to oxidative stress and protein damage are shown to mediate emergence of artemisinin resistance in malaria parasites. Since PfGCN5 was found to be associated with stress responsive and virulence genes, we were interested in investigating the role of PfGCN5 in artemisinin resistance. Previously we showed transcriptional deregulation during artemisinin exposure through RNA sequencing. We looked at the PfGCN5 binding during artemisinin treatment in our ChIP sequencing results. Interestingly, we identified 775 new PfGCN5 bound sites which were acquired under artemisinin stress condition (Figure 3.5A). Moreover, gene ontology of newly acquired PfGCN5 bound genes under artemisinin stress condition includes pathways such as ubiquitin-dependent protein catabolic process, cellular response to stimuli and response to drug, which are known to be deregulated in artemisinin resistant parasites (Figure 3.5B). Hence it is possible that PfGCN5 plays an important role in regulating genes important for the emergence of artemisinin resistance.

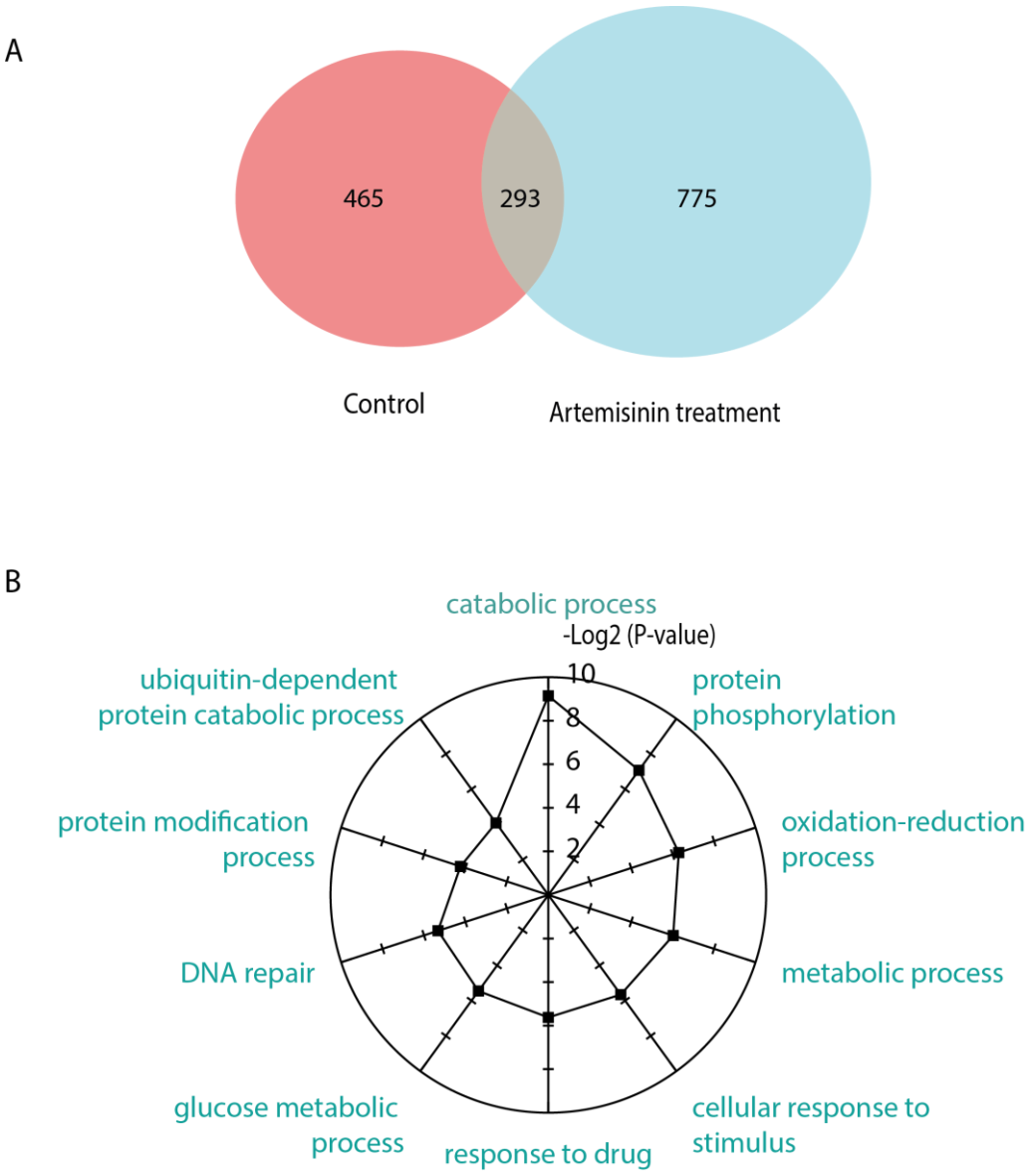
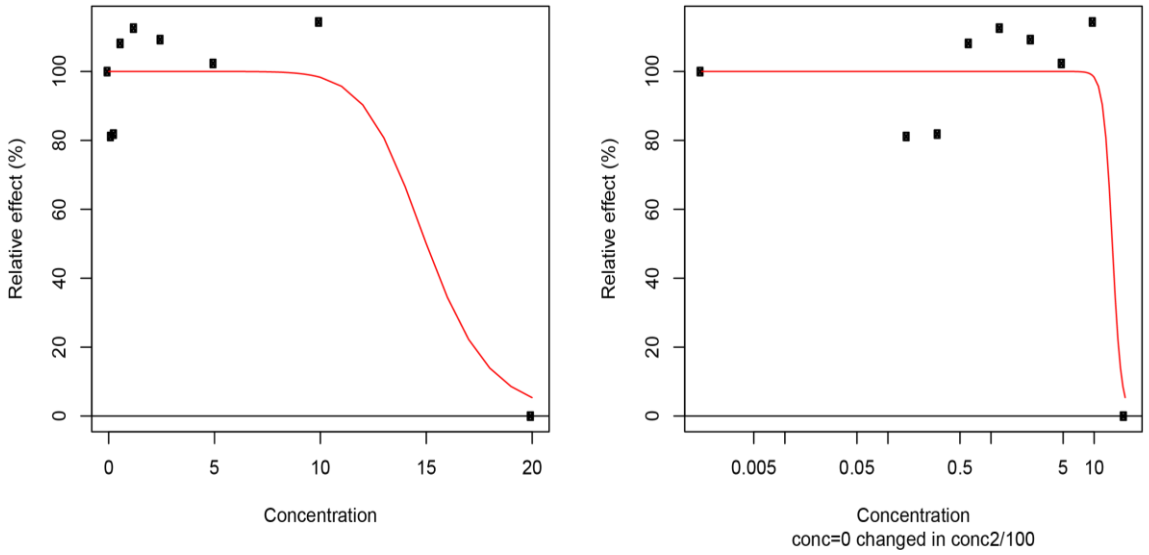


Figure 3.4: PfGCN5 binds to genes involved in artemisinin resistance upon in vitro artemisinin exposure *A) Venn diagram showing the number of genes bound by PfGCN5 during normal conditions and during artemisinin treatment (30nM). B) Gene ontology of the genes which are exclusively bound to PfGCN5 during artemisinin treatment. PfGCN5 is found to be enriched on the genes which are known to be deregulated in artemisinin resistant parasites.*

Of interest is the binding of PfGCN5 at BiP and T-complex protein 1 (TCP1) ring (TRiC) chaperone genes. It is plausible that the higher expression of PfGCN5 upon artemisinin exposure upregulates BiP and TRiC chaperones, thus assisting the unfolded protein response

in artemisinin resistant parasites. This indicates that PfGCN5 might be playing an important role in the emergence of artemisinin resistance by regulating stress responsive pathways in *P. falciparum*.

Furthermore, to understand the role of PfGCN5 in artemisinin drug resistance, we decided to use PfGCN5 inhibitor, garcinol. It is an specific inhibitor of PfGCN5 and showed an IC_{50} of ~15 µM (Figure 3.6).



ICn Estimations

	Estimation	Standard error	Lower CI95%	Upper CI95%	Ratio CI
IC50	15	3.58	6.75	23.25	3.45
IC90	18.69	4.46	8.41	28.97	3.45
IC99	23.75	5.67	10.68	36.82	3.45
Gamma	10	–	–	–	–

Fig. 3.5: IC_{50} calculation of garcinol using dose response assay carried out over a period of 48 hours. *The growth inhibition was measured using the SYBR Green I assay.*

Inhibition of PfGCN5 HAT activity was further confirmed using recombinant PfGCN5-HAT protein in presence and absence of garcinol. Recombinant GST protein was used as a negative control. Histone acetyltransferase activity assay was performed using EpiQuik HAT Activity/Inhibition Assay Kit (Colorimetric). We found significant decrease in the histone acetyltransferase activity in the presence of 10 µM garcinol (Figure 3.7).

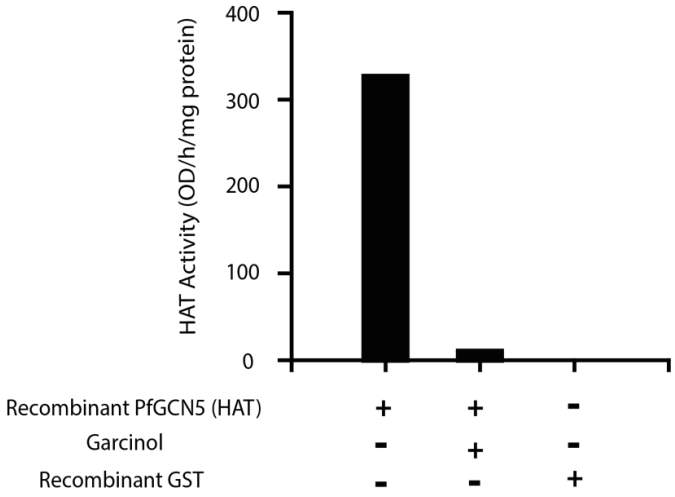


Fig. 3.6: Inhibition of histone acetylation activity of purified recombinant HAT domain of PfGCN5. *10 µM of Garcinol inhibits PfGCN5 HAT activity completely***.**

We found PfGCN5 to be associated with genes like BiP and TCP1 β (T complex protein 1 subunit beta). Since these proteins are known to play critical roles in the artemisinin resistance parasites, we decided to look at the expression level of these genes in presence and absence of garcinol (10 µM) during different stress conditions using quantitative RT-PCR. We found that both BiP and TCP1 β (T complex protein 1 subunit beta) were upregulated during the stress conditions (Figure 3.8). Interestingly, under garcinol treatment there is a significant decrease in expression level of BiP and TCP1 β under stress conditions (Figure 3.8).

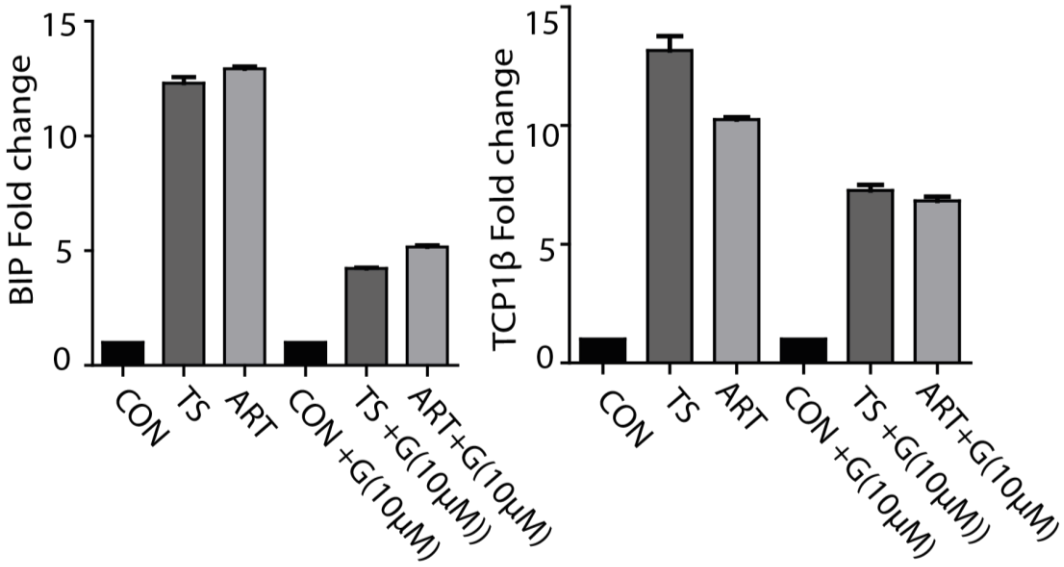


Fig. 3.7: RT-qPCR results showing the upregulation of BiP and TCP1β during different stress conditions and inhibition of upregulation under garcinol treatment (G, 10 μM) respectively. *Data shows the mean ±SEM for three independent experiments. Parasites which are untreated served as control (CON) whereas temperatures stress (TS) and artemisinin treatment (ART, 30 nM) was given for stress induction.*

3.3.2 Inhibition of PfGCN5 in artemisinin resistant lines decreases there resistance level

Further to dissect the role of PfGCN5 in artemisinin drug resistance emergence and maintenance, we looked at transcript level of PfGCN5 in artemisinin resistant lines. Interestingly, PfGCN5 is upregulated in artemisinin resistant lines; K13-I543T (MRA-1241, RSA~25%) and K13-C580Y (MRA-1236, RSA~6%) by 2.5 and 1.5 fold as compared to their sensitive counterparts, respectively (Figure 3.9A). Resistance level for both the resistant parasites lines was calculated using RSA.

We wondered if the inhibition of PfGCN5 activity resulted in change in drug sensitivity of the artemisinin resistant lines: K13-I543T and K13-C580Y. Ring survival assay (RSA) was performed in absence and presence of garcinol (used at a concentration which has no or minimal effect on normal parasite growth). We observed 36.4% decreases in the level of resistance for K13-I543T artemisinin resistant line in the presence of PfGCN5 inhibitor, garcinol (Figure 3.9i). Interestingly, garcinol treatment of the artemisinin resistant parasites, K13-C580Y completely reverses the artemisinin resistance (Fig 3.9ii) indicating that PfGCN5 plays an important role in artemisinin resistance.

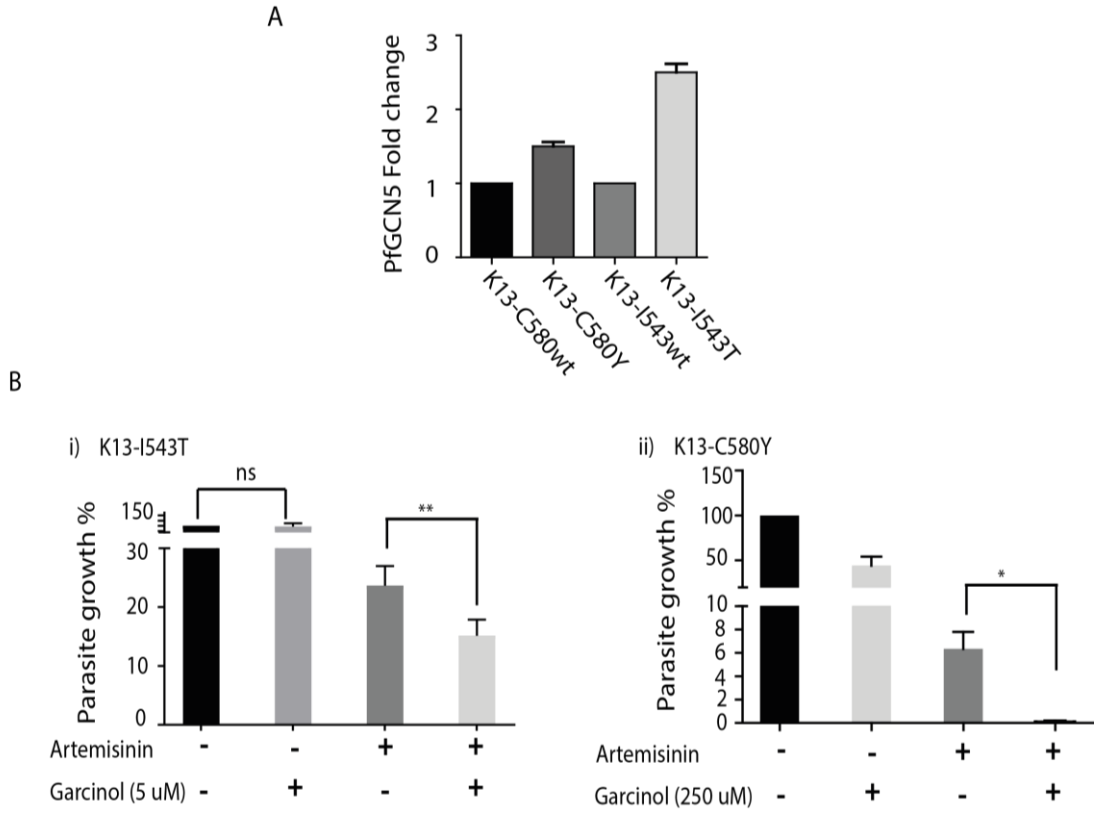


Fig. 3.8: Inhibition of PfGCN5 results in decrease in artemisinin resistance*. A) Transcript level of expression of PfGCN5 in artemisinin resistant strains, K13-I543T (MRA-1241) and K13-C580Y (MRA-1236) in comparison to their sensitive counterparts, K13-I543wt (MRA-1253) and K13-C580wt (MRA-1254), respectively. B) Change in the percentage parasite survival estimated through Ring Survival Assay (RSA) in presence of PfGCN5 inhibitor garcinol. i) K13-I543T (MRA-1241) parasites were treated with 5 µM garcinol and ii) K13-C580Y (MRA-1236) parasites were treated with 250 µM garcinol. Presence of garcinol decreases the artemisinin resistance in K13-I543T (MRA-1241) at a concentration which has otherwise no effect on parasite growth. Higher concentration of garcinol was used with a significant decrease in resistance level in K13-C580Y (MRA-1236) parasites.*

3.3.3 PfGCN5 plays common and strain specific roles in different resistant lines

In order to further understand the role of PfGCN5 in artemisinin resistance, we performed PfGCN5 ChIP sequencing in artemisinin resistant K13-I543T (MRA-1241) and K13-C580Y (MRA-1236) and their sensitive counterparts K13-I543wt (MRA-1253) and K13-C580wt (MRA1254). We investigated the strain specific genes enriched for PfGCN5 binding and

called their associated biological processes through the gene ontology analysis. Several biological processes were found to be conserved between the sensitive and resistant strain. These primarily include cellular adhesion, response to stimulus and antigenic variation, highlighting their regulation by PfGCN5 across strains.

Interestingly, a set of genes are uniquely enriched for PfGCN5 occupancy in the resistant strains. While, PfGCN5 is enriched on cellular metabolism and protein translation associated genes in K13-I543T strain, in K13-C580Y it is enriched on genes involved in vesicle fusion, and morphogenesis (Figure 3.10). Deregulation of these biological pathways has been shown to be crucial for resistance acquisition in the field isolates of *P. falciparum*. Thus, our findings also reiterate an important aspect of resistance emergence posited earlier, that it is highly dynamic and can be shaped by independent underlying genetic and external environmental factors. Together, these results suggest that PfGCN5 plays an important role in the regulation of stress responses, which are associated with drug resistance emergence.

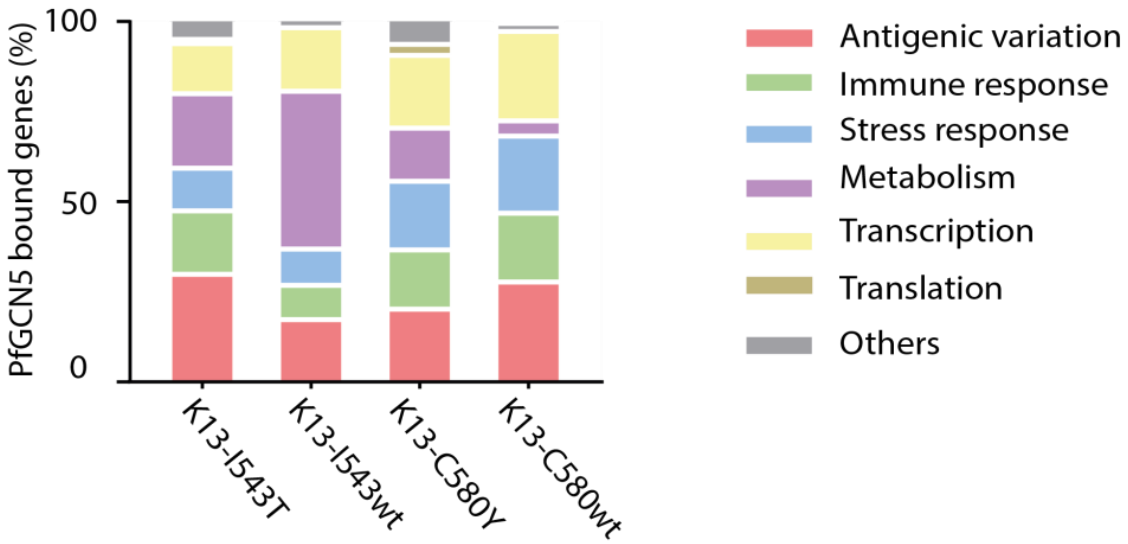


Fig. 3.9: Stacked bar graph showing the genes bound by PfGCN5 in K13-I543T (MRA-1241) and K13-C580Y (MRA-1236) mutant lines and their sensitive counterpart K13-I543wt (MRA-1253) and K13-C580wt (MRA-1254) respectively. *Genes associated with antigenic variation and cellular adhesions were found to be core targets of PfGCN5 in both sensitive and resistant strains. Unique set of genes implicated in resistance in the field were enriched for PfGCN5 occupancy in the two resistant strains.*

3.4 Discussion

Recent studies have suggested the significant role of stress response machinery in artemisinin resistance in *P. falciparum*. Hence to understand the emergence and maintenance of artemisinin resistance it becomes crucial to investigate regulation of stress responses in the parasites. Histone modifiers are known to regulate the expression of genes by modulating the post translation modification on the N terminal tail of histones. We have studied one such histone modifier, histone acetyltransferase PfGCN5. PfGCN5 belongs to the GNAT family of histone acetyltransferase and play crucial role in transcription regulation in higher eukaryotes. GCN5 have been implicated in stress responses in higher eukaryotes like yeast, humans etc. Furthermore, we found that PfGCN5 is associated with stress responsive genes.

In order to understand the role of PfGCN5 specifically during oxidative stress we performed PfGCN5 ChIP sequencing under exposure of artemisinin drug at low concentration. Surprisingly, upon artemisinin treatment PfGCN5 is enriched on the genes important for the development of resistance against artemisinin. Corroborating PfGCN5 genome-wide binding with transcriptome data during stress conditions clearly indicates that PfGCN5 is associated with the genes which are upregulated during stress conditions (e.g. artemisinin exposure). Chemical inhibition of PfGCN5 HAT activity resulted in the downregulation of BIP and TCP1β transcript expression level during stress conditions. Next, we looked at the role of PfGCN5 in mediating artemisinin resistance using artemisinin resistant lines like K13-I543T and K13-C580Y. We looked at the transcripts level of PfGCN5 in these resistant levels. We found higher expression of PfGCN5 transcript in K13-I543T but no significant change in the transcript level of PfGCN5 in K13-C580Y when compared to their sensitive counterpart.

Furthermore, upon interfering with the activity of PfGCN5 using its specific inhibitor, garcinol, we found a significant decrease in the level of artemisinin resistance in the K13-I543T mutant (MR4-1241, RSA-25%) and K13-C580Y (MRA-1236, RSA-6%). This in turn suggests that PfGCN5 is a global regulator of stress responsive genes, and plays an important role in artemisinin resistance maintenance. We further performed ChIP sequencing in resistant lines and their sensitive counterpart to better understand the genes regulated by PfGCN5 in resistance parasites. Interestingly, we found that PfGCN5 regulates strain specific pathways along with the common pathways like adhesion, var gene regulation etc.

Bhattacharjee *et al.* recently reported the amplified presence of PI3P vesicles which helps in mitigating the protein damage due to artemisinin treatment. These vesicles house proteins like Kelch13, PfEMP1, BiP and others proteins required for maintaining homeostasis in artemisinin resistant parasite. Proteome analysis of these vesicles has revealed a list of proteins interacting with each other and possibly helping in emergence of artemisinin resistance. PfGCN5 is one of the proteins detected in the vesicular proteome. We found a significant overlap in the proteins identified in the proteome analysis and PfGCN5 interacting partners (Figure 3.11).

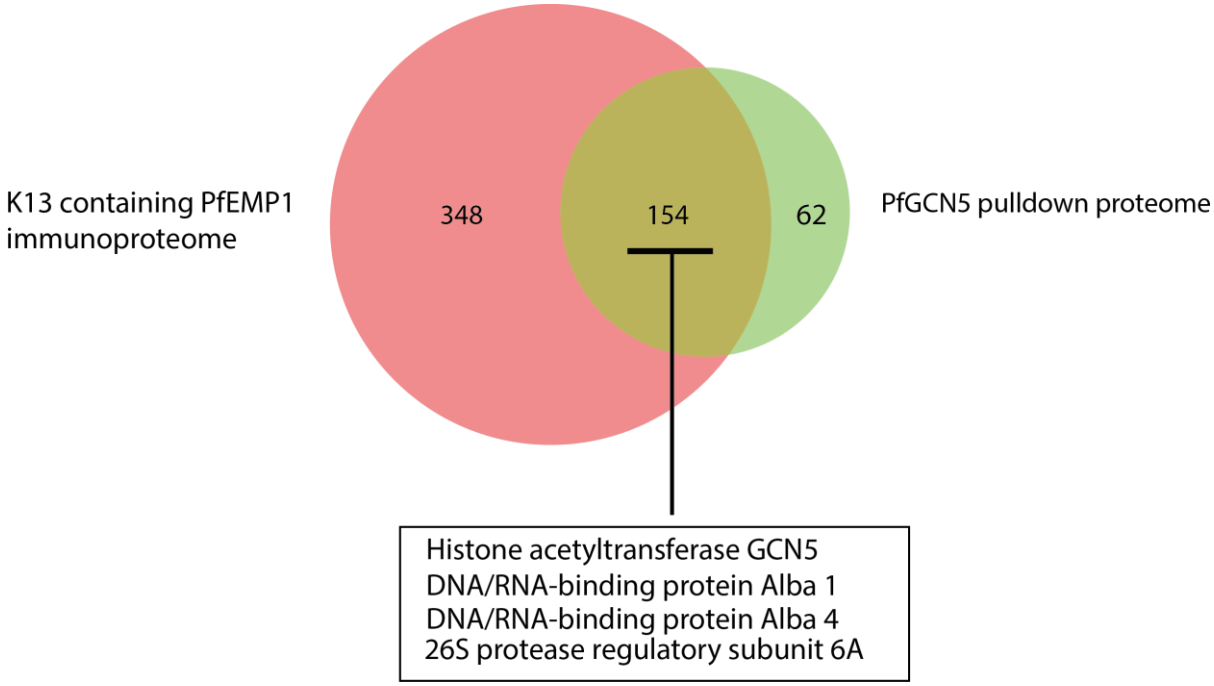


Fig. 3.10: Venn diagram showing the overlap between the proteins which were found in the proteome of the vesicles presents in the artemisinin resistant parasites and proteins interacting with PfGCN5. *Interestingly PfGCN5 was found to be present in theses vesicles.*

In consonance, we also found various stress regulators such as heat shock proteins and Albas as interacting partners of PfGCN5. These interactions may play an important role in activation of stress response pathways upon artemisinin exposure. Moreover, PfGCN5 also regulates transcription of BiP and T complex protein 1 subunit beta under stress conditions. Reports from higher eukaryotic systems have suggested that acetylation of BiP results in its dissociation from the protein kinase RNA-like endoplasmic reticulum kinase (PERK), which further results in phosphorylation of eIF2alpha leading to translation repression. Moreover,

we also found that PfGCN5 is enriched at the promoter of the Kelch13 gene, which possibly hints at its transcriptional regulation.

Summary: This study suggests that PfGCN5 may play an important role in drug resistance generation either by directly regulating the expression of the genes important for emergence/maintenance of artemisinin resistance and/or by interacting with various key stress-regulators involved in resistance generation in *P. falciparum* (Figure 3.12) [231]. Inhibition of PfGCN5 can result in the decreased level of artemisinin resistance.

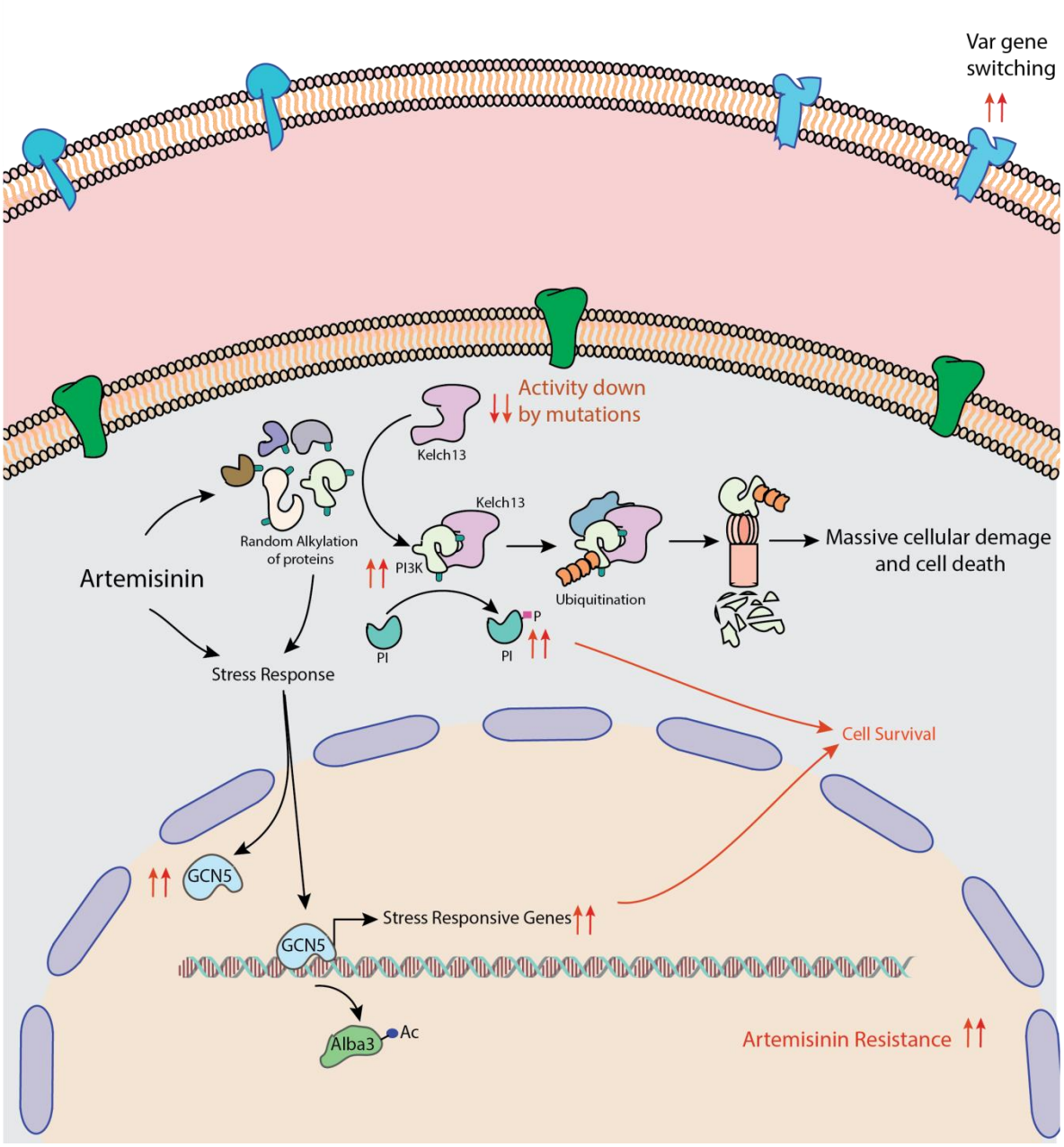


Fig. 3.11: Mechanisms proposed for artemisinin resistance in P. falciparum. Model showing the role and interplay of Kelch13, PI3K and PfGCN5 in artemisinin resistance generation. *Artemisinin treatment leads to random alkylation of proteins, which in turn are ubiquitinated by the protein ubiquitination complex of which Kelch13 is an important ligase adapter component and subjected to degradation by proteosomal degradation. One such protein is the PI3K (phosphatidylinositol 3 kinase), which is implicated in lipid metabolism and cell survival signaling. Massive alkylation by artemisinin exposure and/or oxidative stress activates PfGCN5 in the nucleus, which in turn upregulates the stress-responsive and unfolded protein response pathways. Thus, mutations in Kelch13 as well as upregulation of stress-responsive pathways by PfGCN5 help in artemisinin resistance generation.*

Chapter 4: The role of PfGCN5 in nutrient sensing and transcriptional regulation in *Plasmodium falciparum*

4.1 Introduction

Malaria is a life threatening infectious disease caused by *Plasmodium* leading to millions of deaths worldwide. Despite more than a century of efforts to eradicate malaria, it is still one of the highly widespread parasitic diseases in the tropical and subtropical region of the world. During various developmental cycles the parasites are able to tolerate numerous stresses, experienced in the form of elevated body temperature (fever), parasite density, nutrient fluctuation and drug exposure [163, 164, 168, 169, 231, 232]. These stresses are known to play an instrumental role in increased virulence and emergence of drug resistance in *P. falciparum* [187, 233]. In recent years, it has been well documented that various protozoan pathogens hijack and remodel host cell machinery and metabolism in order to escape immune responses [234-240]. Several studies have shown that the parasite depends upon its host for nutrients as it has lost the ability to synthesize them *de novo* [241, 242]. Moreover, malaria parasite is known to obtain its energy solely from glycolysis and catabolism of host cell haemoglobin during intraerythrocytic life cycle [84, 243, 244]. Since the parasites rely completely on its host for resources, any alterations in the environmental milieu or host cell could have deleterious effects on the parasite growth and virulence [239, 244]. These nutrient fluctuations could be encountered during the process of invading multiple tissues, escaping immune surveillance and induced drug pressure [170, 233, 245-248].

4.1.1 Nutrient acquisition

Nutrient acquisition in *Plasmodium* is known to be mediated by RhopH1 family of proteins encoded by multigene family clag [249-251]. They are known to form channels at the surface of infected RBCs. Patch-clamp studies have confirmed that there is huge increase in the permeability of the erythrocyte membrane for the uptake of nutrients [252, 253]. The RhopH1/clag family is encoded by five genes [254, 255]. Interestingly two members of this family, clag3.1 and clag3.2 are known to be present in the head to tail orientation, next to each other on chromosome 4 [256]. They are known to undergo mutually exclusive expression and switching to the other form takes place at very low frequency [256]. Clag gene

expression is also associated with drug resistance [257]. Studies have shown that switching of clag3.2 to clag3.1 is associated with resistance to blasticidin [257]. Members of this protein family have discrete ability to import different nutrients [254]. Similarly PfACS family involved in lipid metabolism is also known help parasites in adaptation during different nutrient condition [258].

4.1.2 **Essential nutrients for *Plasmodium***

In vitro studies have identified several metabolites which are essential for the survival and development of *Plasmodium* inside the host body [248]. This includes amino acid like isoleucine, cysteine, glutamate, glutamine, tyrosine, proline and methionine [259, 260]. Other nutrients include potassium chloride, sodium chloride, glutathione, magnesium sulphate, hypoxanthine, biotin etc [261-263]. *Plasmodium* contains acetyl-coenzyme A carboxylase which is a biotin dependent enzyme [262]. Study conducted on chickens showed that *Plasmodium* infected chicken, when fed biotin deficient diet had low parasitemia level in comparison to the normal fed chicken [264].

4.1.3 Nutrient stress in *Plasmodium*

Apart from oxidative stress, *Plasmodium* also faces nutrient stress during its growth within the erythrocytes. Malarial infection results in hypoglycaemia in patients where the glucose level decreases to 50 mg/dl [171, 265, 266]. *Plasmodium* lacks the tricarboxylic acid (TCA) cycle during its intra- erythrocytic growth phase, thus the parasite entirely depends on glycolysis for energy [170]. The parasite largely relies on the host for its high glucose demand, owing to which it undergoes nutrient stress when the glucose levels in the infected host drop. *Plasmodium* being a parasite depends on haemoglobin degradation for its amino acid requirements [267]. However, isoleucine is absent in the haemoglobin as the result parasite depends on exogenous source like human serum. Isoleucine is present in more than 90% of *Plasmodium* proteins hence this makes the requirement of this amino acid essential for its survival. Studies have shown that parasite undergoes into hibernatory state when subjected to isoleucine to phosphorylate eIF2alpha resulting in translation repression during amino acid starvation [241]. Moreover, PfMaf1, a regulator of RNA polymerase (Pol) III is known to downregulate the pre-tRNA synthesis under nutrient deprived conditions [268]. Hence, PfMaf1 is necessary for the survival of the parasites during amino acid starvation induced dormancy in *Plasmodium* [268].

Since *Plasmodium* is dependent on host for most of its nutrients, an efficient mechanism for sensing and reacting rapidly to alteration in nutrient composition and adapting to a different environment is required which could entail modulation of replication and differentiation program. This capacity to sense, respond and adapt to changing nutrient composition is evident in *Plasmodium* spp. as it moves between human and mosquito hosts. Importantly, this rapid physiological transitions and fluctuations in nutrients within and across hosts require rapid molecular and cellular reprogramming and could only be achieved by some parasites within a population which are in the state of molecular preparedness to promote differential stress response and antigenic variation. Hence a molecular sensor that is capable of relaying information could be a strategic advancement that enables parasites to adapt and survive in new environments. Earlier we showed that histone acetyltransferase, PfGCN5 is associated with stress and virulence related genes in *P. falciparum* [231]. Since metabolism is known to be a critical determinant of virulence and survival of the parasites, we wanted to understand the role of PfGCN5 in transcriptional regulation during nutrient stress condition. Here, we show that PfGCN5 is associated with the genes that are important for the maintenance of parasite cellular homeostasis upon nutrient stress condition. Thus, our study suggests an important role of PfGCN5 in activation of nutrient stress responsive genes in *Plasmodium falciparum* during nutrient stress condition.

4.2 Material and methods

4.2.1 Nutrient stress induction

Parasites were synchronized at early ring stage using 5% sorbitol. Nutrient stress was given once parasites reach late ring (~16 hpi) for a period of 6 hours (~22 hpi). Nutrient stress was induced by growing the parasites in RPMI1640 medium supplemented with 25 mM HEPES, 0.5% AlbuMAX II, 1.77 mM sodium bicarbonate, 0.5 gram/liters glucose, 100 μM hypoxanthine and 12.5 μg ml^{-1} gentamicin sulfate at 37°C.

4.2.2 Antibodies

Anti-Rabbit IgG (OSB PM035) and previously generated PfGCN5 antibody were used for immunoprecipitation and Western blotting. PfGAPDH antibody was generated using purified

His-tagged PfGAPDH recombinant protein. PfGAPDH antibody was purified using the using affinity chromatography on the sulfolink resin.

4.2.3 PfGAPDH cloning, expression and purification

Full length PfGAPDH DNA sequence was amplified from parasite genomic DNA using gene specific primers. The PCR-amplified fragment was cloned in frame with 6x-His tagged fusion protein in pET28a+ plasmid using NdeI and XhoI restriction enzymes. Cloning was confirmed using DNA Sanger sequencing. The cloned plasmid (pET28a+_PfGAPDH) was transformed into *E.coli* BL21 (DE3) for protein expression. Expression was induced using 0.5 mM isopropyl-1-thio-β-d-galactopyranoside (IPTG) at an optical density of 0.6 at 600 nm for 5 hrs at 25^0C. Bacterial pellet was lysed using sonication buffer (10mM Tris Cl pH 8.0, 300 mM NaCl, 10% Glycerol, 1X PIC, 1X PMSF). Recombinant PfGAPDH was purified using Ni-NTA beads (Qiagen). Protein was eluted using different concentration of imidazole. Protein purification was checked using SDS-PAGE. Protein expression was confirmed using Anti-His Western blotting. After purification, protein was dialyzed in sonication buffer and stored at 4^0C. PfGCN5 (HAT and bromodomain) cloned in pGEX-4T1 plasmid was used for expressing glutathione S-transferase (GST) fusion protein. Expression was induced at an optical density of 0.6 at 600 nm, with 0.5 mM IPTG for 5 hrs at 25^0C. Protein was purified using glutathione sepharose 4B beads (GE healthcare life science). 20 mM concentration of reduced glutathione was used for protein elution. Purified proteins were dialyzed and stored at -20^0C.

4.2.4 Recombinant protein interaction assay

Recombinant purified PfGCN5-HAT and Bromodomain (GST tagged) and PfGAPDH (His tagged) were incubated together overnight. PfGAPDH and GST proteins were also incubated similarly as a control. Anti-GST antibody was added for 12 hours at 4^0C. Dynabeads Protein G (Invitrogen) beads were added to the incubated proteins for 4 hours at 4^0C on slow rotation. Following incubation beads were washed using washing buffer (10 mM Tris-Cl pH 8.0, 300 mM NaCl, 10% Glycerol, 10 mM Imidazole, 1X- PIC, 1X-PMSF) and eluted using 1M glycine, pH 2.5. Eluted sample was run of SDS-PAGE followed by Western blotting. Blot was incubated with Anti-His antibody to test whether PfGAPDH was pulled down using PfGCN5 protein.

4.2.5 Nuclear and cytoplasmic fractionation

Saponin lysed parasite pellet was resuspended in 500 µl of ice-cold lysis buffer (10mM HEPES pH-7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.65% NP40, 1 mM DTT, 2X PMSF) and incubated on ice for 30 min with intermittent mixing. The mix was spun at 13000 rpm for 20 mins at 4°C. The supernatant was collected and labelled as cytoplasmic extract. Parasite pellet was washed three times by re-suspending it in 500 µl of cytoplasmic buffer, incubating on ice for 5 min, spinning it at 13000 rpm for 10 min and discarding the supernatant. The pellet was then washed twice by similar method as mentioned above but by using 500 µl of cold 1X PBS (with 1X PMSF added) per wash. The pellet was resuspended in 3 volumes of nuclear buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA and 1 mM DTT) and incubated on ice for 30 min. This was followed by centrifugation at 13000 rpm for 20 mins at 4°C, the supernatant was removed and labeled as nuclear extract. Histone H3 was used a nuclear and actin as cytoplasmic control.

4.2.6 **Acetylation assay**

Saponin lysed 3D7 parasite pellets were used to isolate nuclear extract using the fractionation protocol. Acetylation assay was performed using recombinant PfGAPDH protein. Since full length PfGCN5 was difficult to clone and express in bacterial system, we performed pull down of PfGCN5 native protein using specific antibody. The pulled complex which contain PfGCN5 was used for the acetylation assay. Acetylation assay was performed using HAT buffer (250 mM Tris-Cl (pH 8), 50% glycerol, 0.5 mM EDTA, 5 mM DTT, 250 mM KCl, 100 mM Na-Butyrate, 1X PIC). Incubation for acetylation assay was done at 30^0C for 1 hour. Acetylation levels on recombinant PfAlba3 protein were assessed using pan acetyl antibody (Abcam, ab22550).

4.3 Results

4.3.1 PfGCN5 is upregulated during nutrient stress condition

Previous study from our lab showed that PfGCN5 regulates expression of stress responsive genes under temperature and artemisinin exposure in *P. falciparum*. Further to gain insights into the role of PfGCN5 under nutrient stress condition, we subjected ring stage parasites (16 hours post synchronization) to nutrient stress condition ($1/4^{th}$ of normal glucose

concentration) for 6 hours followed by which they were harvested (Figure 4.1A). Nutrient stress condition used for the experiment did not affect cell cycle progression of the parasites. Complete removal of glucose from the culture medium resulted in substantial decline in parasites progression, ultimately resulting in their death. Histone acetyltransferases are known to result in gene activation by acetylation of histones N terminal tail. Therefore, we assessed the expression level of all known histone acetyltransferases along with PfGCN5 in *P. falciparum* under nutrient stress condition. Interestingly, PfGCN5 showed highest upregulation in comparison to other histone acetyltransferases (Figure 4.1B).

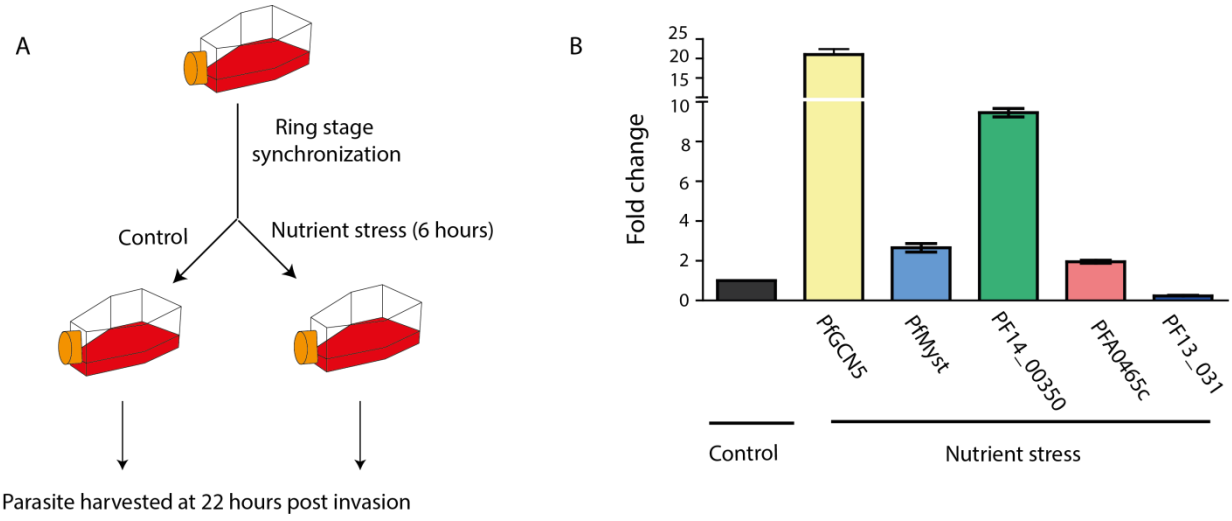


Fig. 4.1: PfGCN5 is upregulated highest among all known HATs during nutrient stress condition. *A) Schematic represents the induction of nutrient stress condition to mimic hypoglycemia observed during malaria infection. Parasites were synchronized during early ring stage using sorbitol treatment. Nutrient stress was given at 16 hpi for period of 6 hours. Parasites were harvested at 22 hpi for qRT-PCR, RNA sequencing and ChIP sequencing. B) RT-qPCR result showing highest upregulation of PfGCN5 among all known HATs under nutrient stress condition. Experiment performed in three biological replicates using 18S rRNA as an endogenous control.*

Further to understand global transcriptional deregulation, we performed RNA-sequencing (RNA-Seq) under nutrient stress and control conditions. In total 303 genes were found to be significantly deregulated (>2 folds); 191 genes upregulated and 112 genes downregulated during nutrient stress condition (Figure 4.2A). Moreover, gene ontology analysis of the upregulated genes revealed enrichment of metabolism related pathways under nutrient stress

condition (Figure 4.2B). Since evidence from previous reports suggested that virulence of the parasites is often modulated under stress condition, we wondered whether var gene expression is altered under nutrient stress condition. Interestingly, several var genes were found to be upregulated and a few displayed switching during the nutrient stress condition (Figure 4.2C). Together, it suggests a significant upregulation of PfGCN5 and global transcriptional deregulation under the nutrient stress condition.

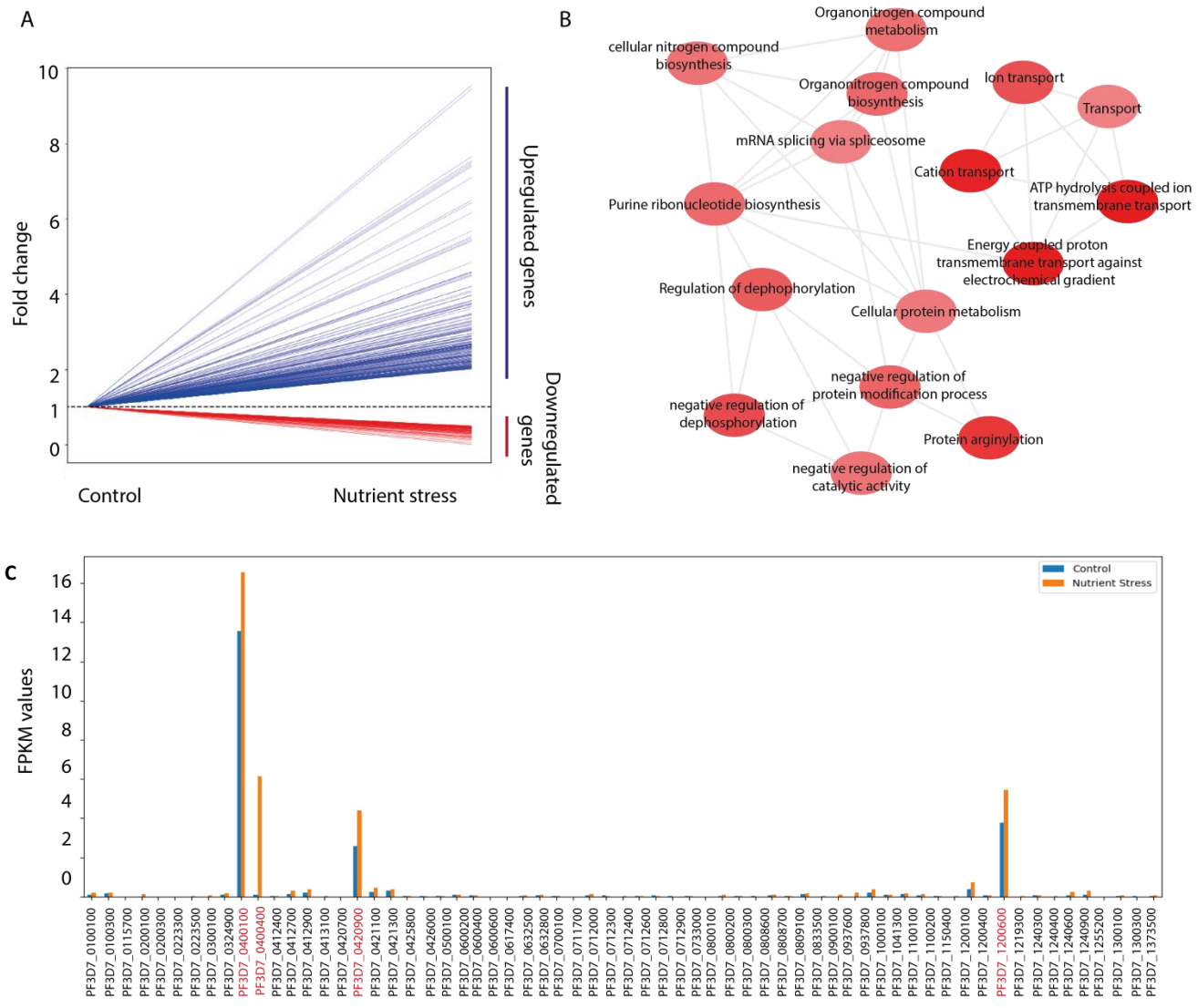


Fig. 4.2: Gene deregulation during nutrient stress condition. *A) Profile plot depicting differential gene expression under nutrient stress condition. RNA sequencing was performed during control and nutrient stress condition. Blue indicates the 191 genes which are upregulated and red indicates 112 down-regulated genes. B) Gene ontology (GO) analysis of*

upregulated genes from RNA seq analysis. GO analysis indicates an overrepresentation of major metabolic pathways like purine ribonucleotide biosynthesis, cellular protein metabolism, organonitrogen compound biosynthesis etc. C) Histogram showing transcriptional deregulation of var genes under nutrient stress condition. Genes which are upregulated during nutrient stress condition are indicated in red color. PF3D7_0400400 was found to be expressed only during nutrient stress condition.

4.3.2 PfGCN5 associates with metabolic genes during nutrient stress condition

Further, to dissect the role of PfGCN5 in nutrient sensing as it is upregulated under nutrient stress condition, we performed chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq). Genome-wide analysis of PfGCN5 suggested that it is associated with 554 genes mainly at gene body and promoter regions during nutrient stress condition (Figure 4.3A). Interestingly, genes associated with antigenic variation and immune responses were found to be targets of PfGCN5 in both control and nutrient stress conditions (Figure 4.3B). However, PfGCN5 was found to be exclusively associated with metabolic genes during nutrient stress condition indicating its involvement in transcriptional regulation of metabolic genes (Figure 4.3C).

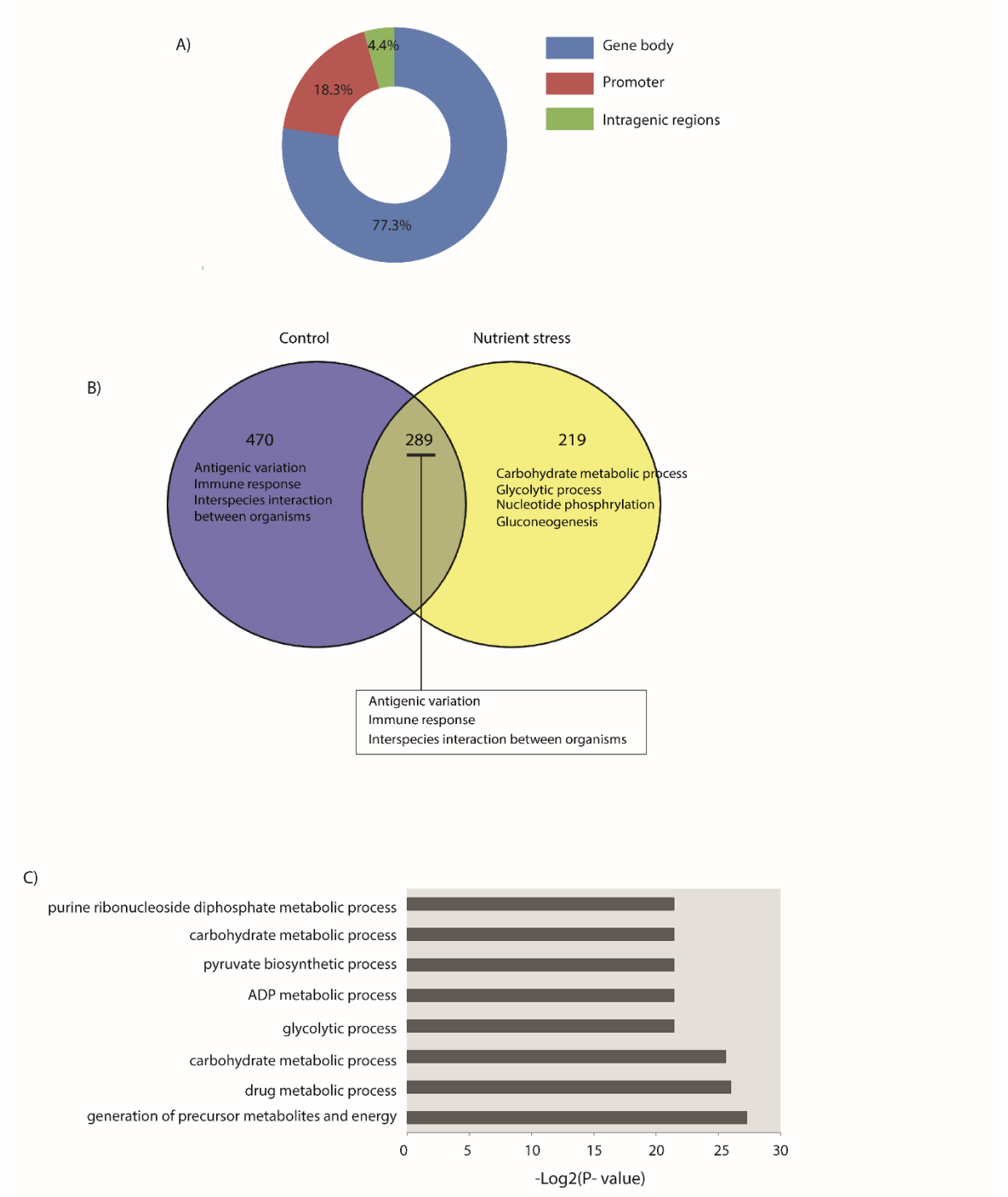


Fig. 4.3: ChIP sequencing of PfGCN5 during nutrient stress condition. *A) Donut chart showing the ChIP-seq based enrichment of PfGCN5 majorly over gene body and promoter regions. Maximum binding sites are present on the gene body followed by promoter and intergenic region. B) Venn diagram showing number of genes bound by PfGCN5 during control and nutrient stress conditions. Genes associated with antigenic variation and immune responses were found to be targets of PfGCN5 in both control and nutrient stress conditions.*

PfGCN5 was bound to metabolic genes exclusively during nutrient stress condition. C) Gene ontology analysis of PfGCN5 bound genes indicates enrichment at metabolic pathways.

Next, we looked at the expression level of PfGCN5 bound genes under nutrient stress condition. Interestingly, expression level of only metabolic genes but not all PfGCN5 bound genes showed significant upregulation upon nutrient stress condition (Figure 4.4A). Furthermore, we measured the expression of PfGCN5 bound genes in the presence and absence of garcinol, an inhibitor of PfGCN5. We observed that PfGCN5 bound genes, which are upregulated upon nutrient stress condition either failed to upregulate or showed downregulation upon garcinol treatment during nutrient stress condition (Figure 4.4B).

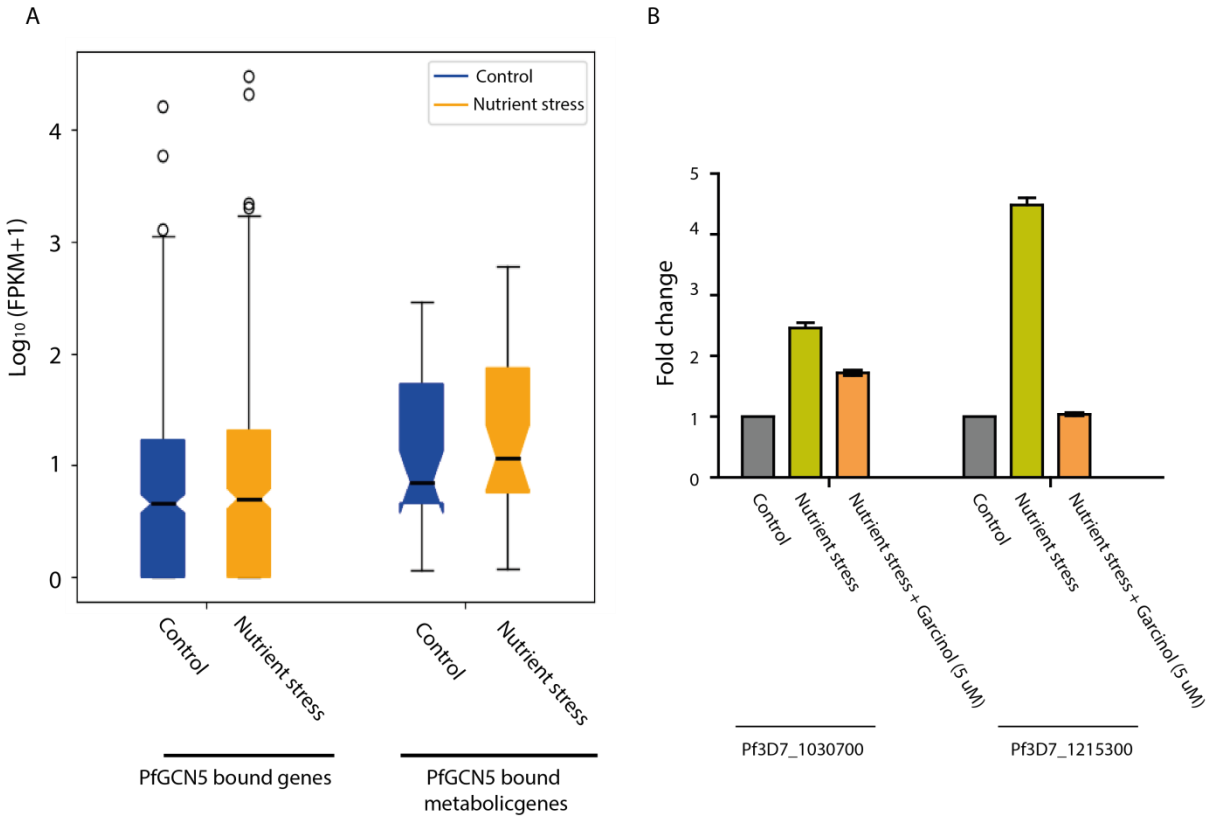


Fig. 4.4: PfGCN5 is associated with metabolic genes. *A) Box and whisker plot showing a significant upregulation of PfGCN5 bound metabolic genes as opposed to all the PfGCN5 bound genes under nutrient stress condition. B) RT-qPCR performed to assess the transcript levels of genes which are upregulated under nutrient stress condition in presence or absence of PfGCN5 inhibitor garcinol (10 µM).*

Thus, our data suggests causative function of PfGCN5 but does not rule out the possibility of inhibition of other histone acetyltransferases by garcinol. Moreover, PfGCN5 was also found to occupy the PfGAPDH locus during the nutrient stress condition, indicating the direct

regulation of PfGAPDH by PfGCN5. These results show that PfGCN5 is bound to metabolism related genes during nutrient stress condition and possibly regulate their expression to maintain homeostasis.

4.3.3 PfGCN5 interacts with different metabolic enzymes

Next, in order to gain insights into the mechanism via which PfGCN5 could be involved in nutrient sensing, we looked at its interacting partners. Earlier, we have identified PfGCN5 interactome using immunoprecipitation followed by mass spectrometry, which comprised of several metabolic proteins as the high confidence hits (Figure 4.5A) along with other expected chromatin organization and stress/stimuli responsive partners. It is well established that glycolytic proteins perform extracellular functions like adherence, modulation of homeostatic and immune system, and nutrient acquisition in parasites [269-271].

We wondered if metabolic proteins perform a moonlighting function of gene regulation in complex with PfGCN5 in *P. falciparum*. Since previously published acetylome data suggested that various proteins involved in glycolysis are acetylated, we decided to examine if PfGCN5 can acetylate metabolic proteins [272]. Our mass spectrometry data showed PfGAPDH as the most confident interacting partner of PfGCN5. Additionally, studies from plant system showed that upon acetylation by GCN5 paralog PCAF, GAPDH translocate into the nucleus and binds to the promoter of glycolytic genes and regulates their expression upon glucose stimulus [273]. Also, we found that the RNA expression level of PfGAPDH increases (>2 folds) during nutrient stress condition (Figure 4.5B). Hence, we narrowed down to PfGAPDH as the metabolic protein of interest for further analysis.

A

Metabolic pathway	Genes
Glycolysis / Gluconeogenesis	phosphoglycerate kinase hexokinase pyruvate kinase glyceraldehyde-3-phosphate dehydrogenase L-lactate dehydrogenase ATP-dependent 6-phosphofructokinase enolase fructose-bisphosphate aldolase
Pentose phosphate pathway	ATP-dependent 6-phosphofructokinase phosphoribosylpyrophosphate synthetase fructose-bisphosphate aldolase
Fructose and mannose metabolism	hexokinase ATP-dependent 6-phosphofructokinase triosephosphate isomerase fructose-bisphosphate aldolase
Purine metabolism	purine nucleoside phosphorylase pyruvate kinase adenylate kinase phosphoribosylpyrophosphate synthetase inosine-5'-monophosphate dehydrogenase hypoxanthine-guanine phosphoribosyltransferase

B

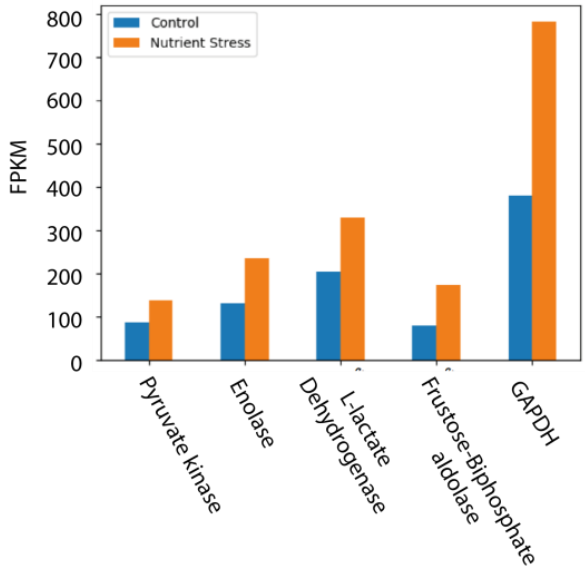


Fig. 4.5: PfGCN5 interacts with different metabolic enzymes *A) Table showing list of PfGCN5 interactome known to play role in different metabolic pathways. B) Histogram showing the RNA expression level of a few metabolic genes under nutrient stress condition.*

In order to see the distribution of PfGAPDH, we performed nuclear cytoplasmic fractionation. As expected, we found PfGAPDH to be majorly localized in the cytoplasm but surprisingly it was also found in the nucleus under normal condition (Figure 4.6A). Moreover,

immunofluorescence analysis using PfGAPDH specific antibody also showed both nuclear and cytoplasmic localization of PfGAPDH protein (Figure 4.6B).

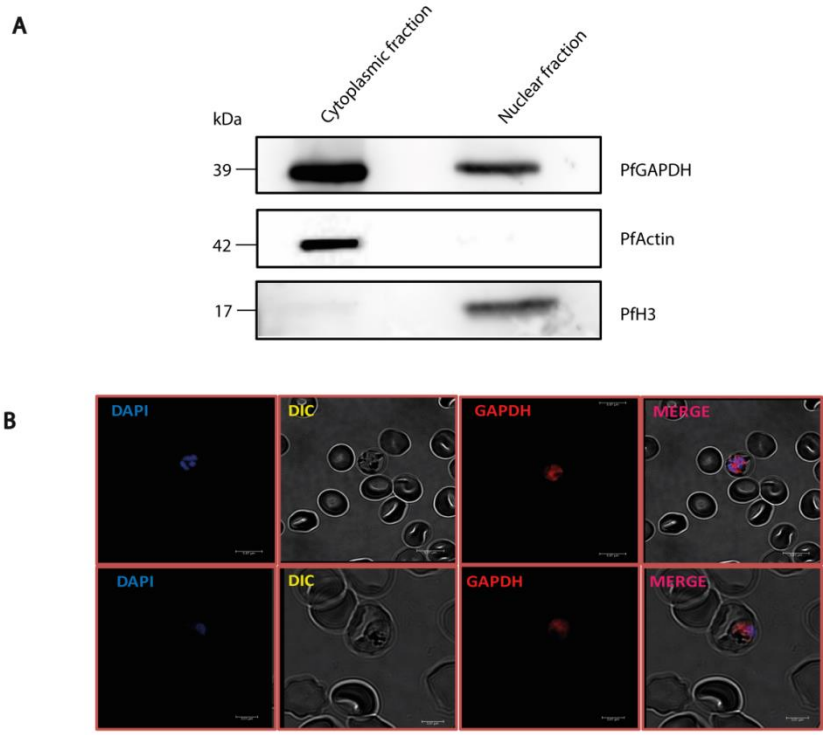


Fig. 4.6: PfGAPDH is localized to both cytoplasm and nucleus *(A) PfGAPDH distribution using nuclear and cytoplasmic fractionation. PfGAPDH was found to be distributed in both cytoplasm and nucleus. Histone H3 and actin are used as nuclear and cytoplasmic controls, respectively. (B) Immunofluorescence analysis showing localization of PfGAPDH in both nucleus and cytoplasm using PfGAPDH specific antibody.*

To further validate the interaction between PfGCN5 (HAT and bromodomain) and PfGAPDH, we performed *in vitro* interaction assay using recombinant PfGAPDH and PfGCN5 proteins. Interestingly, PfGCN5 and PfGAPDH interact *in vitro* (Figure 4.7A). Interaction was also validated by performing immunoprecipitation using PfGAPDH antibody and probing with anti-PfGCN5 antibody. Since PfGAPDH was found to be acetylated in *Plasmodium* acetylome data, we decided to inspect whether PfGCN5 acetylates PfGAPDH. Interestingly, recombinant PfGAPDH was found to be acetylated with PfGCN5 (Figure 4.7B). Thus, PfGCN5 interacts and acetylates PfGAPDH, possibly in the nucleus. Further studies need to be carried out in order to confirm the role of PfGAPDH in the nucleus. It is

possible that PfGAPDH in complex with PfGCN5 modulate gene expression via direct chromatin interactions.

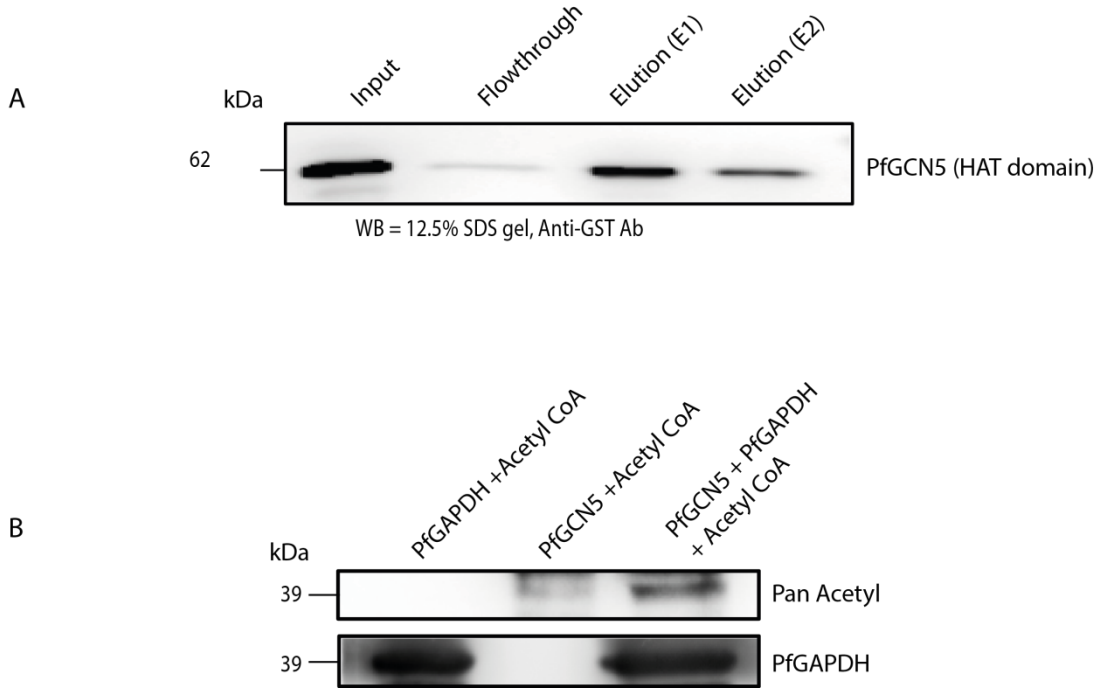


Fig. 4.7: PfGCN5 interacts and acetylate PfGCN5. *A) In vitro interaction assay of recombinant purified PfGCN5 (GST tagged) and PfGAPDH (His tagged). Interaction was confirmed with Western blotting using Anti-His antibody. B) In vitro acetylation assay was performed to test whether PfGCN5 acetylates recombinant PfGAPDH protein. PfGAPDH acetylation levels were assessed using pan-acetyl antibody.*

4.4 Discussion

The life cycle of an intercellular parasite for instance, *Plasmodium falciparum* is intimately connected to its hosts, primarily for obtaining resources in the form of nutrients. Nutrients are required by the parasite as source of energy not only for its development but for also regulating gene expression. Since infection leads to a shared pool of nutrients between the parasite and hosts, any modulations in the critical levels of nutrients can have deleterious effect on both. So in order to cause successful infection, parasites must rapidly sense, respond and adapt to any nutrient fluctuation within the host milieu. It is evident that the parasites are able to take cues from the environment where the glucose levels may fall as low as 50mg/dl. Since canonical nutrient sensing pathways are presumably absent and tricarboxylic acid

(TCA) cycle is known to play a redundant role during the parasite's intra-erythrocytic life cycle, it raises questions as to how the parasites are able to sense the nutrient status of their host. Adding to the complexity, parasites are known to drive energy solely via catabolism of host cell haemoglobin. In this scenario, parasites must strategize low energy – high efficiency mechanisms that help them respond to unanticipated changes in the host nutrient milieu to avoid cost associated in the form of reduced virulence and replication rates. Metabolism related proteins have been implicated in boosting the virulence of the parasites but importantly this requires global transcriptome rearrangement under unfavorable conditions. Only a subset of the heterogeneous population which is pre-programmed to achieve molecular reprogramming is able to survive such nutrient stress condition. Hence it is possible that the surviving population senses the fluctuation in the nutrient milieu, further transmitting the information downstream, which could dictate the expression of genes consequently modulating parasite's virulence.

In this study, we attempted to elucidate the role of a histone acetyltransferase (HAT); PfGCN5 in transcriptional regulation during nutrient stress condition. We found significant upregulation in the transcript level of both PfGCN5 and PfGAPDH during nutrient stress condition. Interestingly, PfGCN5 was found to be associated with different metabolic genes, which are upregulated upon nutrient stress condition. Fractionation and localization studies suggested that PfGAPDH is distributed in both cytoplasm and nucleus. Moreover, PfGCN5 was found to interact with PfGAPDH and also acetylate it *in vitro*. Thus, it is possible that during nutrient stress PfGAPDH is further translocated in the nucleus, where it is acetylated by PfGCN5. Acetylated PfGAPDH, in complex with or without PfGCN5, might regulate the expression of metabolic genes (Figure 4.8). Further systematic investigation of PfGCN5 and PfGAPDH mediated transcriptional regulation during nutrient stress condition would explore some of the unanswered questions i.e. (i) does nutrient stress change the distribution of PfGAPDH in cytoplasm and nucleus? (ii) Whether PfGAPDH interacts with the chromatin to regulate gene expression? (iii) if yes, is it a PfGCN5-PfGAPDH complex? In conclusion, understanding of the nutrient sensing mechanisms would unravel molecular networks modulating the parasite's virulence and pave the way to combat the disease.

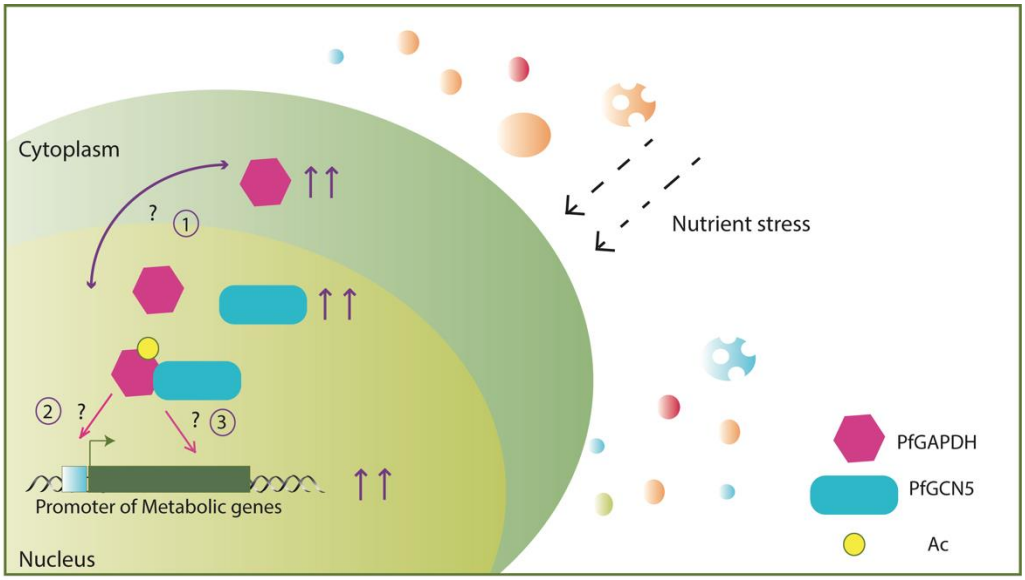


Fig. 4.8: Mechanism proposed for nutrient sensing and transcriptional regulation by PfGCN5 in P. falciparum. *PfGAPDH was found to be present in both nucleus and cytoplasm under normal growth condition. Furthermore, PfGCN5 interacts and acetylates PfGAPDH most probably in nucleus. Possibly, PfGAPDH distribution in cytoplasm and nucleus is altered during nutrient stress condition (1) PfGCN5-PfGAPDH complex binds at the promoters (2) or gene body (3) of metabolic genes leading to upregulation of gene expression.*

Summary: Parasites are exposed to different forms of stresses during erythrocytic life cycle. Nutrient stress is one of the stresses which directly affect the development and virulence of parasite drastically. Understanding transcriptional regulation during nutrient stress conditions can directly helps us in understanding stress response machinery which helps parasite in overcoming unfavorable condition. Interestingly, in our study we found that PfGCN5 is associated with various genes which are activated during nutrient stress condition. Furthermore, PfGCN5 also interacts with various metabolic enzymes. We studied the interaction of PfGCN5 and PfGAPDH. We found that PfGAPDH is present in both nucleus and cytoplasm and it is acetylated via PfGCN5. Further studies need to be conducted to investigate the physiological significance of PfGAPDH acetylation and its role within the nucleus.

Chapter 5: Stress mediated cellular heterogeneity in *P. falciparum*

5.1 Introduction

Almost all cellular systems are heterogeneous in their composition. The cell-to-cell heterogeneity can be attributed to various environmental and physiological conditions such as availability of nutrients, temperature fluctuations, cell-cycle progression and different morphological states [274-279]. Both single and multicellular organisms employ heterogeneity in their population to fight against unfavorable conditions [275, 276] (Figure 5.1). Within cellular level, heterogeneity can exist at different level like genomics, transcriptomics, proteomic etc. Advent of single-cell RNA sequencing (scRNA-seq) has been instrumental in unraveling the cell-to-cell heterogeneity in the population at transcript level. It provides information pertinent to differential gene expression profile of cells within a population leading to cellular heterogeneity.

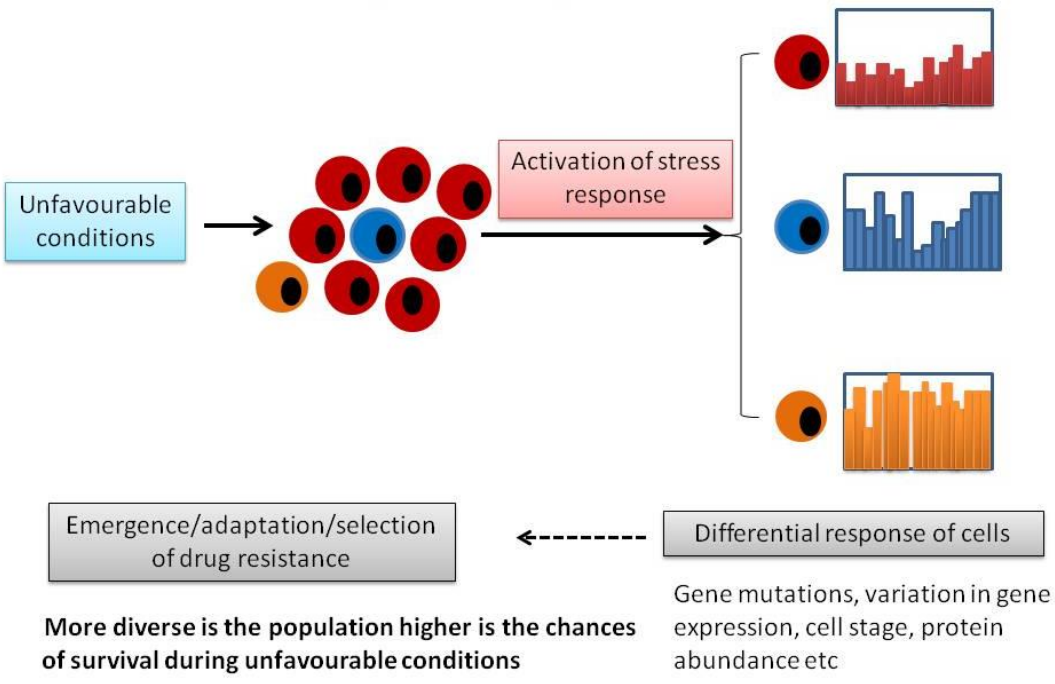


Fig. 5.1: Schematic representation showing importance of heterogeneity within the parasite population. *More heterogeneous is the population better is the chance of survival. Heterogeneous population results in the differential response of the parasites to unfavorable*

condition. Heterogeneity within a population also play crucial role in emergence of resistance.

5.1.1 Single cell RNA sequencing

As the name indicates the single cell RNA sequencing is the technique of obtaining transcriptome information of the individual cells present within a population [280, 281] (Figure 5.2). Bulk RNA sequencing provides the average gene expression profiles of the whole population therefore it undermines the heterogeneity present [280]. It provides information on cell-to-cell variation within the population. It is extensively used for studying the function of heterogeneous population during development and differentiation [280, 282]. It is also used to study cellular response under different physiological conditions. First single cell RNA sequencing (scRNA-seq) was carried out on a blastomere by Azim Surani's lab in 2009 [283]. With the help of scRNA-seq they were able to detect 5270 more genes than previously identified by microarray analysis for hundreds of blastomeres [283].

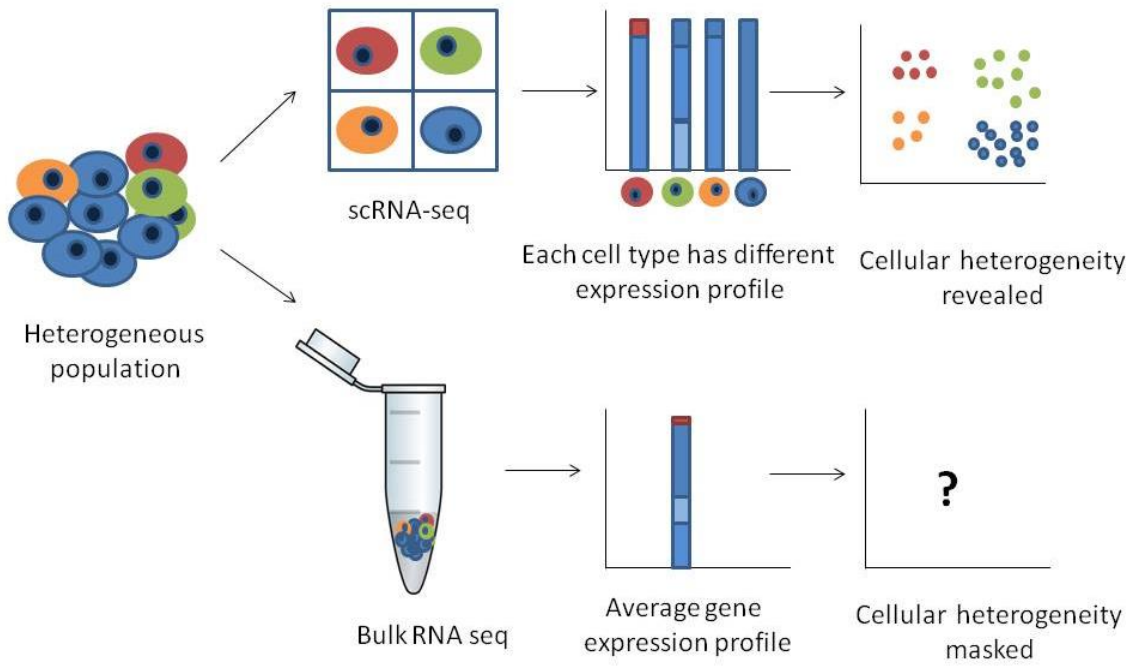


Fig 5.2: Schematic representing advantages of single cell RNA sequencing. *Cellular heterogeneity is masked during bulk RNA transcriptomics. Single cell RNA sequencing provides information about the cellular heterogeneity by sequencing individual cells within the population.*

5.1.2 Single cell isolation techniques

Before proceeding with the RNA sequencing of single cells it is important to isolate single cell from the tissue. There are several tools to isolate single cells. Limiting dilution is one of the most common methods of single cell isolation used earlier [281]. Micromanipulation is another method to obtain single cells [281]. Currently, flow–activated cell sorting (FACS) is used commonly for the isolation of single cells [281]. Inorder to obtain single cells, they are labeled fluorescently either with the help of any surface antibody or with the help of labeling dyes like mitotracker, lysotracker etc. Microfluidic based technologies can be also used for isolation of single cells [281].

5.1.3 10X Genomics

The 10X Genomics Single Cell systems is the platform which performs single cell transcriptomics of many different cells at a time. It provides highest capture efficiency (upto 65%). It allows encapsulation of single cell with micro-bead carrying oligos into an oil droplet. Each micro-bead carries different barcodes (total 750,000) (Figure 5.3). This helps in the maintaining the identity of the single cells. 10X controller allow single-Poisson distribution loading which results in high capture efficiencies in comparison to other available protocol used currently in single cell sequencing. 10X Genomics is much faster as well as eight samples can be processed per batch. Data analysis can be performed using Cell Ranger and Loupe Cell Browser software which are freely available.

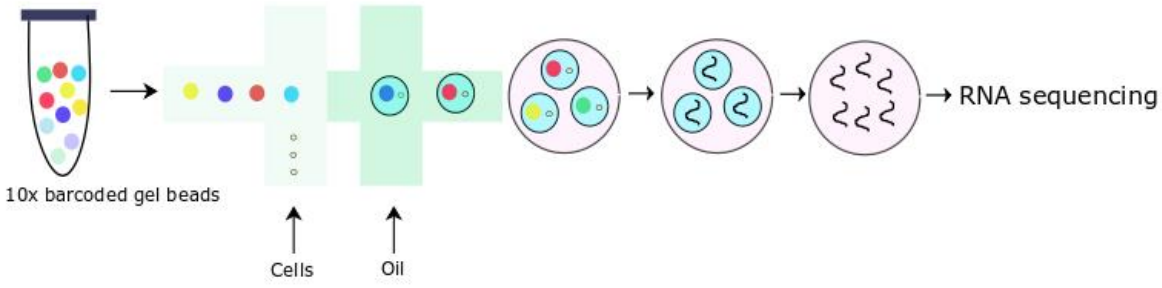


Fig 5.3: Schematic representation of the work flow in 10X Genomics for single cell RNA sequencing. *Each bar-coded gel bead is unique. Oil droplets encapsulate one barcoded beads along with single cell.*

5.1.4 Steps for preparing single cell library using 10x Genomics

Gel bead in emulsion (GEMS) are generated using combination of Single Cell 3' gel beads, partitioning oil and master mix containing cells. Chromium Chip is used to obtain GEMs containing single cell. Inorder to obtain single cell resolution, master mix containing cells is delivered at a limiting dilution such that majority of GEMs (90-99%) contain no cell and rest contain only single cell. Following this, gels beads are dissolved to release primers containing illumine TruSeq Read 1, 16 nucleotide 10x Barcode, 12 nucleotide unique molecular identifier and 30 nucleotide poly (dT) sequence. Once the primers are released, cDNA synthesis is initiated with the help of master mix containing reverse transcriptase. Silane magnetic beads are used for the purification of the first strand of cDNA before synthesis of full length cDNA. This is followed by enzymatic fragmentation; End Repair, A tailing and adaptor ligation. TruSeq read 1 is added during GEM incubation. P5, P7, a sample index and TruSeq Read 2 are added via End Repair, A tailing, adaptor ligation and PCR.

5.1.5 Single cell RNA sequencing in *Plasmodium*

The persistent infection and clinical manifestation of malaria is associated with the development of parasite in red blood cells (RBCs) during asexual intra-erythrocytic cycle (IEC). The transmission of malaria parasite to mosquito depends on intra-erythrocytic differentiation to gametocytes in human host. Thus, parasite maintains balance in transmission and persistent infection by an unknown mechanism. Recent single-cell RNA sequencing (scRNA-seq) suggests commitment to gametocytes during the preceding cycles by epigenetic regulation of master regulator of sexual development, AP2-G [284-286]. However, the environmental cues and the hitherto mechanism therein, which initiate the gametogenesis are not yet clear. AP2-G is considered as the master regulator of sexual development in *Plasmodium* [114]. At a time only a small set of population within the asexually developing parasites are primed for sexual commitment through AP2-G upregulation [287]. Bulk RNA sequencing usually masks the gene expression profile of these sexually committed parasites. Inorder to study the transcriptional changes during induction of AP2-G expression, single cell RNA sequencing was performed using Drop seq. Interestingly they were able to identify other AP2 transcription factors, histone modifying enzymes and regulators of nucleosome positioning upregulated in the sexually committed parasites [285].

Phospholipid LysoPC is known to play an important role in triggering sexual commitment in *Plasmodium*. Earlier studies have suggested that depletion of LysoPC in plasma can result in activation of AP2-G [288]. Brancucci *et al.* investigated the events upstream of master regulator AP2-G activation through depletion of LysoPC [288]. They also identified AP2 transcription factors other than AP2-G and histone methyltransferase to play an instrumental role during sexual commitment and development in parasites. This study also showed the importance of another enzyme, choline kinase in the activation of AP2-G transcription factor [288].

Reid and Talman *et al.* standardized the protocol for single cell RNA sequencing with higher depth to understand both heterogeneity as well as the functional variation [286]. This method enables the full length RNA sequencing instead of 3' RNA sequencing as performed in earlier studies. This kind of RNA sequencing will help in exploring var genes expression and isoform present in different population of the cells. Apart from the transcriptional variation identified through this study, they were able to identify the transcriptional variation between the male and female gametocyte. They also identified the difference in the multigene expression pattern in male and female gametocytes [286]. Recently Howick *et al.* performed single cell RNA analysis across different stages of malaria life cycle in both vertebrate and invertebrate host [289]. This study dissected the transcriptional pattern of marker genes for different stages, host environment and growth and sexual commitment. ScRNA-seq data also enable to infer the possible function of the unannotated genes present in *Plasmodium*. Moreover, investigation of the developmental cycle for three different species enables cross species comparison at single cell level. Furthermore, a protocol for preserving clinical parasites for scRNA-seq to identify the stages and mixed species infection at a single cell level is also developed [289].

5.1.6 Heterogeneity in *Plasmodium* population under stress conditions

P. falciparum is exposed to different environmental conditions during its life cycle in two different hosts. Moreover, it is also constantly exposed to different physiological conditions such as low glucose level (hypoglycemia), high temperature (fever) and oxidative stress (haemoglobin degradation and drug therapy) during IEC cycle [164, 169, 171]. Under such adverse conditions some cells in the isogenic population survive whereas others do not. Previous studies have indicated presence of heterogeneity within parasite population. Such

heterogeneity is present in both unicellular and multicellular organism at different levels and it helps in better survival of the organism during unfavorable conditions [276]. Multicellular organism are more advanced and developed in terms of fighting these fluctuations where heterogeneity is key to differentiation resulting in the diverse functions. In unicellular organism presence of heterogeneity is a population level survival strategy [276]. Single cell organisms need to develop tight machinery for fighting against the unfavorable conditions. Slight change in the ability to respond to different stress conditions can result in competitive advantage over other individuals in the population. Ecological studies have suggested that survival chances of a particular population during extreme conditions are more if they have diverse populations [275]. Thus, cells which are less fit (stress-tolerant) in a particular population can have better survival chances during unfavorable conditions. But whether this stress-tolerant cells have a fully mounted stress response or they emerge from partial response is unclear. Understanding cell-to-cell heterogeneity in stress survival has broad applications in stress adaptation and drug-resistance emergence.

One of the most common stresses experienced by *Plasmodium* is heat stress [167, 169, 290, 291]. During its intraerythrocytic life cycle, there are cyclic episodes of fever due to the release of merozoites. During the fever, temperature peaks as high as 41 degree Celsius for a period of 2-6 hours [167, 169, 291]. Early ring stage of parasite growth is more resistant whereas later stages are more fragile to heat stress. Despite of such variation in temperature during its life cycle, *Plasmodium* manages to overcome these unfavorable conditions resulting in successful infection. Stress response machinery is known to play an important role in establishment of successful infection inside the host. Earlier, population studies have identified various proteins playing an important role in building temperature stress response. Increase in temperature is also known to accelerate the growth and development of the parasites. Moreover, exposure of parasites to higher temperature results in increase of cytoadherence to CD36 and ICAM-1 receptors [169]. This is attributed to the fact that during heat stress there is an increase in virulence (var) gene expression which helps in the binding of infected red blood cells to the endothelial receptors resulting in cytoadherence [168, 169]. Recent reports also suggest stress responses as crucial mediator of artemisinin resistance in *P. falciparum* [187, 292].

Plasmodium is known to have tight gene regulation which is governed at multiple levels including chromatin structure, transcription and translation. This in turn, helps in timely expression of different genes required at various stages of the parasite growth [293]. Despite tight-gene regulation there is a possibility of transcriptional heterogeneity in the parasite population. In this study, we have performed scRNA-seq under control and temperature stress condition to understand the cell-to-cell heterogeneity within the *Plasmodium* population. Using this approach, we identified a combination of gene signatures of cellular heterogeneity and stage transition during stress adaptation. Interestingly, we identified a subset of parasites primed for gametogenesis, which is activated upon temperature stress condition. Moreover, we identified a rare population of cells, which is only emerged during the stress condition, showing the reactive state of the pathogen against the temperature stress condition. Thus, our scRNA-seq analysis reveals important insights into the cell-to-cell heterogeneity in parasite population under temperature stress condition that will be instrumental towards mechanistic understanding of cellular adaptation, population dynamics and drug-resistance emergence in *P. falciparum.*

5.2 Materials and Methods

5.2.1 Synchronization of parasites

Parasites were synchronized using two methods of synchronization. During the schizont stage, parasites were synchronized using 63% percoll density gradient. Parasites were washed once with media before starting the synchronization. Parasites in 3 ml culture were layered over the 5 ml of 63% percoll in a 15 ml falcon. This was followed with centrifugation at 2200g (acceleration was set at 3 and deceleration was set at 2) for 15 minutes. Interface was collected which consist of RBCs having schizont stage parasites. Collected parasites were washed using RPMI media before keeping back in culture along with fresh RBCs. Giemsa smear was prepared to confirm the synchronization. For the early stage synchronization, parasites were washed using RPMI media. Parasites were resuspended in 10 volumes of 5% sorbitol. Parasites were vortexed for 30 sec followed by incubation in 37^0C water bath for 10 mins. Parasites were again vortexed for 15 sec. 5 ml RPMI media was added to dilute the sorbitol followed by centrifugation at 500 g for 5 minutes at RT. Parasites were washed again with RPMI to get rid of any sorbitol and lysed RBCs. Finally the parasites were kept back in culture in a new flask. Fresh RBCs were added to maintain hematocrit. Parasite growth was monitored using Giemsa staining of thin blood smear.

5.2.2 Stress induction

Parasites were synchronized at the early stage of the intraerythrocytic life cycle by sorbitol treatment. Giemsa staining was done to check the synchronization. Parasites were kept back in normal culture condition till they reach 17 hours post invasion. Parasites were kept at 40 degree Celsius for 6 hours. Control parasites were kept under normal culture condition. Parasites were harvested for the experiment at 23 hours post invasion.

5.2.3 Percoll gradient and isolation of single iRBCs

In order to get rid of the uninfected parasites we performed percoll gradient to separate uninfected RBCs from the infected RBC. Different percentages of percoll gradient were tested for the separation of early trophozoite from uninfected RBCs since 63% percoll was used for separation of schizont. 81% Percoll was finally used for the enrichment of the infected RBCs after the experiment. Infected RBCs were washed with 1X PBS twice to get rid of any dead cells and iRBCs were counted before proceeding with bar-coding.

5.2.4 scRNA-sequencing library preparation

Gel Beads-in-emulsion (GEMs) are generated by combining barcoded Single Cell 3' v3 Gel Beads, a master mix containing cells and partitioning oil onto Chromium Chip B. Cells are delivered at a limiting dilution in order to attain single cell resolution. Once the gel bead is dissolved and cells are lysed inside the oil droplet, primers are released which binds to the poly A tail of the mRNAs and helps in the cDNA synthesis. Primers contain an Illumina TruSeq Read 1, 16 nucleotide 10x barcode, 12 nucleotide unique molecular identifier (UMI) and 30 nucleotide poly (dT) sequence. Enzymatic fragmentation and size selection were used to optimize the cDNA amplicon.

5.2.5 scRNA-sequencing at NextSeq550 (Illumina)

Quality of the cDNA was estimated using Bioanalyzer and concentration of the sample was calculated using high sensitivity (HS) DNA Qubit kit. Transcriptome sequencing was performed using Illumina NextSeq 550 system (1x 150 bp read length) at Indian Institute of Science Education and Research, Pune**.**

5.2.6 RNA sequencing and Data analysis

Bulk RNA sequencing was performed using parasites harvested for RNA isolation after the stress induction. Total RNA isolation was performed using TRIzol reagent. Bioanalyzer was performed to analyze the quality of RNA before proceeding for library preparation. Three biological replicates were pooled together for performing RNA sequencing. The cDNA libraries were prepared for samples using Agilent SureSelect strand specific RNA library preparation kit. Transcriptome sequencing was performed using Illumina NextSeq 550 system (2x150 bp read length) at Indian Institute of Science Education and Research, Pune. Quality control of the RNA-sequencing reads was performed using FASTQC and reads were trimmed based on the quality estimates. RNA paired directional reads were mapped to GTF annotation file v41.0 of the *P. falciparum* genome using TopHat. SAMtools [294] were used for file handling and conversion. Cufflinks [295] (cuffmerge, cuffmerge and cuffdiff) programs were used for differential gene expression. MA plot is generated using 'R' software (http://r-project.org/). Gene ontology was performed using Plasmodb [173] (https://plasmodb.org/).

5.2.7 scRNA-sequencing library preparation and sequencing

1000 cells/µL dilution of 80% viable cells was loaded onto the 10x chip for library preparation using Single cell v3 chemistry. The libraries for both the control and the treated sample were multiplexed together and were sequenced using mid output 75 bp single end configuration flow cell on the Illumina NextSeq 550 system. Cell ranger count was run on samples using v41.0 *P. falciparum* genome (PF3D7) to align and summarize per cell barcode (cell) read counts for each sample. For control samples 4949 cells were sequenced at a depth of 88.8 million reads with 88.1% reads in cells assigning a median of 17.9k reads/cell. For treated sample, 6873 cells were sequenced at a depth of 89.3 million reads with 88% reads in cells assigning a median of 13k reads/cell.

5.2.8 scRNA-sequencing quality control and data analysis

The sparse matrices generated by 10x cell ranger count were read into R 3.5.3 using the Seurat 3.0.0 package. In order to remove potential duplets at a frequency of 5% and droplets with background RNA content, we removed cells with the top 5% and bottom 5% of total RNA molecules (or UMIs) from each sample. Thereafter, both the samples were combined using the Canonical correlation analysis using 10 principal components for finding

integration anchors followed by 20 principal components to integrate the data. 20 principal components were used to perform t-SNE projections and clustering at a resolution of 0.5 using the Find Neighbours and Find Clusters functions (Figure 5.4 and 5.5).

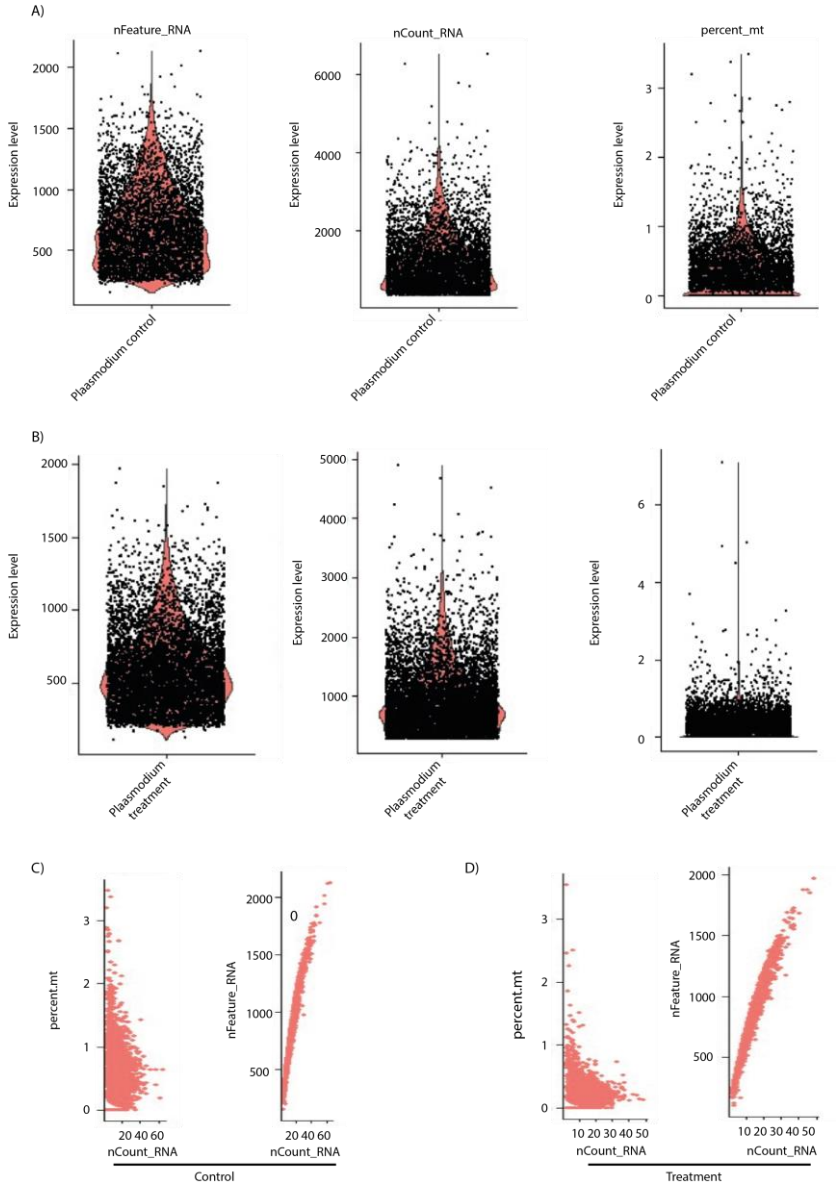


Fig 5.4 Sanity check plots to see the quality of the data obtained by single cell RNA sequencing. *(A and B) Sanity check plots of number of genes (nFeature_RNA), number of RNA molecules (nCount_RNA) and percentage of mitochondrial genes (genes beginning with "mal" in the annotation file) across individual cells in the dataset for both control and temperature stress sample respectively. (C and D) Sanity check plots to see any deviations from the expected relationship between number of RNA molecules (nCount_RNA) and*

percentage of mitochondrial gene expression (percent.mt); expected to be not correlated as shown; and number of genes (nFeature_RNA); expected to be linear because the deeper a cell is sequenced the more number of genes should be detected per cell.

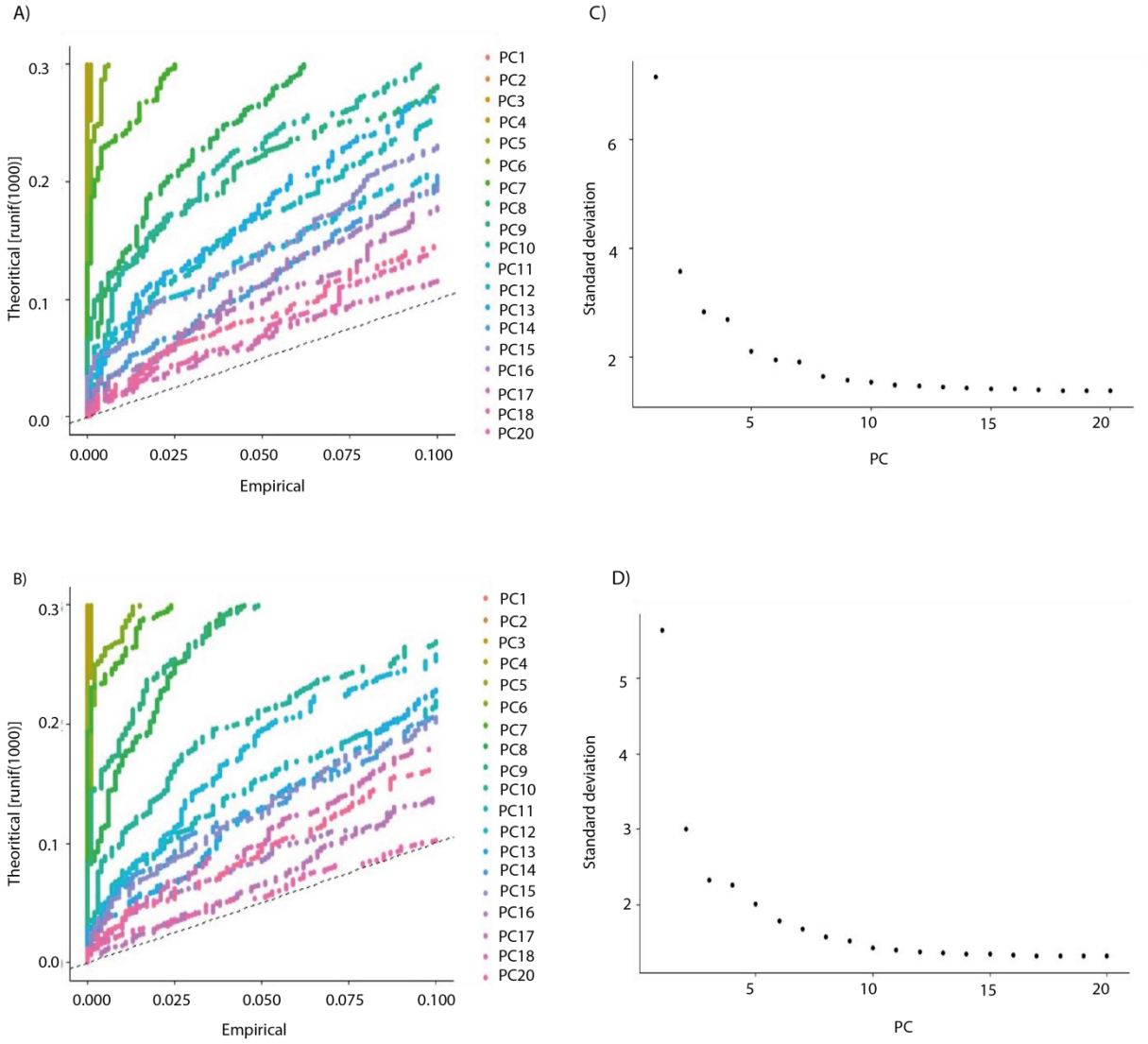


Fig. 5.5: ScRNA-seq data analysis to determine the dimensionality of the datasets. *(A and B) JackStraw procedure was implemented to determine the dimensionality of the datasets (Macosko et al.). 10 significant principal components (PCs) with strong enrichment and low P-value were identified. (C and D) Elbow plots 6 (ranking of principal components based on the percentage of variance) of 20 principal components (PC) for the control and the treated sample show that the percentage variation explained per PC flattened around PC10. Hence, only 10 PCs were taken for downstream analysis like cell clustering. t-Distributed Stochastic Neighbor Embedding (t-SNE) projections of gene expression for control as well as treatment data set using nearest neighbor graph based clustering followed by Louvain algorithm*

suggested 8 clusters across both data set. Both the datasets were combined to explore clusters which are common between the two conditions.

Cluster specific markers were identified using the Find Conserved Markers function (with default parameters) and differentially expressed genes were identified using Find Markers function (with default parameters). Psuedotime analysis was performed using Monocle 2 package assuming a negbionomial distribution with a detection limit of 0.5 and a minimum normalized gene expression of 0.1. The marker genes for different clusters obtained from Seurat analysis were used to calculate psuedotime and order the cells along psuedotime trajectory.

5.2.9 Patient sample data availability and analysis

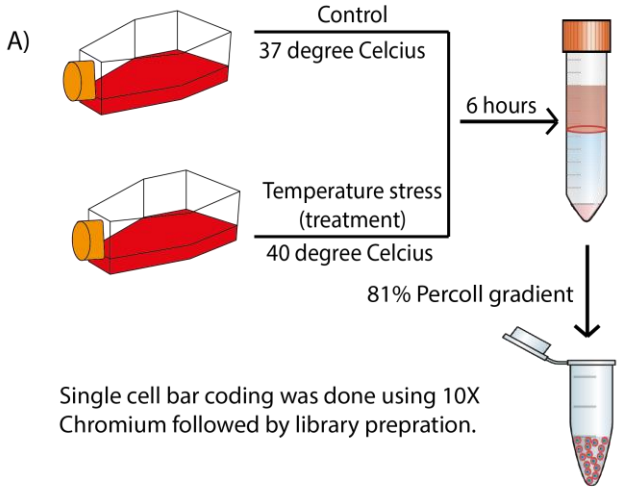
Patient sample data analyzed from the microarray data obtained from Gene Expression Omnibus (GEO), NCBI with accession number GSE59099. Graphpad (www.graphpad.com) was used for plotting the normalized log2 expression ratios of the RNA sample/3D7 RNA reference pool for all probes.

5.3 Results

5.3.1 Single-cell RNA sequencing (scRNA-Seq) during temperature stress in *P. falciparum*

Plasmodium harbored within the human RBCs, gets exposed to various environmental and physiological stresses. To understand the mechanism of stress tolerance and adaptation, the impact of these stresses on gene expression heterogeneity is crucial. To investigate it, we performed single-cell RNA sequencing (scRNA-seq) under control and physiologically stress condition such as high temperature (equivalent to fever) in *P. falciparum*. Parasites were synchronized using sorbitol treatment during early (0-3 hrs) ring stage. Temperature stress was given at 40 degree Celsius approximately at the late ring stage (~17 hrs) for a period of 6 hours. The infected RBCs of control and temperature-treated parasites were around 80% enriched by using Percoll gradient centrifugation and the uninfected RBCs were removed. Enriched parasites under normal and high temperature growth conditions were assayed for their single cell gene expression profiles using the 10X Chromium single cells RNA-sequencing v3 chemistry (Figure 5.6A).

We sequenced 4949 single cells in control and 6873 single cells under temperature stress condition. Median number of genes detected per cells (count ≥1) is 637 and 546 with sequencing depth (mean reads per cell per gene) 17945 and 13001 for control and temperature stress samples, respectively (Figure 5.6B). Further to identify different cell types (Figure 5.6C), markers genes specific to each cluster identified, which are expressed by at least 30% of the population and have 50% higher average expression than other clusters (Figure 5.7).



B)

CONDITION	NUMBER OF CELLS ANALYZED	MEDIAN GENE PER CELLS	MEAN READS PER CELLS
CONTROL	4949	637	17945
TEMPERATURE STRESS	6873	546	13001

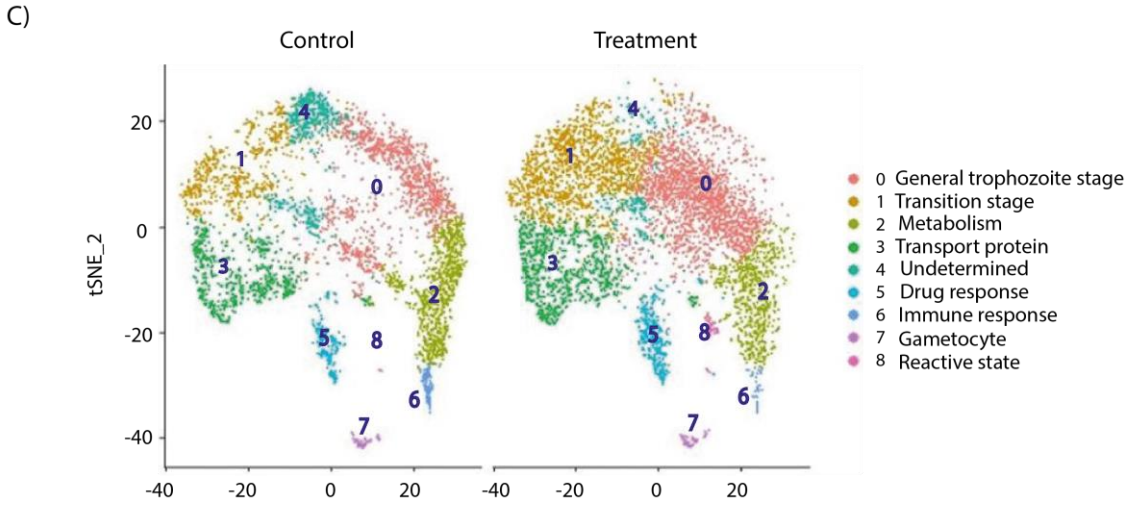


Fig. 5.6: scRNA-seq under temperature stress condition. *A) Schematic representing the pipeline used for stress induction, isolation of single parasites and library preparation for scRNA-seq. Infected RBCs were enriched using 81% percoll gradient centrifugation. Single cell bar coding and library preparation was performed using 10X Chromium protocol as per the manufacturer's instruction. B) Table stating the number of cells sequenced, median genes per cell and mean reads identified per cell under both normal and temperature stress*

conditions. C) t-SNE plot representing the different clusters generated for the Plasmodium population in control and temperature stress conditions.

A)

Cluster	Marker genes
0	hypoxanthine-guanine phosphoribosyltransferase, tetratricopeptide repeat protein
1	translation-enhancing factor, Plasmodium exported protein (PHISTc), EMP1-trafficking protein
2	enolase, thioredoxin reductase, glyceraldehyde-3-phosphate dehydrogenase
3	erythrocyte membrane protein 1, PfEMP1, Plasmodium exported protein (PHISTb), ring-exported protein 1
4	mature parasite-infected erythrocyte surface antigen, early transcribed membrane protein 5
5	multidrug resistance protein 1, E3 ubiquitin-protein ligase, heat shock protein 90, heat shock protein 110
6	phosphatidylinositol 3-kinase, ubiquitin carboxyl-terminal hydrolase UCH54, stearoyl-CoA desaturase, serine repeat antigen 9
7	CPSF subunit A, sexual stage-specific protein precursor, AP2 domain transcription factor AP2-G

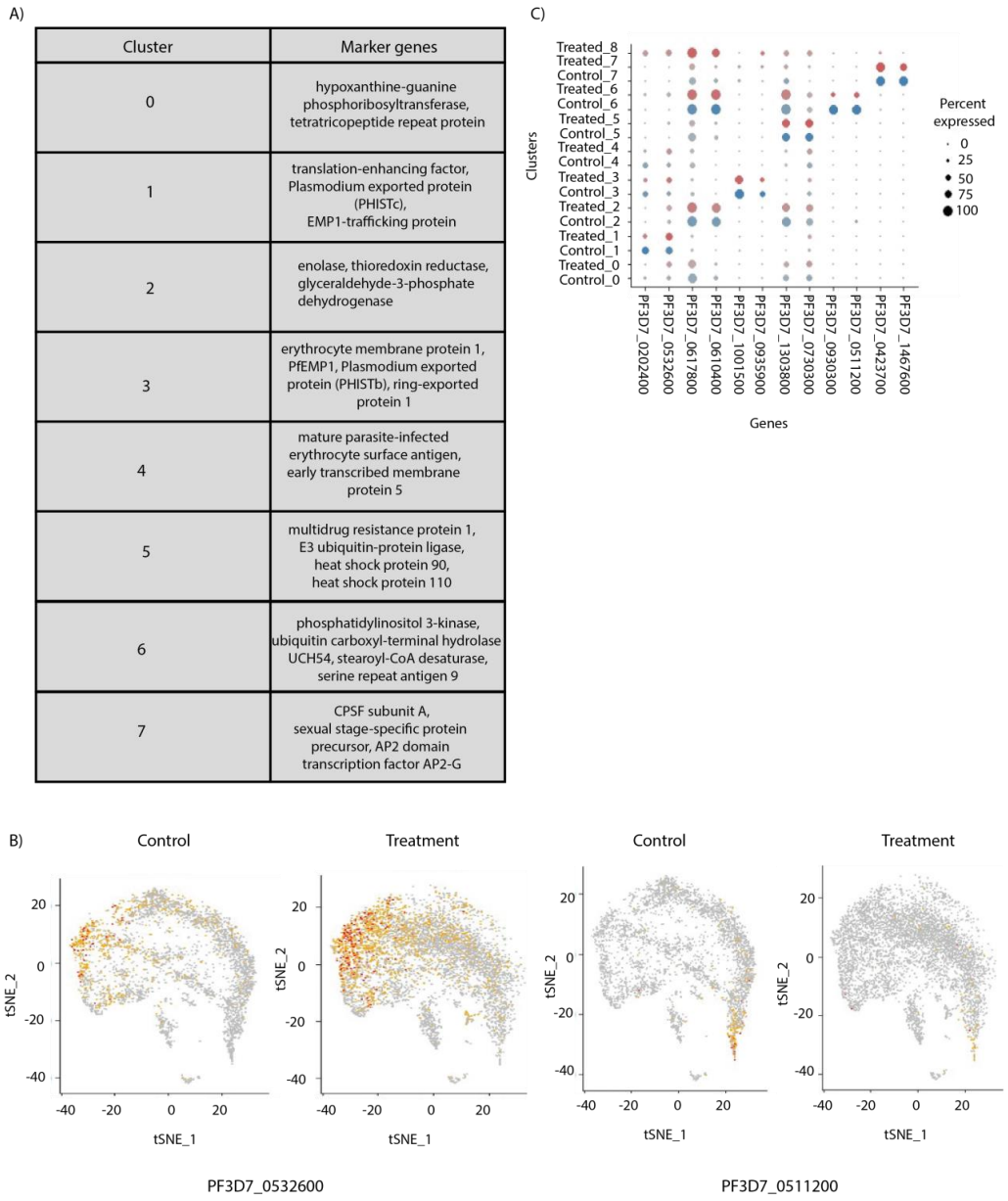


Fig. 5.7: Markers gene identified for each cluster obtained through single cell RNA sequencing. *A) Table showing the markers genes for each cluster obtained through single cell RNA sequencing for control and temperature treatment. B) Representative tSNE plots showing the expression of PF3D7_0532600 and PF3D7_0511200 in control and treatment condition. C) Bubble plot showing the percentage of cells expressing a marker gene under control and temperature stress conditions. Marker genes are specific to each cluster and expressed by at least 30% of the cells and have 50 % or higher average expression than other clusters.*

Interestingly, we found a novel cluster which is present only during the temperature stress condition (Figure 5.6C). Moreover, we also found significant change in the number of cells (%) in different cluster during temperature stress condition (Figure 5.8). Change in the number of cells might be an indicative of how the treatment rewired gene expression and biases the cells to exist in specific states that may be functionally relevant to the treatment (particularly evident for cluster 0, 1 and 4). Interestingly, despite of double synchronization of the parasites we observed significant variation in the control population of the parasite. This is an important observation since most of the experiments performed in the field of parasitology depend on the usual method of synchronization like percoll gradient and sorbitol treatment. In summary, scRNA-seq datasets generated under control and temperature stress conditions indicative of transcriptional heterogeneity in *P. falciparum*, which is analyzed in detail below.

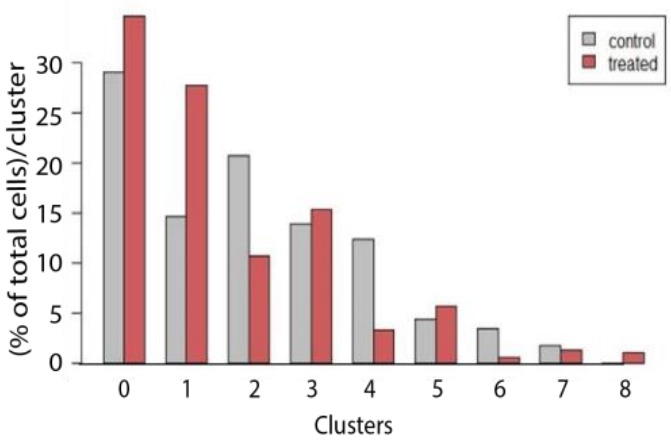


Fig. 5.8: Number of cells in different clusters during control and temperature stress conditions. *Number of cells increased in cluster 0, 1, 3 and 5 and decreased in cluster 2, 4, 6, 7 and 8.*

5.3.2 scRNA-seq identifies transcriptome heterogeneity during temperature stress condition

To understand the cell-to-cell variability of transcriptome regulation we calculated average number of RNA molecules per cell during control and temperature stress conditions. Interestingly, average numbers of RNA molecules per cells are reduced during the temperature stress condition indicative of the fact that parasites slowdown or shutoff their

global transcription machinery during temperature stress (Figure 5.9A). Downregulation can also be observed when RNA molecules per cell are calculated for each cluster except cluster 1 (Figure 5.9B).

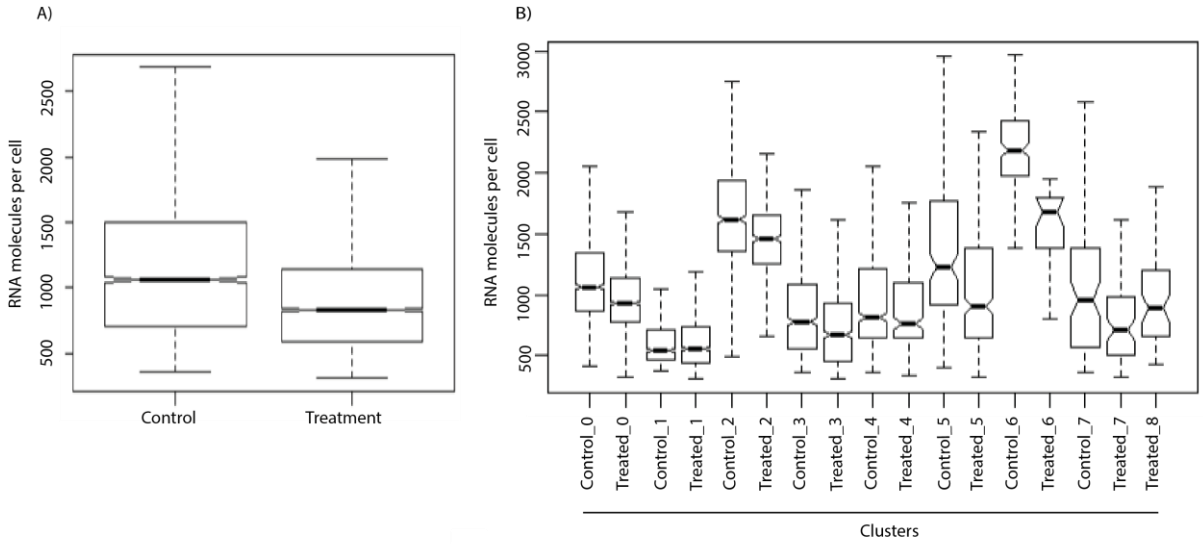


Fig. 5.9: Decrease in the overall transcription during temperature stress *A) Average RNA molecules expressed per cell is plotted for cells under control and temperature stress conditions. Downregulation in the number of RNA molecules was observed during temperature stress. (B) Average number of RNA molecules was plotted for each cluster.*

The number of RNA molecules is particularly affected in clusters associated with stress tolerance, immune response, and gametogenesis (cluster 5-7). Next, we performed bulk RNA sequencing under control and temperature stress conditions to compare global transcription and observed high correlation in relative expression in control (Spearman's correlation, 0.51) and during temperature stress condition (Spearman's correlation, 0.41) in scRNA-seq and bulk RNA sequencing. Moreover, we also observed global transcriptional downregulation during temperature stress condition in bulk RNA-sequencing (Figure 5.10).

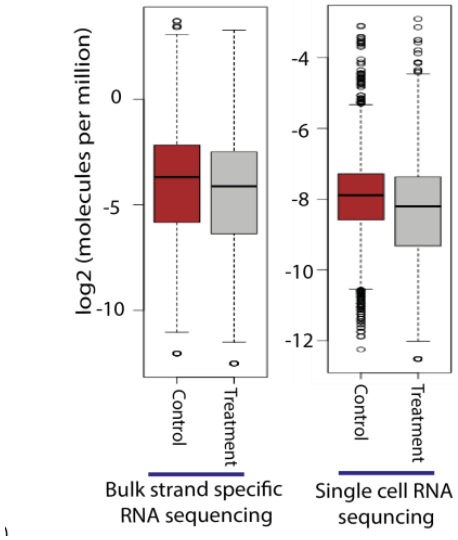


Fig. 5.10: Mean expression level of all the genes expressed during control and temperature stress conditions in both single-cell RNA sequencing and bulk-RNA sequencing. *Overall transcription is suppressed upon temperature stress condition.*

Further to understand the heterogeneity present between the control and temperature stress conditions, we calculated the measure of dispersion within a population using Coefficient of Variation (CV). To check the heterogeneity between the populations we performed the Flinger-Killeen test which checks for homogeneity for variance when data is non-normally distributed. The Flinger-Killeen Test [296] for equal coefficients of variation suggest significant variation between control and stress conditions (CV, 128.19 and 136.32 for control and temperature stress conditions, respectively; p value =2.08e-5) (Figure 5.11A).

Interestingly, genes associated with the gametogenesis, chaperon activity and maintenance of cellular homeostasis showed maximum variation under temperature stress condition (Figure 5.11B and C). This in turn suggests that variability and versatility of the maintenance of cellular homeostasis should enable cells to survive under different stress conditions, and may act as an important stimulator of development of drug-resistance in *P. falciparum*.

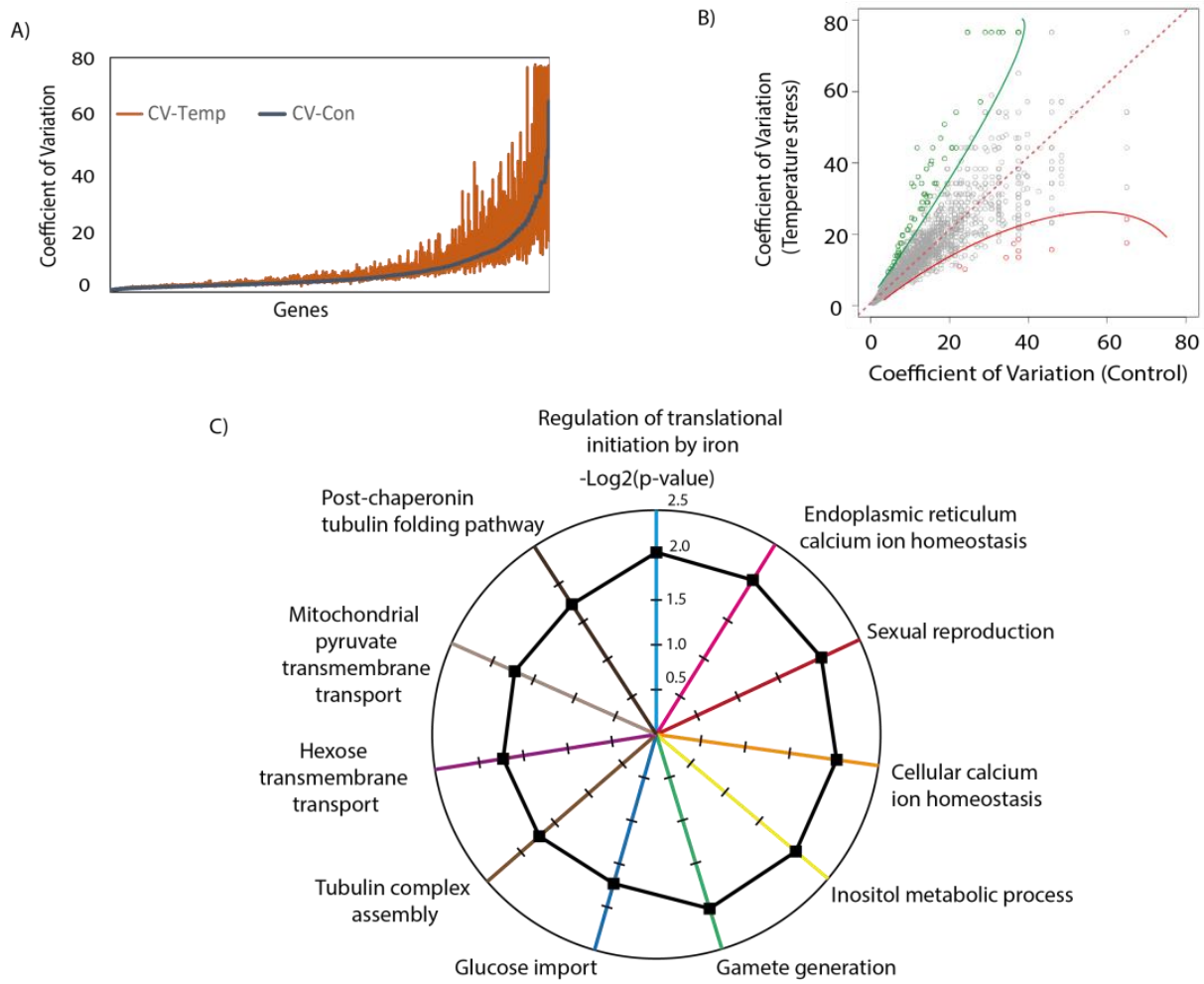


Fig. 5.11: scRNA-seq identifies transcriptome heterogeneity during temperature stress condition. *A) To check the heterogeneity between the two populations, Flinger-Killeen Test is performed which tells the homogeneity for variance when the data is non-normally distributed. The Flinger-Killeen Test suggest significant variation between control and temperature stress condition (CV, 128.19 and 136.32 for control and temperature stress condition, respectively; p value =2.08e-5). Coefficients of variation are plotted for temperature stress condition upon control condition. Under temperature stress condition genes associated with cellular homeostasis show greater variation. B) Coefficient of variation for control is plotted against coefficient of variation for temperature sample for each gene. Genes (red) are genes which have two fold more variation in control sample. Genes (green) are genes which show two fold more variation in temperature stress. C) Radar plot showing the gene ontology terms enriched for the genes which show highest variation under stress conditions.*

5.3.3 Gene expression analysis of stress responsive genes under temperature stress condition

Most of the organisms have evolutionary conserved mechanism of stress response against variety of the environmental fluctuations. These stress response machineries consist of various proteins maintaining cellular homeostasis during unfavorable conditions. One of the most studied class of proteins which play a crucial role in maintenance of cellular homeostasis are heat shock proteins. These proteins are known to play diverse functions like refolding of misfolded proteins and helps in the mRNA processing and maturation. They are considered central to the stress response machinery in higher eukaryotic systems. In order to understand the transcriptional variation in expression of stress responsive genes under temperature stress condition we plotted their expression levels relative to ribosomal protein genes (which were found to be unchanged in all clusters). Though most of the clusters showed upregulation in the expression of stress responsive genes, clusters associated with stress and immune response, and gametogenesis (cluster 5-7) showed highest overall expression and upregulation under temperature stress condition (Figure 5.12A and B).

Interestingly, cluster 8, which is unique to temperature stress condition, showed very high expression of antigenic variation gene-families like var, rifin and stevor. This unique cluster represents cells which express the genes associated with the reactive state of the pathogen against the elevated temperature. Furthermore, we looked at the different stage specific markers for these clusters. Cluster 3 showed the genes specific to ring stage markers whereas cluster 6 shows genes specific to schizont stage (Figure 5.12C).

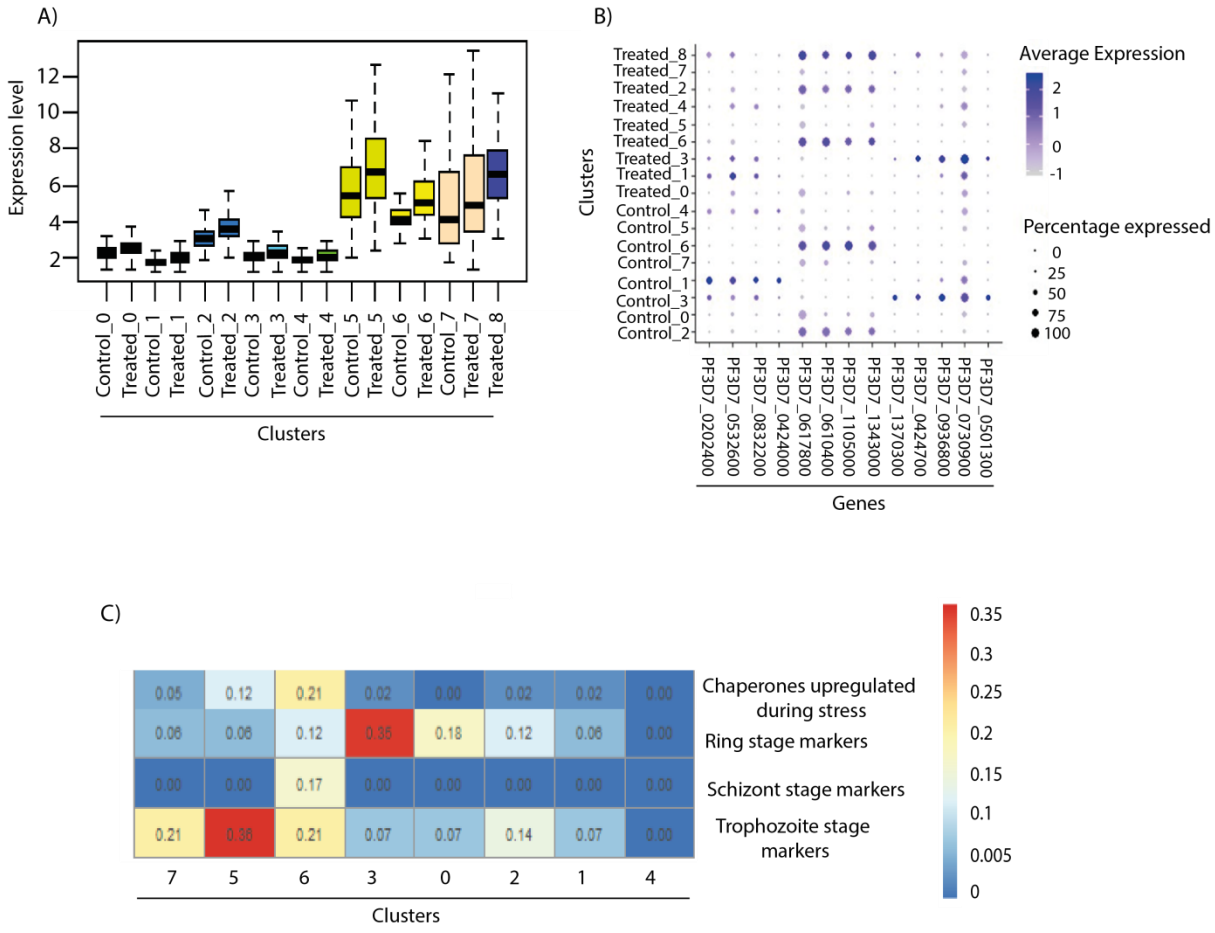


Fig. 5.12: Gene expression analysis of stress responsive genes under temperature stress condition. *(A) Change in the expression level of the stress responsive genes in different clusters. Gene expression is plotted over ribosomal proteins since expression of these genes was found to be similar in all the clusters. (B) Bubble plot showing the percentage of cells and average expression of a particular gene in different cluster. Stress responsive genes were plotted to show gene expression in different clusters. C) Heatmap showing the expression of stage specific gene markers in different clusters. Different stages of parasite growth can be identified using these stage specific markers in various clusters.*

Marker genes from cluster 6 showed expressions of different chaperone proteins which are known to play an important role in the stress response (Figure 5.12C). Gene ontology of the marker genes from these clusters indicate that they play an important role in stress response and immune response functions. Most of the other clusters show gene expression similar to trophozoite stage which is expected since parasites were harvested during trophozoite stage. This indicates that the heterogeneity is an inherent property of *P. falciparum* growth as it is also exhibited under control condition. This is interesting since we have used synchronized

parasites for the study. In order to further validate our observation we compared our clustering with the single cell data available from Malaria Cell Atlas. Since the Malaria Cell Atlas has asynchronized parasites we observed a partial overlap with their data (Figure 5.13). Around 98% of cells of our datasets (both control and temperature stress condition) cluster together with cells of data from Malaria Cell Atlas that lie in clusters containing at least 0.5% cells from the our datasets on the other hand only 49.8 % of the cells of the Malaria Cell Atlas dataset cluster together with cells of our datasets that lie in clusters containing at least 0.5% cells from our dataset.

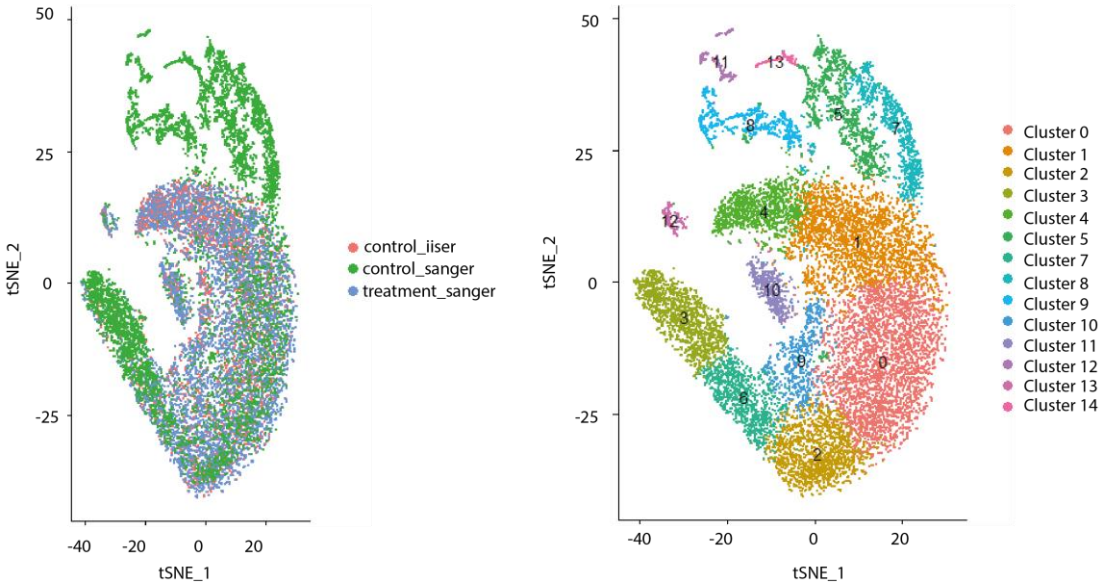


Fig. 5.13: Comparison of the clustering of control and treated sample with the control cells of data obtained from Howick et al. using 10x Genomics.

We wondered if there is any specific transcription factor associated with the regulation of stress and immune response clusters (5-7). ApiAp2 family transcription factors are known to regulate several functions in *Plasmodium*, therefore we looked at the status of AP2 transcription factors in these different clusters (Figure 5.14). Interestingly, we identified an AP2 transcription factor (PF3D7_1239200), which is downregulated in cluster 5. Another AP2 transcription factor (PF3D7_1342900) was specifically found to be upregulated in cluster 6 during the temperature stress condition. PF3D7_1342900, AP2 transcription factor was also found to be highly expressed in other stress responsive clusters like 7 and 8 (Figure 5.14). Other AP2 transcription factors with cluster specific expressions are represented in the heat map (Figure 5.14). Thus, it is plausible that these marker AP2 transcription factors regulating the expression of the genes which are differentially expressed in their respective

clusters. Interestingly, we identified histone deacetylase, HDA1 (PF3D7_1472200) showing high expression in cluster 5, 6, 7 (Figure 5.14). Expression of the histone deacetylase, HDA1 decreases upon temperature treatment suggestive of the fact that it may help in the upregulation of genes related to stress response and gametogenesis.

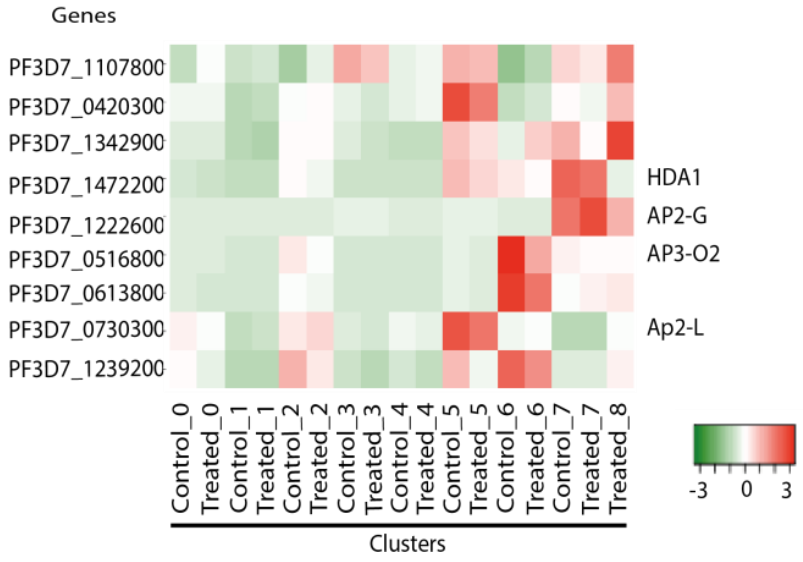


Fig. 5.14: Heat map showing the specific AP2 transcription factors enriched in different clusters. *Different AP2 transcription factors were found to increase or decrease in their expression level in particular cluster. This indicates that these AP2 transcription factors possibly regulate cluster specific gene expression programs.*

5.3.4 Temperature stress results in induction of gametogenesis in *P. falciparum*

Gametogenesis is a genetically encoded phenomenon but it is known to be influenced and triggered by various environmental fluctuations [297, 298]. Previous studies have suggested various factors such as host immune response, high parasitemia load, elevated temperature, drug treatment etc., as triggers for the process of gametogenesis in *P. falciparum* [297-302]. Furthermore, stress conditions are also shown to induce gametogenesis in *Plasmodium*. In similar context, we investigated the enrichment of gametocyte markers in various clusters under temperature stress condition (Figure 5.15A). Interestingly, we found significant upregulation of gametocyte marker (PF3D7_1302100, PF3D7_0406200 and PF3D7_1253000) as well as gametogenesis regulator (PF3D7_1222600) genes in cluster 7 (Figure 5.15B).

Interestingly, genes which are only expressed in cluster 7 include PF3D7_1222600 (AP2 domain transcription factor AP2-G), PF3D7_1466200 (early gametocyte enriched phosphoprotein EGXP), PF3D7_0936600 (gametocyte exported protein 5), PF3D7_1253000 (gametocyte erythrocyte cytosolic protein), PF3D7_1302100 (gamete antigen 27/25) and PF3D7_0406200 (sexual stage-specific protein precursor) (Figure 5.15C).

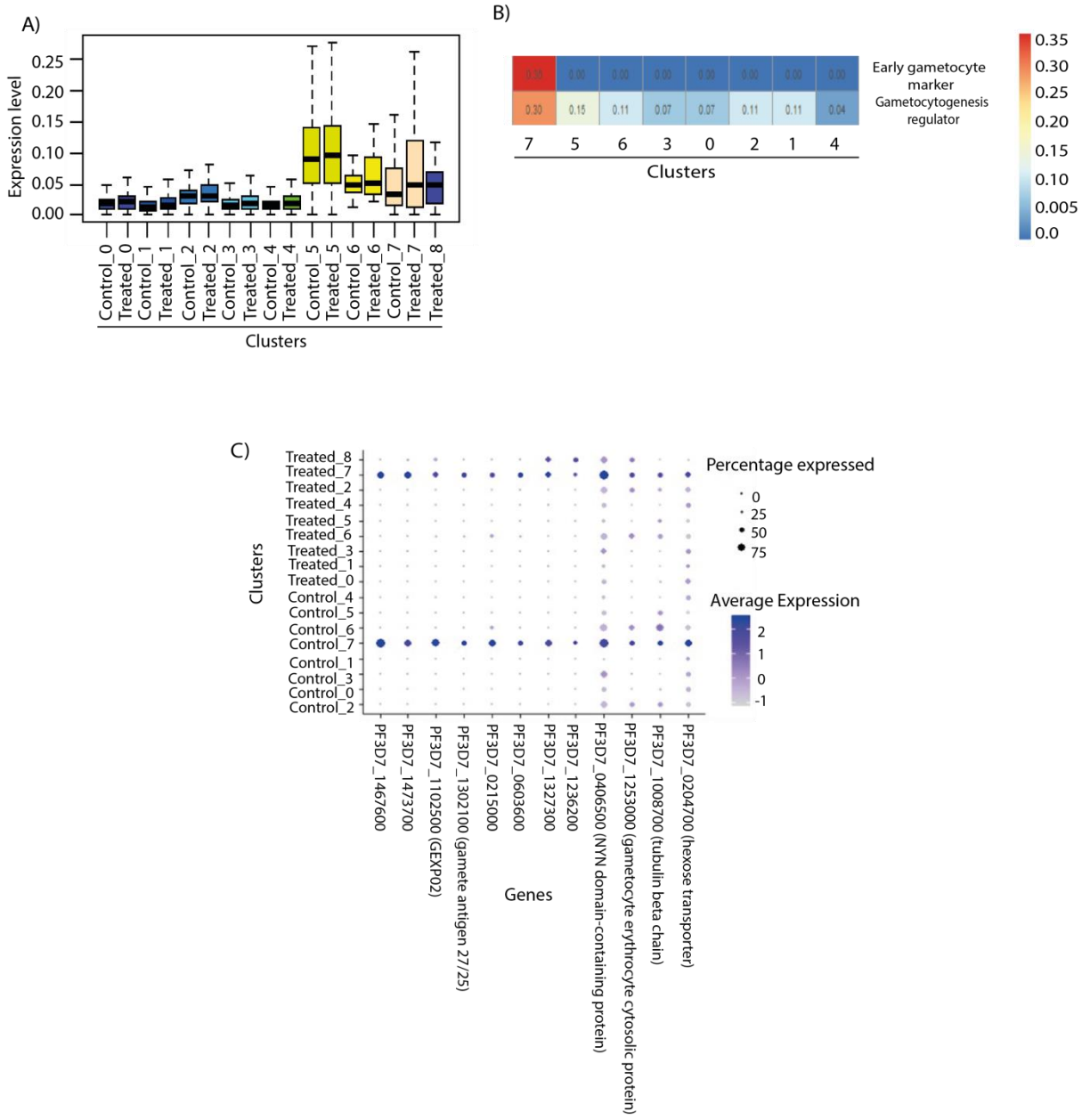


Fig. 5.15: Temperature stress results in induction of gametogenesis in P. falciparum *A) Heat map showing the expression of early gametocyte marker and gametocyte regulator in different clusters. Stage specific gene markers were used to identify the stage of the parasites in different clusters. B) Change in the expression level of the gametogenesis related genes in*

different clusters. Gene expression is plotted over ribosomal protein genes since expression of these genes was found to be similar in all the clusters. C) Bubble plot showing the percentage of cells and average expression of gametocyte related genes.

Recent report in *P. falciparum* identified markers of gametogenesis using scRNA-seq data [285]. We observed significant overlap of these genes, which are upregulated in the gametocyte specific population like surface-associated interspersed protein 8.2 (SURFIN 8.2) (PF3D7_0830800), early transcribed membrane protein 4, serine/threonine protein kinase (PF3D7_1356800), putative NYN domain-containing protein (PF3D7_0406500), putative CPSF (cleavage and polyadenylation specific factor) (PF3D7_0317700), PF3D7_0801900 (lysine-specific histone demethylase) and PF3D7_1472200 in cluster 7. Thus, it is possible that cluster 7 represent a rare population within the parasites, which is already primed for gametogenesis and these parasites show induction of gametogenesis once they are exposed to the higher temperature.

Furthermore, we performed pseudotime analysis across the intraerythrocytic life cycle of *P. falciparum* to understand the cell-fate decisions and stage-transition under temperature stress condition (Figure 5.16). Pseudotime ordering reveals a gradual stage-transition from cluster 3 (early ring stage) to cluster 6 (trophozoite/early schizont) in control as well as temperature stress condition. Interestingly, we observed two branching (indicated by 1 and 2 in Figure 5.16). Branch 1 indicates parasites which failed to diverge successfully from the default development trajectory. While second branch point indicates the parasites in cluster 7 which are primed for gametogenesis (expressing markers of gametogenesis) under control and temperature stress condition. Moreover, there is significant upregulation of gametogenesis related genes upon temperature stress condition (Cluster 7 in Figure 5.15A). This, in turn suggests that though parasites are primed for gametogenesis even in the control condition, temperature stress trigger the process of gametogenesis (Figure 5.15C). Few parasites primed for gametogenesis are also found in the trophozoite stage of parasite development (Figure 5.16). Thus, it is possible that a subset of parasites is primed for gametogenesis and cell-fate decision to gametogenesis can be triggered depending on the environmental conditions.

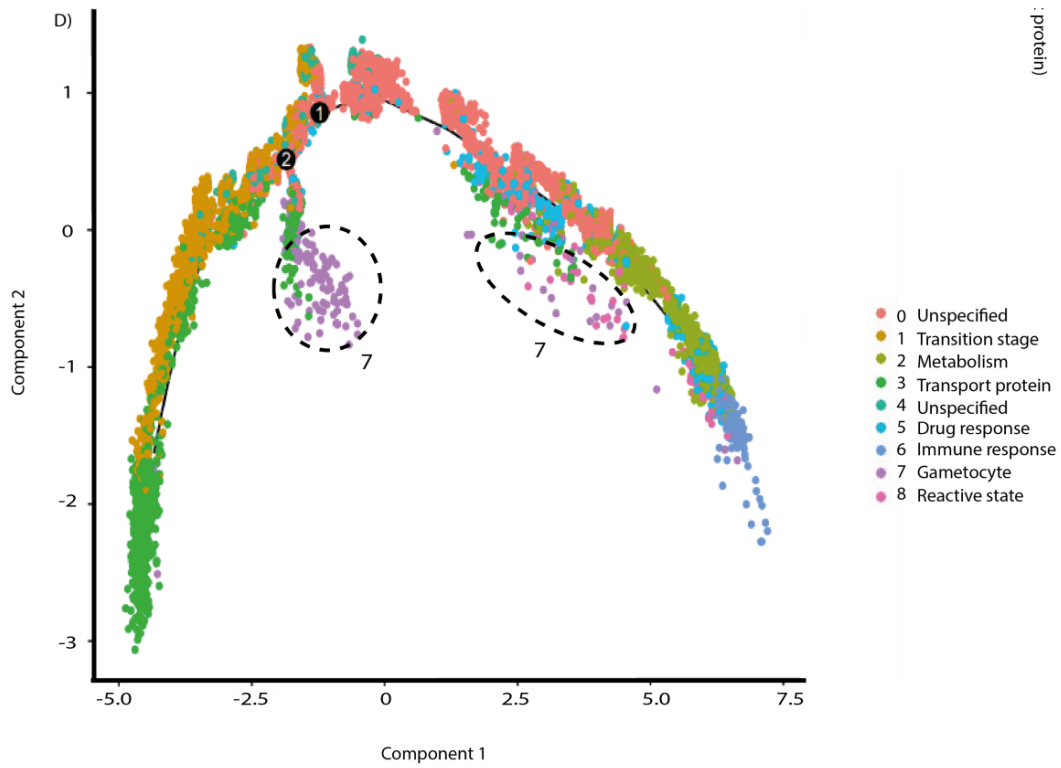


Fig. 5.16: Pseudotime analysis of the parasites sequenced under control and temperature stress conditions. *Pseudotime analysis indicated bifurcation at two stages of parasite growth for gametogenesis.*

5.3.5 Export protein regulation plays an important role in stress response adaptation

Plasmodium during intraerythrocytic life cycle exports various proteins on the surface of the infected erythrocyte [303]. Many of the exported proteins belong to the multivariant gene family, helping the parasites in sequestration by binding to various endothelial receptors [46]. Also, there are export proteins mediate the process of resetting, which helps in avoiding exposure of the parasite to host immune response [27, 49, 50, 55]. Previous studies on gene expression using microarray have suggested that there is a huge increase in the proteins which are known to be either transmembrane or secreted during the temperature stress in *P. falciparum* [169]. Most of these proteins have either PEXEL motif or host target signal sequence. Such transportation is usually mediated during the early stages of intraerythrocytic life cycle. Though transported proteins expressed in almost all clusters, they are significantly upregulated in cluster 3 (Figure 5.17A and B) [169].

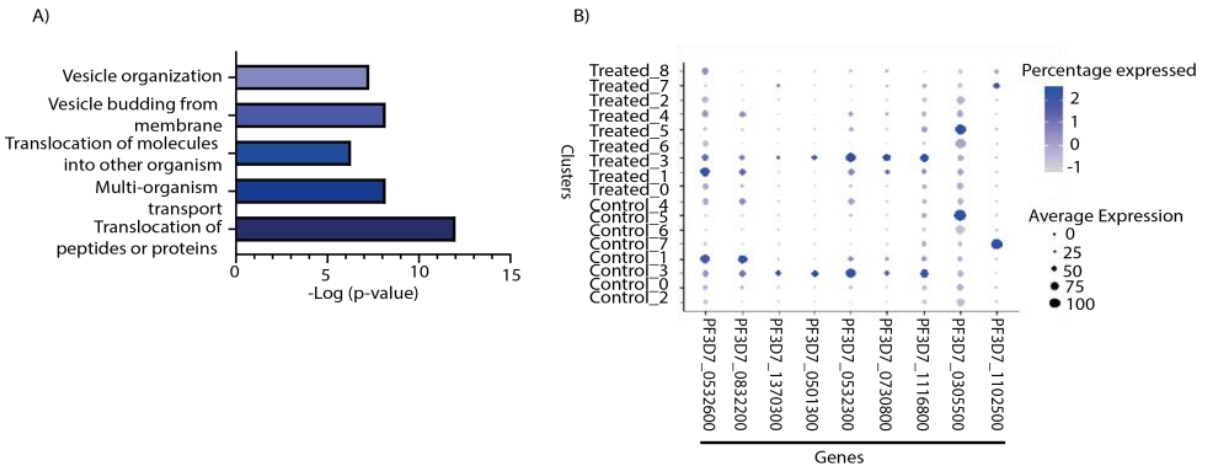


Fig. 5.17: Export proteins regulation plays an important role in stress response adaptation *(A) Gene ontology analysis of the markers genes of cluster 3 indicated that these parasites have higher expression of genes related to transportation and secretion. (B) Bubble plot showing the percentage of cells and average expression of transported proteins.*

Cluster 3 marker genes shows transportation related processes, this is intuitive as most of the transportation related processes take place during ring stage (and cluster 3 also exhibits characteristics of ring stage parasites, Figure 12C). Some of the export proteins which are upregulated in cluster 3 include PfEMP1 (*Plasmodium falciparum* Erythrocyte membrane protein 1) trafficking protein (which helps in the trafficking of PfEMP1 protein on RBC surface) and PF3D7_0424700 (FIKK kinase), which mediate the virulence associated changes over *Plasmodium* infected RBC surface. Interestingly, cluster 3 also shows downregulation of some of the transport proteins like PF3D7_1370300, PF3D7_0730800 and PF3D7_0532600 etc. This indicates mutually exclusive preference for transportation of proteins during the temperature stress condition (Figure 5.18A and B). Various transport proteins showing deregulation during the temperature stress are represented in the heat map plotted for each clusters (Figure 5.18C).

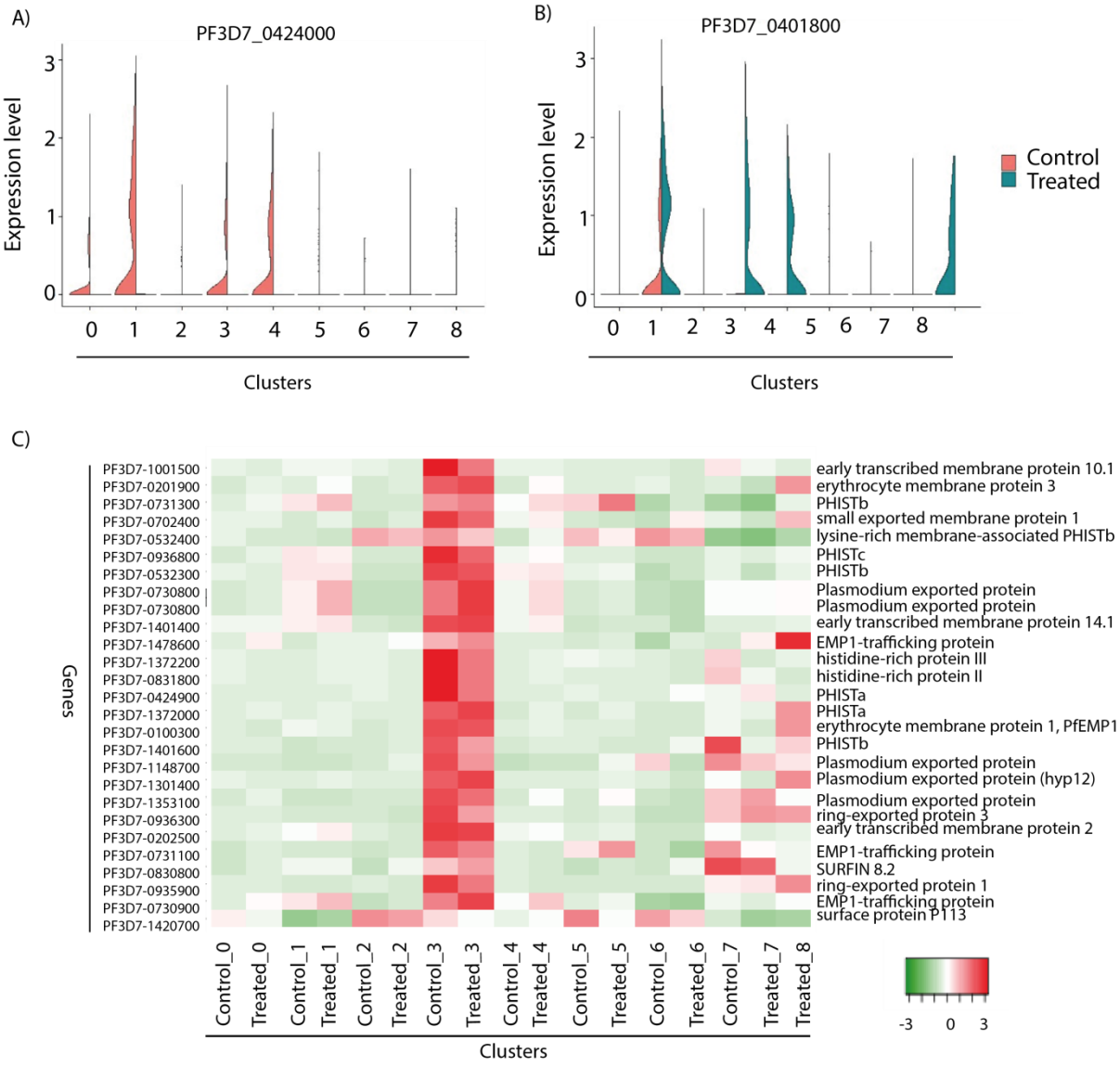


Fig. 5.18: Transport related proteins showing deregulation during temperature stress condition. *A) and B) Mutually exclusive expression of representative transport proteins in control and temperature stress conditions. C) Heat map showing the change in the expression levels of various transport proteins during the temperature stress in different clusters.*

Surprisingly, cluster 1 also shows overrepresentation of genes which helps in transportation of proteins in parasites (Figure 5.17B). This might be the transition cluster, originated from cluster 3 since parasite of these stage show expression of both ring and trophozoite stage genes and helps in transportation of different proteins. Presence of the cluster 1 adjacent to cluster 3 in pseudotime analysis (Figure 5.16) further supports our observation that cluster 1 and cluster 3 parasites show similarity in their gene expression profiles.

5.3.6 Virulence gene regulation during temperature stress condition

Plasmodium falciparum encodes several clonally variant multicopy gene families such as var, rifin and stevor, which are presented on the surface of infected RBCs [304, 305]. These proteins play a central role in enabling host immune evasion and promoting pathogenesis. PfEMP1 is the most commonly expressed immunodominant antigen on iRBCs. PfEMP1, encoded by a family of 60 var genes, believed to exhibit antigenic switching upon immune exposure and/or environmental fluctuation, stress conditions or chromatin organization etc [304-306]. Bulk RNA-sequencing studies have suggested expression of only one var gene in a population. However, the mechanism of selecting a single var gene for expression and mechanism of suppression of the rest of the var genes is still not clear [45, 46]. Recent study using scRNA-seq employing Smart-seq2 has looked into the expression of var genes at the ring stage of the parasite. As per this study majority of parasites (13 out of 17 individual cells) show expression of one dominant var gene belonging to var sub group B [284]. Furthermore, only a few parasites showed expression of two var genes per cell. We decided to look at the expression of var genes per cell using the scRNA sequencing data under temperature stress condition (Figure 5.19A and B).

Interestingly, we found that var genes are predominantly expressed in cluster 3 (transport related cluster) and in cluster 8 (reactive stage of pathogen to elevated temperature) (Figure 5.19A). We further plotted the normalized expression level of different var genes in control and temperature stress conditions and found that few var genes are expressed at a higher level in control condition. On temperature treatment, more var genes are expressed at higher expression level than control condition (Figure 5.19B).

Figure 6

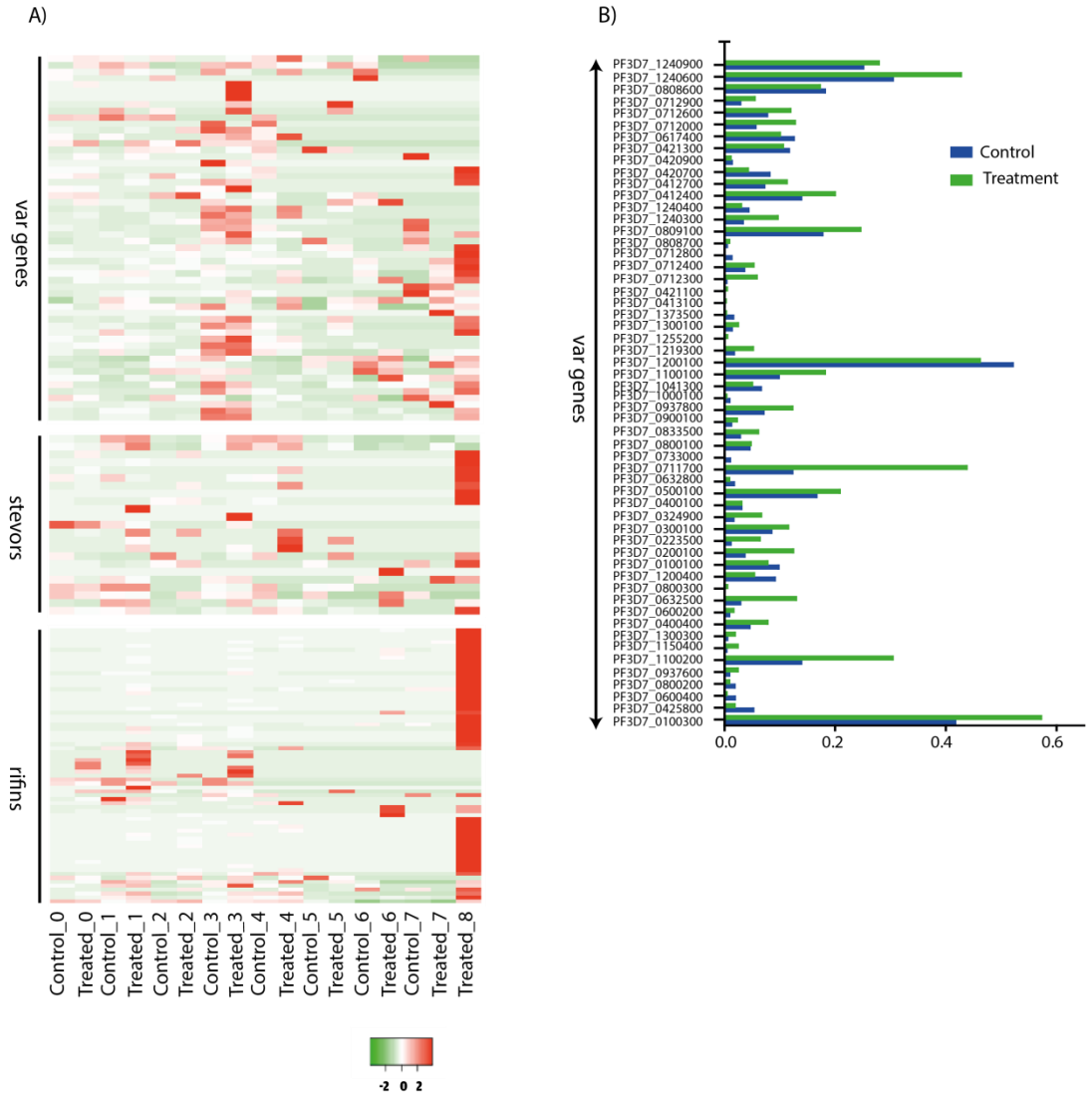


Fig. 5.19: Virulence gene regulation during temperature stress condition. *A) Heat map showing the normalized expression of various var, rifin and stevor genes during the temperature stress condition in different clusters. Cluster 8 shows huge upregulation in the expression of var, rifin and stevor genes. B) Bar plot showing the upregulation of different var genes during temperature stress condition.*

However, it is not clear if multiple var genes are expressed per cell in a population. To investigate the regulation of expression of var genes at single cell level, we performed targeted analysis of var gene expression under control and temperature stress conditions. We observed 1-4 var gene expression per cell under control condition and it goes up to 1-6 var gene expression per cell under temperature stress condition (Figure 5.20A).

It is possible that parasites in order to survive the unfavourable conditions may express more than one var genes per cell to increase the chances of binding to the endothelial cells. Interestingly, basal level of transcription was high for many var genes under temperature stress condition whereas it was comparable to control condition at higher expression level (Figure 5.20B).

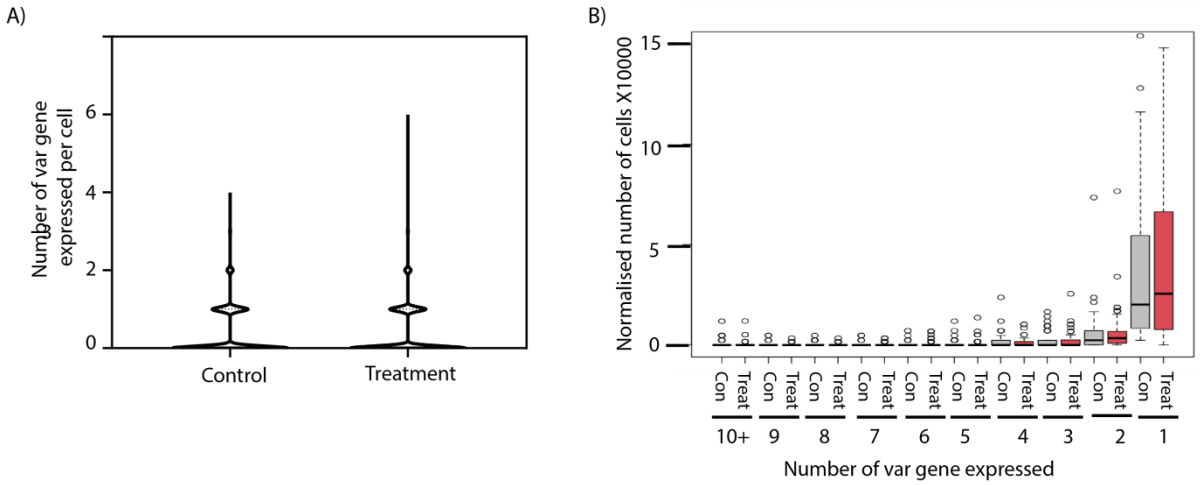


Fig. 5.20: More var genes are expressed per cell in a heterogeneous population. *A) Violin plot indicating the number of var genes expressed per cell. During control cells show 1-4 var genes per cell which is increased to 1-6 var genes per cell under temperature stress condition. B) Normalized number of cells expressing var gene in control and temperature stress condition. Basal level of transcription was high for many var genes under temperature stress condition whereas it was comparable to control condition at higher expression level.*

Thus, it can only be speculated that parasite under unfavourable conditions express multiple var genes at basal level and only a few of them are selected for higher expression. Since several studies have suggested the role of various epigenetic modifiers, intronic non-coding RNAs in var gene regulation, future functional analyses under normal and temperature stress conditions will discern mechanism of var gene selection and activation.

We next investigated the expression levels of different groups of var gene in scRNA-seq data from control and temperature stress conditions. Interestingly, we observed that var group B and C are highly expressed under both the conditions (Figure 5.19B). Moreover, bulk RNA-sequencing also showed upregulation of var genes during temperature stress condition which

are also validated RT-qPCR (Figure 5.21).

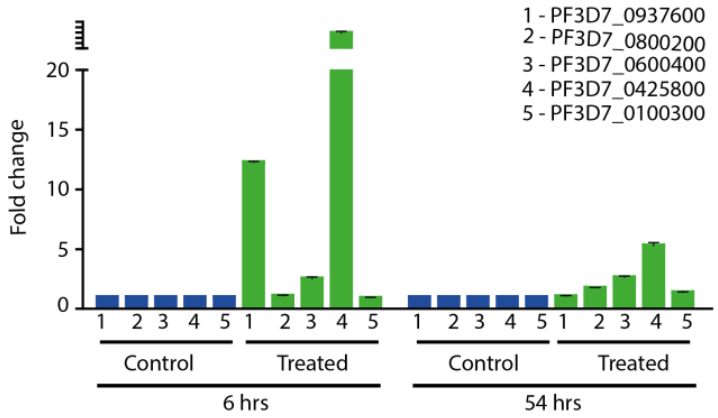


Fig. 5.21: Var gene expression analysis using RT-qPCR under control and temperature stress condition. *Heat stress was given for 6 hours and expression level was analyzed after 6 hours and 54 hours post heat treatment. Interestingly, even after 54 hours of treatment few var genes show slight upregulation in the expression level in comparison to control.*

Expression level of var genes was quantified after 6 hours of heat treatment (40^0C). Expression level was also checked after 54 hours (same time point in next cycle) to check the effect of heat treatment at later hours. Interestingly, we found var group B gene PF3D7_1200100 to be highly upregulated in control condition. We wondered if the expression of var group B is higher in patient samples as indicated by our study and earlier studies and if it is geographical location dependent. Interestingly, we observed that indeed var group B is highly expressed in patient samples and it is independent of geographical location as we have observed similar profile from African and south Asian patient samples (Figure 5.22) indicating that there is preference for expression of var group B over other var groups in *P. falciparum*. This can be further exploited to specifically design vaccines targeting members of var group B in *P. falciparum*. Other members of multivariant gene family like rifin and stevor are also known to play an important role during the malaria pathogenesis. However, nothing much is known about their expression and role under different conditions. We studied expression of rifins and stevors under temperature stress condition. Interestingly, we observed a marked increase in number of rifins and stevors expressed during temperature stress condition (Figure 5.19A). Thus, in future studies temperature stress condition can be used to activate the expression of rifins and stevors to further characterize their role in *P. falciparum*.

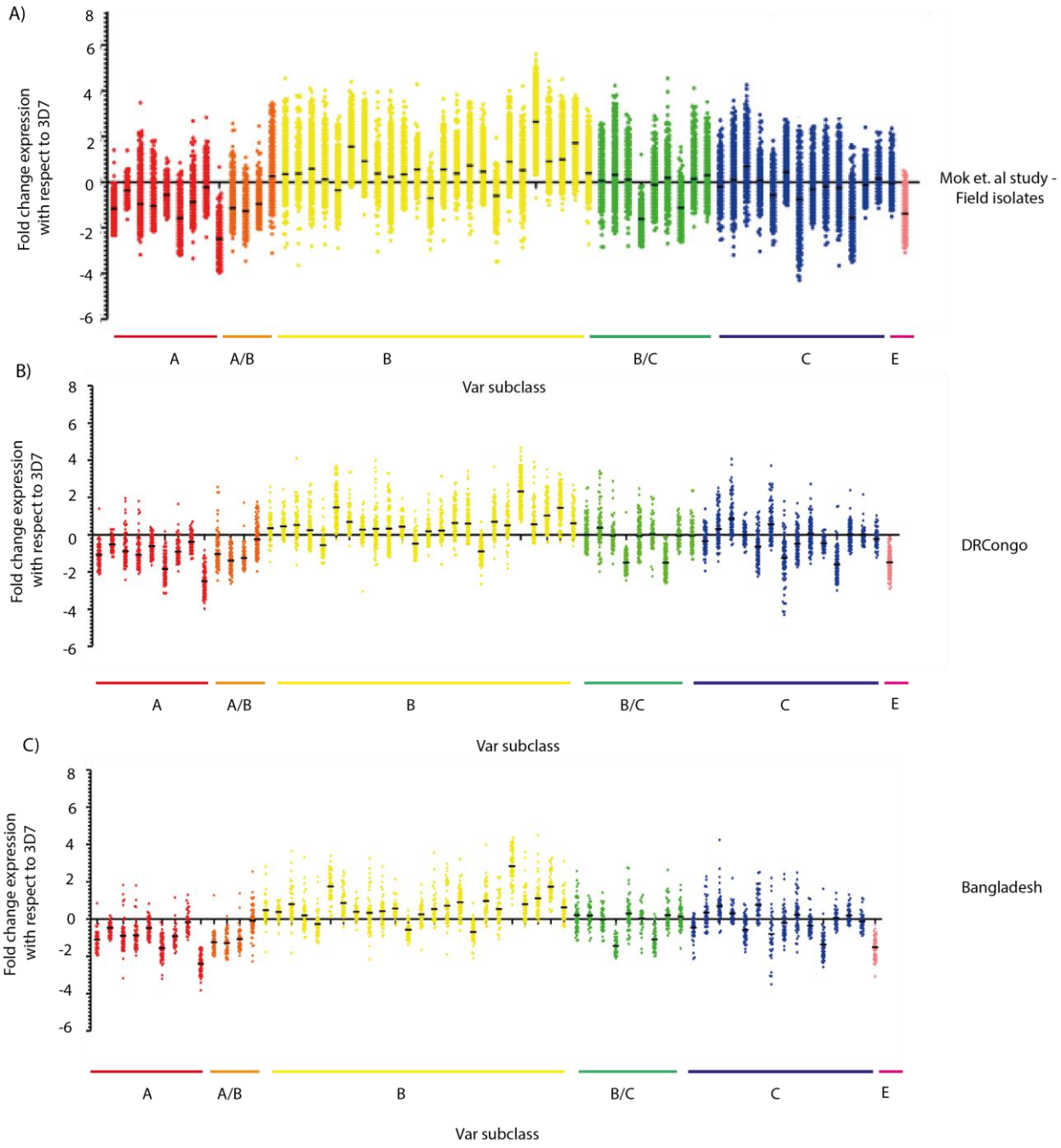


Fig. 5.22: Expression of var gene subgroup in patient samples. *A) Fold change in expression level of the var genes (normalized to 3D7 strain) in patient samples. B) Fold change in expression level of the var genes (normalized to 3D7 strain) in patient samples from Congo. C) Fold change in expression level of the var genes (normalized to 3D7 stain) in patient samples from Bangladesh. A, A/B, B, B/C, C and E are the different sub-classes of the var gene family in P. falciparum.*

5.4 Discussions

Parasites constantly face various environmental fluctuations during their life cycle which can have different effects on parasite growth and development [307-309]. Most organisms have well developed stress response machinery that helps the organism to cope up with the unfavorable conditions. Such machinery is not well studied and characterized in *Plasmodium falciparum*. Hence, cell-to-cell heterogeneity present in the population and how it affects the response of the parasites to different stress conditions is still unclear. With recent studies appreciating the role of stress responses in drug resistance generation in *P. falciparum*, it is important to look at the effect of stress conditions on cell-to-cell variability using single cell RNA sequencing [187, 292]. Unlike previous scRNA-seq studies that were performed on mixed parasite populations at different stages of growth; we performed scRNA sequencing of more number of cells at higher depth to better understand the composition and complexity of the synchronized *Plasmodium* cultures. Since synchronization is performed on a regular basis for different *in vitro* experiments, it is crucial to understand the transcriptional variation between sub-populations which can bias experimental outcomes. We found nine transcriptionally distinct cell populations all of which were represented in the Malaria Cell Atlas (Figure 5.13) [289]. Thus, though parasites are morphologically synchronized, they exhibit a transcriptionally heterogeneous population.

Under temperature stress, few parasites (<1%) adapt to a reactive state. They have higher expression level of stress responsive genes, gametocyte markers and multivariant gene family like var, rifin and stevor. Since rifin and stevor genes are expressed only in very few cells, not much is known about their role in parasitology. Therefore, temperature stress may be used to dissect the physiological and molecular role of rifins and stevors in *P. falciparum*. About 10% of the parasites are primed for stress adaptation under normal condition. Stress responsiveness is an important determinant for drug resistance in *P. falciparum*. This is also evident from the fact that even a clonal population of resistant parasites does not show complete resistance (varying Ring Survival Assay (RSA) levels, a measure of resistance against artemisinin). Thus, this sub-population (~10% of the total population), which exhibits upregulation of stress response pathways might be responsible for the possible artemisinin resistance in the parasites. This finding was averaged out in previously bulk transcriptomic studies and may have hampered dissection of drug-resistance mechanism in *P. falciparum*.

Exposure to stress conditions can trigger the process of gametogenesis in *Plasmodium*. Here, we have identified a sup-population (< 5%) of parasites which express various gametocyte markers and regulator genes and thus committed for gametogenesis. Interestingly, this sub-population does not change upon temperature exposure but upregulates expression of gametocyte marker and regulator genes. This in turn suggests that a sub-population of the parasites is primed for gametogenesis and the temperature acts as a cue to activate the marker genes and induces the gametogenesis. Furthermore, bulk RNA sequencing performed during temperature stress does not show any change in the levels of gametogenesis related genes indicating that there are very few parasites which are primed and identification of this rare population can only be done using single cell RNA sequencing techniques. Interestingly, we found a bifurcation for cell-fate decision to gametogenesis at an early and at a later stage of intra-erythrocytic cycle, illustrating that a sub-population of the parasites is always committed to gametogenesis. This may be a mechanism evolved by parasites to ensure continues transmission during each intra-erythrocytic cycle.

Summary: scRNA-seq transcriptional profiling generated under temperature stress condition improved our understanding of the parasite stress response pathways under unfavorable condition. Our study also identifies the heterogeneity in the parasite population and how such diversity in their cellular states responds discreetly upon stress conditions [310]. Importantly, the knowledge generated in this study could potentially be used to better understand the molecular mechanism of drug resistance, pathogenesis and virulence of the parasite.

Chapter 6: Conclusions and Future prospective

Malaria is one of the most common infections in tropical and subtropical regions of the world. Despite of several efforts to eradicate the disease, it is still responsible for more than 200 million cases every year [11]. Among several species capable of causing malaria in humans, *P. falciparum* infections are most lethal. One of the major problems in controlling malaria is the emergence of resistance against most of the antimalarial drugs. Several drugs like chloroquine, sulfadoxine-pyrimethamine, and mefloquine are no longer effective against *P. falciparum* [64, 206, 311]. Currently, artemisinin based combination therapy is considered as the first line of defense against malaria. However, 2009 onwards, alarming incidents of resistance against artemisinin have been reported in south east Asia, which is the epicenter for emergence of anti-malarial drug-resistance [211]. The trajectory of artemisinin resistance spread is following an expected trend as recently definitive report of artemisinin resistance has appeared from Bengal, Eastern India [228].

Artemisinin-resistant parasites are characterized by slow growth and reduced drug-susceptibility at the ring stage of the asexual growth [312]. Population genomics and transcriptomic studies have identified mutations in Kelch13 gene as a molecular marker for artemisinin resistance [312]. However, several reports thereafter indicated that Kelch13 is not the main mediator rather oxidative stress and protein damage responses are the main regulators of the artemisinin resistance [187, 292]. Multiple reviews in last few years have hinted for an unknown yet important transcriptional player as the global regulator of artemisinin resistance.

In this study, for the first time we have identified PfGCN5, as a global regulator of stress responsive genes by performing RNA-sequencing and corroborating our findings by ChIP-sequencing under various stress conditions including artemisinin exposure. We also found that PfGCN5 is not a general transcriptional coactivator and it is present in the poised form on stimuli/stress responsive genes in *P. falciparum*. In this study, we have also dissected the mechanism of PfGCN5 binding and regulation of drug-responsive genes. Also, we have identified PfAlba3 (Acetylation lower binding affinity) as one of the interacting partners of PfGCN5. Finally, we have demonstrated that inhibition of PfGCN5 can reverse the sensitivity of the parasites to artemisinin in two different artemisinin-resistant parasite lines (Kelch13-C580Y and Klech13-R539T). Together, these findings elucidate the role of PfGCN5 as a global chromatin regulator of stress-responses in general and artemisinin resistance in

particular and pave the way to discovery of novel drug targets against PfGCN5 in artemisinin resistant *P. falciparum.* This study is a first of its kind where chromatin regulator has been identified as a global modulator of artemisinin resistance and connected the link between other mechanisms (including Kelch13 mutations) of artemisinin resistance in *P. falciparum*

Nutrient stress haven been known to modulate parasite virulence [233]. Since PfGCN5 was found to be associated with antigenic variation genes, we performed ChIP sequencing of PfGCN5 under nutrient stress condition. Interestingly, PfGCN5 was found to be associated with the genes important for several metabolic pathways. Furthermore transcript level of metabolic genes bound by PfGCN5 was found to be upregulated during nutrient stress condition. Recently, several metabolic enzymes are shown to regulate gene expression through direct chromatin binding. Therefore, we looked into the biological significance of PfGAPDH and PfGCN5 interaction. Localization studies showed that PfGAPDH is localized to both cytoplasm as well as nucleus. PfGAPDH was found to be acetylated by PfGCN5 possibly within the nucleus due to PfGCN5 nuclear localization. Further studies need to be carried out to investigate whether PfGAPDH regulates genes expression through chromatin binding. Furthermore, it will be interesting to understand the physiological role of PfGAPDH acetylation via PfGCN5.

Further, we also tried to dissect cellular heterogeneity present within parasite population during stress conditions. To investigate cellular heterogeneity determined by gene expression, we performed single-cell RNA sequencing (scRNA-seq) of about 12,000 synchronized *Plasmodium* cells in physiologically relevant normal (37^0 C) and temperature stress (40^0 C) conditions phenocopying the cyclic bouts of fever experienced during malarial infection. Unlike previous scRNA-seq studies that were performed on mixed parasite populations at different stages of growth, our study would be of immediate relevance to parasite biology as it presents results on synchronized *Plasmodium* cultures, which is currently the only way researchers manipulate and study the parasite's molecular biology.

We found synchronized cultures of *P. falciparum* to have heterogeneous cell populations. Since synchronization is performed on a regular basis for different *in vitro* experiments, it is crucial to understand the transcriptional variation between sub-populations which can bias experimental outcomes. We found nine transcriptionally distinct cell populations all of which were represented in the malaria cell atlas. Parasitic transmission during intra-erythrocytic

cycles may be regulated by continued commitment and activation to gametogenesis. We found that about 5% of *P. falciparum* cells primed for gametogenesis and temperature stress inducted the process of gametogenesis through the upregulation of AP2-G (the master regulator of sexual conversion). About 10% of the parasites are primed for stress adaptation under normal conditions. Stress responsiveness is an important determinant for drug resistance in *P. falciparum*. We have identified a subpopulation of the parasite which has gene expression signatures associated with artemisinin resistance. Moreover, high temperature induces about 1% of parasites to adapt to a reactive state. This population has a high expression of antigenic variation genes such as var, rifin and stevor. Since rifin and stevor genes are expressed only in a few cells not much is known about their role in parasitology. Therefore, temperature stress may be used to dissect the molecular biology of rifins and stevors in *P. falciparum*.

Several experiments can be performed further to understand the transcriptional regulation during stress conditions in *Plasmodium*

1) Since PfGCN5 is an essential enzyme for survival of Plasmodium, conditional deletion of PfGCN5 can be performed to further validate its role in artemisinin resistance. For conditional knockdown several strategies can be used like glms mediated degradation of mRNA.
2) PfGCN5 transcripts were found to be upregulated during stress conditions. Hence it will be interesting to decipher the mechanism of PfGCN5 activation in response to unfavorable conditions. We can identify the signaling molecules which are crucial for sensing the stress condition from the environment and further relay information to PfGCN5 which is present in the poised state over stress responsive genes.
3) Currently there is no PfGCN5 specific inhibitor available. PfGCN5 specific inhibitors can be screened as it is a potential molecular target against artemisinin-resistant parasites.
4) PfGCN5 was found to interact and acetylate PfGAPDH. It will be interesting to understand the physiological significance of PfGAPDH acetylation. Whether

PfGAPDH regulate gene expression through chromatin binding? This can be addressed through chromatin immunoprecipitation followed by sequencing.

5) Synchronized parasites can be FACS sorted (through staining of marker gene for stress response) to obtain parasite population which show higher stress responsiveness. Further in-depth RNA sequencing as well as PfGCN5 ChIP sequencing can be performed to understand the role of PfGCN5 in the emergence of artemisinin resistance.
6) Temperature stress resulted in the emergence of a unique population which has higher expression of multivariant family genes. Further studies can be performed on this subpopulation to understand the deregulation of multivariant family genes using temperature stress condition.
7) Temperature stress results in upregulation of AP2-G and downregulation of HDA1. This suggests that fever conditions during malarial infection can play an important role in triggering parasites for gametocytogenesis. Role of temperature stress in induction of sexual development can be further studied.

APPENDIX

1 PfGCN5 interacting proteins. PfGCN5 interacting proteins were identified using the both PfGCN5 α-HAT and α-peptide antibodies

Gene ID	Product Description
Proteins identified in both PfGCN5 HAT and PfGCN5 Peptide antibody pull-down	
PF3D7_0209800	ATP-dependent RNA helicase UAP56
PF3D7_0306300	glutaredoxin 1
PF3D7_0306800	T-complex protein 1 subunit beta
PF3D7_0307100	40S ribosomal protein S12, putative
PF3D7_0307200	60S ribosomal protein L7, putative
PF3D7_0309600	60S acidic ribosomal protein P2
PF3D7_0317600	40S ribosomal protein S11, putative
PF3D7_0320900	histone H2A.Z
PF3D7_0322000	peptidyl-prolyl cis-trans isomerise
PF3D7_0406100	V-type proton ATPase subunit B
PF3D7_0416800	small GTP-binding protein sar1
PF3D7_0422400	40S ribosomal protein S19
PF3D7_0500800	mature parasite-infected erythrocyte surface antigen
PF3D7_0503400	actin-depolymerizing factor 1
PF3D7_0517000	60S ribosomal protein L12, putative
PF3D7_0519400	40S ribosomal protein S24
PF3D7_0520900	Adenosylhomocysteinase
PF3D7_0523000	multidrug resistance protein 1
PF3D7_0608700	T-complex protein 1 subunit zeta
PF3D7_0608800	ornithine aminotransferase
PF3D7_0610400	histone H3

PF3D7_0617800	histone H2A
PF3D7_0617900	histone H3 variant
PF3D7_0618300	60S ribosomal protein L27a, putative
PF3D7_0619400	cell division cycle protein 48 homologue, putative
PF3D7_0621200	pyridoxine biosynthesis protein PDX1
PF3D7_0624000	Hexokinase
PF3D7_0626800	pyruvate kinase
PF3D7_0627500	protein DJ-1
PF3D7_0708400	heat shock protein 90
PF3D7_0713700	conserved *Plasmodium* protein, unknown function
PF3D7_0714000	histone H2B variant
PF3D7_0719600	60S ribosomal protein L11a, putative
PF3D7_0719700	40S ribosomal protein S10, putative
PF3D7_0727400	proteasome subunit alpha type-5, putative
PF3D7_0812400	karyopherin alpha
PF3D7_0813900	40S ribosomal protein S16, putative
PF3D7_0814000	60S ribosomal protein L13-2, putative
PF3D7_0814200	DNA/RNA-binding protein Alba 1
PF3D7_0818200	14-3-3 protein
PF3D7_0818900	heat shock protein 70
PF3D7_0821700	60S ribosomal protein L22, putative
PF3D7_0827900	protein disulfide isomerise
PF3D7_0903700	alpha tubulin 1
PF3D7_0913200	elongation factor 1-beta
PF3D7_0915400	ATP-dependent 6-phosphofructokinase
PF3D7_0917900	heat shock protein 70
PF3D7_0919000	nucleosome assembly protein

PF3D7_0922200	S-adenosylmethionine synthetase
PF3D7_0922500	phosphoglycerate kinase
PF3D7_0925900	parasitophorous vacuolar protein 5, putative
PF3D7_0929400	high molecular weight rhoptry protein 2
PF3D7_0930300	merozoite surface protein 1
PF3D7_1004000	60S ribosomal protein L13, putative
PF3D7_1008700	tubulin beta chain
PF3D7_1012400	hypoxanthine-guanine phosphoribosyltransferase
PF3D7_1015600	heat shock protein 60
PF3D7_1015900	Enolase
PF3D7_1026800	40S ribosomal protein S2
PF3D7_1027300	Peroxiredoxin
PF3D7_1037300	ADP/ATP transporter on adenylate translocase
PF3D7_1103100	60S acidic ribosomal protein P1, putative
PF3D7_1104400	thioredoxin-like mero protein
PF3D7_1105000	histone H4
PF3D7_1105100	histone H2B
PF3D7_1105400	40S ribosomal protein S4, putative
PF3D7_1108400	casein kinase 2, alpha subunit
PF3D7_1108600	endoplasmic reticulum-resident calcium binding protein
PF3D7_1115600	peptidyl-prolyl cis-trans isomerise
PF3D7_1116800	heat shock protein 101
PF3D7_1117700	GTP-binding nuclear protein RAN/TC4
PF3D7_1120100	phosphoglycerate mutase, putative
PF3D7_1126200	40S ribosomal protein S18, putative
PF3D7_1127100	deoxyuridine 5'-triphosphate nucleotidohydrolase
PF3D7_1130100	60S ribosomal protein L38

PF3D7_1130200	60S ribosomal protein P0
PF3D7_1132200	T-complex protein 1 subunit alpha
PF3D7_1134000	heat shock protein 70
PF3D7_1202900	high mobility group protein B1
PF3D7_1203700	nucleosome assembly protein
PF3D7_1204300	eukaryotic translation initiation factor 5A
PF3D7_1222300	endoplasmin, putative
PF3D7_1223100	cAMP-dependent protein kinase regulatory subunit
PF3D7_1224300	polyadenylate-binding protein 1, putative
PF3D7_1238100	calcyclin binding protein, putative
PF3D7_1242700	40S ribosomal protein S17, putative
PF3D7_1246200	actin I
PF3D7_1302100	gamete antigen 27/25
PF3D7_1302800	40S ribosomal protein S7, putative
PF3D7_1311900	V-type proton ATPase catalytic subunit A
PF3D7_1323400	60S ribosomal protein L23
PF3D7_1324900	L-lactate dehydrogenase
PF3D7_1325100	phosphoribosyl pyrophosphate synthetase
PF3D7_1330800	RNA-binding protein, putative
PF3D7_1331800	60S ribosomal protein L23, putative
PF3D7_1338200	60S ribosomal protein L6, putative
PF3D7_1341200	60S ribosomal protein L18, putative
PF3D7_1341300	60S ribosomal protein L18-2, putative
PF3D7_1342000	40S ribosomal protein S6
PF3D7_1343000	phosphoethanolamine N-methyltransferase
PF3D7_1347500	DNA/RNA-binding protein Alba 4
PF3D7_1351400	60S ribosomal protein L17, putative

PF3D7_1357900	pyrroline-5-carboxylate reductase, putative
PF3D7_1358800	40S ribosomal protein S15
PF3D7_1360900	RNA-binding protein, putative
PF3D7_1408600	40S ribosomal protein S8e, putative
PF3D7_1410400	rhoptry-associated protein 1
PF3D7_1414300	60S ribosomal protein L10, putative
PF3D7_1421200	40S ribosomal protein S25
PF3D7_1424100	60S ribosomal protein L5, putative
PF3D7_1427900	leucine-rich repeat protein
PF3D7_1438900	thioredoxin peroxidase 1
PF3D7_1441200	60S ribosomal protein L1, putative
PF3D7_1444800	fructose-bisphosphate aldolase
PF3D7_1447000	40S ribosomal protein S5
PF3D7_1451100	elongation factor 2
PF3D7_1451700	calcineurin subunit B
PF3D7_1454400	aminopeptidase P
PF3D7_1460700	60S ribosomal protein L27
PF3D7_1461300	40S ribosomal protein S28e, putative
PF3D7_1462800	glyceraldehyde-3-phosphate dehydrogenase
PF3D7_1465900	40S ribosomal protein S3
PF3D7_1468700	eukaryotic initiation factor 4A
Only identified with PfGCN5 HAT antibody	
PF3D7_0305700	ubiquitin-conjugating enzyme E2, putative
PF3D7_0316800	40S ribosomal protein S15A, putative
PF3D7_0501500	rhoptry-associated protein 3
PF3D7_0503800	60S ribosomal protein L31
PF3D7_0511000	translationally-controlled tumour protein homolog

PF3D7_0511800	inositol-3-phosphate synthase
PF3D7_0513300	purine nucleoside phosphorylase
PF3D7_0524000	karyopherin beta
PF3D7_0614500	60S ribosomal protein L19
PF3D7_0617300	conserved *Plasmodium* protein, unknown function
PF3D7_0618000	conserved *Plasmodium* membrane protein, unknown function
PF3D7_0708800	heat shock protein 110
PF3D7_0813300	conserved protein, unknown function
PF3D7_0813400	conserved protein, unknown function
PF3D7_0819500	conserved protein, unknown function
PF3D7_0819900	U6 snRNA-associated Sm-like protein LSm3, putative
PF3D7_0822300	small nuclear ribonucleoprotein G, putative
PF3D7_0823300	histone acetyltransferase GCN5
PF3D7_0826700	receptor for activated c kinase
PF3D7_0905400	high molecular weight rhoptry protein 3
PF3D7_0918000	glideosome-associated protein 50
PF3D7_0932200	Profiling
PF3D7_1008900	adenylate kinase
PF3D7_1020900	ADP-ribosylation factor
PF3D7_1102800	early transcribed membrane protein 11.2
PF3D7_1105800	conserved Apicomplexan protein, unknown function
PF3D7_1113400	ubiquitin domain-containing protein DSK2, putative
PF3D7_1121600	exported protein 1
PF3D7_1124600	ethanolamine kinase
PF3D7_1127000	protein phosphatase, putative
PF3D7_1130400	26S protease regulatory subunit 6A, putative
PF3D7_1144000	40S ribosomal protein S21

PF3D7_1235600	serine hydroxymethyltransferase
PF3D7_1238800	acyl-CoA synthetase
PF3D7_1323100	60S ribosomal protein L6, putative
PF3D7_1344200	heat shock protein 110, putative
PF3D7_1348300	elongation factor Tu, putative
PF3D7_1352500	thioredoxin-related protein, putative
PF3D7_1361900	proliferating cell nuclear antigen 1
PF3D7_1407800	plasmepsin IV
PF3D7_1407900	plasmepsin I
PF3D7_1408100	plasmepsin III
PF3D7_1410700	conserved protein, unknown function
PF3D7_1416500	NADP-specific glutamate dehydrogenase
PF3D7_1426100	transcription factor BTF3, putative
PF3D7_1431700	60S ribosomal protein L14, putative
PF3D7_1432100	voltage-dependent anion-selective channel protein, putative
PF3D7_1434300	Hsp70/Hsp90 organizing protein
PF3D7_1439900	triosephosphate isomerise
PF3D7_1471100	exported protein 2
Identified only with PfGCN5 peptide antibody	
PF3D7_0213100	protein SIS1
PF3D7_0312400	glycogen synthase kinase 3
PF3D7_0322900	40S ribosomal protein S3A, putative
PF3D7_0507100	60S ribosomal protein L4
PF3D7_0516200	40S ribosomal protein S11
PF3D7_0520000	40S ribosomal protein S9, putative
PF3D7_0602900	conserved *Plasmodium* protein, unknown function
PF3D7_0710600	60S ribosomal protein L34

PF3D7_0716800	eukaryotic translation initiation factor 3 subunit I, putative
PF3D7_0721600	40S ribosomal protein S5, putative
PF3D7_0722200	rhoptry-associated leucine zipper-like protein 1
PF3D7_0802200	1-cys peroxiredoxin
PF3D7_0802800	serine/threonine protein phosphatase 2B catalytic subunit A
PF3D7_0810000	acyl-CoA binding protein, putative
PF3D7_0920800	inosine-5'-monophosphate dehydrogenase
PF3D7_0922100	ubiquitin-like protein, putative
PF3D7_1006200	DNA/RNA-binding protein Alba 3
PF3D7_1011800	PRE-binding protein
PF3D7_1019400	60S ribosomal protein L30e, putative
PF3D7_1021900	PHAX domain-containing protein, putative
PF3D7_1027800	60S ribosomal protein L3
PF3D7_1033400	haloacid dehalogenase-like hydrolase
PF3D7_1035800	probable protein, unknown function
PF3D7_1103800	CCR4-NOT transcription complex subunit 1, putative
PF3D7_1109900	60S ribosomal protein L36
PF3D7_1116100	serine esterase, putative
PF3D7_1129100	parasitophorous vacuolar protein 1
PF3D7_1215400	conserved *Plasmodium* protein, unknown function
PF3D7_1218500	dynamin-like protein, putative
PF3D7_1219100	clathrin heavy chain, putative
PF3D7_1229500	T-complex protein 1 subunit gamma
PF3D7_1306400	26S protease regulatory subunit 10B, putative
PF3D7_1317800	40S ribosomal protein S19
PF3D7_1320800	dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex
PF3D7_1336900	tryptophan--tRNA ligase

PF3D7_1353900	proteasome subunit alpha type-7, putative
PF3D7_1360800	Falcilysin
PF3D7_1364800	DNA-directed RNA polymerases I, II, and III subunit RPABC1, putative
PF3D7_1407100	rRNA 2'-O-methyltransferase fibrillarin, putative
PF3D7_1410600	eukaryotic translation initiation factor 2 subunit gamma, putative
PF3D7_1422500	ERAD-associated E3 ubiquitin-protein ligase HRD1
PF3D7_1423700	conserved *Plasmodium* protein, unknown function
PF3D7_1428300	proliferation-associated protein 2g4, putative

2 List of oligonucleotide used for RT-qPCR

Gene	Forward Primer	Reverse Primer
GCN5	TGCTGATAATAAAGGGGCTGC	AATGGCCATGCAGACTGTTG
GCN5	GGAATCGGGCATATCGATTAAC	CCGCATCTCTGTGCAAATATC
Glutathione S transferase	ATGCAAGGGGTAAAGCTGAA	TCAACAAAAGCATCACCGTTT
HSP70	TGCTGATAACCAACCAGGTG	TTTCTTGGTGCAGGTGGAAT
Superoxide dismutase	CAAGGTGGAGGAATGCCATA	CCACCCACTTCCAAAATGAC
PF3D7_1370500	GGATAGCTGTTCTGACCACATTT	GGTACTTAACCCTCCCCGTAG
PF3D7_0832000	TGGTAATGTTGCTGCTGCTT	TGGGGCTGAAAAAGCACTTA
PF3D7_0531800	CGATGGATGTCTTGGTTCCT	AGTACCACTATTCCCCGAAGG
PF3D7_1001300	AAGAAATTCCACCAAAACGTG	TCACAATCATATGGGTTATTCTT
tRNA synthetase	TGGAACAATGGTAGCTGCACAAA	ATGGGCGCAATTTTTCAGGA

18s RNA	GCTGACTACGTCCCTGCCC	ACAATTCATCATATCTTTCAATCGGTA
PF3D7_020010	ATGTGCGCTACAAGAAGCTG	TTGATCTCCCCATTCAGTCA
PF3D7_0420900	AGAGGGTTATGGGAATGCAG	GCATTCTTTGGCAATTCCTT
PF3D7_0500100	GAAGCTGGTGGTACTGACGA	TATTTTCCCACCAGGAGGAG
PF3D7_0809100	TGCAAGGGTGCTAATGGTAA	CCTGCATTTTGACATTCGTC
PF3D7_1200600	TGGTGATGGTACTGCTGGAT	TTTATTTTCGGCAGCATTTG

3 List of oligonucleotide used for ChIP-qPCR

Coordinates	Forward primer	Reverse Primer
PF3D7_0506800	TGTTGGGTTAAACATACCACCA	GGAGGCATCCATATGCTGTT
PF3D7_0202000	AACTGTTCCCTGGGGTTGTT	TCAACACCAACACCAAGCTC
PF3D7_1410600	TGTGGCTCATGGAAAATCAA	TTCAGGTGGTAAGCAATCAGG
PF3D7_1022400	TGGGTCATCTGGAAAGTGTG	CATGGACTAGCCAATCAAGAA
PF3D7_0807500	GTTTTTGTGCGGGATATCGT	GCTTCAATTGCCAAAATGGT
PF3D7_1200600	TGGTGATGGTACTGCTGGAT	TTTATTTTCGGCAGCATTTG
PF3D7_1200100	CGGAGGAGGAAAAACAAGAG	TGCCGTATTTGAGACCACAT
PF3D7_1410600	TGTGGCTCATGGAAAATCAA	TTCAGGTGGTAAGCAATCAGG
PF3D7_1400500	TTTGCTAGTGCTATTGTTGCAGA	TGGTATAGCTTGAGATGCTGGA

4 List of oligonucleotide used for Cloning of PfGCN5, PfGAPDH and PfAlba3

Clone information	Forward Primer	Reverse Primer
GST tagged GCN5_HAT and Bromodomain protein	CAGTGGATCCATAACATTTGAATGT	CATCTCGAGTTTTGCTGTATCAGT
His tagged GCN5_HAT and Bromodomain protein	GCTGCTAGCATAACATTTGAATGTATA	GATGGATCCGGTTTTGCTGTATCAGT
pARL 1a PfGCN5	AGGGGTACCATGGATTATT	AGGGCTAGCTTTTGCTGTATCAGTT

cloning – Truncated	TGATTAGGAATAAT	ATAGCTT
pARL 1a PfGCN5 cloning – Full length	AGGGGTACCACAAATAAT AATTCTGATCCTATA	AGGGCTAGCTTTTGCTGTATCAGTT ATAGCTT
His tagged PfGAPDH	ACGCATATGATGGCAGTA ACAAAACTTG	ATGCCTCGAGGTTGTTAGTAATGTG TACGG
His tagged PfAlba3	ACGCATATGATGGCAAGC ACAGAAGAAG	ATGCCTCGAGGTTTGCTACAAAATC TGGGT

LIST OF PUBLICATIONS

1) Mukul Rawat*, Rashim Malhotra*, Sharvani Shintre, Samarendra Pani and Krishanpal Karmodiya. The role of PfGCN5 in nutrient sensing and transcriptional regulation in *Plasmodium falciparum. Journal of Biosciences* (*equal contribution)
2) Mukul Rawat, Ashish Srivastava, Ishaan Gupta and Krishanpal Karmodiya. Single Cell RNA-sequencing reveals cellular heterogeneity, stage transition and antigenic variation during stress adaptation in synchronized *Plasmodium falciparum*. BioRxiv. doi: https://doi.org/10.1101/752543
3) Mukul Rawat, Abhishek Kanyal, Aishwarya Sahasrabudhe, Shruthi S. Vembar, Jose-Juan Lopez-Rubio and Krishanpal Karmodiya (2019) PfGCN5, a global regulator of stress responsive genes, modulates artemisinin resistance in *Plasmodium falciparum*. BioRxiv. doi: https://doi.org/10.1101/679100
4) Abhishek Kanyal*, Mukul Rawat*, Pratima Gurung, Deepak Choubey, Krishanpal Anamika and Krishanpal Karmodiya (2018) Genome-wide survey and phylogenetic analysis of histone acetyltransferases and histone deacetylases of *Plasmodium falciparum*. FEBS J, 285(10):1767-1782 (*equal contribution)
5) Suyog Ubhe, Mukul Rawat, Srikant Verma, Krishanpal Anamika and Krishanpal Karmodiya (2017). Genome-wide identification and functional characterization of novel intergenic enhancer-like elements: implications in the regulation of transcription in *Plasmodium falciparum*. BMC Genomics 23; 18(1):656.
6) Mukul Rawat, Madhvi A. Bhosale, Krishanpal Karmodiya, (2016). *Plasmodium falciparum* epigenome: A distinct dynamic epigenetic regulation of gene expression. Genomics Data, 7, 79-81.

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