PfHDAC1 is a regulator of cell cycle in *Plasmodium falciparum* and drives the artemisinin resistance associated transcriptome

A thesis

Submitted in partial fulfilment of the requirements

Of the degree of

Doctor of Philosophy

By

Abhishek Kanyal

ID: 20153385

Under the guidance of

Dr. Krishanpal Karmodiya

At

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

2021

This piece of work is dedicated to all those who have believed in me, Always.

CERTIFICATE

Certified that the work incorporated in the thesis entitled "**PfHDAC1 is a regulator of cell cycle in *Plasmodium falciparum* and drives the artemisinin resistance associated transcriptome***"* Submitted by Abhishek Kanyal 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: 11th August, 2021

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.

(Signature)

Abhishek Kanyal

Roll No. - 20153385

Date: 11th August, 2021

ACKNOWLEDGEMENT

There is an endless wave of folk I would like to thank for helping and guiding me through my times at IISER, Pune. Each one has contributed uniquely and inspired me to keep up the pursuit. Taking up a research project is like embarking on a quest. The path is often a maze, winding, twisting and the end unknown. But it always helps to have a good company. People who can inspire you, motivate you and lift-up your spirits when things make little sense and I have been blessed with such company.

My foremost and heartwarming thanks to my supervisor Dr. Krishanpal Karmodiya for being the beacon of endless inspiration and hope throughout my doctoral journey. He has shaped me with his constant motivation for exploration, critical thought and expanding the possibilities in my work. He is a perfect example to me on how to balance work and life outside the lab. I shall remember him for his immaculate organisation, kindness, understanding and patience throughout the journey. Special thanks to Anamika, Ankrisha and li'l Kiyaan for always making us feel at home.

Huge thanks to my research advisory committee members Dr. Dhanasekaran Shanmugam and Dr. Girish Ratnaparkhi for their critical and constant guidance. Special thanks to Dr. Nishad Matange for keeping me on my toes with your sharp questions and thoughtful inputs. You have all motivated me to think out of the box and envision the larger picture. My note of appreciation for Dr. Sanjeev Galande for supporting us through the early phases of establishment of Krish lab and welcoming us to his journal club which has always been an arena of profound scientific discussions and brainstorming.

I would like to extend a note of appreciation for the support I received through EMBO for the short-term research visit to the Francis Crick Institute at London. My heartiest appreciation to Dr Moritz Treeck and his lab members especially Dr. Heledd Davies for helping us with generation of transgenic lines for our research and being so welcoming even in the midst of a pandemic. Thanks to CSIR-SPMF and MHRD for supporting me through the term of my PhD with their fellowship schemes.

A note of appreciation for the diligent staff members at IISER, Pune. Special shoutout to Mr. Tushar Kurulkar and Ms. Sayali Damle for their constant and prompt support for all official matters. A note of great admiration to the spirit of IISER for being thoughtful to the needs of the students and supporting one and all through various initiatives. To the ever-diligent housekeeping, engineering, IT staff, Covid taskforce at IISER, who took half the toil on our behalf and kept things smooth for us. Love to the MDP café staff for their perfect concoctions to keep us up and running.

My thanks to the Krish lab members (both current and past) who have created such a wonderful atmosphere for my academic and personal growth. Love and remembrance to Pratima and Madhavi, my earliest mentors in the lab. Thanks for helping me through the baby steps and patiently guiding me through the initial months. Affection and best wishes to the lovely ladies Bhagyashree, Anjani, Mamatha, Devatrisha, Disha and Rashim. It has been wonderful the last few months with you all. I shall remember our times together (and the movie nights and pizza parties). Over the years I have had the opportunity to work with some brilliant graduate students in the lab. It has been a joy discussing, understanding, and learning things with you all. My special remembrance to Vijay, Sharvani, Dilsha, Sucharita, Sayantan, Mohit, Shweta, Shikha, Vandana, Haripriya, Deepa, Prachi, Feba, and Yajush. You folks have been amazing to interact with.

To my nearest and dearest with whom life would have been unimaginably different. To Deepak, Ishwar, Mrinmoy and Ashish for keeping my faith in kind and gentle men. You guys are some of the loveliest people I have ever had the chance of calling family. To members of Sanjeev lab with whom we made the original "SG-KK gang". To Manu, Ankitha, Rini for being the quintessential crazy, class-apart folk who also happened to be the most fun people on the planet. I shall always remember our deep conversation on all aspects of life. To my soul-sisters Sneha, Nilam and Sudipta, thanks for tolerating my energy. To the group at large Greg, Keshav, Prateek, Anirudh, Ayush, Indu, Akhila, Amit, Rahul and Rahul, Ankita. Special thanks to Saurabh for his support and kind advice during our NGS work.

To my brother from another mother, Mukul, you deserve a paragraph of your own. I am one of the countless souls who have flourished under your brilliant guidance. You have been a constant companion from day one. Discussing work and troubleshooting the obstacles in work has been an amazing experience. Thanks for always inspiring me to stretch my limits and yet be the comfy support to fall back to. It has been a brilliant adventure all along and I am grateful for having shared it with you dearo! Thanks for being my faith in humanity and inspiration to strife for silent excellence. You have nothing but love and appreciation from me.

To Ketakee, for all the lovely times we have spent together. It's a joy discussing life and more with you, day after day, every day. And finally, at the bedrock, to Maa and Papa, who have always been the silent support and motivation. Thanks for always believing in me, motivating me, and always supporting me through my highs and lows, unquestioning. I am what I am because of you! Love, Always!

Life in Pune is a blurry mess of emotions these past six years. I have grown beyond my wildest thought over this time. It has taken me through familiar and strange lands (be it the corridors of the lab, the stairs of MDP with a late night gathering or a late monsoon trek somewhere in

the Western Ghats) and given me the chance to meet some of the loveliest folk. I shall take with me a part of everyone I have met and had the joy of sharing a few moments of love and laughter, with tea, whiskey, and good food around.

Dhanyavaad/Shukriya!

ABSTRACT

Plasmodium falciparum is a deadly protozoan parasite and the causative agent of malaria which accounts for close to 200 million cases and 400,000 deaths every year (WHO World Malaria Report 2020). It has been identified to possess a tightly regulated gene expression profile which is integrally linked to its timely development during the intraerythrocytic stage. Epigenetic modifiers of the histone acetylation code have been identified as key regulators of the parasite's transcriptome. In this study, we have characterised the solitary class I histone deacetylase, PfHDAC1 as an important regulator of numerous parasite genes/pathways implicated in antigenic variation/immune evasion, proteostasis and cell cycle regulation. Furthermore, inhibition of PfHDAC1 leads to a delayed progression through the intraerythrocytic development and even lower infection rates over the subsequent cycle. Conversely, overexpression of PfHDAC1 reversibly enhances the proliferation of the parasites. Interestingly, changes in cell cycle progression have been associated with resistance to artemisinin (ART) drug in *P. falciparum*. Population transcriptomics studies of artemisinin resistant parasites from the field have shown deregulation in the levels of PfHDAC1. We find that genetic manipulation as well as pharmacological targeting of PfHDAC1 abundance/activity resulted in enhanced survival of parasites under therapeutic ART dosage, essentially aiding drug resistance. To identify the transcriptional links between ART resistance and deregulation of PfHDAC1, we compared the transcriptional profile of ART resistant field isolates and parasites with deregulation of PfHDAC1. We identified a core set of biological pathways (upregulation of proteostasis, fatty acid biosynthesis, translation and downregulation of cell cycle, antigenic variation, host-pathogen interaction) matches with the transcriptome of PfHDAC1 deregulation. This in turn suggests that PfHDAC1 deregulation can account for the

core conserved transcriptional changes that are the hallmark of ART resistance. In synopsis, PfHDAC1 is critically linked to governance of a host of important biological pathways in the parasite including cell cycle and its deregulation can facilitate rewiring of the parasite transcriptome in a way that allows drug resistance to emerge.

SYNOPSIS

Chapter 1: Discusses the fundamentals of the devastating disease malaria, the history and burden of the disease across the world. The causative agent *Plasmodium* (especially *Plasmodium falciparum)* and its life cycle are described at length. A brief note on the antimalarial compounds (especially artemisinin-based combination therapy) currently used in the market and the emerging threat of multidrug resistance is presented. In the recent years multi omics NGS studies have been utilised to understand the distinct genetic and epigenetic factors at play in *Plasmodium.* We summarise a fraction of those investigations condensed as discussion of the interesting details of the *Plasmodium* genome and basic transcription regulatory apparatus.

Chapter 2: *Plasmodium falciparum* has a well-defined set of epigenetic regulators that are at play in governing transcriptional patterns in the parasite. These help in governing the stage specific gene expression in the parasite. In the second chapter we review the set of epigenetic modification factors (histone acetyltransferase and histone deacetylases) governing the histone acetylation code in *Plasmodium falciparum*. This chapter is a compilation of a review-cum-analysis where we screened the *P. falciparum* genome for genes bearing HAT and HDAC-like sequence motifs. A phylogenetic analysis to investigate the sequence similarity of the various HATs and HDACs in *P. falciparum* and corresponding molecules from other clades of life has been done. Finally, we have checked the expression profile of HATs and HDACs in artemisinin resistant *Plasmodium falciparum* to see if some of these factors are skewed in resistance and may hence contribute to the resistance associated transcriptome.

Chapter 3: In this chapter we briefly discuss the pharmacological targeting of the *P. falciparum* epigenetic machinery and how histone deacetylase inhibitors are at the forefront of future range of antimalarials. A solitary member of Class I HDAC, PfHDAC1 has been identified as a target of a list of these inhibitors with great promise, but the HDAC itself lacks characterisation of its biological function in the parasite. This chapter thus characterises the PfHDAC1 in relation to its enzymatic activity, its regulation by a posttranslational modification, the genomic occupancy, and gene targets of PfHDAC1 and the biological functions that seem to lie at the core of PfHDAC1 regulation. We also discuss how PfHDAC1 seems to be an important regulator of cell cycle progression in *P. falciparum*. The opposing effects of its pharmacological inhibition and overexpression (by genetic manipulation) on the cell cycle progression and rate of proliferation have also been discussed.

Chapter 4: This chapter discusses the fundamentals of drug resistance, especially artemisinin resistance in *Plasmodium falciparum*. We note that artemisinin resistant parasites show similar cell cycle defects as observed in PfHDAC1 inhibited cells. PfHDAC1 levels have been reported to be deregulated in artemisinin resistant parasites and we make similar observations in resistant parasites. We further discuss the effects of pharmacological inhibition of PfHDAC1 on artemisinin sensitivity in PfKelch13 wildtype and mutant parasites (artemisinin sensitive and resistant parasites respectively). Furthermore, we discuss the effects of genetic manipulation of PfHDAC1 abundance on artemisinin sensitivity of parasites. We also discuss the differences in genomic occupancy of PfHDAC1 and its effects on the expression of proteostasis associated genes in artemisinin resistant lines. Thus, the differential abundance of PfHDAC1 is associated with differential gene occupancy and downstream changes in biological pathways important to resistance.

Chapter 5: This chapter discusses the transcriptional landscape that is characteristic of artemisinin resistance and how the parasite tends to rewire its gene expression profile. We characterise the transcriptome of three PfKelch13 mutant strains and identify the deregulation of biological pathways that characterises them. We observe that artemisinin resistance may be characterised by a core set of gene expression patterns (focussed on slowing down drug activation and repairing drug induced damage) and some strain specific variations that evolve due to distinct genetic/epigenetic and environmental influences. We finally profile the effect of PfHDAC1 depletion on gene expression in parasites and identify that it leads to changes in expression of cell cycle, invasion, and proteostasis associated genes which are reminiscent of artemisinin resistance. Thus, tweaks in the levels of PfHDAC1 may transcriptionally prime parasites to acquire resistance-like features and drive, in part, the resistance transcriptome.

Chapter 6: Discusses the conclusion to this study and the major questions that have arisen from it including scope of future experiments.

ABBREVIATIONS

RBC	Red blood cell
IDC	Intraerythrocytic developmental cycle
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
DDT	Dichloro-diphenyl-trichloroethane
DNA	Deoxyribonucleic acid
CRT	Chloroquine resistance transporter
MDR	Multidrug resistance
DHFR	Dihydrofolate reductase
DHTS	Dihydropteroate synthase
ACT	Artemisinin based combination therapy
TNF	Tumour necrosis factor
MHC	Major histocompatibility complex
CD	Cluster of differentiation
EMP1	Erythrocyte membrane protein 1
ICAM-1	Intercellular adhesion molecule 1
Pfs	*Plasmodium falciparum* surface protein
CSP	Circumsporozoite protein
SPZ	Sporozoite
AMA-1	Apical membrane antigen 1
MSP-1	Merozoite surface protein 1
TSS	Transcription start site
TES	Transcription end site
UTR	Untranslated region
TAD	Topologically associated domain
TBP	TATA binding protein
TAP	TBP associated protein
PIC	Pre-initiation complex
PIC	Protease inhibitor cocktail
AP2	Apetala 2

GDV-1	Gametocyte development protein 1
HP1	Heterochromatin protein 1
HDAC1	Histone deacetylase 1
GCN5	General control non-derepressible 5
ATP	Adenosine triphosphate
SWI/SNF	Switch sucrose non-fermentable
PHD	Plant homeodomain
PTM	Post translational modification
GNAT	GCN5 related N-acetyl transferase
CBP	CREB binding protein
SAGA	Spt-Ada-Gcn5 acetyltransferase
ADA	Adenosine deaminase
PV	Parasitophorous vacuole
PEXEL	*Plasmodium* export element
SAM	S-adenosyl methionine
CD	Chromodomain
NAD	Nicotinamide adenine dinucleotide
Alba	Acetylation lowers binding affinity
NURD	Nucleosome remodelling and deacetylase complex
SAHA	Suberoylanilide hydroxamic acid
ChIP	Chromatin immunoprecipitation
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
PBS	Phosphate Buffered Saline
RPM	Rotation per minute
IPTG	isopropyl-1-thio-β-d-galactopyranoside
PMSF	Phenylmethylsulphonyl fluoride
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
GST	Glutathione S transferase
NTA	Nitrilotriacetic acid
NP-40	Nonyl phenoxypolyethoxylethanol -40
PVDF	Polyvinylidene difluoride
TBST	Tris buffered saline Tween-20
DAPI	4′,6-diamidino-2-phenylindole
BSA	Bovine serum albumin
PCR	Polymerase chain reaction

DTT	Dithiothreitol
FDR	False discovery rate
RT-qPCR	Real time quantitative polymerase chain reaction
CKII	Casein Kinase II
ATP	Adenosine triphosphate
TBB	4,5,6,7-tetrabromo-1H-benzotriazole
EDTA	Ethylenediaminetetraacetic acid
GFP	Green fluorescence protein
SLI	Selection linked integration
HSP	Heat shock protein
BiP	Immunoglobulin binding protein
GMS	Greater Mekong Subregion
DHA	Dihydroartemisinin
SURFIN	Surface associated interspersed gene
SNP	Single nucleotide polymorphism
WGS	Whole genome sequencing
CRISPR	Clustered regularly interspaced repeats
TRAC	Tracking resistance to artemisinin consortium
DMSO	Dimethyl sulphoxide
RSA	Ringstage survival assay
ERAD	Endoplasmic reticulum associated degradation

TABLE OF CONTENTS

CHAPTER 1: Introduction to malaria and *Plasmodium*

1.1 Malaria

Malaria is an infectious disease that is caused in humans by the protozoan parasites belonging to the genus *Plasmodium*. There are five species of *Plasmodium* that infect humans and cause malaria including *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi [1, 2]*. Of these, *Plasmodium falciparum* is associated with maximum morbidity and mortality in humans. The parasite itself is transmitted by the bite of an infected female *Anopheles* mosquito as it feeds on the human blood during a meal. The parasite upon entering the human body first hides in the liver cells where it undergoes rapid multiplication for a duration of 7 to 30 days. From there the infection spills over into the bloodstream and the cyclic parasite invasion of RBC commences [3]. It is in this erythrocytic stage where the disease manifests most of its symptoms notably chills, shivering followed by episodes of fever, joint pain, and vomiting. The disease has global implications and is strongly prevalent in the equatorial and sub/tropical countries. These areas of the world are characterised by high population pressures and generally poor health infrastructure and environmental hygiene. This, along with the favourable climate allows for rapid expansion of the disease [4]. Thus, malaria is not only a disease affecting individuals, but it also shapes the policies of governments at large, making it a strong socio-economic challenge.

1.1.1 History of malaria

Malaria has a fascinating historical link with humanity and has dramatically shaped how civilisations have evolved over the millennia. The very first evidence of the parasites have been

found precariously preserved in petrified mosquitoes found from amber dating from the Palaeogene period, roughly 30 million years old [5]. The parasite itself diversified its repertoire of hosts over eons and came to infect reptiles, birds, rodents and primates [6]. Malaria parasites in primate populations may have zoonotically skipped over to prehistoric human populations even prior to their migration out of Africa [7]. Around the Neolithic age and the advent of agriculture, malaria became of major consequence to human survival. This is emphasised by selection of otherwise debilitating blood disorders like sickle cell anaemia, thalassemia, Glucose 6-phosphate-dehydrogenase deficiency and the loss of Glycophorin C and Duffy antigen on RBC surface [8]. Molecular evidence has certified the prevalence of malaria in the hay days of the Egyptian civilisation (around 2000 BCE) with the first evidence of bed nets as a means of vector control (then unknown for its role in transmission of the disease) coming from the accounts of the Pharaohs [9].

The disease became widely acknowledged by the Greek in 4th century BC with the Hippocrates of Kos associating the disease with bad fumes (miasma) that originated from swampy, marshy regions [10]. The Chinese around 300 BC made great advances in the understanding and treatment of the disease that they associated with periodic fevers and enlarged spleens [11]. It is around 168 BC that the herbal remedy Quing Hao (a derivative of the sweet wormwood *Artemisinin annua*) gained popularity thus establishing the earliest use of the compound in treatment of malaria [12]. It would be years later that these ancient texts would be referred to by modern scientists and revitalised for modern therapy. The disease may have been largely responsible for the fall of the Roman empire in the 4th century AD, with a major outbreak of "Roman fever" documented around the time [13]. The Italians were the first to coin the term malaria (based on Mal: foul Aria: Air) due to rampant occurrence of the disease in marshy areas. This finds its roots in the ancient Greek Miasma theory. Unbeknownst to them, the marshes were actually the breeding grounds of the mosquito vectors of the disease [14].

The first documented investigation into the cause of the disease comes with the medical study of Charles Laveran stationed at the military hospital in Constantinople (Fig. 1). He noticed brown pigment granules in the red blood cells of malaria patients. He was the first to document exflagellation parasites (which he coined 'Oscillaria malariae') in the blood and showed that quinine was able to kill off these forms [15]. He was awarded the Nobel Prize in Physiology (1907) for his contribution to the discovery of the causative agent of the disease. Sir Ronal Ross, a surgeon in the British Indian army was the first documented person to finally establish the link between mosquito vector and the malaria pathogen by artificially feeding the blood of malaria patients to mosquitoes [16]. Bastianelli, Bignami and Grassi became the first people to document the entire life cycle of *P. falciparum* [17].

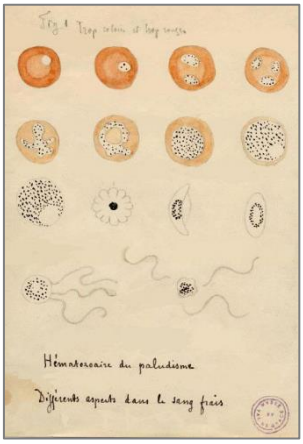


Fig. 1: Illustrations sketched by Charles Laveran for the stages of Plasmodium observed in fresh blood smear. Dark pigmented hemozoin granules are prominent through all forms. Source: Centre for Disease Control.

1.1.2 Burden of malaria

The World Health Organisation which maintains a strict monitoring of the disease burden and documents it in the form of the World Malaria Report annually. The World Malaria Report

2020, documents close to 229 million cases of malaria in 87 malaria endemic countries in 2019 [4]. Countries in Africa (including Nigeria, Congo, Niger, Uganda, and Mozambique) accounted for 95% of the total cases. 409,000 deaths were recorded and directly attributed to malaria in the year 2019 (Fig. 2). Most of these deaths are still reported in children under the age of 5, owing to a naive immune system as well as pregnant women. On a brighter note, over the past two decades there has been a significant reduction in the overall incidence of malaria globally (60% drop over 2000-2019) with government efforts being strongly driven by active investment in malaria research. 21 countries have been declared malaria free in the last twenty years including recent additions of Sri Lanka and Timor-Leste [4].

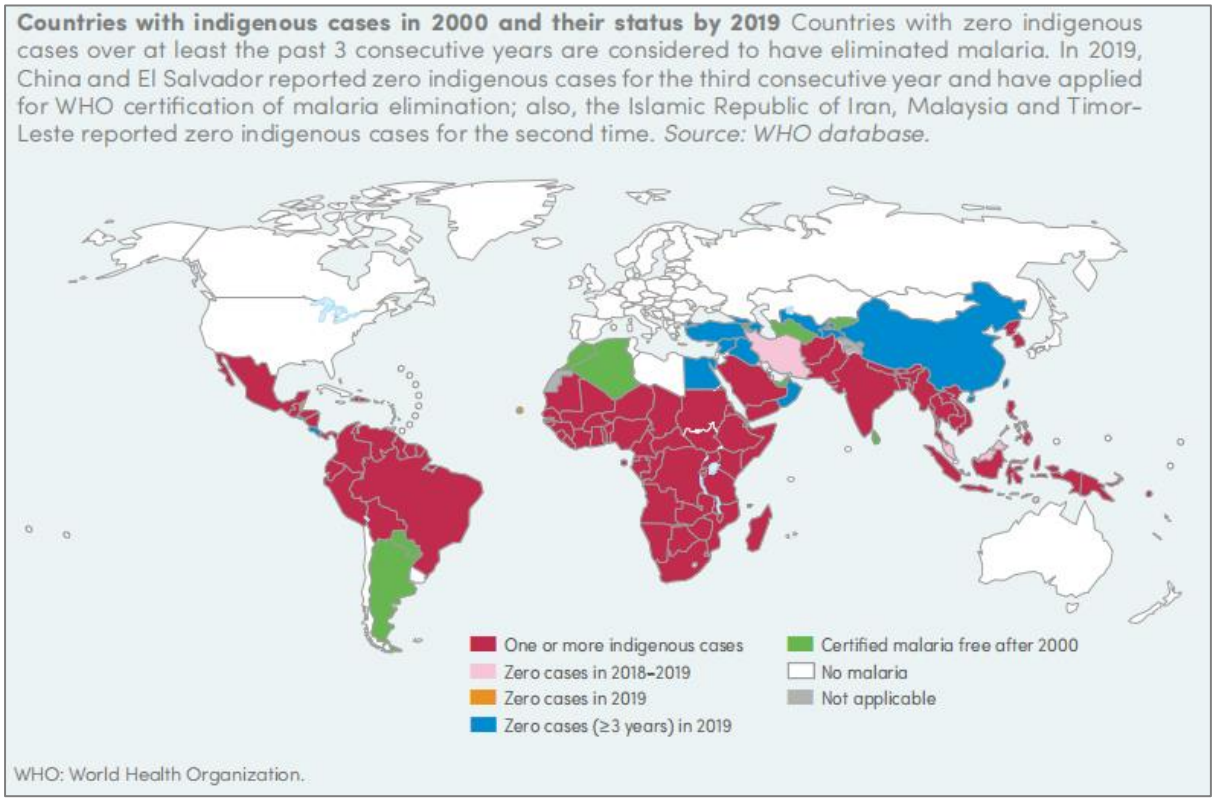


Fig. 2: World map of the global incidence of malaria from 2000-2019. Source: WHO World Malaria Report 2020 [4].

1.1.3 Malaria in India

India accounts for around 3% of the global burden and a major chunk of the infections in the region of Southeast Asia [4]. The disease puts roughly 1.26 billion people at risk in the country. Owing to a wide disparity in the availability and logistic access to counter measures, many of the states in the country with extensive rural populations lead the infection counts (including Chhattisgarh, Orissa, Madhya Pradesh, Jharkhand, Meghalaya, Mizoram). *P. falciparum* and *P. vivax* are the two major parasite species prevalent in the region [18]. The country has started to report an overall drop in cases of malaria but an alarming trend of rise in proportion of *P. falciparum* malaria among the number of existing cases puts the country at risk of the more virulent form of the disease [19].

The country has however taken huge strides in the control and elimination of the disease over the decades. As per the WHO, India has had a sustained reduction in the Annual Parasitic Index since 2012, with an API of 17.6% in 2019 and 27.6% in 2018. It has contributed to the largest drop in region wide (Southeast Asia) cases (72%) and mortality (74%) in the last two decades [4]. The National Vector Borne Disease Control Programme has consolidated itself as the nodal point for addressing the burden of malaria in the country. Through coordination with field stations and clinics spanning the country the organisation collects and assesses the situation of malaria in the country and directly coordinates with the Ministry of Health to advise and consolidate its policies.

1.2 Incubation period and symptoms of malaria

Upon being bitten by an infected mosquito, the parasite enters the human host and goes into an incubation period that may last anywhere between 7 to 30 days. Brief incubation periods are reported for *P. falciparum* and longer ones for *P. malariae*. Upon entering the body, the parasite

gains access to the bloodstream and immediately seeks access to the liver through specific receptors. There it undergoes a brief phase of replication coinciding with the incubation period before finally effluxing into the bloodstream and showing symptoms. Prophylactic drugs can often lengthen the duration of the incubation period leading to inaccurate diagnoses. In the blood-infective stage *Plasmodium* shows periodic cycles of invasion, growth, egress, and reinvasion. The classic malaria attack is characterised by episodes of chills, shivering followed by rapidly rising body temperature, and finally a sweating phase which returns the body temperature back to normal but is characterised by lethargy. Other symptoms include bouts of nausea, vomiting, headache, and joint pain [20].

1.3 Complicated, severe malaria

Malaria symptoms are quite toilsome but if left untreated or by a combination of host physiological factors the disease may sometimes progress to a more systemic form leading to multiple organ failure and death. This is called complicated malaria and has a host of manifestations. Cerebral malaria characterised by cognitive and behavioural abnormalities is one such form. Severe anemia, blood coagulation defects and hematuria are also reported in severe malaria. These defects eventually increase the toil of key organs in the body including spleen, liver and kidney which are critically involved in detoxification of the body. This splenomegaly, hepatomegaly and kidney failure are manifestations frequently found in severe instances of malaria. Morbid forms of the disease are attributed to especially virulent forms of *Plasmodium* that frequently report antigenic variation and cytoadherence through a special class of parasite virulence factors [21].

1.4 The causative agent: *Plasmodium*

Malaria is caused by unicellular eukaryotic protozoa of *Plasmodium* species. *Plasmodium* belongs to the phylum Apicomplexa. The phylum is named Apicomplexa owing to the presence of the apical complex structure. The genus reports around 250 species known to be infectious in a wide variety of organism clades including reptiles, birds, rodents and primates [6]. There are five species of *Plasmodium* which can infect human beings. These include *P. falciparum, P. malariae, P. vivax, P. ovale, and P. knowlesi* (Fig. 3) [1]. Out of these five species, *P. falciparum* is the most lethal and is associated with highest mortality [22].

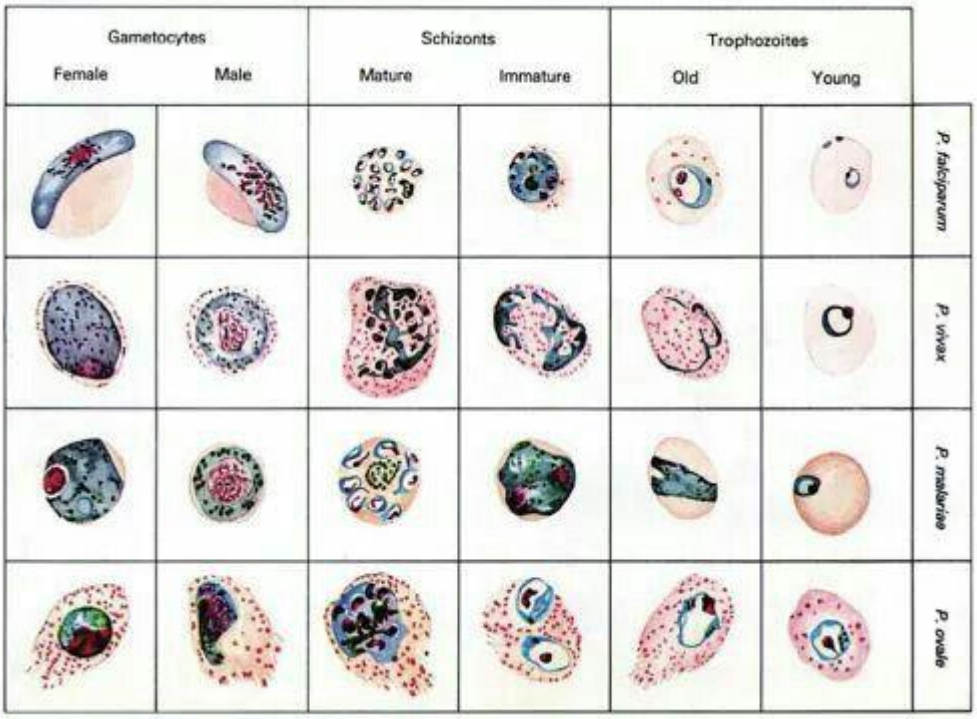


Fig. 3: Illustrations of the blood stage smears of various Plasmodium species. Source: Jennifer Lana Roa illustrations.

P. falciparum infections are predominant in tropical countries (in Africa, Asia and Latin America) [4]. Infection results in high titre of parasites in the blood which can result in severe manifestations of malaria or even death of the host. The species *P. vivax* is predominant in

tropical as well as some temperate countries in Latin America and the Indian subcontinent [23]. *P. ovale* is conspicuous by its presence mostly in West Africa [24]. *P. vivax* and *P. ovale* are known to have dormant stages in the liver resulting in relapse of malaria. This makes treatment as well as adequate diagnosis of the disease uncertain [25]. *P. knowlesi* was originally thought to infect only non-human primates but recent findings have proven that it has passed a zoonotic event and transitioned into infecting humans as well [26]. *P. knowlesi* completes its asexual life cycle in a mere 24 hours and can hence result in high parasitemia leading to disease exacerbation and death in humans [27].

Plasmodium is known to possess the apical complex structure situated at the apical pole of the organism (Fig. 4). It houses machinery like granules, micronemes and rhoptries, necessary for the invasion of the parasite into the host cells. It carries an apicoplast which is likely the product of secondary endosymbiosis of red alga by *Plasmodium* ancestor. This organelle carries enzymes that are crucial for key metabolic processes in the organism including fatty acid biosynthesis, and generation of iron-sulphur clusters necessary for heme metabolism [28]. The food vacuole is the site where the digestion of the host haemoglobin is carried out along with additional detoxification of haem into non-toxic hemozoin [29]. *Plasmodium* also contains other organelles like the Golgi apparatus, endoplasmic reticulum and a single mitochondrion which coordinates its replication along with the cell division [30].

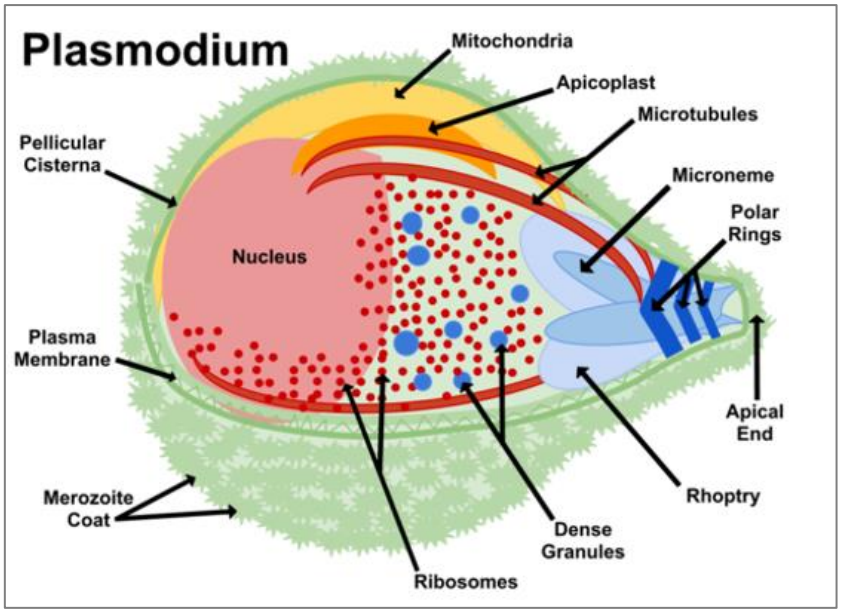


Fig. 4: A cellular cross section of the P. falciparum merozoite displaying individual components of the apical complex including the outer coat of merozoite. Source: Microscope Master.

1.5 Life cycle of *Plasmodium*

The *Plasmodium* life cycle consists of a sexual and asexual phase (Fig. 5). The sexual phase is carried out in the mosquito (which also serves as the transmission host) and the asexual phase in human host [31]. The asexual phase commences when a female *Anopheles* feeds on human blood and injects a load of sporozoites with its saliva. The saliva also carries vasodilators which enhance the porosity of blood vessels and allows the sporozoites to easily traverse into them. The blood flow is then used to passively transport the parasite to the liver where it tethers onto the hepatocyte cells using specialised receptors. Sporozoites persist in the hepatocytes for one to two weeks during which they undergo rapid division to generate thousands of merozoites (hepatic schizogony) [32]. A small portion of these parasites form dormant hypnozoites which persist in the liver cell for a long time and are responsible for relapse of malaria if not identified timely and treated [25]. The merozoites released from the hepatocyte upon rupture enter the

blood circulation where they commence the erythrocytic phase and start infecting red blood cells in an episodic manner. The merozoites tether to the red blood cells using the apical complex and invade into it forming a parasitophorous vacuole [33]. Upon establishing infection, they undergo transition through different morphological stages: the ring, trophozoite and schizont. During early stages in RBCs, parasites are referred to as rings due to their signet ring shape appearance. They then become metabolically active and start feeding upon the host cell hemoglobin channelled through endocytosis into the food vacuole. The hemoglobin serves to provide and replenish the amino acids required for the growth. It is during the trophozoite stage that the parasite also enters into the erythrocytic schizogony, whereby it rapidly multiplies its genetic material in an endomitotic fashion [34]. Finally, during the schizont stage, parasites undergo final rounds of nuclear division followed by a cytokinesis which results in the formation of segmented schizonts. The final mature parasite in the RBC on the cusp of rupture and egress has around 16-32 merozoites [34]. This is followed by rupture of the infected RBCs and release of the merozoites which can now potentially re-invade new RBCs and renew the cycle. Eventually over the course of infection the parasite count shoots up dramatically and leads to the periodic episodes of chills, fever and sweat as the host immune system releases a flurry of immunomodulators in response to the parasite.

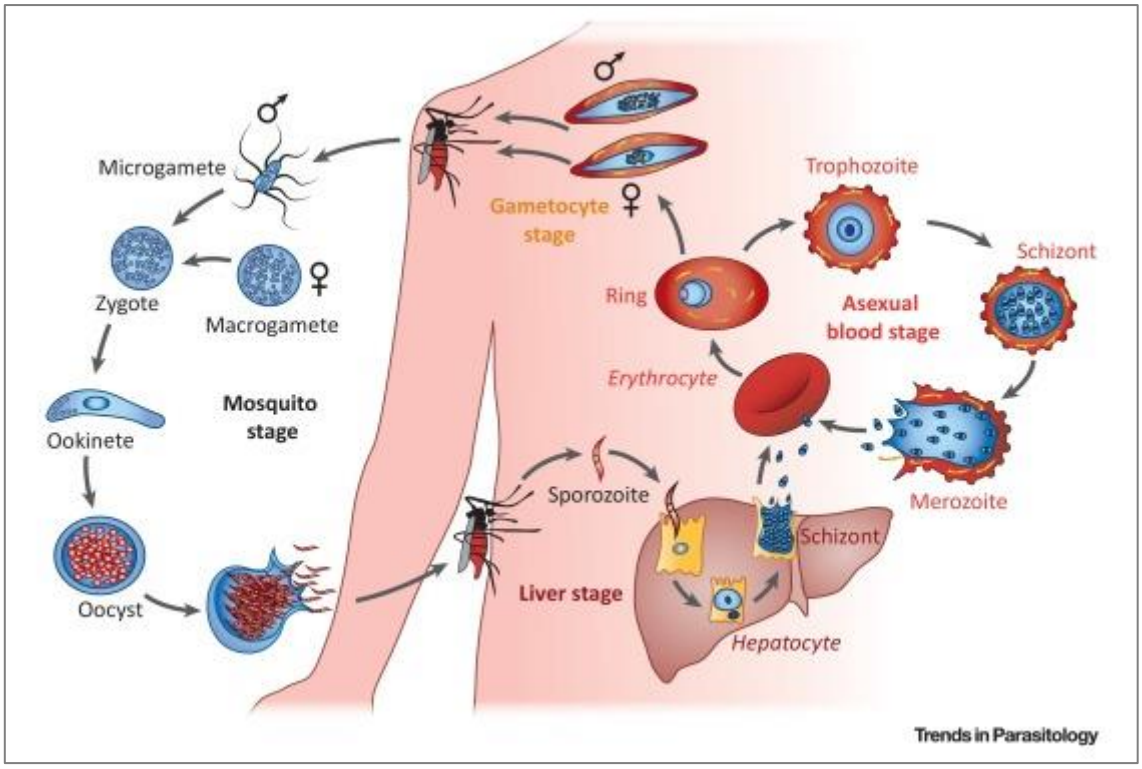


Fig. 5: Life cycle of Plasmodium falciparum spread across the mosquito and human host. Source: Maier et. al., 2019; Trends in Parasitology [35].

When the parasitemia in an infected host becomes too high, leading to depletion of nutrients in the host, molecular signalling mechanisms kick in and reprogram the freshly invading merozoites to express genes relevant to sexual conversion. This response essentially mimics a "jumping ship" strategy of the parasite to evade the host which does not provide optimal conditions for parasite growth. The infected RBCs harbouring the sexual form of the parasite are ingested by the female *Anopheles* while feeding on a malaria infected human [36]. Within the *Anopheles* midgut, gametocytes are released and develop into mature flagellar motile male (microgamete) and female (macrogamete) gametes. Fusion of the mature gametes results in a zygote which matures to form the ookinete stage that travels to the salivary gland to form oocysts [37]. Each oocyst serves as the precursor to thousands of sporozoites. The sporozoites in the salivary gland get transmitted into the human host anew when the mosquito releases its vasodilator rich saliva prior to taking in a blood meal [38].

1.6 Prevention and control of malaria

Vector control, elimination, prophylactic medication and prevention are the major arcs of malaria prevention [39]. The parasite transmission is a critical link in its continued survival in a region and hence methods to curb that are very important. Unsuitable conditions for infection propagation have thus resulted in elimination of malaria from Europe and Northern Africa. Vector control strategies can be effective in the long run but prove to be economically non-viable for most people living in the worst affected areas hence requiring extensive investment and public education on part of the government [40].

1.6.1 Vector control

Vector control strategies rely on utilisation of methods to control the mosquito populations in the community. Insecticide treated mosquito nets and indoor residual spraying are the two most common effective strategies for controlling vector populations. Nets coated with insecticides (pyrethroids) have been proven to be almost twice as effective in preventing mosquito-human contact than non-coated nets [41]. Data gathered over the last few decades has shown insecticide treated nets to be increasingly available to populations in Africa. This has strongly correlated with better prognosis for pregnant ladies in the region and overall lower incidences of malaria among the younger generation, especially infants [42]. Indoor residual spraying involves spraying household boundaries and surfaces with insecticides so as to kill off mosquitoes as they settle down to rest after a blood meal [43]. DDT and the pyrethroids including deltamethrin have been widely used in the past for the purpose of spraying but in recent years emergence of insecticide resistant strains of mosquitoes and the adverse health effects of the chemicals on humans has dampened the efficacy of this strategy. Modifications to households and communities at large has also helped address the problem with people being increasingly made aware of the hazard of stagnant water sites being the breeding grounds of

mosquitoes. This has promoted the practice of measures to either clear away or periodically cleanse such zones of danger [44].

1.6.2 Prophylactic medication

Antimalarial compounds frequently used for treatment of malaria can also be used for preventive measures. This is especially useful for immunocompromised individuals, pregnant women and travellers visiting malaria endemic regions. In pregnant women it has been shown to be associated with improved infant weight at birth and low risk of anemia [45, 46]. Intermittent preventive therapy in infants can improve their chances of staving off infections especially when their immunity is still naïve [47]. Cotrimoxazole and mefloquine are some of the prominently used medicines for prophylaxis [48].

1.6.3 Antimalarial compounds for treatment

Owing to lack of any definitive vaccine against malaria in the market, efforts to control the disease are focussed on preventive action and vector control. Antimalarial medication can be categorised as therapeutic (for confirmed infections), prophylactic (taken as a pre-emptive measure) and intermittent preventive (periodic dosage in endemic areas). Once infection has occurred there are several therapeutic options at hand. Cinchona alkaloids (quinine), amino-quinolones (chloroquine), pyrimidines (pyrimethamine), sesquiterpene lactones (artemisinin), sulphonamides (sulphadoxine), quinolone derivatives (mefloquine), and biguanides (proguanil) are some of the frequently employed drugs (Table 1) [49]. These drugs have varying mechanisms of action and target different aspects of the parasite biology. Chloroquine targets the bio-crystallisation of toxic heme into hemozoin in the parasite food vacuole,

pyrimethamine blocks the nucleotide biosynthesis thus hampering DNA replication and cell cycle, while primaquine is an effective gametocide thus blocking further transmission [50].

Antimalarial Drug	Mechanism of Action	Site of Action	Mechanism of Resistance
Antifolates ((pyrimethamine (PYR) and cycloguanil (CYC))	Inhibition of dihydrofolate reductase (DHFR)	Cytosol	Mutations in dihydrofolate reductase (DHFR)
Antifolates (sulfadoxine (SDX))	Inhibition dihydropteroate synthetase (DHPS)	Cytosol	Dihydropteroate synthetase (DHPS)
Naphthoquinones (Atovaquone (ATQ))	Inhibits mitochondrial electron transport	Mitochondria	A single point mutation in the cytochrome b subunit (CYTb) of the bc1 complex
Antibiotics (Clindamycin (CLD) and Doxycycline (DOX))	Inhibit protein translation inside the apicoplast	Inside the apicoplast	A point mutation in the apicoplast encoded 23S rRNA (CLD)
Artemisinin (ART)	Alkylation of proteins and lipids	ER, vesicular structures	Mutation in K13
4- aminoquinolines (CQ, AQ, PPQ, Mannich base pyronaridine (PND))	They bind reactive heme and interfere with its detoxification through incorporation into chemically inert hemozoin.	Digestive vacuole	Point mutations in the transporters PfCRT and PfMDR1, increased expression of the hemoglobinases plasmepsin 2 and 3 (PM2/PM3, in the digestive vacuole), and might in some instances involve mutant PfCRT

Table 1: List of commonly employed antimalarial compounds, their mechanism/site of action and the mechanism of resistance. Source: Shibeshi et. al., 2020; DovePress [51].

1.7 Resistance to antimalarial compounds

The one reason behind the persistence of malaria is its tenacity to the therapeutic options that are deployed against it. Despite decades of use of a diverse array of antimalarial medication *Plasmodium* has shown great adaptability and evolves resistance to the drugs. Parasites from Southeast Asia especially the greater Mekong subregion have served as the progenitors of drug resistant strains that have spread across the world [52]. Due to rampant uncontrolled usage of drugs over decades resistance started to emerge against the classically favoured drugs like chloroquine, sulphadoxine and pyrimethamine. The drug policies for usage of these compounds were not strict often leading to indiscriminate monotherapeutic usage. Since many

of the classically used drugs target specific pathways of the parasite biology it did not take long for resistance to emerge in the field. Chloroquine resistance emerged by mutation of the parasite transporter Pfcrt and Pfmdr1 which allowed for the drug to be pumped out of the food vacuole and hence rendered function less. Similarly, mutations in the gene encoding Pfdhfr and Pfdhps were enough to suppress the targeting of these molecules by sulphadoxine and pyrimethamine leading to enhanced parasite survival. Mutations in the mitochondrial electron transport chain proteins cytochrome bc1 complex rendered the drug atovaquone ineffective [53]. Owing to the prolific replication the parasite undergoes in the bloodstream every 48 hours it takes a relatively short duration for mutants to emerge in the field that show resilience to drugs. The problem has been greatly accelerated by the usage of drugs in monotherapy format for decades, which allows for mutants to establish, persist and spread easily in populations [54].

1.8 Artemisinin based combination therapy

During the late 20th century artemisinin was reintroduced into mainstream therapy by the research led by Tu Youyou (Fig. 6) [55]. It is a sesquiterpene lactone that is derived from the sweet wormwood plant *Artemisia annua*. It is a potent source of free radicals once activated in the system which often requires a metabolised version of heme that is only generated in the parasite food vacuole. Upon activation the drug generates a burst of free radicals in the parasitise and forms adducts with a host of cellular molecules. This causes a collapse of the system and parasite death [56]. Owing to its selectivity and high efficacy it quickly gained prominence in the field and has been used extensively in regions facing multidrug resistance problems. In order to make it more bioavailable chemical derivatization has led to development of water soluble artesunate and lipid soluble artemether [57]. Learning from previous mistakes

international drug policies have been revised to use this compound only in combinatorial format. It is coupled now with a longer lasting (slow metabolising) partner drug like mefloquine, sulphadoxine/pyrimethamine or lumefantrine to form what is known as Artemisinin based combination therapy. While the short-lived artemisinin kills off the major parasitic load. The longer-lived partner drug prevents the resurgence of infection from the residual parasite population [58].

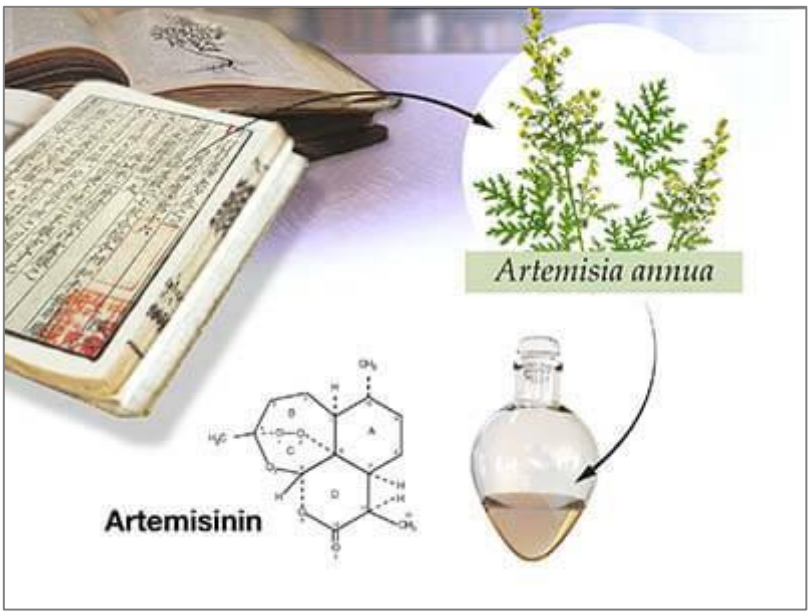


Fig. 6: The rediscovery of artemisinin as a potent antimalarial compound derived from Artemisia annua based on ancient Chinese herbal medicine scriptures. Source: Pharmacopodia.

However, in 2008 the first report of artemisinin resistance came from Southeast Asia and raised alarms everywhere [59]. Studies eventually traced mutations in the PfKelch13 gene to be causative to resistance but this marker was not found to be universally linked to resistance [60]. Extensive research is underway to trace the molecular mechanism of artemisinin resistance which seems to be multifactorial in nature.

1.9 Acquired immunity against *P. falciparum* infection

The parasite in the intraerythrocytic stages is essentially hidden from the immune system. However, antibodies designed against merozoites can prevent them from invading the RBCs or in conjunction with complement factors lead to phagocytosis of infected cells or agglutination/clearance of infected RBCs [61]. Antibody cell dependent inhibition is a mode in which merozoite specific IgG1 or IgG3 antibodies are generated which stimulate phagocytes to release TNF-alpha and promote the killing of infected cells [62]. Infected RBCs present a challenge to instigate and execute a good immune response since these parasites are essentially hidden in cells that present no MHCs. Despite this T cell response to iRBCs is observed [63]. Infected RBC specific CD4 helper T cells are important for generation of cytokines that can in turn boost the antibody generation responses from B cells [64]. The right type of IgG isotype (IgG3) is important for maintaining a robust immune response against the malaria pathogen in the form of antibody-cell dependent inhibition [65]. Interferon-gamma secreting CD8 T cells have also been experimentally proven to reduce the severity of infection in tested mice [66].

1.10 Antigenic variation in *P. falciparum*

Contrary to antigenic polymorphism where allelic variants of the antigens can be used to weaken immune responses antigenic variation is a strategy where a single clonal population of parasites relies upon using different versions of multigene family encoded antigenic factors [67]. With the availability of the whole genome sequencing data the molecular function of antigenic variation in *P. falciparum* became evident.

P. falciparum has evolved to possess a set of factors encoded by the multigene families belonging to the vir family. These include the *var*, *rifin* and *stevor*. These genes exist as multicopy gene families in sub-telomeric or central clusters [68]. Upon expression they are

exported to the surface of the RBC where they tether onto the RBC membrane and expose themselves. A number cell-cell adhesion phenotypes such as agglutination (clumping of infected RBCs by platelet junctions) and rosetting (clustering of uninfected RBCs around a single infected RBC) are proposed to be mediated by these factors [69]. Furthermore, these exported proteins are known to interact with endothelial receptors deep in the microvasculature of organs like the brain and bone marrow. The PfEMP1 protein encoded by the *var* genes possess hypervariable domains called Duffy Binding Like. These domains interact with the ICAM-1, CD36, and chondroitin sulphate A on endothelial cells (Fig. 7) [70]. Here they can often allow persister parasite populations to hide out and survive immune system pressure or drug exposure. Excess clumping of these cells in fine microvasculature is proposed to be a mechanism via which complicated cerebral malaria emerges as they can potentially clog blood vessels supplying nutrition to key areas of the brain [71]. Orthologs of the vir genes go by the name of pir in *P. vivax* and *P. knowlesi* [72].

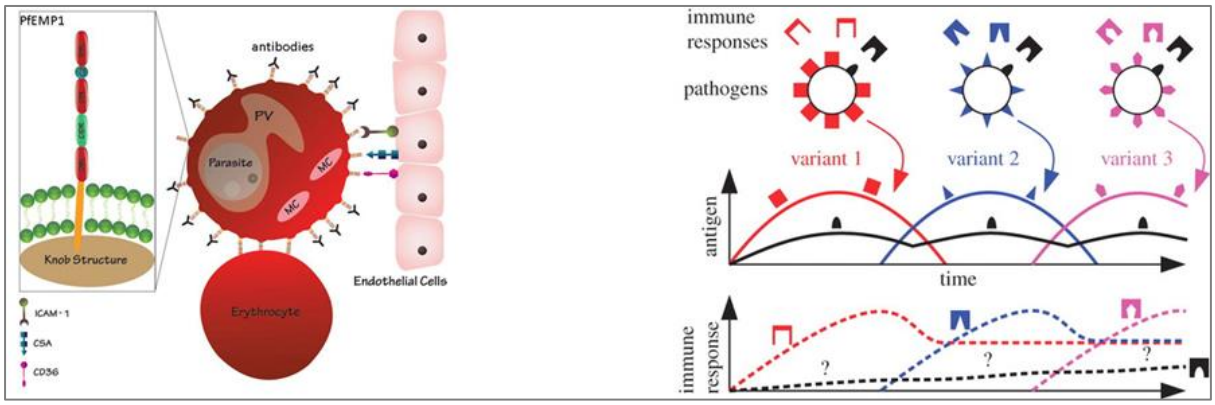


Fig. 7: (Left) The var gene encoded PfEMP1 protein that can influence RBC cytoadherence by interacting with endothelial membrane receptors ICAM, CSA. (Right) Wave-like expression of virulence gene variants over time contributing to immune evasion. Source: (Left) Pasternak et. al., 2009; International J Biochem Cell Biol. (Right) Johnson et. al., 2012; Proceedings of the Royal Society B [73, 74].

While genomics studies gave us molecular insights into the organisation and sequence diversity of multigene families in *P. falciparum* transcriptomics studies have helped us understand how the antigenic variation mechanism is played out. The expression of these multigene families is highly controlled in a mutually exclusive expression pattern where only one member of the *var* gene family is expressed at a time while the rest are silenced by transcriptional regulatory mechanisms. Every cycle there is a stochastic change in expression of these *var* genes at the rate of roughly 2% [75]. Upon encountering an immune pressure, viz. In the event of immune detection of the parasite, the expression of the existing *var* genes is suppressed and a new one is executed. With a repertoire of close to 350 virulence genes including *var*, *rifin* and *stevor* the parasite has a number of options at its disposal [76]. The study of the expression profile and mechanism of regulation of vir genes is of utmost importance since they can determine the efficacy of vaccination trials across the globe. Furthermore, certain virulence genes like the var2csa are specifically implicated in certain aspects of human biology. Var2csa mediates a strong cytoadherence of RBCs to placenta in pregnant women leading to complications and stillbirths [77].

1.11 Vaccination for malaria

Even though we address and cure malaria infection there is still the lack of definitive vaccines against the disease. One reason behind this is the extreme level of antigenic polymorphism and variation the parasite has evolved to deploy against our immune system. There are a few vaccines that exist targeting various stages and aspects of the parasite biology: namely i) transmission blocking, ii) anti-infection, and iii) blood-stage vaccines (Fig. 8). Transmission blocking vaccines (against Pfs25, Pfs45/48, Pfs230) are designed to identify sexual states of the malaria parasite in the blood and neutralize them before they are picked up by mosquitoes and transmitted to the next host [78]. ii) Anti Infection vaccines are some of the most invested

in since they aim to neutralise the early infective form of parasites (sporozoites) that enter the human system. These come as subunit vaccines designed from protein subunits of the sporozoites (full length CSP, RTS,S/AS01E) or whole sporozoite vaccines that use neutralised (often irradiated or genetically attenuated sporozoites) including the PfSPZ vaccine [79, 80]. iii) Blood-stage vaccines designed against merozoite antigens (AMA1, MSP1/3, Rh5) that propagate RBC infection via reinvasion or against other antigens like the var2csa expressed in *P. falciparum* infecting pregnant women [81].

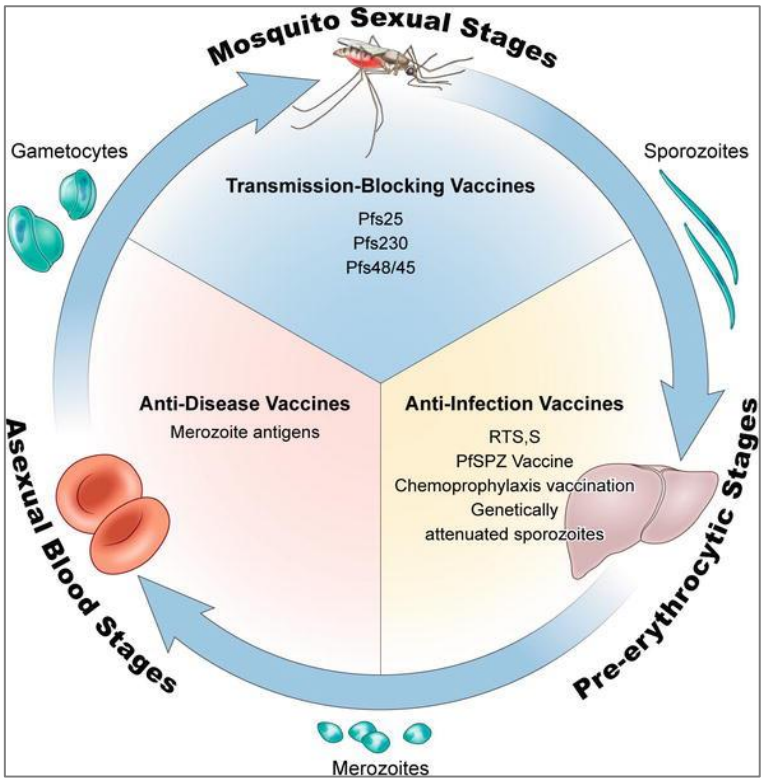


Fig. 8: The vaccine candidates from various stages of Plasmodium falciparum life cycle. Source: Duffy and Gorres, 2020; NPJ Vaccines [82].

The RTS, S vaccine is developed by the collaboration of PATH Malaria Vaccines Initiative and GlaxoSmithKline supported by the Bill and Melinda Gates foundation. The latest version of the vaccine fuses the CSP antigen of *P. falciparum* and the Hepatitis B surface antigen to provide robust immunity against the hepatocyte invading forms of the parasite [83]. Clinical

trials carried out in young children have shown 30% immunising efficacy in children of age 4 and above but limited potential for infants [80].

1.12 Multiomics investigation pave way for understanding the complex biology of *P. falciparum*

Plasmodium has over the millennia proven to be a hard adversary to eliminate. Despite decades of investigation into its biology, discovery of a wide range of compounds with antimalarial properties and even use of therapy that can effectively cure patients, the disease itself has not faded into obscurity unlike other pathogenic diseases [84]. We have come to understand that part of the problem lies with the extreme resilience of the parasite and its adaptability in the face of environmental challenges. The secret to its resilience lies in its genetic code, what genes it possesses and how the parasite chooses to express its repertoire of genes. With the advent of next generation sequencing, multi omics studies have been implemented to understand the curious biology of this parasite to great success [85].

1.12.1 *Plasmodium* genomics

The first draft of the *Plasmodium falciparum* genome sequence was published in 2002 [86]. *Plasmodium* parasites mostly spend a haploid life cycle except for a short diploid phase post fertilisation in mosquitoes. The genome size for the various species is on average 2-3 times larger than the *Saccharomyces cerevisiae, ranging* at around 25Mb. *Plasmodium* species have 14 chromosomes, a circular plastid genome at 35Kb and a mitochondrial genome at 6kb size. Pan species comparison has shown abundance of homologous genes arranged as syntenic blocks across the chromosomes. A distinctive feature of the genomes of *Plasmodium* species is the abundance of AT rich sequences ranging from about 80% in *P. falciparum* to around

60% in *P. vivax* [87]. This AT rich genome is part of the explanation for simple repeat sequences, low complexity regions, a highly skewed codon usage bias and abundance of similar looking multigene families [87]. *P. falciparum* is known to possess around 5770 genes but due to the abundant AT rich sequences many of these genes are shown to have no orthologues in other organisms and are thus uncharacterised. This extreme AT bias in the *Plasmodium* genome establishes it to have rather uncanonical gene organisation as well as possibly different strategies to regulate the expression of genes [86].

1.12.2 Transcriptomics of the malaria parasite

The investigations into the gene expression in *P falciparum* began with microarray investigations in the early 2000s in two pioneering studies done by Karine le Roche and Zbynek Bozdech [88, 89]. The studies were focussed on identification of the genes that are expressed in different erythrocytic stages of development. These studies identified that a major chunk of the genes is expressed at one point or the other in the parasite (60-97% of all genes) in a highly coordinated stage specific fashion which is synchronised to address the specific biological pathways in the stage. For instance, the trophozoite stage is characterised by extensive expression of DNA replication associated genes (required for increasing the parasite ploidy in the stage) while the late schizont stage was characterised by expression of RBC invasion related genes (so as to prepare the merozoites for the next round of invasion into fresh RBCs) [88]. Bozdech et al. also discovered a peculiar feature of the gene expression that while a significant proportion of the nuclear genome is arranged as contiguous gene units, they are rarely coregulated. The plastid genome on the other hand is highly correlated owing to its polycistronic organisation. The overall understanding of the gene expression profile of the

parasite at that time gave rise to the "just in time" transcriptional expression mode where genes were switched on right before they are functionally relevant (Fig. 9) [88].

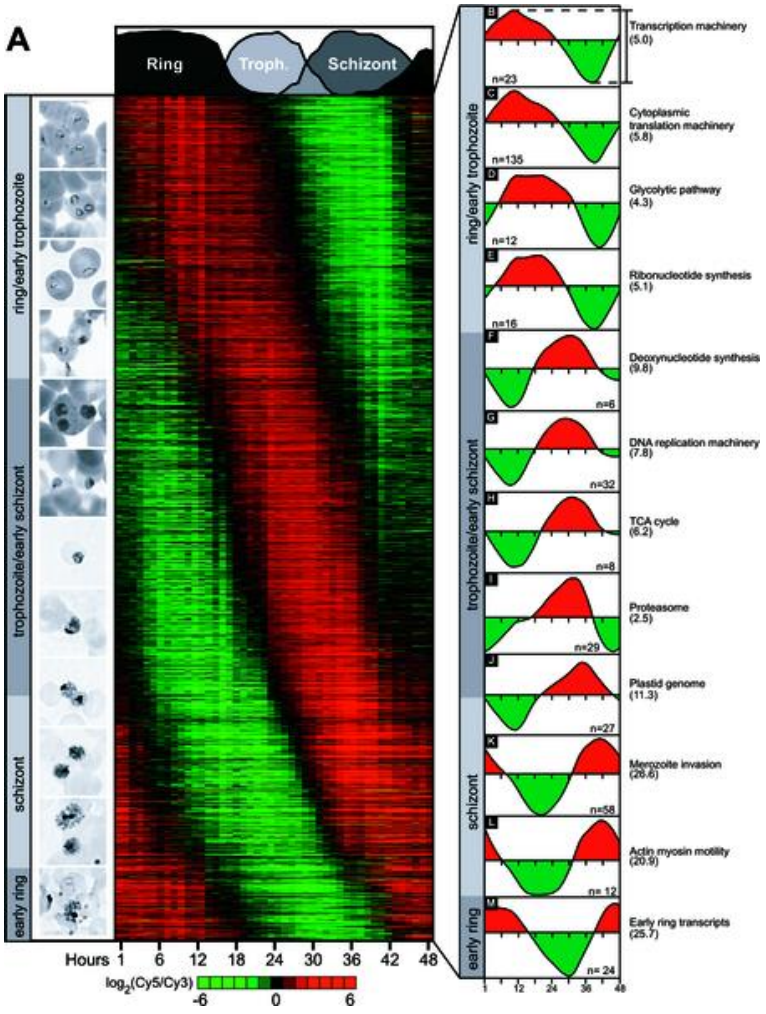


Fig. 9: The synchronised wave-like stage specific expression of P. falciparum gene sets supports a "just in time" model for gene expression. Source: Bozdech et. al., 2003; PLoS Biology [88].

1.12.3 Regulatory DNA elements in *Plasmodium*

P. falciparum possesses the bipartite gene organisation which calls for the recruitment of the RNA polymerase II to the transcription start site. Both promoter and enhancer cis regulatory regions have been discovered in *P. falciparum* but further evidence for the role of the latter in

gene expression regulation is required [90]. The spatial location of the transcriptional start site (TSS) is characteristically distant from the first exon on average leading to an exceptionally longer 5'UTR region [91]. Promoters possess DNA sequences that can help in selection of the transcriptional start site and promoter strength [92]. Recent *in silico* studies have hinted at presence of cis regulatory elements that are highly dissimilar from eukaryotic counterparts and are abundantly available for every gene [93]. Thus, a model of multiple regulatory elements coordinating the expression of several genes emerges for *Plasmodium* rather than a one gene one regulator model [94].

1.12.4 Nucleosome landscape in *P. falciparum*

The basic genomic organisation in most eukaryotes begins with the DNA double-helix wrapped around the octameric histone core formed by interaction of the histone proteins H2A, H2B, H3 and H4 [95]. A number of non-standard histone variants have been documented in *P. falciparum* including H2Az, H2Bz, H3.3 and CenH3 [96, 97]. These variants can readily exchange with the standard histones and add to the differential gene expression profile. The linker histone H1, which is responsible for higher order packaging of the genomic content, is missing in *P. falciparum* which results in an "open chromatin" environment that is significantly less compact than standard eukaryotic genomes [86].

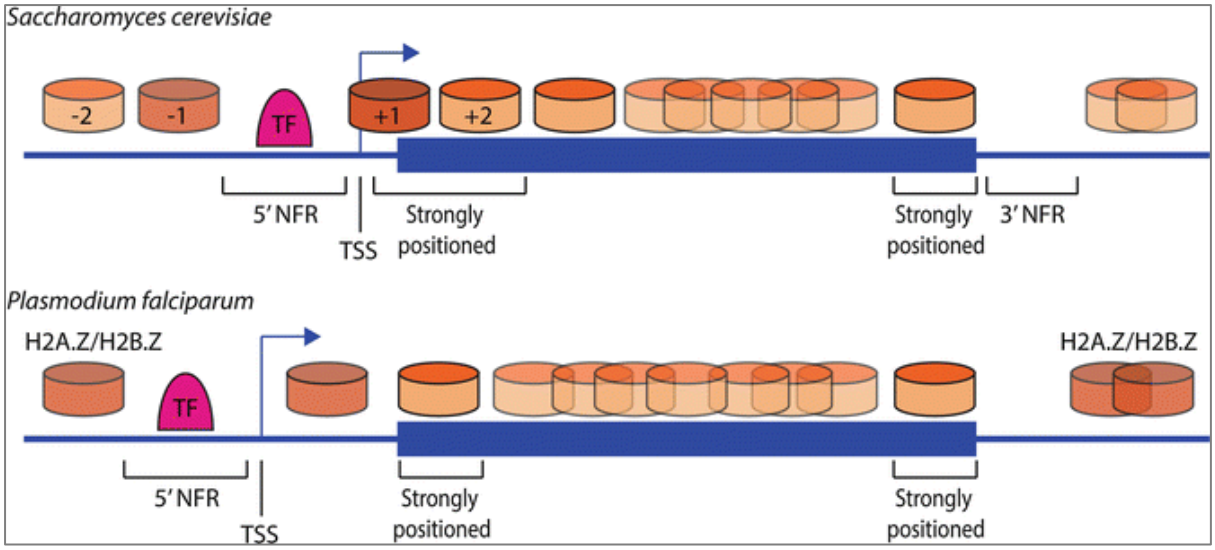


Fig. 10: The nucleosome landscape of P. falciparum presents features that are different from other eukaryotic organisms. Longer 5' UTR and lack of positionally defined nucleosomes flanking the TSS are some distinctive features. Source: Bunnick and le Roche, 2014; Encyclopedia of Malaria [98].

The positioning of the nucleosome also offers a means to regulate gene expression. Its occupancy at the gene promoter regions especially immediately upstream of the transcription start site can demarcate genes that are ready for transcription (nucleosome depletion) vs genes that need to be kept silenced (nucleosome enriched). The nucleosome position at the +1 position of the TSS is however non-standard and defined by a weakly positioned +1 nucleosome (Fig. 10) [98]. The intergenic regions of the genome are also highly depleted of nucleosomes and show an abundance of H2A.Z and H2B.Z both of which are often found to coexist [99]. Thus, a distinctive nucleosome landscape in the parasite is a starting point of the diversity of gene regulation landscape in *P. falciparum*.

1.12.5 Chromatin organisation in *P. falciparum*

Techniques ranging from chromatin conformation capture (3C) to Hi-C have been utilised to study the 3-dimensional layout of the genome in *P. falciparum* [100]. Studies have shown the presence of a transcriptionally suppressed telomeric, heterochromatic genome that is pushed to the periphery of the nucleus while the euchromatin mostly occupies a more central and away from telomere organisation [100]. Much of the genomic organisation is thought to be driven by the presence of the multicopy virulence gene families in the sub-telomeric regions that dominate the genome organisation landscape [101]. One characteristic feature of the *P. falciparum* genome is the absence of the classically observed Topologically Associated Domains (TADs). Gene expression was found to strongly correlate with the overall genomic organisation from the centromeric to the telomeric clusters [101].

1.12.6 Transcription machinery and transcription factors in *P. falciparum*

The fundamental unit of transcriptional machinery assembles as a Pre-Initiation Complex (PIC) on the gene promoter. The PIC includes the TATA binding protein (TBP), TBP associated factors (TAFs), general transcription factors (TFs) and RNA polymerase II [102]. Furthermore, the occupancy of the PIC components doesn't seem to correlate universally with gene expression. For instance, TBP and RNA polymerase II have been located on promoters of both transcriptionally active and silenced genes. Which adds to the possibility of other transcriptional regulatory mechanisms at play [103].

The varied transcriptional regulatory landscape is perhaps the most evident when TFs are taken into consideration. Among eukaryotes, *Plasmodium* has some of the lowest count of specific TFs known [104]. Only one dominant family of transcription factors has been identified in *P. falciparum* known as the Apicomplexan apetala 2 (ApiAP2), being closely related to the plant apetala 2 family of TFs [105]. There are close to 8 helix-turn-helix, 34 C2H2 type zinc proteins

in *P. falciparum*. Other prominent transcription factors belonging to the basic homeodomain, leucine zipper motif, nuclear hormone receptor family are as yet unidentified in *P. falciparum* [106]. The 27 ApiAP2 TFs show sequence specific DNA binding and are thought to drive gene expression at specific stages of the parasite which also forms a basis for their nomenclature. For instance, PfAP2-L drives gene expression at liver stage of the parasite while PfAP2-G is responsible for the transcriptional cascade that triggers the conversion of asexual erythrocytic parasites to sexual stages [107, 108]. PfAP2-I is thought to drive the expression of invasion associated genes while PfAP2-O is important for ookinete specific gene expression [109].

1.12.7 Epigenetic regulation of gene expression in *Plasmodium*

Epigenetic factors serve as an additional layer for gene expression regulation and are highly relevant for a proper spatio-temporal gene expression profile. Epigenetic factors include chromatin remodellers and modifiers of the histone tail post translational modification code and non-coding RNA.

1.12.8 Non-coding RNAs in *P. falciparum*

Non-coding RNAs provide an additional layer of transcriptional regulation and while these have been reported abundantly in multiple studies, they have still not been functionally characterised in *P. falciparum*. A few instances known for functional relevance of noncoding RNAs in *Plasmodium* biology come from the observations of non-coding RNAs derived from introns in virulence genes that are directly shown to be responsible for simulating expression of the associated gene [110]. Another study that greatly highlights the role of noncoding RNAs shows the role of gametocyte development protein (*gdv1*) non-coding antisense RNA that silences the expression of GDV1 gene. The GDV1 usually forms a complex with

heterochromatin protein 1 (HP1) and its suppression allows the eviction of HP1 from the PfAP2-G gene, thus tipping the gene expression circuit in parasites for sexual conversion (Fig. 11) [111].

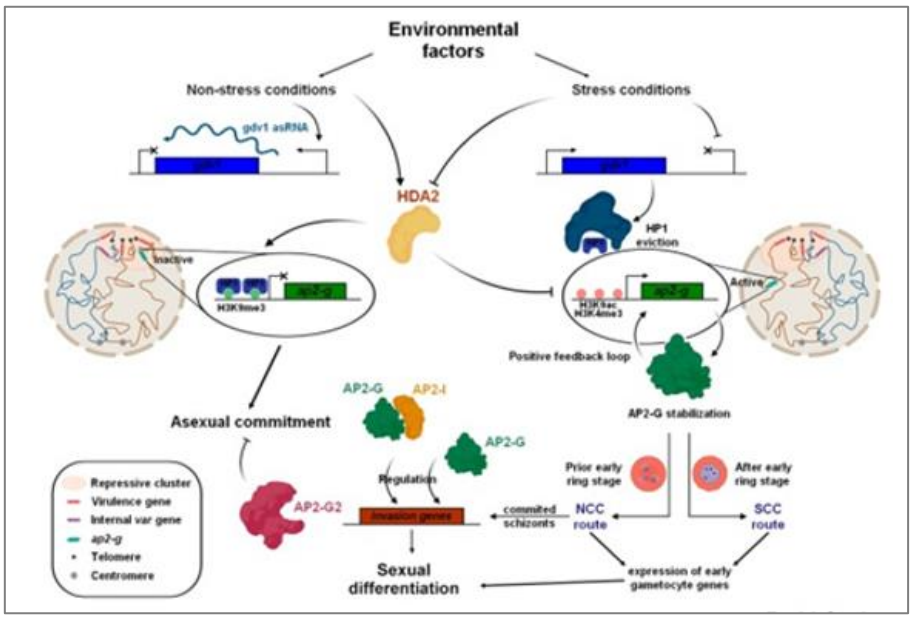


Fig. 11: Environmental triggers and epigenetic factors (including Pfgdv-1 antisense, PfHP1 and Pfap2g) regulate the expression of sexual conversion specific genes in Plasmodium falciparum. Source: Hollin et. al., 2020; Trends in Genetics [112].

1.12.9 Chromatin remodellers

While chromatin modifiers function by chemically modifying histone tails, chromatin remodellers function by moving nucleosomes around the DNA elements (in an ATPase dependent fashion) thus regulating the accessibility of genes to transcriptional regulatory factors [113]. The factors are additionally responsible for eviction and exchange of histones from the octameric core adding another layer to transcriptional control. Hidden Markov Model studies have predicted the presence of 10 genes encoding the SWI/SNF ATPases in *Plasmodium* [90]. While higher eukaryotes have evolved redundancy in the remodelling factors by having multiple genes belonging to the same family of remodellers, *Plasmodium* has only

one remodeller for every family [114]. This lack of diversification is further reflected in the absence of domain variety (PHD, Chromo and SANT) in *Plasmodium* specific remodellers. Only a handful of studies have investigated apicomplexan remodellers [115]. Many of the factors have been shown to be dispensable in mutagenesis studies hinting at their possible non-essential role in the early transcriptional regulatory landscape of apicomplexans.

1.12.10 Chromatin modifiers

The histones not only serve as the structural basis of the genome but also provide a key regulatory context. The N terminal tails of the histone protrude out from the octameric core and can be modified by several post-translational modifications like acetylation, methylation, phosphorylation, ubiquitin and crotonylation among others (Fig. 12). Mass Spectrometry studies have identified around 160 PTMs in *Plasmodium* 60 of which have never been documented in other organisms [116]. These modifications can modulate the electrostatic dynamics that govern the wrapping of the DNA on the core making them more or less accessible to transcriptional machinery but more importantly these modifications serve as docking points for the various proteins to assemble and interact [117]. Factors that can add these modifications to the histone tails are termed as writers, those that remove them are termed as erasers and the factors that bind to them and grant a regulatory context are termed as readers [118]. The various modifications and the complexes that assemble in the histone tails serve as a code for governing the transcriptional dynamics in the parasite and these have been deemed especially important given the apparent dearth of sequence specific TFs in *Plasmodium*.

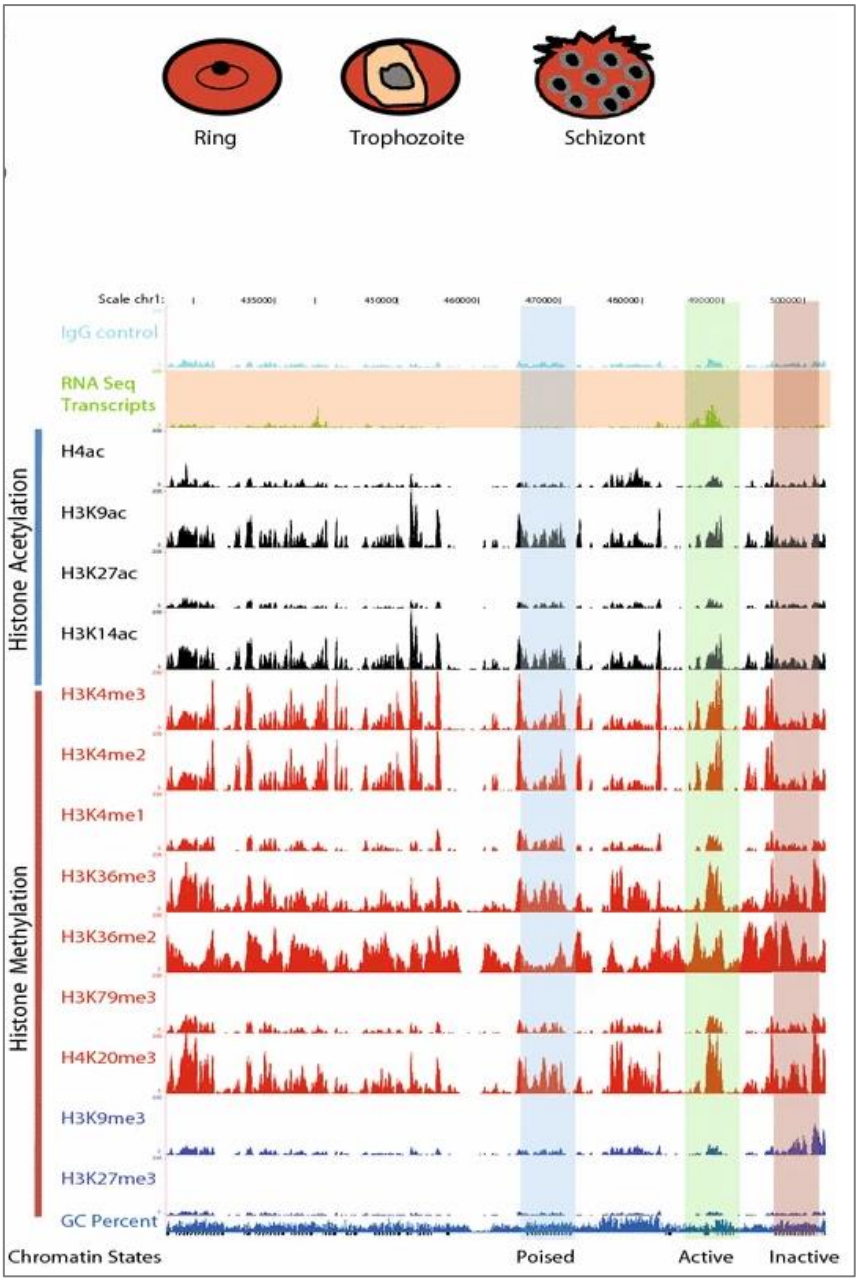


Fig. 12: A host of histone post-translational modifications shape the epigenetic landscape and determine the transcriptional states of target genes. Source: Karmodiya et. al., 2015; Epigenetic and chromatin [119].

The histone modifications have been proposed to play a role in key biological processes in the parasite including antigenic variation, sexual conversion, stress responses, DNA replication and cell cycle [120]. For instance, the H3K9 modification status can dictate the expression or repression of genes. If this residue is tri-methylated by histone methyltransferase SET proteins

it leads to a transcriptionally repressed heterochromatic environment. While removal of the tri-methyl mark and acetylation of the same by histone acetyltransferases can promote transcriptional activation and euchromatic environment in the genomic loci. This is how the methyl and acetyl PTM are known to regulate antigenic variation associated with gene loci in the parasite [121].

Plasmodium possesses a distinct genomic and transcriptional landscape than that is observed for most of the lower eukaryotes. *Plasmodium* chromatin is purported to be “open” and lacks a definitive higher order organisation. The immense AT richness of the genome and the lack of diversification of the chromatin associated machinery may be contributively to this. In fact, the one set of factors that *Plasmodium* doesn't seem to have dearth of are chromatin modifiers and these may play crucial roles in the transcriptional regulation of the parasite worth further dissecting.

CHAPTER 2: Genome-wide survey and phylogenetic analysis of histone acetyltransferases and histone deacetylases of *Plasmodium falciparum*

2.1 A review of the histone acetyl modification landscape regulators

Malaria remains a major problem in many developing countries, with *Plasmodium falciparum* causing most malaria associated mortality [122]. The first definitive map of *P. falciparum* genome was published in 2002, which described a significantly AT rich, 23 MB genome distributed into 14 chromosomes featuring around 5300 genes [86]. Genomic and epigenomic studies have given us considerable insights into the mechanism of transcriptional regulation of phenomena which can influence virulence and pathogenicity of the parasite [119, 123-126]. These studies demonstrate that as the parasite transits through distinct stages during its intraerythrocytic developmental cycle, a fraction of the genome also orchestrates a stage specific expression profile. This oscillatory protein expression profile helps the parasite to rapidly switch and utilize its transcriptome/proteome in a stage dependent fashion. This can only be possible through a remarkably fine-tuned transcriptional and/or post-transcriptional gene regulation paradigm. Thus, gene expression analysis lies at the core of understanding how the parasite can utilize its limited repertoire of genes and flexibly utilize them for a plethora of cellular functions to adapt and evolve to its ever changing and challenging environment. The DNA of *Plasmodium* like all eukaryotes is compacted into condensed structures of increasing order of chromatin organization [127]. 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. The modifications are catalysed 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 (HDACs), histone methyltransferase/demethylases, and proteins that specifically recognize and target these PTMs along with a number of ATP dependent chromatin remodellers [128].

2.2 Histone acetyltransferase and deacetylases: crucial modulators of the chromatin landscape

Reversible acetylation of histones by HATs and histone deacetylases (HDACs) is among the well-studied covalent histone modifications [117]. These enzymes serve as the 'writers' and 'erasers' of acetyl marks on histone tails, and hence manipulate the histone code. Histone acetylation is shown to play an important role in a plethora of processes in *P. falciparum*. Characterization of histone modifying enzymes will further strengthen our understanding of how the plastic nature of transcriptional control allows host–pathogen interactions, stage switching, immune evasion, stress adaptation, and evolution of drug-resistance. 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 antimalarial agents [129].

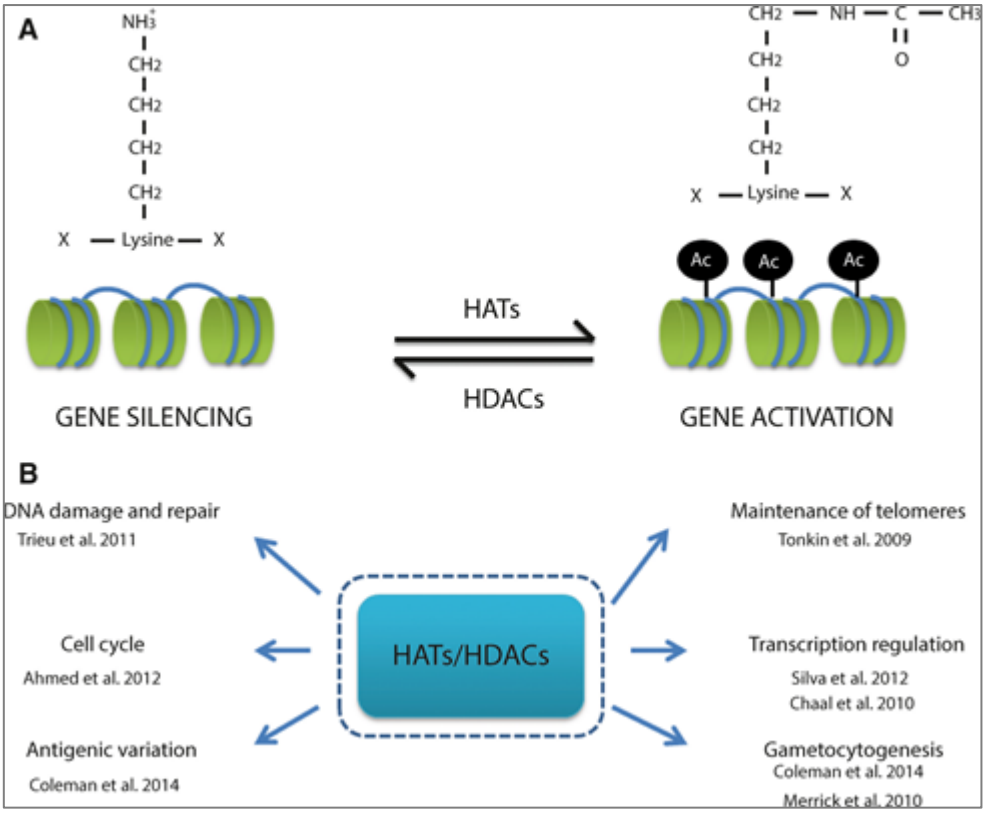


Fig. 13: (A) Schematic representation of the role of HATs and HDACs. Cartoon illustrating reversible acetylation of a lysine (Lys) residue. HAT catalyses the transfer of an acetyl group from acetyl CoA to the ε-amino group of the lysine residue. With a water molecule, an HDAC promotes the removal of the acetyl group from acetyl lysine (Ac-Lys), regenerating the ε-amino group and releasing an acetate molecule. (B) Schematic illustration of the prevalence of reversible lysine (K) acetylation in diverse cellular processes reported in Plasmodium falciparum. Source: Kanyal et. al., 2018; FEBS Journal [120].

In this study, we present a genome-wide survey and a comprehensive review of repertoire of HATs and HDACs in *P. falciparum*, to understand their crucial roles in regulating the cellular biology of the parasite. Phylogenetic analysis of HATs/HDACs was performed to assess their relative closeness as well as homology with their functional/structural counterparts in humans, which can be eventually used as potential targets for therapeutic purposes. Furthermore, we have provided a brief overview of the transcriptional regulation of HATs/HDACs during drug-

resistance generation. Thus, this analysis-cum-review is an effort to analyse and summarize the current knowledge and creates an intellectual repository on repertoire of HATs and HDACs in *P. falciparum.*

2.3 Material and methods

2.3.1 Identification of HATs and HDACs

All the seed sequences of HDAC (Histone deacetylase) and HAT (Histone acetyltransferase) from Pfam (http://pfam.xfam.org/) were downloaded. *Plasmodium falciparum* protein data was downloaded from PlasmoDB (release-10.0) (http://plasmodb.org/common/downloads/release-10.0/P*falciparum*3D7/fasta/data/). PSIBLAST with E-value set at 0.001 was performed, making seed sequences as database and *P. falciparum* protein data as query. Hits found were examined and filtered based upon 60% subject coverage and 20% identity. Hits not passing the coverage and identity cut-offs were eliminated from the analysis. Based upon the above criteria, 9 proteins were identified as putative HDACs and 16 proteins as putative HATs. Identified HATs and HDACs were further *in silico* validated using Phyre for putative fold recognition (Protein Homology/analogY Recognition Engine) and HMMER using the Pfam database for the sequence homology.

2.3.2 Domain organization and phylogenetic analysis of HATs and HDACs

The protein sequences of the various HATs and HDACs were extracted from PlasmoDB and domain organization was identified using the InterPro search tool. For corroboration the NCBI Conserved Domain Database for the individual HATs and HDACs was also referenced. Based upon the HMMER result, HAT and HDAC domains were extracted. To exclude false positives, we further performed BLAST using HAT and HDAC domain as a query against non-redundant

protein databases (nrdb). The complete set of protein sequences of HATs and HDACs for prokaryotes and eukaryotes organisms were retrieved from the NCBI Protein database. For further analysis, we also included the SIR family, which belongs to the Class III family of HDACs. Phylogenetic analysis using MEGA tool (version 7) was performed using HAT and HDAC domain sequence from all eukaryotic and prokaryotic organisms using the Pfam. ClustalW was used for alignment and trees were generated using three different methods: neighbour joining, UPGMA and Maximum likelihood for performing an in-depth analysis. Bootstrap method was used to test phylogeny by keeping the number of bootstrap methods 1000. The analysis of HDACs was performed in two steps i.e., one which solely contains HDAC sequence and other which contain HDACs sequence along with SIR2 family sequences.

2.4 Genome-wide survey and classification of HATs and HDACs in *P. falciparum*

Genome-wide survey and classification of HATs on the basis of protein sequence and domain organization recognizes five major families of HATs; Gcn5-related N-acetyltransferases (GNATs), MYST, p300/CREB binding protein (CBP), nuclear receptor coactivator and other transcription associated families [130]. Our genome-wide survey of HATs in *P. falciparum* genome identified ten putative HATs of which only PF08_0034 (GCN5) and PF11_0192 (MYST) have been experimentally demonstrated to have HAT activity (Fig. 14).

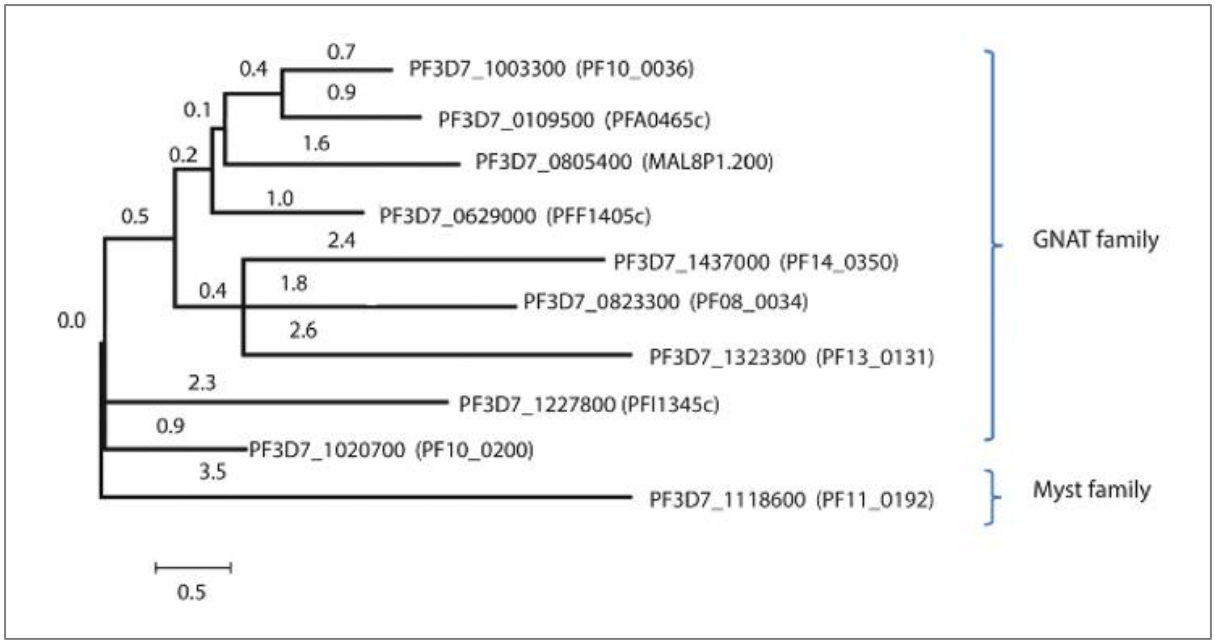


Fig. 14: Plasmodium falciparum HATs, identified in our in-silico analysis, are divided between two families, GNAT and MYST. Source: Kanyal et. al., 2018; FEBS Journal [120].

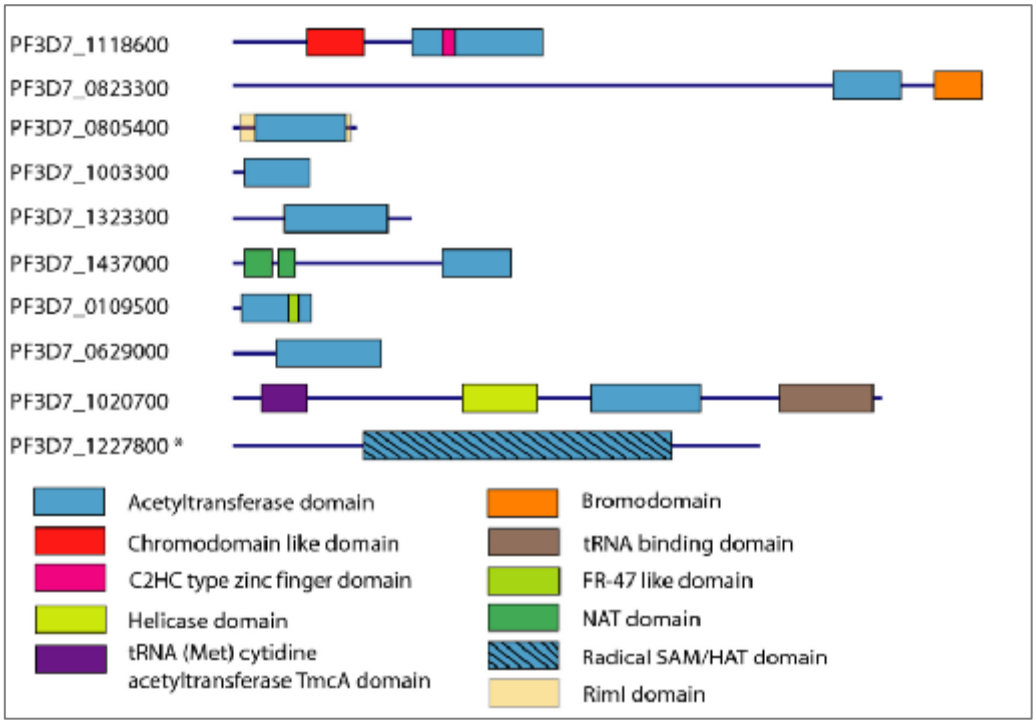


Fig. 15: The domain organisation of the various P. falciparum HATs. Source: Kanyal et. al., 2018; FEBS Journal [120].

2.4.1 Gcn5-related N-acetyltransferases (GNATs) family

GCN5 related N-acetyltransferase family is a superfamily of acetyltransferases that is known to acetylate a diverse set of proteins including histones and nonhistone substrates. This family includes a set of proteins which have been clustered together based on acetylation related motifs. In higher organisms, this group includes Gcn5, Hat1, elongator complex protein 3 (Elp3), and Hpa2. The GNAT proteins share a domain composed of four conserved sequence motifs A-D. Among these motifs, motif A, which is involved in the recognition and binding of the Acetyl Coenzyme A substrate, is highly conserved and it is present in GNAT, MYST, and p300/CBP family of HATs [131]. Motif C on the other hand is shared uniquely among the Gcn5 family HATs. The GNAT members play a critical role in the regulation of transcription, DNA repair, cell growth, and development [131].

2.4.1.1 PF08_0034 (PF3D7_0823300/PfGCN5)

The gene PF08_0034 (PfGCN5) was identified as a homolog of yeast GCN5 in *P. falciparum* [132]. PfGCN5 has a catalytic GNAT and a bromo-domain (Fig. 15). The N terminal region of PfGCN5 is longer than GCN5 in any other organism and bears little similarity with them. It is postulated to be involved in protein-protein interaction and recognition of nucleosome substrates [132]. PfGCN5 is constitutively expressed at the asexual stages and transcription can be triggered from multiple transcription start sites [132-134]. Recombinant PfGCN5 possess conserved *in vitro* HAT activity with preference to H3 histone at K9 and K14 and to certain extent toward histone H4 [132]. Presence of PfADA2, homologs of which are also present in other organisms, indicates minimal functional SAGA complex in *P. falciparum* [135]. In yeast, GCN5 and ADA proteins are part of the SAGA complex, which plays an important role in regulation of stress-induced genes [136].

ChIP-on-chip analysis indicates a weak yet positive correlation between PfGCN5, H3K9ac mark and gene expression [137]. PfGCN5 is found to be enriched at all the chromosomes and it is associated with processes such as binding, catalytic activity, transcription/translation, enzyme regulation, signalling, and transport [137]. 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 [138, 139].

2.4.1.2 MAL8P1.200 (PF3D7_0805400)

MAL8P1.200 has a conserved GNAT domain and it is identified as a putative acetyltransferase in in silico and biological survey of proteins implicated in transcriptional machinery during asexual stages of *P. falciparum* [106]. MAL8P1.200 contains a Riml domain (Fig. 15), indicating that it might be associated with mitochondrial DNA replication and genome maintenance. MAL8P1.200 is highly expressed in gametocyte (sexual) and trophozoite (asexual) stages [134].

2.4.1.3 PF10_0036 (PF3D7_1003300)

PF10_0036 bears a canonical GNAT domain and is recognized as homolog of Ard1p which is a part of NatD protein complex (Fig. 15). It is believed to acetylate the PEXEL motif which is responsible for targeted export of parasite proteins to the host erythrocyte during blood infection stages [140]. These acetylation marks also help putative translocases to differentiate between proteins that need to be retained in the parasitophorous vacuole (PV) and those that need to be targeted beyond the PV membrane. The Ard1p and Nat1p proteins along with other factors form a supramolecular complex in *Saccharomyces cerevisiae*, the likes of which have

yet to be identified in *P. falciparum* [141]. PF10_0036 shows upregulated expression at trophozoite, schizont, and gametocyte stages [134].

2.4.1.4 PF13_0131 (PF3D7_1323300)

PF13_0131 has a canonical GNAT domain and it is homologous of the HAT, Elp-3. Studies in yeast have shown that Elp3 protein is the HAT component of the RNA Polymerase II complex [142]. This suggests the role of PF13_0131 in progression of transcriptional elongation in *P. falciparum*. It is among the top 60–80% of highly expressed genes at gametocyte stage [134].

2.4.1.5 PFA0465c (PF3D7_0109500)

PFA0465c, a member of the GNAT family, bears the ribosomal protein S18 acetylase, RimI related domain and a FR-47 like domain (Fig. 15) resembling the C- terminal region of the *Drosophila melanogaster* hypothetical protein FR47, making it distinct from other *P. falciparum* and human HATs. This enzyme serves as the homolog of NatD protein, Nat3p in *P. falciparum*. This protein, along with another acetyltransferase, PF10_0036, function to target and acetylate the cleaved N terminal PEXEL motif of proteins undergoing translocation across the PVM into the host cell cytoplasm [140, 141]. This is another example of an acetyltransferase, which has non histone targets, primarily exported proteins and peptides. The ring and trophozoite stage of the parasite show peak expression of PFA0465c, which is expected as this stage of parasite growth is associated with the transport of protein to the cytoplasm of iRBCs [143].

2.4.1.6 PFF1405c (PF3D7_0629000)

PFF1405c is a GNAT domain containing novel HAT identified in this study and it is believed to catalyse N-terminal protein amino acid acetylation, thus having nonhistone targets (Fig. 15).

The protein is moderately expressed during the mid-trophozoite to late schizont stages [134, 141].

2.4.1.7 PFL1345c (PF3D7_1227800)

PFL1345c is unique in possessing two unusual domains: a radical SAM domain (elongator protein 3, MiaB family, component of the RNA polymerase elongator complex) and a HAT domain (Fig. 15). PFL1345c is believed to play a role in complementing PfGCN5 mediated H3K9ac *in vivo* and this functional overlap is in the context of its role in SAGA complex [137, 144]. It is also speculated to be useful for acetylation of the cleaved N-terminal regions of proteins undergoing transport across PVM [145]. The presence of the HAT domain and the transmembrane domain makes them suitable candidates for entry into endoplasmic reticulum (ER) where this catalysis occurs [145]. PFL1345c is also proposed to have RNA polymerase I nucleolus activity [146]. PFL1345c might also assist RNA Polymerase II during transcriptional elongation by providing a conducive chromatin confirmation for transcriptional elongation.

2.4.1.8 PF10_0200 (PF3D7_1020700)

PF10_0200 is a putative HAT, which features a prominent GNAT domain, among other domains populated along its sequence (Fig. 15). It has an N- terminal located tRNA (Met) cytidine acetyltransferase (TmcA) domain. TmcA catalyses the formation of N (4)-acetylcytidine (ac4C) at the wobble position of tRNA (Met) and ensures precise recognition of the AUG codon [147]. This might be a unique instance of a HAT enzyme being involved in ensuring the fidelity of protein translation. The PF10_0200 belongs to a cluster of uncharacterized proteins that have a known role in RNA polymerase I nucleolus activity. It interacts with many nucleolus ribosome assembly proteins [146].

2.4.1.9 PF14_0350 (PF3D7_1437000)

PF14_0350 has a GNAT HAT domain (Fig. 15) and it is downregulated during switching of invasion pathways from sialic acid dependent receptors to independent ones, suggesting its role in determining the selection of parasite invasion routes and modulating host parasite interaction [148]. PF14_0350 is also significantly upregulated in artemisinin-resistant parasites [149]. It is also possible that PF14_0350 might be involved in the N-acetylation of cleaved host targeting (HT) motif of proteins in ER prior to their transport across the PVM into the host cytoplasm [145]. The protein has a helical transmembrane domain unlike any other HAT and maybe important for its transport and localization in the ER lumen, where the catalysis needs to be performed. The N terminal of the protein, which is largely uncharacterized with mostly unintegrated signatures and bears little sequence similarity with other proteins, may be crucial for protein-protein interactions, trafficking and various other functions.

2.4.2 MYST Family

The MYST family of HATs is named after its flagship members MOZ, Ybf/Sas3, Sas2 and Tip60. The defining feature of the MYST family members is a conserved MYST domain, which comprises an acetyl-CoA binding motif and a C2HC zinc finger domain. Members of this family are involved in regulating a host of cellular functions including, silencing of telomeric genes, governing progression through pre mitotic phase of cell cycle, X-chromosome dosage compensation in higher eukaryotes etc. [150].

2.4.2.1 PF11_0192 (PF3D7_1118600/PfMYST)

PF11_0192 (PfMYST) is the only MYST family gene found in *P. falciparum*. It has a highly conserved MYST domain, C2HC- type zinc finger domain and chromo-domain (CD) which binds to single stranded nucleic acids and methylated histone peptides [151]. The protein bears

significant similarity with the yeast Esa1 protein, whose N- and C-terminal segments directly flank the catalytic core and play an important role in histone substrate binding PfMYST has two isoforms, long (~72 kDa) and short (~ 37 kDa), expressed during different erythrocytic stages [152, 153]. The short version is less abundant and lacks N-terminal low complexity sequence, which does not have any apparent homology to any protein or domain [152]. The function of low complexity N- terminal region is not understood well; however, it is assumed to play an important role in assembly of complexes [152]. Since PfMYST lacks a recognizable nuclear localization signal, it is possibly recruited into complexes that help with its trafficking [152, 154]. The domain organization, sequence similarity, and substrate specificity of PfMYST suggest it to be an ortholog of yeast Esa1 and human Tip60. The Zn finger motif is mostly found in metazoan enzymes and absent in yeast Esa1, thereby suggesting that this motif in *Plasmodium* might be an ancestral feature in MYST family proteins. TAP tagged purified endogenous PfMYST has been reported to show activity on recombinant histones H4 (K5, K8 and K12) in *P. falciparum* and HeLa cell [152].

PfMYST plays an important role in transcription regulation, cell cycle progression and DNA damage repair highlight a conserved function in evolutionarily divergent eukaryotes [152]. There is evidence of interaction of PfMYST with PfRuvB3 protein, which is involved in cell-cycle checkpoint activations [155]. PfMYST is specifically recruited to the active virulence gene promoter, which probably facilitates transcription initiation [152]. Thus, PfMYST has a key regulatory role in a wide array of cellular functions. PfMYST was found to be the synthetic lethality (SL) partner of PfMDR1 and PfDHFR-TS, two genes which are implicated to be involved in antimalarial drug-resistance [156]. Two genes are SL if the mutation of either gene is compatible with viability, whereas simultaneous mutations in both the genes are lethal. Consequently, if mutation in one gene of SL gene pair causes antimalarial drug-resistance, targeting the other gene of the pair may serve as an effective treatment strategy for drug-

resistance [156]. This raises the possibility of using PfMYST as a target for development of antimalarial compounds against drug-resistance parasites.

2.4.3 p300/CBP family

The p300/CBP are a pair of transcriptional coactivators having roles in cell cycle regulation, differentiation, and apoptosis. No known members of p300/CBP have been identified in *P. falciparum*. However, it is possible that the functions of p300/CBP may be performed by other members of the GNAT family of acetyltransferases in *P. falciparum*.

2.4.4 Nuclear receptors coactivators and other transcription factors

Nuclear receptor and other transcription factor associated HATs could not be identified in *P. falciparum*. It is highly likely that some uncharacterized proteins might be acting as substitutes for these HATs as this class of HATs lack HAT domain and are mostly identified by functional assays.

2.5 Genome-wide survey and classification of HDACs

The HDACs are the antagonists of HATs and they maintain the balance of acetyl lysine on histone tails. The HDAC family has been divided into five classes (I–IV and U); the U-class sirtuins are found only in Gram-positive bacteria [157]. *Plasmodium falciparum* has only Class I, II and III of HDACs (Fig. 16).

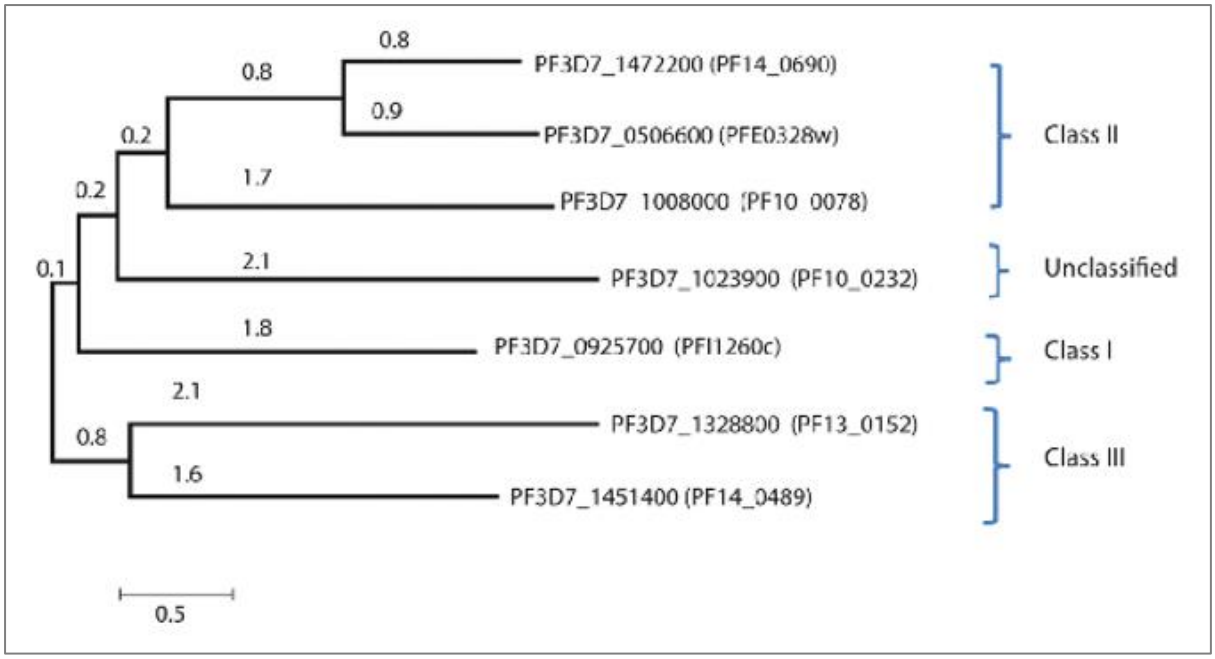


Fig. 16. Plasmodium falciparum HDACs identified in our analysis belong to three major classes. One unclassified HDAC was also identified. P. falciparum lacks the Class IV HDACs. Source: Kanyal et. al., 2018; FEBS Journal [120].

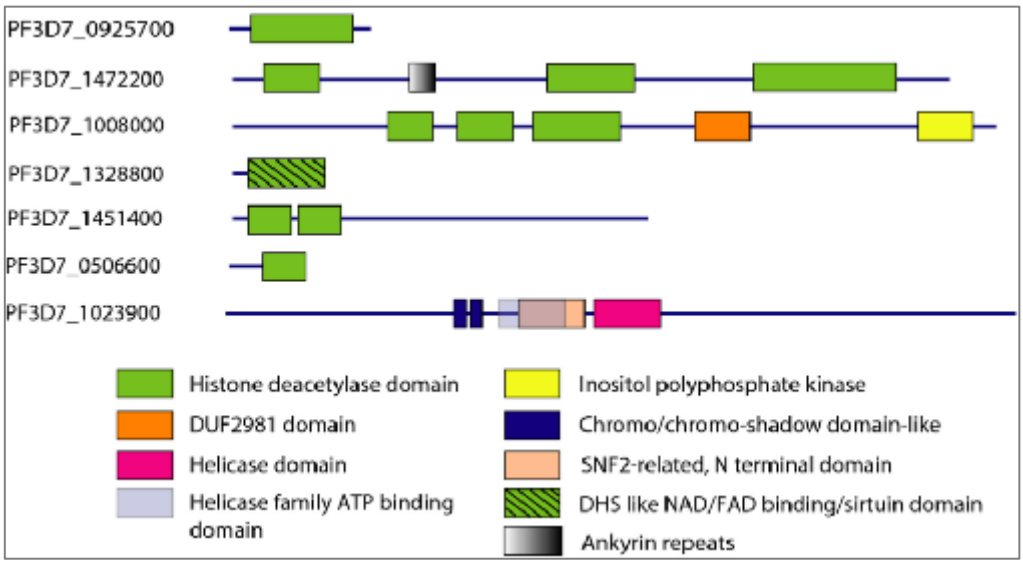


Fig. 17: The domain organisation of the P. falciparum HDACs identified in our analysis. Source: Kanyal et. al., 2018; FEBS Journal [120].

2.5.1 Class I

Class I HDACs utilize Zn^{2+} ions as cofactors for catalytic activity. They have nucleo-cytoplasmic localization and in higher eukaryotes are involved in transcriptional repression, cell cycle regulation, cell differentiation and the DNA damage response [158-160].

2.5.1.1 PFI1260c (PF3D7_0925700)

PFI1260c (PfHDAC1) has a Class I HDAC domain, and it belongs to the arginase and histone-like hydrolase supercluster. PfHDAC1 exhibits stage specific transcriptional activity and shown to specifically regulate developmental processes like schizogony, gametocytogenesis, and hepatocyte invasion [161]. Interestingly, most of the PfHDAC1 activity centres at the intrachromosomal-region and it is distinct from sirtuins, which act along peripheral chromosomal regions [161]. PfHDAC1 also serves as the key interface for PfHsp90 chaperon protein, allowing for the *P. falciparum* chaperone complex to play a role in the process of chromatin remodelling and protein expression [162, 163]. Importantly, artemisinin resistant *P. falciparum* has shown downregulation of PfHDAC1 indicating its role as a crucial regulator of cellular transcriptional rewiring during drug-resistance emergence [149].

2.5.2 Class II

Members of Class II HDACs in higher eukaryotes utilize Zn^{2+} ionic cofactor and have nucleo-cytoplasmic localization with significant enrichment at Golgi apparatus and cytosolic caveola [164]. They are regulators of transcription and cell differentiation, poly-ubiquitinated protein recruitment to aggresomes and macro-autophagy [165-167].

2.5.2.1 PF14_0690 (PF3D7_1472200)

PF14_0690 (PfHDA1) is a member of Class II HDACs and it has three canonical HDAC domains and several interesting and unique structural signatures including the left-handed beta roll motif (implicated in bacterial virulence) and the Bin/Amphiphysin/Rvs (BAR) domain (Fig. 17) [168]. PfHDA1 also contains ankyrin repeats which mediate protein-protein interactions [169]. Domain wise sequence similarity search shows that domain 1 is a unique characteristic of the *Plasmodium* family. PfHDA1 is not yet characterized in *P. falciparum*.

2.5.2.2 PF10_0078 (PF3D7_1008000)

PF10_0078 (PfHDA2) has three HDAC domains and a unique (for a HDAC) inositol phosphate kinase (IPK) domain toward the C terminus (Fig. 17). The IPK domain canonically catalyses the phosphorylation of soluble secondary messengers using ATP as the Pi donor. PfHDA2 has also a DUF2981 protein domain with little known functions. PfHDA2 lacks homology with PfHDA1 outside of the HDA core. Immunofluorescence studies have revealed the accumulation of PfHDA2 in the nuclear periphery [170]. This region features a heterochromatic sub-compartment which regulates the switching of clonally variant genes [171]. Knockdown of PfHDA2 also causes defects in DNA replication and dysregulation of a limited set of genes associated with gametocytogenesis and clonal variation [170]. PfHDA2 knockdown shows that it is the global regulator of the *var* gene family (upregulated) and the *rif/stevor* multigene family [170]. The ApiAP2 transcription factor crucial for transcriptional switch to gametocytogenesis is shown to be strongly dysregulated upon PfHDA2 knockdown with a three-fold increase in phenotypic conversion into gametocytes [170]. PfHDA2 hence forms a link between expression of virulence factors and the decision to form gametocytes, which is fundamentally linked with control over virulence gene expression and antigenic variation.

2.5.2.3 PFE0328w (PF3D7_0506600)

The PF3D7_0506600 is a pseudogene, which is considered as a putative HDAC based on the presence of an HDAC domain toward the C-terminus (Fig. 17) The sequence features multiple stop codons, which upon manual removal and sequence assessment reveals a single HDAC domain shifted toward the C-terminus of the protein (164-228aa). It is possible that the protein in the distant evolutionary past had been a functional HDAC and after accumulation of mutational events transformed into a non-expressing pseudogene.

2.5.3 Class III (sirtuins)

Sirtuins are unusual HDACs as they require an alternative co-factor in the form of NAD+ for their activation. Their distribution is nucleo-cytoplasmic and are essential for mediating histone deacetylation and regulation of double-strand break repair via homologous recombination [172]. In *P. falciparum* sirtuins coordinate their function (possibly in a complex) to regulate the maintenance and hetero-chromatinization of telomeric DNA-ends and governing the expression of peripheral virulence genes [173]. Sirtuins have also been identified as sensors of nutrient fluxes within host cells and may lie at the interface of environmental factors with gene expression.

2.5.3.1 PF13_0152 (PF3D7_1328800)

PF13_0152 (Sir2A) bears a catalytic core domain of sirtuin family and a deoxyhypusine synthase (DHS) like NAD/FAD binding domain (Fig. 17). Sir2A is also found to localize at telomeres and regulate their length and may have roles in its maintenance and hetero-chromatinization in *P. falciparum* [174]. PfSir2A is trimeric in solution but dissociates to monomeric forms upon NAD+ contact. The C terminal region of the protein has been

implicated in trimerization, while the N terminus is important for catalytic efficiency [175]. PfSir2A has been reported to be involved in regulating subtelomeric *var* genes (ups A and ups E) but not the centrally placed upsB [176]. The PfSir2A protein is highly expressed in patients with severe forms of malaria and is correlated with upregulation of *var* gene [177]. Also, field isolates of *P. falciparum* have revealed increased expression of PfSir2a in response to starvation and elevated blood lactate in a subset of a study group, confirming that sirtuins respond to nutritional and metabolic status via the NAD+ cofactor. Thus raises the possibility that environmental stresses may directly affect the levels of Sir2A providing an interface for transcriptional control by external factors [177, 178]. PfSir2A interacts with PfAlba3 and deacetylates its N-terminal peptide specific for DNA binding and they colocalize at the telomeric region [179]. Possibly PfAlba3 mediates telomeric position effect and may contribute to regulation of gene expression mediated by PfSir2A [179]. PfSir2A shows significant enrichment in the nucleolus parallel with the rDNA locus, with almost three-fold overexpression of A and S type rRNA transcripts upon PfSir2A knockdown [180]. There is an overall increase in the 18s rRNA and cytoplasmic ribosome particles in the mutants of PfSir2A, which also report increased parasite proliferation [180]. This lends credence to the hypothesis that PfSir2A can sense the metabolic and energy status of parasites based on NAD+ levels and couple them with regulation of rRNA expression and cell proliferation [180].

2.5.3.2 PF14_0489 (PF3D7_1451400)

PF14_0489 (PfSir2B) is a distant relative of PfSir2A but is almost five times larger in size and highly divergent in function. The PfSir2 paralogs together regulate the virulence genes and antigenic variation [174]. The PfSir2B mutants are enriched with PfH2Z variants at the early blood stage of the parasite [181]. The knockouts of PfSir2B have little influence on telomere length [174]. Knockdown of PfSir2B relaxes the proximal *var* gene expression (controlled by

upsB promoter) [174]. This is nonoverlapping with regulation of UpsA, UpsC, and UpsE controlled *var* genes regulated by PfSir2A. Thus, subtle differences in the HDAC domain specificity of the two paralogs for the different promoter types of *var* genes may be crucial for regulation of distinct subsets of *var* genes.

2.5.4 Class IV

Class IV HDACs rely on Zn^{2+} cofactors and demonstrate nuclear distribution [182]. They are primarily involved in histone deacetylation mediated transcriptional regulation [182, 183]. This class of proteins has not yet been identified in *P. falciparum*.

2.5.5 PF3D7_1023900

PF10_0232 [CD-helicase-DNA-binding protein 1 homolog (CHD1)] presents several features which are non-canonical for a chromatin modifier. It possesses a chromo/chromo-shadow like domain; a Helicase family ATP binding domain; a Helicase C terminal like domain involved in ATP dependent DNA unwinding; and a SNF-2 N terminal related domain (Fig. 17). These subunits fit the functional description of a chromatin remodelling protein. It is well-known that members of the CHD subfamily II form nucleosome remodelling and deacetylase complexes (NURD) alongside HDAC1 and HDAC2 in humans and are associated with deacetylase activity [184]. The lack of any recognizable domain makes the classification of this protein a challenging task. However, the existence of this protein lends credence to the possibility of a primitive form of a multiprotein assembly showing histone modification activity.

Thus, in this study, a total of ten HAT proteins have been identified in *P. falciparum*, which includes well known PfGCN5 and PfMYST and eight previously unclassified HATs. While most HATs are clustered into the GNAT family, the MYST family has just one representative

member (PfMYST). Furthermore, *P. falciparum* lacks members of p300/CBP, nuclear receptor and transcription factor associated HATs. Genome-wide survey of HDACs has re-confirmed presence of five known HDACs and identified one novel putative HDAC and one putative HDAC pseudogene in *P. falciparum*. There are no Class IV HDAC members in *P. falciparum*. Interestingly, several PfHDACs have multiple HDAC domains of varying complexity. A possible reason for such diversity is that molecular evolution could have allowed for a smaller number of HDACs to accumulate multiple domains with flexible roles via domain duplication and neofunctionalization, hence cutting down the need for different HDACs.

2.6 Phylogenetic analysis of HATs and HDACs of *P. falciparum*

We performed a comprehensive phylogenetic analysis to assess the evolutionary distance of *P. falciparum* HATs/HDACs with members from other organisms ranging from primitive microbes to higher eukaryotes. Orthologs with a higher percentage similarity with human proteins cannot be used as therapeutic targets for risk of collateral damage they pose. Conversely, HATs/HDACs that are farthest from human proteins on the evolutionary scale can be taken forward confidently for translational pharmacological research. These include the proteins that are closer to those found in primitive microscopic forms of life.

2.6.1 HATs

The HATs, PF3D7_1003300, PF3D7_0629000, PF3D7_1323300, PF3D7_1020700, and PF3D7_1437000 constitute a distinct subcluster in our phylogenetic analysis and thus occupy a distinct evolutionary niche (Fig. 18). As this subcluster of HATs is a direct offshoot from the root of the phylogenetic tree, these HATs must have diverged from the rest of the HATs quite early during the course of diversification. The HAT domain of PF3D7_0805400 on the other

hand shares closer resemblance with that of *Pseudomonas* and *Synechococcus* HATs. The HAT domain of PF3D7_0109500 also bears similarity with the respective domain belonging to *Crocosphaera watsonii* and *Cytophaga hutchinsonii* pro HATs (Fig. 18). Interestingly, PF3D7_1118600 (PfMYST) is proximal to the HATs found in higher organisms. It is very closely related to the HAT-KAT6A from *Danio rerio* and the MYST from *Homo sapiens*. This suggests that PfMYST HAT is evolutionarily conserved from lower to higher eukaryotes. PF3D7_0823300 (PfGCN5) is also grouped alongside HATs belonging to *H. sapiens* (Human GCN5 and PCAF) as well as HATs from plants viz. *Arabidopsis thaliana* and *Zea mays* (Fig. 18).

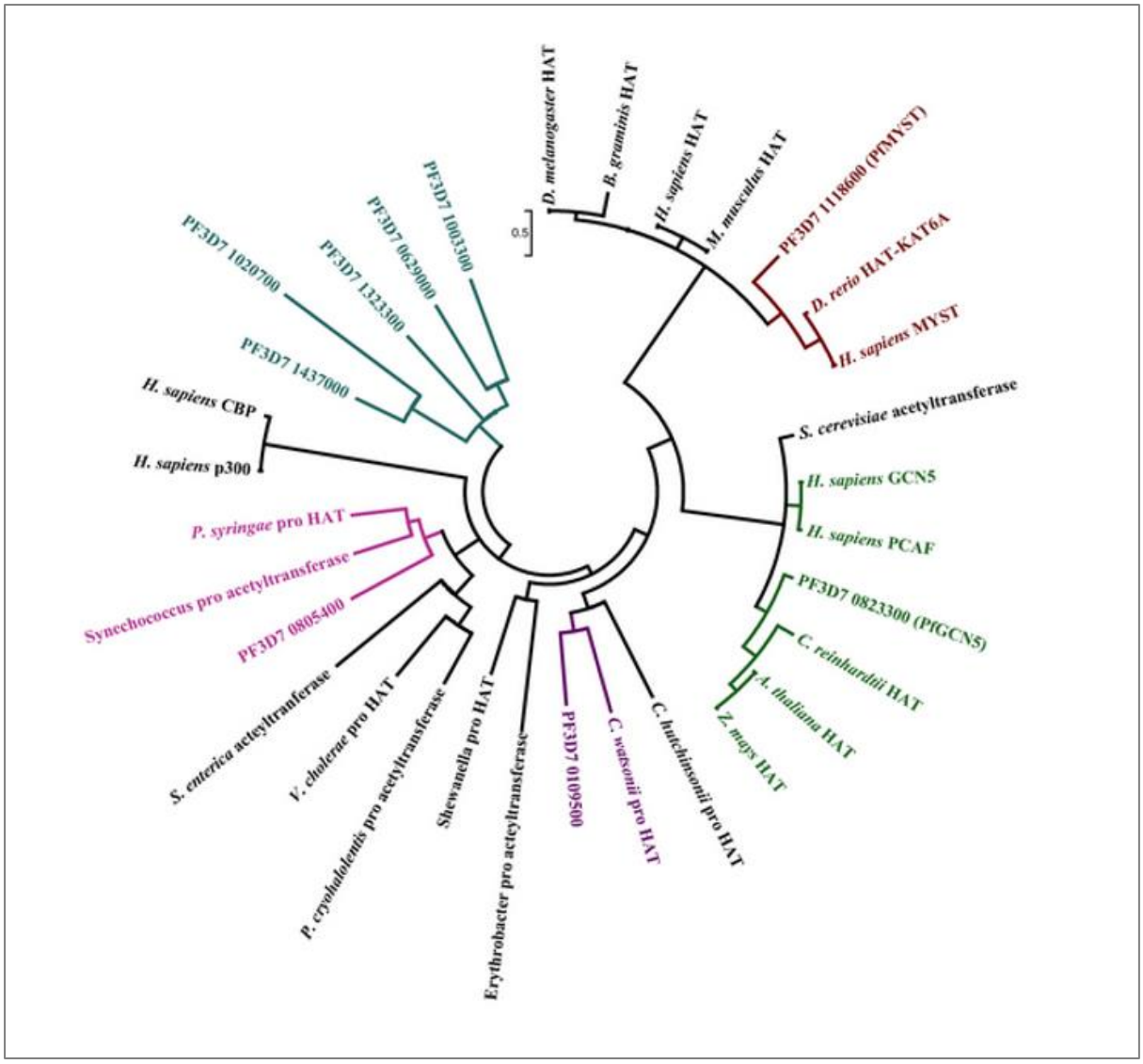


Fig. 18: Dendrogram representing the phylogenetic association of P. falciparum HATs with major HATs observed from other clades of life. Source: Kanyal et. al., 2018; FEBS Journal [120].

2.6.2 HDACs

The dendrogram for HDACs shows marked separation of classes, which are also clearly segregated across different domains of life (Fig. 19). There is also a high level of disparity in the extent of genetic sequence divergence that has been accumulated among the HDACs, with few showing divergence while others showing evolutionary leaps. There are a few proteins

with multiple HDAC domains which individually bear similarity with HDACs from organisms belonging to diverse taxa of life and have also evolved to different extent. For instance, HDA2 (PF3D7_1008000) has three HDAC domains, of which the first domain (PF3D7_1008000_1) has a distinctly high scale of divergence and shares high similarities with *Arabidopsis thaliana* HDAC2 (Fig. 19). Its second HDAC domain (PF3D7_1008000_2) is closer to microbial HDAC domains like those found in *Chloroflexus aurantiacus* (proHDAC2) and *Vibrio cholerae* (proHDAC). The third HDAC domain (PF3D7_1008000_3) is closer to HDAC domains of extremophile microbes like *Thermus thermophilus* and *Deinococcus radiodurans* (Fig. 19). The pseudogene PF3D7_0506600 also falls in the same cluster as Domain 3 of HDA2 (PF3D7_1008000_3). Interestingly, it is related to the HDAC domain of sirtuins of ancient extremophiles such as *D. radiodurans* and *Thermus thermophilus*. It can be reasonably speculated that in the evolutionary past PF3D7_0506600 could have served as a fully functional HDAC, possibly a sirtuin and has subsequently undergone nullification of function over the course of time. The PF3D7_147220 (putative HDA1) is closer to the Domain 2 of HDA2 (PF3D7_1008000_2) and bears close similarity with the HDAC domains from primitive microbial *Chromobacterium violaceum* pro deacetylase and *V. cholerae* pro HDAC.

Interestingly, the two HDAC domains of PfSir2A (PF3D7_1328800) have evolved distinctly, domain 1 closer to microbe, *Caulobacter crescentus*, while the domain 2 sharing features with *A. thaliana* HDAC2 and *Vibrio vulnificus* sirtuin (Fig. 19). Furthermore, PfHDAC1 belongs to a unique evolutionary niche which bears HDACs from various taxa of life indicating that the protein is highly conserved from unicellular to advanced multicellular forms of life like *H. sapiens, D. rerio, Gallus gallus,* and *A. thaliana*. Interestingly, the novel HDAC identified, PF10_0232 shows significant similarity with *Synechococcus sirtuin* and the HDAC4 from *G. gallus* further supporting it to be a putative novel HDAC in *P. falciparum.*

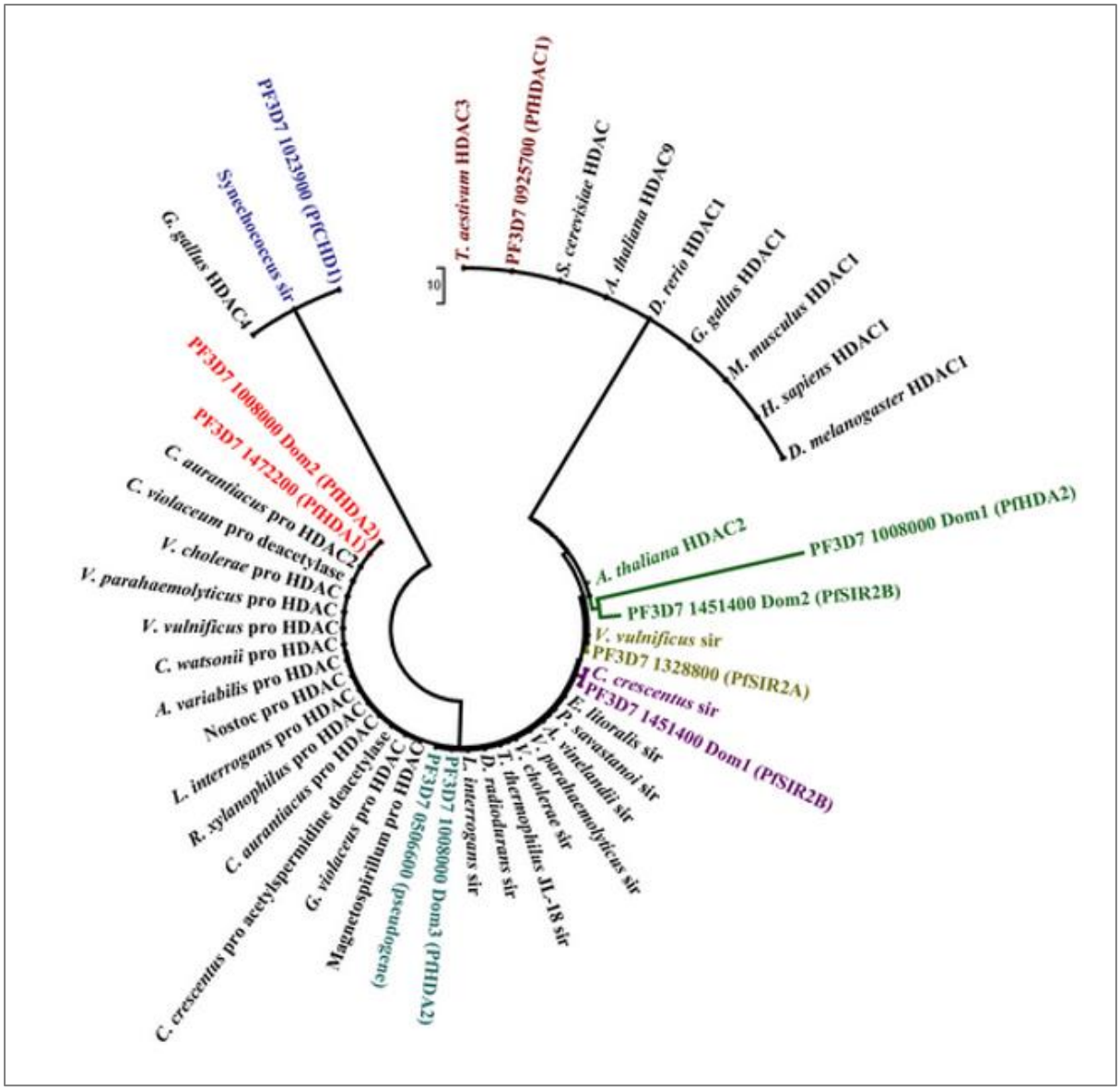


Fig. 19: Dendrogram representing the phylogenetic relation of P. falciparum HDACs with major HDACs observed in other clades of life. Source: Kanyal et. al., 2018; FEBS Journal [120].

2.7 Emergence of drug-resistance and transcription regulation

Recent reports suggest emergence and spread of drug resistance against Artemisinin based Combination Therapy (ACT), which is currently a frontline drug against *P. falciparum* [185]. Drug-exposure of parasites leads to comprehensive repatterning of the transcriptome profile in

artemisinin resistant parasites, which is thought to be due to differential expression of key regulatory proteins such as transcription factors and the proteins associated with histone modification and chromatin remodelling [149]. Here, we analysed the expression status of HATs and HDACs in drug-resistant parasites using GEO2R tool from Gene Expression Omnibus (GEO) [149, 186, 187]. Among the HATs, PfMYST (PF11_0192) and PfGCN5 are consistently downregulated throughout the intraerythrocytic developmental cycle (Fig. 20A). The downregulation of these HATs could be contributing to the deceleration of the basal metabolism, which is observed during the emergence of drug-resistance. Among the HATs that show the significant upregulation are PFI1345c and PF10_0200. The elevated levels of these proteins may have a crucial role to play in establishment of a conducive chromatin micro-environment as well as increased translational fidelity for better survival of the parasite against drug-exposure.

Among the HDACs, we observed a remarkable down-regulation of PfHDAC1 (Fig. 20B), which may contribute to the suppression of the parasite metabolism leading to a dormant phenotype reported in artemisinin resistant parasites. Interestingly, transcription profile of artemisinin resistant parasites was strikingly similar to sensitive parasites that have been exposed to HDAC inhibitors [149]. Moreover, PfCHD1 was also observed to be consistently downregulated throughout the parasite IDC (Fig. 20B), which may contribute to lower chromatin accessibility of transcriptional machinery and depleted enrichment of histone modifiers. In the later stages of IDC, however, the level of dysregulation reduces which may allow for increased transcriptional output. Overall, this constitutes another method of restricting gene expression in drug-resistant parasites, which show a dormant phenotype.

2.8 Perspective: HAT/HDAC drug development

Histone acetyltransferases and HDACs lie at the core of a complex network of biological processes including gene expression, DNA replication, cell cycle progression, differentiation and cell death making them suitable targets for drug-development. Over the years several HAT/HDAC inhibitors have been profiled for their anti-plasmodial activity. Curcumin, anacardic acid and several novel compounds have been shown to have anti-PfGCN5 activity and cause subsequent deregulation of gene expression [137, 188, 189]. Recently, garcinol, a natural compound was reported to suppress chloroquine resistant and sensitive *P. falciparum*, asexual replication with submicromolar IC_{50} [190]. Similarly, HDAC inhibitors have been found to cause large-scale generic dysregulation of the parasite transcriptome and inhibit parasite growth. Apicidin inhibits parasite growth both *in vitro* and *in vivo* at nanomolar range and induces hyper-acetylation of histones in direct conjugation with suppression of HDAC activity [191]. Interestingly, in silico approaches have also been used to elucidate the molecular structure and docking properties of PfHDAC1 enzyme using human HDACs as structural references [192-194]. Many of the compounds identified in such screens have shown significantly more toxicity against *Plasmodium* parasites than to human cells [195]. Moreover, during the parasite IDC, trophozoite and schizont stages of development are the most vulnerable owing to their extensive metabolic flux, and high DNA replication respectively. Multiple HDAC inhibitors, which are approved for T-cell lymphoma treatment including, suberoylanilide hydroxamic acid (SAHA), romidepsin and belinostat, exhibit potent antimalarial activity with IC_{50} in the range of 10–200nM against the asexual stages of *P. falciparum* [196]. Thus, HATs and HDACs have excellent potential to serve as future drug targets, especially in the light of emergence of multidrug resistant *P. falciparum* strains.

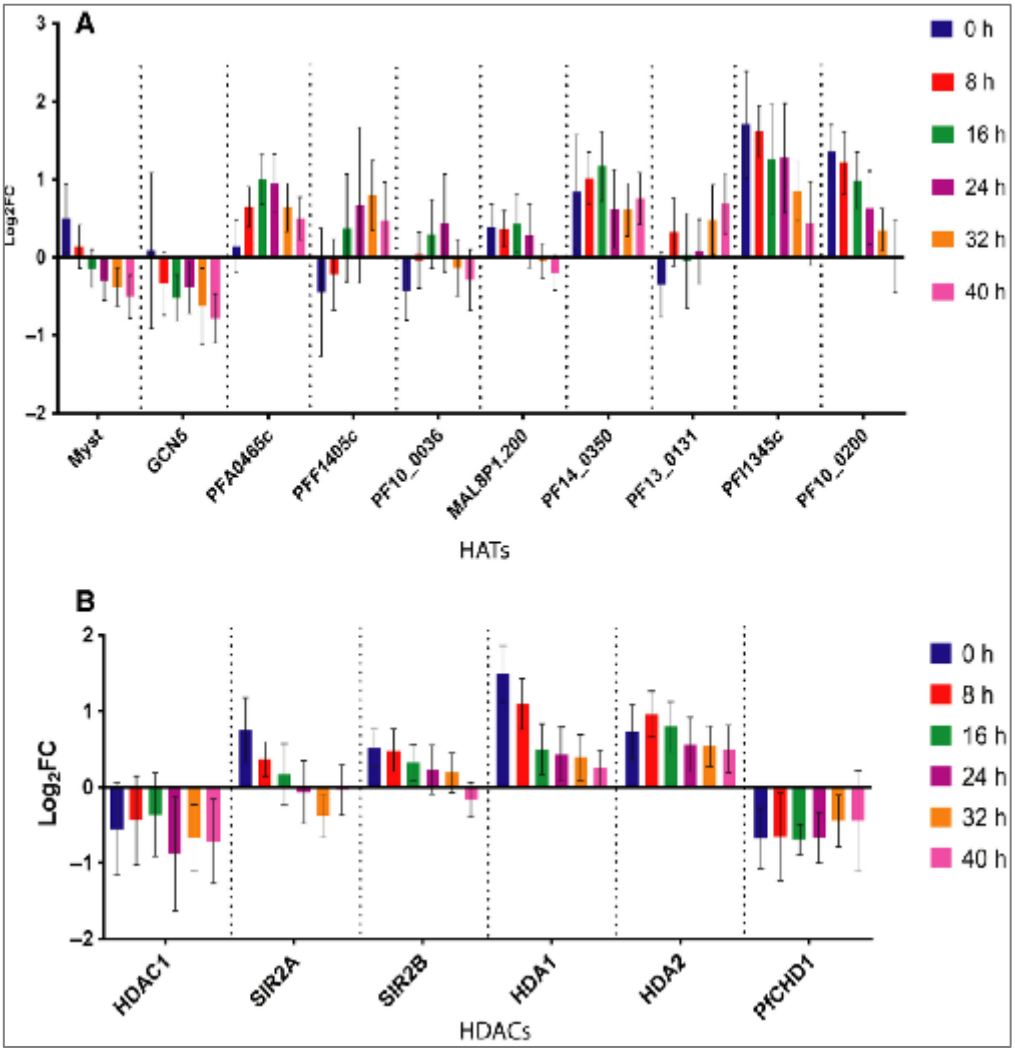


Fig. 20: Expression status of HATs and HDACs in drug-resistant parasites. Gene expression analysis was performed using the GEO2R tool on the microarray data from ex vivo cultivated drug-resistant parasites at different time points of 48 h IDC. The values plotted are Log2 fold change (FC) in expression of (A) HATs and (B) HDACs in drug-resistant parasites to Plasmodium falciparum 3D7 reference strain. The error bars represent the standard deviation of 16 ex vivo cultivated drug-resistant parasite isolates. Source: Kanyal et. al., 2018; FEBS Journal [120, 197].

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

Gene	Class	Gene ID	Maximum sequence similarity of full length/domain with	Percent identity
B. HDACs				
PfHDAC1	I	PF3D7_0925700	*Drosophila melanogaster* HDAC1	67.43
PfHDA1	II	PF3D7_1472200	*Leptospira interrogans* proHDAC	28.12
PfHDA2	II	PF3D7_1008000	Domain 1 with *Homo sapiens* HDAC1	25
			Domain 2 with *Leptospira interrogans* proHDAC	27.84
			Domain 3 with *Magnetospirillum* proHDAC	37
PfSir2A	III	PF3D7_1328800	*Thermus thermophilus* JL-18 sir	37.13
PfSir2B	III	PF3D7_1451400	Domain 1 with *Caulobacter crescentus_sir*	27.27
			Domain 2 with *Leptospira interrogans* sir	25
CHD1	II	PF3D7_1023900	*Caulobacter crescentus* pro acetylspermidine deacetylase	18.93
PFE0328W	Unclassified	PF3D7_0506600	*Crocosphaera watsonii* pro HDAC	21

Table 2: Table enlisting the major HATs and HDACs identified and discussed in the review and their maximum sequence similarity with HATs/HDACs in the eukaryotic pool. Source: Kanyal et. al., 2018; FEBS Journal [120].

2.9 Discussion

In this study, we have elucidated the distribution and role of these distinct families and classes of HATs and HDACs (enlisted in Table 2). We have also discovered the existence of unique domains and motifs within these molecules that hold the key to their flexible nature and diversity of targets apart from the canonical chromatin modifiers. With domain organization study we have proposed putative functions of relatively less characterized HATs. Phylogenetic assessment of these proteins across an array of organisms depicts their relations with members from other organisms, most importantly from humans. This has given us several candidates for future research as therapeutic targets with minimal effects on the host to ensure safe and precise treatment. Transcriptome analysis of drug-resistant parasites has also reflected upon the critical role of the HATs/HDACs in morphing the metabolic pathways of the parasite to help it adapt to drug stress. HATs/HDACs have potential of being among the master regulators of the parasite biology, which also raises the intriguing possibility of using them as targets for drug resistant malaria in the face of declining efficiency of modern ACT.

CHAPTER 3: Characterization of PfHDAC1 as a regulator of housekeeping and stress response genes in *P. falciparum* and its role in governing the parasite life cycle

3.1 *Plasmodium falciparum* epigenetic machinery is targetable

Plasmodium falciparum has a limited subset of histone deacetylases to regulate its transcription (Fig. 21) [120]. These factors in Zn or NAD+ dependent fashion carry out systematic acetylation and deacetylation of histones associated with specific genes in the parasite. Their biological functions have been identified in regulation of genes involved in antigenic variation (PfSir2A and PfSir2B) and gametocytogenesis (PfHda2) [174, 198]. Numerous stage specific gene expression study datasets show highly coordinated stage specific expression of these factors which hints at their roles in different stages of parasite development [88]. While studies have investigated the effects of the depletion of these HDACs on the parasite biology, to date a thorough characterisation of these factors is still missing.

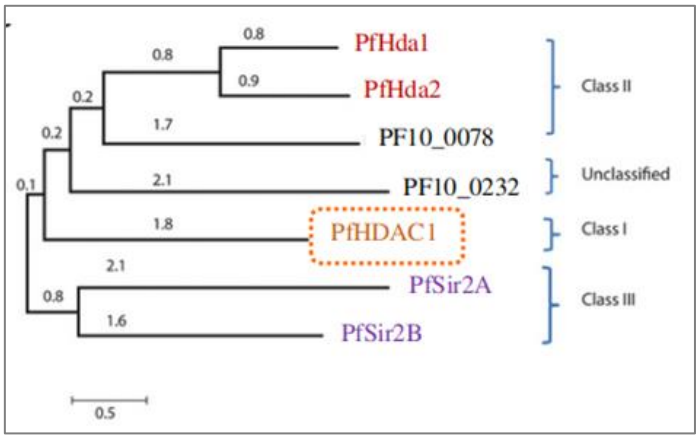


Fig. 21: Cladogram of the major HDACs found in Plasmodium falciparum, with a solitary uncharacterised HDAC belonging to Class I. Source: Kanyal et. al., 2018; FEBS Journal [120].

Among the host of antimalarial compounds at our disposal and those that have been *in vitro* characterised for their anti-*Plasmodium* activity, epigenetic inhibitors are perhaps some of the most important. Wide range of pharmacological compounds have been tested for their parasiticidal efficacy and are lead candidates for future drug development [199]. A recent study with a number of Cayman chemical library compounds tested for anti-*Plasmodium* activity showed high efficacy of compounds that target various stages of parasite development (sexual and asexual). A vast majority of these compounds are identified to be HDAC inhibitors (Fig. 22) [200]. In fact, multiple studies have shown the utility of repurposing HDAC inhibitors into antimalarial applications. Many of these compounds have been piggy-backed from human trials in cancer research (with human cancers showing skewed, often overexpressed levels of HDACs) [196]. Multiple such compounds have been shown to be effective in the submicromolar dosage and safe for use in humans. While multiple HDAC inhibitors have shown pan-stage activity which makes them especially effective in clearing off infections, some are rather stage specific [201].

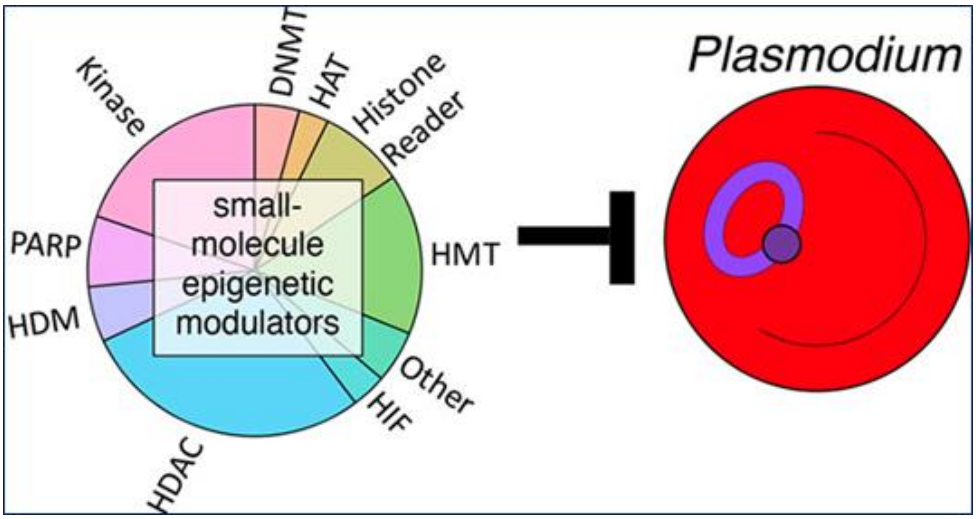


Fig. 22: A host of epigenetic machinery targeting inhibitors show antimalarial promise. A vast majority of these compounds are HDAC inhibitors. Source: Van Heer et. al., 2019; Antimicrobial Agents and Chemotherapy [201].

3.2 Effect of HDAC inhibitors on gene expression profile and histone acetylation landscape in *Plasmodium*

To characterise the effect of HDAC inhibitors on the transcriptional landscape Chaal et. al. performed microarray and ChIP on chip on parasites treated with various HDAC inhibitors. They observed widespread deregulation of the parasite biological pathways with nearly an equal number of genes undergoing up and downregulation in almost all the stages. The effect was understood to be a generic one owing to the non-specific nature of the inhibitor used. These inhibitors were also shown to be associated with a global increase in the histone acetylation particularly H3K9ac and H4K8ac. Furthermore, the inhibitors also affected the levels of histone methyl modifications hinting at a probable crosstalk between these modifications. Much of these changes were focused on the promoter regions of the genes indicating their role in gene expression control. Several transcriptional regulatory proteins including ApiAP2 transcription factors were found to be under regulation of HDACs. This study was performed using broad specificity HDAC inhibitors and while it does hint at the broadscale role of HDACs in the *P. falciparum* biology the roles played by individual HDACs was missing in information (Fig. 23) [161]. The step forward from this study was understood to be a gene specific investigation of their role in transcription regulation.

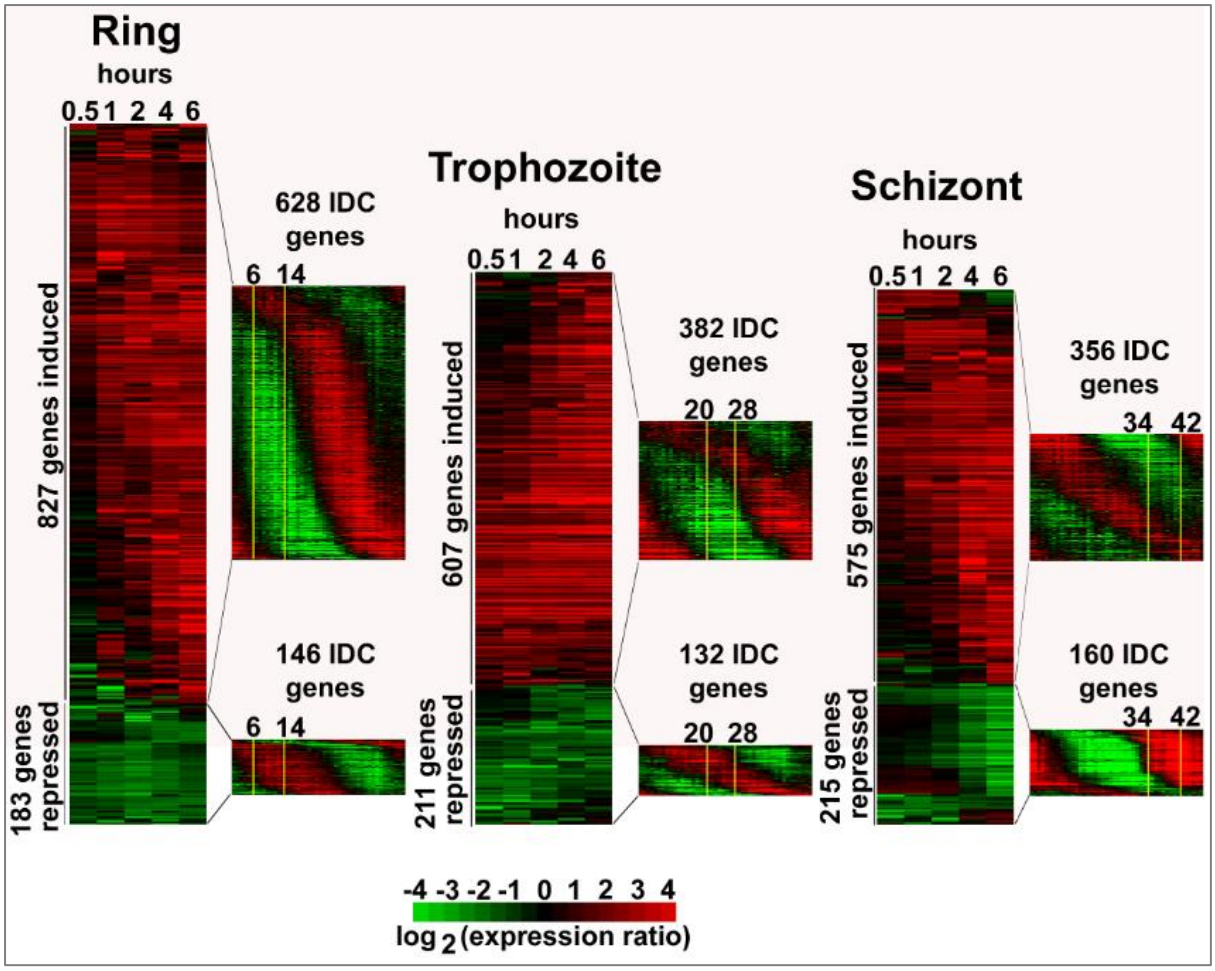


Fig. 23: Heatmaps showing the deregulated transcription of a significant proportion of the parasite transcriptome across all the three blood stages, upon treatment with pan HDAC inhibitor apicidin. Source: Chaal et. al. 2010; PLoS Pathogen [161].

3.3 PfHDAC1 is a solitary class I HDAC in *P. falciparum*

Plasmodium falciparum has three distinct classes of HDACs namely Zn^{2+} dependent Class I and Class II and the NAD^{+} dependent Class III. Class I has a single HDAC also known as PfHDAC1 based on its homology with the yeast HDAC Rpd3 [202]. There are two Class II HDACs namely PfHda1 and PfHda2 and two class III Sirtuins PfSir2A and PfSir2B. Among the class II members only PfHda2 has been characterised and it is shown to be strongly responsible for governing the expression of transcription factor ApiAP2-G which governs the

transition to sexual parasite stages [170]. PfHda1 is yet to be characterised. The Sir2A and Sir2B have been characterized extensively for their role in regulation of antigenic variation associated with *var* genes. They are believed to work in a coordinated fashion in regulating the expression of distal and proximal *var* genes [174]. The Class I HDAC, PfHDAC1 is made conspicuous by being the solitary member in its class. It is thoroughly lacking in characterisation apart from some initial localisation and expression studies [202]. Class I in a number of biological systems is reported to have multiple HDACs (HDAC1 and HDAC2 being the most prominent) which function in a coordinated fashion to control several key biological roles in organisms [182]. PfHDAC1 is an essential gene in *P. falciparum* and PbHDAC1 in *P. berghei* in mice models as shown by a couple of mutagenesis studies (Fig. 24) [203, 204]. This further highlights the crucial role it plays in parasite biology.

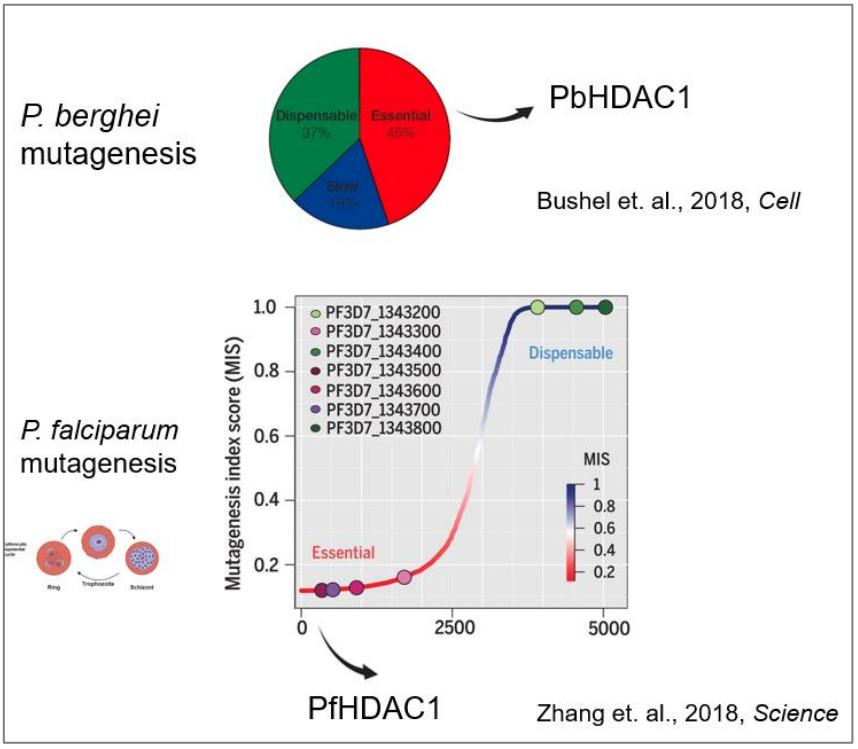


Fig. 24: Mutagenesis experiments in mice and human malaria parasites reveal HDAC1 counterparts to be essential for survival. Source: Bushell et. al., 2018; Cell and Zhang et. al., 2018; Science [204].

3.4 PfHDAC1 is identified as a target for multiple anti-*Plasmodium* inhibitors

Over the years several anti-*Plasmodium* inhibitors have been identified to function through targeting of PfHDAC1. PfHDAC1 molecular structure model was generated based on its high-level similarity with human hsHDAC2. Docking studies were performed and it was identified that the highly effective inhibitor valproic acid operates through interaction and possible inhibition of PfHDAC1 (Fig. 25) [193]. The human HDAC4 and HDAC5 inhibitor LMK 235 has been repurposed for use in anti-*Plasmodium* research and has been identified to inhibit the *in vitro* activity of recombinant PfHDAC1 [205]. The alkoxyamide class of inhibitors tested in this study were shown to have effectiveness against a range of asexual and sexual parasite stages [205]. Another study also profiled the targeting of the Zn binding pocket of the PfHDAC1 by a library of 26 compounds containing cinnamic acid or NSAID active ingredients and found PfHDAC1 inhibition (IC_{50} range of <100nM) [206]. A recent study thoroughly investigated the effect of quisinostat derived compound that possessed activity against the entire asexual stage of parasite as well as the gametocytes. *In vivo* experiments also provided evidence of activity against *P. yoelii.* RNA sequencing to compare the gene expression effects of the inhibitor with the effect of PfHDAC1 and PfHda1 knockdown proved that the compound operated through inactivation of PfHDAC1 in *P. falciparum* and not PfHda1 [207].

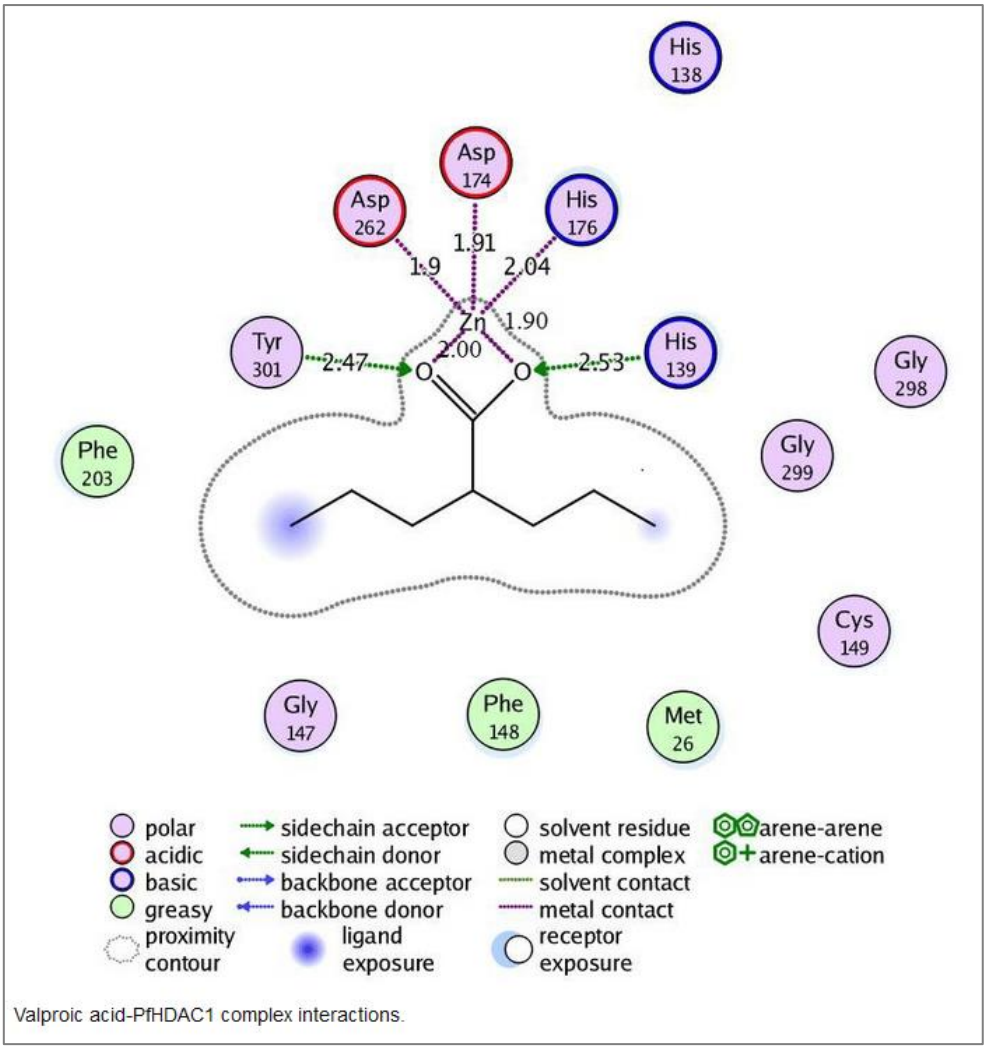


Fig. 25: The interaction map of the active site of PfHDAC1 displaying valproic acid inhibitor docking. Source: Elbadawi et. al., 2015; International Journal of Molecular Sciences [193].

3.5 Characterisation of PfHDAC1: An investigation into the protein activity, regulation, localisation, and gene targets

Despite extensively being reported as a target of multiple highly potent inhibitors a thorough characterisation of PfHDAC1 is lacking. Thus, we sought to perform a basic characterisation of PfHDAC1 especially in context of its gene regulatory role. We aimed at profiling its deacetylase activity and its potential regulation, identification of gene targets that it binds to and the biological pathways it functionally regulates (Fig. 26). Over the years evidence has

accumulated which hints at the possible role of interacting partners of HDACs and post translational modifications of the protein playing a broader role in determining substrate selection and activity [208]. *In vitro* activity for PfHDAC1 has been reported from commercially available PfHDAC1 expressed in insect cell expression systems. How this activity itself is regulated is unknown for *P. falciparum* PfHDAC1. A broader understanding of the deacetylase activity of PfHDAC1 and its regulation may be obtained from investigation of PfHDAC1 interacting partners and post translational modifications it undergoes.

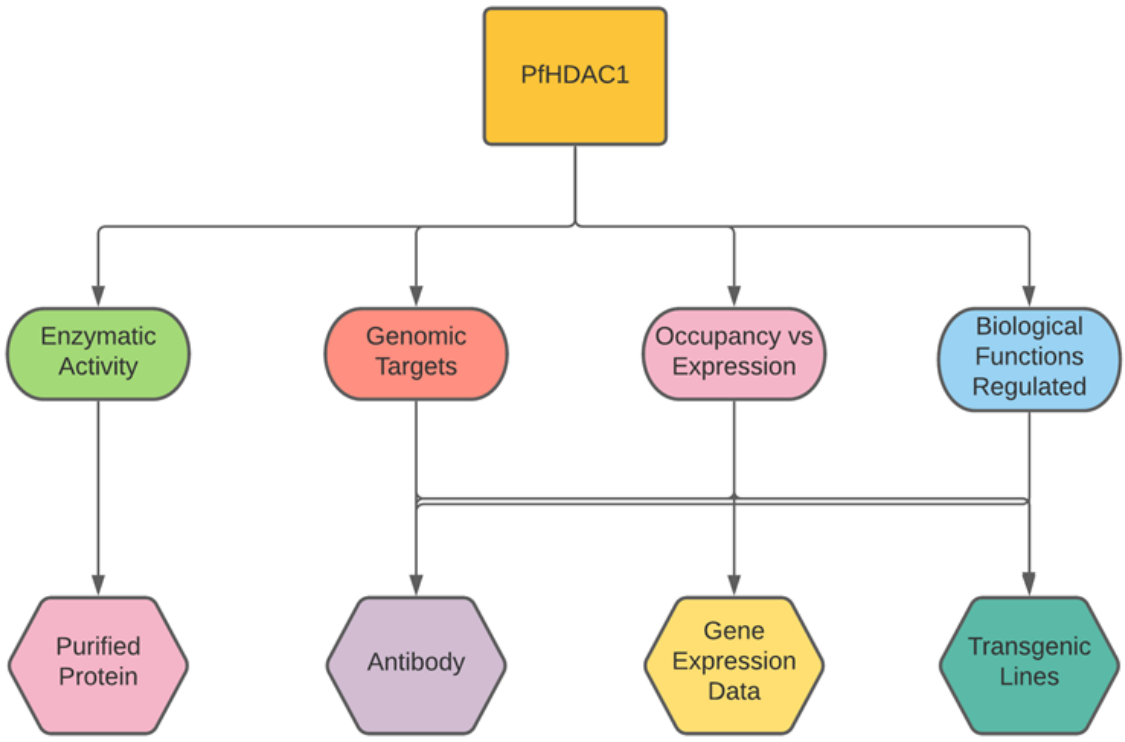


Fig. 26: Characterisation of PfHDAC1 enzymatic activity and gene regulation and generation of requisite molecular tools.

HDACs have been most prominently associated with their roles in regulating gene expression. Class I HDACs have been identified to regulate a host of biological functions in eukaryotes. In higher organisms they have been associated with supporting developmental programs in tissues especially nervous, skeletal, cardiac [209-211]. Knockouts of Class I HDACs are reported to

be deleterious, further highlighting their essence in cell survival and developmental biology. HDACs are known to regulate the expression of genes involved in embryonic development as well [212]. Aberrant expression of HDACs is associated with growth defects often leading to uncontrolled cell division. Human cancers often report overexpression of HDACs and utilisation of HDAC inhibitors has proven to be useful in controlling cancerous progression [213]. The inhibition of parasite development upon inhibitor-based targeting of PfHDAC1 also indicated a strong role of the protein in regulating cell cycle in the parasite which we wanted to characterise [196]. HDAC1 has been reported as a strong modulator of cell cycle associated genes in other organisms, especially mammalian cells [158]. All this evidence puts in a strong case for investigating the role PfHDAC1 in cell cycle and developmental progression in *P. falciparum*. By virtue of their high placement in the gene regulatory hierarchy, HDACs can regulate hundreds of genes linked into several biological pathways (Fig. 27). It would be interesting to follow what key gene targets underlie PfHDAC1 regulation in the parasite. Investigations in other eukaryotic systems have hinted at the strong regulation of genes involved in cell cycle, DNA replication, DNA damage repair, and apoptosis [214-216].

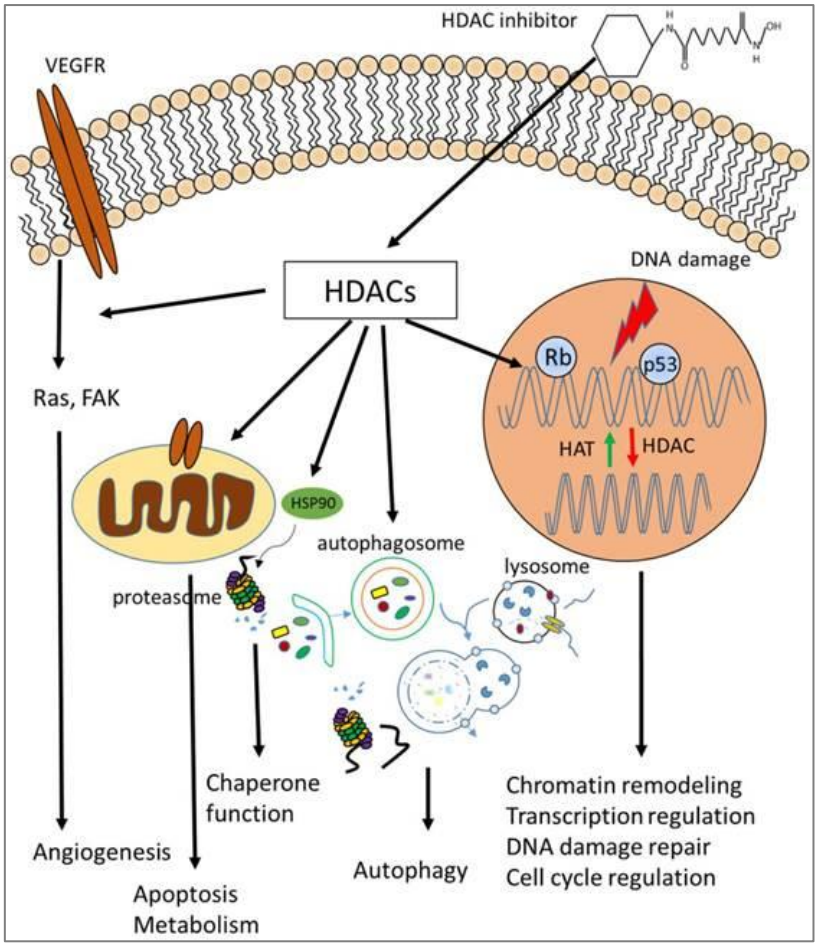


Fig. 27: The various biological roles that are governed by HDAC1 in higher eukaryotes. Source: Li and Zhu, 2014; International Journal of Biological Sciences [217].

We sought to characterise the biological functions of PfHDAC1 in the parasite with special emphasis on the deacetylase activity and its regulation, identification of the gene targets that underlie PfHDAC1 regulation and the effect of PfHDAC1 inhibition on parasite development in general.

3.6 Material and methods

3.6.1 *Plasmodium falciparum in vitro* culture

P. falciparum strain 3D7, the various PfKelch13 mutant/resistant strains from the field and transgenic lines for GFP-tagging or overexpression of PfHDAC1 were cultured as previously described [218]. Briefly, parasites were cultured in RPMI1640 medium supplemented with 25mM HEPES, 0.5 % AlbuMAX I, 1.77 mM sodium bicarbonate, 100µM hypoxanthine and 12.5µg ml^{-1} gentamicin sulphate at 37 °C. Parasites were sub-cultured after every two days. Subculturing was done by splitting the flask into multiple flasks to maintain parasitemia around 5%. Hematocrit was maintained to 1-2% by adding freshly washed O +ve human RBC isolated from healthy human donors. Synchronization was done with the help of a 5% sorbitol at ring stage. Synchronization was performed using the Percoll density gradient method (63%). Parasitemia was monitored using Giemsa staining of thin blood smear.

3.6.2 Freezing of parasites

Parasites were frozen according to the protocol mentioned in Radfar et al., 2009 [218]. 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. Freezing solution was added drop wise to the pellet and mixed properly before aliquoting 500µl of the mix in each cryovial. Cryovials were immediately stored in liquid nitrogen.

3.6.3 Thawing of the *Plasmodium* culture

Parasites were thawed using two reviving solutions, A and B. The frozen pellet was thawed for 1 minute in a water bath at 37 ℃. Solution A (12% NaCl solution) was added drop wise to the *Plasmodium* pellet to a total volume 1/10 of the pellet. It was kept for 5 minutes at room

temperature. Solution B (10x volume of parasite pellet) was added subsequently to the mix and the suspension was centrifuged. Parasites were washed using incomplete media and kept back in culture after adding fresh RBCs to maintain hematocrit.

Alternatively, the transgenic parasite lines were thawed in a 37 ℃ water bath for a minute followed by dropwise addition of 3.5% NaCl solution (equal to the pellet volume) while gently swirling the tube. The mix was incubated for 1 minutes and then 10 ml of incomplete media was added (1ml at a time) on top. The suspension was then centrifuged, and the supernatant removed. Fresh RBC equal to the pellet volume and media was added to make a 1% hematocrit solution and kept in the CO2 incubator.

3.6.4 Harvesting of parasites via saponin lysis of the infected RBCs

Parasites were harvested from the infected RBCs 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/lyse the RBC pellet and release the internal parasites, followed by incubation at 37 ℃ for 15 minutes. After the incubation the mix was centrifuged at 6000RPM for 15 minutes at 4 ℃. This was followed by 1x PBS washes to the pellet to get rid of the lysed RBC. Parasite pellets were stored at -80 ℃ immediately after the washes.

3.6.5 Cloning, overexpression, and purification of recombinant PfHDAC1

The full length PfHDAC1 sequence was PCR amplified from the genomic DNA using gene specific primers. The amplicon was then cloned into the pET28a+ bacterial expression vector using the NheI and XhoI restriction enzyme sites and into the pGEX4T1 expression vector using the BamHI and XhoI restriction enzyme sites to get a 6xHis tagged and GST-tagged

version of the gene respectively (Primer details in Table 3/Appendix 1). The overexpression constructs were transformed independently into the BL21 (DE3) E. coli expression system. Bacterial culture growing at 0.5 optical density at 600 nm was induced with 0.5 mM isopropyl-1-thio-β-d-galactopyranoside IPTG for 5 hrs at 25 ℃. The bacterial pellet was resuspended in sonication buffer (10 mM Tris-Cl pH 8.0, 150mM NaCl, 10% Glycerol, 1XPIC and 1XPMSF) 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 14,000RPM for 30 minutes at 4ºC. The supernatant was stored at -80℃ and the pellet was resuspended in 8M Urea prepared in the sonication buffer. Pellet was kept in shaking condition for 1 hour at room temperature followed by centrifugation. The 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 or anti-GST antibody). Once the expression was confirmed protein purification was performed. The GST tagged PfHDAC1 was purified from the soluble fraction with glutathione sepharose 4B beads (GE Healthcare) using 20mM reduced glutathione. The 6xHis tagged protein was purified from the insoluble fraction of a separately induced culture using Ni-NTA beads (GE Healthcare) with 250 mM imidazole. Purification of proteins was checked using SDS PAGE and western blotting. Purified proteins were dialyzed and stored at -20 ℃.

The PfCKII-α was PCR amplified from genomic DNA and cloned into the pEX4T1 vector for bacterial expression (Primer details in Table 3/Appendix 1). GST tagged CKII alpha clone was transformed into BL21 DE3 E. coli competent cells. Protein induction was performed with 1M IPTG (at OD 0.6) and protein expression was allowed for 12hrs. at 18ºC. Cell lysate was prepared as described for PfHDAC1-GST tagged above and purification of protein was done using glutathione beads. The purified protein was eluted with 10mM reduced glutathione and dialysed subsequently.

3.6.6 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 (Invitrogen A21245), Goat anti-Rat Alexa Fluor 488 (Invitrogen A11006), Goat Anti-Rabbit Alexa Fluor 488 (Invitrogen A11034) were used for immunofluorescence. Rabbit polyclonal antibodies against PfHDAC1 were generated by immunizing rabbits with purified his-tagged recombinant PfHDAC1 (conjugated to Freund's Incomplete and Complete adjuvant). The New Zealand White rabbits (3-4 months old) were used for antibody generation at the Jawaharlal Nehru Centre for Advanced Research animal house facility. 200µg protein was intramuscularly injected for the first round followed by 4 booster doses of 15µg each. Five bleeds were collected via femoral bleeds and centrifuged for obtaining the antisera from whole blood. The antibodies were affinity purified using the GST-tagged recombinant PfHDAC1 conjugated to sulpholink resin.

3.6.7 Western blotting

Parasites were harvested using 0.15% saponin. Parasite 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 (MgCl2), 1X protease inhibitor cocktail (PIC), 1 mM phenylmethylsulphonyl fluoride (PMSF)). Three freeze thaw cycles were performed using liquid nitrogen to achieve complete lysis of the parasites. To get rid of debris, parasites were spun at 17949x g for 30 minutes. Supernatant was transferred to another tube. The lysate proteins were separated on 7.5% -12%

polyacrylamide gels and transferred to the PVDF membrane. The membrane was blocked using 5% skimmed milk and probed using relevant primary antibody overnight at 4℃. After overnight incubation the membrane was 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).

3.6.8 Immunofluorescence assay

Parasites were fixed using 4% PFA and 0.00075% glutaraldehyde for 30 minutes at 37 ℃ Permeabilization 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).

3.6.9 RNA isolation

Parasite pellets were homogenized in 1 ml TRIzol (Biorad) reagent properly. A 0.2x volume of chloroform was then added to the TRIzol suspension. Tubes were properly mixed and kept at room temperature for 10 minutes. Samples were centrifuged at 13,000RPM for 12 minutes at 4ºC. The upper aqueous phase was collected in a separate tube. Isopropanol (0.8x volume) was added to the tubes and properly mixed. Samples were incubated for 15-20 minutes at room temperature for precipitation followed by centrifugation at 13,000RPM for 12minutes at 4℃. The supernatant was removed, and the pellet was washed once with 75% ethanol. Pellet was

air dried before resuspending in nuclease free water. RNA quantity and quality were estimated using a Nanodrop or Qubit fluorometer.

3.6.10 Quantitative RT-PCR

2µg of DNAse free RNA was used for cDNA synthesis using ImProm-II Reverse transcription system (Promega), as per the manufacturer'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 seryl-tRNA synthetase were used as internal controls to normalize for variability across samples. Quantification of the expression was done with the help of fluorescence readout of SYBR green dye incorporation into the amplifying targets using a Biorad thermo-cycler. Each experiment included three technical replicates and was performed for three independent biological replicates.

3.6.11 Immunoprecipitation and mass spectrometry

To harvest the parasites, infected RBCs were lysed using 0.15% saponin at 37°C. The harvested parasites were then lysed using ice cold parasite lysis buffer (20mM Tris-Cl pH 8.0, 150mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 0.1mM EDTA, 1.5mM 55mM MgCl2, 1X PIC, 1mM PMSF). Lysed parasites were then centrifuged at 20817xg for 30 min at 4°C. Pre-clearing was performed using recombinant protein G conjugated sepharose beads for 1 hour at 4ºC. Precleared lysate was then used for overnight incubation with antibody at 4ºC. After the overnight incubation of lysate with antibody, sepharose protein G beads were added to the lysate for 4hours incubation. Washes were done using immunoprecipitation buffer (25mM Tris pH 7.9, 5mM MgCl2, 10% glycerol, 100mM KCl, 0.1% NP-40, 0.3mM DTT) followed by elution of the proteins using glycine (pH –2.5). Eluted proteins were neutralized using 1M Tris pH 8.8. For mass-spectrometry analysis samples were digested with trypsin for 16 hrs at 37ºC.

The digested samples were cleared using C18 silica cartridge. Peptides were then analysed 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 analysed with Proteome Discoverer and MaxQuant against the Uniprot P. falciparum reference proteome database. For Sequest search, the precursor and fragment mass tolerances were set at 10ppm and 0.5Da, respectively. The protease used to generate peptides, i.e., enzyme specificity was set for trypsin/P (cleavage at the ℃ terminus of "K/R: unless followed by "P") along with maximum missed cleavage value of two. 0.001 FDR was set for both peptide spectrum match and protein false discovery rate.

3.6.12 Chromatin immunoprecipitation

Parasitized RBCs were crosslinked using 1% formaldehyde (Thermo Scientific, 28908) for 10 mins at RT. 150mM 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 (25mM Tris-Cl pH 7.9, 1.5mM MgCl2, 10mM KCL, 0.1% NP-40, 1mM DTT, 0.5mM PMSF, 1xPIC) 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 200-400bp. Pre-clearing was performed for 1hr at 4℃ using recombinant protein G conjugated sepharose beads with continuous gentle inverting. 30µg purified chromatin was used per immunoprecipitation reaction (α-PfHDAC1 antibody) and incubated for 12h at 4 ℃. Samples were then incubated with saturated Protein G Sepharose beads for 4 hours at 4 ℃. Bound chromatin was finally washed with low salt, high salt, LiCl wash buffers followed by TE buffer

wash and eluted using ChIP elution buffer (1 % SDS, 0.1 M sodium bicarbonate). Both the ChIP and input samples were reverse crosslinked using 0.3M NaCl overnight at 65 ℃ along with RNAse A treatment. Proteinase K treatment was performed at 42 ℃ next day for 1 hour. Finally, DNA was purified using phenol chloroform isoamyl alcohol precipitation. Target sites identified from ChIP sequencing analysis were further validated by ChIP-qPCR using the Biorad SYBR Green Master Mix (Biorad).

3.6.13 ChIP-sequencing library preparation and sequencing

ChIP-sequencing libraries for PfHDAC1 ChIP in 3D7 strain (and later for the artemisinin resistant and sensitive isolates) were prepared from 5-10 ng of DNA using the NEBNext 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). For PfHDAC1 ChIP seq in artemisinin resistant and sensitive isolates, the library was prepared as described above. The sequencing for these was done at the Next Generation Sequencing facility at IISER, Pune using the Illumina NextSeq 550 sequencer with average read length of 75 bp.

3.6.14 ChIP-Seq data analysis

The sequencing reads were demultiplexed using the bcl2fastq tool in the Linux platform. Read quality check was performed using FASTQC and sub quality reads (not passing Q30 filter) and adaptor sequences were trimmed using Trim_Glaore. Reads were aligned onto the *Plasmodium*

falciparum 3D7 reference genome using Bowtie aligner. SAMTools was used to further process the data (sorting, etc.). The sorted ChIP and Input BAM files were then used for peak calling in MACS2 software. Background subtraction was performed using the bdgcmp command in MACS. Peak annotation was performed using Bedtools and the peaks were visualised using IGV genome browser.

Deeptools suite was used for additional ChIP data analysis and visualization. The ChIP and input BAM files were converted into bigwig format using BamCoverage option. BamCompare was used to generate an input normalized ChIP bigwig file. The computeMatrix option was used to generate an enrichment matrix for the PfHDAC1 over average gene body flanked by 500bp of upstream and downstream UTR. The matrix was then fed to the plotHeatmap tool to generate the PfHDAC1 occupancy heatmap on the target genes. PlasmoDB gene ontology tool was used to identify the biological pathways enriched for PfHDAC1 occupancy gene targets.

For additional comparison of the PfHDAC1 occupancy (ChIP data) and gene expression (RNA seq data) in artemisinin resistant/sensitive isolates computeMatrix command was used to generate the gene expression reads density matrix over the PfHDAC1 enrichment/depletion loci (fed in the reference BED file) and then visualized using the plotHeatmap option.

3.6.15 Additional NGS data source and analysis

The ChIP-seq dataset for the H3K9ac and H3K9me3 were obtained and downloaded from the GEO server link GSE63369. Analysis and representation of the datasets was performed similar to the methodology described for PfHDAC1 ChIP-seq. computeMatrix command on Deeptools was used to generate the average gene body and gene flanks occupancy matrix for H3K9ac and H3K9me3. This matrix was imputed into the plotProfile command to perform a comparative assessment of the occupancy patterns of these modifications relative to PfHDAC1.

3.6.16 Drug sensitivity assays to determine IC_{50} of class I HDAC specific inhibitors

The drug/compound sensitivity assays were adapted from the WHO Mark II test. In brief, parasite culture at 2% parasitemia and 2% hematocrit was cultured in 96 well plates in 2X serially diluted doses of the compound for 48 hr. The parasites were then lysed with the SYBR Green I lysis buffer (20 mM Tris pH 7.5, 5mM EDTA, 0.008% (wt/vol) saponin, and 0.08% (vol/vol) Triton X-100, 1 x SYBR Green I) and then incubated in dark at room temperature for 40 min. Fluorescence readouts were taken for parasite viability using the Thermo Scientific 96 well plate reader in fluorescence mode (excitation: 495 nm; emission: 520 nm). The readouts were fed into the Graphpad Prism IC_{50} estimator which uses the linear regression model to calculate the compound's IC_{50} concentration.

3.6.17 PfHDAC1 specific inhibitor treatment for RT-qPCR and protein analysis

Parasite culture grown at 5% parasitemia and 2% hematocrit was treated at $2xIC_{50}$ dosage of entinostat or romidepsin for 4 hours prior to harvesting for gene expression analysis using RT-qPCR. The same treatment was carried on for 8 hours prior to harvesting for estimation of effect on protein levels with western blotting.

3.6.18 Investigation of the effect of PfHDAC1 inhibition upon cell cycle

Highly synchronized (Percoll density gradient centrifugation) parasites were cultured at ~5% parasitemia and 2% hematocrit and treated with $0.5xIC_{50}$ concentration of PfHDAC1 inhibitors (Entinostat or Romidepsin) at roughly 6 hours post invasion (regarded as 0 hr post treatment). The parasites were then sampled off at every 8 hour for the next 48 hours and frozen for either SYBR fluorometry to estimate the DNA content or prepared into thin Giemsa-stained smears

for microscopic examination of morphology and stage progression. The SYBR fluorometry samples were lysed with the SYBR Green I lysis buffer and processed for fluorometry as described previously for the compound sensitivity assay. The relative DNA signal for inhibitor treatment estimated by fluorescence was plotted as a percentage against mock sample readouts. For parasite proliferation estimation parasitemia was recounted upon completion of one cycle in the mock vs inhibitor treated samples. Relative percentage of parasites from each of the developmental stages of *P. falciparum* was calculated and plotted on histograms. Each time-point is validated with at least 1000 cell count observations.

3.6.19 *In vitro* protein-protein interaction assay

The recombinant purified PfHDAC1 and PfCKII-α were incubated together in an interaction buffer overnight for 4 hours at 4 ℃. The incubation reaction was split into two sets. Immunoprecipitation with anti PfHDAC1 antibodies was performed for one set and IgG for another at 4 ℃ overnight (constant rolling). These served as the test and control pull down respectively. The next day, washed recombinant protein-G sepharose beads were added to the two reactions and incubated on rolling for 4 hours at 4 ℃. The bound complex was washed and eluted from the beads using 1M glycine (pH 2.5) and neutralized with Tris pH 8.8. The complex was resolved on an SDS-PAGE gel and western blotting was done using anti-GST antibody to identify the presence of tagged co immunoprecipitated proteins.

3.6.20 *In vitro* kinase activity assay

Highly purified recombinant PfHDAC1 and PfCKII-α were used as substrate and enzyme for *in vitro* kinase assay. 500 ng of PfHDAC1 was incubated with increasing amounts (360ng, 780ng, 1080ng) of PfCKII-α in the presence of a kinase activity buffer (20 mM Tris HCl pH

7.5, 20 mM MgCl2, 2 mM MnCl2,0.1mM PMSF) with 10µM ATP. For a baseline control PfHDAC1 incubated without PfCKII-α was taken. Furthermore, two reactions with increasing dosages of a PfCKII-α specific inhibitor TBB were taken to check for possible suppression of the phosphoryl group transfer. Furthermore, to probe the effect of artemisinin treatment on the kinase reaction we set up an additional reaction with 100nM artemisinin. The reaction was incubated at 37 ℃ on a thermal mixer and then run on SDS-PAGE gel followed by western blotting with anti-phospho-serine antibody.

3.6.21 Histone isolation from parasite

Histones were purified from *Plasmodium falciparum* using urea-salt lysis and acid extraction method. The *P. falciparum* 3D7 harvested pellet was resuspended in 2x volume of Hb removal buffer (25mM Tris-HCl pH 7.8, 1 mM EDTA, 0.2% NP40) to remove contaminating membrane and Hb. Spin was given at 12000RPM and the pellet was washed again with Hb removal buffer till the supernatant was clear. It was then washed with ice cold distilled water and then with 0.8M NaCl. Acid extraction step was done by adding 8 volumes of 0.25M HCl and giving vigorous vortexing. It was incubated for 2 hrs and then the acid soluble supernatant was collected. Equal volume of 20% trichloroacetic acid was added to the supernatant and mixed by inverting and incubating on ice for 15 mins. The pellet after spin at 12000RPM was washed with acetone and was dissolved in dH2O + 0.1M beta mercaptoethanol. Histones were then validated by loading onto a 15% SDS-PAGE gel.

3.6.22 *In vitro* deacetylase activity assay

Highly purified recombinant PfHDAC1 was first phosphorylated in the *in vitro* kinase assay. The phosphorylated PfHDAC1 was used as the enzyme and histones isolated from the *P. falciparum* pellet were used as substrate for this reaction. 2µg of histones were incubated with

increasing amounts of PfHDAC1 in the presence of a deacetylase buffer (25mM Tris HCl, pH8.0, 137mM NaCl, 2.7mM KCl, 1mM MgCl2, 0.1mM ZnCl2). For control reaction only purified histones were incubated in the deacetylase reaction mix (san PfHDAC1). A class I HDAC specific inhibitor Entinostat was used in an additional reaction to inhibit the potential deacetylase activity of PfHDAC1. PfHDAC1 obtained from kinase assay (incubated with kinase inhibitor) was used as a non-phosphorylated enzyme and essentially served as a control.

3.6.23 Generation of overexpression constructs for PfHDAC1

The full-length sequence of PfHDAC1 was PCR amplified from genomic DNA using sequence specific primers and cloned into the pDC2 overexpression vector using the AvrII and NheI restriction sites (Primer details in Table 3/Appendix 1). This put the PfHDAC1 under control of the calmodulin promoter and in frame with a C-terminal GFP tag. Furthermore, to add a layer of regulatability to the overexpression system we cloned a glmS ribozyme sequence (PCR amplified from pHSP101 plasmid) in frame with the GFP tag using XhoI enzyme. This resulted in an overexpression system synthesising PfHDAC1-GFP -glmS fusion transcript.

3.6.24 *P. falciparum* transfections

Lonza nucleofector 4D was used for transfection of plasmids into highly synchronised parasites (obtained from the London School of Tropical Health and Medicine). 30µg of episomal plasmid was dissolved in 100µl of P3 primary solution. Double synchronised (Percoll centrifugation) segmented schizonts were mixed with the DNA/P3 solution mix and nucleoporated in the nucleofector 4D machine with the pulse program FP 158 (designed for *P. berghei*). The zapped cells were immediately transferred to a T25 flask with 3 ml of media and 200µl fresh RBCs and set on a shaker incubator at 37 ℃ for 2 hours to allow the ruptured

merozoites to invade the fresh cells. The flask was later supplemented with additional media to make it to 2% hematocrit. Drug selection was started 24-hour post transfection with 2µg/ml Blasticidin-S. Appearance of transgenic lines was checked initially via Giemsa smear. The cells were then checked for GFP-tagged PfHDAC1 expression via confocal microscopy. Parasite lysates were also tested for PfHDAC1-GFP expression via western blotting. The transgenics were generated and tested upon at Dr. Moritz Treeck's lab at The Francis Crick Institute, London.

3.6.25 Clonal selection of the transgenic parasite lines

The validated transgenic lines were serially diluted in a 96 well flat plate to obtain 200µl cultures with finally 1 parasite per well. These were confirmed with plaque formations and then transferred onto round bottom 96 well plates for expansion. The cells were then microscopically confirmed for GFP expression and then expanded into flasks.

3.6.26 Validation of reversible overexpression of PfHDAC1-GFP

Validation of PfHDAC1-GFP overexpression and reversible depletion was validated with treatment of culture with 0, 2.5 and 5 mM of glucosamine-HCl followed by western blotting for depletion of GFP tagged PfHDAC1 signal.

3.6.27 Parasite growth curve assay

The GFP-glmS ctrl overexpression and PfHDAC1-GFP-glmS overexpression were tightly synchronised using two rounds of Percoll density gradient centrifugation. Parasitemia was estimated by staining cells with SYBR Green I dye and subjecting them to flow cytometry. The cultures were diluted to 2% starting parasitemia and 2% hematocrit in 96 well plates and

allowed to proliferate over a duration of 6 days/3 IDC. For testing the effect of glucosamine dosage (and episomal expression suppression) on the growth trend, wells with 2.5mM and 5mM glucosamine treatment were set up in parallel with mock treated parasites for both PfHDAC1-GFP-glmS overexpression and GFP-glmS overexpression lines. Media was replenished carefully after the third day with accurate replacement for mock and drugs. Each strain and condition were set in triplicate on the 96 well plate. The culture in the wells were sampled off every 24 hours until the finish of the assay and subjected to SYBR Green I flow cytometry to calculate parasitemia progression over the course of the experiment. Growth curves were plotted on GraphPad Prism software. Baseline toxicity of glucosamine was calculated based on the viability of the GFP-glmS lines and then subtracted from the cognate readouts of PfHDAC1-GFP-glmS to represent toxicity corrected growth curves.

3.7 Results

3.7.1 General features of PfHDAC1

The PfHDAC1 is a mono-exonic gene, 1349 bp in length and located on the 9th chromosome, encoding a 449 amino acid protein of 51 kDa molecular weight. It features a single prominent deacetylase domain spanning almost the entire length of the gene (24-314 aa) identified by the Pfam and Interpro database (Fig. 28). No signalling peptide or transmembrane domain has been detected for this gene. Global RNA seq studies have shown low (but non null) expression of PfHDAC1 in the ring stage and heightened expression during trophozoite stage which persists till schizonts (Fig. 29) [97]. This hints at a possible association with developmental progression through the intraerythrocytic development cycle as well as its involvement in transcriptional regulation since trophozoite stage parasites show enhanced transcriptional activity. Protein

evidence from mass spec studies have identified phosphorylation post-translational modifications on PfHDAC1 [219].

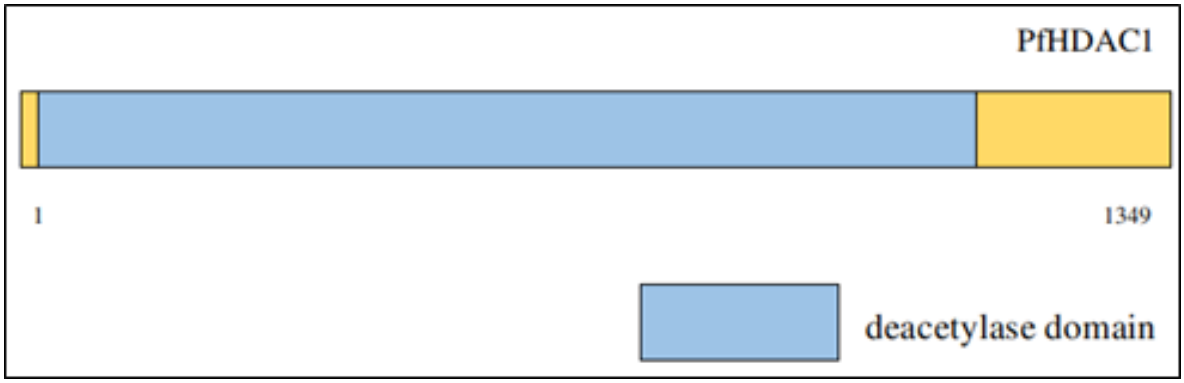


Fig. 28: The domain organisation of PfHDAC1 shows a single prominent deacetylase domain.

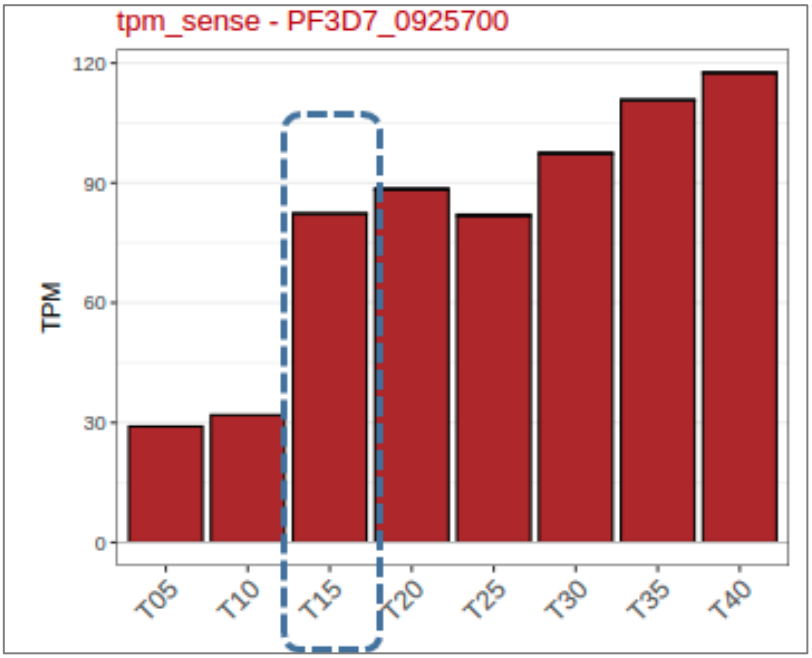


Fig. 29: Bar plots of the gene expression of PfHDAC1 during the intraerythrocytic developmental cycle show a spike in expression during transition from ring to trophozoite stage. Source: Toenhake et. al., 2018; Cell Host and Microbe [97].

3.7.2 Recombinant PfHDAC1 protein overexpression, purification, and antibody generation

The full length PfHDAC1 gene sequence was PCR amplified and cloned into the pET28a+ and the pGEX4T1 expression vectors to obtain C-terminal 6x-His tagged and GST tagged version of PfHDAC1 (Primer details in Table 3/Appendix 1). The proteins were overexpressed and purified from BL21 bacterial expression systems. SDS-PAGE and western blotting using anti-His and anti-GST antibody confirmed the purified proteins which were obtained at the expected fusion product size. The His tagged PfHDAC1 was obtained from the insoluble fraction of bacterial lysate while the GST tagged PfHDAC1 was purified from the soluble fraction (Fig. 30). Owing to the smaller tag and greater overall yield the His tagged recombinant PfHDAC1 was used for antisera generation in a rabbit over a course of 5 inoculations/boosters and bleeds. Higher solubility of the GST tagged PfHDAC1 made it suitable for *in vitro* activity assays as well as for purification of anti PfHDAC1 antibody obtained from rabbits. Sulpholink resin conjugated with purified PfHDAC1-GST was used for affinity chromatography purification of anti PfHDAC1 antibody. The purified antibody detected PfHDAC1 at the expected size from the parasite protein lysate resolved on SDS-PAGE followed by western blot (Fig. 31). The purified antibody was further validated for detection of PfHDAC1 using protein immunoprecipitation followed by mass spectrometry.

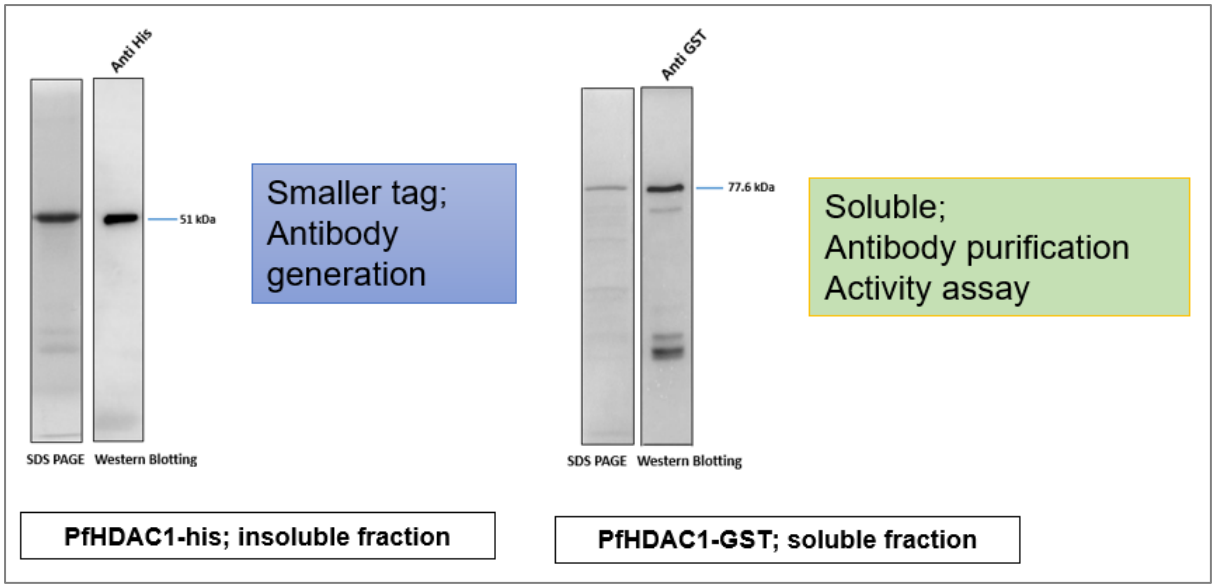


Fig. 30: (Left) SDS-PAGE and anti-His western blot to validate the purity of 6x-His tagged PfHDAC1 obtained from insoluble pellet fraction. (Right) SDS-PAGE and anti-GST western blot to validate the purity of GST-tagged PfHDAC1 obtained from soluble cytosolic fraction.

The PfHDAC1 antibody was utilized further for characterisation of PfHDAC1 genomic occupancy (via ChIP-seq) and investigation of associated biological pathways, while the purified GST-tagged protein was used for *in vitro* activity assays.

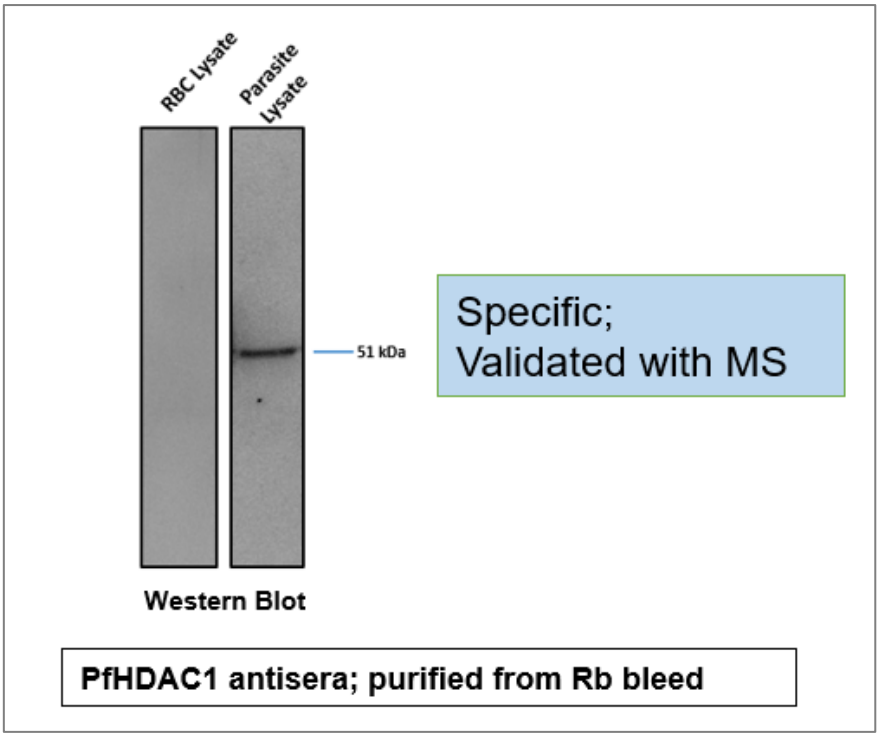


Fig. 31: Characterisation of the specificity of PfHDAC1 antibody with western blot on uninfected RBC and parasite lysates. Band at 51kDa represents the parasite PfHDAC1 as detected by the antibody.

3.7.3 Validation of the deacetylase activity of PfHDAC1 with *in vitro* activity assays

To confirm the histone deacetylase activity of PfHDAC1 we performed an *in vitro* deacetylase activity assay with histones extracted from *P. falciparum* pellet used as substrate and purified recombinant PfHDAC1 as the enzyme. We probed for the H3K9ac modification on the histones upon completion of the assay but did not observe any significant drop in acetylation signal. A brief literature survey gave us a strong indication that a post translational phosphorylation modification is required for the deacetylase activity of HDAC1 in mammalian systems. Furthermore, the phosphorylation PTM of HDACs is reported to regulate their cellular localisation and protein-protein interactions which dictate their complex formations and functional recruitment to specific gene sets [220].

In our search for the kinase responsible for the phosphorylation of PfHDAC1 we came across strong evidence in literature for the interaction of HDAC1 from a number of organisms with casein kinase 2-α (CKII-α) [221]. We thus sought to confirm if the same was true in *P. falciparum* as well. We expressed a recombinant PfCKII-α subunit in the *E. coli* expression system to be used for further *in vitro* studies.

3.7.4 PfHDAC1 and PfCKII-α interact in *in vitro* settings

The recombinant PfHDAC1 and PfCKII-α were expressed and purified from bacterial lysate and co-incubated. Following this anti PfHDAC1 antibody was used to pull down PfHDAC1 and in a control IP IgG was used. The immunoprecipitated fractions in PfHDAC1 and IgG pulldowns were probed with α-GST antibody to detect for CKII-α co-immunoprecipitation. We detected an enrichment of PfCKII-α in the PfHDAC1 IP but not in IgG IP. This validates the interaction of PfHDAC1 and PfCKII-α (Fig. 32).

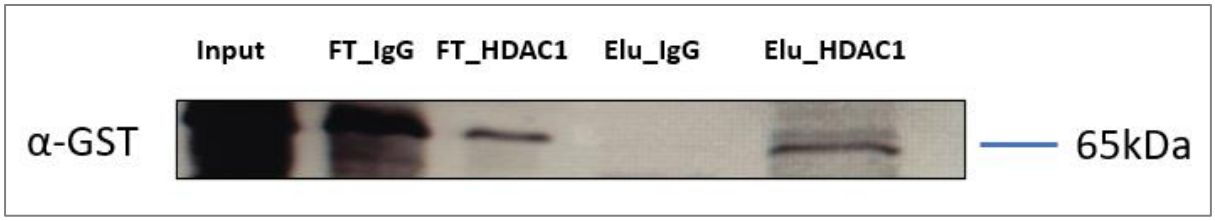
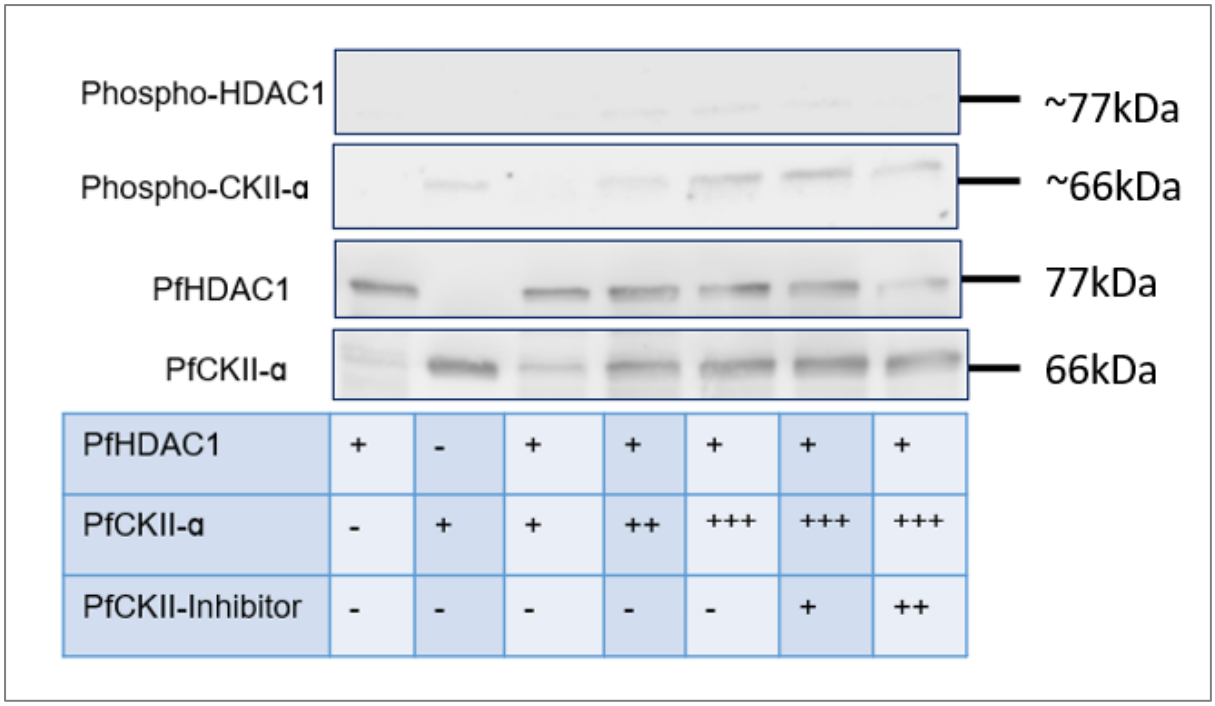


Fig. 32: PfCKII-α interacts in vitro with PfHDAC1. Western blot for IgG immunoprecipitation and PfHDAC1 immunoprecipitation shows pull down of GST-tagged PfCKII-α in the latter fraction. (Lanes in order: Input, Flow through IgG, Flowthrough PfHDAC1, Elution IgG, Elution PfHDAC1).

3.7.5 The PfCKII-α imparts a phospho-serine PTM on PfHDAC1

Having confirmed that PfCKII-α and PfHDAC1 interact together in *in vitro* settings we next sought to validate if this interaction was functionally relevant and resulted in phosphorylation of PfHDAC1. We set up an *in vitro* kinase activity assay where recombinant PfHDAC1 was incubated with either null or progressively increasing amounts of PfCKII-α. We also set up two control tubes where the kinase reaction was spiked with increasing doses of PfCKII-α specific inhibitor TBB.

The incubated mixes were subsequently probed with a α-phosphoserine antibody to recognise any phosphorylation modified forms of PfHDAC1. We observed an appearance of the phosphorylated PfHDAC1 which increased in intensity in a PfCKII- α in a dose dependent manner. The control reaction with no PfCKII- α did not report any band for phospho-PfHDAC1. In the reaction mixes incubated with the PfCKII- α inhibitor we observed a dose dependent inhibition of the phosphorylated PfHDAC1 band. Interestingly we also noticed that PfCKII-α has auto-phosphorylation activity which gets inhibited in the presence of the TBB inhibitor (Fig. 33).



PfHDAC1	+	-	+	+	+	+	+
PfCKII-α	-	+	+	++	+++	+++	+++
PfCKII-Inhibitor	-	-	-	-	-	+	++

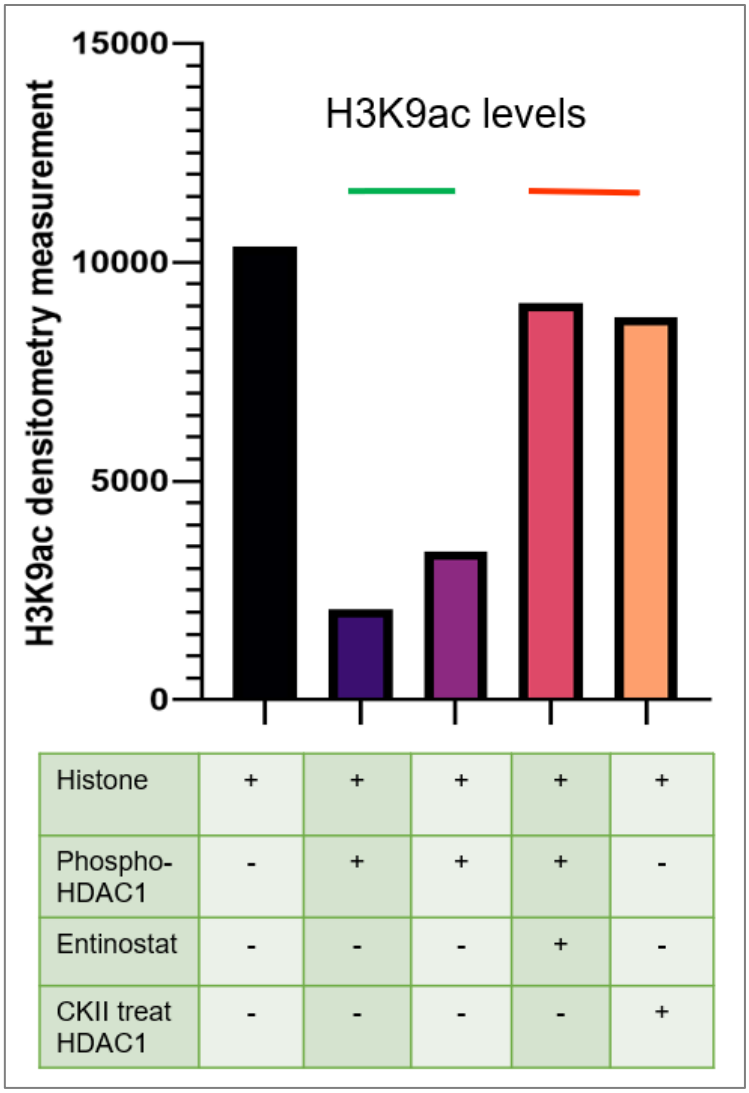
Fig. 33: PfCKII-α phosphorylates PfHDAC1 in a dose dependent fashion and is inhibited by CKII specific inhibitor TBB. The western blot represents probing for phospho-serine modified proteins. The reaction components are as defined in the table below the blot.

These results indicate that PfHDAC1 and PfCKII-α interact and this interaction results in the addition of a phospho-serine post translational modification on PfHDAC1. This interaction raises further possibilities into the role of phosphorylation PTM of PfHDAC1 into regulating deacetylase activity.

3.7.6 Phosphorylated PfHDAC1 shows histone deacetylase activity *in vitro*

Having confirmed the phosphorylation PTM imparted on PfHDAC1 by PfCKII-α we sought to check if this modification could contribute to catalytic activity of PfHDAC1. We set up an *in vitro* histone deacetylase activity using *P. falciparum* isolated histones as substrate and PfCKII-α treated PfHDAC1 (phosphorylated) as the enzyme. Phosphorylated PfHDAC1 was able to perform deacetylase activity and we observed a depletion of H3K9ac histone

modification on the substrate confirmed via western blotting. Further, we used a Class I specific histone deacetylase inhibitor Entinostat (MS-275) and observed that it was able to suppress the deacetylase activity associated with phosphorylated PfHDAC1. Moreover, a reaction with non-phosphorylated recombinant PfHDAC1 (not incubated with PfCKII-α prior to deacetylase assay) resulted in no deacetylase activity (Fig. 34).



Histone	+	+	+	+	+
Phospho-HDAC1	-	+	+	+	-
Entinostat	-	-	-	+	-
CKII treat HDAC1	-	-	-	-	+

Fig. 34: Phosphorylated version of PfHDAC1 shows robust histone deacetylase activity in an in vitro activity assay. Densitometry plot for the H3K9ac modification on purified P. falciparum histones in a deacetylase activity assay using phosphorylated vs unphosphorylated PfHDAC1. The composition of the reaction mix is as described in the table.

Thus, our results suggest that PfCKII-α dependent phosphorylation of PfHDAC1 is important for robust deacetylase activity of the latter. This activity was also shown to be inhibited by Class I specific histone deacetylase inhibitors.

3.7.7 Localisation study of PfHDAC1 using immunofluorescence

Immunofluorescence assay was performed to validate the localisation of PfHDAC1 in the parasite cell. We observed a strong colocalization of PfHDAC1 with the DAPI stained parasite nuclei. We did also notice a fainter distribution of the PfHDAC1 in the cytoplasmic compartment of the parasite (demarcated by the lack of DAPI signal) (Fig. 35). Class I HDACs are classically acknowledged to be predominantly nuclear histone deacetylases, but there are reports of their non-canonical distribution in non-nuclear compartments which raises an interesting possibility of their roles in deacetylating non histone substrate proteins [222, 223]. Thus, our observations indicate that apart from the nuclear functions performed by PfHDAC1 it could have interactors and client proteins in the parasite cytoplasm regulating aspects of parasite biology other than transcriptional regulation. These possibilities have not been discussed in our work, however.

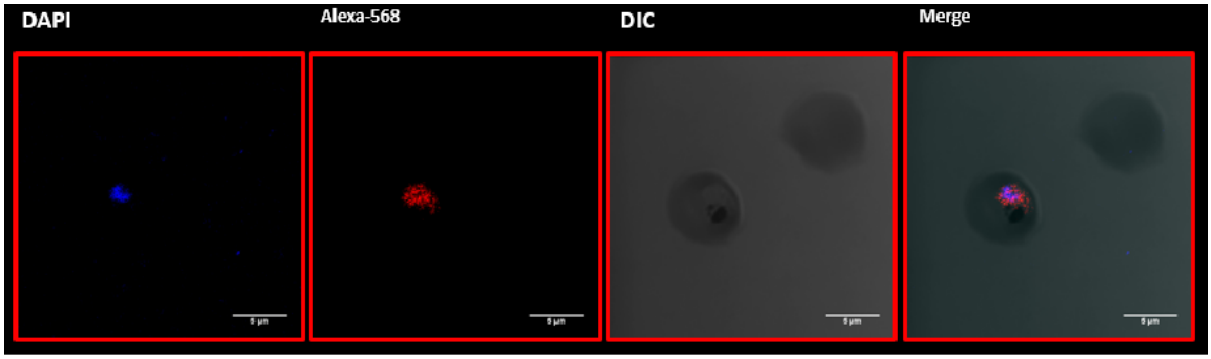


Fig. 35: Immunofluorescence assay showing predominantly nuclear and some cytoplasmic localisation of PfHDAC1. (Blue: DAPI channel for nuclear signal; Red: Alexa-568 channel for PfHDAC1 signal).

3.7.8 PfHDAC1 Chromatin immunoprecipitation-sequencing (ChIP-sequencing)

To identify the genome-wide targets of PfHDAC1, we performed chromatin immunoprecipitation and sequencing with anti-PfHDAC1 antibody. The PfHDAC1 peaks were enriched predominantly over the gene body with very sparse distribution recorded for intergenic regions. A total of 669 genes were identified as enriched (>=1.8fold) for PfHDAC1 occupancy (Fig. 36) (Enlisted in Table 4/Appendix 2). Gene Ontology Enrichment Analysis of the target genes revealed a vast majority of PfHDAC1 target genes are associated with the biological function of antigenic variation and cytoadherence. Other major biological pathways under PfHDAC1 regulation were identified to be translation, response to drug, generation of metabolites/precursor of energy, chromosome organisation, host-pathogen interaction (entry and egress), response to stress, cell cycle, DNA repair/replication, protein-refolding, macro-autophagy, etc (Fig. 37).

3.7.9 PfHDAC1 distribution profile over the gene-body

We investigated the enrichment profile of PfHDAC1 on genes and flanking 1 kb upstream/downstream regulatory region. Strong enrichment covering the entire gene body and the regulatory flanks was observed for a cluster of target genes involved in ribosomal rRNA and transfer tRNA synthesis (since these genes are predominantly polycistronic). For a vast majority of the gene targets however, the enrichment was observed at the gene body predominantly and very little over the regulatory upstream and downstream regulatory regions.

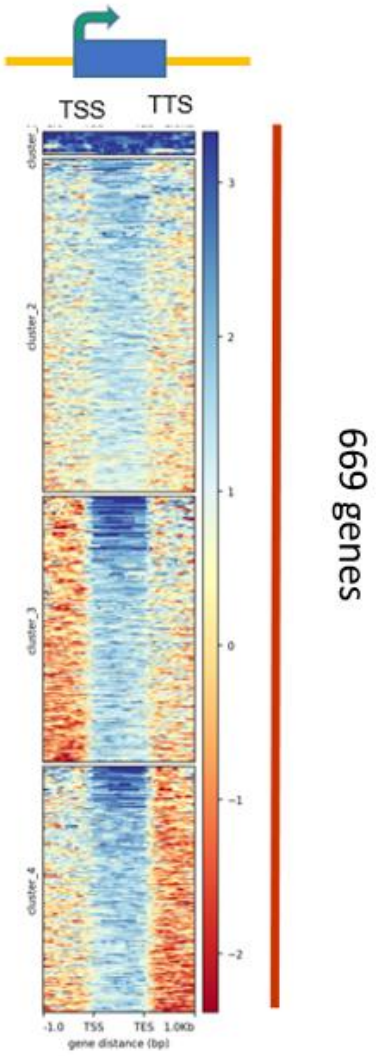


Fig. 36: Heatmap representing the >=1.8fold enriched gene occupancy of PfHDAC1 on the 669 target genes identified as its direct targets.

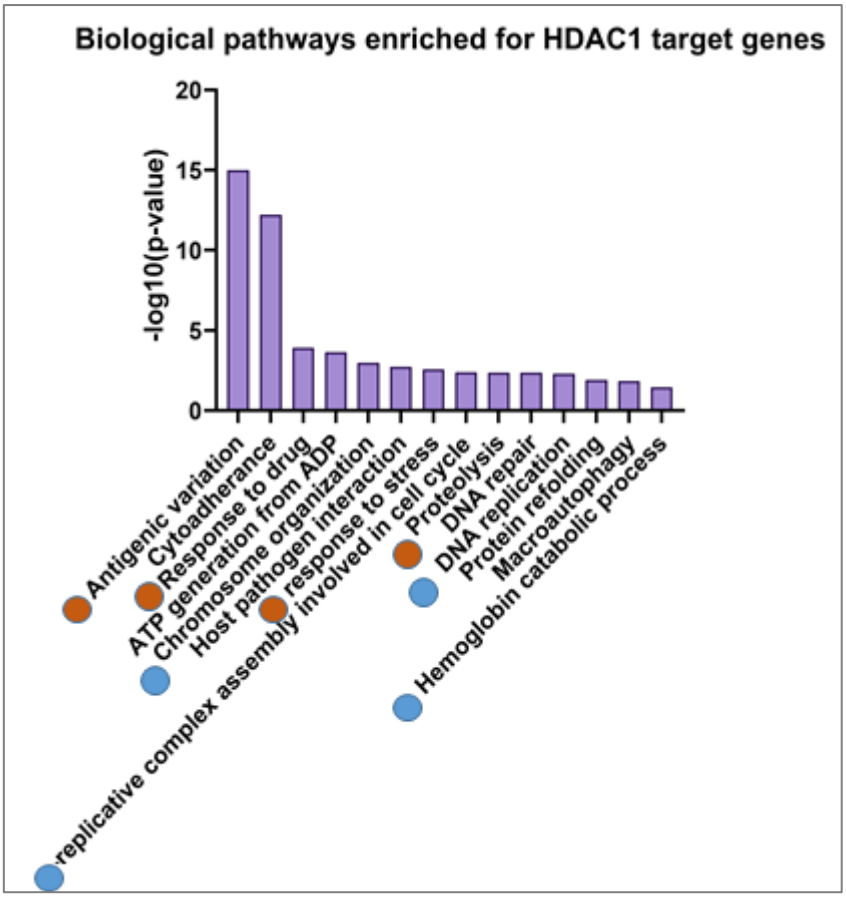


Fig. 37: Bar plot representing the Gene Ontology enrichment analysis for the major biological pathways that are bound by PfHDAC1. Both housekeeping pathways (Blue dots) and stress responsive pathways (Red dots) are associated with PfHDAC1.

3.7.10 Correlation of PfHDAC1 occupancy with gene expression

We next checked the correlation of PfHDAC1 occupancy with the expression level of target genes. Despite being a histone deacetylase, which are classically viewed as transcriptionally repressive in function, PfHDAC1 occupancy was not found to correlate with a strong suppressive effect on gene expression. In fact, PfHDAC1 occupancy was observed on both highly and lowly expressed genes. The mean expression of PfHDAC1 target genes was marginally higher than the mean expression of the entire *P. falciparum* transcriptome. A correlation analysis of the PfHDAC1 enrichment vs gene expression done on genomic bins of

50 bp interval revealed a spearman correlation coefficient R=0.36 (p-value 2.2 e-16), indicating a weak positive correlation (Fig. 38).

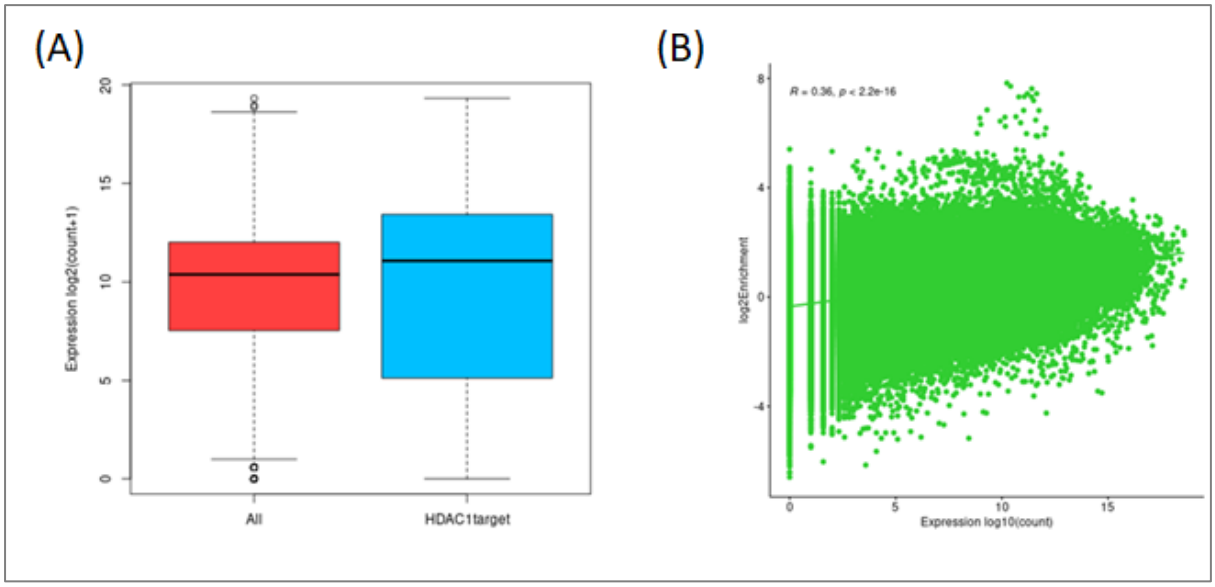


Fig 38: PfHDAC1 occupancy is not universally suppressive to expression of gene targets. (A) Boxplot comparing expression of All genes (Red) vs PfHDAC1 bound genes (Blue). (B) Scatter plot comparing PfHDAC1 enrichment and gene expression across the genome. (Spearman's R=0.36; p-value <2.2e-16).

We compared the enrichment profile of PfHDAC1 with two epigenetic histone modifications H3K9ac and H3K14ac that are associated principally with gene activation [224]. PfHDAC1 enrichment was found to be exclusive of either of the histone modifications. While PfHDAC1 was highly enriched at target gene bodies, H3K9/14ac were found to be depleted. The converse was true for the 5' regulatory region where the activating epigenetic modifications were abundant (Fig. 39). Curiously, the PfHDAC1 presence dropped dramatically in the regulatory region immediately upstream of the TSS and remained low in the UTR while the levels of the activating modification were found to be higher in upstream regulatory regions and peak just prior to TSS. These regions of the genome are known to be crucial for dictating the balance between gene expression vs repression. Correlative analysis of pan genome enrichment of

H3K9ac vs PfHDAC1 yielded a negative Spearman coefficient of correlation, R= -0.36 (Fig. 40).

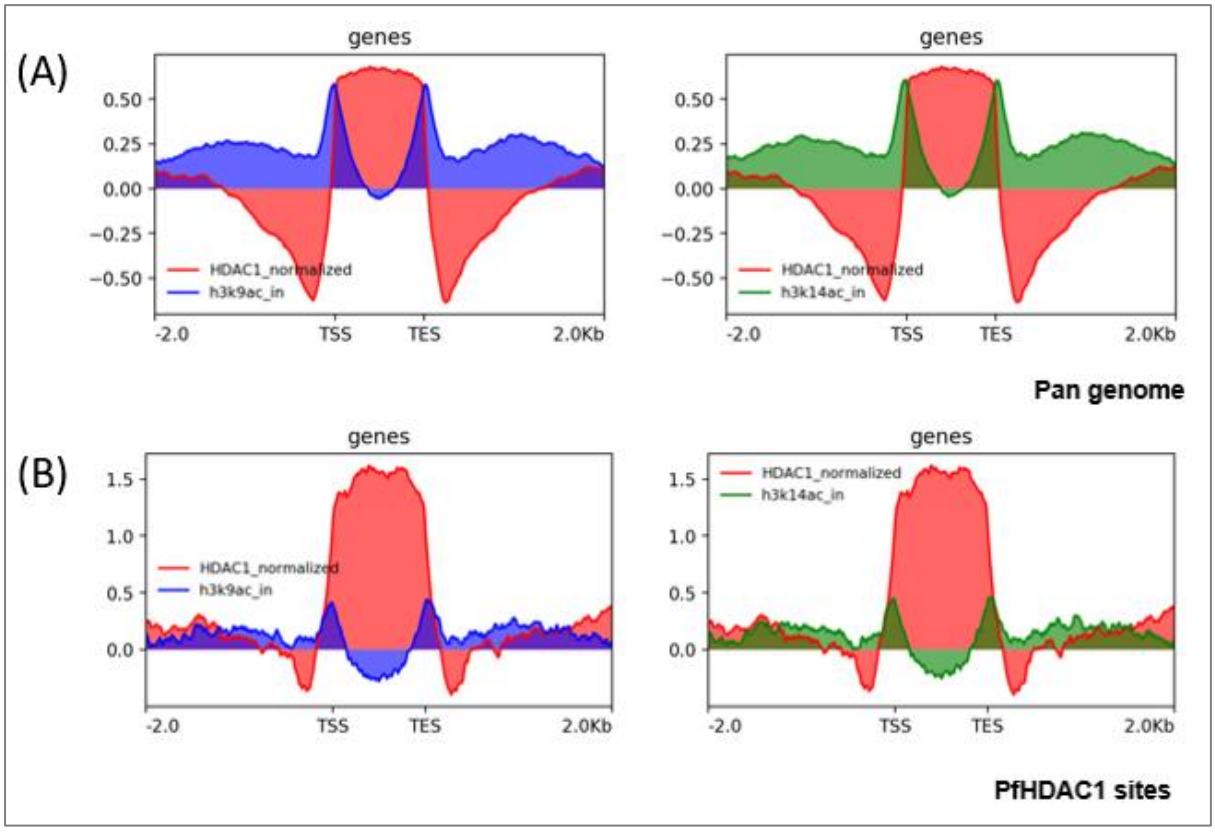


Fig. 39: PfHDAC1 genomic occupancy is exclusive of the H4K9ac and H3K14ac epigenetic marks. Profile plots of PfHDAC1 occupancy (Red) on the average gene body and 500 bp UTR flanks compared with the same for H3K9ac (Blue) and H3K14ac (Green) on pan genome scale (A) and PfHDAC1 occupancy peaks (B). The histone modifications are highly depleted on gene body but prevalent on UTRs, inverse of PfHDAC1.

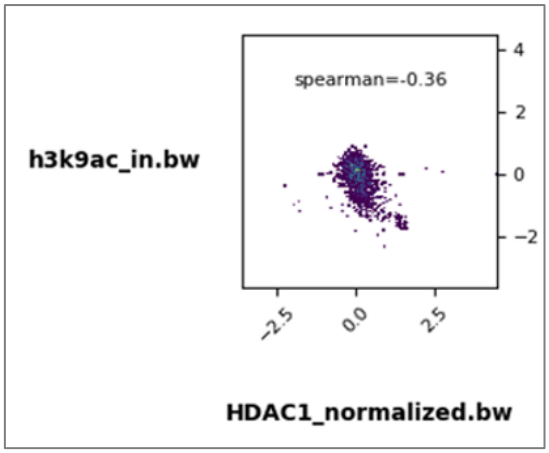
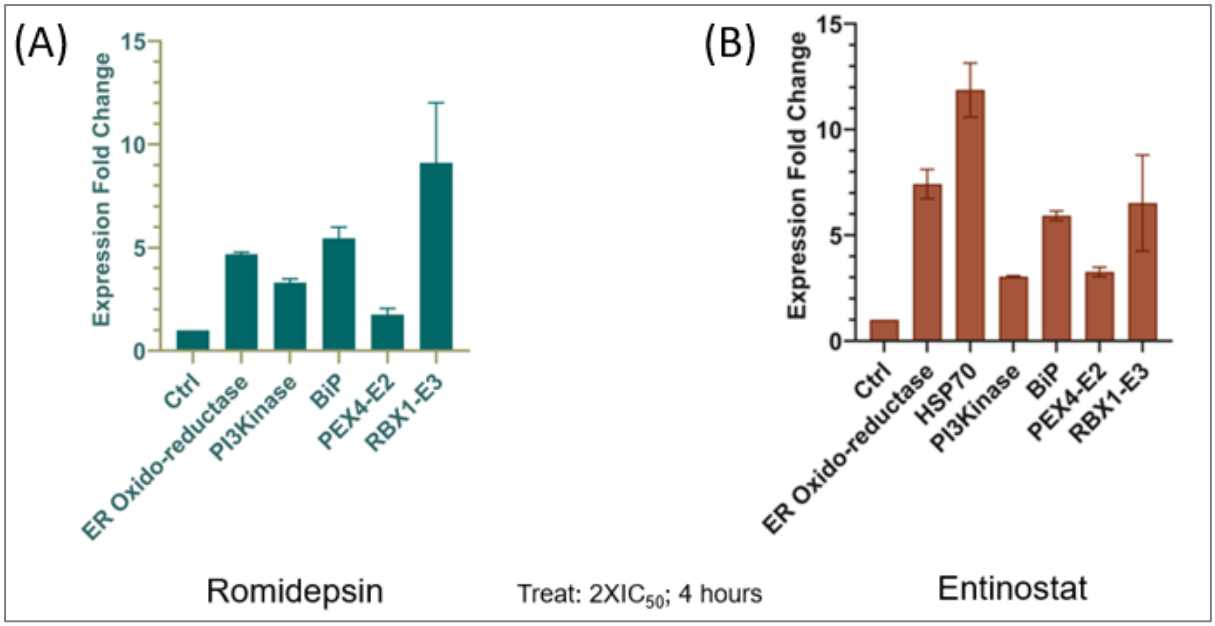


Fig. 40: PfHDAC1 genomic occupancy is negatively correlated with the H3K9ac epigenetic modification. Scatter plot showing negative correlation of the PfHDAC1 and H3K9ac histone modification across the P. falciparum genome.

3.7.11 Validation of PfHDAC1 ChIP targets

For further functional validation of the target genes, we selected a few genes implicated in stress response pathways (protein refolding, protein ubiquitination and redox pathway) and performed RT-qPCR in mock vs HDAC1 inhibitor treated parasites (Primer details in Table 3/Appendix 1). The expression of the chaperones heat shock protein 70 (PfHSP70), PfBiP; the redox response protein ER-oxidoreductase and ubiquitination associated PfPEX-E2 and PfRBX-E3 was found to be highly upregulated upon PfHDAC1 inhibitor treatment (Fig. 41). The upregulation of two of these genes (PfHSP70 and PfBiP) upon PfHDAC1 inhibition was further validated at the protein level through western blotting (Fig. 41).



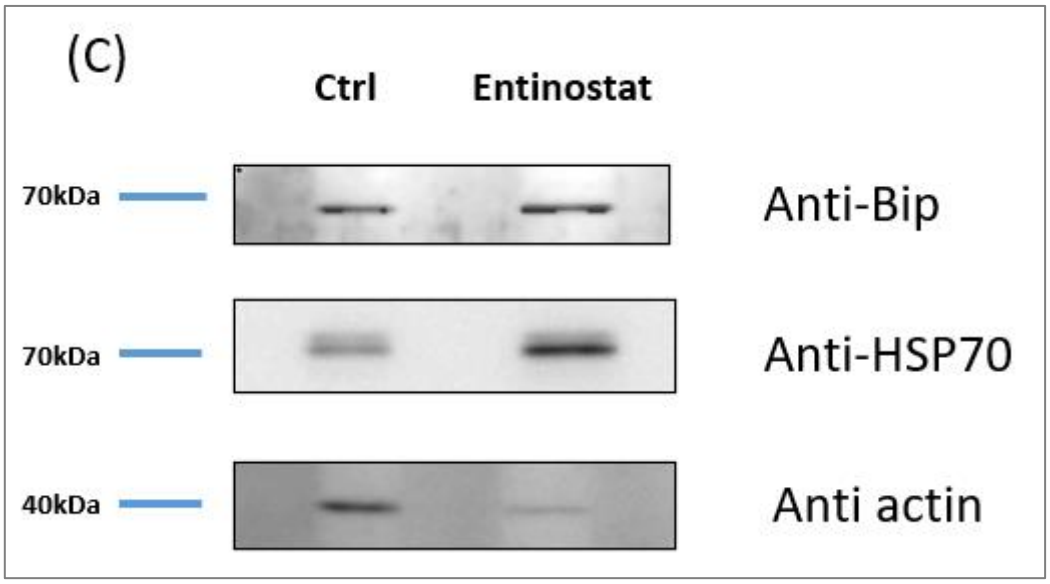


Fig. 41: PfHDAC1 inhibition evokes the expression of stress responsive proteostasis genes. Bar plot showing the expression fold change in (A) entinostat and (B) romidepsin PfHDAC1 inhibitor treated vs mock treated parasites. Increased protein abundance for some of the chaperones upon entinostat treatment in parasites was validated by western blotting (C).

Around 25% of the targets identified for PfHDAC1 were implicated to be associated with antigenic variation and cytoadherence. These include members of the multigene families *var* (encoding PfEMP), *rifin* and *stevor*. Members of these gene families are usually associated

with immune evasion of the parasite in host circulation through mutually exclusive patterns of expression that are designed to present polymorphic targets to the host immune system. As such the basal expression of majority of these genes is null or very low and transcripts for these are hard to detect in native conditions except for a predominantly expressed *var* gene. We performed RT-qPCR for a set of PfEMP1 encoding var genes in mock vs romidepsin/entinostat treated parasites (Primer details in Table 3/Appendix 1). While all the chosen *var* genes were barely detected in mock treated cells we did observe an enhanced expression of many of these genes upon HDAC1 inhibition (Fig. 42). This indicates that PfHDAC1 activity at *var* genes may contribute to their silencing.

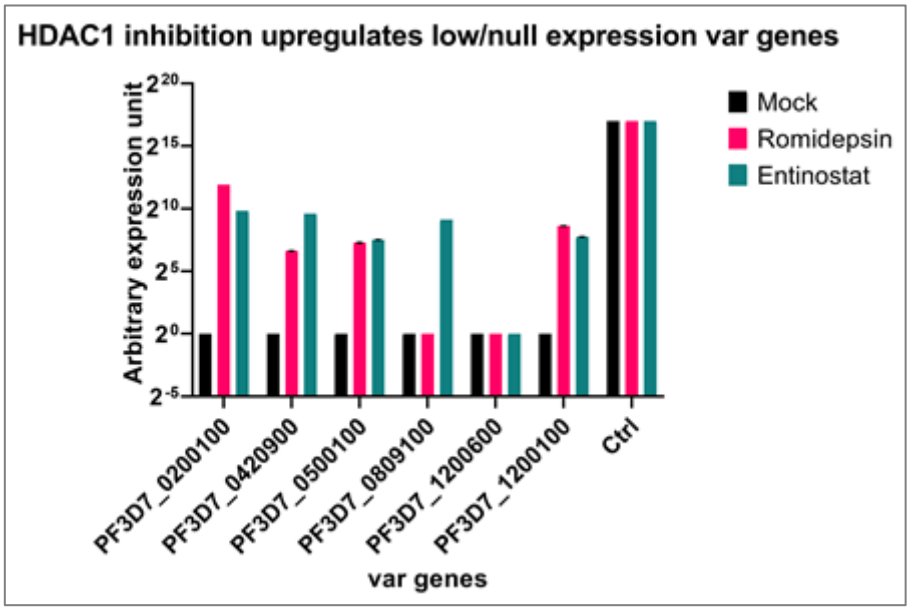


Fig. 42: Pharmacological inhibition of PfHDAC1 deregulated the expression of virulence associated var genes. Bar plot representing the fold change in expression upon mock (black), entinostat (green), and romidepsin treatment (red).

We investigated the epigenetic modification landscape at the virulence gene clusters and observed the enrichment of the heterochromatic mark H3K9me3. PfHDAC1 was also found to be strongly enriched upon the gene cluster. The activating epigenetic mark H3K9ac was

however observed to be significantly depleted across these genes, even in the upstream and downstream regulatory sequences (unlike the non *vir* gene sets) (Fig. 43).

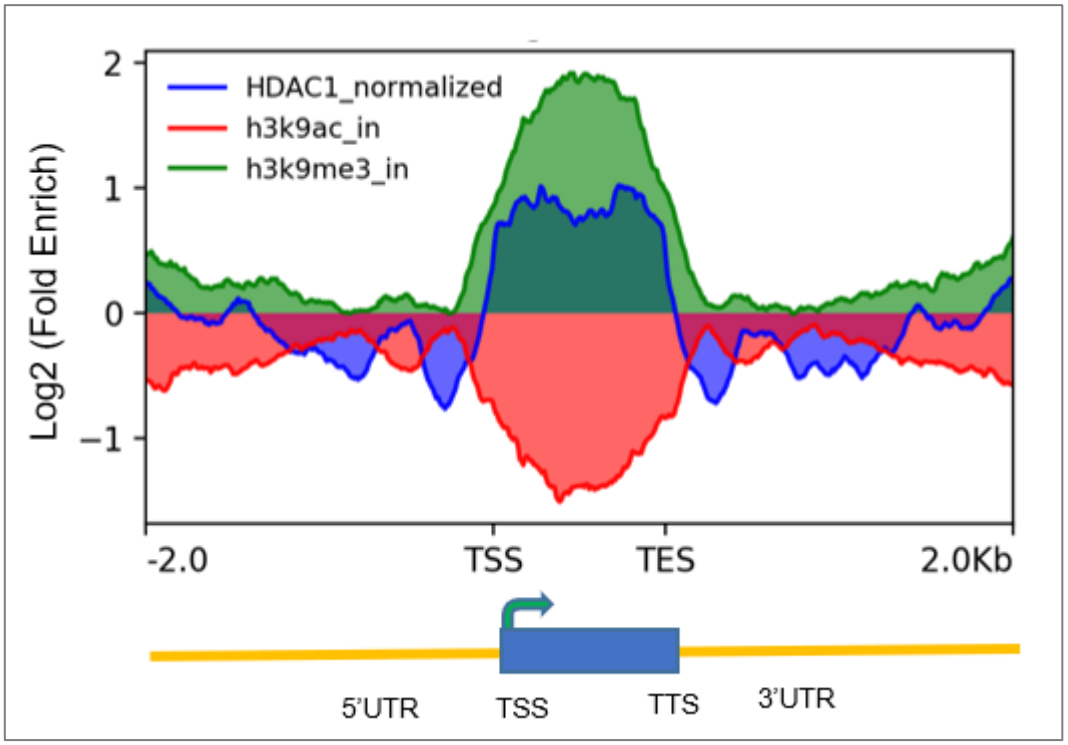


Fig. 43: PfHDAC1 has strong occupancy over heterochromatic virulence genes in P. falciparum. Plot profile representing prevalence of PfHDAC1 (blue), and H3K9me3 (green) and depletion of H3K9ac on virulence associated var loci.

3.7.12 PfHDAC1 as a modulator of parasite intraerythrocytic developmental cycle

Our initial assessment of biological pathways enriched for PfHDAC1 targets showed prevalence of cell cycle and DNA replication associated genes. Class I HDACs have been well characterised for their role in cell cycle regulation in a variety of biological systems. HDAC1 is known for regulating expression of cyclins and cyclin-dependent kinases and forms an integral factor governing cell cycle checkpoint dynamics [158]. HDAC inhibitors have also been characterised for their anti-proliferative functions and effects on slowing down/arrest of cell cycle. Furthermore, cancerous cells report overexpression of HDAC1 associated with drug

therapy resistance and survival [225]. These reports clearly establish a significant role of HDAC1 in cell cycle regulation. We were thus curious to investigate whether the same holds true for PfHDAC1.

We performed an experiment involving parallel mock vs romidepsin-treated parasite culture (starting with a highly synchronised population of cells) and followed the intraerythrocytic cell cycle progression via microscopic examination at 8-hour intervals of the intraerythrocytic development cycle (starting at 6-hour post invasion mark) (Fig. 44). Since the cell cycle progression in *P. falciparum* is also linked with progressive DNA replication in the process of erythrocytic schizogony, we estimated the DNA content changes through SYBR fluorescence readout at each of these timepoints.

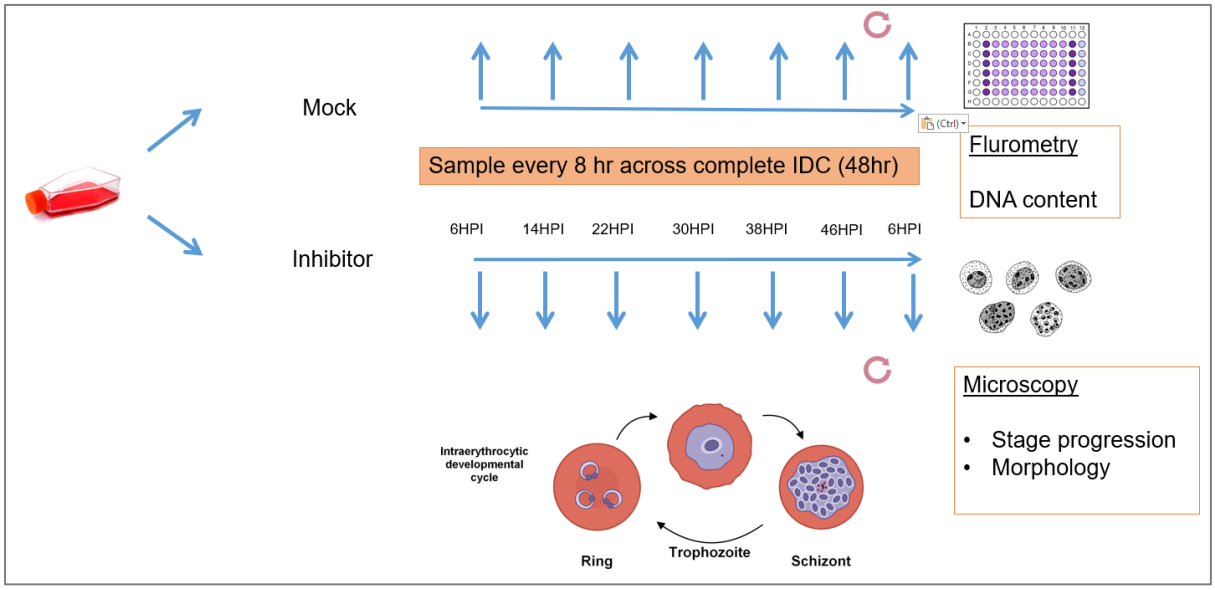


Fig. 44: Schematic of the intraerythrocytic developmental cycle follow-up for investigating the effects of PfHDAC1 inhibitor treatment on parasites.

We counted for the individual stages (ring/trophozoite/schizont) present at each timepoint and estimated the stage progression (or inhibition) of parasites over time (Fig. 45 and 46). At 22 hours post invasion the mock treated parasites had almost completely transitioned from ring to

the trophozoite stage whereas a small proportion of the inhibitor treated cells was still found to be lagging in the ring stage or not having fully transitioned into trophozoite morphology. The trophozoite development was closely monitored in mock treated culture and it showed gradual enlargement of the nuclear volume associated with parasite DNA replication along with maturation of the trophozoite (noted at 30 HPI). In the inhibitor treated culture a vast majority of trophozoites showed stunted maturation and only a few of those went on to develop into full sized trophozoites (as referenced in mock treated culture). This hinted at a strong inhibition of morphological development associated with stunted DNA replication in PfHDAC1 inhibited parasites. Furthermore, segmented schizonts were observed at the 46 hours post invasion time point in mock treated parasites along with just egressing parasites. On the other hand, the romidepsin treated culture showed a large proportion of immature schizonts with delayed segmentation. While a significant proportion of the mock treated cells had started to undergo egress and reinvasion by 54 hours post invasion/6 hour post invasion of cycle 2, the romidepsin treated cells showed a comparatively lesser cells with parasite egress and reinvasion into fresh RBCs. Schizonts persisted in romidepsin treated culture at least 6-8 hours after the second invasion cycle had already begun in the mock treatment culture, resulting in a delayed second wave of infection.

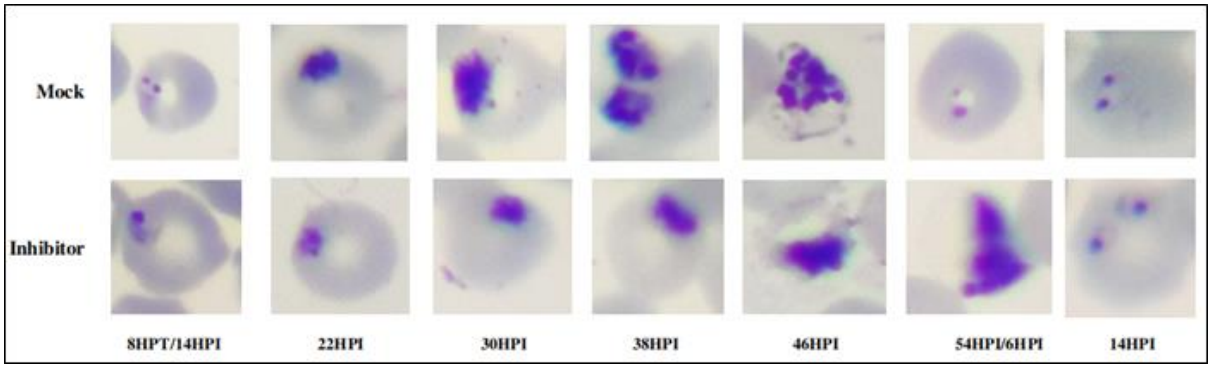


Fig. 45: PfHDAC1 inhibition causes morphological defects visible in development of trophozoites, maturation and segmentation of schizonts and timely reinvasion of merozoites. Microscopy images of Giemsa smeared parasites transitioning normally and aberrantly through the 48 hr IDC in Mock vs Romidepsin treated culture.

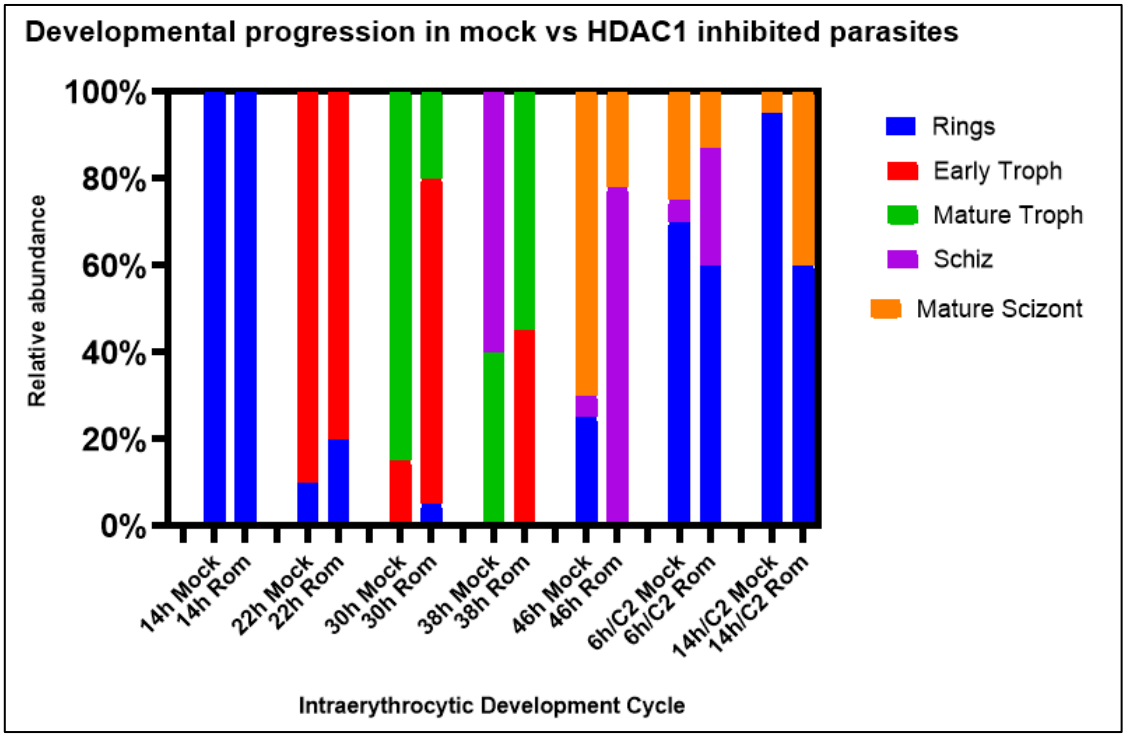


Fig 46: PfHDAC1 inhibition causes a delayed progression of parasite cell cycle. Compared to mock treated parasites, inhibitor treated parasites show delayed stage-wise progression through the IDC.

Fluorometry readings for the DNA content of the mock vs romidepsin treated parasites revealed a dramatic drop in the DNA content of romidepsin treated parasites relative to the mock within 16 hours of treatment. This could be accounted for by the normal morphological development (and DNA replication) in mock treated parasites which progress normally into the trophozoite stage while the romidepsin treated parasites tend to lag partly in their transition from "DNA light" ring stage into "DNA heavy" trophozoites and fail to properly mature into full sized trophozoites (Fig. 47). The romidepsin/mock normalised DNA signal then gradually rises as the parasites transition into trophozoites and finally settles to a relative value of 75% around 38 hours post invasion. This 25% deficit of DNA fluorescence in romidepsin treated parasites reflects the loss of DNA replication observed upon PfHDAC1 inhibition.

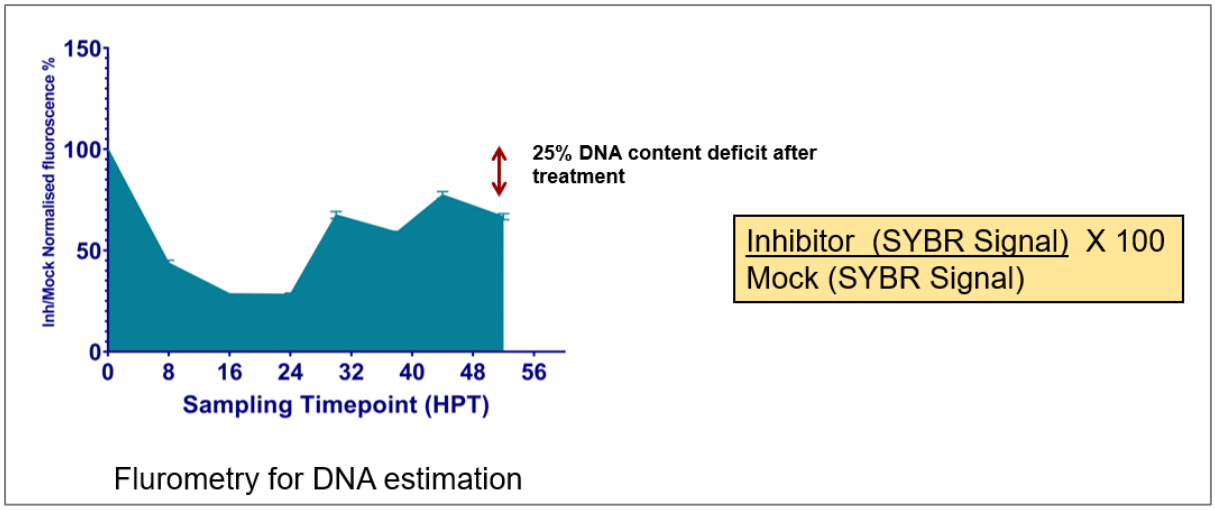


Fig. 47: PfHDAC1 inhibition results in defective DNA replication in parasites. A filled line plot of relative DNA content observed in romidepsin vs mock treated parasites as measured by fluorometry.

We checked the parasitemia in the mock vs romidepsin treated culture into the second intraerythrocytic developmental cycle and estimated that while the parasitemia during the second cycle in mock treated culture was at 11%, it was recorded to drop in the romidepsin treated culture at 7%. Thus, PfHDAC1 inhibition was shown to lead to a delayed cell cycle

progression compounded with defects in proper morphological development of parasites and reduction in proliferation over to the next cycle of the intraerythrocytic development (Fig. 48). Thus, PfHDAC1 presents as a suitable target for suppressing parasite growth and proliferation through pharmacological targeting.

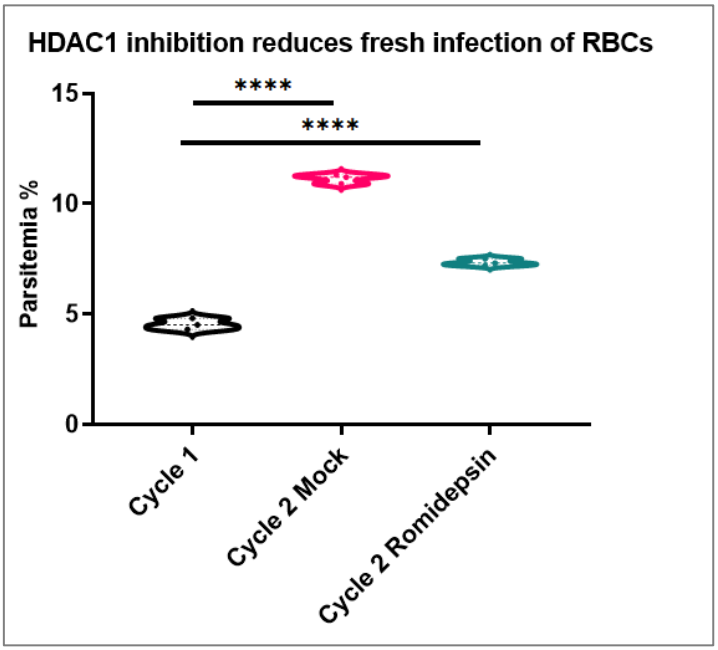


Fig. 48: PfHDAC1 inhibition results in substantially lower reinvasion to the next cycle of IDC. Violin plot showing a 2.5-fold increase in parasitemia for cycle 2 in mock treated culture, compared to the <2-fold increase observed for romidepsin treated culture.

3.7.13 PfHDAC1 regulates the expression of cell cycle and DNA replication associated genes

PfHDAC1 was found to be enriched upon numerous cell cycle and DNA replication associated genes. We investigated the expression of these gene targets under conditions of PfHDAC1 knockdown using data from Huang et. al. Majority of the cell cycle (including anaphase promoting complex subunit 11, calcium dependent protein kinase 4, cyclin dependent kinase regulatory subunit) and DNA replication associated genes (including origin recognition

complex subunit 2 and two conserved *Plasmodium* proteins) were found to be downregulated under conditions of PfHDAC1 knockdown further proving that PfHDAC1 is a strong regulator of cell cycle and DNA replication in the parasite (Fig. 49 and 50) [207].

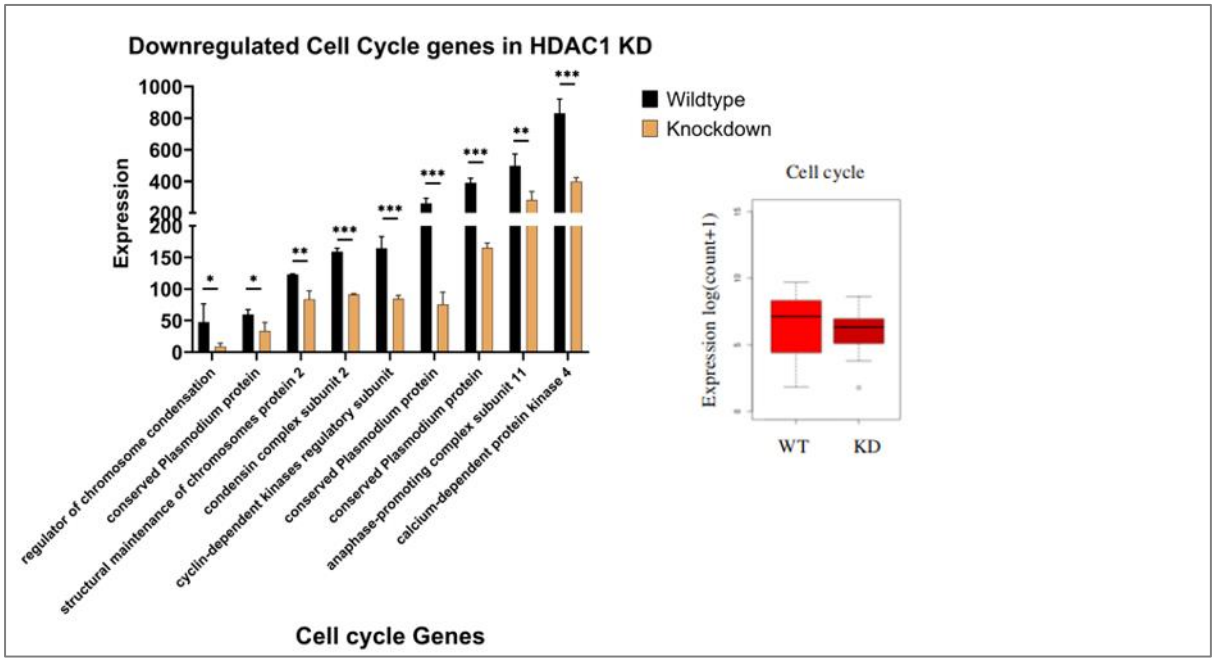


Fig. 49: PfHDAC1 depletion is associated with downregulation of key cell cycle associated genes in parasites. Inset: Boxplot representing the average expression of cell cycle associated genes in PfHDAC1 wildtype vs knockdown line. Source: Analysed from Huang et. al. 2020; Cell Discovery [207].

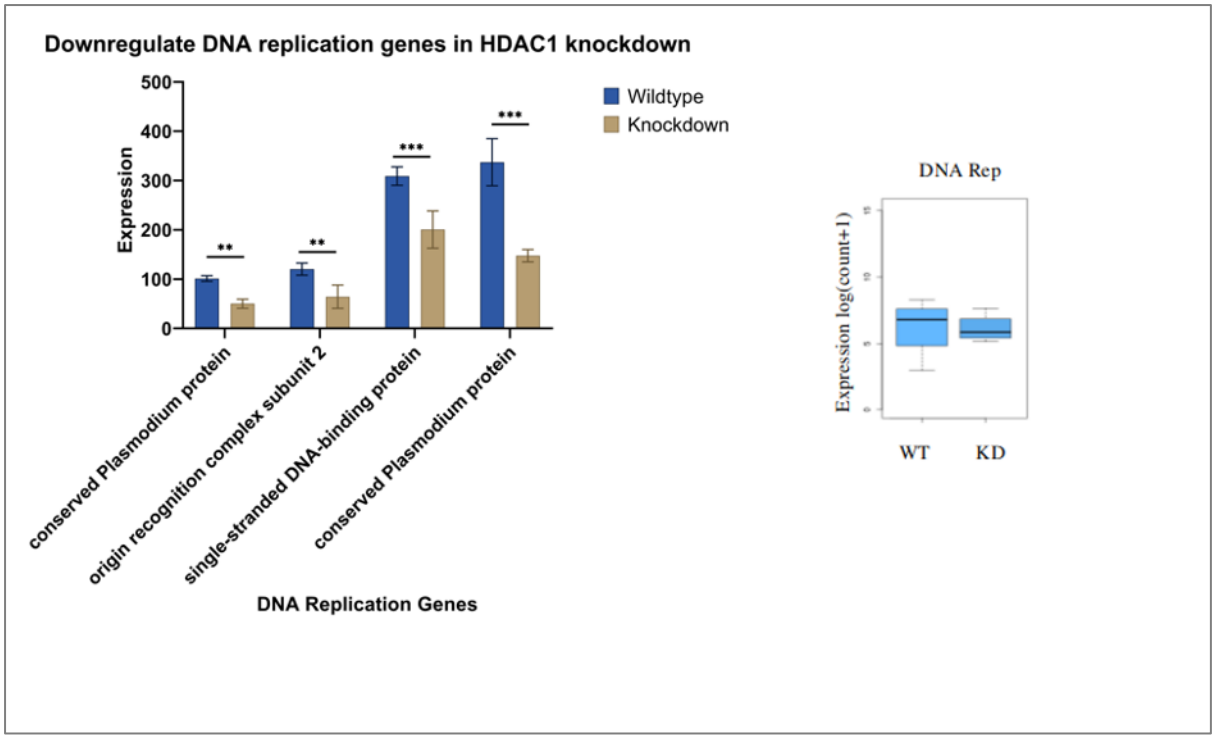


Fig. 50: PfHDAC1 depletion is associated with downregulation of key DNA replication associated genes in parasites. Inset: Boxplot representing the average expression of DNA replication associated genes in PfHDAC1 wildtype vs knockdown line. Source: Analysed from Huang et. al. 2020; Cell Discovery [207].

3.7.14 Overexpression of PfHDAC1 reversibly enhances cell proliferation in *P. falciparum*

The overexpression of HDAC1 is reported to be associated with hyperproliferative disorders like cancers in humans. It has been directly implicated in the enhanced cellular proliferation, and cell survival under drug pressure and to contribute to chemotherapy resistance. Since we had already observed an inhibitory effect of PfHDAC1 inhibition upon cell cycle progression and reinvasion in *P. falciparum* we decided to see if the overexpression of PfHDAC1 would have converse effects on the growth and proliferation of the parasites. For this purpose, we utilised a strategy to episomally overexpress a GFP tagged version of PfHDAC1 using the pDC2 overexpression plasmid transfected into *P. falciparum* (Fig. 51). To grant a degree of

regulatability to the design we introduced a glmS ribozyme sequence downstream of the PfHDAC1-GFP gene block resulting in a PfHDAC1-GFP-glmS overexpression construct that could be regulated at the post-transcriptional level by supplementing different dosages of glucosamine ligand to the culture media resulting in overexpressed to basal PfHDAC1 presence in the parasite [226]. A GFP-glmS control line was also generated to account for glucosamine toxicity.

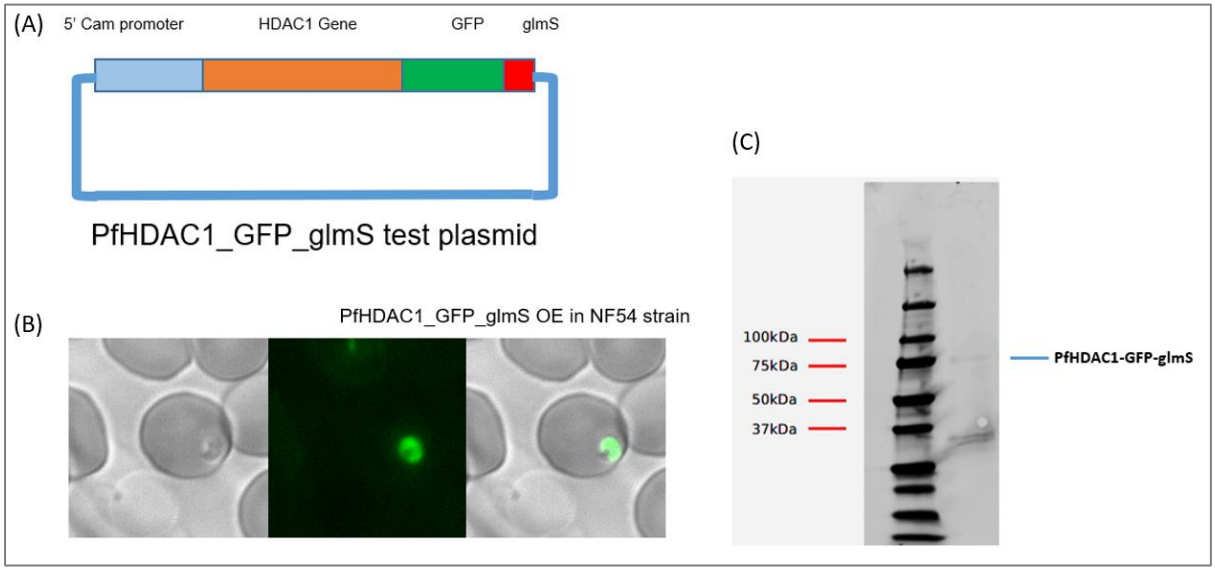


Fig. 51: Establishment of PfHDAC1 overexpression P. falciparum line. (A) The plasmid construct used for in-frame expression of PfHDAC1, GFP and glmS. (B) Confocal microscopy images validating the expression of GFP tagged PfHDAC1. (C) Western blot-based validation of the fusion product PfHDAC1-GFP-glmS expression in transgenic lines (probed with α-GFP)

3.7.15 Growth curve analysis to assess the effect of PfHDAC1 overexpression on parasite growth dynamics

We seeded the test (PfHDAC1-GFP-glmS) and control (GFP-glmS) parasite culture at 2% starting parasitemia and 2% hematocrit and followed their progress of infection over a 3 cycle/6-day duration. The growth was recorded as a fold change in parasitemia relative to the

starting Day 0 parasitemia of 1%. The PfHDAC1 overexpression line was observed to proliferate at a faster rate than the GFP control line. The increase in parasitemia was observable even after cycle 1/day 2 and was especially noticeable on day 3. We suspect a mild shift in the invasion time point of both the parasite lines (observed as a delay in their second cycle invasion). Since samples were taken once every 24 hour this accounts for observation of a significant growth difference associated with PfHDAC1 overexpression on day 3 rather than day 2. The proliferation promoting effect of PfHDAC1 overexpression relative to the control was observed to be mildly enhanced at the end of cycle 2 with around 2-fold higher change in parasitemia for the PfHDAC1 overexpression line and furthermore at the end of cycle 3 with a 3-fold higher change in parasitemia. Thus, the overexpression of PfHDAC1 was found to be associated with an enhanced cellular proliferation in the parasites which compounded at the end of each cycle (Fig. 52).

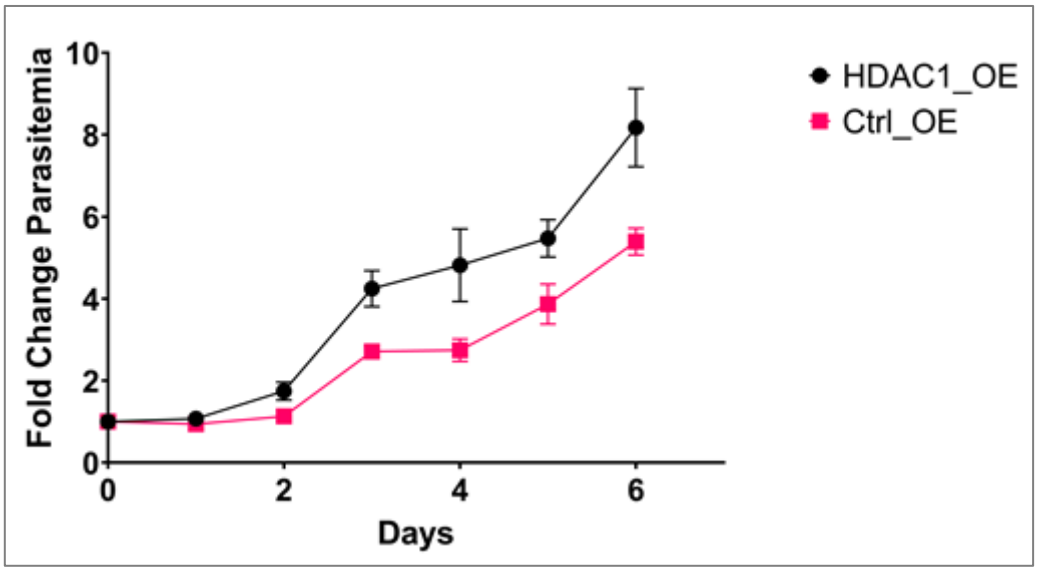


Fig. 52: PfHDAC1 overexpression is associated with enhanced proliferation in parasites. Line plot representing growth trend of PfHDAC1-GFP-glmS overexpression line (Black) vs the control GFP-glmS overexpression line (Red) over a 6-day growth assay.

To test the reversibility of the overexpression system we optimized the glucosamine dosage required to repress the episomal overexpression of PfHDAC1. Glucosamine inducible autocatalytic destruction of the glmS ribozyme tagged transcripts while simple in implementation faces one challenge namely the basal toxicity of glucosamine to the parasites. We tested two doses of glucosamine (2.5mM and 5mM) which were found to partially and almost completely deplete the overexpressed PfHDAC1 transcripts respectively (Fig. 53).

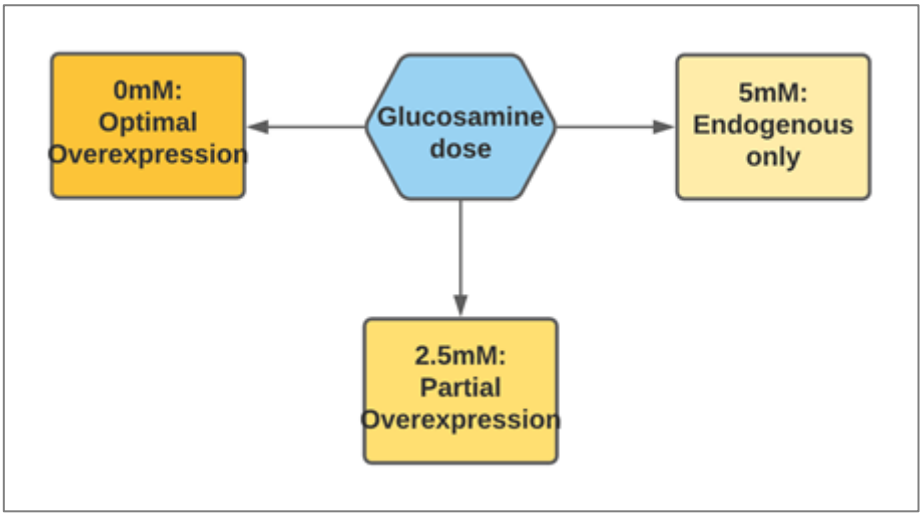


Fig. 53: A glucosamine dose dependent regulation of the episomally overexpressed PfHDAC1-GFP-glmS construct.

We checked the viability of parasite lines under glucosamine treatment with 2.5mM and 5mM glucosamine added to the PfHDAC1-GFP-glmS overexpression line and the GFP-glmS overexpression line. Glucosamine treatment at 2.5mM concentration resulted in 17% parasite death in the GFP-glmS line (relative to the non-treated GFP-glmS line) which was the basal toxicity of glucosamine on the parasites. The same dosage on the PfHDAC1-GFP-glmS line resulted in a ~30% cell death relative to the non-treated PfHDAC1-GFP-glmS line (Fig. 54). Therefore, an additional 13% decline in parasite viability was attributed to the partial depletion of episomal PfHDAC1. 5mM glucosamine treatment was associated with a basal toxicity of

21% on the GFP-glmS line while an additional 23% decline in viability was attributed to PfHDAC1 depletion in the PfHDAC1-GFP-glmS line. These figures were recorded at 48hr post treatment.

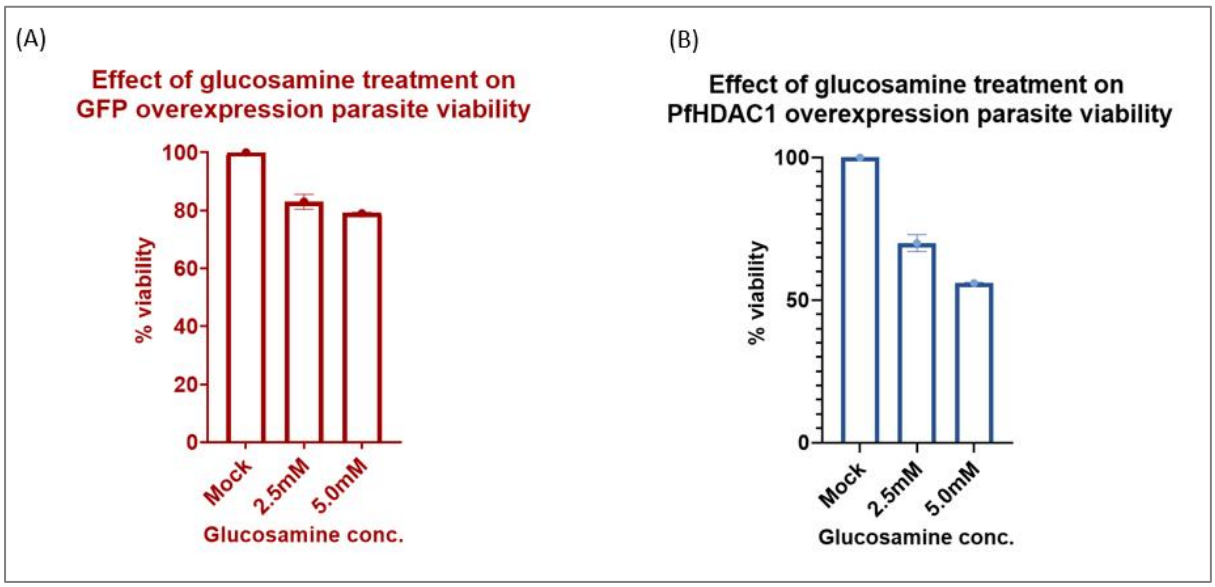


Fig. 54: Glucosamine treatment to regulate PfHDAC1 overexpression is associated with some basal toxicity. (A) Glucosamine treatment at 2.5mM concentration results in 17% drop in parasitemia in the GFP-glmS ctrl overexpression line whereas treatment at 5mM conc. results in 21% drop in parasitemia in the GFP-glmS ctrl overexpression line on day 2 of the growth assay. (B) Treatment at 2.5mM conc. causes 30% loss of viability while at 5 mM causes 44% loss in PfHDAC1 overexpression lines on day 2 of growth assay.

A complete curve for a 6-day growth assay with glucosamine treatments was prepared. We plotted the GFP-glmS subtracted progression in parasitemia of the PfHDAC1-GFP-glmS line and found that 2.5mM and 5.0mM glucosamine treatment were associated with drop in parasite viability of the PfHDAC1-GFP-glmS attributable to the progressive reduction of overexpressed PfHDAC1 back to basal levels (Fig. 55).

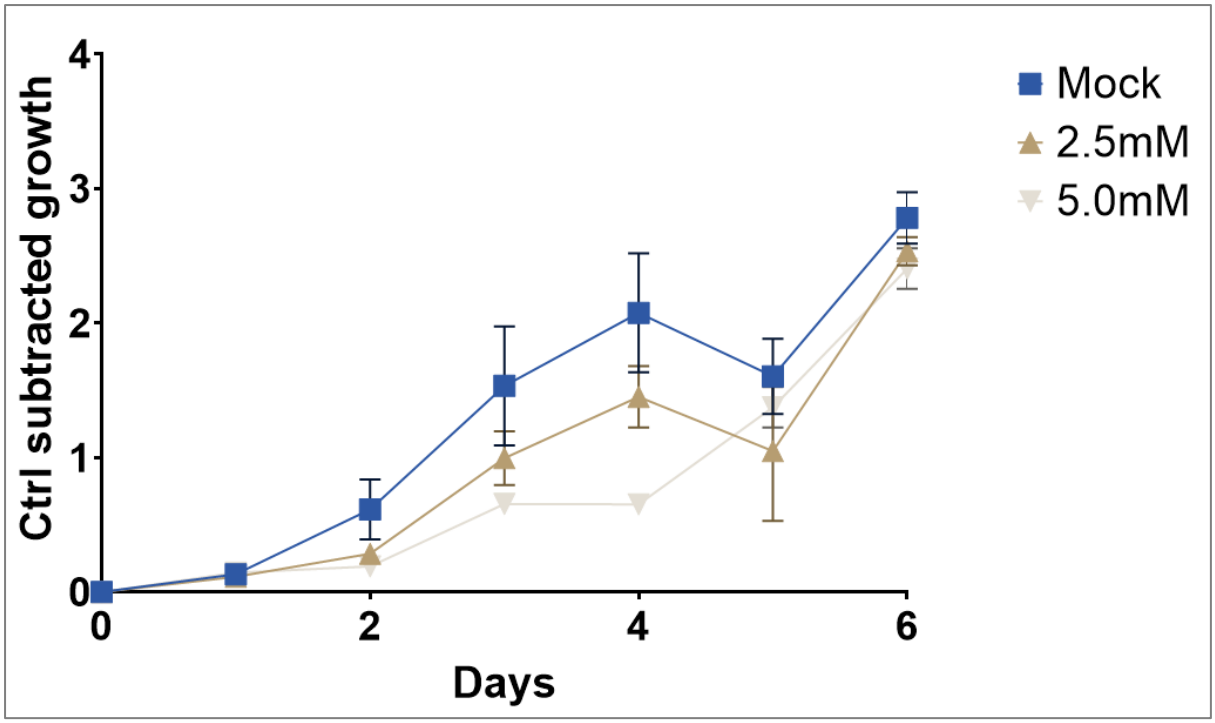


Fig. 55: Suppression of PfHDAC1 overexpression reverts the growth advantage offered by it. Line plot showing the GFP-glmS growth subtracted plot for PfHDAC1-GFP-glmS over 6-day growth curve.

3.8 Discussion

PfHDAC1 is a solitary Class I histone deacetylase that has been identified as a potent target for a number of antimalarial compounds [206]. Early data implicates its role in cell survival and establishes its role in regulating a number of biological processes in cells. We expressed recombinant PfHDAC1 protein in the *E. coli* bacterial expression system and used it to generate antiserum in rabbits for further characterisation. We observed poor *in vitro* enzymatic activity with recombinant PfHDAC1 and through literature survey raised that post translational modifications may be necessary for regulating its enzymatic activity. Recombinant PfCKII-α was found to interact with PfHDAC1 in *in vitro* settings. We identified this interaction could impart a phospho-PTM on PfHDAC1 as well as carry out a self-phosphorylation reaction as well. It is possible that the autocatalysis may help fine tune the activity of PfCKII-α just like it

does for PfHDAC1. The kinase activity of PfCKII-α could be inhibited using specific inhibitor TBB. Whether the interaction of the two protein allows for a deacetylation of PfCKII-α was not investigated by us, although acetyl modifications of PfCKII-α have been reported. So, a crosstalk of the kinase-deacetylase arc offers complex regulatory points in cell communications. We further observed that the phosphorylated version (and not non-phosphorylated) of PfHDAC1 could deacetylate H3K9ac modifications from histones. Thus, activity was shown to be inhibited by Class specific HDAC inhibitor entinostat, highlighting its utility in epigenetic mechanism control. The serine-phosphorylation of PfHDAC1 has already been identified in mass spec studies and the associated kinases are poorly understood, thus we show PfCKII-α as a potential kinase regulating PfHDAC1 cellular activity [219].

Our ChIP sequencing data reveals the control of PfHDAC1 over a slew of critical basic biological functions in cells like host-cell invasion/egress, protein synthesis and homeostasis, energy metabolism, chromosome organisation, cell cycle and DNA replication. This may be the basis of the essential nature of PfHDAC1 for parasite survival and death upon knockout [204]. We furthermore observe occupancy of PfHDAC1 on both genes with high and low levels of expression. Thus, our data suggests that PfHDAC1 is not universally suppressive of gene function. This observation is further supported by data published by Huang et. al. shows that depletion of PfHDAC1 causes both upregulation and downregulation of roughly equal numbers of genes [207]. PfHDAC1 may be involved in maintaining a low basal expression of its target genes. HATs and HDACs could participate in cyclic setting/resetting of histone modifications supporting basal transcriptional activity in coordination with other factors [227]. Abundance of PfHDAC1 on the gene body may restrict the spread of activating histone modifications and keep transcription at bay. The balance of levels of PfHDAC1 and H3K9ac imparting HATs (vi. PfGCN5) at the regulatory regions (esp. immediately upstream of TSS) may dictate the subtle balance between a repressed vs activated chromatin. While the presence of HDAC1 may be

only mildly permissive to transcription, its eviction may allow for transcription promoting machinery to establish/spread across the gene body and enhance gene expression.

Our ChIP data also hints at the strong role of HDAC1 in regulating stress responses and antigenic variation gene expression in the parasite. This establishes the role of PfHDAC1 in governing responses to both biotic as well as abiotic stresses in the parasite. Protein refolding and ubiquitination are two important mechanisms of proteostasis in biological systems [228]. A coordinated function of the two can protect cells from harmful stresses that directly target the proteome. Furthermore, the observation that PfHDAC1 inhibition can evoke the expression of 'silent' *var* genes raises exciting possibilities for its role in contributing to the mutually exclusive expression of *var* genes. Indeed, its enrichment upon a majority of *var* genes may keep them silenced while a highly coordinated removal from a select few genes may allow only those to be expressed at a time. Of note is the differential epigenetic landscape between the vir and non vir gene sets where the H3K9ac dynamics driven by PfHDAC1 and PfGCN5 may be unique. H3K9ac is an important driver of *var* gene activation, and its enrichment profile seems strongly dependent on PfHDAC1. This opens the venues for investigating PfHDAC1's role in driving antigenic variation and associated processes.

We observe a strong effect of inhibition of PfHDAC1 on the cell cycle progression, morphological maturation as well as DNA replication in the parasite. Analysis of data published by Huang et. al. shows the deregulation in the expression of cell cycle and DNA replication associated genes that could explain the aberrant development of the parasites in the cells. Owing to the significant number of these regulators like cyclin dependent kinase regulatory subunit, anaphase promoting complex 11, and calcium dependent protein kinase 4, it is possible that the inhibition of PfHDAC1 and consequently all these crucial regulators causes a shutdown in the cell thereby explaining the strong potency of compounds that target

PfHDAC1 on the parasites. Increase in ploidy upon DNA replication characteristic of the developmental progression through the IDC. With PfHDAC1 depletion the morphological defect observed in the Giemsa-stained cells and SYBR-DNA fluorometry data clearly reflects a depleted DNA abundance in PfHDAC1 inhibited cells. The effects of this defective DNA replication could also resonate onto the next cycle as it would hamper the number of maturing merozoites within the segmenting schizonts resulting in lower overall numbers of invading merozoites for the subsequent cycle. Our gene target data for PfHDAC1 also suggests that it may regulate numerous genes that regulate host-pathogen interactions (namely RBC egress and reinvasion). This may be another contributing factor to a lower overall egress from and reinvasion into RBCs resulting in a reduction in infection. While PfHDAC1 inhibition is associated with growth defects and decreased infection over subsequent cell cycle, the overexpression of PfHDAC1 was found to be associated with an enhanced proliferation of parasites. Our data implicates roles of PfHDAC1 in DNA replication and an overexpression of the protein may enhance the process resulting in higher DNA content in trophozoites which gets segmented into more merozoites during and after schizogony. This would result in enhanced infection rate over successive cycles and the observed growth promotion in case of the PfHDAC1-GFP-glmS line.

CHAPTER 4: Investigating the potential regulation of artemisinin sensitivity phenotype by PfHDAC1

4.1 Cell cycle dynamics of PfHDAC1 inhibited parasites mimic that in artemisinin resistant parasites

Inhibition of PfHDAC1 by Class specific inhibitors showed a skew of cell cycle development in the parasite. We observed a small fraction of inhibitor treated parasites to have longer persistence in the ring stage. The development of trophozoite which is the metabolically active stage of the parasite was observed to be strongly stunted, hinted at lower DNA replication, and impeded development. The appearance of schizonts in these parasites was also observed to be delayed. These findings match certain phenotypic characteristics of artemisinin drug resistant parasites. Artemisinin resistant parasites are reported to have prolonged ring stage development and a stunted trophozoite phase, phenotype which purported to have benefits for parasite survival under artemisinin drug stress (Fig. 56) [229]. Ring stage of parasites is associated with lower transcriptional and metabolic activity. Metabolic activity, especially intake of hemoglobin and its digestion in the food vacuole, is responsible for release of free iron in cells that directly cleaves the endoperoxide bridge in artemisinin and causes it to undergo a free radical cascade [230]. Hence it is advantageous for the parasites to lengthen the metabolically inactive ring stage and suppress trophozoite development to minimise drug activation. We were thus curious to further investigate whether PfHDAC1 could have a role to play in determining drug sensitivity of the parasites to artemisinin.

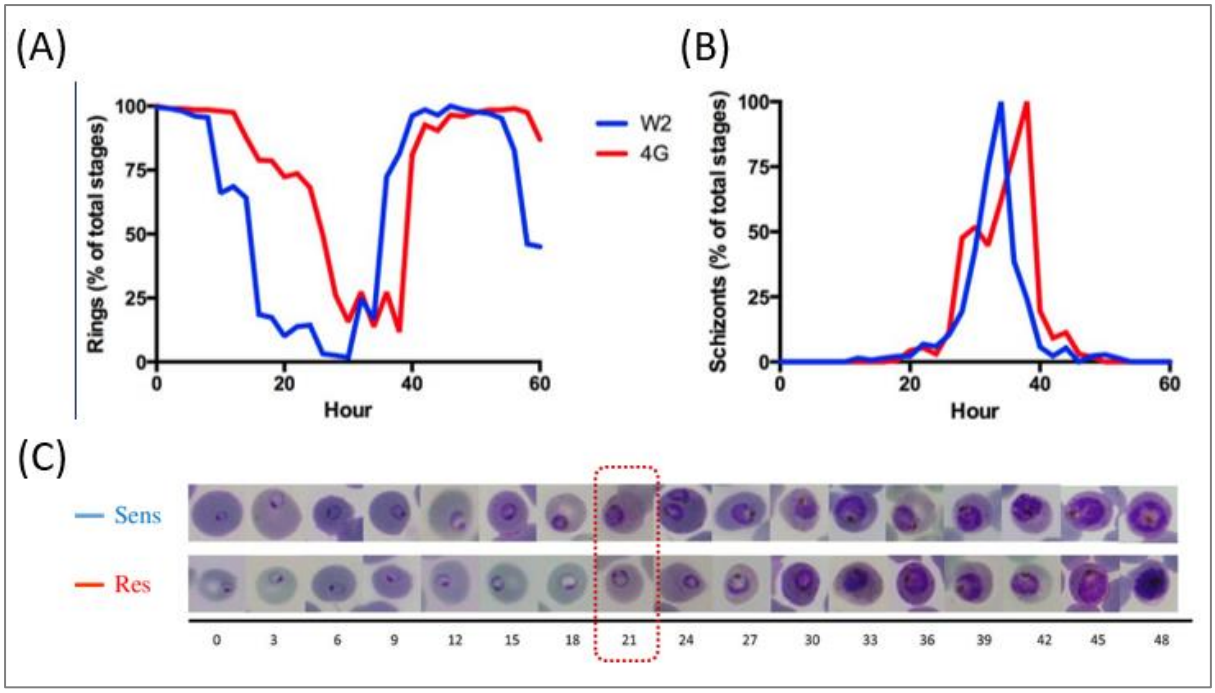


Fig. 56: The cell cycle of artemisinin resistant P. falciparum is skewed with delayed transition to trophozoite stage and prolonger persistence of schizonts. Line plots showing longer prevalence of ring stage (A) parasite in resistant (Red, 4G) vs sensitive (Blue, W2) strains and a delayed appearance of schizonts as well (B). A thin smear profile of the data across IDC is presented in (C). Source: Hott et. al., 2015; Antimicrobial Agents and Chemotherapy [229].

4.2 Drug resistance in *P. falciparum*

Despite the plethora of drugs available against *Plasmodium* it has been extremely hard to eliminate or even control the parasite owing to its extremely complicated and little understood biology (Fig. 57) [231]. The parasite is extremely adept at evolving rapidly and displays adaptability to strenuous environments [232]. Antimalarial drug resistance is characterised by survival and proliferation of the parasite despite administration of recommended or higher dosage of therapeutic agent. Antimalarial drug resistance has gradually rendered treatment with once common drugs increasingly futile. Chloroquine which was once hailed as the gold

standard of medication in the field is now no longer effective against *falciparum* malaria [233]. Other prominent drugs like Mefloquine, Sulphadoxine and Pyrimethamine have also gradually been reported to be increasingly ineffective [234].

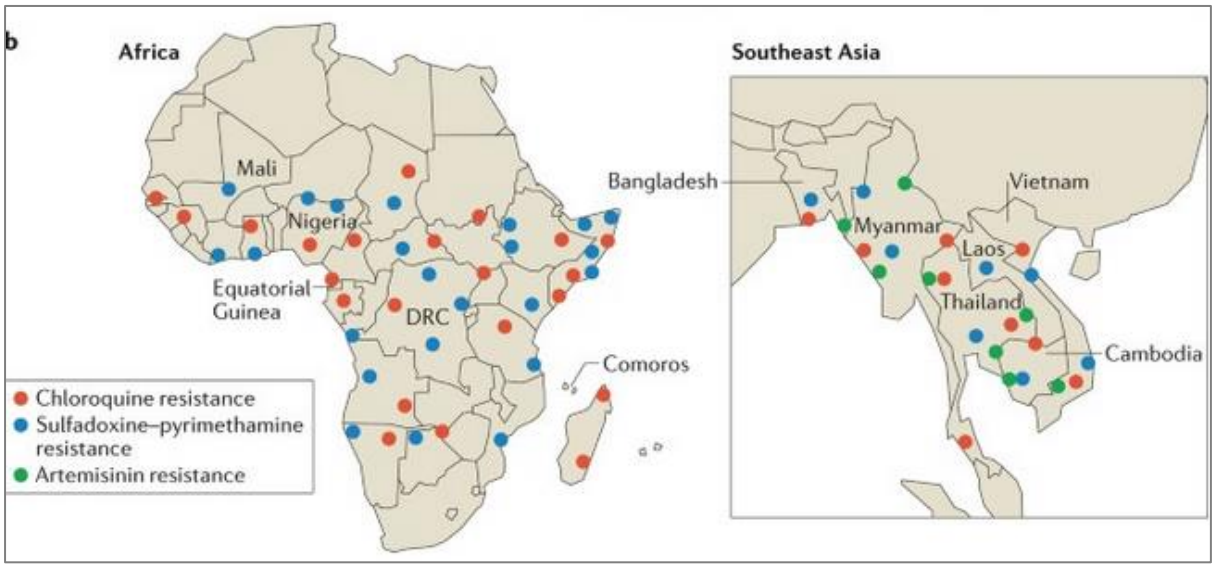


Fig. 57: Map of Africa and Southeast Asia depicting the prevalence of drug resistant P. falciparum strains. Source: Haldar et. al., 2018; Nature Reviews Microbiology [235].

Due to its peculiar genetics the *Plasmodium* genome is susceptible to frequent mutational events. Mutations tend to appear randomly either *de novo* or due to direct genotoxic effects from environmental cues and can serendipitously confer tolerance to drugs, offering selective advantage to a cohort of parasites [236]. As the susceptible population begins to wane out under drug pressure, the resistant strains become predominant. In regions such as Southeast Asia, low transmission and immunity allow parasitemia to reach very high levels. Symptomatic nature of infections provokes people to seek medication which allows for the parasites to be exposed to drugs that aren't often sufficient in dosage to match the parasitemia levels. The problem is augmented by poor pharmacokinetics of fake drugs and reinfections during the recovery phase. Endemic regions such as Africa on the other hand show less emergence of drug resistance. In

these areas of higher transmission people get infected repeatedly and develop a baseline immune response against the parasite (pre-immunity). Such infections present no symptoms and people rarely seek treatment thus limiting exposure to drugs [237].

4.2.1 History of drug resistance in *P. falciparum*

Drug resistant strains of *Plasmodium* are now known to have evolved out of geographical regions with low transmission rates like Southeast Asia and then spilled over to highly endemic pockets like Africa [238]. In the 1950s WHO launched the Global Malaria Eradication Programme which featured the first mass usage of the then prevalent drug Chloroquine for both therapy as well as pre-emptive prophylaxis [239]. As a direct consequence of mass administration and monotherapy Chloroquine resistance first established in this region in the 1950s. Owing to migrant worker populations resistance soon reached Myanmar and Bangladesh in the late 1960s [240]. By the 1980s CQ resistance had spilled overseas into Sub-Saharan Africa where it formed major foci within the endemic pockets [241].

With a wane in the efficiency of Chloroquine (CQ) other drugs were introduced in succession in Southeast Asia but no change in usage policy resulted in similar fate as resistance against Sulphadoxine-Pyrimethamine appearing in 1960s [242]. Extensive monotherapy and unregulated drug administration have been responsible for emergence of resistance against most of these drugs. Artemisinin gained extensive usage in the 1970s as the first line treatment in China, Cambodia, and Vietnam. In order to sustain the efficiency of this miracle drug, WHO imposed a ban on Artemisinin based monotherapy in 1998 and recommended Artemisinin based Combination Therapy in 2001 [243]. However, reports around 2008 started to arise out of Southeast Asia again on alarming rise in IC_{50} values of Artemisinin based compounds and delayed parasite clearance rates [59].

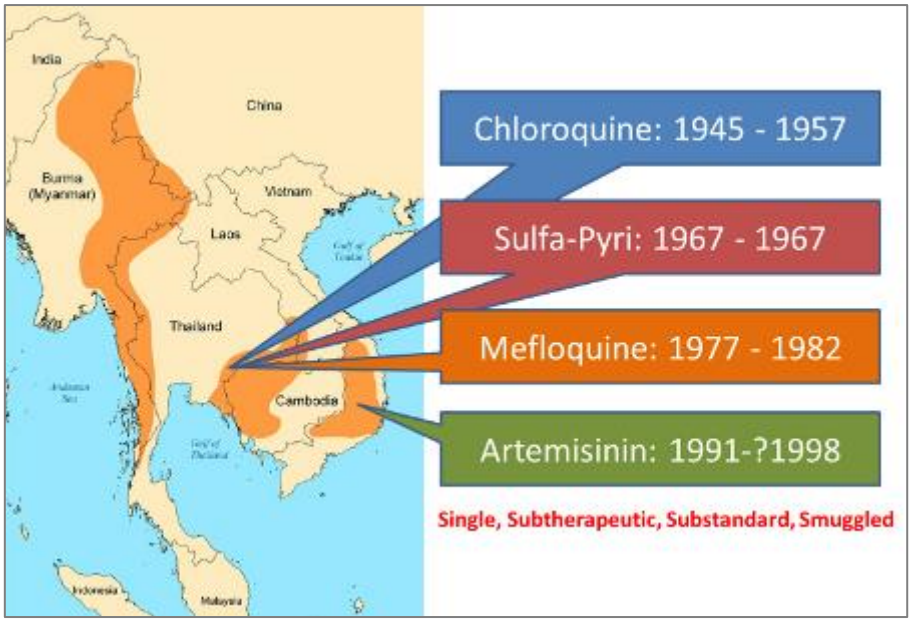


Fig. 58: Timeline of emergence of drug resistance in Southeast Asia Greater Mekong Subregion. Source: Malaria Site.

There are several factors which coalesce to make the Greater Mekong Sub-region (GMS) a prime hotspot for major episodes of drug resistances (Fig. 58). The mass prophylaxis medication campaigns (medicated salt projects) led to supplementation of often unregulated suboptimal dosage of drug in monotherapy format to large factions of the population allowing resistance to evolve in parasites [244]. In the non-endemic regions of GMS malaria is more often contracted by occasional bites of mosquitoes which are not conducive to evoke a robust immune response leading to better survival of parasites *in vivo*. Most importantly the ecological and population genetic structure of the parasite population in GMS is very distinct from that found elsewhere. The population size is small and well-structured and predisposed to mutational events [236]. Ecological cues such as hemoglobinopathies in the host human population can impart a stress to these parasites allowing them to adapt and evolve rapidly [245].

4.2.2 Artemisinin based combination therapy

Currently Artemisinin based Combination Therapy (ACT) is the most effective regimen of treatment of *falciparum* malaria. Artemisinin is a sesquiterpene lactone derived from the herb *Artemisia annua* (Fig. 59). At the heart of the molecule's efficacy lies a peculiar endo-peroxide bridge, which forms the basis of its mode of action [246]. Artemisinin is extremely efficient at reducing parasite load in the bloodstream, shows no adverse effects and as of yet has little significant resistance. Yet it has a short half-life and poor bioavailability. Chemical derivatization of the parent molecule has yielded a lot of semisynthetic versions (Artesunate, Artemether) with good bioavailability and the drug is now commonly accompanied by a secondary drug with a longer half-life to ensure persistence of therapy and preventing relapse. The secondary drug is chosen with a mode of action different from Artemisinin to ensure more stringent control over the possibility of cross resistance. Artemether + Lumefantrine, Artesunate + Pyrimethamine and Artesunate + Piperaquine in a fixed ratio are amongst the most effective treatment options available in market and can reduce the parasite load one hundred million fold over a 3 day standard ACT regime [247].

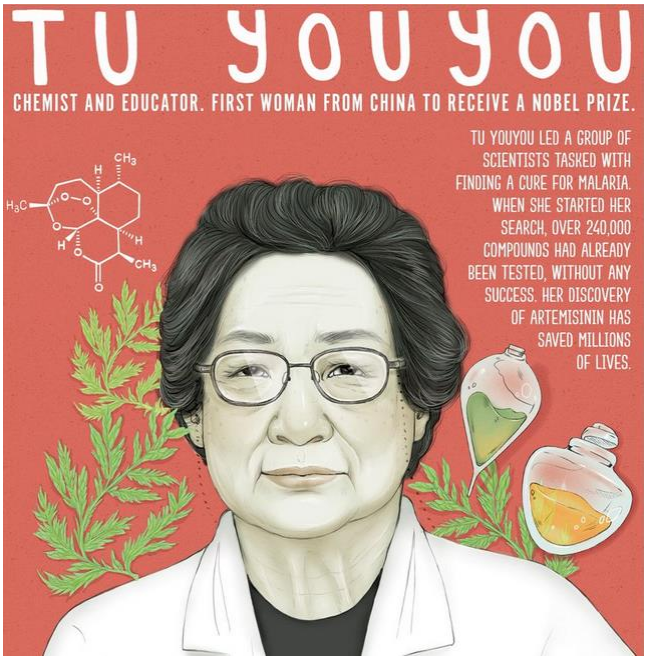


Fig. 59: Tu Youyou depicted against the herb Artemisia annua and the derivative antimalarial compound Artemisinin. Source: Xu Hui archives.

4.2.3 Emergence of artemisinin resistance

In the recent decades there have been numerous reports of decreased artemisinin sensitivity of *Plasmodium falciparum* in the Southeast Asian countries. The Greater Mekong Sub-region has emerged as the epicentre of artemisinin tolerant strain of *P. falciparum*, with clinical observations of delayed parasite clearance rates and rise in the frequency of treatment failures [59]. There is no evidence for full-blown artemisinin resistance yet. However, partial artemisinin resistance (tolerance) could facilitate the selection of partner drug resistance. Since ACT serves as our last line of defence against the *falciparum* malaria these findings pose great threat to worldwide efforts to manage and eliminate malaria [248].

In wake of the appearance of artemisinin tolerant strains in GMS, research focused on accurate assessment of the status of drug resistance in the field. Initial data was primarily built on clinical

reports which relied on unreliable parameters like parasite clearance time and treatment failure. Thereby it was acknowledged that more extensive research into understanding the genotype (mutations that were causing resistance) of the parasites was required.

To track down the mutations that underlie the resistance phenotype population genetics studies were undertaken (Fig. 60). Resistance inducing mutations often leave signatures in the flanking genomic regions and are vigorously selected for under continuous drug pressure. This results in low genetic variation, high linkage disequilibrium, and high haplotype homozygosity. Researchers have utilised statistical parameters like Extended Haplotype Homozygosity (EHH) and Cross Population EHH (XP-EHH) to detect regions of *Plasmodium falciparum* genome which are under selective sweeps [249].

Chemical screens have been widely employed in order to assess the response of parasites to panels of endo-peroxide drugs (from NIH Chemical Genomics Centre Pharmaceutical Collection) that resemble artemisinin structurally. Genotypic and phenotypic assessment has revealed the involvement of several key genes in response to these compounds including *pfa0655w* (SURFIN), *pf11_0188* (a heat shock protein 90), and *mal13p1.268* (a *Plasmodium* conserved protein) [250]. Another genotyping study revealed the prevalence of SNPs (1 per 7kb) in culture adapted parasites from Asia. The same study has revealed novel loci on chromosome 1, 3 and 8 to be involved in response to Di-Hydro Artemisinin (DHA). The same study confirmed selection around known resistance genes like *Pfdhfr*, *Pfcrt* and *Pfmdr* [251].

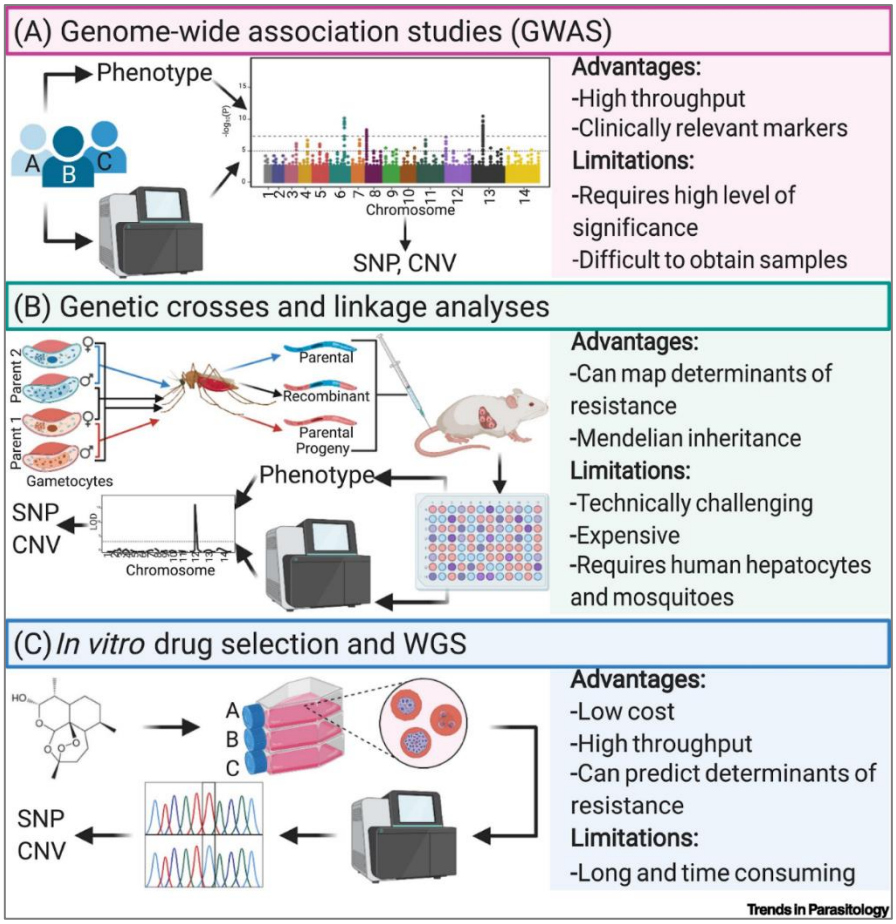


Fig. 60: A summary of the molecular and genetic techniques implemented for the identification of markers of artemisinin resistance in P. falciparum. Source: Okombo et. al., 2021; Trends in Parasitology [252].

In 2012, a major genome region underlying artemisinin resistance in 91 *P. falciparum* isolates from the Greater Mekong Sub-region was characterised using microarray data and microsatellite based fine mapping [253]. Their hypothesis states Artemisinin resistance didn't originate out of an isolated event (temporally or geographically). A 35 kb region on chromosome 13 turned up as a strong candidate for selection of artemisinin resistance. 331 parasite samples from Pailin, Thailand, Wang Pha were genotyped over SNP arrays. Of the four SNPs identified two ranks among the top contenders for recent positive selection [254]. Miotto et. al. in 2013 found a peculiar substructure within the parasite population in Cambodia.

They identified three distinct sub-clusters of *P. falciparum* concomitant with possible three different populations with low haplotype diversity and clearance rates. These studies were further able to pinpoint a region on Chromosome 13 as a major genomic region being selectively maintained for artemisinin resistance [255].

4.3 Next generation sequencing studies to identify the markers of artemisinin resistance

Artemisinin resistance marker identification has remained at the crest of research for the past few years. The scientific community is still hard at finding genetic markers definitively associated with resistance and also glean into the possible molecular pathways by which artemisinin action and resistance operate through. Genome wide association studies using next generation sequencing and improved microarray platforms allow us a peek into the genomic landscape of this challenging organism.

Ariey et. al. 2014 has been successful at identifying specific loci associated with artemisinin resistance. Using a combination of next generation sequencing of *in-vitro* selected artemisinin resistant parasite lines a mutation (C580Y) in the PfKelch13 propeller containing protein (K-13) was strongly correlated with significant artemisinin tolerance (Fig. 61). The PfKelch13 mutation has since then become the hallmark of artemisinin resistance for assessment of field strains [60]. In one of the follow-up studies to this discovery Ghorbal et. al. utilised the CRISPR-Cas9 genome editing tool, modified for use in *Plasmodium* to introduce the C580Y mutation into the propeller domain of PfKelch13 in NF54 drug sensitive strains of *Plasmodium falciparum*. The engineered parasites showed slow clearance phenotype giving a direct experimental proof of the involvement of the locus in artemisinin resistance [256]. Recent study by Ashley et. al. members of the Tracking Resistance to Artemisinin Consortium (TRAC) demonstrates that although PfKelch13 is a marker associated with artemisinin resistance, it

isn't a universal marker since parasite populations are tolerant to artemisinin in Africa do not show this genotypic feature [185].

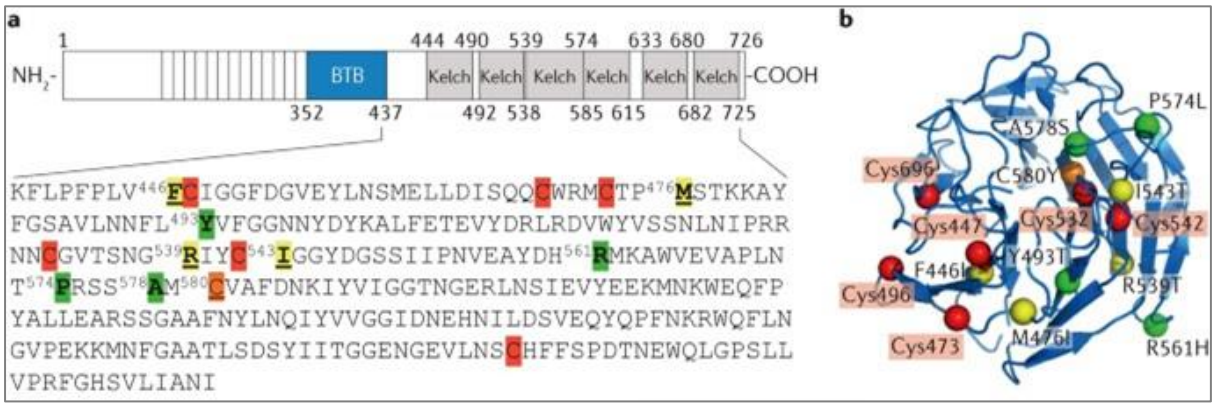


Fig. 61: A molecular map of the SNPs in the beta-propeller domain of PfKelch13 gene (a) and amino acid substitutions in protein structure (b) that are associated with decreased sensitivity to artemisinin. Source: Ariey et. al., 2014; Nature [60].

4.4 Mechanism of action of artemisinin

The mechanism of artemisinin resistance has taken a while to be decoded. Given its trigger of free radical cascade in the cell it was understood initially that the effect of artemisinin is highly pleomorphic. Click chemistry-based studies identified that artemisinin tends to interact, and form adducts with numerous biomolecules in the parasite. A number of these molecules are proteins involved in crucial biological processes in the parasite. Among these hemoglobin degradation pathways, protein folding associated machinery and DNA replication complexes were prominently identified [257]. It was thus understood that artemisinin targets and leads to the collapse of critical biological function in cells which could not be recovered from (Fig. 62). Tilley et. al. later showed that artemisinin can directly target proteasomal machinery in the cell thereby leading to accumulation of misfolded proteins. This eventually leads to cellular toxicity and death of cells [258].

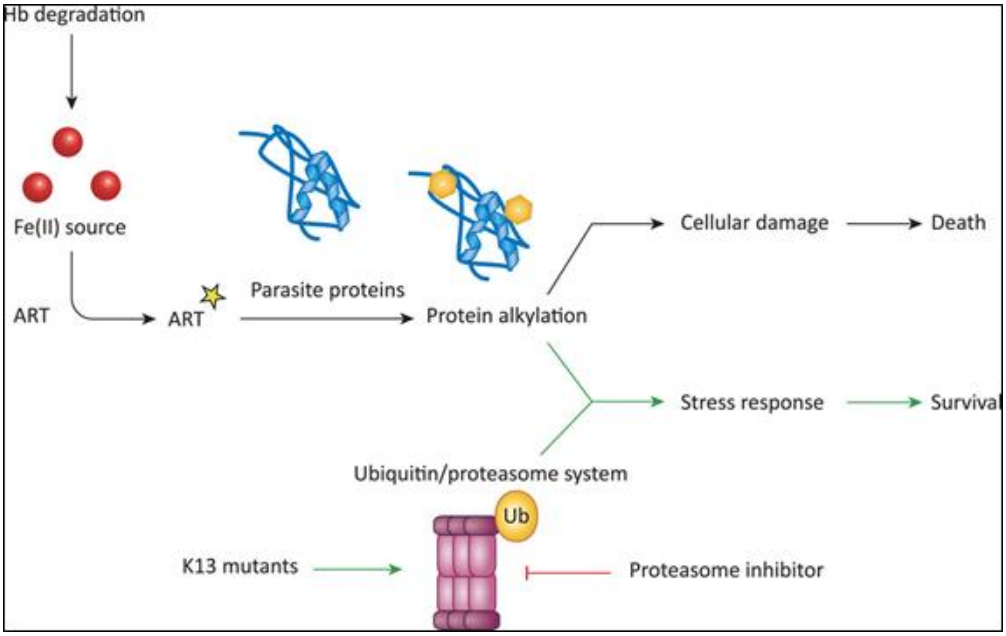


Fig. 62: Molecular mechanism of action of artemisinin and emergence of PfKelch13 mediated resistance to it. Source: Dogovski et. al., 2015; PLoS Biology [259].

4.5 Mechanism of artemisinin resistance

One of the first studies to definitively identify the role of PfKelch13 mutations in artemisinin resistance came from the work of Mbengue et. al. The study identified PfPI3K to be one of the targets of PfKelch13 and the protein was identified to be responsible for its ubiquitination and proteasomal degradation. With mutation in the PfKelch13 protein, PfPI3K evaded ubiquitination mediated proteasomal degradation. Its higher abundance in the cell was found to be directly correlated with artemisinin resistance. The group speculates that the increase in PfPI3K levels leads to an enhanced lipid metabolism in cells [260]. A subsequent study led to the identification of lipid bound vesicles that carry proteostasis factors across the cell, possibly to alleviate the damage caused by the drug (Fig. 63) [261]. A more definitive link of artemisinin resistance with PfKelch13 was established by Tobias Spielmann's group in 2019 when they showed the presence of PfKelch13 in vesicular compartments associated with host cytoplasm endocytosis. PfKelch13 and several other proteins (including Eps15) were identified to

determine the influx of hemoglobin into the cell (Fig. 64). Mutations or even lower levels of PfKelch13 and few of its compartmental interacting partners were identified with severe depletion of endocytosis leading to enhanced resistance to artemisinin. The group's finding hinted strongly that any mechanism that could regulate the bioavailability of heme/iron in cells could alter the artemisinin susceptibility [262].

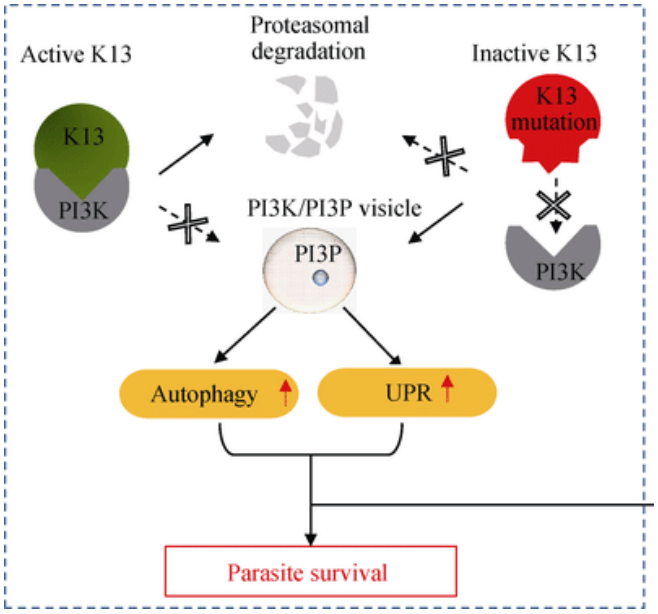


Fig. 63: Mechanism of artemisinin resistance conferred by PfKelch13 mutation leading to enhanced PI3P vesicles in the parasite. Source: Lu et. al, 2019; Chinese Journal of Natural Medicines [263].

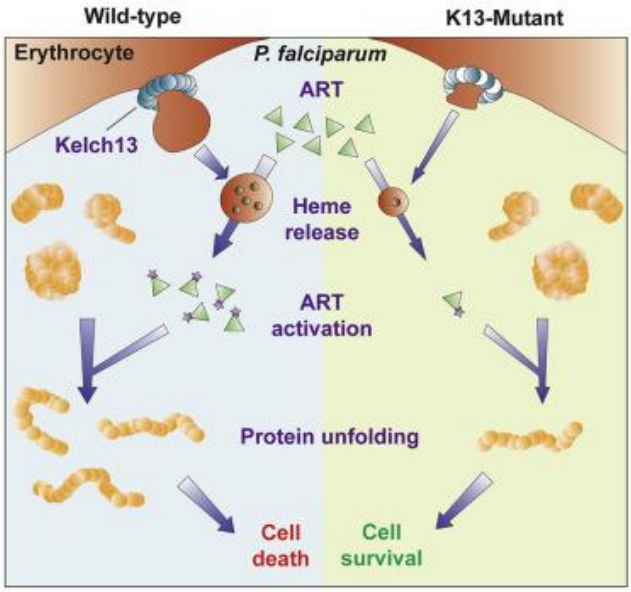


Fig. 64: PfKelch13 depletion/mutation is associated with reduced endocytosis and subsequent activation of artemisinin leading to resistance. Source: Yang et. al., 2019; Cell Reports [264].

4.6 Transcriptomics studies provide a system wide insight into the drug response and resistance mechanism mounted by parasites

With the advent of RNA sequencing a systems wide insight into the mechanism of artemisinin resistance could be made. Over the decade a number of population transcriptomics studies have investigated the gene expression changes that occur in artemisinin resistant parasites. The population transcriptomics studies were driven majorly by Zbynek Bozdech's group. The studies highlight that artemisinin resistance is marked by a strong rewiring of the parasite transcriptome hinting at a systemic change in gene expression. Artemisinin resistant parasites were shown to have a delay in their transcriptional progression which could be correlated with a stalling of the cellular dynamics. This delayed progression of the transcriptional profile has also been observed (and can be manifested) at a phenotype scale where artemisinin resistant isolates show delayed progression through the ring stage. Furthermore, there is a mobilisation

(through enhanced expression) of cellular homeostasis associated genes involved in Unfolded Protein response, ER associated degradation pathways, RNA stability, transport, protein refolding and transport (Fig. 65). Collectively these transcriptional changes are believed to contribute to less activation of artemisinin in the cell while allowing for a primed state ready to mitigate any damage that may be caused by residual artemisinin activation [149, 186].

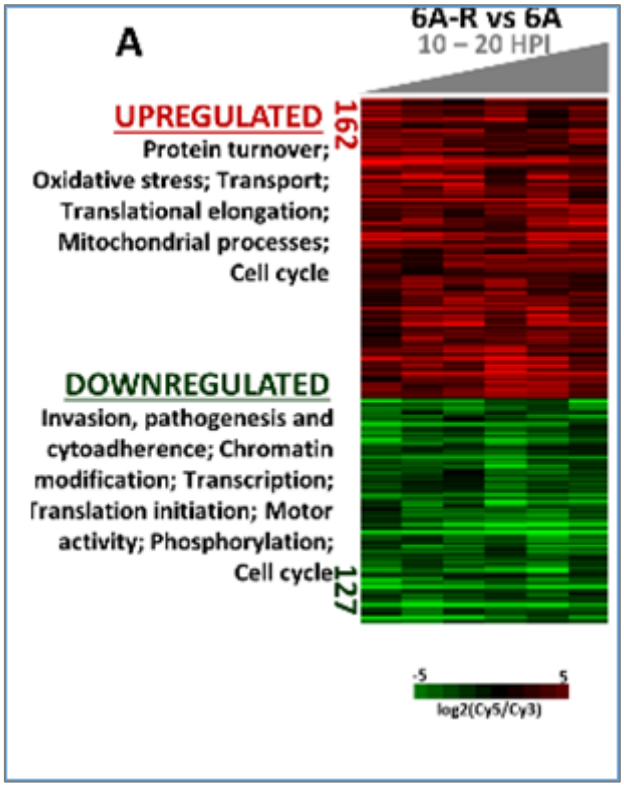


Fig. 65: Artemisinin resistance is driven by widespread changes in gene expression profile of protein metabolism, cytoadherence associated genes. Heatmap of the gene expression deregulation observed in in vitro selected artemisinin resistant parasites. Source: Rocamora et. al., 2018; PLoS Pathogen [265].

4.6.1 Transcriptional regulatory proteins may drive resistance associated programme

The widespread transcriptional changes observed in artemisinin resistance hints at a skewed regulatory profile in the resistant parasites. A few studies have investigated the expression profile of regulatory factors in resistant isolates and have led to the speculation that a change in the expression level of these factors could cause a transcriptional cascade that could have widespread effects on the downstream gene expression networks. Gene expression regulatory factors lie higher up in the expression regulation hierarchy any change in the expression profile of few such factors could explain the widespread gene deregulation observed with artemisinin resistance [149]. Study by Rawat et. al. has further elaborated upon the role of transcription regulatory histone modifiers in governing stress responses and artemisinin response/ resistance. The histone acetyltransferase PfGCN5 has been shown to respond to artemisinin exposure and bind to genes that govern oxidative (glutathione-S-transferase) and proteotoxic (PfBiP) stresses. Suppression of PfGCN5 activity by garcinol treatment suppresses stress responsive gene expression and was found to suppress artemisinin resistance in ring-stage survival assay [197]. Thus, epigenetic factors have a strong precedence in governance of complex biological functions in parasites including drug associated phenotype.

4.6.2 Histone modification enzyme PfHDAC1 as a potential regulator of artemisinin resistance associated transcriptome

PfHDAC1 was identified to be one of the most strongly downregulated genes in a population transcriptomics study of artemisinin resistant parasites by Zbynek Bozdek's group. It was in fact discovered to be among the bottom 5% of genes arranged by their level of deregulation expressed as a z-score (Fig. 66) [149]. We were thus curious to investigate whether PfHDAC1 could serve as a potential contributor to the resistance landscape associated with artemisinin resistance.

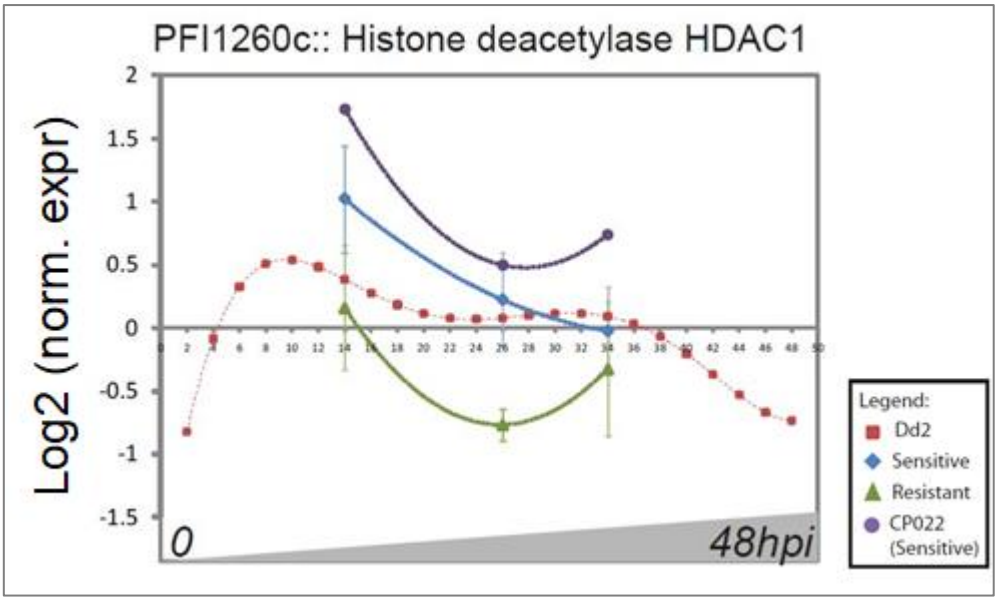


Fig. 66: PfHDAC1 is downregulated in artemisinin resistant parasites obtained from the field in Southeast Asia. Source: Mok et. al., 2011; BMC Genomics [149, 186].

One of the strongest evidence in support of a potential regulatory role of PfHDAC1 in artemisinin resistance comes from our observation that inhibition of the PfHDAC1 activity is associated with delayed progression of the parasites through the life cycle which mimics the cell cycle dynamics observed in artemisinin resistant parasites [229]. Furthermore, inhibition of PfHDAC1 was also seen to upregulate the expression of several proteostasis associated genes that have been shown to be associated with artemisinin resistance (namely upregulation of cytosolic chaperone PfHSP70 and the ER-resident chaperone PfBiP) [186]. The combined effect of slower cell cycle progression and enhanced proteostasis associated genes may lead to better resilience against artemisinin in the parasite (Fig. 67).

We thus sought to validate the association of deregulation of PfHDAC1 with artemisinin resistance and see if the same could contribute to artemisinin resistance associated transcriptome as well.

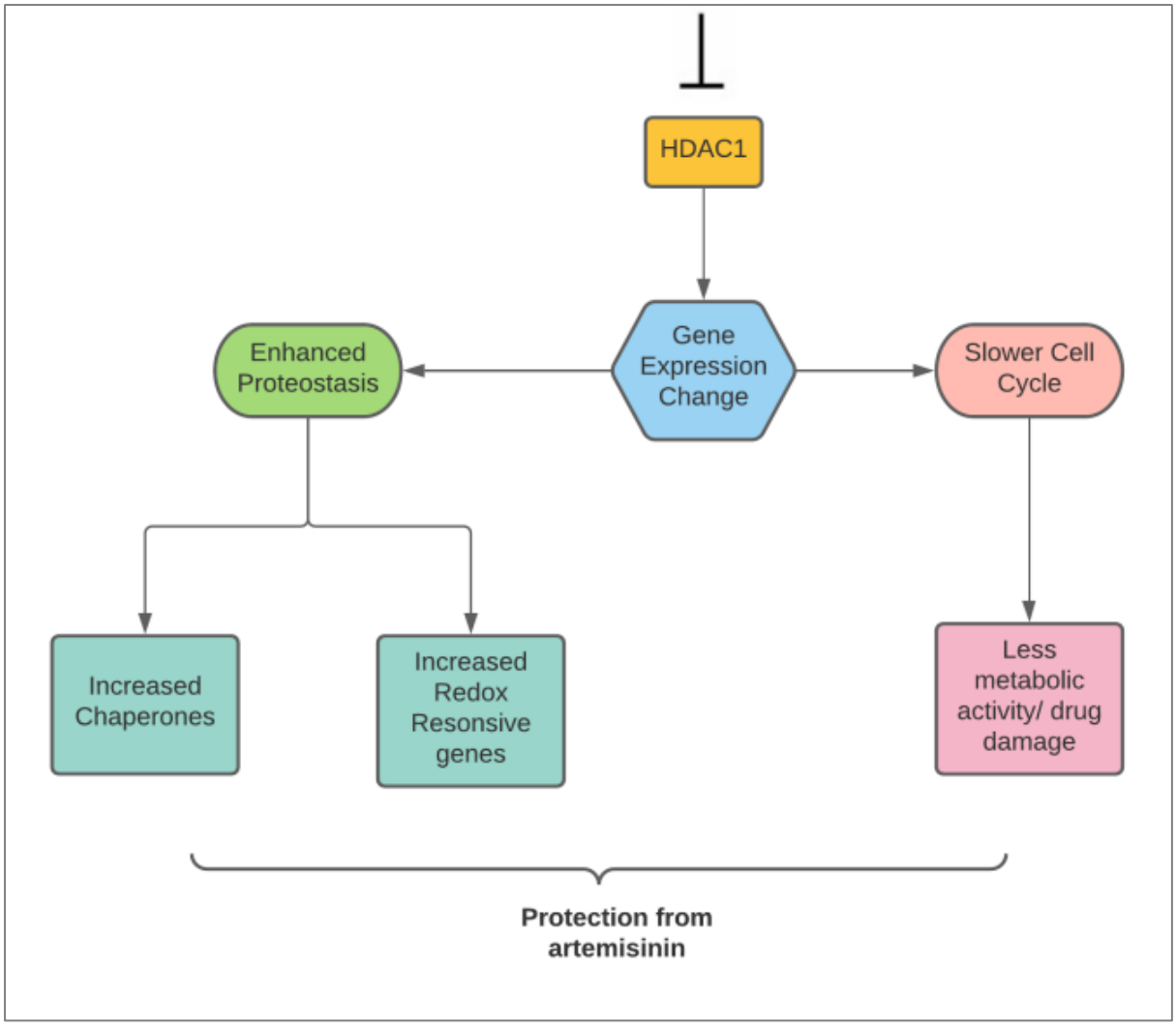


Fig. 67: PfHDAC1 regulates biological pathways whose deregulation (as observed in case of pharmacological inhibition of PfHDAC1) is implicated in artemisinin resistance.

4.7 Material and methods

4.7.1 Ring-stage survival assay

Tightly synchronised (double Percoll density gradient centrifugation) 0-3 hr 3D7 *P. falciparum* ring stage parasites were taken at 1.5% parasitemia and 2% hematocrit in 200µl volume 96 well plates. Parasites were then either treated with DMSO mock or with 700 nM dihydroartemisinin for a duration of 6 hrs following which the mock/drug was washed off and the parasites were reinstated in culture. They were allowed to grow for another 66 hrs. At the

end of 72 hours live parasites were counted using Giemsa smears for at least 10,000 cells in both mock and DHA treated cultures. The percentage of cells surviving DHA treatment relative to the mock treatment were logged as % RSA. For the transgenic lines (generated at the Crick institute), the RSA was performed similarly, however the parasitemia was calculated by flow cytometry for SYBR Green I and MitoTracker signal in the DHA vs mock treated culture.

For checking the effect of PfHDAC1 inhibition tightly synchronised freshly invaded ring stages were treated with 0.25x IC_{50} dosage of the selected PfHDAC1 inhibitor and then half an hour later followed by DHA or mock treatment which was continued for 6 hrs. The drugs/mock were washed off at the end of 6 hrs DHA treatment however the inhibitors were readded to the culture at the original conc. till the completion of the assay at 66 hr post DHA wash off. The percentage of cells surviving concomitant inhibitor + DHA treatment relative to those surviving inhibitor + mock treatment was taken as the %RSA. The assays were carried out in technical triplicates and repeated for two biological replicates.

RSA on transgenic (PfHDAC1-GFP-glmS or GFP-glmS) artemisinin sensitive (PfKelch13 R539WT) or resistant (PfKelch13 R539T) lines was performed the same was as described for the 3D7 lab strain of P. falciparum. The original lines were obtained from London School of Tropical Health and Medicine.

4.7.2 Chromatin Immunoprecipitation

Parasitized RBCs were crosslinked using 1% formaldehyde (Thermo Scientific, 28908) for 10 mins at RT. 150mM glycine was added for quenching the cross-linking reaction. The samples were washed using 1XPBS (chilled) before proceeding with lysis. Sample homogenization was performed using swelling buffer (25mM Tris-Cl pH 7.9, 1.5 mM MgCl2, 10mM KCL, 0.1%

NP 40, 1mM DTT, 0.5mM PMSF, 1xPIC) 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 200-400bp. Pre-clearing was performed for 1 hour at 4 ºC using recombinant protein G conjugated sepharose beads with continuous gentle inverting. 30µg purified chromatin was used per immunoprecipitation reaction (α-PfHDAC1 antibodies) and incubated for 12h at 4 ºC. Samples were then incubated with saturated Protein G Sepharose beads for 4 hours at 4 ºC. Bound chromatin was finally washed with low salt, high salt, LiCl wash buffers followed by TE buffer wash and eluted using ChIP elution buffer (1%SDS, 0.1M sodium bicarbonate). Both the IPed sample and input were reverse crosslinked using 0.3M NaCl overnight at 65 ºC along with RNAse A treatment. Proteinase K treatment was performed at 42 ºC next day for 1 hour. Finally, DNA was purified using phenol chloroform isoamyl alcohol precipitation. Target sites identified from ChIP sequencing analysis were further validated by ChIP-qPCR using the Biorad SYBR Green Master Mix (Biorad).

4.7.3 ChIP-sequencing library preparation and sequencing

ChIP-sequencing libraries for PfHDAC1 ChIP in 3D7 strain (and later for the artemisinin resistant and sensitive isolates) were prepared from 5-10 ng of DNA using the NEBNext 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. For PfHDAC1 ChIP seq in artemisinin resistant and sensitive isolates, the library was prepared as described above. The sequencing for these was done at the Next Generation Sequencing

facility at IISER, Pune using the Illumina NextSeq 550 sequencer with average read length of 75 bp.

4.7.4 ChIP-Seq data analysis

The sequencing reads were demultiplexed using the bcl2fastq tool in the Linux platform. Read quality check was performed using FASTQC and sub quality reads (not passing Q30 filter) and adaptor sequences were trimmed using Trim_Glaore. Reads were aligned onto the *Plasmodium falciparum* 3D7 reference genome using Bowtie aligner. SAMTools was used to further process the data (sorting, etc.). The sorted ChIP and Input BAM files were then used for peak calling in MACS2 software. Background subtraction was performed using the bdgcmp command in MACS. Peak annotation was performed using Bedtools and the peaks were visualised using IGV genome browser.

Deeptools suite was used for additional ChIP data analysis and visualization. The ChIP and input BAM files were converted into bigwig format using BamCoverage option. BamCompare was used to generate an input normalized ChIP bigwig file. The computeMatrix option was used to generate an enrichment matrix for the PfHDAC1 over average gene body flanked by 500bp of upstream and downstream UTR. The matrix was then fed to the plotHeatmap tool to generate the PfHDAC1 occupancy heatmap on the target genes. PlasmoDB gene ontology tool was used to identify the biological pathways enriched for PfHDAC1 occupancy gene targets.

For additional comparison of the PfHDAC1 occupancy (ChIP data) and gene expression (RNA seq data) in artemisinin resistant/sensitive isolated computeMatrix command was used to generate the gene expression reads density matrix over the PfHDAC1 enrichment/depletion loci (fed in the reference BED file) and then visualized using the plotHeatmap option.

4.7.5 Generation of overexpression constructs for PfHDAC1

The full-length sequence of PfHDAC1 was PCR amplified from genomic DNA using sequence specific primers and cloned into the pDC2 overexpression vector using the AvrII and NheI restriction sites (Primer details in Table 3/Appendix 1). This put the PfHDAC1 under control of the calmodulin promoter and in frame with a C-terminal GFP tag. Furthermore, to add a layer of regulatability to the overexpression system we cloned a glmS ribozyme sequence (PCR amplified from pHSP101 plasmid) in frame with the GFP tag using XhoI enzyme (Primer details in Table 3/Appendix 1). This resulted in an overexpression system synthesising PfHDAC1-GFP -glmS fusion transcript.

4.7.6 *P. falciparum* transfections

Lonza nucleofector 4D was used for transfection of plasmids into highly synchronised parasites (*P. falciparum* PfKelch13 R53T mutant and R539 wildtype lines obtained from the London School of Tropical Health and Medicine). 30µg of episomal plasmid was dissolved in 100µl of P3 primary solution. Double synchronised (Percoll centrifugation) segmented schizonts were mixed with the DNA/P3 solution mix and nucleoporated in the nucleofector 4D machine with the pulse program FP 158 (designed for *P. berghei*). The zapped cells were immediately transferred to a T25 flask with 3 ml of media and 200µl fresh RBC and set on a shaker incubator at 37ºC for 2 hours to allow the ruptured merozoites to invade the fresh cells. The flask was later supplemented with additional media to make it to 2% hematocrit. Drug selection was started 24 hours post transfection. Appearance of transgenic lines was checked initially via Giemsa smear. The cells were then checked for GFP-tagged PfHDAC1 expression via confocal microscopy. The transgenics were generated and tested upon at Dr. Moritz Treeck's lab at The Francis Crick Institute, London.

4.7.7 Cloning of the transgenic parasite lines

The validated transgenic lines were serially diluted in a 96 well flat plate to obtain 200µl cultures with finally 1 parasite per well. These were confirmed with plaque formations and then transferred onto round bottom 96 well plates for expansion. The cells were then microscopically confirmed for GFP expression and then expanded into flasks.

4.7.8 Validation of reversible overexpression of PfHDAC1-GFP

Validation of PfHDAC1-GFP overexpression and reversible depletion was validated with treatment of culture with 0, 2.5 and 5 mM of glucosamine-HCl followed by western blotting for depletion of GFP tagged PfHDAC1 signal.

4.7.9 Parasite growth curve assay

The GFP-glmS ctrl overexpression and PfHDAC1-GFP-glmS overexpression were tightly synchronised using two rounds of Percoll density gradient centrifugation. Parasitemia was estimated by staining cells with SYBR Green I dye and subjecting them to flow cytometry. The cultures were diluted to 2% starting parasitemia and 2% hematocrit in 96 well plates and allowed to proliferate over a duration of 6 days/3 IDC. For testing the effect of glucosamine dosage (and episomal expression suppression) on the growth trend, wells with 2.5mM and 5mM glucosamine treatment were set up in parallel with mock treated parasites for both PfHDAC1-GFP-glmS overexpression and GFP-glmS overexpression lines. Media was replenished carefully after the third day with accurate replacement for mock and drugs. Each strain and condition were set in triplicate on the 96 well plate. The culture in the wells were sampled off every 24 hours until the finish of the assay and subject to SYBR Green I flow

cytometry to calculate parasitemia progression over the course of the experiment. Growth curves were plotted on GraphPad Prism software. Baseline toxicity of glucosamine was calculated based on the viability of the GFP-glmS lines and then subtracted from the cognate readouts of PfHDAC1-GFP-glmS to represent toxicity corrected growth curves.

4.8 Results

4.8.1 Investigating the level of PfHDAC1 in artemisinin resistant vs sensitive *P. falciparum* lines

Artemisinin resistant parasites have been extensively studied for their transcriptional signatures in several studies. Mok et. al. in a population transcriptomics studies implicated widespread transcriptional changes laid out on top of Kelch13 mutation may have a role in shaping the artemisinin sensitivity of parasites [149]. The study mentions that changes in the expression profile of key regulatory proteins (like transcription factors and epigenetic modifiers) that lie high up in the gene network hierarchy could cause rewiring of the parasite transcriptome, essentially laying ground for reduced artemisinin sensitivity. Interestingly, the study identified PfHDAC1 as one of the most significantly downregulated genes (among the bottom 5% of the genes arranged by their z-score) in resistant parasites [149]. We checked if it was the same for a number of artemisinin resistant/sensitive isolates (essentially PfKelch13 mutant and WT counterparts) which we procured from MR4 repository, originally sourced from Cambodia [266].

We observed downregulation of PfHDAC1 protein in the C580Y artemisinin resistant line compared to the WT counterpart strain (Fig. 68). Thus, we corroborated that indeed PfHDAC1 down regulation is associated with artemisinin resistant parasites. We were curious to see if

this depletion of the PfHDAC1 in resistant lines was a consequence of resistance or if it was a contributing factor in the phenotype.

Fig. 68: PfHDAC1 was found to be downregulated in artemisinin PfKelch13 mutant resistant parasites. Western blot for PfHDAC1 protein levels in artemisinin resistant vs sensitive strain and upon PfHDAC1 inhibitor romidepsin treatment for same.

4.8.2 Investigating the effect of PfHDAC1 inhibition on artemisinin (drug) sensitivity in *P. falciparum*

The observed effects of PfHDAC1 inhibition on cell cycle progression in *P. falciparum* presented two very interesting aspects. First was the delayed maturation of trophozoite stage in romidepsin treated culture. Second was the delayed appearance of the schizont stage of parasites. Furthermore, several of the genes and associated biological pathways that we identified under PfHDAC1 regulation (cytoadherence, heat shock proteins, DNA damage response, hemoglobin catabolism, and autophagy) are strongly implicated in artemisinin resistance by various transcriptome wide association studies of artemisinin resistant field isolates [186]. We were thus curious to see if the pharmacological inhibition of PfHDAC1 could tweak the artemisinin sensitivity of *P. falciparum*.

4.8.3 Ring-stage survival assays to assess artemisinin sensitivity of parasites under PfHDAC1 inhibition

Ring-stage Survival Assay (RSA) is the gold standard of artemisinin sensitivity assessment since it mimics *in vitro* the dosage and pharmacological exposure routines observed in clinical settings of artemisinin therapy (Fig. 69) [267]. To establish the baseline RSA of the lines tested for artemisinin sensitivity, highly synchronised <3hr rings stage parasites were exposed to 700nM of DHA or DMSO mock for 6 hours. The drug was then washed off and the parasites were put back in culture for another 66hrs. %RSA was calculated as the percentage of parasites surviving in DHA vs DMSO treatments. To assess the effect of PfHDAC1 inhibition on artemisinin sensitivity, the parasites were either exposed to DHA along with an inhibitor (romidepsin/entinostat) as test vs only DMSO + inhibitor as mock. Post 6 hrs DHA was washed off and the parasites were allowed to grow back in culture (with inhibitor alone) for another 66 hr. %RSA was calculated as the percentage of parasites surviving in DHA and inhibitor treatment vs DMSO and inhibitor ctrl. We thus compared the basal RSA% of DHA/DMSO treatment regime with the RSA% from the DHA + Inhibitor/DMSO + Inhibitor regimes.

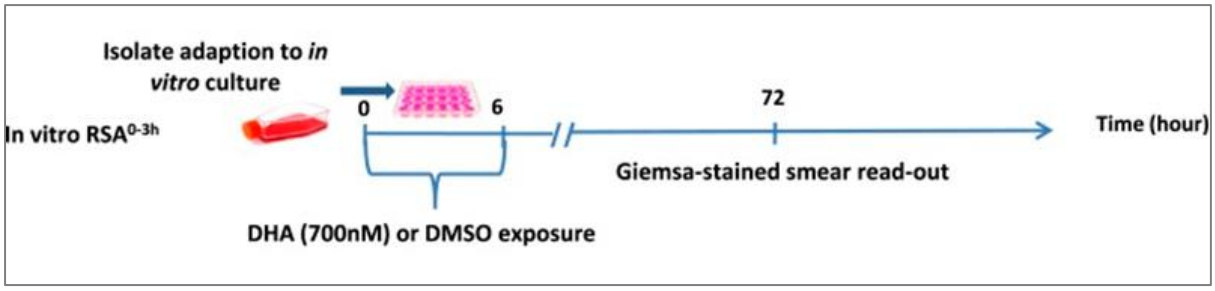


Fig. 69: An outline of the Ring-stage Survival Assay used to characterise sensitivity of parasites to artemisinin. Source: Niare et. al., 2018; American Journal of Tropical Medicine and Health [268].

We first tested the %RSA of PfKelch13 wild type *P. falciparum* 3D7 strain, which was found to be at an expected <1 % RSA. The artemisinin survival in parasites concomitantly treated with DHA and entinostat was reported at 4.2% and that for DHA and romidepsin at 5% relative to the respective inhibitor only mock treatment (Fig. 70A). Thus, we discovered that PfHDAC1 inhibition during DHA exposure can enhance artemisinin resistance in otherwise drug sensitive parasites.

We further investigated the effect of PfHDAC1 inhibition on drug sensitivity of various PfKelch13 mutant *P. falciparum* isolates. The C580Y mutant reported a baseline RSA of 4.2% which increased marginally to 7% under concomitant romidepsin treatment and 6.5% under entinostat treatment alongside DHA. We tested another artemisinin resistant *P. falciparum* Dd2 strain that harboured an R539T mutation. The baseline RSA was observed at 8.5% for the strain while RSA under concomitant DHA and romidepsin treatment was observed to be 11% (Fig. 70B and 70C).

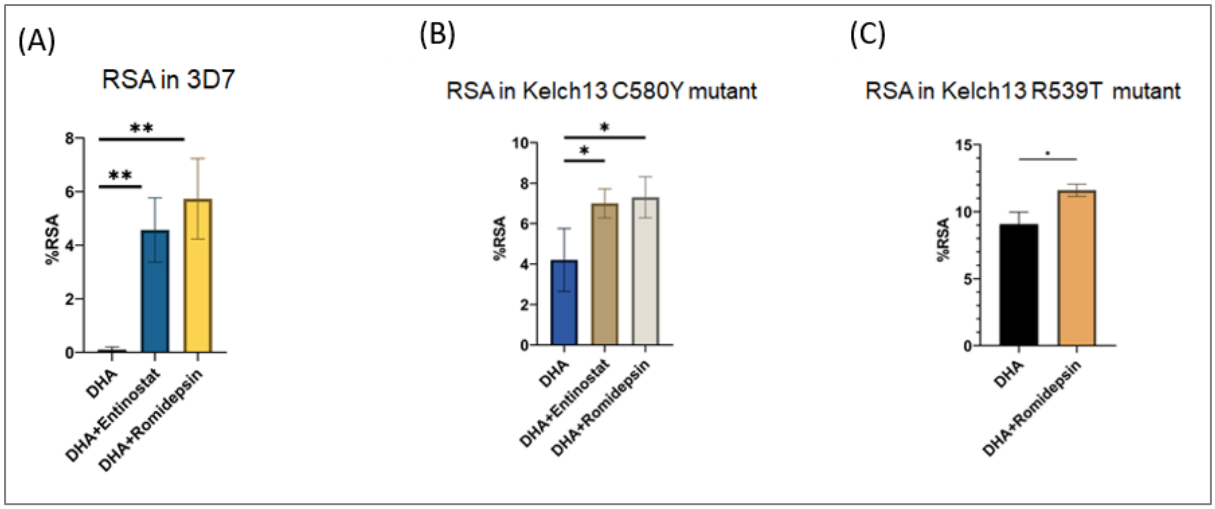


Fig. 70: PfHDAC1 inhibition establishes artemisinin resistance in P. falciparum 3D7 strain (observed as an increase in %RSA) and further enhances the level of resistance observed in PfKelch13 mutant parasites. (A) Bar plots for %RSA of 3D7 parasites under DHA only and DHA + entinostat (blue) or DHA + romidepsin treatment (yellow). Bar plots for %RSA of PfKelch13 C580Y (B) and R539T (C) mutant strains from Southeast Asia under DHA and DHA + inhibitor treatment.

Thus, our observations highlight that PfHDAC1 inhibition can not only establish clinically significant artemisinin resistance in PfKelch13 wildtype isolates, but it can also enhance the level of resistance observed in already resistant PfKelch13 mutant strains.

We have detected enrichment of PfHDAC1 on PfGCN5 which has been identified as an important stress responsive factor in *Plasmodium* [197]. Pharmacological inhibition of PfHDAC1 evokes the expression of PfGCN5 in our study. Based on findings of Rawat et. al. this could serve to benefit the cells by increased expression of stress responsive genes. Hypothesising on the observation that PfGCN5 inhibition lowers RSA% in parasites, any factor that can enhance the levels of PfGCN5 (including PfHDAC1 inhibition/depletion) could lead to resistance (Fig. 71). Thus, the functions of PfHDAC1 could be also visualised as linked and independent from PfGCN5 functionality in cells. Additionally, depletion of PfHDAC1 could

allow for eviction of the factor from nucleosome thus making space for PfGCN5 for its activity. Thus, the crosstalk of the HAT and HDAC factors in parasites is a strong focus of transcriptional regulation.

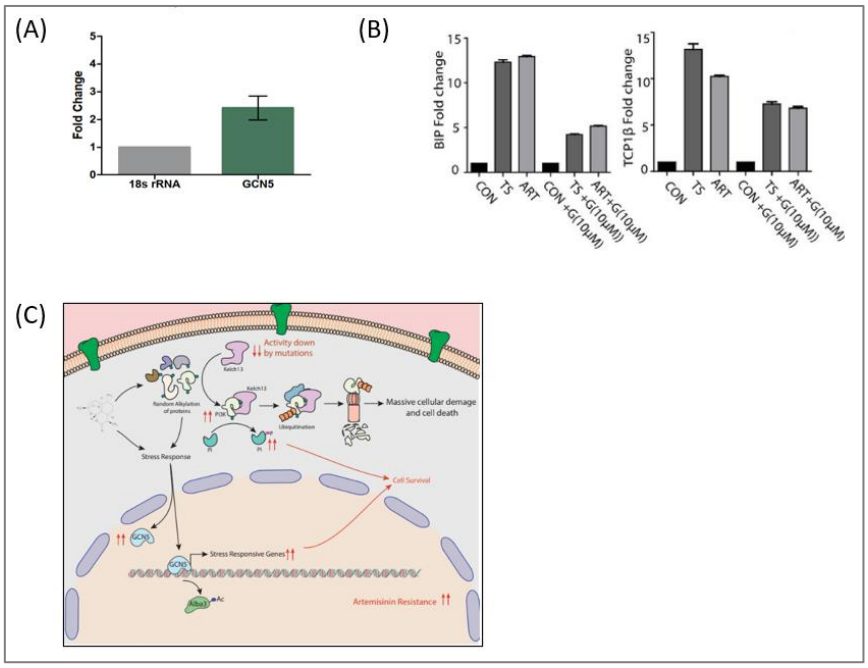


Fig. 71: PfHDAC1 governs the expression of another epigenetic regulator PfGCN5. (A) Pharmacological inhibition of PfHDAC1 evokes the expression of PfGCN5 in our study. (B) Rawat et. al. study shows enhanced expression of PfGCN5 target stress responsive genes under artemisinin stress (C) Model of enhanced PfGCN5 mediated resistance to artemisinin. Source for (B) and (C) Rawat et. al. 2021, Scientific Reports [186, 197, 269].

4.8.4 Investigating the effect of PfHDAC1 overexpression on artemisinin (drug) sensitivity in *P. falciparum*

We discovered an enhancement of resistance in parasites treated with HDAC1 inhibitors. We could correlate this with the stunted morphological progression of the parasites through the IDC. PfHDAC1 overexpression on the other hand enhanced parasite proliferation. We were thus curious to investigate the effects of PfHDAC1 overexpression on parasite sensitivity to

artemisinin. Overexpression of HDAC1 has been associated with enhanced survival under a variety of stress conditions including but not limited to therapeutic drugs. Human malignancies often report overexpression of HDAC1 in association with drug therapy failure [225]. The genes we identified under PfHDAC1 regulation control biological pathways that are crucial for dictating drug sensitivity in parasites. These pathways include and are not limited to cell cycle, heat shock response, DNA damage response, autophagy and haemoglobin metabolism [270].

4.8.5 Ring-stage Survival Assays to assess artemisinin sensitivity of parasites under PfHDAC1 overexpression

We had observed enhancement of RSA% in parasites treated with PfHDAC1 inhibitor. By this logic overexpression of PfHDAC1 would potentially result in suppression of RSA%. We thus investigated the effect of PfHDAC1 overexpression on a PfKelch13 mutant line (R539T) which was already resistant to artemisinin. We generated transgenic lines for episomal overexpression of PfHDAC1-GFP-glmS and GFP-glmS control and subjected them to RSA. Highly synchronised <3hr rings of PfHDAC1-GFP-glmS overexpression and GFP-glmS control overexpression parasite lines were exposed to 700nM of DHA or DMSO for 6 hours. The drug was then washed off and the parasites could grow back in culture for another 66 hr. Finally, the survival of the GFP and PfHDAC1 overexpression parasite lines was individually assessed as a percentage of parasitemia (%RSA) observed in DHA vs mock treatment. The GFP-glmS control transgenic line (with PfKelch13 R539T mutation) reported 29.05% RSA. To our surprise, the PfHDAC1-GFP-glmS overexpression control line reported an enhanced survival under DHA exposure with 42.38% RSA. Another clonal line for PfHDAC1-GFP-glmS overexpression was assessed for its artemisinin sensitivity and reported 39.79% RSA (Fig. 72). While the GFP overexpression control line reported a growth change of 2.36-fold the two

clones for PfHDAC1-GFP overexpression reported higher growth rates with 4.23- and 3.27-fold growth over the course of the assay.

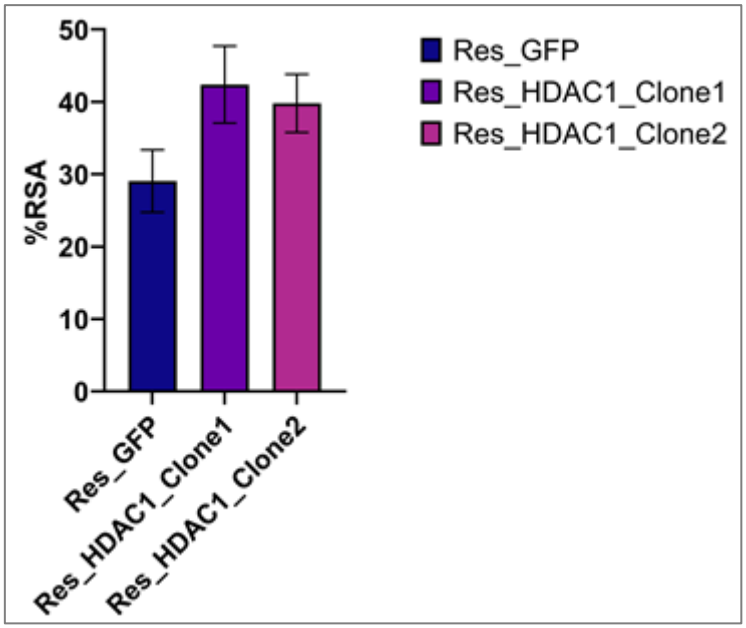


Fig. 72: PfHDAC1 overexpression is associated with enhanced resistance to artemisinin in PfKelch13 mutant strains. Bar plot for %RSA in GFP-glmS control and PfHDAC1-GFP-glmS overexpression in PfKelch13 mutant strains.

Driven by this curious observation we decided to check the effect of PfHDAC1 overexpression on artemisinin sensitivity in PfKelch13 wildtype parasites. The RSA was repeated on artemisinin sensitive parasites overexpressing either PfHDAC1-GFP-glmS or GFP-glmS alone. The ring stage survival percentage was recorded at 0.17% for the GFP overexpression control lines, while it was 16.51% for PfHDAC1-GFP overexpression line (Fig. 73). Analysis of the fold growth over the 72hr duration of the assay showed that the GFP overexpression line grew 3.2-fold while the PfHDAC1-GFP overexpression line grew 4.13-fold. Thus, enhanced levels of PfHDAC1 in *P. falciparum* were found to be associated not only with an increase in growth of parasites but also with establishment of resistance to artemisinin in PfKelch13 wildtype parasites. Whether this is by the virtue of enhanced proliferation of parasites offered

by PfHDAC1 or associated with the transcriptional changes that the parasites undergo hasn't yet been determined by us.

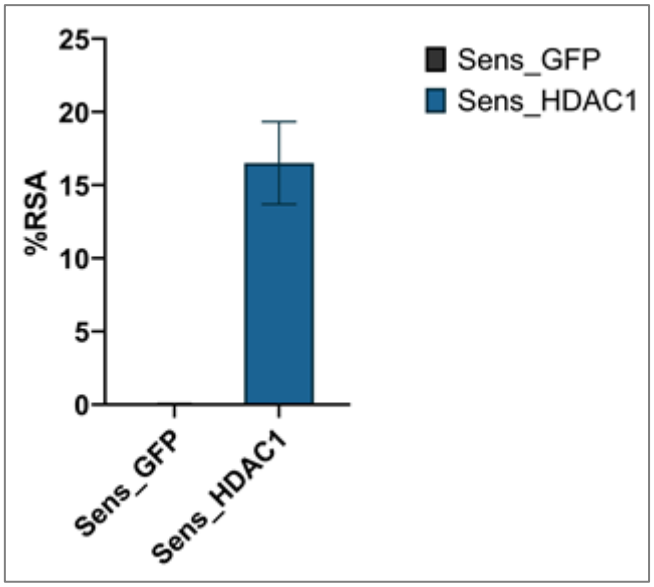


Fig. 73: PfHDAC1 overexpression is associated with establishment of resistance to artemisinin in previously sensitive strains. Bar plot for %RSA in GFP-glmS control and PfHDAC1-GFP-glmS overexpression strains.

Thus, our experiments with both the PfKelch13 wildtype and mutant parasite lines demonstrate that PfHDAC1 overexpression can not only establish artemisinin resistance in the wildtype sensitive isolates but also enhance the existing level of resistance in mutant resistant isolates. The enhanced artemisinin resistance in PfHDAC1-GFP overexpressing lines could be associated with increased growth rates offered by PfHDAC1 and potentially additional transcriptional changes associated with its increased availability.

These observed change in sensitivity to artemisinin reported under PfHDAC1 overexpression and pharmacological inhibition in addition to its role in regulating the parasite cell cycle and expression of key genes implicated in resistance (cytoadherence, proteostasis and hemoglobin digestion) lay credence to the possibility that PfHDAC1 may be an important regulator of not

only basic biological functions in the parasites but also modulate its sensitivity to artemisinin.

4.8.6 Effect of PfHDAC1 overexpression on the growth dynamics of artemisinin resistant *P. falciparum*

Our growth curve studies on the PfKelch13 wild type artemisinin sensitive strains of *P. falciparum* under conditions of PfHDAC1 overexpression had demonstrated a strong proliferation promoting phenotype. We were curious to see if the same held true under conditions of transgenic overexpression of PfHDAC1 in PfKelch13 mutant artemisinin resistant *P. falciparum* strains. 6-day growth progression curve revealed a mild increase in the proliferation of PfHDAC1-GFP-glmS overexpression cell line. The fold change in growth was assessed to be lesser than what was imparted by PfHDAC1 overexpression on the PfKelch13 sensitive lines which reported a 2-3-fold increase in proliferation from starting parasitemia (Fig. 74).

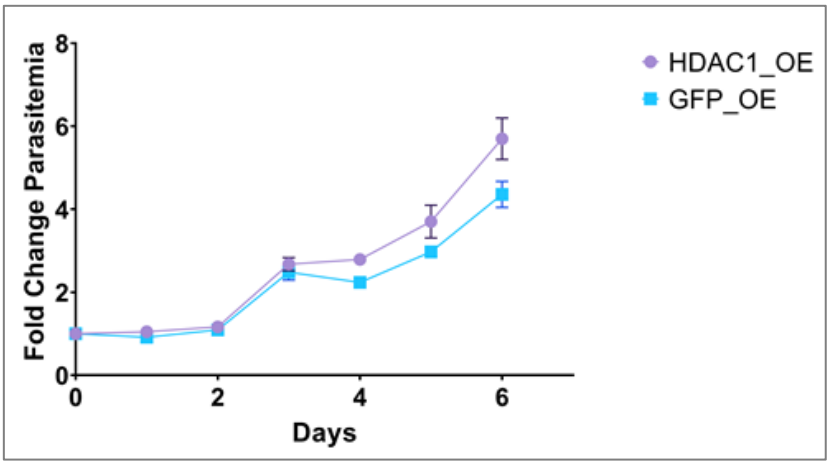


Fig. 74: PfHDAC1 overexpression is associated with mildly enhanced proliferation in PfKelch13 mutant, artemisinin resistant parasites. Line plot representing growth trend of PfHDAC1-GFP-glmS overexpression line (Purple) vs the control GFP-glmS overexpression line (Blue) over a 6-day growth assay.

In parallel cultures grown with 2.5mM glucosamine (to inhibit the transgenic overexpression of PfHDAC1-GFP-glmS or control GFP-glmS) we found a minor decline in the fold growth compared to the non-glucosamine treated culture. This decline in growth was however attributed to the basal toxicity of 2.5mM glucosamine as observed in the GFP-glmS control line (resulting in 15% drop in viability). No additional decline in parasite viability was observed upon 2.5mM glucosamine treatment in PfHDAC1-GFP-glmS overexpression in R539T lines. Furthermore, PfHDAC1-GFP-glmS overexpression vs GFP-glmS ctrl overexpression cultures were treated with 5.0mM glucosamine. We observed a basal toxicity of around 25% in the GFP-glmS culture while an additional 25% decline in viability could be observed in PfHDAC1-GFP-glmS culture. We generated the GFP-glmS growth subtracted curves for PfHDAC1-GFP-glmS in 0mM, 2.5mM and 5.0mM glucosamine treatment regime over the growth curve analysis (Fig. 75). While the subtracted curve for 2.5mM glucosamine surprisingly revealed little to no reversal of growth upon suppression of PfHDAC1 overexpression, the subtracted curve for 5.0mM glucosamine treatment did show the reversal of the growth attributed by PfHDAC1 overexpression.

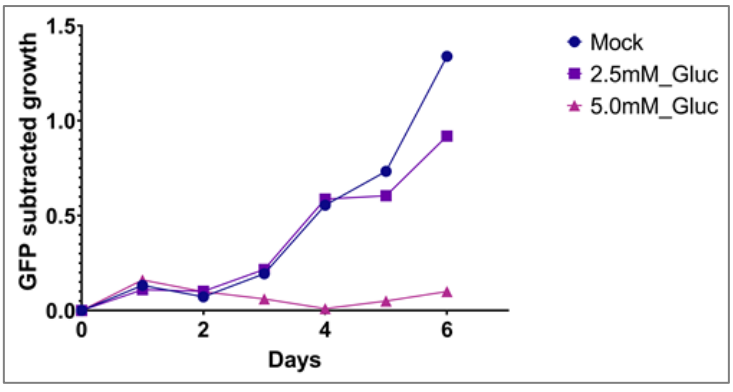


Fig. 75: Suppression of PfHDAC1 overexpression reverts the growth advantage offered by it. Line plot showing the GFP-glmS growth subtracted plot for PfHDAC1-GFP-glmS over a 6day growth curve.

Artemisinin resistant (PfKelch13 mutant) parasites are reported to have cellular proliferation defects. This is believed to be a mode of protection from the fatal effects of drug activation in the cell. To validate this, we compared the growth profile of artemisinin resistant and sensitive parasite lines for a total of 6 days/3 cycles. There was a small yet statistically significant difference between the growth dynamics of the two lines which started to appear towards the end of cycle 2, with the resistant line observed to be growing slower in comparison to the sensitive. At the end of the assay the sensitive line (PfKelch13 wildtype) had registered a close to 6-fold increase in parasitemia while the resistant line (PfKelch13 R539T) had grown by a factor of 4 (Fig. 76).

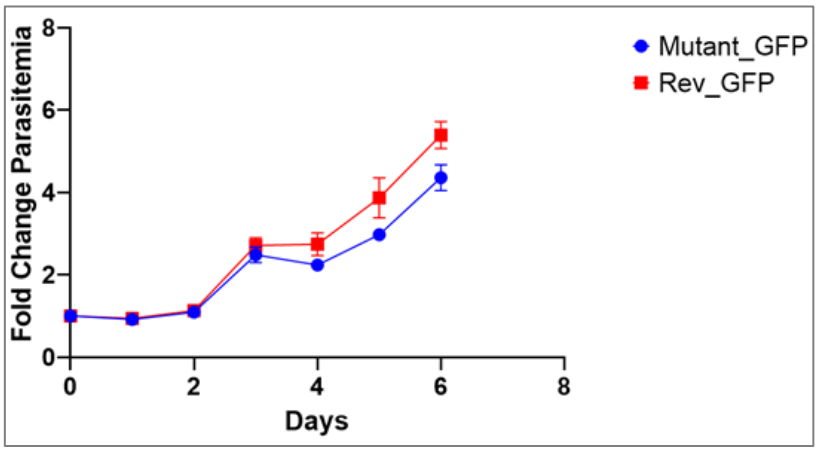


Fig. 76: Artemisinin resistant parasites have slower growth dynamics as compared to sensitive parasites. Line plot representing the fold growth increment from 1% starting parasitemia for PfKelch13 mutant (Blue) and wildtype (Red) parasites over a 6-day long growth assay.

Thus, we confirmed that the resistant line in our possession showed slower growth proliferation as reported in the field. We then compared the growth trend of the PfHDAC1-GFP-glmS overexpression on resistant line with the GFP-glmS control overexpression on sensitive line and found them to be comparable. Thus, PfHDAC1 overexpression in artemisinin resistant

lines can annul the growth defect observed in them and make them grow at a competent pace with sensitive parasites (Fig. 77).

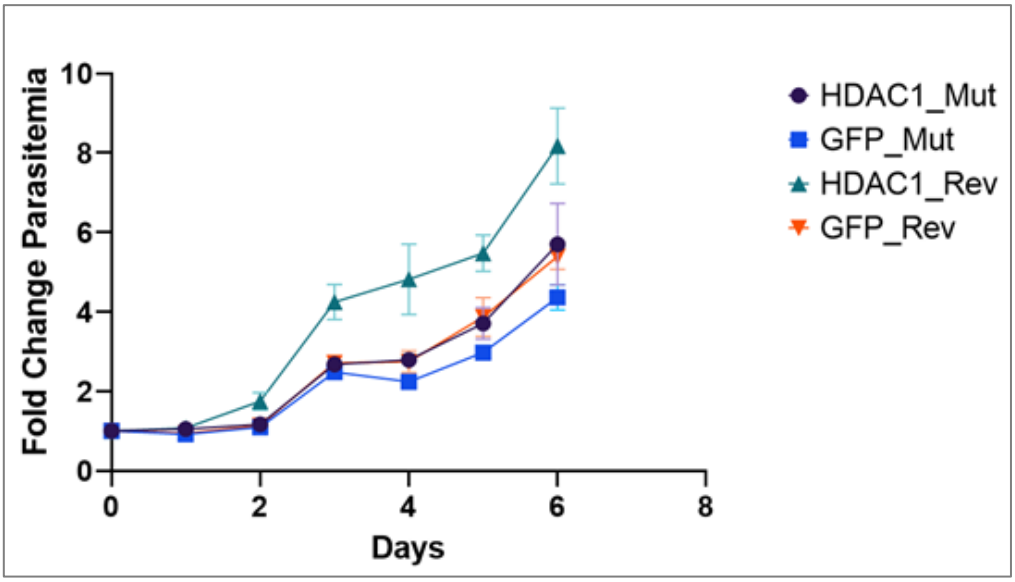


Fig. 77: Episomal PfHDAC1 overexpression can enhance the growth dynamics of artemisinin resistant parasites and revert the growth defect observed in the strains. Line plots representing the fold growth of PfHDAC1 overexpression resistant parasite (Black) vs GFP overexpression sensitive parasite (Red) show a comparable growth trend. PfHDAC1 overexpression grants growth advantage in both sensitive and resistant lines albeit higher in the former.

4.8.7 Comparative analysis of PfHDAC1 occupancy in artemisinin resistant vs sensitive strain

Since artemisinin resistant parasites reported a depletion of PfHDAC1 we were interested to check if this could cause changes in its genomic occupancy. A differential abundance of PfHDAC1 can potentially lead to differential gene occupancy in artemisinin resistant vs sensitive strains. Loss or acquisition of gene targets could lead to interesting gene expression/regulation dynamics. We performed ChIP-sequencing for PfHDAC1 in artemisinin resistant and sensitive *P. falciparum*. We identified a total of 299 genes under PfHDAC1

regulation as compared to 599 genes under its regulation in sensitive strain. This difference in gene target number falls in line with the lower abundance of PfHDAC1 in resistant strains. We observed 455 unique gene targets in sensitive strain and 155 in resistant strain. Only 44 gene targets were found to be common between the two. So, 411 genes were lost from PfHDAC1 occupancy during acquisition of resistance while 155 new targets were acquired.

Gene Ontology Enrichment analysis was performed for the gene sets under PfHDAC1 regulation in resistant and sensitive strains. The genes under PfHDAC1 in sensitive strains were associated with response to drug, proteolysis, carbohydrate metabolism, hemoglobin catabolism, oxidation-reduction, phosphorylation, cell cycle regulation and ERAD. Processes under PfHDAC1 regulation in resistant strains were mainly restricted to antigenic variation and translation (Fig. 78).

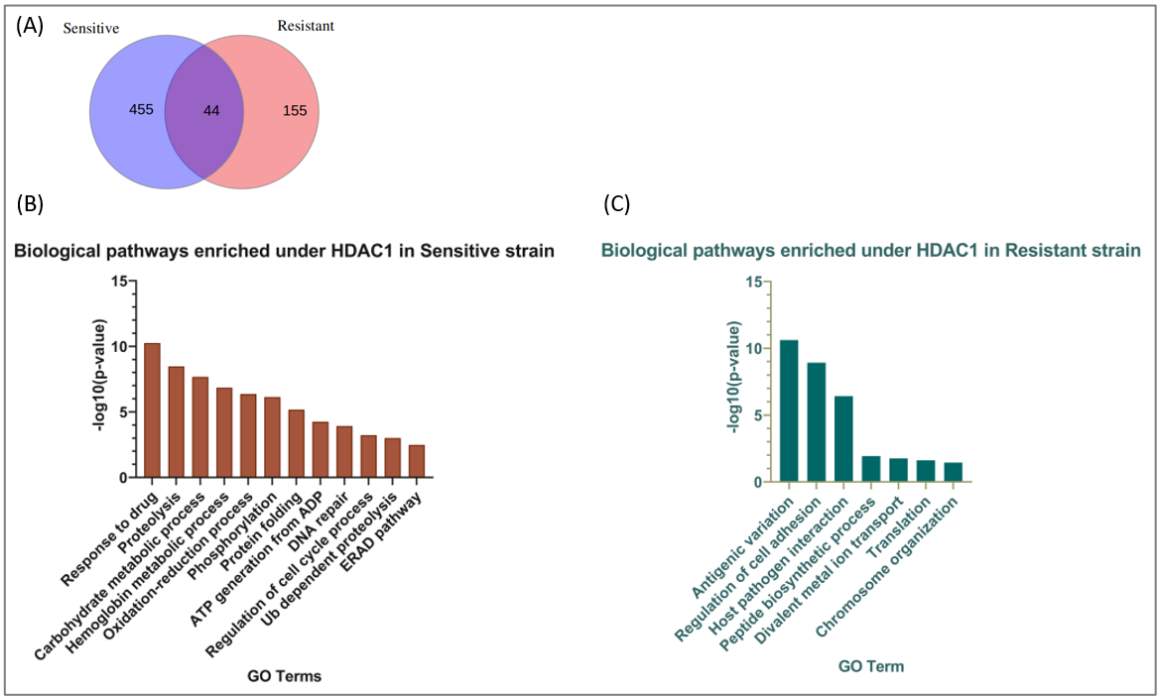


Fig. 78: PfHDAC1 is associated with a distinct set of genes in artemisinin sensitive vs artemisinin resistant parasites. (A) Venn diagram comparing the number of PfHDAC1 associated gene targets in artemisinin sensitive vs resistant parasites. Gene Ontology of

biological pathways under PfHDAC1 regulation in artemisinin sensitive (B) vs resistant strains (C).

We were curious to investigate the association of PfHDAC1 occupancy with the expression of the target genes in the two strains. Considering that PfHDAC1 occupied a higher number of gene targets in sensitive strain and lost a substantial fraction of those during the acquisition of resistance by parasites, we wanted to see what happened to the expression of the genes from which PfHDAC1 got evicted. In artemisinin resistant strain compared to the average pan genome expression profile, PfHDAC1 target genes showed a lower mean expression. This showed that PfHDAC1 occupancy was associated with reduced gene expression on the genes in resistant strains. We then checked the expression profile of the genes which were originally targets of PfHDAC1 in sensitive strain but were lost in resistant strain. The average expression profile of these genes was found to be higher than the pan transcriptome expression average. Thus, eviction of PfHDAC1 from these genes is associated with higher expression in the resistant strain. Among the genes that were most significantly upregulated in the resistant strains were chaperones PfHSP70, PfHSP90, PfBiP, PfTCP, Pfcis-trans isomerase and the multidrug resistance associated transporter MDR1 (Fig. 79). Interestingly, PfKelch13 was found to be a target of PfHDAC1 in the sensitive strain but not in the resistant strain. The expression of PfKelch13 however was not found to be variable between the two strains.

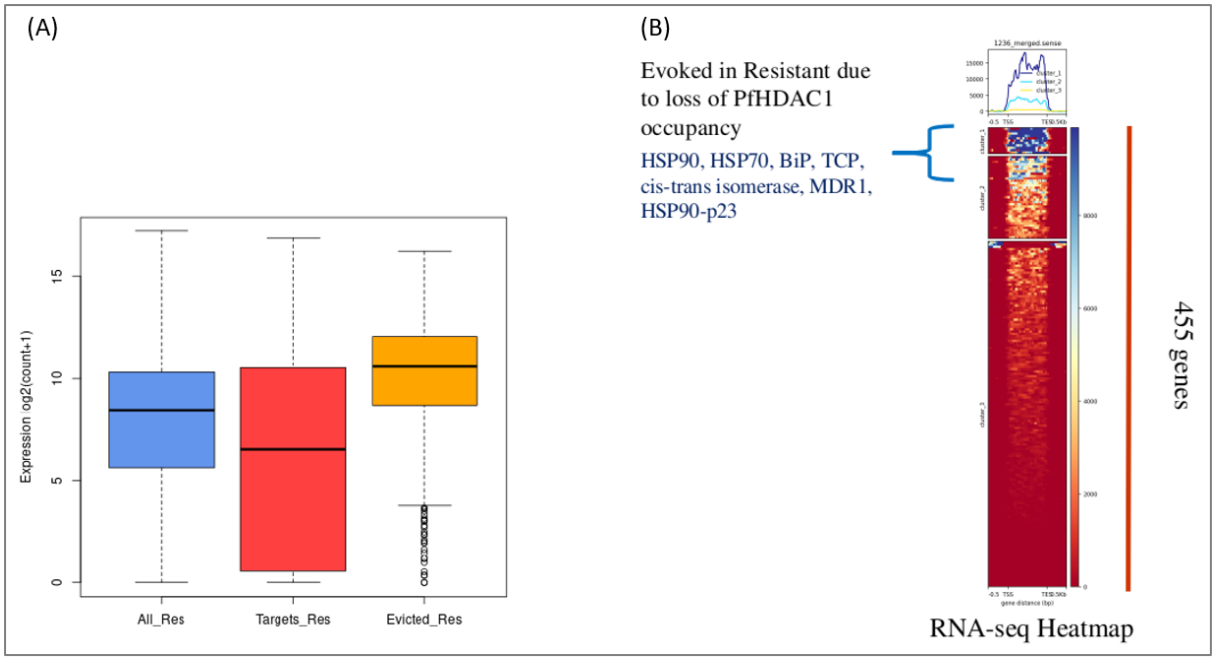


Fig. 79: Differential gene occupancy of PfHDAC1 in artemisinin sensitive vs resistant strains allows for enhanced expression of protein stress response associated genes. (A) Boxplot showing PfHDAC1 enriched genes (Red) in artemisinin resistant parasites are expressed lower than average total gene expression (Blue). Genes occupied by PfHDAC1 in sensitive strain but evicted in resistant strain show higher expression (Yellow). (B) Heatmap of gene expression of genes in resistant parasites that were occupied by PfHDAC1 in the sensitive strain shows the highest expression of protein folding assisting chaperones.

Thus, we observe a differential gene occupancy of PfHDAC1 with a lower abundance of the protein directly correlated with fewer genomic targets. The differential occupancy of PfHDAC1 in artemisinin resistance vs sensitive strain seems to influence the expression of protein homeostasis, carbohydrate metabolism and antigenic variation associated genes leading to highly enhanced expression of chaperones in resistant strains. This follows our observations with PfHDAC1 inhibition experiments where we observe an upregulation in stress response associated pathways.

4.9 Discussion

The defective cell cycle dynamics we observed for PfHDAC1 inhibition in the parasite were very similar to the stage progression defects observed in artemisinin resistant parasites [229]. A slower progression of the metabolically calm ring stage to an active trophozoite stage allows for lesser activation of the prodrug artemisinin into the active form dihydroartemisinin upon iron released from hemoglobin endocytosis and digestion in food vacuole [186]. PfHDAC1 down regulation has been reported in artemisinin resistant parasites in the field which report a diverse array of strategies to adopt resistance to the drug. Our investigation found PfHDAC1 to be lower in artemisinin resistant PfKelch13 mutant isolates thus reiterating the observations made previously. Furthermore, it's pharmacological inhibition was found to enhance artemisinin resistance in *in vitro* RSA. Thus, a slowing down of the cell cycle upon PfHDAC1 inhibition and reduced development of metabolically active trophozoites could be directly responsible for lower activation of the drug and hence improve chances of survival under drug pressure in the assay. The shift in RSA % upon concomitant PfHDAC1 inhibition seems to be independent of the PfKelch13 genotype and was observable (to different extent) in R539T and C580Y mutant lines as well as PfKelch13 wildtype lines. Interestingly, the shift in RSA% observed for parasites that were already resistant to artemisinin was found to be lesser than the sensitive parasites. A possible explanation behind this could lie in the different levels of PfHDAC1 in the parasites. Since resistant parasites already have lower PfHDAC1 the advantage brought about by this may not be that observable as compared to sensitive parasites where PfHDAC1 levels are higher and thus the activity can be affected by the inhibitor. There could be a limit to the extent to which PfHDAC1 may be able to modulate artemisinin resistance by controlling the cell cycle and stage transitions.

We had anticipated that the overexpression may revert the advantages offered by the depletion or inhibition of PfHDAC1 (slower cell cycle and enhanced homeostasis) and hence might be

associated with a drop in the level of resistance in parasites. We surprisingly observed that even overexpression of PfHDAC1 was associated with a dramatic increase in artemisinin resistance in parasites. The effect was observable on both artemisinin sensitive and artemisinin resistant parasite lines. We do not have a firm explanation for this observation, but extension of knowledge gained from overexpression of PfHDAC1 in other systems may help us better understand the observation. HDAC1 overexpression in cancers is associated with chemoresistance and enhanced cell survival under stressful environments. Higher levels of HDAC1 have been shown to be associated with robust expression of survival associated cell signalling [166, 271, 272]. These pathways have not been very well characterised in *Plasmodium*. Our observations that PfHDAC1 overexpression is associated with enhanced proliferation may be an extension of this pro survival hypothesis thus allowing for parasites to resist the effects of artemisinin. Investigation of the effects on PfHDAC1 overexpression on gene expression may allow for the explanation of this curious aspect of *P. falciparum* biology.

Furthermore, it is curious to note the differential effects of PfHDAC1 overexpression on the cellular proliferation in PfKelch13 wildtype vs mutant background. We observed a far stronger effect on growth in the wildtype background and a very nominal one in the mutant. It is possible that since the levels of endogenous PfHDAC1 are lower in the mutant vs sensitive line an overexpression of episomal PfHDAC1 results in lower overall levels of PfHDAC1 (endogenous +episomal) in the mutant line compared to the sensitive. It is also possible that the extent of overexpression permitted in the mutant strain could possibly be regulated by a feedback based on the total amount of endogenous and episomal PfHDAC1 tolerated in the parasites. Upon comparing the cell proliferation trends in artemisinin sensitive vs resistant parasites we observed that the mutant parasites tend to grow marginally slower. Overexpression of PfHDAC1 in the resistant line was however found to be sufficient to compensate for this and allow for growth trends like artemisinin sensitive parasites. This further highlights the need

to understand the transcriptional consequences of PfHDAC1 overexpression in cells. PfHDAC1 overexpression annuls the growth defect that is characteristically observable in sensitive parasites but still allows for resistance to emerge. There ought to be transcriptional consequences of its enhanced presence in cells that allow for resistance.

The differential abundance of PfHDAC1 in artemisinin resistant vs sensitive parasites is directly reflected in the genomic occupancy of the molecule with far fewer genes under PfHDAC1 regulation in resistant parasites. This differential occupancy seems to allow for suppression of stress responsive genes like chaperones (HSP70, HSP90, cis-trans isomerases) and only allow for their expression in resistant lines where they are more functionally relevant. Thus, the eviction of PfHDAC1 from key gene targets may allow for transcriptional changes that are protective to parasites upon drug exposure. It is also logical to keep the expression of these genes robust and constitutive in resistant strains since they are frequently exposed to the drug and may thus require higher protein quality control.

CHAPTER 5: Investigating the transcriptional deregulation underlying artemisinin resistance and PfHDAC1 deregulation

5.1 Population transcriptomics identify the major gene expression changes accompanying artemisinin resistance

Artemisinin resistance was identified to be multifactorial in nature owing to the large number of cellular targets it showed. Classical drugs like chloroquine or sulphadoxine and pyrimethamine target very specific aspects of the parasite biology. For instance, chloroquine targets the conversion of toxic hemozoin to a non-toxic by-product, thereby leading to its accumulation and cell death [273]. This is overcome by merely overexpressing a transporter in the food vacuole membrane and shuttling out the chloroquine into the cytoplasm where it is useless [274]. Artemisinin on the other hand causes a systemic response in the cell owing to the multiple biological pathways it targets. The parasites that evolve resistance to artemisinin show deregulation of key gene expression programs in cells. Some of the earliest population transcriptomics studies gave an insight into the possible mechanism of artemisinin resistance. Artemisinin resistant parasites were observed to have enhanced expression of proteostasis associated genes. Crucially genes of the Unfolded Protein Response pathway hinged around the ER resident chaperone BiP. Both cytoplasmic and ER- resident chaperones were reported to be highly upregulated. Furthermore, members of the *Plasmodium* reactive oxidative stress complex (PROSC) and TCP-1 ring complex (TRiC) were reported to be highly upregulated in artemisinin resistant parasites. This was observed as a strategy of the cell to repair the damage that may be caused upon artemisinin activation in the cell. Importantly by carefully profiling the transcriptional profile of the parasite every 8 hour during the IDC and comparing it with a

reference *P. falciparum* transcriptome the group was able to establish that artemisinin resistant parasites are transcriptionally slowed down compared to sensitive isolates sampled during the same interval (Fig. 80). This delay in development allowed for slower metabolic state which suppressed the activation of artemisinin. Genes responsible for glycolysis, TCA cycle were also observed to be downregulated [186].

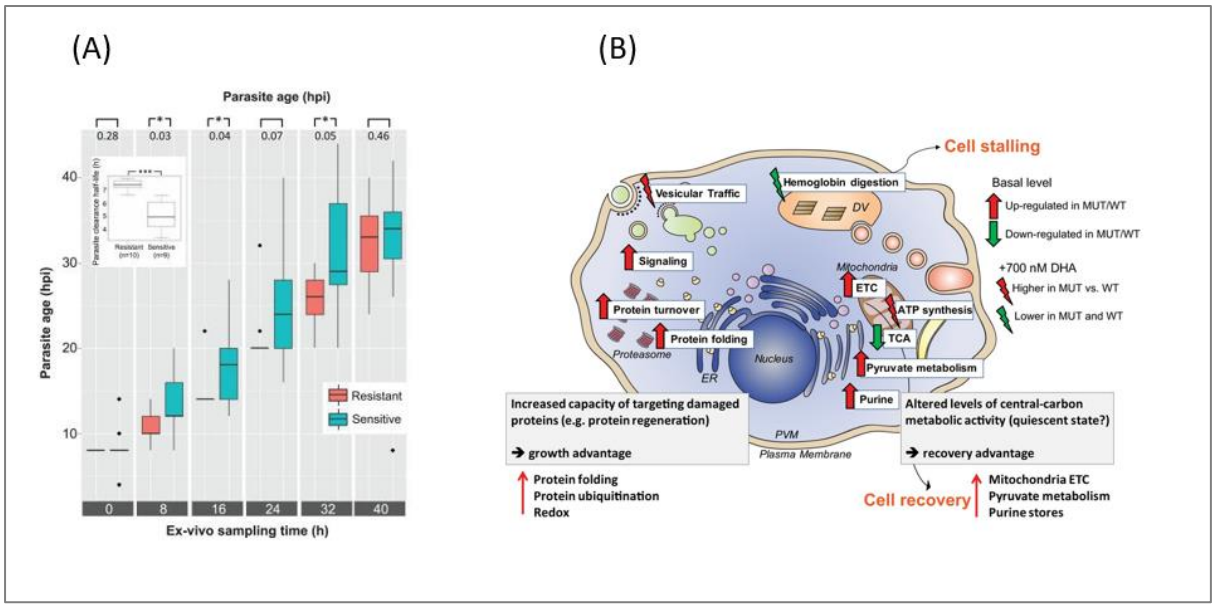


Fig. 80: A skewed cell cycle (A) and host of gene expression changes (B) underlie artemisinin resistance in P. falciparum. Resource: (A) Mok et. al., 2015; Science. (B) Mok et. al., 2021; Nature Communications [186, 269].

5.2 *In vitro* generation of artemisinin resistance reiterates the *in vivo* findings of a skewed transcriptional profile in artemisinin resistance

To further emphasise findings observed in the field, parasites were selected *in vitro* under increasing artemisinin pressure to study their phenotype and transcriptional profile. Lines that were generated resistant independently reported deregulation of some common biological pathways, namely upregulation of genes related to oxidative stress response, protein transport

and translation, autophagy, cytoadherence and mitochondrial processes. Among the pathways that were downregulated included host cell invasion, chromatin modification, phosphorylation and cell cycle [265].

Both *in vivo* and *in vitro* generated artemisinin resistant isolates reiterate the observations that the parasite on a global level tries to shut down major metabolic pathways and evokes the expression of genes responsible for damage repair and control. In the face of artemisinin treatment this seems a good enough strategy to deploy. Recent work by Bozdech's group has shown that the basal transcriptional responses that get established in artemisinin resistant parasites originate from the changes in gene expression changes observed upon artemisinin exposure in sensitive parasites. Thus, consolidation of these responses over repeated exposures may have given rise to the resistance transcriptome. Over years of selection under suboptimal drug pressure, epigenetic and mutational changes may have allowed parasites into a transcriptionally primed state that can fight off the drug's effects quicker with minimal damage to the cell. The group further shows that under-artemisinin pressure resistant parasites show a lesser degree of differential gene expression compared to sensitive parasites, possibly due to this aforementioned primed state. These transcriptional changes are focused around the redox and proteostasis mechanisms in the cell [275].

5.3 PfKelch13 mutations and the trade-off between growth vs resistance

Artemisinin resistance was identified for the first time in Southeast Asia and shown to be causally linked with mutations in the PfKelch13 gene. Multiple mutations in the propeller domain (up to 26 non synonymous mutations) of the gene have been associated with artemisinin resistance in the field (Fig. 81) [276]. The most prominent of these are C580Y followed by R539T and I543T. These mutations are spread across the Southeast Asian region

at different frequencies [277]. The mutations are largely mutually exclusive with only one major mutation existing in the parasite. It is believed that the mutations exert a strong destabilising effect on the protein stability and thus only one mutation is tolerated in an isolate [278]. Independently though multiple mutations have evolved in the region, each associated with a definite survival score (represented as %RSA). PfKelch13 mutations are believed to destabilise the protein leading to less activity/levels in cells. The protein has been associated with endosomal compartments in cells that help take in the hemoglobin host cytoplasm which is late digested in the food vacuole. Thus, the PfKelch13 availability is directly associated with the level of host cytoplasm endocytosis which supplies hemoglobin for growth and development of the parasites. However, the digestion of hemoglobin releases iron which can activate artemisinin in cells. Thus, artemisinin resistance is established as a fine balance between the depleted bioavailability of heme (for parasite growth) vs enhanced survival under artemisinin stress. So, while the parasite grows slowly it is able to withstand the deleterious effects of the drug [279]. Over the years different mutants of PfKelch13 have been associated with different levels of artemisinin resistance and have spread across Southeast Asia in a dynamic, population reshaping process driven by this cost vs benefit effect [280].

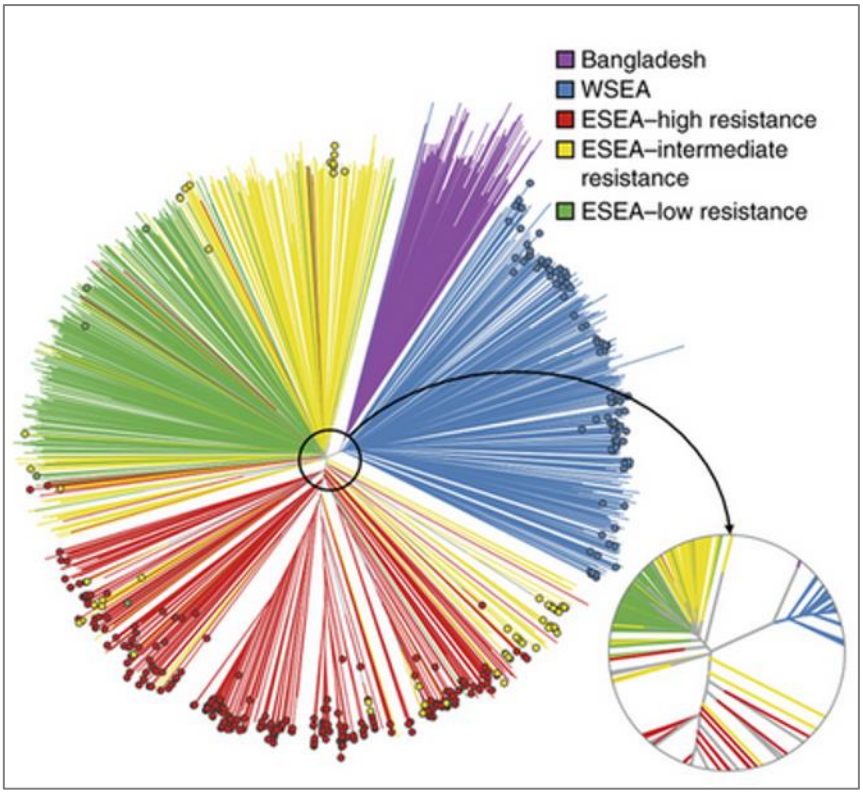


Fig. 81: Diversity in the population of the P. falciparum strains from Southeast Asian countries. Population structure and distribution of PfKelch13 mutants in Southeast Asia. (a) Neighbour-joining tree showing population structure across the 15 Asian sampling sites. Branches with coloured tip symbols indicate that the samples are PfKelch13 mutants, whereas those without tip symbols are wild type for PfKelch13. Source: Miotto et. al., 2015; Nature Genetics [281].

5.4 Heterogeneous gene expression underlies phenotypic plasticity in parasites showing artemisinin resistance in Southeast Asia

With thorough investigation of the transcriptional states of artemisinin resistant parasite populations in Southeast Asia another theory has emerged which speculates an additional layer of transcriptional regulation defining differential artemisinin resistance observed in the field. Artemisinin resistant parasites in the region show segregated populations that cluster distinctly based on their gross transcriptional profile. So, there is an inherent heterogeneity at the gene expression level in the parasite cohorts of Southeast Asia.

Comprehensive datasets of parasite genotype (SNPs) and gene expression patterns from different cohorts of artemisinin resistant parasites have bolstered GWAS, TWAS and eQTL surveys. There is evidence that specific transcriptional profiles may exist in parasites that could potentially drive the cellular/physiological programs within parasite cells. Subtle changes in the transcriptional profile may possibly be driving the establishment of distinct physiological states in the region (Fig. 82). These transcriptional profiles are overlaid on a complex interaction of environmental pressures and selective evolution of genotypes over decades long environmental pressures. Not only is the transcriptional variation regulated genetically it is also governed epigenetically by factors that can sense environmental perturbations in real time and evoke rapid responses in parasites. Such gene expression regulatory mechanisms are pruned and shaped by environmental variables and can often confer survival advantage to pathogens under adverse conditions [270].

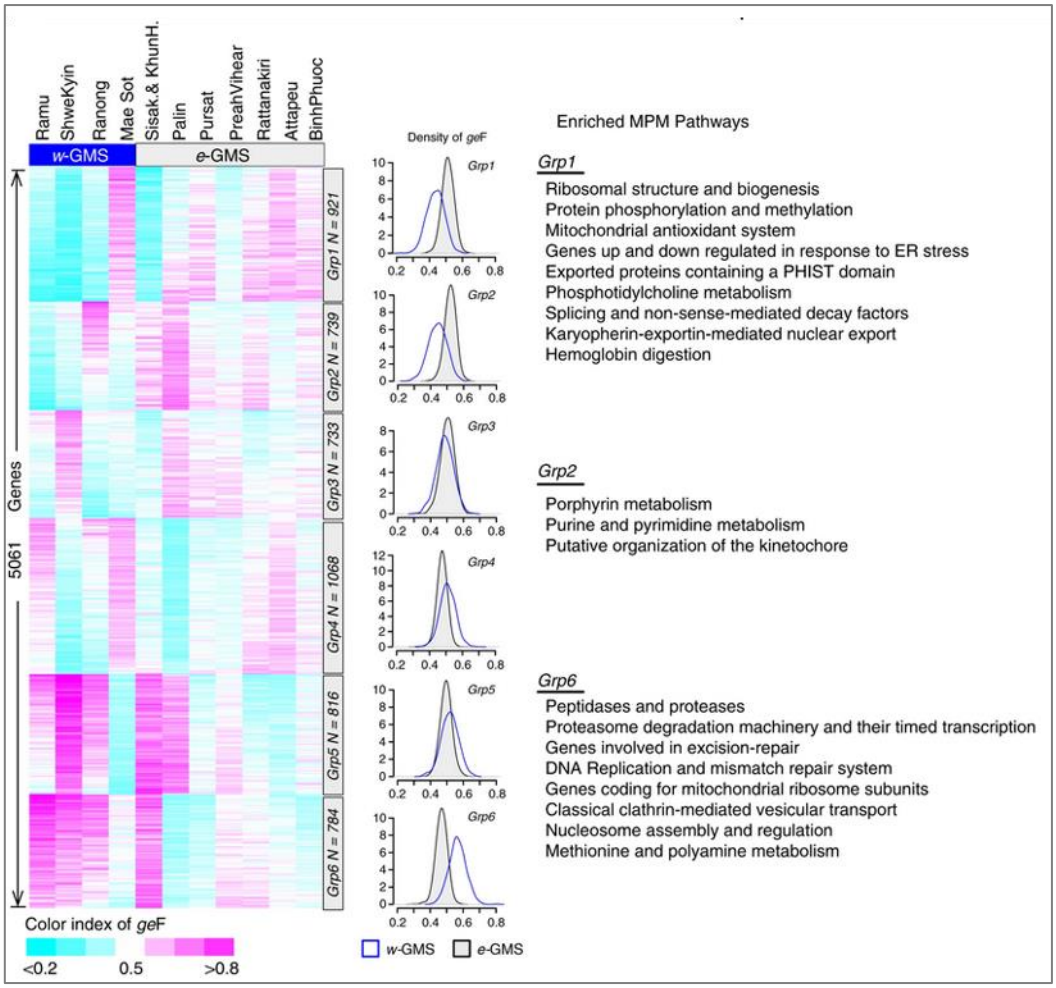


Fig. 82: Transcriptomic population structure of P. falciparum isolates, by geography in Southeast Asia. Source: Zhu et. al., 2018; Nature Communications [270].

This school of thought is further supported by the emergence of artemisinin resistant isolates from other parts of Asia independent of mutations in PfKelch13 [282]. There is evidence that the genetic background of the parasite may harbour mutations which allows for selection of PfKelch13 mutations in a drawn-out evolutionary process. These mutations may also drive differential expression of genes by serving as eQTLs [270]. Thus, the complex transcriptomic landscape of artemisinin resistance demands further inspection. The role of epigenetic factors that may potentially allow deregulation of gene expression for better resilience to artemisinin needs further validation.

5.5 A complex interaction of cell cycle dynamics, genetic background, transcriptional responses and PfKelch13 mutations may underlie artemisinin resistance

Recent multi omics studies have condensed our decade worth of understanding of artemisinin resistance. A crosstalk between genetic mutations in parasites may supplement the establishment of PfKelch13 mutations (perhaps to balance out its effects on cell). This may be overlaid with transcriptional and proteomic changes that emerge and strengthened over time to provide protection from the harmful effects of the drug (by enhanced proteostasis) (Fig. 83). Furthermore, slower progression of the cell development dynamics may allow for artemisinin resistance to emerge. Thus, artemisinin resistance by its very nature is highly pleomorphic. Any factor that can aid in suppression of drug activation and enhance the defence mechanisms in the parasite may contribute to it. Reports of PfKelch13 mutation independent emergence of artemisinin resistance hint at additional mechanisms that may stimulate artemisinin resistance in the field.

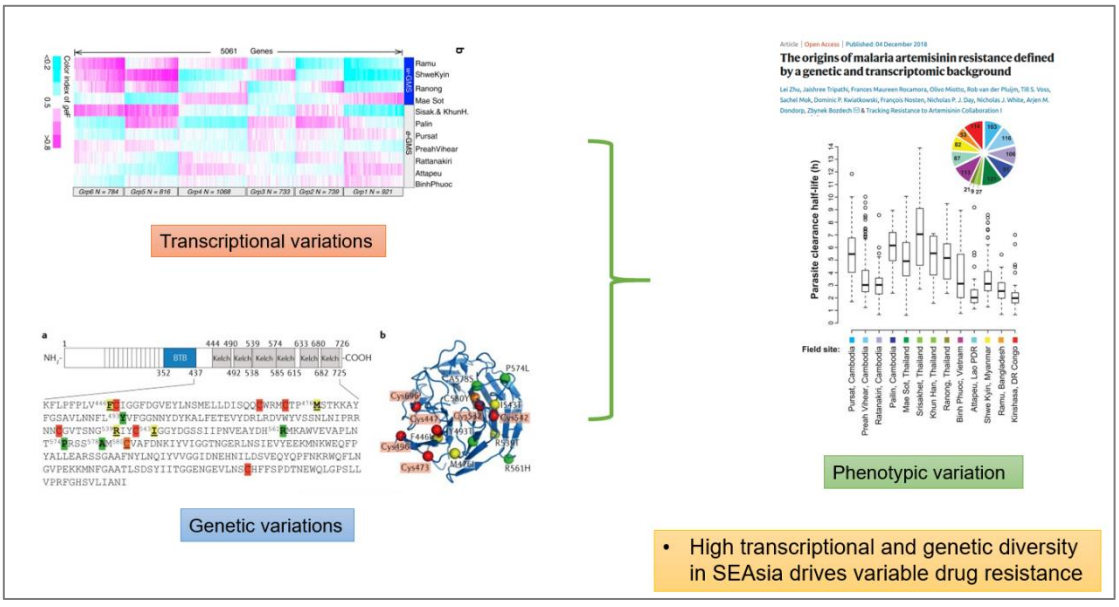


Fig. 83: A combination of genetic and transcriptomic elements drives the phenotypic diversity in artemisinin resistant P. falciparum.

5.6 PfHDAC1 regulated gene targets are highly reminiscent of pathways deregulated in artemisinin resistance

A brief introspection of the gene targets that underlie PfHDAC1 regulation (implicated in cytoadherence, protein refolding, ubiquitin mediated protein catabolism, autophagy, and cell cycle) reveals they are very similar to the genes and pathways that are implicated in resistance [269]. Furthermore, our data suggests that inhibition of PfHDAC1 can skew the cell cycle to slow down the progression from ring to trophozoite stage of the parasites. Furthermore, enhanced proteostasis responses may also allow for improved combat against the effect of artemisinin. Thus, we were curious to further investigate if the deregulation of PfHDAC1 could lead to transcriptional changes that could prime the parasites to withstand the effects of artemisinin and develop resistance. A comparative transcriptome analysis was performed to investigate the transcriptional effects of PfHDAC1 depletion in parasites and compare them with the transcriptional changes observed in artemisinin resistance (Fig. 84).

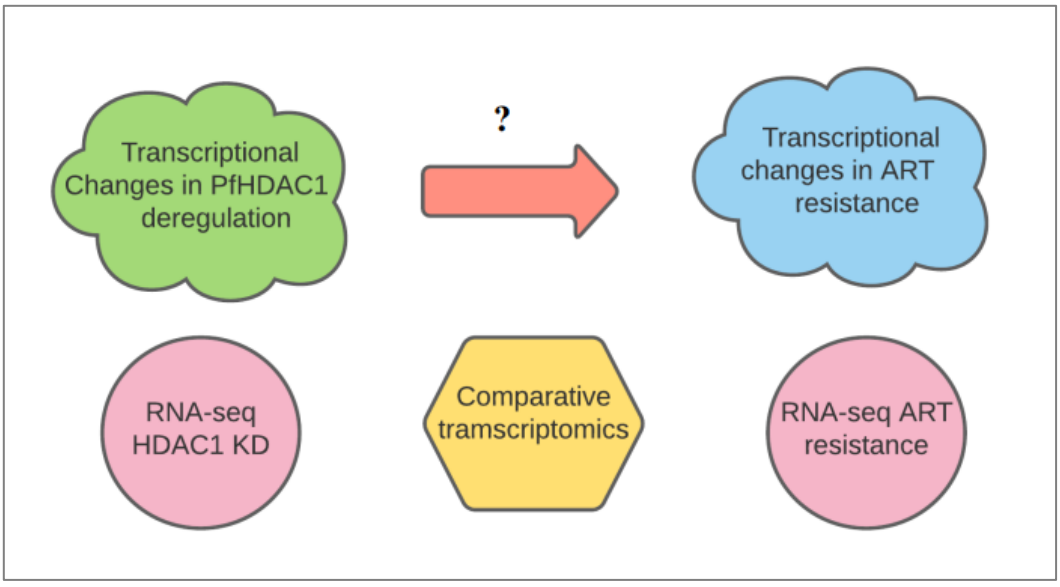


Fig. 84: Comparative transcriptomics of artemisinin resistant P. falciparum and PfHDAC1 depleted P. falciparum to trace the level of similarity between the two.

5.7 Material and methods

5.7.1 Generation of knock sideways constructs for PfHDAC1

For the knock sideways construct, 750bp of the C-terminal region of PfHDAC1 was cloned into the pSLI-2xFKBP-GFP plasmid generated by Dr. Tobias Spielmann team and deposited to Addgene [283] (Primer details in Table 3/Appendix 1).

5.7.2 *P. falciparum* transfections

Lonza nucleofector 4D was used for transfection of plasmids into highly synchronised parasites. 80µg of knockin plasmid was dissolved in 100µl of P3 primary solution. Double synchronised (Percoll centrifugation) segmented schizonts were mixed with the DNA/P3 solution mix and nucleoporated in the nucleofector 4D machine with the pulse program FP 158 (designed for *P. berghei*). The zapped cells were immediately transferred to a T25 flask with 3 ml of media and 200µl fresh RBC and set on a shaker incubator at 37ºC for 2 hours to allow the ruptured merozoites to invade the fresh cells. The flask was later supplemented with additional media to make it to 2% hematocrit. Drug selection was started 24 hours post transfection. Appearance of transgenic lines was checked initially via Giemsa smear.

5.7.3 Cloning of the transgenic parasite lines

The validated transgenic lines were serially diluted in a 96 well flat plate to obtain 200µl cultures with finally 1 parasite per well. These were confirmed with plaque formations and then transferred onto round bottom 96 well plates for expansion. The cells were then microscopically confirmed for GFP expression and then expanded into flasks.

5.7.4 Validation of PfHDAC1-2xFKBP-GFP transgenic line

Confocal microscopy and western blotting using anti-GFP primary antibody was used to validate the PfHDAC1-2xFKBP-GFP transgenic line

5.7.5 Ring-stage survival assay

Tightly synchronised (double Percoll density gradient centrifugation) 0-3 hr ring stage parasites were taken at 1.5% parasitemia and 2% hematocrit in 200 ul volume in 96 well plates. Parasites were then either treated with DMSO mock or with 700nM dihydroartemisinin for a duration of 6 hrs following which the mock/drug was washed off and the parasites were reinstated in culture. They were allowed to grow for another 66 hrs. At the end of 72 hours live parasites were counted using Giemsa smears for at least 10,000 cells in both mock and DHA treated cultures. The percentage of cells surviving DHA treatment relative to the mock treatment were logged as % RSA. For the transgenic lines generated at the Crick institute the RSA was performed similarly the parasitemia was however calculated by flow cytometry for SYBR Green I and MitoTracker signal in the DHA vs mock treated culture.

5.7.6 Culture, RNA isolation and sequencing of PfKelch13 mutant/WT strains from MR4

The MR4 isolates were cultured as per the standard protocol. For RNA sequencing each strain was cultivated in triplicate at 5% parasitemia and 2% hematocrit after double Percoll synchronisation. At 24 hr post invasion the culture was harvested by saponin lysis method and the pellet was resuspended and stored in TRIzol until further processing. RNA isolation from the TRIzol samples was carried out as per standard operating protocol described previously. The RNA was quantified using Nanodrop and Qubit fluorometer. 500ng-1.5ug of RNA was used for cDNA library preparation using the Agilent SureSelect XT strand specific RNA library

preparation kit. The size of the libraries was verified using Agilent Bioanalyzer to be around 350bp. Libraries were quantified and multiplexed at a final pool concentration of 4nM (individual and total pool). 1.5pM of 1% PhiX control spiked library was finally sequenced on the Illumina NextSeq 550 sequencer using 150bp paired end mode.

5.7.7 RNA sequencing data analysis

The sequencing reads were demultiplexed using the bcl2fastq tool. Read quality check was performed using the FASTQC tool in Linux. Subpar reads (not passing the Q30 score) and the adapter sequences were trimmed off using Trim_Galore. The quality checked reads were then aligned onto the *P. falciparum* genome using Hisat2. The aligned reads were then sorted with SAMTools into BAM files. HTseq-count was used to quantify the gene expression counts in individual samples in union reverse stranded mode. Differential gene expression analysis was performed in DESeq2 on the R platform. MAPlot was generated using the MAPlot command in DESeq2. Volcano plots to visualize differentially expressed genes were generated in R using ggplot2. Principal component analysis was performed in R using the PlotPCA command in DESeq2 (using the normalized gene counts generated by DESeq2). Gene ontology enrichment analysis was performed on the online widget offered by PlasmoDB database.

5.7.8 Additional RNA sequencing dataset for PfHDAC1 knockdown

The RNA sequencing dataset produced by Huang et. al. is still not in the public domain. However, we utilized their supplementary dataset containing the gene expression counts for PfHDAC1 wildtype and knockdown parasite lines [207]. Gene Ontology analysis was done on the list of upregulated and downregulated genes using the PlasmoDB Gene Ontology widget.

Boxplots for comparison of genes from specific pathways in WT vs KO condition were generated in R using the boxplot command.

5.8 Results

5.8.1 Ring-stage survival assay on MR4 isolates with different PfKelch13 background

We procured 3 pairs of PfKelch13 mutant vs wildtype strains from the MR4 repository (submitted by David Fidock's team). These were the MR4 isolates MRA-1236/MRA-1254 with the C580Y/WT genotype; MRA-1241/MRA-1253 with the I543T/WT genotype and MRA-1240/MRA-1252 with the R539T/WT genotype. These isolates were originally sourced from different geographical regions of Southeast Asia, genetically edited at the PfKelch13 locus and submitted to MR4 repository [266]. The C580Y isolate was procured from Pailin in western Cambodia, the I543T from Mondulkiri in eastern Cambodia and the R539T from Battambang in eastern Cambodia.

Ring-stage survival assay was performed on highly synchronised ring stage parasites. The PfKelch13 wildtype isolates in each pair reported a sensitive phenotype with <1% RSA. The mutant strains however reported different levels of artemisinin resistance unique to each genotype. The C580Y line reported the minimal ring-stage survival of 4.5% associated with early low-level resistance. I543T was found to be associated with an average ring-stage survival of 29% while the R539T mutant reported a strong 45% RSA. We thus observed varying degrees of resistance associated with specific PfKelch13 mutations and geographical origins of these isolates (Fig. 85).

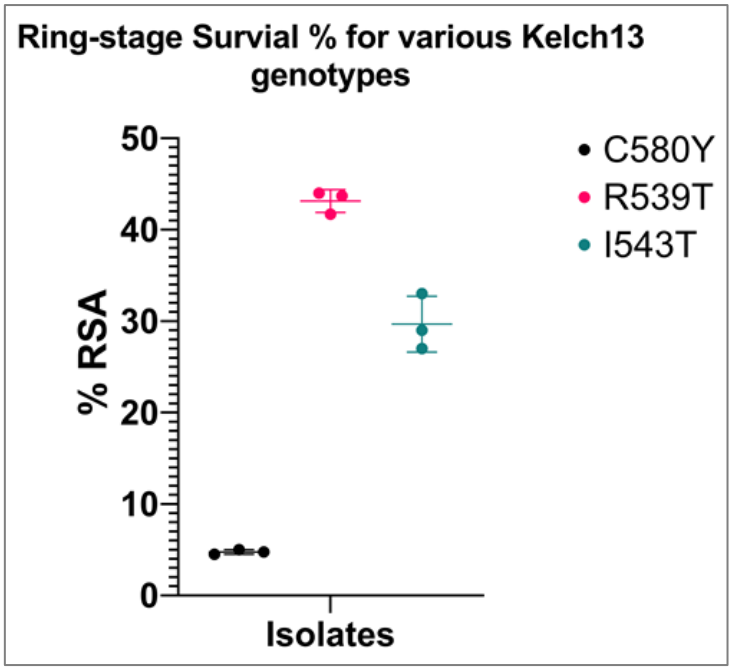


Fig. 85: RSA% of the artemisinin resistant, PfKelch13 mutants procured from MR4 resources displays a diversity in the level of resistance to artemisinin. C580Y mutation was associated with low level resistance, while both I543T and R539T were found to be associated with higher grades of resistance.

5.8.2 Whole transcriptome analysis and principal component analysis of PfKelch13 isolates

We performed strand-specific whole transcriptome sequencing of the isolates and assessed their similarity based on global gene expression trend using principal component analysis (Fig. 86). The biological replicates sequenced for the individual strains were found to cluster compactly indicating their similarity. An exception to this observation was the I543T_Rep3 which was found to cluster distantly. This was found to be due to sequencing coverage discrepancy for the sample.

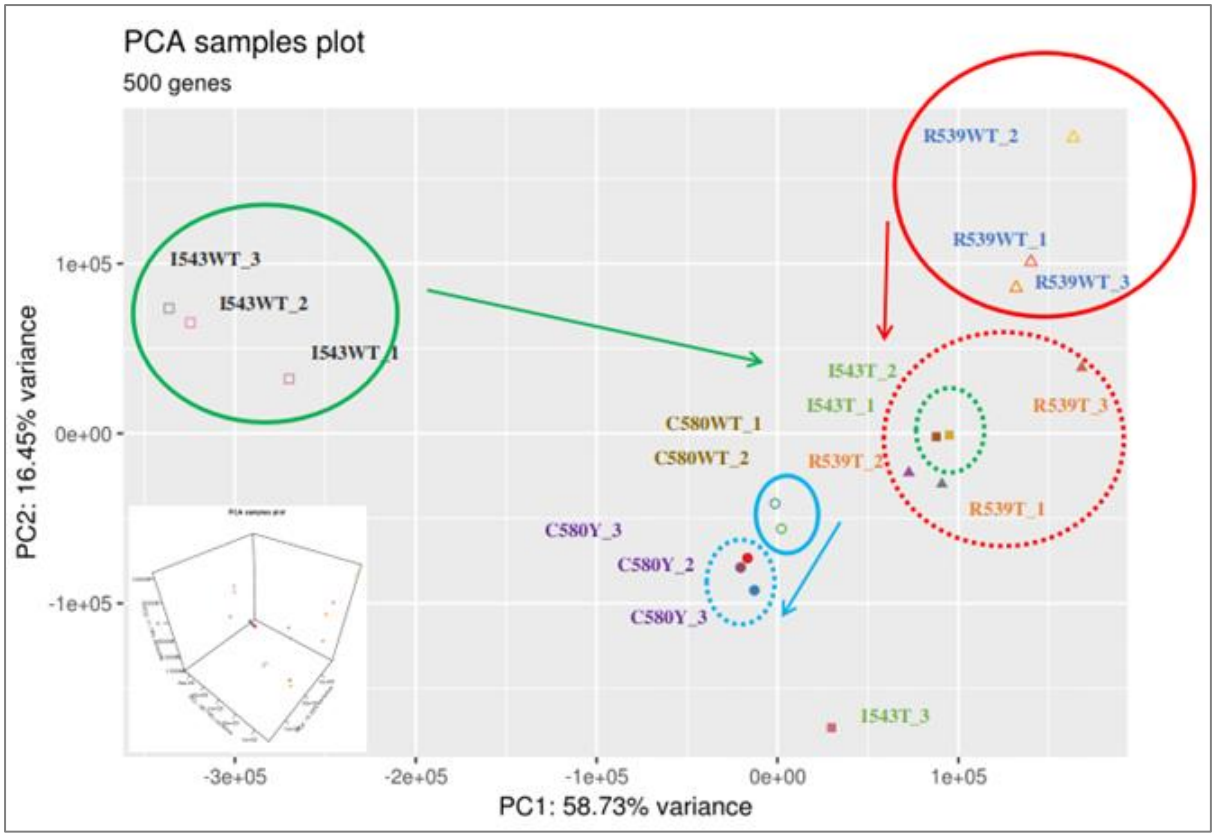


Fig. 86: Principal component analysis plot to depict the transcriptional similarity between the three PfKelch13 mutant vs sensitive isolates. Sensitive isolates are segregated farther apart whereas the mutant resistant isolated drift to cluster closer together.

The sensitive strains with wildtype PfKelch13 genotype were clustered distantly while their PfKelch13 mutant counterparts were found to cluster closer to each in the 2D space. This indicated a pattern of confluence where the originally segregated sensitive strains were converging in their gene expression trends to appear closer to each other. The extent of shift observed in the 2D space was different for each of the sensitive/mutant pairs. The C580Y was found to shift minimally from the parental C580WT strain, while the shift was more pronounced for the R539T from the parental R539WT. The starkest shift was observed for the I543T mutant line which seemed to indicate a considerable degree of transcriptional change.

Differential gene expression analysis was performed using HTSeq-count and DESeq2 algorithm for the individual sensitive vs resistant strains. A total of 636 (250 upregulated, 386 downregulated) genes were deregulated in the C580Y isolate, 983 genes (348 upregulated, 635 downregulated) for the R539T isolate and 2360 genes (1170 upregulated, 1190 downregulated) for the I543T isolate (Fig. 87). In general, we found a higher number of genes deregulated with isolates reporting stronger resistance grades (I543T and R539T) although an absolute linear correlation between the number of genes deregulated and grade of resistance (%RSA) couldn't be drawn. The extent to which the sensitive vs resistant counterparts were segregated in the 2D principal component space was reflected by the number of genes deregulated between them. For instance, maximum shift was noted between I543T and I543WT (with a total of 2360 genes deregulated between them), moderate for R539T vs R539WT (with a modest 983 genes deregulated) and the minimum observed between C580Y and C580WT (with only 636 genes deregulated between them) (Fig. 88).

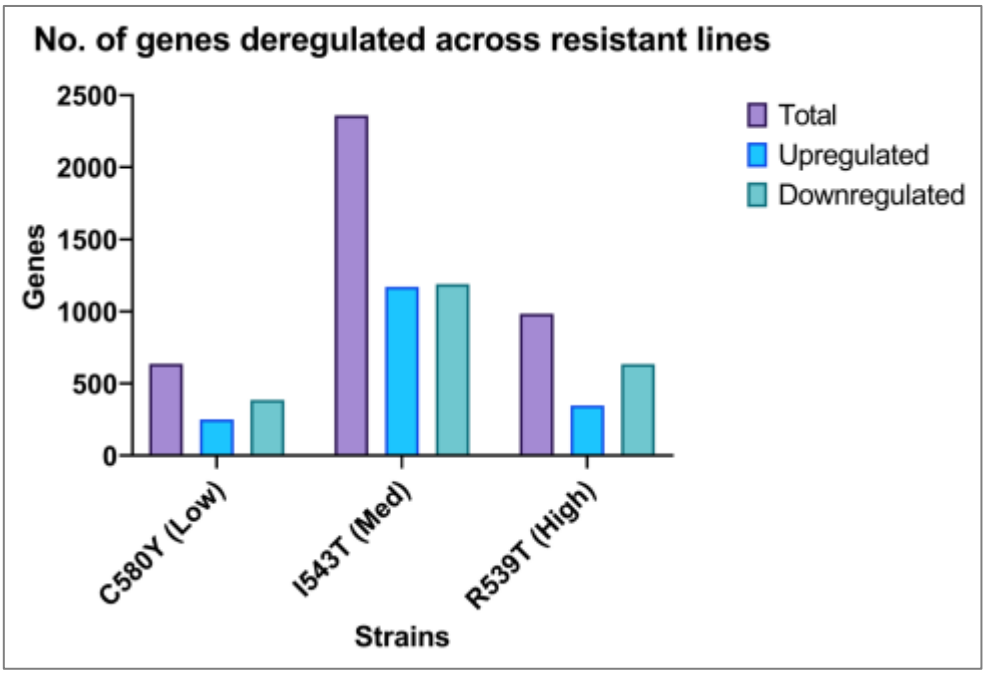


Fig. 87: Bar plots depicting the extent of gene deregulation (total, upregulated and downregulated) observed in the various PfKelch13 mutants. Maximum gene deregulation is

observed for PfKelch13 I543T mutant which shows around 29% moderate resistance to artemisinin.

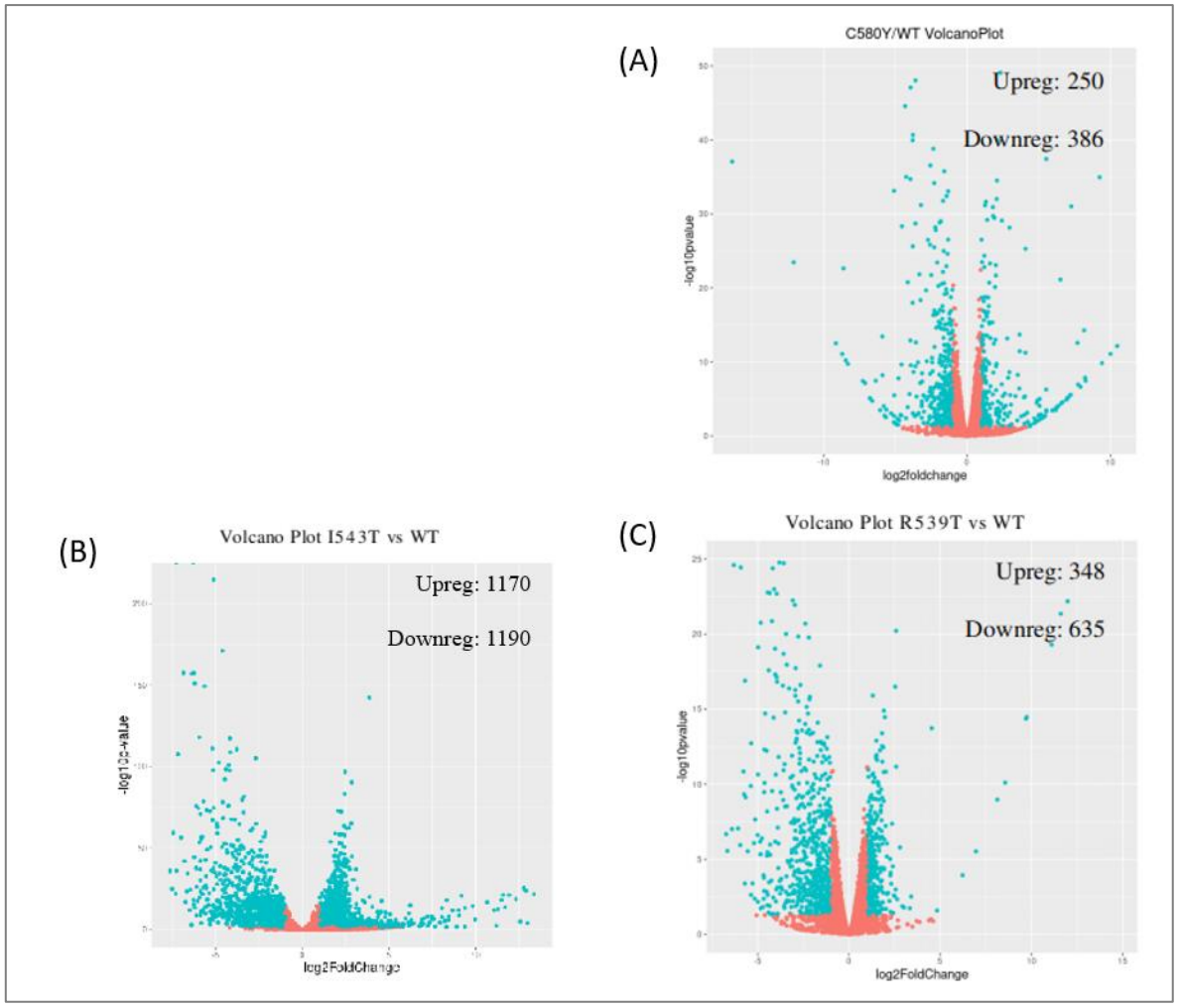


Fig. 88: Volcano plots depicting the gene deregulation across the three pairs of PfKelch13 mutant/resistant isolates [C580Y/WT (A), I543T/WT (B) and R539T/WT (C)].

5.8.3 Assessment of the gene deregulation underlying artemisinin resistance in different PfKelch13 isolates

To identify the genes and the biological pathways that get deregulated in the three resistant isolates we performed differential gene expression analysis followed by gene ontology enrichment analysis. We were particularly interested to see if there were a common set of

biological pathways whose deregulation was conserved in all artemisinin resistant field isolates and if there were any unique biological pathways whose deregulation could be associated with different grades of resistance observed across the three isolates. We checked the top 50 most significantly deregulated genes (based on adjusted p-value) in each of the three pairs of resistant sensitive isolates. Interestingly, these genes were enriched for biological pathways related to host pathogen interactions (Fig. 89). Enrichment for the terms cytoadherence, host cell invasion was highlighted for the most significantly deregulated genes at all grades of resistance.

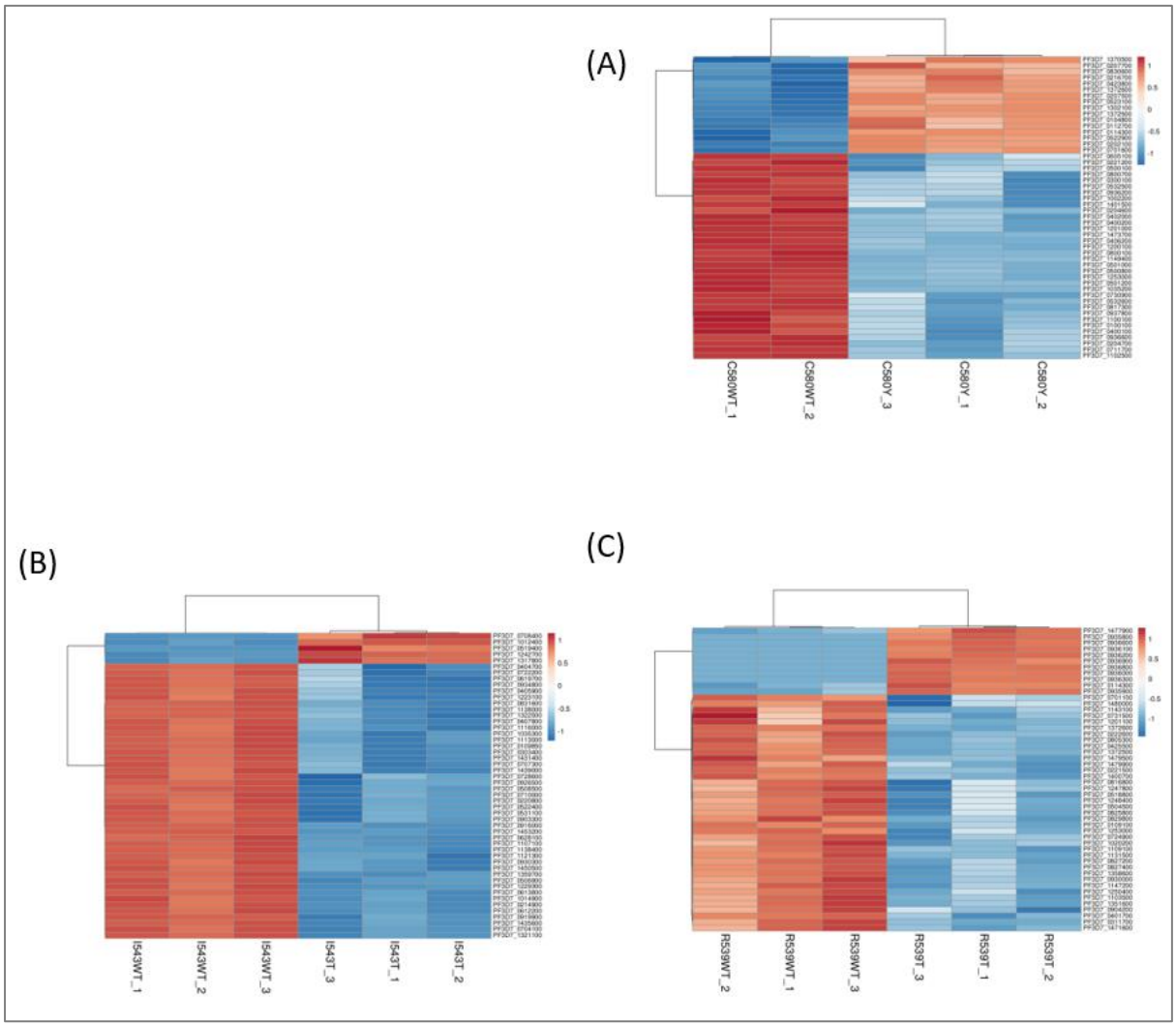
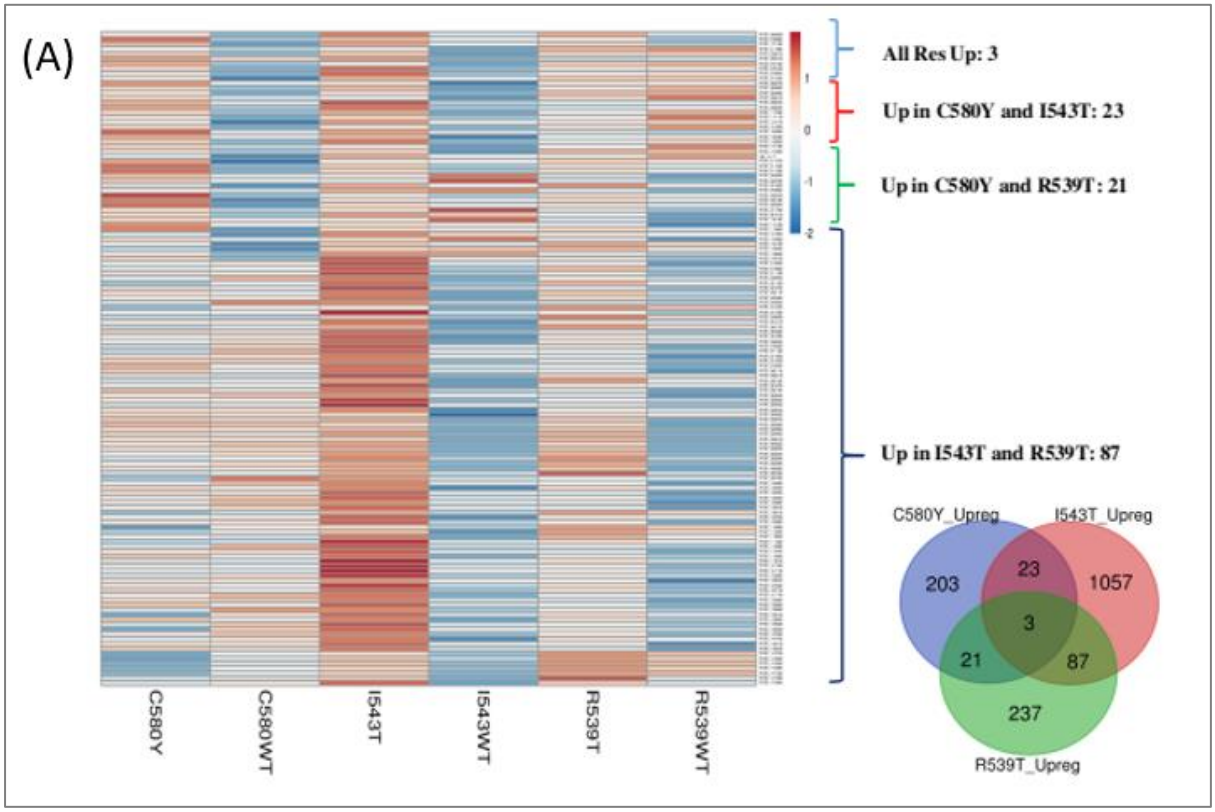


Fig. 89: Artemisinin resistance is associated strongly with changes in the expression profile of antigenic variation associated genes. Heatmaps depicting the top 50 genes deregulated across

the three pairs of PfKelch13 mutant/WT isolates characterized [C580Y/WT (A), I543T/WT (B) and R539T/WT (C)]. A vast majority of these genes are associated with host pathogen interaction and antigenic variation.

We next checked the number of genes that were deregulated commonly across all the three resistant isolates. Among the upregulated common genes, only 3 genes were found to be upregulated in all the three resistant isolates. 23 genes were upregulated in C580Y and I543T, 21 in C580Y and R539T and 87 in I543T and R539T. Among the commonly downregulated genes across the resistant isolates 15 genes were downregulated across all the three resistant isolates. 21 genes were downregulated in C580Y and I543T, 91 genes were downregulated for C580Y and R539T and 186 genes for I543T and R539T. A higher number of genes were found to be commonly deregulated for R539T and I543T than C580Y, but this could simply be because of the greater number of genes that are deregulated for the two resistant isolates (Fig. 90).



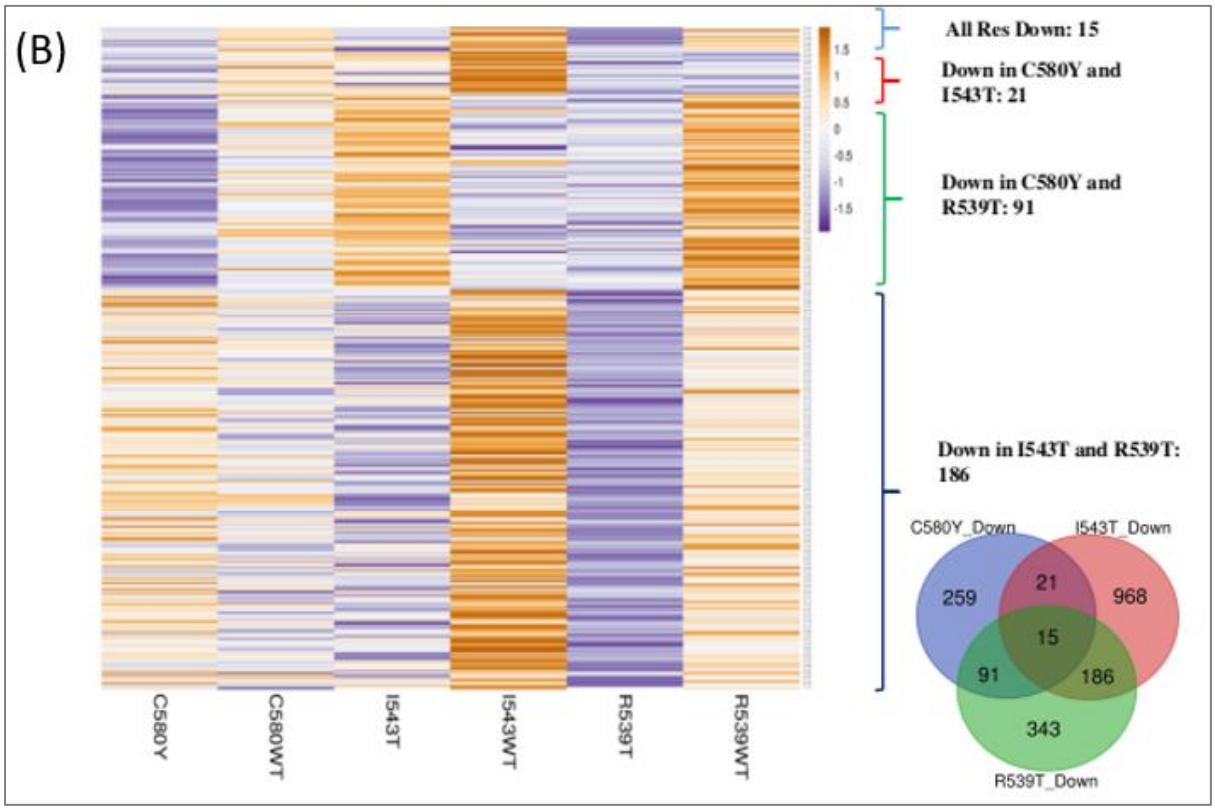


Fig. 90: Similarity in the genes deregulated across PfKelch13 mutant/sensitive pairs. Heatmaps depicting common gene sets deregulated (upregulated in (A) and downregulated in (B)) across the three pairs of PfKelch13 mutant/sensitive isolates. Inset: Venn diagrams show the numbers of upregulated or downregulated genes common across the three PfKelch13 mutant sets.

To further identify the biological pathways that are commonly vs uniquely deregulated across each of the resistant/sensitive isolates showing different levels of resistance Gene Ontology enrichment analysis was performed on the set of deregulated genes (Fig. 91). We discovered several pathways that were enriched in the three isolates but to varying extent. Genes related to entry into host cells were deregulated across all the three resistant isolates, but the enrichment was the strongest for R539T followed by I543T and finally C580Y. The C580Y isolate showed very strong enrichment for genes related to antigenic variation, while a weaker enrichment was observed in the R539T and I543T isolates. The C580Y and R539T isolates further commonly

displayed enrichment for genes related to phosphorylation. All the isolates showed a small but significant enrichment of genes associated with cell cycle. Elsewhere, R539T and I543T both had enrichment for lipid metabolism related genes to similar extent.

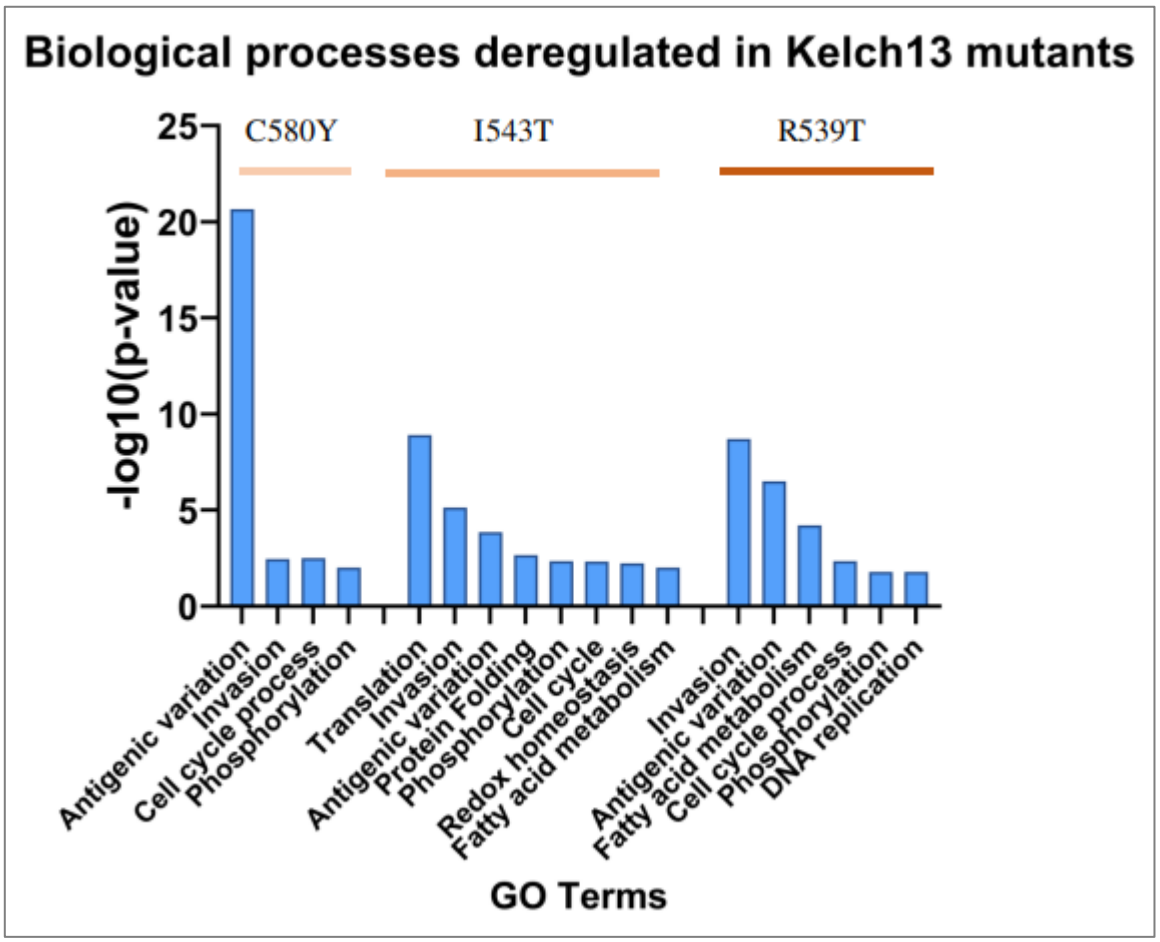


Fig. 91: Artemisinin resistance across different grades and PfKelch13 genotypes is characterised by deregulation of genes related to a core set of biological processes (antigenic variation, host pathogen interaction, cell cycle and fatty acid metabolism). Bar plots depicting the GO terms for biological processes deregulated commonly across the different PfKelch13 mutants.

Thus, antigenic variation, host pathogen interaction, phosphorylation and lipid metabolism associated genes are found to be highly deregulated across the three distinct artemisinin resistant isolates. These pathways may constitute a core set of responses that are enacted in

response to the threat of artemisinin in *P. falciparum*. We found that the extent to which individual PfKelch13 mutant strains rely on these pathways may differ with some showing higher preference/deregulation of antigenic variation associated genes (viz. C580Y).

We next investigated which biological pathways were exclusively deregulated in the specific PfKelch13 mutants (Fig. 92). The C580Y mutant line reported a strong deregulation of genes associated with antigenic variation followed by in translocation of peptides into the host. While we did observe antigenic variation related genes to be deregulated in other strains as well, the enrichment in C580Y far exceeds that in the other two strains. Thus, C580Y seems to rely very strongly on the antigenic variation (apart from the few common pathways mentioned previously). This gene set may not provide very direct protective benefits from artemisinin hence the low %RSA reported for the strain. The I543T mutant on the other hand reported exclusive deregulation of genes associated with ribosome biogenesis, translation, protein import into mitochondria, protein folding, and redox response. These are the biological pathways that have been very strongly implicated in resistance against artemisinin. The I543T strain in our RSA reported strong survival (29%) out of the three strains under investigation. Thus, these additional deregulated genes may allow the strain to fight directly against the proteotoxic effects of artemisinin thus conferring a significant degree of resistance. Finally, the R539T strain reported deregulation of an additional set of genes related to exit from host cell, motility, and DNA recombination/repair. It is unclear to us as to how these additional gene sets may be responsible for conferring resistance in this strain although it is the one reporting the highest level of resistance at 45% RSA.

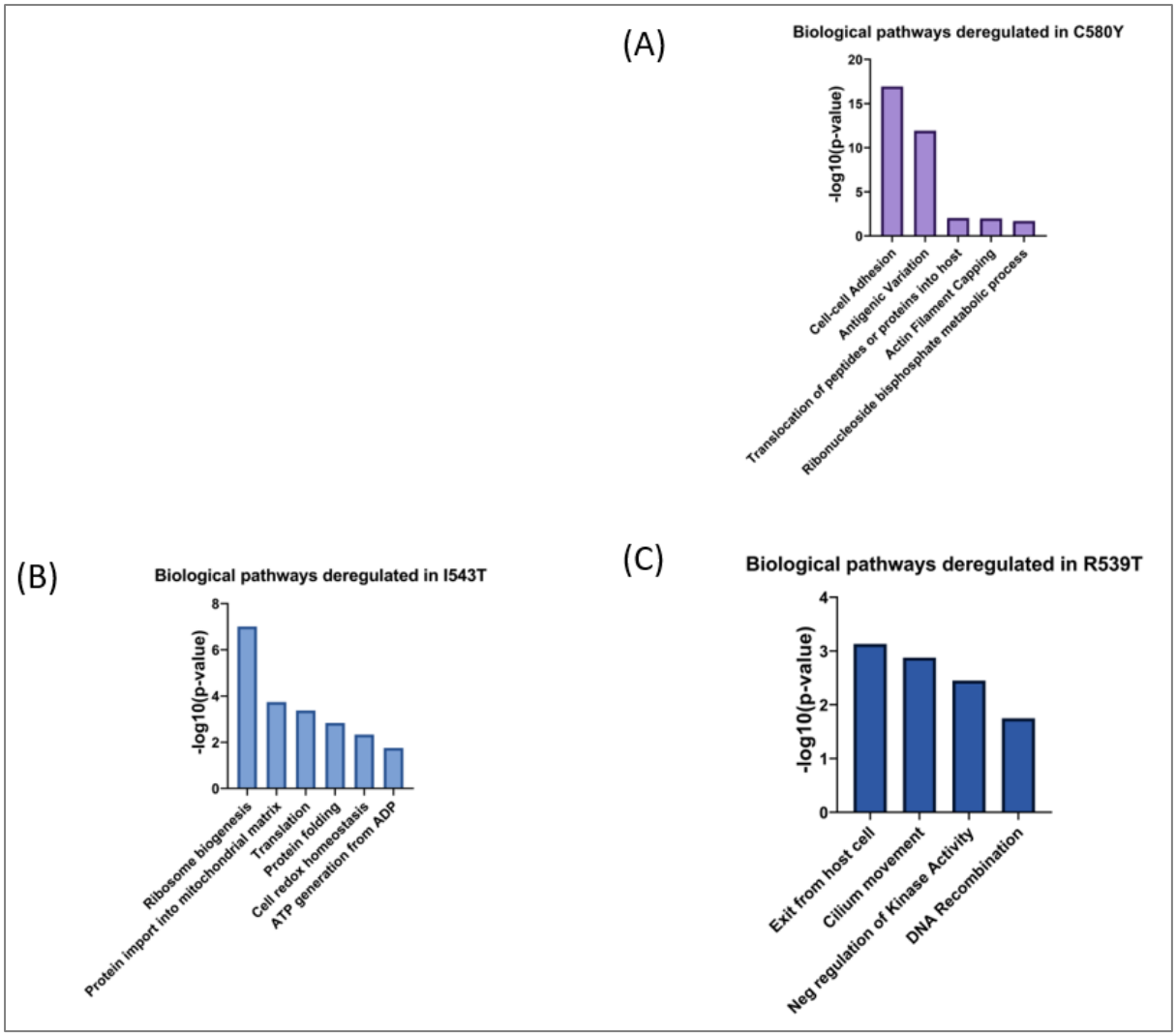


Fig. 92: PfKelch13 mutant isolates with different grades of resistance show deregulation of distinct biological pathways as well. Bar plots displaying the biological processes deregulated uniquely in each of the PfKelch13 mutant strains.

Thus, we find that artemisinin resistance is associated with deregulation of a core set of biological pathways in different PfKelch13 mutants reporting a spectrum of resistance grades to artemisinin. The extent to which the individual strain may show deregulation of any of these pathways is however variable. Each of these strains however also displays an additional layer of transcriptional heterogeneity by showing deregulation of unique biological pathways. These may additionally help the parasites to survive the attack of artemisinin and thus contribute to variation in the grade of resistance reported for the PfKelch13 mutant. These unique biological

responses may be a derivative of the distinct transcriptional states of the parasites displayed originally and thus sculpted by unique physiological factors.

5.8.4 Discrete set of genes show progressive deregulation with increasing grades of artemisinin resistance

The three PfKelch13 mutant isolates present three different grades of artemisinin resistance (ranging from low, mid to high) which gives us the unique opportunity to investigate for any transcriptional changes that could be linked to progressive increase in the level of resistance. We screened for genes that were either upregulated or downregulated across all the grades of resistance and whose level of deregulation progressed with the increasing resistance grade across the three PfKelch13 mutant isolates.

We identified 69 genes showing progressive upregulation and 39 genes with progressive downregulation in expression across the resistance grades (Fig. 93 and 94). A strong enrichment of any specific biological pathway could be detected. We did however note that genes implicated in protein folding (TCiP complex subunits, HSPs), gametocyte stages (gametocyte exported protein 2 and 3, early gametocyte enriched phosphoprotein EGXP), and transcription factors (PfAP2-G, PfAP2-O4) and a putative histone deacetylase were progressively upregulated in resistance. Among the genes that showed progressive decrease in expression were associated with cell cycle (putative cell cycle associated protein, SMC protein 4) and glycosylphosphatidylinositol metabolism proteins (GPI-anchor transamidase, GPI1).

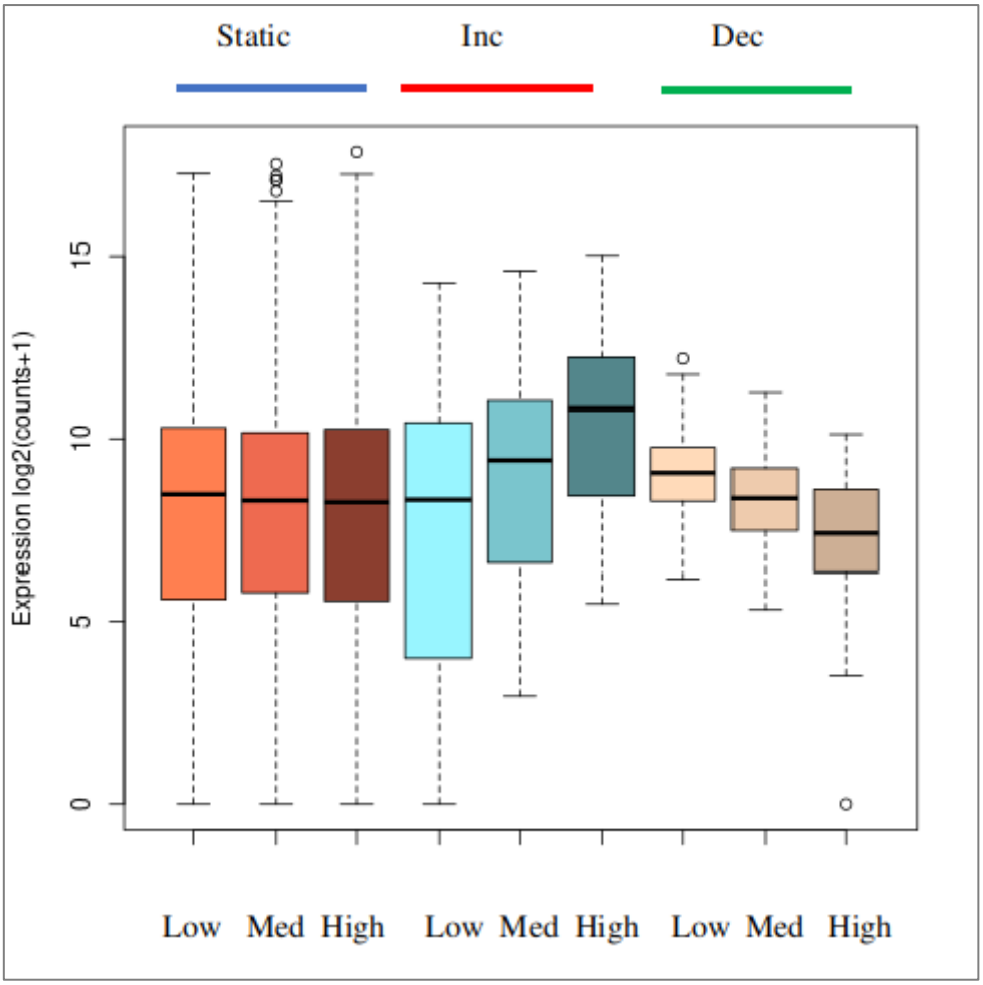


Fig. 93: Discrete set of genes show progressive increase or decrease in expression across low, medium and high grades of artemisinin resistance. Box Plot depicting gene sets showing neutral, progressive increase or decrease in low, medium and high grade of resistance. Genes encoding for chaperones are among those progressively upregulated.

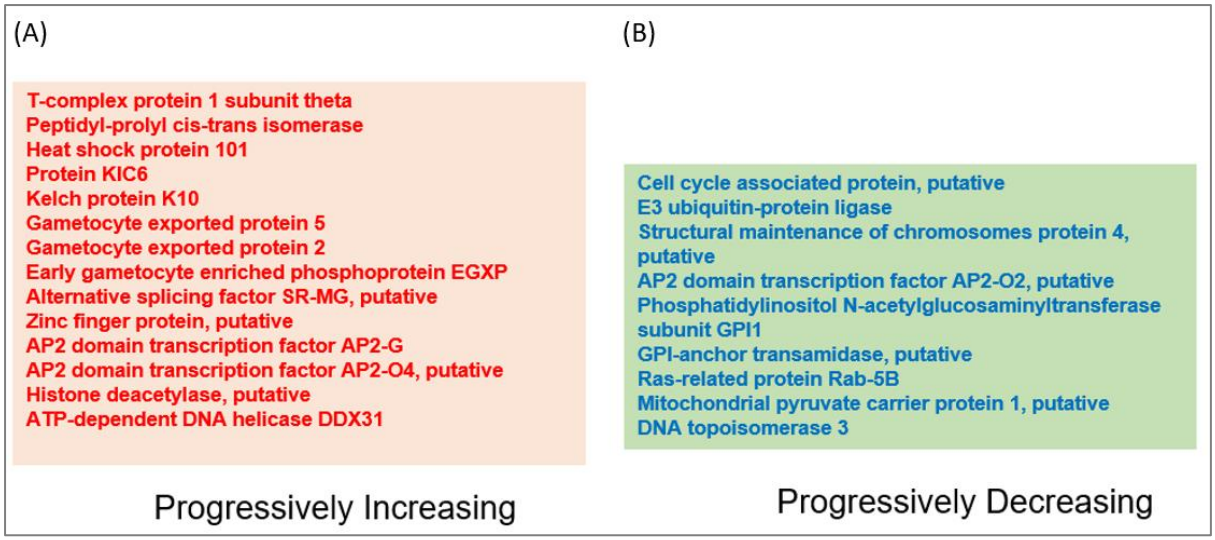


Fig. 94: Genes associated with gametocytogenesis and protein chaperones are progressively upregulated with increasing grades of resistance (A). Genes associated with cell cycle and GPI anchor metabolism are progressively downregulated with increasing grades of resistance.

5.8.5 Transcriptional deregulation associated with downregulation of PfHDAC1

Our ChIP sequencing data revealed enrichment of PfHDAC1 on a host of genes associated with crucial biological function both basic (translation, cell cycle, DNA replication, hemoglobin endocytosis) as well as response to external stimuli (protein refolding, DNA damage repair). Using pharmacological inhibitors, we experimentally evaluated the effect of PfHDAC1 deregulation on the expression of genes associated with proteostasis and cytoadherence. We were interested in finding out how the gene expression profile of these targets would change with suppression/depletion or conversely, enhancement of PfHDAC1 abundance in the cells. The genes and biological networks under PfHDAC1 seem to be like the ones that are deregulated in artemisinin resistance. Our data already suggests that deregulation of PfHDAC1 levels and activity in the cell can modulate the susceptibility of the parasites to artemisinin. PfHDAC1 deregulation was also shown to be causative of skew in the parasite cell cycle progression and proliferation which has been implicated in establishment of artemisinin

resistance. To gain transcriptional mechanistic insights into this we decided to see if the transcriptional deregulation following PfHDAC1 depletion can mimic the artemisinin resistance transcriptome.

We used selection linked integration to generate transgenic lines tagging the endogenous PfHDAC1 locus with 2x FKBP-GFP and transfected the line with an episomal construct expressing pLyn-FRB-mCherry. The GFP tag would help in visualisation of the endogenous PfHDAC1. The 2x FKBP tag on endogenous PfHDAC1 would allow for rapalog inducible dimerisation with episomal pLyn-FRB-mCherry which carries a PPM signal peptide (pLyn) causing the freshly translated PfHDAC1 to mislocalize to the parasite plasma-membrane and hence deplete in the nucleus (Fig. 95 and 96). While the transgenic lines were obtained and validated by confocal microscopy and western blotting, we could not achieve satisfactory mis-localization of the tagged PfHDAC1 to the PPM. However, a recent study by Huang et al. investigated the potency of an antimalarial compound and using RNA sequencing of PfHDAC1 knockdown using the glmS ribozyme system validated that PfHDAC1 was the specific target of the compound. We decided to analyse the knockdown data further for our purpose.

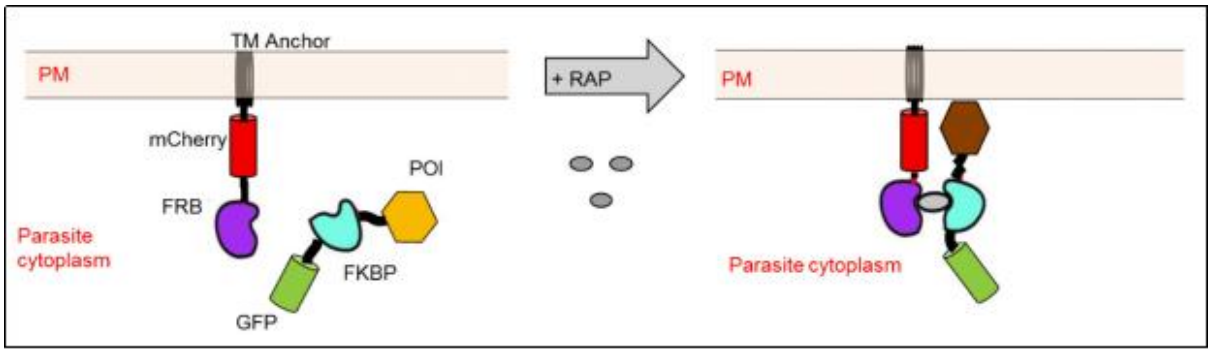


Fig. 95: A schematic of the rapalog inducible depletion of protein of interest from the cellular compartment (knock sideways) of its action.

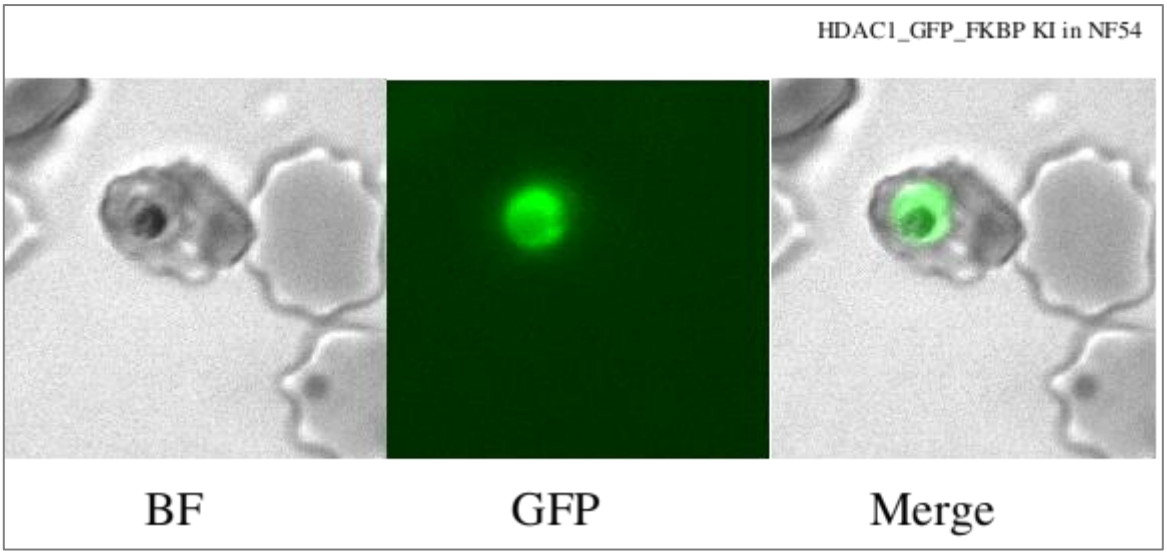


Fig. 96: Confocal microscopy images validating the endogenous GFP-2xFKBP tagged PfHDAC1 transgenic line generated for the purpose of rapalog inducible knock sideways.

We checked for genes that were deregulated more/less than equal to 2-fold (p-value <=0.05) and found 761 genes to be upregulated upon PfHDAC1 knockdown and 806 genes to be downregulated (Fig. 97). While our analysis found PfHDAC1 enrichment to be inversely correlated with the gene expression activating epigenetic mark H3K9ac and its inhibition resulted in enhanced H3K9ac, the knockdown data indicates that PfHDAC1 depletion isn't exclusively associated with upregulation of genes expression, since almost an equal number of genes were also downregulated. This fits with our correlation assessment of PfHDAC1 occupancy vs expression and in turn indicates that PfHDAC1 is not a general repressor of transcription in *P. falciparum*.

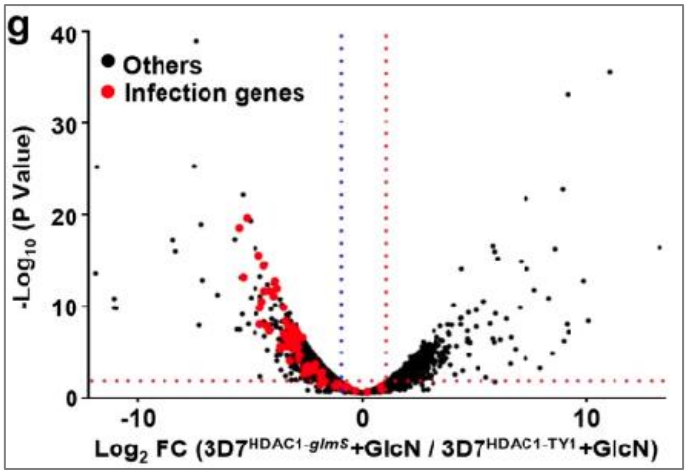


Fig. 97: Volcano plot depicting the deregulation of P. falciparum genes upon PfHDAC1 depletion in transgenic parasite lines. A significant proportion of genes downregulated regulate host cell invasion. Source: Huang et. al., 2020; Cell Discovery [207].

We performed Gene Ontology enrichment analysis of the biological pathways enriched among the deregulated gene set for PfHDAC1 knockdown. Genes associated with cell cycle regulation, antigenic variation, endocytosis, host pathogen interaction (host cell egress and invasion) and phosphorylation were found to be strongly downregulated in PfHDAC1 knockdown condition. Conversely, genes associated with protein refolding (chaperones), fatty acid biosynthesis and ribosome biosynthesis (translation) were found to be upregulated with PfHDAC1 knockdown (Fig. 98). We found a significant overlap of the PfHDAC1 target genes in our ChIP seq data with the genes deregulated in the knockdown dataset further validating our ChIP-seq targets.

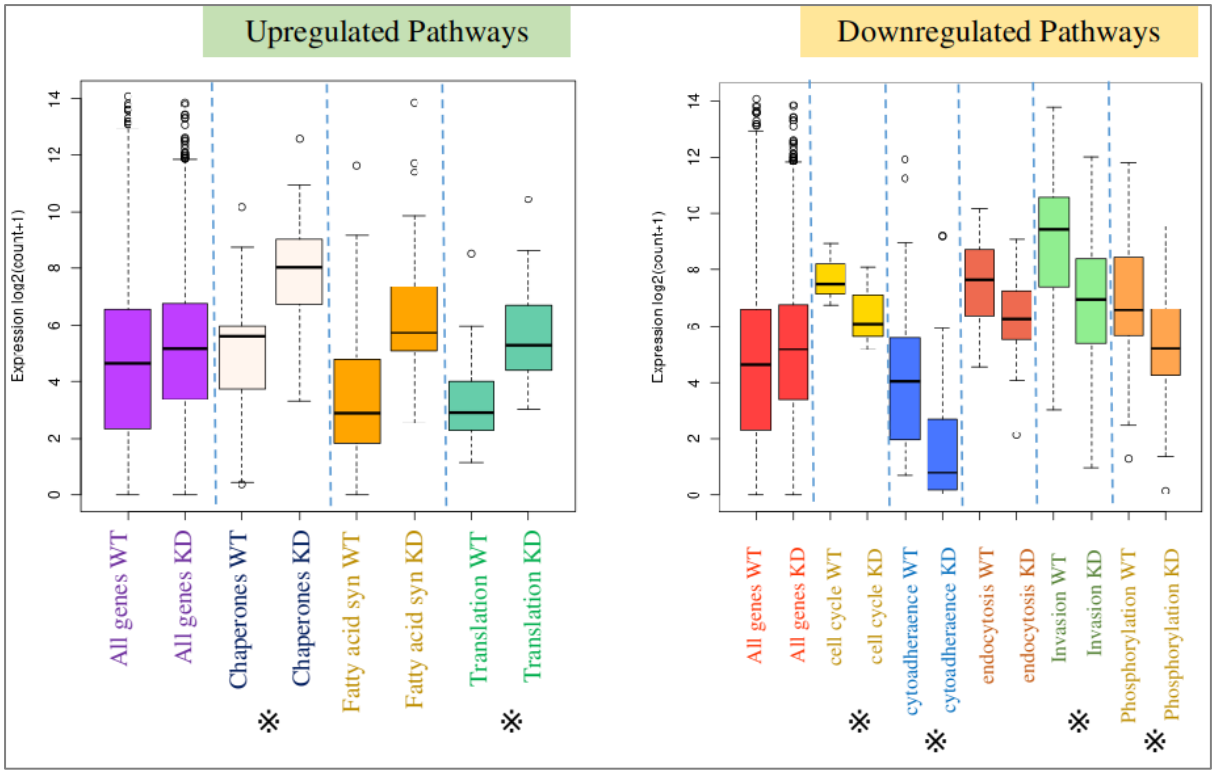


Fig. 98 PfHDAC1 depletion in P. falciparum is associated with deregulation of major biological processes implicated in artemisinin resistance. Boxplots depicting the upregulation of chaperones fatty acid biosynthesis, protein translation and downregulation of cell cycle, endocytosis, phosphorylation and cytoadherence associated genes with PfHDAC1 depletion.

Interestingly genes from the endocytosis pathway majorly appeared downregulated with PfHDAC1 depletion. Taking lead from a recent publication exploring the role of endocytosis in artemisinin resistance we investigated the effect of PfHDAC1 depletion on expression of endocytosis compartments marked by PfKelch13 protein. Depletion of these PfKelch13 interacting candidates (KICs) has been associated with enhanced artemisinin resistance [262]. We found that PfHDAC1 depletion in the Huang et. al. study was associated with reduced expression of 8 KIC members, 4 of which (Eps15 like protein, AP2-μ, KIC 4 and metacaspase-2) have been strongly identified as deterministic of artemisinin resistance (Fig. 99). Thus, PfHDAC1 depletion allows for a suppression of these genes and in turn could lead to reduced levels of hemoglobin endocytosis in cells and reduced artemisinin activation.

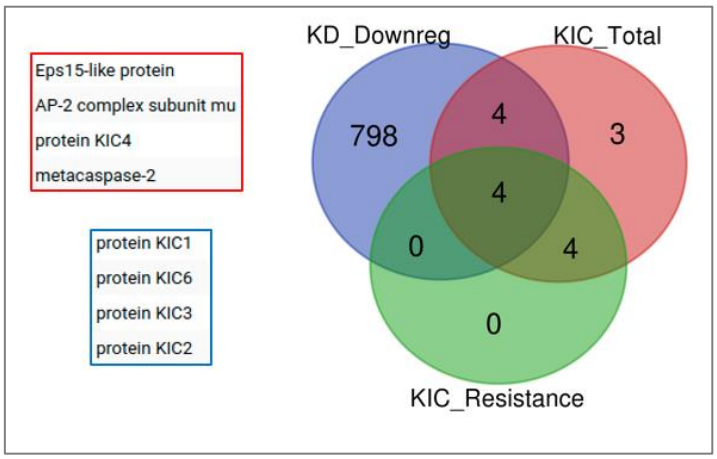


Fig. 99: PfHDAC1 depletion is associated with reduced expression of genes controlling hemoglobin endocytosis. A Venn diagram displaying the intersection of genes downregulated upon PfHDAC1 knockdown (Purple), KIC proteins (Red) and KIC proteins whose levels are deterministic of RSA%. Of the 8 KIC factors deregulated in PfHDAC1 depletion 4 are shown to regulate artemisinin sensitivity.

Even a cursory glance at the major pathways deregulated under condition of PfHDAC1 depletion becomes interesting since these are the key biological pathways that have been primarily shown to be deregulated with emergence of artemisinin resistance in the field. For a comparison of the gene deregulation in PfHDAC1 depleted parasites vs artemisinin resistant parasites we performed a correlative assessment of the transcriptional deregulation under conditions of PfHDAC1 downregulation with the gene expression profile observed in each of the three grades of artemisinin resistant PfKelch13 mutant lines. Parasite responses to artemisinin tend to be systemic in nature and engage a wide variety of biological pathways. Our primary objective in this analysis was to identify which profile of resistance associated transcriptome could be best explained with a downregulation of PfHDAC1 levels and if conserved aspects of gene deregulation observed across all the resistant isolates could be explained (even if in part) by the deregulation of PfHDAC1.

We compared the number of genes deregulated individually in the three PfKelch13 mutant lines vs the PfHDAC1 knockdown and checked for the overlap of genes that were commonly deregulated between them. Of the total 631 genes deregulated in C580Y, 221 were common with PfHDAC1 knockdown. Of the 982 genes deregulated in the R539T isolate 334 were common with PfHDAC1 knockdown. Interestingly, 809 genes out of the total 2342 genes deregulated in the I543T isolate were common with the PfHDAC1 knockdown. This was the maximum overlap of deregulated genes shared by PfHDAC1 knockdown line with any of the PfKelch13 isolates. We also performed spearman correlation coefficient analysis to assess the degree of gene expression (or deregulation) correlation between the individual mutants and the PfHDAC1 knockdown.

Globally the coefficient of correlation R between the deregulated transcriptome of C580Y/WT and PfHDAC1 knockdown transcriptome was R= 0.051 (p-value= 0.00019) indicating a neutral correlation when comparing the entire transcriptome. This can be explained by the fact that there is a very limited transcriptional deregulation of the C580Y mutant line from the wildtype line. Thus, a majority of the transcriptome is still unperturbed in C580Y line compared to the PfHDAC1 knockdown and this doesn't compare on the global scale. Genes relevant to response to abiotic stimulus, ribosome biogenesis, chaperone mediated protein-folding and pyruvate metabolism were strongly enriched for in the PfHDAC1 knockdown dataset but not in C580Y. We then refined our search to identify the genes that were deregulated along the same trend (upregulated/ downregulated in both datasets). Significant correlation was found for genes encoding for cytoadherence and antigenic variation associated biological functions (R=0.66 and p-value= 2.2e-16). It is worth noting that these are the prime pathways that were enriched for deregulation in the C580Y strain specifically. C580Y strain per se does not display a strong deregulation of its transcriptome from the basal sensitive counterpart

except for cytoadherence functionality which may be helpful in acquisition of resistance in a limited way, hence allowing for small %RSA benefit.

While comparing the transcriptome of PfHDAC1 knockdown with artemisinin resistant R539T/WT isolate, we again observed a neutral correlation globally (R=-0.091, p-value= 3.7e-11). Upon inspection we noted that a number of genes related to chaperone mediated protein folding function, host pathogen interaction and fatty acid metabolism were similarly enriched for. The R539T isolate however had additional downregulation of a number of genes that could not be explained by PfHDAC1, protein targeting to the mitochondrion and nucleotide metabolism were among these pathways. Hence PfHDAC1 deregulation could only explain a very limited perspective of deregulation observed uniquely in the R539T isolate.

Perhaps the strongest correlation between PfHDAC1 knockdown and artemisinin resistance could be drawn from the I543T isolate. There was a good positive correlation globally for the deregulation observed in both the datasets (R=0.46, p-value= 2.2e-16). Significant overlap was observed for genes associated with ribosome biogenesis, translation, entry into host cells (host pathogen interaction) and chaperone mediated protein folding (Fig. 100 and 101). Deregulation of these pathways is the most profitable in association with combating the damage caused by artemisinin thus affording a better ring-stage survival to these parasites. Thus, PfHDAC1 depletion leads to a transcriptional profile that matches with the core, conserved profile of artemisinin resistance but it may show little correlation with additional adaptive changes that occur uniquely in certain parasites that show stronger levels of resistance.

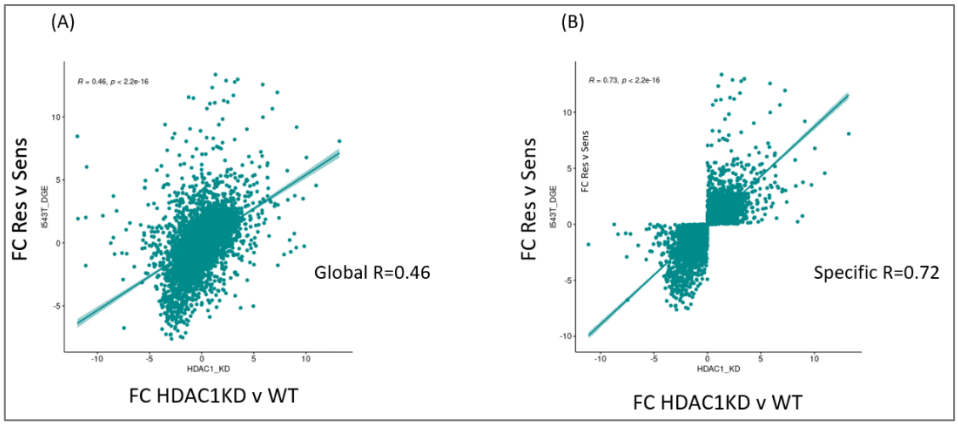


Fig. 100 Transcriptomics changes occurring in artemisinin resistant parasites resemble those observed upon PfHDAC1 depletion. Scatter plot depicting the correlation of gene expression changes in (PfKelch13 I543T mutant) artemisinin resistance (y-axis) vs PfHDAC1 depletion (x-axis) on a global scale (A) and specific key conserved processes observed common in artemisinin resistance (B).

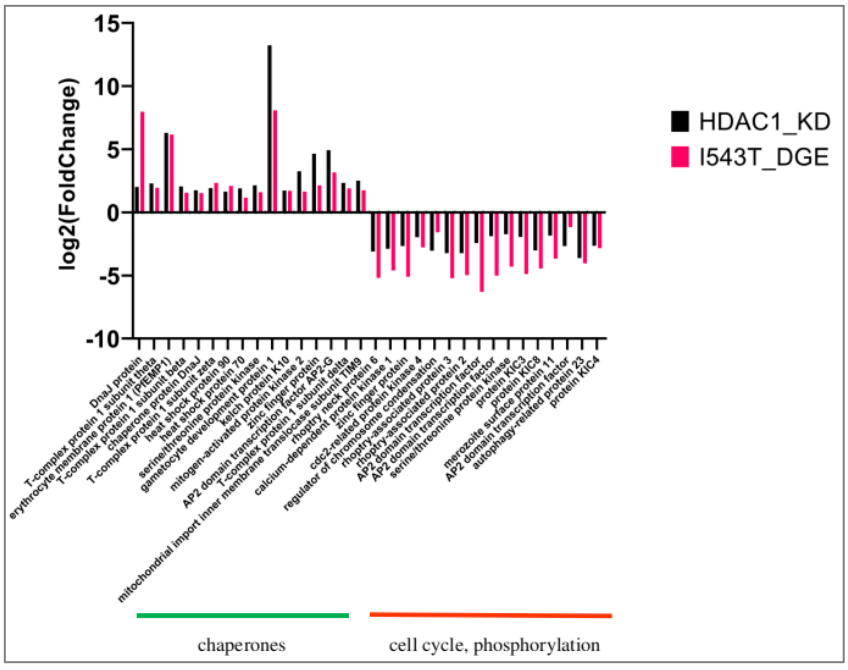
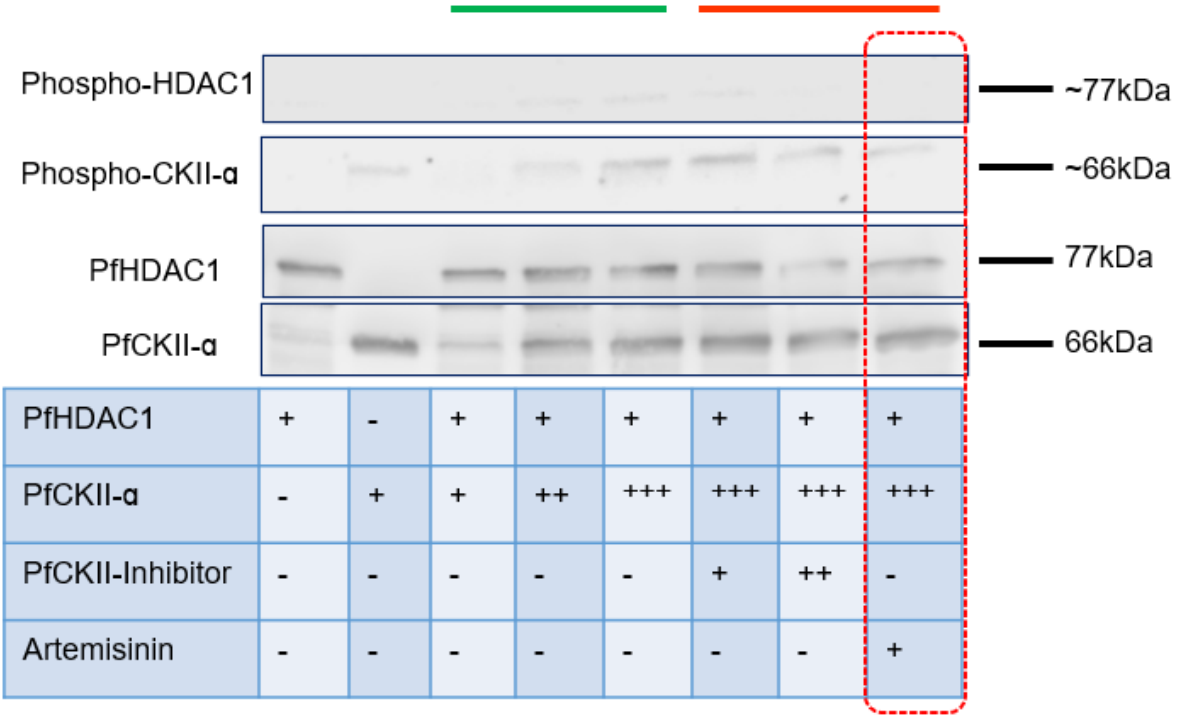


Fig. 101: Bar plots depicting the fold change in expression of genes associated with cell cycle and chaperones, etc. in artemisinin resistant parasites vs PfHDAC1 depleted cells.

5.8.6 Artemisinin treatment inhibits the PfCKII-α dependent phosphorylation of PfHDAC1

The antimalarial drug artemisinin has shown to target a number of biological pathways in the parasite and as such can especially affect the kinase signalling cascade. We were curious to know how artemisinin treatment could affect the activity of PfCKII-α and the phosphorylation of its target PfHDAC1. We carried out a kinase activity assay with PfHDAC1 as the substrate and PfCKII-α as the enzyme. We observed a robust phosphorylation of PfHDAC1 in the artemisinin null reaction. However, depletion of PfHDAC1 phosphorylation was recorded in reactions with artemisinin coincubation (Fig. 102). Our data already shows that depletion of phospho-PTM on PfHDAC1 suppresses its catalytic activity.



PfHDAC1	+	-	+	+	+	+	+	+
PfCKII-α	-	+	+	++	+++	+++	+++	+++
PfCKII-Inhibitor	-	-	-	-	-	+	++	-
Artemisinin	-	-	-	-	-	-	-	+

Fig. 102: Artemisinin treatment inhibits PfCKII-α mediated phosphorylation of PfHDAC1 (highlighted lane). The western blot represents probing for phospho-serine modified proteins. The reaction components are as defined in the table below the blot.

5.9 Discussion

Our investigation of the various PfKelch13 mutant transcriptomes highlights the heterogeneous mechanisms underlying artemisinin resistance. High levels of transcriptional diversity have been reported in the region from where these isolates were originally procured. The clustering of the isolates in the 2D principal component space is dependent on the gene expression profile. The higher disparity among the three sensitive strains is possibly owing to their unique geographical subregions of origin which feeds into the unique transcriptomic composition. Transcriptional rewiring is observed for isolates when they transition from artemisinin sensitive to resistant status. Our observations with the convergence of the transcriptional profiles of the isolates during the acquisition of resistance (or PfKelch13 mutations) suggests that artemisinin resistant parasites are more transcriptionally similar than their sensitive counterparts. This indicates that some crucial gene expression changes (or reconfiguration of biological pathways) may be required essentially for the establishment of resistance. This is further hinted by population transcriptomics studies. Furthermore, different strains display different degrees of transcriptional reprogramming to acquire resistance. Thus, the extent to which each sensitive strain has to deregulate gene expression to acquire artemisinin resistance may depend on the initial gene expression profile of the sensitive isolate as well as the final level of resistance it gets selected for. There may be a correlation between the grade of resistance with the percentage of transcriptome rewired. Deregulation of more genes may allow for engagement of a higher number of biological pathways to contribute to minimising and mitigating the effects of artemisinin on the cell.

Apart from the common biology we observe to be deregulated at the gene expression level we observe some processes that are unique or more strongly enriched for specific strains. It is

possible that each of these pathways may in their own way deregulate to afford a certain degree of protection from artemisinin and that may be in turn strongly linked with the level of resistance acquired by the strain displaying the changes. These unique changes may also be reflective of the unique environmental conditions different strains may be exposed to and hence evolve their own way to cope up with drug stress to variable extents. Deregulation of cytoadherence associated genes (which is strongly observed for the C580Y PfKelch13 mutant line) may grant a lower degree of resilience from artemisinin as compared to genes associated with ribosome biogenesis, protein folding and redox responses (which are directly implicated in mitigating the damage posed by artemisinin) observed in PfKelch13 I543T mutant line. Thus, the gross feature of the biological pathways contributing to protection from artemisinin may finally decide the grade of resistance from low to high. Despite having origin in distinct geographical cohorts and their own unique gene expression profile, artemisinin resistance isolates reprime themselves with some similar as well as a few distinct strategies to acquire resistance. Since artemisinin has widespread effects on a large number of biological pathways in the cell, how the individual cell adapts may depend on the initial state of the cell.

It is interesting to note that genes associated with antigenic variation, immune evasion and host -parasite interaction are not only deregulated for in each of the three isolates they are also among the most deregulated genes for all the three isolates the most significantly deregulated genes in each of the three isolates. Multigene families in *P. falciparum* are associated with host cell remodelling and export of parasite specific proteins onto the RBC surface that can be utilized to tether the infected cells to the endothelial layer of blood vessels. These parasites can thus be sequestered away from the peripheral circulation where the therapeutic dosages of artemisinin may reach peak concentrations. These “micro-pockets” of evasive parasites may find themselves in a niche where they can relatively stay safe from drug exposure and survive.

Cytoadherence has only recently been credited to be associated with emergence of drug resistance in *P. falciparum* and adequate experimental proof is still lacking [261]. It is a complex biological phenomenon whose mechanisms of gene expression regulation are relatively poorly understood but believed to be vastly crucial for controlling population dynamics and resilience to biotic and abiotic stresses like artemisinin. Our findings further highlight the implication of antigenic variation associated genes in the emergence of drug resistance. Another interesting observation is that while the number of genes that are commonly deregulated for all the three isolates are low, different genes still pertaining to the broader function of cytoadherence and host pathogen interaction are deregulated across all isolates.

We further identified a subset of genes that shows progressive change in expression in increasing grades of resistance. The genes showing progressive expression changes represent factors whose deregulation is classically associated with resistance (proteostasis and cell cycle) and several transcription regulatory proteins. While an increment in the protein refolding machinery reverses the damage caused by artemisinin, decrease in cell cycle regulatory factors stalls progression and renders artemisinin inert. Interestingly, the few gametocytogenesis associated proteins and the gametocytogenesis promoting transcription factor (PfAP2-G) raise the interesting possibility of enhanced tendency of the parasites to convert from the replicative asexual stages to the transmissive sexual stages. This has been experimentally proven in *P. falciparum* in previous studies [284, 285].

In our analysis with the PfKelch13 isolates displaying different grades of artemisinin resistance we identified a set of core biological pathways that undergo changes in all the artemisinin resistant isolates and may be thus critically linked to resistance. Interestingly, a majority of these biological pathways (antigenic variation/cytoadherence, host-pathogen interaction, phosphorylation, lipid biosynthesis) were deregulated in PfHDAC1 knockdown condition as

well (as analysed from Huang et. al. 2020 dataset). Furthermore, PfHDAC1 seems to cover more ground with deregulation of genes in pathways that have been heavily implicated in resistance in previous studies (cell cycle, protein refolding, endocytosis, fatty acid metabolism and protein translation). Thus, PfHDAC1 deregulation accounts for a significant proportion of the core conserved transcriptional changes in parasites that are required for protection from the damage of artemisinin.

The deregulation of pathways caused by PfHDAC1 depletion is such that it actually fits well with the mechanics that have been proposed for addressing artemisinin resistance [186, 269]. For instance, the downregulation of the cell cycle and endocytosis allows for slower metabolism and lesser activation of artemisinin, decreased host cell egress and invasion comes as a cost of the benefit associated with slower proliferation of the sensitive parasite in environments where drug exposure is regular. Conversely, enhanced protein expression and chaperone expression allows for better repairing the cellular damages that are caused by the drug in the cell. Of note was the deregulation of endocytosis associated proteins called PfKelch13 interaction candidates which have been shown to be associated with reduced endocytosis in parasites and thus a higher RSA%. 4 KIC genes were found to be downregulated upon PfHDAC1 depletion adding further evidence how PfHDAC1 could be transcriptionally regulating artemisinin resistance. Interestingly, all these pathways are associated with the core features we have identified to be conserved (albeit to a variable degree) across all the artemisinin resistance grades. On a global level, we found a variable degree of association between the various PfKelch13 isolate transcriptomes and the effects of PfHDAC1. PfHDAC1 deregulation did account for changes in isolates with low and medium grade of resistance as observed in C580Y and I543T isolates but failed to do so for the R539T strain. Thus, the mechanistic aspects of resistance classically understood may offer protection only up to a

certain degree (low to medium grade RSA) while additional poorly understood changes fuel transition of parasites to high grade resistance. Additional factors may influence these changes.

We finally explored the effect of artemisinin on PfHDAC1 phosphorylation. The phosphorylation of HDACs is reported to regulate catalytic activity as well as protein-protein interactions [220]. The latter is important for functional activity of the protein (as opposed to catalytic activity) where the protein interaction network can often dictate the recruitment of the protein to specific cellular locations. For HDACs the phosphorylated state can define the interaction with additional co-repressor/activator molecules and recruitment over specific gene targets. Thus, the phosphorylated form of PfHDAC1 could have widely different regulatory behaviour in cells than non-phosphorylated PfHDAC1 and any factor that can control the phosphorylation of PfHDAC1 can impose strong gene regulatory effects in the cell. This becomes very pertinent with our observation that artemisinin can suppress the phosphorylation of PfHDAC1. This raises interesting possibilities for the mechanism of transcriptional regulation by active PfHDAC1 under artemisinin pressure. Under artemisinin stress phospho-depleted PfHDAC1 could have very different regulatory effects on the transcriptome which could also mimic an overall lesser availability of catalytic PfHDAC1. Furthermore, the protein -protein interactions of PfHDAC1 under governance of its phosphorylated state would also be altered under artemisinin stress leading to reorganisation of the PfHDAC1 interactome in the cell and as a result lead to change in its regulation via recruitment to specific gene loci. Interestingly, artemisinin resistant *P. falciparum* report a downregulation of a number of kinases (including PfCKII-α in our data). This could again dramatically affect the catalytic state and interactome of PfHDAC1 and thus its gene regulatory functions in artemisinin resistant parasites.

6. Conclusion and Future Directions

Plasmodium falciparum is a tenacious parasite that has been a scourge of humanity for millennia [286]. Despite decades of study and advances into its biology and employment of countless therapeutic agents against it, the parasite persists. It is especially adept at fooling our adaptive immune responses and thus a definitive vaccine for protection from malaria remains a distant reality [287]. A large part of the puzzle lies at the heart of the parasite's genome and the way in which it selectively utilises it to invade host cells. Much of the functional status of the parasite genome remains elusive and uncharacterised [86]. The expression of these genes is also a highly coordinated process allowing for only the functionally important set of genes to be expressed in certain stages of the parasite. The transcriptional machinery in the parasite is very basic but highly effective in orchestrating great control over gene expression [288]. Epigenetic modifiers of the histone code seem to be crucial regulators in this process. *P. falciparum* has a well-defined set of HATs and HDACs which have been associated with numerous critical biological pathways in the parasite [120]. These molecules are also at the forefront of antimalarial drug sets being discovered and evaluated by scientific studies worldwide [201].

In this study we focus on the under characterised, solitary, Class I HDAC, PfHDAC1 which is a prime antimalarial compound target and is critical for parasite viability. We identify that the enzymatic activity of PfHDAC1 is strictly governed by its post translational modification (serine-phosphorylation) status, which is not unlike what is observed for higher eukaryotic HDACs. We identify PfCKII-α as an important interactor of PfHDAC1 and responsible for imparting the serine-phosphorylation PTM. The phosphorylated form of PfHDAC1 can perform the deacetylase activity which is associated with changes in the histone acetyl PTM code leading to downstream gene expression changes. Thus, the PTM of PfHDAC1 adds an

important layer towards the regulation of its activity and offers leads into investigation of the effects of compounds that can potentially inhibit this and lead to loss of its functionality. One such compound that we have identified is the antimalarial drug artemisinin which is reported to directly target PfCKII-α and thus inhibit the phosphorylation of PfHDAC1. This additionally hints at the potential impact of artemisinin through suppression of PfHDAC1 phosphorylation leading to a collapse of basic functions under the molecule.

We identify several critical biological processes under the regulation of PfHDAC1, namely cytoadherence, cell cycle, protein folding, hemoglobin metabolism and endocytosis. Our data hints at a strong role of PfHDAC1 in maintenance of the gene suppressive environment at the telomeric heterochromatin clusters in strong correlation with H3K9me3 heterochromatin mark and in stark opposition to the H3K9ac activating mark. Inhibition of PfHDAC1 was found to deregulate the silenced state of multiple *var* genes. PfHDAC1 is strongly enriched on the gene body of target genes and is depleted from the UTR regulatory regions. Thus, the gene regulatory activity of PfHDAC1 may be focussed more on histone modification dynamics on the TSS and gene body and allow for activating marks like H3K9ac/H3K14ac to set in on the promoter regions. Further investigation of the dynamics of PfHDAC1 and its cognate HAT (probably PfGCN5) may lead to a better understanding of the histone acetyl code regulation by them. We identify that PfHDAC1 binds to both highly and lowly expressed genes and thus may not be strictly involved in suppression of all its gene targets. This is further established by observations that HDAC1 inhibition often leads to both upregulation and downregulation of the gene expression [207]. There is an interesting possibility that PfHDAC1 could be driving basal expression of housekeeping genes it binds to by continually resetting the histone acetylation on these genes. On the other hand, it could have a generally suppressive effect on the stress response genes like chaperones and redox metabolism genes, which are only required upon specific environmental triggers.

Using Class specific HDAC inhibitors we observed a deceleration of parasite progression through the IDC. This was also found to correlate with distortion in proper morphological development (especially DNA replication) and carry over of the infection to the next cycle. This may explain why knockouts of PfHDAC1 and PbHDAC1 are deleterious in the respective organisms, owing to the central position HDAC1 may occupy for cell cycle progression and viability. Using data published by Huang et. al. we identified a host of critical cell cycle regulatory and DNA replication factors under PfHDAC1 control which get suppressed upon PfHDAC1 knockdown. This explains the defective cell cycle dynamics of parasites treated with inhibitors of PfHDAC1 and highlights its targetability in *Plasmodium.* Using a genetic system to overexpress PfHDAC1 in transgenic lines we observe enhancement in the proliferation of the parasite which could be owing to enhanced DNA replication or better invasion of RBCs.

The effect of PfHDAC1 inhibition on cell cycle mimics the cell cycle dynamics of artemisinin resistant parasites, especially the slower progression of a fraction of PfHDAC1 inhibitor treated rings to trophozoite stage and immature development of trophozoites. Survey of literature related to artemisinin resistance gave us a lead that PfHDAC1 was reported to be downregulated in the PfKelch13 mutant artemisinin resistant parasite [149]. We were able to observe a depletion of PfHDAC1 protein in the PfKelch13 C580Y mutant parasites compared to the wildtype sensitive counterpart strain. Pharmacological inhibition of PfHDAC1 which we found to be correlated with slower cell cycle and increased expression of chaperones and was found to enhance the resistance of parasites to artemisinin. This could explain how artemisinin resistant strains could have offset the effects of artemisinin by evolving for reduced levels of PfHDAC1. This would allow for slower growth but offer a protective advantage from the damage caused by artemisinin. We were further able to functionally correlate the depletion of

PfHDAC1 in artemisinin resistant parasites by showing that PfHDAC1 occupies far fewer genomic sites in artemisinin resistant lines than sensitive. The eviction of PfHDAC1 from chaperone genes in resistant strains was found to be associated with high expression of these factors, which could be important for drug resistance. We did note the enrichment of PfHDAC1 on PfKelch13 gene in sensitive parasites but not in resistant parasites. This differential occupancy could not be associated with differential expression of PfKelch13 between the two strains, however.

Surprisingly even overexpression of PfHDAC1 in both PfKelch13 mutant and wildtype parasites was found to enhance resistance to artemisinin. As of now we lack a definitive answer to this observation but future studies investigating the exact molecular effects of overabundance of PfHDAC1 may be needed for the same. HDAC1 overexpression has long been associated with enhanced cellular proliferation as observed in numerous human cancers. An additional effect associated with HDAC1 overexpression is tolerance to numerous stresses in cancer, especially therapeutic. HDAC1 overexpression is reported to enhance survival signalling pathways, which could be an alternative mode of resistance to artemisinin in PfHDAC1 overexpression parasites.

To understand the potential transcription regulatory effects PfHDAC1 deregulation may have in artemisinin resistance, we first sought to characterise the transcriptomes of PfKelch13 mutant artemisinin resistant parasites. Strains with different grades of resistance to artemisinin displayed transcriptional convergence to allow certain common gene expression traits to emerge. We speculate that despite the diverse genetic and transcriptomic background of the parent isolates, certain core, conserved transcriptional programs may allow for protection from artemisinin. The existence of such transcriptional circuits have been hinted in recent studies [275]. Some of the common transcription trends involving changes in the expression of

cytoadherence, lipid biosynthesis, host cell invasion and phosphorylation were observed, and these have been highlighted in previous transcriptomics studies [269, 275]. Cytoadherence could allow for parasites to sequester in microvasculature deep within tissues and thus evade peripheral circulation where drug concentrations may peak dramatically. Recent work has hinted at the potential role of cytoadherence in artemisinin resistance, but a thorough investigation remains to be done [261].

Additional factors could contribute to the differences in levels of artemisinin resistance including the effects of the individual distinct SNPs on the stability of PfKelch13 and its various posited roles in endocytosis, ubiquitination of target proteins and mitochondrial functions that are hitherto poorly understood. We also provide evidence that there are strain specific transcriptional changes that are enriched for specific biological pathways in each resistant strain (viz. higher deregulation of proteostasis associated genes in one strain vs deregulation of fatty acid metabolism associated genes in other strain). These transcriptional changes may contribute differently to resistance and thus allow for different %RSAs.

Thus, diverse transcriptional profiles are a means to an end. As recognised by pioneering studies in *Plasmodium* drug resistance, anything that can allow a slower activation of artemisinin and compensate for the damage caused can modulate artemisinin sensitivity. PfHDAC1 with its regulation of several critical pathways could be one such regulatory element that could fine tune the parasite's responses to artemisinin. To dissect the effects of deregulation of PfHDAC1 may have on transcription that could potentially explain the changes to artemisinin sensitivity, we finally delved into the transcriptional consequences of PfHDAC1 deregulation and compare them with the transcriptome of resistant isolates. Using Huang et. al. study dataset we identified that PfHDAC1 depletion is accompanied by broad-scale gene

expression changes. Expression of genes related to protein translation, chaperone, fatty acid biosynthesis tends to go up, while genes related to cell cycle, phosphorylation, endocytosis and cytoadherence tend to be downregulated. Much of the transcriptional profile associated with artemisinin resistance matched the profile that is classically associated with resistance in Southeast Asia. Thus, PfHDAC1 deregulation could drive gene expression patterns in parasites that allow for emergence of resistance (summarised in Fig. 103). The extent to which these changes are finally adopted in the individual parasite strains may be variable. It is worth noting that not all artemisinin resistant parasites report downregulation of PfHDAC1. In such scenarios alternative regulatory mechanisms may be in place. Thus, deregulation of PfHDAC1 may be a means to acquire resistance if not the only means to do so.

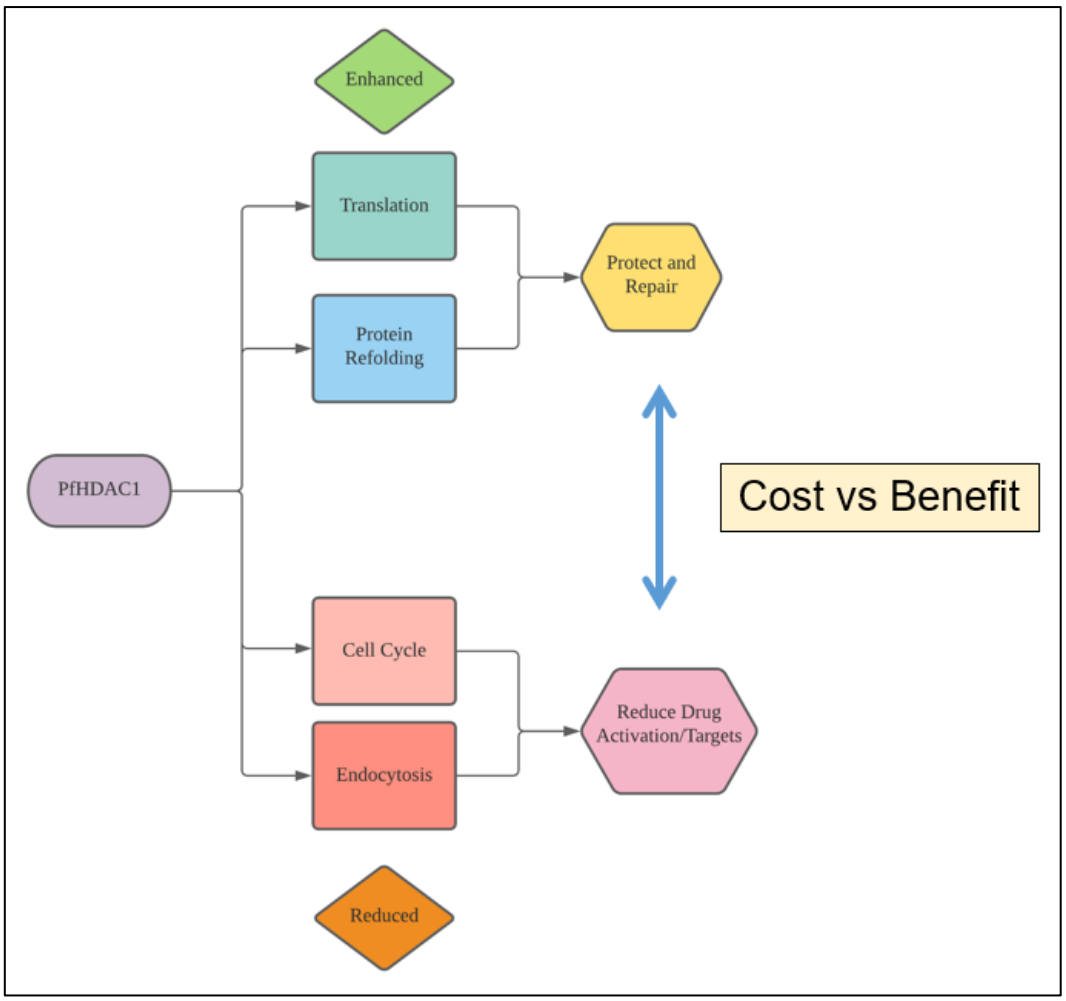


Fig. 103: A model of the effects of PfHDAC1 deregulation and its effects on the basal and stress responsive cellular processes that may contribute to artemisinin resistance.

This study like any other answers some questions and raises a lot many more. We have barely begun to scratch the surface of the evidently complex repertoire of transcription regulatory factors in *Plasmodium* which may be far more invested in different aspects of the parasite biology than previously expected. Some of the pressing questions that came to us that we may not have been able to address yet are summarised as follows:

1. **Further dissection of the PfHDAC1 serine-phosphorylation PTM and its mediator in *Plasmodium***
    1. Identification of the residues that are phosphorylated by PfCKII-α on PfHDAC1. This can be achieved by pull down of PfCKII-α treated PfHDAC1 and then followed by enrichment for phosphorylated peptides using trap columns or phospho-modification antibodies. Mass spectrometry would help identify the residues crucial for PfHDAC1 phosphorylation
    2. Does the phospho-PTM state of PfHDAC1 dictate its cellular localisation? We have detected PfHDAC1 predominantly in the nuclear but also in the cytoplasmic compartment. This hints at a possible shuttling of the protein and additional non histone substrates. The phospho-serine modified PfHDAC1 is a known target of 14-3-3 transporter proteins that can dictate the nucleo-cytoplasmic distribution of PfHDAC1. This can be investigated using inhibition of *in vivo* phosphorylation of PfHDAC1 by specific inhibitors of the cognate kinase and studying its nucleocytoplasmic distribution using fractionation followed by western blot or immunofluorescence.
2. **Characterisation of the molecular and transcriptomic consequences of PfHDAC1 overexpression in *P. falciparum***

1. Study of the effects of overexpression of PfHDAC1 on the progression of cell cycle in the blood stage and investigation of nuclear replication dynamics is something that needs to be followed. This will help answer if PfHDAC1 overexpression can drive converse changes in cell morphology and cycle progression as observed for PfHDAC1 inhibition.
2. RNA-seq of PfHDAC1 overexpression lines shall help answer what are the effects of PfHDAC1 overexpression on gene expression in the parasite. We have observed the conference of resistance in parasites upon overexpression of PfHDAC1, whether this is mediated by specific transcriptional changes (viz. enhancement of cell survival pathways in parasites needs to be well studied).

3. **Identification of the cell cycle regulatory network that underlies PfHDAC1 regulation:** We have identified several important cell cycle regulatory factors that get down regulated upon PfHDAC1 depletion in parasites. A proper characterisation of the direct functional targets of PfHDAC1 that drive cell cycle in *P. falciparum* may be helpful to understand the primary network of factors that can govern cell cycle processes under PfHDAC1
4. **Identification of the components of the protein-protein interactome of PfHDAC1:** An understanding of the interacting partners of PfHDAC1 is important since they can define its functional activity in cells. HDAC1 usually exists as a part of the Sin3 complex in higher eukaryotes. While we have done mass spectrometry of PfHDAC1 and identified several its interacting partner, a thorough scrutiny of these factors may be more helpful in revealing how these partner proteins may modulate the activity, cellular localisation, and target selection of PfHDAC1. If the protein-protein interactome of PfHDAC1 changes during drug stress or other environmental pressures is also worth investigating to better understand the regulation of the protein.

5. **Investigation of the acetylation landscape (histone as well as non-histone) under PfHDAC1 regulation:** We show that PfHDAC1 can dictate the presence of H3K9ac in specific regions of the genome. Using a knockdown line for PfHDAC1 we can investigate the changes to the acetylation PTM on histone and non-histone proteins in the parasite. The acetylation landscape on histones has shown to be highly dynamic in *Plasmodium falciparum* and quite connected with gene expression changes in stage specific fashion. PfHDAC1 thus stands as an important factor to study for its control on the epigenetic acetylome.

List of Publications

1. Exceptionally high sequence-level variation in the transcriptome of *Plasmodium falciparum*. Dave B, **Kanyal A**, Mamatha Rani DV, Karmodiya K (BioRxiv 2021)

2. Chromo domain protein interacts with H3K9me3 and controls rosette formation by regulating the expression of a subset of RIFINs in the malaria parasite. Sethumadhavan DV, Tiburcio M, **Kanyal A**, Jabeena CA, Govindaraju G, Karmodiya K, Moritz Treeck, and Arumugam Rajavelu. (MS under review, 2021)

3. Analysis of drug resistance marker genes of *Plasmodium falciparum* after implementation of artemisinin-based combination therapy in and around Pune, India. J Biosci. (2021), 46:77. Ozarkar A, **Kanyal A**; Dass S, Deobagkar D, Deshpande P, Karmodiya K.

4. Revisiting the population genomics and transcriptomics landscape of artemisinin resistant *Plasmodium falciparum* to identify key compensatory mutational and gene-expression trends. Rawat M*, **Kanyal A***, Choubey D*, Deshmukh B, Malhotra R, Mamatha Rani DV, Rao A and Karmodiya K. (MS under review)

5. PfGCN5, a global regulator of stress responsive genes, modulates artemisinin resistance in *Plasmodium falciparum*. Scientific Reports (2021), 11(1):852. Rawat M, **Kanyal A**, Sahasrabudhe A, Vembar SS, Lopez-Rubio JJ and Karmodiya K.

6. Prognostic Epigenetics (2019), Chapter 7, Volume 15, 1° Edition, pp. 171-192. Epigenetics in Infectious diseases. eBook ISBN: 9780128142608 (Book Chapter) **Kanyal A**, Nahata S and Karmodiya K.

7. Ru-Catalysed dehydrogenative synthesis of antimalarial arylidene oxindoles. Organic & biomolecular chemistry (2018), 16(39), pp.7223-7229. Bisht GS, Pandey AM, Chaudhari MB, Agalave SG, **Kanyal A**, Karmodiya K and Gnanaprakasam B.

8. Genome-wide survey and phylogenetic analysis of histone acetyltransferases and histone deacetylases of *Plasmodium falciparum*. The FEBS journal (2018), 285(10), pp.1767-1782. **Kanyal A***, Rawat M*, Gurung P, Choubey D, Anamika K and Karmodiya K.

* Shared First author

Appendix 1

Table 3: Primer used for various applications in this study

Protein Overexpresssion			
S No.		**Fwd Primer**	**Rev Primer**
1	PfHDAC1_His	ctgGCTAGCATGTCTAATAGAAAAAAGG	acgtCTCGAGATATGGTACAATAGATTG
2	PfHDAC1_GST	ACGGGATCCATGTCTAATAGAAAAAAGG	acgtCTCGAGATATGGTACAATAGATTG
3	PfCKII-α_GST	GGGGGAATTCATGTCGGTTAGCTCAATTAATAAAAA	GGGGCTCGAGTGATTCCTCACGGACTTCTCAAA
PfHDAC1_GFP_glmS Overexpression			
S No.		**Fwd Primer**	**Rev Primer**
1	PfHDAC1_OE	catCCTAGGATGTCTAATAGAAAAAAGGTTGCCTA	catGCTAGCATATGGTACAATAGATTGATCCCTG
2	glmS_tag	catcCTCGAGTACCCGTACGACGTCCC	catcCTCGAGAGATCATGTGATTTCTCTTTG
PfHDAC1 Knock sideway			
S No.		**Fwd Primer**	**Rev Primer**
1	PfHDAC1_KS_pSLI	AGGCGGCCGCTAGTGCCAGATGTGTAGAACACG	ttgCCTAGGATATGGTACAATAGATTGATCCCTG
qRT-PCR Primers			
S No.		**Fwd Primer**	**Rev Primer**
1	BiP	TGAGGGACCCGTTATTGGTA	TGCCTCACCAACTTTCCTTT
2	PfHSP70	TGCTGTCATTACCGTTCCAG	AGCAGCTGCAGTAGGTTCATT
3	ER-Oxidoreductin	TGAGTGACGAACATATTGGCT	AGAATCTGCAAAGTAGTATAGGGC
4	E3 ubiquitin protein ligase RBX1	CAGTAGCAGCCTGGTCATGG	TCTGTTGTTTTAGCTTGGCATTC
5	E2 ubiquitin conjugating enzyme	TGAACCAAATGCTGACAGTCC	TCTTGCCATCGACTGAAATCC
6	PI3Kinase	CGTGTTTGGGCGATAAACTGA	CCTAACGAGGAAACGCCTGA
qRT-PCR var gene Primers			

S No.		Fwd Primer	Rev Primer
1	PF3D7_0200100	ATGTGCGCTACAAGAAGCTG	TTGATCTCCCCATTCAGTCA
2	PF3D7_0420900	AGAGGGTTATGGGAATGCAG	GCATTCTTTGGCAATTCCTT
3	PF3D7_0500100	GAAGCTGGTGGTACTGACGA	TATTTTCCCACCAGGAGGAG
4	PF3D7_0809100	TGCAAGGGTGCTAATGGTAA	CCTGCATTTTGACATTCGTC
5	PF3D7_1200600	TGGTGATGGTACTGCTGGAT	TTTATTTTCGGCAGCATTTG
6	PF3D7_1200100	CGGAGGAGGAAAAACAAGAG	TGCCGTATTTGAGACCACAT

Appendix 2

Table 4: Genes with at least 1.8-fold enrichment for PfHDAC1 in *P. falciparum* 3D7

Gene ID	**Gene Name**
PF3D7_0100800	rifin
PF3D7_0101900	rifin
PF3D7_0102200	ring-infected erythrocyte surface antigen
PF3D7_0102500	erythrocyte binding antigen-181
PF3D7_0104300	ubiquitin carboxyl-terminal hydrolase 1, putative
PF3D7_0106100	V-type proton ATPase subunit C, putative
PF3D7_0106300	calcium-transporting ATPase
PF3D7_0106700	small ribosomal subunit assembling AARP2 protein
PF3D7_0107500	Niemann-Pick type C1-related protein
PF3D7_0108700	secreted ookinete protein, putative
PF3D7_0109000	photosensitized INA-labeled protein PHIL1
PF3D7_0109800	phenylalanine--tRNA ligase alpha subunit
PF3D7_0112300	18S ribosomal RNA
PF3D7_0112700	28S ribosomal RNA
PF3D7_0113300	Plasmodium exported protein (hyp1), unknown function
PF3D7_0113600	surface-associated interspersed protein 1.2 (SURFIN 1.2), pseudogene
PF3D7_0113800	DBL containing protein, unknown function
PF3D7_0114200	exported protein family 3
PF3D7_0115200	rifin
PF3D7_0115300	rifin
PF3D7_0115700	erythrocyte membrane protein 1, PfEMP1
PF3D7_0200400	stevor
PF3D7_0200500	rifin
PF3D7_0200700	rifin
PF3D7_0201200	rifin
PF3D7_0201300	stevor
PF3D7_0202100	liver stage associated protein 2
PF3D7_0205900	26S proteasome regulatory subunit RPN1, putative
PF3D7_0206500	conserved Plasmodium protein, unknown function

PF3D7_0206700	adenylosuccinate lyase
PF3D7_0207500	serine repeat antigen 6
PF3D7_0207600	serine repeat antigen 5
PF3D7_0207700	serine repeat antigen 4
PF3D7_0207800	serine repeat antigen 3
PF3D7_0209000	6-cysteine protein P230
PF3D7_0209100	patatin-like phospholipase 1
PF3D7_0211800	asparagine--tRNA ligase
PF3D7_0212100	conserved Plasmodium protein, unknown function
PF3D7_0212300	eukaryotic peptide chain release factor subunit 1, putative
PF3D7_0212500	conserved Plasmodium protein, unknown function
PF3D7_0212600	secreted protein with altered thrombospondin repeat domain
PF3D7_0212800	multidrug efflux pump, putative
PF3D7_0214100	protein transport protein SEC31
PF3D7_0214300	conserved Plasmodium protein, unknown function
PF3D7_0215000	acyl-CoA synthetase
PF3D7_0215700	DNA-directed RNA polymerase II subunit RPB2, putative
PF3D7_0217900	thioesterase/thiol ester dehydrase-isomerase, putative
PF3D7_0218000	replication factor C subunit 2, putative
PF3D7_0220200	Plasmodium exported protein, unknown function
PF3D7_0220800	cytoadherence linked asexual protein 2
PF3D7_0221400	stevor
PF3D7_0222400	rifin, pseudogene
PF3D7_0222500	rifin
PF3D7_0223100	rifin
PF3D7_0300700	rifin
PF3D7_0300800	rifin
PF3D7_0301600	Plasmodium exported protein (hyp1), unknown function
PF3D7_0302100	serine/threonine protein kinase
PF3D7_0302200	cytoadherence linked asexual protein 3.2
PF3D7_0302300	erythrocyte membrane protein 1 (PfEMP1), pseudogene
PF3D7_0302900	exportin-1, putative
PF3D7_0303000	N-ethylmaleimide-sensitive fusion protein
PF3D7_0305100	conserved Plasmodium protein, unknown function
PF3D7_0305500	protein dopey homolog, putative

PF3D7_0306800	T-complex protein 1 subunit beta
PF3D7_0307700	conserved Plasmodium protein, unknown function
PF3D7_0308200	T-complex protein 1 subunit eta
PF3D7_0308400	U4 spliceosomal RNA
PF3D7_0309300	N2227-like protein, putative
PF3D7_0309500	asparagine synthetase [glutamine-hydrolyzing], putative
PF3D7_0310200	phd finger protein, putative
PF3D7_0311800	ATP synthase F0 subunit d-like protein, putative
PF3D7_0312700	tRNA Isoleucine
PF3D7_0314700	RING finger protein RNF1
PF3D7_0315200	circumsporozoite- and TRAP-related protein
PF3D7_0316800	40S ribosomal protein S15A, putative
PF3D7_0317100	6-cysteine protein
PF3D7_0317200	cdc2-related protein kinase 4
PF3D7_0318200	DNA-directed RNA polymerase II subunit RPB1
PF3D7_0318300	conserved Plasmodium protein, unknown function
PF3D7_0319000	P-type ATPase, putative
PF3D7_0320300	T-complex protein 1 subunit epsilon
PF3D7_0320400	oocyst capsule protein Cap380
PF3D7_0320800	ATP-dependent RNA helicase DDX6
PF3D7_0323400	Rh5 interacting protein
PF3D7_0323900	erythrocyte membrane protein 1 (PfEMP1), exon 2, pseudogene
PF3D7_0324400	rifin
PF3D7_0324500	rifin
PF3D7_0400900	rifin
PF3D7_0401200	rifin
PF3D7_0401300	rifin
PF3D7_0402500	rifin, pseudogene
PF3D7_0402600	stevor
PF3D7_0402700	rifin, pseudogene
PF3D7_0402800	erythrocyte membrane protein 1 (PfEMP1), pseudogene
PF3D7_0403000	tRNA Asparagine
PF3D7_0403600	conserved Plasmodium protein, unknown function
PF3D7_0405400	pre-mRNA-processing-splicing factor 8, putative
PF3D7_0405700	lysine decarboxylase, putative

PF3D7_0406000	conserved protein, unknown function
PF3D7_0406100	V-type proton ATPase subunit B
PF3D7_0406200	parasitophorous vacuole membrane protein S16
PF3D7_0407800	protein CINCH
PF3D7_0409200	protein SOF1, putative
PF3D7_0409600	replication protein A1, large subunit
PF3D7_0410100	tRNA Serine
PF3D7_0410200	tRNA Isoleucine
PF3D7_0410600	armadillo-type repeat protein ATRP
PF3D7_0411000	conserved Plasmodium protein, unknown function
PF3D7_0411400	DEAD box ATP-dependent RNA helicase, putative
PF3D7_0411500	tRNA Alanine
PF3D7_0411600	tRNA Glutamic acid
PF3D7_0412000	LITAF-like zinc finger protein, putative
PF3D7_0412600	rifin, pseudogene
PF3D7_0413200	rifin
PF3D7_0413300	rifin
PF3D7_0413400	erythrocyte membrane protein 1 (PfEMP1), exon 1, pseudogene
PF3D7_0413900	ubiquitin carboxyl-terminal hydrolase 13, putative
PF3D7_0416300	DNA helicase MCM9, putative
PF3D7_0416800	small GTP-binding protein sar1
PF3D7_0417100	mRNA-binding protein PUF2
PF3D7_0418200	eukaryotic translation initiation factor 3 subunit M, putative
PF3D7_0418300	conserved Plasmodium protein, unknown function
PF3D7_0421600	erythrocyte membrane protein 1 (PfEMP1)-like pseudogene
PF3D7_0422500	pre-mRNA-splicing helicase BRR2, putative
PF3D7_0424700	serine/threonine protein kinase, FIKK family
PF3D7_0425300	Plasmodium exported protein (PHISTa), unknown function, pseudogene
PF3D7_0425700	rifin
PF3D7_0425800	erythrocyte membrane protein 1, PfEMP1
PF3D7_0501200	parasite-infected erythrocyte surface protein
PF3D7_0501300	skeleton-binding protein 1
PF3D7_0505100	trafficking protein particle complex subunit 8, putative
PF3D7_0507600	protein CAF40, putative
PF3D7_0508000	6-cysteine protein

PF3D7_0509400	RNA polymerase I
PF3D7_0510200	peptidyl-prolyl cis-trans isomerase
PF3D7_0510800	WD repeat-containing protein, putative
PF3D7_0510900	small nucleolar RNA snoR04
PF3D7_0511000	translationally-controlled tumor protein homolog
PF3D7_0511800	inositol-3-phosphate synthase
PF3D7_0513000	conserved protein, unknown function
PF3D7_0513300	purine nucleoside phosphorylase
PF3D7_0513600	deoxyribodipyrimidine photo-lyase, putative
PF3D7_0513900	conserved protein, unknown function
PF3D7_0515300	phosphatidylinositol 3-kinase
PF3D7_0515700	glideosome-associated protein 40, putative
PF3D7_0516100	cation-transporting ATPase 1
PF3D7_0516300	tRNA pseudouridine synthase, putative
PF3D7_0516700	ubiquitin carboxyl-terminal hydrolase 2, putative
PF3D7_0517000	60S ribosomal protein L12, putative
PF3D7_0517400	FACT complex subunit SPT16, putative
PF3D7_0518200	SWIB/MDM2 domain-containing protein
PF3D7_0519300	protoheme IX farnesyltransferase
PF3D7_0522400	conserved Plasmodium protein, unknown function
PF3D7_0522900	zinc finger protein, putative
PF3D7_0524400	ribosome-interacting GTPase 1, putative
PF3D7_0525000	zinc finger protein, putative
PF3D7_0525100	acyl-CoA synthetase
PF3D7_0526300	WD repeat-containing protein 55, putative
PF3D7_0526600	conserved Plasmodium protein, unknown function
PF3D7_0527700	tRNA Glutamic acid
PF3D7_0527800	tRNA Leucine
PF3D7_0528100	AP-1/2 complex subunit beta, putative
PF3D7_0529600	tRNA Arginine
PF3D7_0530400	conserved Plasmodium protein, unknown function
PF3D7_0531500	non-coding RNA
PF3D7_0531600	18S ribosomal RNA
PF3D7_0531700	unspecified product
PF3D7_0531800	5.8S ribosomal RNA

PF3D7_0531900	unspecified product
PF3D7_0532000	28S ribosomal RNA
PF3D7_0532100	early transcribed membrane protein 5
PF3D7_0600400	erythrocyte membrane protein 1, PfEMP1
PF3D7_0606600	conserved Plasmodium protein, unknown function
PF3D7_0606800	VFT protein
PF3D7_0607800	GTP-binding protein, putative
PF3D7_0608100	conserved Plasmodium protein, unknown function
PF3D7_0608500	proteasome subunit alpha type-2, putative
PF3D7_0608700	T-complex protein 1 subunit zeta
PF3D7_0608800	ornithine aminotransferase
PF3D7_0609100	zinc transporter ZIP1, putative
PF3D7_0609900	conserved Plasmodium protein, unknown function
PF3D7_0610400	histone H3
PF3D7_0611200	AP2 domain transcription factor, putative
PF3D7_0612700	6-cysteine protein P12
PF3D7_0613600	conserved Plasmodium protein, unknown function
PF3D7_0615600	zinc finger protein, putative
PF3D7_0615800	conserved oligomeric Golgi complex subunit 4, putative
PF3D7_0617700	rifin
PF3D7_0617800	histone H2A
PF3D7_0619400	cell division cycle protein 48 homologue, putative
PF3D7_0620800	tRNA Alanine
PF3D7_0620900	tRNA Leucine
PF3D7_0621100	conserved Plasmodium protein, unknown function
PF3D7_0621400	Pf77 protein
PF3D7_0621600	tRNA Serine
PF3D7_0623700	ATP-dependent RNA helicase SUV3, putative
PF3D7_0624600	SNF2 helicase, putative
PF3D7_0626800	pyruvate kinase
PF3D7_0626840	small nucleolar RNA snoR35
PF3D7_0627100	ankyrin-repeat protein, putative
PF3D7_0627800	acetyl-CoA synthetase, putative
PF3D7_0628100	HECT-domain (ubiquitin-transferase), putative
PF3D7_0629400	polyadenylate-binding protein 3, putative

PF3D7_0631800	rifin
PF3D7_0631900	stevor
PF3D7_0632100	rifin
PF3D7_0700300	rifin
PF3D7_0700400	stevor
PF3D7_0700500	rifin
PF3D7_0700600	Plasmodium exported protein, unknown function, pseudogene
PF3D7_0701800	rifin
PF3D7_0702800	tRNA Tyrosine
PF3D7_0703500	erythrocyte membrane-associated antigen
PF3D7_0705400	DNA replication licensing factor MCM7
PF3D7_0706700	DNA mismatch repair protein MSH2, putative
PF3D7_0706800	tRNA Threonine
PF3D7_0706900	tRNA Histidine
PF3D7_0707000	tRNA Lysine
PF3D7_0707100	tRNA Lysine
PF3D7_0707700	E3 ubiquitin-protein ligase, putative
PF3D7_0708000	cytoskeleton associated protein, putative
PF3D7_0709000	chloroquine resistance transporter
PF3D7_0709100	Cg1 protein
PF3D7_0711900	rifin, pseudogene
PF3D7_0712200	rifin, pseudogene
PF3D7_0712400	erythrocyte membrane protein 1, PfEMP1
PF3D7_0712500	rifin, pseudogene
PF3D7_0713000	rifin
PF3D7_0713300	erythrocyte membrane protein 1 (PfEMP1), pseudogene
PF3D7_0714700	tRNA Asparagine
PF3D7_0714800	tRNA Leucine
PF3D7_0714900	tRNA Serine
PF3D7_0715200	conserved Plasmodium protein, unknown function
PF3D7_0717100	conserved Plasmodium protein, unknown function
PF3D7_0717200	conserved Plasmodium protein, unknown function
PF3D7_0717700	serine--tRNA ligase, putative
PF3D7_0718300	cysteine repeat modular protein 2
PF3D7_0719300	actin-related protein, putative

PF3D7_0720300	conserved Plasmodium protein, unknown function
PF3D7_0720700	phosphoinositide-binding protein, putative
PF3D7_0721600	40S ribosomal protein S5, putative
PF3D7_0723700	metallo-hydrolase/oxidoreductase, putative
PF3D7_0725300	conserved protein, unknown function
PF3D7_0725600	18S ribosomal RNA
PF3D7_0725700	unspecified product
PF3D7_0725800	5.8S ribosomal RNA
PF3D7_0725900	unspecified product
PF3D7_0726000	28S ribosomal RNA
PF3D7_0726100	Plasmodium exported protein, unknown function
PF3D7_0726300	DNA mismatch repair protein PMS1, putative
PF3D7_0726400	conserved Plasmodium membrane protein, unknown function
PF3D7_0726700	conserved Plasmodium protein, unknown function
PF3D7_0727800	cation transporting ATPase, putative
PF3D7_0728100	anaphase-promoting complex subunit 1, putative
PF3D7_0730300	AP2 domain transcription factor AP2-L, putative
PF3D7_0730600	tRNA Valine
PF3D7_0730700	tRNA Threonine
PF3D7_0731100	EMP1-trafficking protein
PF3D7_0731300	Plasmodium exported protein (PHISTb), unknown function
PF3D7_0731500	erythrocyte binding antigen-175
PF3D7_0731600	acyl-CoA synthetase
PF3D7_0731800	alpha/beta hydrolase, putative
PF3D7_0732300	rifin
PF3D7_0732400	rifin
PF3D7_0732500	rifin
PF3D7_0732700	rifin
PF3D7_0800200	erythrocyte membrane protein 1, PfEMP1
PF3D7_0800500	rifin
PF3D7_0801000	Plasmodium exported protein (PHISTc), unknown function
PF3D7_0801100	28S ribosomal RNA
PF3D7_0801200	5.8S ribosomal RNA
PF3D7_0802300	periodic tryptophan protein 2, putative
PF3D7_0802600	adenylyl cyclase beta

PF3D7_0803400	DNA repair and recombination protein RAD54, putative
PF3D7_0803700	tubulin gamma chain
PF3D7_0804400	methionine aminopeptidase 1c, putative
PF3D7_0804500	conserved Plasmodium membrane protein, unknown function
PF3D7_0805500	conserved Plasmodium protein, unknown function
PF3D7_0806500	DnaJ protein, putative
PF3D7_0807300	ras-related protein Rab-18
PF3D7_0807500	proteasome subunit alpha type-6, putative
PF3D7_0807900	tyrosine--tRNA ligase
PF3D7_0808100	AP-3 complex subunit delta, putative
PF3D7_0808600	erythrocyte membrane protein 1, PfEMP1
PF3D7_0808900	rifin
PF3D7_0811300	CCR4-associated factor 1
PF3D7_0812100	proteasome activator complex subunit 4, putative
PF3D7_0812400	karyopherin alpha
PF3D7_0813700	ABC transporter F family member 1
PF3D7_0814200	DNA/RNA-binding protein Alba 1
PF3D7_0818900	heat shock protein 70
PF3D7_0820700	2-oxoglutarate dehydrogenase E1 component
PF3D7_0821300	ATP-dependent RNA helicase DHX36, putative
PF3D7_0822600	protein transport protein SEC23
PF3D7_0823200	RNA-binding protein, putative
PF3D7_0823300	histone acetyltransferase GCN5
PF3D7_0826700	receptor for activated c kinase
PF3D7_0827800	SET domain protein, putative
PF3D7_0827900	protein disulfide-isomerase
PF3D7_0830200	28S ribosomal RNA
PF3D7_0831400	Plasmodium exported protein, unknown function
PF3D7_0831700	heat shock protein 70
PF3D7_0900800	erythrocyte membrane protein 1 (PfEMP1), exon 2, pseudogene
PF3D7_0901100	rifin
PF3D7_0901600	stevor
PF3D7_0902300	serine/threonine protein kinase, FIKK family
PF3D7_0902800	serine repeat antigen 9
PF3D7_0903700	alpha tubulin 1

PF3D7_0904600	ubiquitin specific protease, putative
PF3D7_0904900	copper-transporting ATPase
PF3D7_0906600	zinc finger protein, putative
PF3D7_0908500	conserved Plasmodium protein, unknown function
PF3D7_0909400	exoribonuclease, putative
PF3D7_0909500	subpellicular microtubule protein 1, putative
PF3D7_0909900	helicase SKI2W, putative
PF3D7_0910800	cytosolic Fe-S cluster assembly factor NBP35, putative
PF3D7_0912400	alkaline phosphatase, putative
PF3D7_0914200	phospholipid or glycerol acyltransferase, putative
PF3D7_0915400	ATP-dependent 6-phosphofructokinase
PF3D7_0917500	conserved protein, unknown function
PF3D7_0917600	pre-mRNA-splicing factor ATP-dependent RNA helicase PRP43, putative
PF3D7_0918800	dihydrouridine synthase, putative
PF3D7_0919800	TLD domain-containing protein
PF3D7_0920800	inosine-5'-monophosphate dehydrogenase
PF3D7_0922200	S-adenosylmethionine synthetase
PF3D7_0922500	phosphoglycerate kinase
PF3D7_0923300	perforin-like protein 3
PF3D7_0924300	thiamine pyrophosphokinase
PF3D7_0925700	histone deacetylase 1
PF3D7_0926100	protein kinase, putative
PF3D7_0926600	conserved Plasmodium membrane protein, unknown function
PF3D7_0927200	zinc finger protein, putative
PF3D7_0927300	fumarate hydratase
PF3D7_0927700	serine/threonine protein phosphatase 4, putative
PF3D7_0929400	high molecular weight rhoptry protein 2
PF3D7_0930800	conserved Plasmodium membrane protein, unknown function
PF3D7_0932700	conserved Plasmodium protein, unknown function
PF3D7_0934400	AP2 domain transcription factor, putative
PF3D7_0936900	Plasmodium exported protein (PHISTb), unknown function, pseudogene
PF3D7_0937300	rifin
PF3D7_0937600	erythrocyte membrane protein 1, PfEMP1
PF3D7_0937800	erythrocyte membrane protein 1, PfEMP1
PF3D7_1000600	rifin

PF3D7_1000900	erythrocyte membrane protein 1 (PfEMP1), pseudogene
PF3D7_1001600	exported lipase 2
PF3D7_1001700	Plasmodium exported protein (PHISTc), unknown function
PF3D7_1004300	E3 ubiquitin-protein ligase, putative
PF3D7_1004400	RNA-binding protein, putative
PF3D7_1007200	rho GTPase-activating protein, putative
PF3D7_1007600	conserved protein, unknown function
PF3D7_1007700	AP2 domain transcription factor AP2-I
PF3D7_1008400	26S protease regulatory subunit 4, putative
PF3D7_1008800	nucleolar protein 5, putative
PF3D7_1008900	adenylate kinase
PF3D7_1009200	ribonuclease, putative
PF3D7_1009700	tubulin--tyrosine ligase, putative
PF3D7_1010200	DNA2/NAM7 helicase, putative
PF3D7_1011700	DNA repair protein RAD23, putative
PF3D7_1012400	hypoxanthine-guanine phosphoribosyltransferase
PF3D7_1012500	phosphoglucomutase, putative
PF3D7_1012800	conserved Plasmodium protein, unknown function
PF3D7_1014200	male gamete fusion factor HAP2, putative
PF3D7_1015300	methionine aminopeptidase 1b, putative
PF3D7_1015600	heat shock protein 60
PF3D7_1015900	enolase
PF3D7_1016700	Plasmodium exported protein (PHISTc), unknown function
PF3D7_1017000	DNA polymerase delta catalytic subunit
PF3D7_1018200	serine/threonine protein phosphatase 8, putative
PF3D7_1020500	ribosomal silencing factor RsfS, putative
PF3D7_1022100	methyltransferase, putative
PF3D7_1024800	exported protein 3
PF3D7_1025200	ATP synthase mitochondrial F1 complex assembly factor 2, putative
PF3D7_1025400	conserved Plasmodium membrane protein, unknown function
PF3D7_1026800	40S ribosomal protein S2
PF3D7_1028700	merozoite TRAP-like protein
PF3D7_1029400	LEM3/CDC50 family protein, putative
PF3D7_1031000	ookinete surface protein P25
PF3D7_1033200	early transcribed membrane protein 10.2

PF3D7_1034500	conserved Plasmodium protein, unknown function
PF3D7_1034900	methionine--tRNA ligase
PF3D7_1035600	merozoite surface protein
PF3D7_1036300	duffy binding-like merozoite surface protein 2
PF3D7_1037000	DNA polymerase zeta catalytic subunit, putative
PF3D7_1037500	dynamin-like protein
PF3D7_1037600	TFIIH basal transcription factor complex helicase XPB subunit, putative
PF3D7_1040100	rifin
PF3D7_1040200	stevor
PF3D7_1040600	rifin
PF3D7_1040700	rifin
PF3D7_1040900	rifin
PF3D7_1100400	rifin
PF3D7_1101300	rifin
PF3D7_1101400	rifin, pseudogene
PF3D7_1102500	gametocyte exported protein 2
PF3D7_1103200	tRNA Glycine
PF3D7_1103300	tRNA Leucine
PF3D7_1104200	chromatin remodeling protein
PF3D7_1105000	histone H4
PF3D7_1105100	histone H2B
PF3D7_1105600	translocon component PTEX88
PF3D7_1105700	tRNA-splicing ligase RtcB, putative
PF3D7_1108100	conserved Plasmodium protein, unknown function
PF3D7_1108500	succinyl-CoA synthetase alpha subunit, putative
PF3D7_1108700	heat shock protein J2
PF3D7_1110200	pre-mRNA-processing factor 6, putative
PF3D7_1113900	mitogen-activated protein kinase 2
PF3D7_1116600	dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3, putative
PF3D7_1116700	dipeptidyl aminopeptidase 1
PF3D7_1116800	heat shock protein 101
PF3D7_1117900	conserved Plasmodium protein, unknown function
PF3D7_1118200	heat shock protein 90, putative
PF3D7_1118300	insulinase, putative
PF3D7_1120100	phosphoglycerate mutase, putative

PF3D7_1122800	calcium-dependent protein kinase 6
PF3D7_1124200	thioredoxin-like protein, putative
PF3D7_1126100	autophagy-related protein 7, putative
PF3D7_1126600	steryl ester hydrolase, putative
PF3D7_1130200	60S ribosomal protein P0
PF3D7_1130400	26S protease regulatory subunit 6A, putative
PF3D7_1131800	oxysterol-binding protein, putative
PF3D7_1133400	apical membrane antigen 1
PF3D7_1134000	heat shock protein 70
PF3D7_1134100	protein disulfide-isomerase, putative
PF3D7_1134700	DNA-directed RNA polymerase I subunit RPA2, putative
PF3D7_1135100	protein phosphatase PPM8, putative
PF3D7_1135400	conserved Plasmodium protein, unknown function
PF3D7_1136900	subtilisin-like protease 2
PF3D7_1137000	U2 spliceosomal RNA
PF3D7_1144400	conserved Plasmodium protein, unknown function
PF3D7_1145400	dynamin-like protein
PF3D7_1147200	tubulin--tyrosine ligase, putative
PF3D7_1148600	18S ribosomal RNA
PF3D7_1148640	28S ribosomal RNA
PF3D7_1149000	antigen 332, DBL-like protein
PF3D7_1149600	DnaJ protein, putative
PF3D7_1149800	rifin
PF3D7_1149900	stevor
PF3D7_1150100	rifin, pseudogene
PF3D7_1150300	rifin
PF3D7_1200300	rifin
PF3D7_1200400	erythrocyte membrane protein 1, PfEMP1
PF3D7_1200600	erythrocyte membrane protein 1, PfEMP1
PF3D7_1204300	eukaryotic translation initiation factor 5A
PF3D7_1207000	conserved Plasmodium protein, unknown function
PF3D7_1208900	protein phosphatase PPM11, putative
PF3D7_1210600	conserved Plasmodium protein, unknown function
PF3D7_1211700	DNA replication licensing factor MCM5, putative
PF3D7_1212900	bromodomain protein 2, putative

PF3D7_1213000	non-coding RNA
PF3D7_1213100	U1 spliceosomal RNA
PF3D7_1216800	tRNA Proline
PF3D7_1219500	erythrocyte membrane protein 1 (PfEMP1), exon 2, pseudogene
PF3D7_1219700	raf kinase inhibitor
PF3D7_1219900	ribulose-phosphate 3-epimerase, putative
PF3D7_1221000	histone-lysine N-methyltransferase, H3 lysine-4 specific
PF3D7_1223500	conserved Plasmodium protein, unknown function
PF3D7_1224300	polyadenylate-binding protein 1, putative
PF3D7_1224400	WD repeat-containing protein, putative
PF3D7_1225800	ubiquitin-activating enzyme E1
PF3D7_1229500	T-complex protein 1 subunit gamma
PF3D7_1230500	WD repeat-containing protein, putative
PF3D7_1232200	dihydrolipoyl dehydrogenase, mitochondrial
PF3D7_1233600	asparagine and aspartate rich protein 1
PF3D7_1233900	sentrin-specific protease 1
PF3D7_1234600	protein TOC75, putative
PF3D7_1234700	upregulated in late gametocytes ULG8
PF3D7_1234900	CHCH domain-containing protein, putative
PF3D7_1235600	serine hydroxymethyltransferase
PF3D7_1236200	conserved Plasmodium protein, unknown function
PF3D7_1237100	conserved Plasmodium protein, unknown function
PF3D7_1237900	conserved Plasmodium protein, unknown function
PF3D7_1238500	conserved Plasmodium protein, unknown function
PF3D7_1240200	erythrocyte membrane protein 1 (PfEMP1), pseudogene
PF3D7_1242700	40S ribosomal protein S17, putative
PF3D7_1242800	rab specific GDP dissociation inhibitor
PF3D7_1244300	ACEA small nucleolar RNA U3
PF3D7_1246200	actin I
PF3D7_1246900	RAC-beta serine/threonine protein kinase
PF3D7_1247300	GPI mannosyltransferase 2, putative
PF3D7_1247500	serine/threonine protein kinase, putative
PF3D7_1248500	bax inhibitor 1, putative
PF3D7_1248900	26S protease regulatory subunit 8, putative
PF3D7_1249300	protein phosphatase PPM4, putative

PF3D7_1250100	osmiophilic body protein G377
PF3D7_1251900	tRNA Valine
PF3D7_1252000	tRNA Glutamine
PF3D7_1252400	reticulocyte binding protein homologue 3, pseudogene
PF3D7_1252600	esterase, putative
PF3D7_1253700	rifin
PF3D7_1253900	Plasmodium exported protein (PHISTa), unknown function, pseudogene
PF3D7_1254000	rifin
PF3D7_1254400	rifin
PF3D7_1254500	rifin
PF3D7_1254700	rifin
PF3D7_1254800	rifin
PF3D7_1300300	erythrocyte membrane protein 1, PfEMP1
PF3D7_1300800	erythrocyte membrane protein 1 (PfEMP1), pseudogene
PF3D7_1301000	rifin, pseudogene
PF3D7_1301600	erythrocyte binding antigen-140
PF3D7_1302000	EMP1-trafficking protein
PF3D7_1303500	sodium/hydrogen exchanger
PF3D7_1303800	conserved Plasmodium protein, unknown function
PF3D7_1306900	U1 small nuclear ribonucleoprotein A, putative
PF3D7_1307000	exosome complex component RRP40, putative
PF3D7_1307600	DNA-directed RNA polymerase subunit alpha, putative
PF3D7_1308900	mRNA-decapping enzyme 2, putative
PF3D7_1309000	conserved protein, unknown function
PF3D7_1309700	vacuolar protein sorting-associated protein 18, putative
PF3D7_1311400	AP-1 complex subunit mu-1
PF3D7_1311800	M1-family alanyl aminopeptidase
PF3D7_1311900	V-type proton ATPase catalytic subunit A
PF3D7_1316600	choline-phosphate cytidylyltransferase
PF3D7_1317100	DNA replication licensing factor MCM4
PF3D7_1318900	conserved Plasmodium protein, unknown function
PF3D7_1322100	histone-lysine N-methyltransferase SET2
PF3D7_1322200	TOG domain-containing protein, putative
PF3D7_1322300	conserved Plasmodium protein, unknown function
PF3D7_1323300	N-acetyltransferase, GNAT family, putative

PF3D7_1323500	plasmepsin V
PF3D7_1323600	conserved protein, unknown function
PF3D7_1324900	L-lactate dehydrogenase
PF3D7_1325900	conserved Plasmodium protein, unknown function
PF3D7_1327800	ribose-phosphate pyrophosphokinase, putative
PF3D7_1328000	conserved Plasmodium protein, unknown function
PF3D7_1328100	proteasome subunit beta type-7, putative
PF3D7_1328800	transcriptional regulatory protein sir2a
PF3D7_1329000	DNA-directed RNA polymerase III subunit RPC1, putative
PF3D7_1331100	DNA polymerase theta, putative
PF3D7_1331400	CPW-WPC family protein
PF3D7_1331700	glutamine--tRNA ligase, putative
PF3D7_1335000	MSP7-like protein
PF3D7_1337200	1-deoxy-D-xylulose 5-phosphate synthase
PF3D7_1337300	exoribonuclease, putative
PF3D7_1337600	tRNA Serine
PF3D7_1338600	conserved protein, unknown function
PF3D7_1338700	conserved protein, unknown function
PF3D7_1339100	tRNA Methionine
PF3D7_1339200	tRNA Proline
PF3D7_1339300	conserved protein, unknown function
PF3D7_1341500	inner membrane complex suture component, putative
PF3D7_1342400	casein kinase II beta chain
PF3D7_1342600	myosin A
PF3D7_1344300	zinc finger protein, putative
PF3D7_1345600	inner membrane complex protein
PF3D7_1345700	isocitrate dehydrogenase [NADP], mitochondrial
PF3D7_1346100	protein transport protein SEC61 subunit alpha
PF3D7_1346400	VPS13 domain-containing protein, putative
PF3D7_1347200	nucleoside transporter 1
PF3D7_1349200	glutamate--tRNA ligase, putative
PF3D7_1349300	tyrosine kinase-like protein
PF3D7_1350500	conserved Plasmodium protein, unknown function
PF3D7_1352500	thioredoxin-related protein, putative
PF3D7_1356600	regulator of chromosome condensation, putative

PF3D7_1357000	elongation factor 1-alpha
PF3D7_1357100	elongation factor 1-alpha
PF3D7_1357500	DNA helicase, putative
PF3D7_1359400	CUGBP Elav-like family member 1
PF3D7_1361100	protein transport protein Sec24A
PF3D7_1361700	cytochrome c oxidase subunit 2A, putative
PF3D7_1361800	glideosome-associated connector
PF3D7_1362200	RuvB-like helicase 3
PF3D7_1364100	6-cysteine protein P92
PF3D7_1367700	alanine--tRNA ligase
PF3D7_1367800	secreted ookinete protein, putative
PF3D7_1368200	ABC transporter E family member 1, putative
PF3D7_1369200	conserved Plasmodium protein, unknown function
PF3D7_1369800	tRNA Arginine
PF3D7_1369900	tRNA Tryptophan
PF3D7_1370100	tRNA Cysteine
PF3D7_1370200	tRNA Glycine
PF3D7_1371000	18S ribosomal RNA
PF3D7_1371300	28S ribosomal RNA
PF3D7_1372100	Plasmodium exported protein (PHISTb), unknown function
PF3D7_1372600	rifin
PF3D7_1372700	rifin
PF3D7_1373000	rifin
PF3D7_1373500	erythrocyte membrane protein 1, PfEMP1
PF3D7_1400500	rifin
PF3D7_1401000	GBPH protein
PF3D7_1401050	erythrocyte membrane protein 1 (PfEMP1), exon 2, pseudogene
PF3D7_1401300	epoxide hydrolase 2
PF3D7_1404300	secreted ookinete adhesive protein, putative
PF3D7_1404400	ribosomal protein L16, mitochondrial, putative
PF3D7_1407000	LCCL domain-containing protein
PF3D7_1407100	rRNA 2'-O-methyltransferase fibrillarin, putative
PF3D7_1408000	plasmepsin II
PF3D7_1408100	plasmepsin III
PF3D7_1408700	conserved protein, unknown function

PF3D7_1415300	RNA-binding protein Nova-1, putative
PF3D7_1415700	serine palmitoyltransferase, putative
PF3D7_1417000	inner membrane complex protein 1l
PF3D7_1417600	conserved Plasmodium protein, unknown function
PF3D7_1417800	DNA replication licensing factor MCM2
PF3D7_1418400	tRNA Proline
PF3D7_1418500	5S ribosomal RNA
PF3D7_1418600	5S ribosomal RNA
PF3D7_1418700	5S ribosomal RNA
PF3D7_1418800	signal recognition particle RNA
PF3D7_1421900	copper transporter, putative
PF3D7_1424100	60S ribosomal protein L5, putative
PF3D7_1426000	60S ribosomal protein L21
PF3D7_1426200	protein arginine N-methyltransferase 1
PF3D7_1430300	acid phosphatase, putative
PF3D7_1430900	cytochrome c oxidase subunit 2B, putative
PF3D7_1434200	calmodulin
PF3D7_1434500	dynein-related AAA-type ATPase, putative
PF3D7_1434800	mitochondrial acidic protein MAM33, putative
PF3D7_1435000	cytochrome c oxidase assembly protein COX15, putative
PF3D7_1435300	glutamate synthase [NADH], putative
PF3D7_1436600	cGMP-dependent protein kinase
PF3D7_1437200	ribonucleoside-diphosphate reductase large subunit, putative
PF3D7_1438100	translocation protein SEC62, putative
PF3D7_1438300	tRNA Methionine
PF3D7_1438400	metacaspase-2
PF3D7_1439600	cytochrome c oxidase subunit ApiCOX26, putative
PF3D7_1441400	FACT complex subunit SSRP1, putative
PF3D7_1443600	gamma-tubulin complex component, putative
PF3D7_1443900	heat shock protein 90, putative
PF3D7_1444800	fructose-bisphosphate aldolase
PF3D7_1446000	U5 spliceosomal RNA
PF3D7_1446100	conserved Plasmodium protein, unknown function
PF3D7_1446400	pyruvate dehydrogenase E1 component subunit beta
PF3D7_1447900	multidrug resistance protein 2

PF3D7_1449500	AP2 domain transcription factor AP2-O5, putative
PF3D7_1451100	elongation factor 2
PF3D7_1451200	conserved Plasmodium protein, unknown function
PF3D7_1452000	rhoptry neck protein 2
PF3D7_1454400	aminopeptidase P
PF3D7_1455800	LCCL domain-containing protein
PF3D7_1456100	serine hydroxymethyltransferase
PF3D7_1458000	cysteine proteinase falcipain 1
PF3D7_1458300	alpha/beta hydrolase, putative
PF3D7_1458700	exonuclease V, mitochondrial, putative
PF3D7_1459900	rhoptry protein, putative
PF3D7_1460200	conserved protein, unknown function
PF3D7_1461800	conserved Plasmodium protein, unknown function
PF3D7_1462300	GTP-binding protein, putative
PF3D7_1462800	glyceraldehyde-3-phosphate dehydrogenase
PF3D7_1463200	replication factor C subunit 3, putative
PF3D7_1464600	serine/threonine protein phosphatase UIS2, putative
PF3D7_1464900	ATP-dependent zinc metalloprotease FTSH, putative
PF3D7_1465000	cytochrome c oxidase subunit ApiCOX25, putative
PF3D7_1465800	dynein beta chain, putative
PF3D7_1466400	AP2 domain transcription factor AP2-EXP
PF3D7_1468100	MORC family protein, putative
PF3D7_1472200	histone deacetylase, putative
PF3D7_1473200	DnaJ protein, putative
PF3D7_1475400	cysteine repeat modular protein 4
PF3D7_1475500	LCCL domain-containing protein
PF3D7_1476400	serine/threonine protein kinase, FIKK family, pseudogene
PF3D7_1476600	Plasmodium exported protein, unknown function
PF3D7_1477300	Plasmodium exported protein (PHIST), unknown function
PF3D7_1479600	rifin
PF3D7_1479900	stevor
PF3D7_API05200	tRNA Valine
PF3D7_API05300	tRNA Arginine
PF3D7_API05400	tRNA Leucine
PF3D7_API05500	tRNA Asparagine

PF3D7_API05600	tRNA Alanine
PF3D7_API05700	small subunit ribosomal RNA
PF3D7_API05900	small subunit ribosomal RNA
PF3D7_API06000	tRNA Alanine
PF3D7_API06100	tRNA Asparagine
PF3D7_API06200	tRNA Leucine
PF3D7_API06300	tRNA Arginine
PF3D7_API06400	tRNA Valine
PF3D7_API06500	tRNA Arginine
PF3D7_API06600	tRNA Methionine

Bibliography

1. Miguel-Oteo, M., Jiram, A. I., Ta-Tang, T. H., Lanza, M., Hisam, S. & Rubio, J. M. (2017) Nested multiplex PCR for identification and detection of human Plasmodium species including Plasmodium knowlesi, *Asian Pacific Journal of Tropical Medicine.* **10**, 299-304.

2. Warrell, D. A., Cox, T. M., Benz, E. J. & Firth, J. D. (2003) *Oxford textbook of medicine*, Oxford University Press, USA.

3. Tuteja, R. (2007) Malaria− an overview, *The FEBS Journal.* **274**, 4670-4679.

4. WHO (2020) World Malaria Report 2020: 20 years of global progress and challenges in *World Malaria Report 2020: 20 years of global progress and challenges*

5. Poinar, G. (2005) Plasmodium dominicana n. sp.(Plasmodiidae: Haemospororida) from Tertiary Dominican amber, *Systematic Parasitology.* **61**, 47-52.

6. Baird, J. K. (2009) Malaria zoonoses, *Travel Medicine and Infectious Disease.* **7**, 269-277.

7. Sharp, P. M., Plenderleith, L. J. & Hahn, B. H. (2020) Ape origins of human malaria, *Annual Review of Microbiology.* **74**, 39-63.

8. Canali, S. (2008) Researches on Thalassemia and Malaria in Italy and the Origins of the"Haldane Hypothesis", *Medicina nei Secoli.* **20**, 827-846.

9. Brier, B. (2004) Infectious diseases in ancient Egypt, *Infectious Disease Clinics.* **18**, 17-27.

10. Pappas, G., Kiriaze, I. J. & Falagas, M. E. (2008) Insights into infectious disease in the era of Hippocrates, *International Journal of Infectious Diseases.* **12**, 347-350.

11. Cox, F. E. (2002) History of human parasitology, *Clinical Microbiology Reviews.* **15**, 595-612.

12. Li, Y. & Wu, Y.-L. (2003) An over four millennium story behind qinghaosu (artemisinin)-a fantastic antimalarial drug from a traditional Chinese herb, *Current Medicinal Chemistry.* **10**, 2197-2230.

13. Sallares, R. (2002) *Malaria and Rome: a history of malaria in ancient Italy*, Oxford University Press on Demand.

14. Hempelmann, E. & Krafts, K. (2013) Bad air, amulets and mosquitoes: 2,000 years of changing perspectives on malaria, *Malaria Journal.* **12**, 1-14.

15. Cox, F. E. (2010) History of the discovery of the malaria parasites and their vectors, *Parasites.* **3**, 1-9.

16. Chernin, E. (1988) Sir Ronald Ross, malaria, and the rewards of research, *Medical History.* **32**, 119-141.

17. Grassi, G. B., Bignami, A. & Bastianelli, G. (1899) *Ulteriori ricerche sul ciclo dei parassiti malarici umani nel corpo del zanzarone*, Rome R. accad. d. Lincei.

18. Ghosh, S. K. & Rahi, M. (2019) Malaria elimination in India—the way forward, *Journal of Vector Borne Diseases.* **56**, 32.

19. Singh, N., Nagpal, A., Saxena, A. & Singh, M. (2004) Changing scenario of malaria in central India, the replacement of Plasmodium vivax by Plasmodium falciparum (1986–2000), *Tropical Medicine International Health.* **9**, 364-371.

20. Tangpukdee, N., Duangdee, C., Wilairatana, P. & Krudsood, S. (2009) Malaria diagnosis: a brief review, *The Korean Journal of Parasitology.* **47**, 93.

21. Bartoloni, A. & Zammarchi, L. (2012) Clinical aspects of uncomplicated and severe malaria, *Mediterranean Journal of Hematology Infectious Diseases.* **4**.

22. Ross, A., Maire, N., Molineaux, L. & Smith, T. (2006) An epidemiologic model of severe morbidity and mortality caused by Plasmodium falciparum, *The American Journal of Tropical Medicine Hygiene.* **75**, 63-73.

23. Howes, R. E., Battle, K. E., Mendis, K. N., Smith, D. L., Cibulskis, R. E., Baird, J. K. & Hay, S. I. (2016) Global epidemiology of Plasmodium vivax, *The American Journal of Tropical Medicine Hygiene.* **95**, 15-34.

24. Mitchell, C. L., Brazeau, N. F., Keeler, C., Mwandagalirwa, M. K., Tshefu, A. K., Juliano, J. J. & Meshnick, S. R. (2021) Under the radar: epidemiology of Plasmodium ovale in the Democratic Republic of the Congo, *The Journal of Infectious Diseases.* **223**, 1005-1014.

25. Richter, J., Franken, G., Holtfreter, M. C., Walter, S., Labisch, A. & Mehlhorn, H. (2016) Clinical implications of a gradual dormancy concept in malaria, *Parasitology Research.* **115**, 2139-2148.

26. White, N. (2008) Plasmodium knowlesi: the fifth human malaria parasite, *Clinical Infectious Diseases*.

27. Ahmed, M. & Cox-Singh, J. (2015) Plasmodium knowlesi–an emerging pathogen, *ISBT Science Series.* **10**, 134-140.

28. Foth, B. J., Ralph, S. A., Tonkin, C. J., Struck, N. S., Fraunholz, M., Roos, D. S., Cowman, A. F. & McFadden, G. I. (2003) Dissecting apicoplast targeting in the malaria parasite Plasmodium falciparum, *Science.* **299**, 705-708.

29. Banerjee, R. & Goldberg, D. E. (2001) The Plasmodium food vacuole in *Antimalarial Chemotherapy* pp. 43-63, Springer.

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

31. Soulard, V., Bosson-Vanga, H., Lorthiois, A., Roucher, C., Franetich, J.-F., Zanghi, G., Bordessoulles, M., Tefit, M., Thellier, M. & Morosan, S. (2015) Plasmodium falciparum full life cycle and Plasmodium ovale liver stages in humanized mice, *Nature Communications.* **6**, 1-9.

32. Vaughan, A. M. & Kappe, S. H. (2017) Malaria parasite liver infection and exoerythrocytic biology, *Cold Spring Harbor Perspectives in Medicine.* **7**, a025486.

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

34. Gerald, N., Mahajan, B. & Kumar, S. (2011) Mitosis in the human malaria parasite Plasmodium falciparum, *Eukaryotic Cell.* **10**, 474-482.

35. Maier, A. G., Matuschewski, K., Zhang, M. & Rug, M. (2019) Plasmodium falciparum, *Trends in Parasitology.* **35**, 481-482.

36. Talman, A. M., Domarle, O., McKenzie, F. E., Ariey, F. & Robert, V. (2004) Gametocytogenesis: the puberty of Plasmodium falciparum, *Malaria Journal.* **3**, 1-14.

37. Sinden, R., Canning, E. U., Bray, R. & Smalley, M. (1978) Gametocyte and gamete development in Plasmodium falciparum, *Proceedings of the Royal Society of London Series B Biological Sciences.* **201**, 375-399.

38. Aly, A. S., Vaughan, A. M. & Kappe, S. (2009) Malaria parasite development in the mosquito and infection of the mammalian host, *Annual Review of Microbiology.* **63**, 195-221.

39. Kajfasz, P. (2009) Malaria prevention, *International Maritime Health.* **60**, 67-70.

40. Sabot, O., Cohen, J. M., Hsiang, M. S., Kahn, J. G., Basu, S., Tang, L., Zheng, B., Gao, Q., Zou, L. & Tatarsky, A. (2010) Costs and financial feasibility of malaria elimination, *The Lancet.* **376**, 1604-1615.

41. Pryce, J., Richardson, M. & Lengeler, C. (2018) Insecticide-treated nets for preventing malaria, *Cochrane Database of Systematic Reviews*.

42. WHO (2015) *Achieving the malaria MDG target: reversing the incidence of malaria 2000-2015*, World Health Organization.

43. Enayati, A. & Hemingway, J. (2010) Malaria management: past, present, and future, *Annual Review of Entomology.* **55**, 569-591.

44. Furnival-Adams, J., Olanga, E. A., Napier, M. & Garner, P. (2021) House modifications for preventing malaria, *Cochrane Database of Systematic Reviews*.

45. Fernando, S. D., Rodrigo, C. & Rajapakse, S. (2011) Chemoprophylaxis in malaria: drugs, evidence of efficacy and costs, *Asian Pacific Journal of Tropical Medicine.* **4**, 330-336.

46. Radeva-Petrova, D., Kayentao, K., ter Kuile, F. O., Sinclair, D. & Garner, P. (2014) Drugs for preventing malaria in pregnant women in endemic areas: any drug regimen versus placebo or no treatment, *Cochrane Database of Systematic Reviews*.

47. Esu, E. B., Oringanje, C. & Meremikwu, M. M. (2021) Intermittent preventive treatment for malaria in infants, *Cochrane Database of Systematic Reviews*.

48. González, R., Pons-Duran, C., Piqueras, M., Aponte, J. J., Ter Kuile, F. O. & Menéndez, C. (2018) Mefloquine for preventing malaria in pregnant women, *Cochrane Database of Systematic Reviews*.

49. Wiesner, J., Ortmann, R., Jomaa, H. & Schlitzer, M. (2003) New antimalarial drugs, *Angewandte Chemie International Edition.* **42**, 5274-5293.

50. Müller, I. B. & Hyde, J. E. (2010) Antimalarial drugs: modes of action and mechanisms of parasite resistance, *Future Microbiology.* **5**, 1857-1873.

51. Shibeshi, M. A., Kifle, Z. D. & Atnafie, S. A. (2020) Antimalarial drug resistance and novel targets for antimalarial drug discovery, *Infection Drug Resistance.* **13**, 4047.

52. Imwong, M., Dhorda, M., Tun, K. M., Thu, A. M., Phyo, A. P., Proux, S., Suwannasin, K., Kunasol, C., Srisutham, S. & Duanguppama, J. (2020) Molecular epidemiology of resistance to antimalarial drugs in the Greater Mekong subregion: an observational study, *The Lancet Infectious Diseases.* **20**, 1470-1480.

53. White, N. (1998) Drug resistance in malaria, *British Medical Bulletin.* **54**, 703-715.

54. Menard, D. & Dondorp, A. (2017) Antimalarial drug resistance: a threat to malaria elimination, *Cold Spring Harbor Perspectives in Medicine.* **7**, a025619.

55. Tu, Y. (2011) The discovery of artemisinin (qinghaosu) and gifts from Chinese medicine, *Nature Medicine.* **17**, 1217-1220.

56. Kamchonwongpaisan, S. & Meshnick, S. R. (1996) The mode of action of the antimalarial artemisinin and its derivatives, *General Pharmacology: The Vascular System.* **27**, 587-592.

57. Price, R., Nosten, F., Luxemburger, C., Kham, A., Brockman, A., Chongsuphajaisiddhi, T. & White, N. (1995) Artesunate versus artemether in combination with mefloquine for the treatment of multidrug-resistant falciparum malaria, *Transactions of the Royal Society of Tropical Medicine Hygiene.* **89**, 523-527.

58. WHO (2016) Artemisinin and artemisinin-based combination therapy resistance: status report in, World Health Organization,

59. Dondorp, A. M., Nosten, F., Yi, P., Das, D., Phyo, A. P., Tarning, J., Lwin, K. M., Ariey, F., Hanpithakpong, W. & Lee, S. J. (2009) Artemisinin resistance in Plasmodium falciparum malaria, *New England Journal of Medicine.* **361**, 455-467.

60. Ariey, F., Witkowski, B., Amaratunga, C., Beghain, J., Langlois, A.-C., Khim, N., Kim, S., Duru, V., Bouchier, C. & Ma, L. (2014) A molecular marker of artemisinin-resistant Plasmodium falciparum malaria, *Nature.* **505**, 50-55.

61. Tijani, M. K., Lugaajju, A. & Persson, K. E. (2021) Naturally Acquired Antibodies against Plasmodium falciparum: Friend or Foe?, *Pathogens.* **10**, 832.

62. Bouharoun-Tayoun, H., Oeuvray, C., Lunel, F. & Druilhe, P. (1995) Mechanisms underlying the monocyte-mediated antibody-dependent killing of Plasmodium falciparum asexual blood stages, *Journal of Experimental Medicine.* **182**, 409-418.

63. Langhorne, J., Ndungu, F. M., Sponaas, A.-M. & Marsh, K. (2008) Immunity to malaria: more questions than answers, *Nature Immunology.* **9**, 725-732.

64. von der Weid, T., Kopf, M., Köhler, G. & Langhorne, J. (1994) The immune response to Plasmodium chabaudi malaria in interleukin-4-deficient mice, *European Journal of Immunology.* **24**, 2285-2293.

65. Podoba, J. & Stevenson, M. (1991) CD4+ and CD8+ T lymphocytes both contribute to acquired immunity to blood-stage Plasmodium chabaudi AS, *Infection Immunity.* **59**, 51-58.

66. Horne-Debets, J. M., Karunarathne, D. S., Faleiro, R. J., Poh, C. M., Renia, L. & Wykes, M. N. (2016) Mice lacking programmed cell death-1 show a role for CD8+ T cells in long-term immunity against blood-stage malaria, *Scientific Reports.* **6**, 1-9.

67. Mendis, K. N., David, P. H. & Carter, R. (1991) Antigenic polymorphism in malaria: is it an important mechanism for immune evasion?, *Immunology Today.* **12**, A34-A37.

68. Brendan, S. & Alen, F. (2002) Plasmodium falciparum virulence determinants unveiled genome, *Genome Biology.* **3**.

69. Fernandez, V. & Wahlgren, M. (2002) Rosetting and autoagglutination in Plas modium falciparum, *Clinical Immunology.* **80**, 163-187.

70. Mahamar, A., Attaher, O., Swihart, B., Barry, A., Diarra, B. S., Kanoute, M. B., Cisse, K. B., Dembele, A. B., Keita, S. & Gamain, B. (2017) Host factors that modify Plasmodium falciparum adhesion to endothelial receptors, *Scientific Reports.* **7**, 1-8.

71. Barrett, M. P., Kyle, D. E., Sibley, L. D., Radke, J. B. & Tarleton, R. L. (2019) Protozoan persister-like cells and drug treatment failure, *Nature Reviews Microbiology.* **17**, 607-620.

72. Frech, C. & Chen, N. (2013) Variant surface antigens of malaria parasites: functional and evolutionary insights from comparative gene family classification and analysis, *BMC Genomics.* **14**, 1-23.

73. Johnson, P. L., Kochin, B. F., Ahmed, R. & Antia, R. (2012) How do antigenically varying pathogens avoid cross-reactive responses to invariant antigens?, *Proceedings of the Royal Society B: Biological Sciences.* **279**, 2777-2785.

74. Pasternak, N. D. & Dzikowski, R. (2009) PfEMP1: an antigen that plays a key role in the pathogenicity and immune evasion of the malaria parasite Plasmodium falciparum, *The International Journal of Biochemistry Cell Biology.* **41**, 1463-1466.

75. Roberts, D. J., Berendt, A. R., Pinches, R., Nash, G., Marsh, K. & Newbold, C. I. (1992) Rapid switching to multiple antigenic and adhesive phenotypes in malaria, *Nature.* **357**, 689-692.

76. Neafsey, D. E., Galinsky, K., Jiang, R. H., Young, L., Sykes, S. M., Saif, S., Gujja, S., Goldberg, J. M., Young, S. & Zeng, Q. (2012) The malaria parasite Plasmodium vivax exhibits greater genetic diversity than Plasmodium falciparum, *Nature Genetics.* **44**, 1046-1050.

77. Srivastava, A., Gangnard, S., Round, A., Dechavanne, S., Juillerat, A., Raynal, B., Faure, G., Baron, B., Ramboarina, S. & Singh, S. K. (2010) Full-length extracellular region of the var2CSA variant of PfEMP1 is required for specific, high-affinity binding to CSA, *Proceedings of the National Academy of Sciences.* **107**, 4884-4889.

78. Kapulu, M., Da, D., Miura, K., Li, Y., Blagborough, A., Churcher, T., Nikolaeva, D., Williams, A., Goodman, A. & Sangare, I. (2015) Comparative assessment of transmission-blocking vaccine candidates against Plasmodium falciparum, *Scientific Reports.* **5**, 1-15.

79. Richie, T. L., Billingsley, P. F., Sim, B. K. L., James, E. R., Chakravarty, S., Epstein, J. E., Lyke, K. E., Mordmüller, B., Alonso, P. & Duffy, P. E. (2015) Progress with Plasmodium falciparum sporozoite (PfSPZ)-based malaria vaccines, *Vaccine.* **33**, 7452-7461.

80. Rts, S. (2015) Efficacy and safety of RTS, S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial, *The Lancet.* **386**, 31-45.

81. Salanti, A., Staalsoe, T., Lavstsen, T., Jensen, A. T., Sowa, M. K., Arnot, D. E., Hviid, L. & Theander, T. G. (2003) Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering Plasmodium falciparum involved in pregnancy-associated malaria, *Molecular Microbiology.* **49**, 179-191.

82. Duffy, P. E. & Gorres, J. P. (2020) Malaria vaccines since 2000: progress, priorities, products, *NPJ Vaccines.* **5**, 1-9.

83. Partnership, R. S. C. T. (2012) A phase 3 trial of RTS, S/AS01 malaria vaccine in African infants, *New England Journal of Medicine.* **367**, 2284-2295.

84. Hyde, J. E. (2005) Drug-resistant malaria, *Trends in Parasitology.* **21**, 494-498.

85. Su, X.-z., Lane, K. D., Xia, L., Sá, J. M. & Wellems, T. E. (2019) Plasmodium genomics and genetics: new insights into malaria pathogenesis, drug resistance, epidemiology, and evolution, *Clinical Microbiology reviews.* **32**, e00019-19.

86. Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., Carlton, J. M., Pain, A., Nelson, K. E. & Bowman, S. (2002) Genome sequence of the human malaria parasite Plasmodium falciparum, *Nature.* **419**, 498-511.

87. Frech, C. & Chen, N. (2011) Genome comparison of human and non-human malaria parasites reveals species subset-specific genes potentially linked to human disease, *PLoS Computational Biology.* **7**, e1002320.

88. Bozdech, Z., Llinás, M., Pulliam, B. L., Wong, E. D., Zhu, J., DeRisi, J. L. & Ward, G. (2003) The transcriptome of the intraerythrocytic developmental cycle of Plasmodium falciparum, *PLoS Biology.* **1**, e5.

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

90. Horrocks, P., Wong, E., Russell, K. & Emes, R. D. (2009) Control of gene expression in Plasmodium falciparum–ten years on, *Molecular Biochemical Parasitology.* **164**, 9-25.

91. Watanabe, J., Sasaki, M., Suzuki, Y. & Sugano, S. (2002) Analysis of transcriptomes of human malaria parasite Plasmodium falciparum using full-length enriched library: identification of novel genes and diverse transcription start sites of messenger RNAs, *Gene.* **291**, 105-113.

92. Toenhake, C. G. & Bártfai, R. (2019) What functional genomics has taught us about transcriptional regulation in malaria parasites, *Briefings in Functional Genomics.* **18**, 290-301.

93. Wu, J., Sieglaff, D. H., Gervin, J. & Xie, X. S. (2008) Discovering regulatory motifs in the Plasmodium genome using comparative genomics, *Bioinformatics.* **24**, 1843-1849.

94. Wang, C., Gibbons, J., Adapa, S. R., Oberstaller, J., Liao, X., Zhang, M., Adams, J. H. & Jiang, R. H. (2020) The human malaria parasite genome is configured into thousands of coexpressed linear regulatory units, *Journal of Genetics Genomics data.* **47**, 513-521.

95. García, A., González, S. & Antequera, F. (2017) Nucleosomal organization and DNA base composition patterns, *Nucleus.* **8**, 469-474.

96. Sullivan Jr, W. J. (2003) Histone H3 and H3. 3 variants in the protozoan pathogens Plasmodium falciparum and Toxoplasma gondii, *DNA Sequence.* **14**, 227-231.

97. Toenhake, C. G., Fraschka, S. A.-K., Vijayabaskar, M. S., Westhead, D. R., van Heeringen, S. J. & Bártfai, R. (2018) Chromatin accessibility-based characterization of the gene regulatory network underlying Plasmodium falciparum blood-stage development, *Cell Host Microbe.* **23**, 557-569. e9.

98. Bunnik, E. M., Polishko, A., Prudhomme, J., Ponts, N., Gill, S. S., Lonardi, S. & Le Roch, K. G. (2014) DNA-encoded nucleosome occupancy is associated with transcription levels in the human malaria parasite Plasmodium falciparum, *BMC Genomics.* **15**, 1-15.

99. Hoeijmakers, W. A., Salcedo-Amaya, A. M., Smits, A. H., Françoijs, K. J., Treeck, M., Gilberger, T. W., Stunnenberg, H. G. & Bártfai, R. (2013) H 2 A. Z/H 2 B. Z double-variant nucleosomes inhabit the AT-rich promoter regions of the P lasmodium falciparum genome, *Molecular microbiology.* **87**, 1061-1073.

100. Bunnik, E. M., Cook, K. B., Varoquaux, N., Batugedara, G., Prudhomme, J., Cort, A., Shi, L., Andolina, C., Ross, L. S. & Brady, D. (2018) Changes in genome organization of parasite-specific gene families during the Plasmodium transmission stages, *Nature Communications.* **9**, 1-15.

101. Bunnik, E. M., Venkat, A., Shao, J., McGovern, K. E., Batugedara, G., Worth, D., Prudhomme, J., Lapp, S. A., Andolina, C. & Ross, L. S. (2019) Comparative 3D genome organization in apicomplexan parasites, *Proceedings of the National Academy of Sciences.* **116**, 3183-3192.

102. Luse, D. S. (2014) The RNA polymerase II preinitiation complex: Through what pathway is the complex assembled?, *Transcription.* **5**, e27050.

103. Rai, R., Zhu, L., Chen, H., Gupta, A. P., Sze, S. K., Zheng, J., Ruedl, C., Bozdech, Z. & Featherstone, M. (2014) Genome-wide analysis in Plasmodium falciparum reveals early and late phases of RNA polymerase II occupancy during the infectious cycle, *BMC genomics.* **15**, 1-19.

104. Templeton, T. J., Iyer, L. M., Anantharaman, V., Enomoto, S., Abrahante, J. E., Subramanian, G., Hoffman, S. L., Abrahamsen, M. S. & Aravind, L. (2004) Comparative analysis of apicomplexa and genomic diversity in eukaryotes, *Genome Research.* **14**, 1686-1695.

105. Campbell, T. L., De Silva, E. K., Olszewski, K. L., Elemento, O. & Llinás, M. (2010) Identification and genome-wide prediction of DNA binding specificities for the ApiAP2 family of regulators from the malaria parasite, *PLoS Pathogens.* **6**, e1001165.

106. Bischoff, E. & Vaquero, C. (2010) In silico and biological survey of transcription-associated proteins implicated in the transcriptional machinery during the erythrocytic development of Plasmodium falciparum, *BMC Genomics.* **11**, 1-20.

107. Iwanaga, S., Kaneko, I., Kato, T. & Yuda, M. (2012) Identification of an AP2-family protein that is critical for malaria liver stage development, *PLoS One.* **7**, e47557.

108. Sinha, A., Hughes, K. R., Modrzynska, K. K., Otto, T. D., Pfander, C., Dickens, N. J., Religa, A. A., Bushell, E., Graham, A. L. & Cameron, R. (2014) A cascade of DNA-binding proteins for sexual commitment and development in Plasmodium, *Nature.* **507**, 253-257.

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

110. Jing, Q., Cao, L., Zhang, L., Cheng, X., Gilbert, N., Dai, X., Sun, M., Liang, S. & Jiang, L. (2018) Plasmodium falciparum var gene is activated by its antisense long noncoding RNA, *Frontiers in Microbiology.* **9**, 3117.

111. Filarsky, M., Fraschka, S. A., Niederwieser, I., Brancucci, N. M., Carrington, E., Carrió, E., Moes, S., Jenoe, P., Bártfai, R. & Voss, T. S. (2018) GDV1 induces sexual commitment of malaria parasites by antagonizing HP1-dependent gene silencing, *Science.* **359**, 1259-1263.

112. Hollin, T., Gupta, M., Lenz, T. & Le Roch, K. G. (2020) Dynamic chromatin structure and epigenetics control the fate of malaria parasites, *Trends in Genetics*.

113. Clapier, C. R., Iwasa, J., Cairns, B. R. & Peterson, C. L. (2017) Mechanisms of action and regulation of ATP-dependent chromatin-remodelling complexes, *Nature Reviews Molecular Cell Biology.* **18**, 407-422.

114. Flaus, A., Martin, D. M., Barton, G. J. & Owen-Hughes, T. (2006) Identification of multiple distinct Snf2 subfamilies with conserved structural motifs, *Nucleic Acids Research.* **34**, 2887-2905.

115. Bryant, J. M., Baumgarten, S., Dingli, F., Loew, D., Sinha, A., Claës, A., Preiser, P. R., Dedon, P. C. & Scherf, A. (2020) Exploring the virulence gene interactome with CRISPR/dC as9 in the human malaria parasite, *Molecular Systems Biology.* **16**, e9569.

116. Saraf, A., Cervantes, S., Bunnik, E. M., Ponts, N., Sardiu, M. E., Chung, D.-W. D., Prudhomme, J., Varberg, J. M., Wen, Z. & Washburn, M. P. (2016) Dynamic and combinatorial landscape of histone modifications during the intraerythrocytic developmental cycle of the malaria parasite, *Journal of Proteome Research.* **15**, 2787-2801.

117. Bannister, A. J. & Kouzarides, T. (2011) Regulation of chromatin by histone modifications, *Cell Research.* **21**, 381-395.

118. Gillette, T. G. & Hill, J. A. (2015) Readers, writers, and erasers: chromatin as the whiteboard of heart disease, *Circulation Research.* **116**, 1245-1253.

119. Karmodiya, K., Pradhan, S. J., Joshi, B., Jangid, R., Reddy, P. C. & Galande, S. (2015) A comprehensive epigenome map of Plasmodium falciparum reveals unique mechanisms of transcriptional regulation and identifies H3K36me2 as a global mark of gene suppression, *Epigenetics Chromatin.* **8**, 1-18.

120. Kanyal, A., Rawat, M., Gurung, P., Choubey, D., Anamika, K. & Karmodiya, K. (2018) Genome-wide survey and phylogenetic analysis of histone acetyltransferases and histone deacetylases of Plasmodium falciparum, *The FEBS Journal.* **285**, 1767-1782.

121. Duraisingh, M. T. & Horn, D. (2016) Epigenetic regulation of virulence gene expression in parasitic protozoa, *Cell Host Microbe.* **19**, 629-640.

122. WHO (2016) World Malaria Report 2015 in, World Health Organization,

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

124. Coulson, R. M., Hall, N. & Ouzounis, C. A. (2004) Comparative genomics of transcriptional control in the human malaria parasite Plasmodium falciparum, *Genome Research.* **14**, 1548-1554.

125. Rawat, M., Bhosale, M. A. & Karmodiya, K. (2016) Plasmodium falciparum epigenome: a distinct dynamic epigenetic regulation of gene expression, *Genomics Data.* **7**, 79-81.

126. Ubhe, S., Rawat, M., Verma, S., Anamika, K. & Karmodiya, K. (2017) Genome-wide identification of novel intergenic enhancer-like elements: implications in the regulation of transcription in Plasmodium falciparum, *BMC Genomics.* **18**, 1-16.

127. Cary, C., Lamont, D., Dalton, J. P. & Doerig, C. (1994) Plasmodium falciparum chromatin: nucleosomal organisation and histone-like proteins, *Parasitology Research.* **80**, 255-258.

128. Cui, L. & Miao, J. (2010) Chromatin-mediated epigenetic regulation in the malaria parasite Plasmodium falciparum, *Eukaryotic Cell.* **9**, 1138-1149.

129. Andrews, K. T., Tran, T. N., Wheatley, N. C. & Fairlie, D. P. (2009) Targeting histone deacetylase inhibitors for anti-malarial therapy, *Current Topics in Medicinal Chemistry.* **9**, 292-308.

130. Karmodiya, K., Anamika, K., Muley, V., Pradhan, S. J., Bhide, Y. & Galande, S. (2014) Camello, a novel family of Histone Acetyltransferases that acetylate histone H4 and is essential for zebrafish development, *Scientific Reports.* **4**, 1-9.

131. Roth, S. Y., Denu, J. M. & Allis, C. D. (2001) Histone acetyltransferases, *Annual Review of Biochemistry.* **70**, 81-120.

132. Fan, Q., An, L. & Cui, L. (2004) Plasmodium falciparum histone acetyltransferase, a yeast GCN5 homologue involved in chromatin remodeling, *Eukaryotic Cell.* **3**, 264-276.

133. Adjalley, S. H., Chabbert, C. D., Klaus, B., Pelechano, V. & Steinmetz, L. M. (2016) Landscape and dynamics of transcription initiation in the malaria parasite Plasmodium falciparum, *Cell reports.* **14**, 2463-2475.

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

135. Fan, Q., An, L. & Cui, L. (2004) PfADA2, a Plasmodium falciparum homologue of the transcriptional coactivator ADA2 and its in vivo association with the histone acetyltransferase PfGCN5, *Gene.* **336**, 251-261.

136. Huisinga, K. L. & Pugh, B. F. (2004) A genome-wide housekeeping role for TFIID and a highly regulated stress-related role for SAGA in Saccharomyces cerevisiae, *Molecular Cell.* **13**, 573-585.

137. Cui, L., Miao, J., Furuya, T., Li, X., Su, X.-z. & Cui, L. (2007) PfGCN5-mediated histone H3 acetylation plays a key role in gene expression in Plasmodium falciparum, *Eukaryotic Cell.* **6**, 1219-1227.

138. Jiang, H., Patel, J. J., Yi, M., Mu, J., Ding, J., Stephens, R., Cooper, R. A., Ferdig, M. T. & Su, X. (2008) Genome-wide compensatory changes accompany drug-selected mutations in the Plasmodium falciparum crt gene, *PLoS One.* **3**, e2484.

139. Trieu, A., Kayala, M. A., Burk, C., Molina, D. M., Freilich, D. A., Richie, T. L., Baldi, P., Felgner, P. L. & Doolan, D. L. (2011) Sterile protective immunity to malaria is associated with a panel of novel P. falciparum antigens, *Molecular Cellular Proteomics.* **10**.

140. Chang, H. H., Falick, A. M., Carlton, P. M., Sedat, J. W., DeRisi, J. L. & Marletta, M. A. J. M. (2008) N-terminal processing of proteins exported by malaria parasites, *Molecular Biochemical Parasitology.* **160**, 107-115.

141. Polevoda, B. & Sherman, F. (2003) Composition and function of the eukaryotic N-terminal acetyltransferase subunits, *Biochemical Biophysical Research Communications.* **308**, 1-11.

142. Wittschieben, B. Ø., Otero, G., de Bizemont, T., Fellows, J., Erdjument-Bromage, H., Ohba, R., Li, Y., Allis, C. D., Tempst, P. & Svejstrup, J. Q. (1999) A novel histone

acetyltransferase is an integral subunit of elongating RNA polymerase II holoenzyme, *Molecular Cell.* **4**, 123-128.

143. Otto, T. D., Wilinski, D., Assefa, S., Keane, T. M., Sarry, L. R., Böhme, U., Lemieux, J., Barrell, B., Pain, A. & Berriman, M. (2010) New insights into the blood-stage transcriptome of Plasmodium falciparum using RNA-Seq, *Molecular Microbiology.* **76**, 12-24.

144. Wittschieben, B. Ø., Fellows, J., Du, W., Stillman, D. J. & Svejstrup, J. Q. (2000) Overlapping roles for the histone acetyltransferase activities of SAGA and elongator in vivo, *The EMBO Journal.* **19**, 3060-3068.

145. Osborne, A. R., Speicher, K. D., Tamez, P. A., Bhattacharjee, S., Speicher, D. W. & Haldar, K. (2010) The host targeting motif in exported Plasmodium proteins is cleaved in the parasite endoplasmic reticulum, *Molecular Biochemical Parasitology.* **171**, 25-31.

146. Zhou, Y., Ramachandran, V., Kumar, K. A., Westenberger, S., Refour, P., Zhou, B., Li, F., Young, J. A., Chen, K. & Plouffe, D. (2008) Evidence-based annotation of the malaria parasite's genome using comparative expression profiling, *PLoS One.* **3**, e1570.

147. Chimnaronk, S., Suzuki, T., Manita, T., Ikeuchi, Y., Yao, M., Suzuki, T. & Tanaka, I. (2009) RNA helicase module in an acetyltransferase that modifies a specific tRNA anticodon, *The EMBO Journal.* **28**, 1362-1373.

148. Kuss, C., Gan, C. S., Gunalan, K., Bozdech, Z., Sze, S. K. & Preiser, P. R. (2012) Quantitative proteomics reveals new insights into erythrocyte invasion by Plasmodium falciparum, *Molecular Cellular Proteomics.* **11**.

149. Mok, S., Imwong, M., Mackinnon, M. J., Sim, J., Ramadoss, R., Yi, P., Mayxay, M., Chotivanich, K., Liong, K.-Y. & Russell, B. (2011) Artemisinin resistance in Plasmodium falciparum is associated with an altered temporal pattern of transcription, *BMC Genomics.* **12**, 1-14.

150. Gómez, E. B., Espinosa, J. M. & Forsburg, S. L. (2005) Schizosaccharomyces pombe mst2+ encodes a MYST family histone acetyltransferase that negatively regulates telomere silencing, *Molecular Cellular Biology.* **25**, 8887-8903.

151. Kim, J., Daniel, J., Espejo, A., Lake, A., Krishna, M., Xia, L., Zhang, Y. & Bedford, M. T. (2006) Tudor, MBT and chromo domains gauge the degree of lysine methylation, *EMBO Reports.* **7**, 397-403.

152. Miao, J., Fan, Q., Cui, L., Li, X., Wang, H., Ning, G., Reese, J. C. & Cui, L. (2010) The MYST family histone acetyltransferase regulates gene expression and cell cycle in malaria parasite Plasmodium falciparum, *Molecular Microbiology.* **78**, 883-902.

153. Yan, Y., Barlev, N. A., Haley, R. H., Berger, S. L. & Marmorstein, R. (2000) Crystal structure of yeast Esa1 suggests a unified mechanism for catalysis and substrate binding by histone acetyltransferases, *Molecular Cell.* **6**, 1195-1205.

154. Miao, J., Lawrence, M., Jeffers, V., Zhao, F., Parker, D., Ge, Y., Sullivan Jr, W. J. & Cui, L. (2013) Extensive lysine acetylation occurs in evolutionarily conserved metabolic pathways and parasite-specific functions during P lasmodium falciparum intraerythrocytic development, *Molecular Microbiology.* **89**, 660-675.

155. Ahmad, M. & Tuteja, R. (2012) Plasmodium falciparum RuvB proteins: emerging importance and expectations beyond cell cycle progression, *Communicative Integrative Biology.* **5**, 350-361.

156. Lee, S. J., Seo, E. & Cho, Y. (2013) Proposal for a new therapy for drug-resistant malaria using Plasmodium synthetic lethality inference, *International Journal for Parasitology: Drugs Drug Resistance.* **3**, 119-128.

157. Frye, R. A. (2000) Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins, *Biochemical Biophysical Research Communications.* **273**, 793-798.

158. Wilting, R. H., Yanover, E., Heideman, M. R., Jacobs, H., Horner, J., Van Der Torre, J., DePinho, R. A. & Dannenberg, J. H. (2010) Overlapping functions of Hdac1 and Hdac2 in cell cycle regulation and haematopoiesis, *The EMBO Journal.* **29**, 2586-2597.

159. Miller, K. M., Tjeertes, J. V., Coates, J., Legube, G., Polo, S. E., Britton, S. & Jackson, S. P. (2010) Human HDAC1 and HDAC2 function in the DNA-damage response to promote DNA nonhomologous end-joining, *Nature Structural Molecular Biology.* **17**, 1144-1151.

160. Haberland, M., Montgomery, R. L. & Olson, E. N. (2009) The many roles of histone deacetylases in development and physiology: implications for disease and therapy, *Nature Reviews Genetics.* **10**, 32-42.

161. Chaal, B. K., Gupta, A. P., Wastuwidyaningtyas, B. D., Luah, Y.-H. & Bozdech, Z. (2010) Histone deacetylases play a major role in the transcriptional regulation of the Plasmodium falciparum life cycle, *PLoS Pathogens.* **6**, e1000737.

162. Pavithra, S. R., Kumar, R. & Tatu, U. (2007) Systems analysis of chaperone networks in the malarial parasite Plasmodium falciparum, *PLoS Computational Biology.* **3**, e168.

163. Kumar, R., Pavithra, S. R. & Tatu, U. (2007) Three-dimensional structure of heat shock protein 90 from Plasmodium falciparum: molecular modelling approach to rational drug design against malaria, *Journal of Biosciences.* **32**, 531-536.

164. Martin, M., Kettmann, R. & Dequiedt, F. (2007) Class IIa histone deacetylases: regulating the regulators, *Oncogene.* **26**, 5450-5467.

165. Yang, X.-J. & Grégoire, S. (2005) Class II histone deacetylases: from sequence to function, regulation, and clinical implication, *Molecular Cellular Biology.* **25**, 2873-2884.
166. Oehme, I., Linke, J.-P., Böck, B. C., Milde, T., Lodrini, M., Hartenstein, B., Wiegand, I., Eckert, C., Roth, W. & Kool, M. (2013) Histone deacetylase 10 promotes autophagy-mediated cell survival, *Proceedings of the National Academy of Sciences.* **110**, E2592-E2601.
167. Kawaguchi, Y., Kovacs, J. J., McLaurin, A., Vance, J. M., Ito, A. & Yao, T.-P. (2003) The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress, *Cell.* **115**, 727-738.
168. Marchler-Bauer, A., Bo, Y., Han, L., He, J., Lanczycki, C. J., Lu, S., Chitsaz, F., Derbyshire, M. K., Geer, R. C. & Gonzales, N. R. (2017) CDD/SPARCLE: functional classification of proteins via subfamily domain architectures, *Nucleic Acids Research.* **45**, D200-D203.
169. Bork, P. (1993) Hundreds of ankyrin-like repeats in functionally diverse proteins: mobile modules that cross phyla horizontally?, *Proteins: Structure, Function, Bioinformatics.* **17**, 363-374.
170. Coleman, B. I., Skillman, K. M., Jiang, R. H., Childs, L. M., Altenhofen, L. M., Ganter, M., Leung, Y., Goldowitz, I., Kafsack, B. F. & Marti, M. J. C. h. (2014) A Plasmodium falciparum histone deacetylase regulates antigenic variation and gametocyte conversion, *Cell Host Microbe.* **16**, 177-186.
171. Ralph, S. A., Scheidig-Benatar, C. & Scherf, A. (2005) Antigenic variation in Plasmodium falciparum is associated with movement of var loci between subnuclear locations, *Proceedings of the National Academy of Sciences.* **102**, 5414-5419.
172. Choi, J.-E. & Mostoslavsky, R. (2014) Sirtuins, metabolism, and DNA repair, *Current Opinion in Genetics Development.* **26**, 24-32.
173. Duraisingh, M. T., Voss, T. S., Marty, A. J., Duffy, M. F., Good, R. T., Thompson, J. K., Freitas-Junior, L. H., Scherf, A., Crabb, B. S. & Cowman, A. F. (2005) Heterochromatin silencing and locus repositioning linked to regulation of virulence genes in Plasmodium falciparum, *Cell.* **121**, 13-24.
174. Tonkin, C. J., Carret, C. K., Duraisingh, M. T., Voss, T. S., Ralph, S. A., Hommel, M., Duffy, M. F., Silva, L. M. d., Scherf, A. & Ivens, A. (2009) Sir2 paralogues cooperate to regulate virulence genes and antigenic variation in Plasmodium falciparum, *PLoS Biology.* **7**, e1000084.

175. Chakrabarty, S. P., Saikumari, Y. K., Bopanna, M. P. & Balaram, H. (2008) Biochemical characterization of Plasmodium falciparum Sir2, a NAD+-dependent deacetylase, *Molecular Biochemical Parasitology.* **158**, 139-151.

176. Freitas-Junior, L. H., Hernandez-Rivas, R., Ralph, S. A., Montiel-Condado, D., Ruvalcaba-Salazar, O. K., Rojas-Meza, A. P., Mâncio-Silva, L., Leal-Silvestre, R. J., Gontijo, A. M. & Shorte, S. (2005) Telomeric heterochromatin propagation and histone acetylation control mutually exclusive expression of antigenic variation genes in malaria parasites, *Cell.* **121**, 25-36.

177. Merrick, C. J., Huttenhower, C., Buckee, C., Amambua-Ngwa, A., Gomez-Escobar, N., Walther, M., Conway, D. J. & Duraisingh, M. T. (2012) Epigenetic dysregulation of virulence gene expression in severe Plasmodium falciparum malaria, *The Journal of Infectious Diseases.* **205**, 1593-1600.

178. Daily, J. á., Scanfeld, D., Pochet, N., Le Roch, K., Plouffe, D., Kamal, M., Sarr, O., Mboup, S., Ndir, O. & Wypij, D. (2007) Distinct physiological states of Plasmodium falciparum in malaria-infected patients, *Nature.* **450**, 1091-1095.

179. Goyal, M., Alam, A., Iqbal, M. S., Dey, S., Bindu, S., Pal, C., Banerjee, A., Chakrabarti, S. & Bandyopadhyay, U. (2012) Identification and molecular characterization of an Alba-family protein from human malaria parasite Plasmodium falciparum, *Nucleic Acids Research.* **40**, 1174-1190.

180. Mancio-Silva, L., Lopez-Rubio, J. J., Claes, A. & Scherf, A. (2013) Sir2a regulates rDNA transcription and multiplication rate in the human malaria parasite Plasmodium falciparum, *Nature Communications.* **4**, 1-6.

181. Petter, M., Lee, C. C., Byrne, T. J., Boysen, K. E., Volz, J., Ralph, S. A., Cowman, A. F., Brown, G. V. & Duffy, M. F. (2011) Expression of P. falciparum var genes involves exchange of the histone variant H2A. Z at the promoter, *PLoS Pathogens.* **7**, e1001292.

182. Seto, E. & Yoshida, M. (2014) Erasers of histone acetylation: the histone deacetylase enzymes, *Cold Spring Harbor Perspectives in Biology.* **6**, a018713.

183. Villagra, A., Cheng, F., Wang, H.-W., Suarez, I., Glozak, M., Maurin, M., Nguyen, D., Wright, K. L., Atadja, P. W. & Bhalla, K. (2009) The histone deacetylase HDAC11 regulates the expression of interleukin 10 and immune tolerance, *Nature Immunology.* **10**, 92-100.

184. Marfella, C. G. & Imbalzano, A. N. (2007) The Chd family of chromatin remodelers, *Mutation Research Fundamental Molecular Mechanisms of Mutagenesis.* **618**, 30-40.

185. Ashley, E. A., Dhorda, M., Fairhurst, R. M., Amaratunga, C., Lim, P., Suon, S., Sreng, S., Anderson, J. M., Mao, S. & Sam, B. (2014) Spread of artemisinin resistance in Plasmodium falciparum malaria, *New England Journal of Medicine.* **371**, 411-423.

186. Mok, S., Ashley, E. A., Ferreira, P. E., Zhu, L., Lin, Z., Yeo, T., Chotivanich, K., Imwong, M., Pukrittayakamee, S. & Dhorda, M. (2015) Population transcriptomics of human malaria parasites reveals the mechanism of artemisinin resistance, *Science.* **347**, 431-435.

187. Barrett, T., Wilhite, S. E., Ledoux, P., Evangelista, C., Kim, I. F., Tomashevsky, M., Marshall, K. A., Phillippy, K. H., Sherman, P. M. & Holko, M. (2012) NCBI GEO: archive for functional genomics data sets—update, *Nucleic Acids Research.* **41**, D991-D995.

188. Cui, L., Miao, J., Furuya, T., Fan, Q., Li, X., Rathod, P. K., Su, X.-z. & Cui, L. (2008) Histone acetyltransferase inhibitor anacardic acid causes changes in global gene expression during in vitro Plasmodium falciparum development, *Eukaryotic Cell.* **7**, 1200-1210.

189. Kumar, A., Bhowmick, K., Vikramdeo, K. S., Mondal, N., Subbarao, N. & Dhar, S. K. (2017) Designing novel inhibitors against histone acetyltransferase (HAT: GCN5) of Plasmodium falciparum, *European Journal of Medicinal Chemistry.* **138**, 26-37.

190. Jeffers, V., Gao, H., Checkley, L. A., Liu, Y., Ferdig, M. T. & Sullivan Jr, W. J. (2016) Garcinol inhibits GCN5-mediated lysine acetyltransferase activity and prevents replication of the parasite Toxoplasma gondii, *Antimicrobial Agents Chemotherapy.* **60**, 2164-2170.

191. Darkin-Rattray, S. J., Gurnett, A. M., Myers, R. W., Dulski, P. M., Crumley, T. M., Allocco, J. J., Cannova, C., Meinke, P. T., Colletti, S. L. & Bednarek, M. A. (1996) Apicidin: a novel antiprotozoal agent that inhibits parasite histone deacetylase, *Proceedings of the National Academy of Sciences.* **93**, 13143-13147.

192. Mukherjee, P., Pradhan, A., Shah, F., Tekwani, B. L. & Avery, M. A. (2008) Structural insights into the Plasmodium falciparum histone deacetylase 1 (PfHDAC-1): A novel target for the development of antimalarial therapy, *Bioorganic Medicinal Chemistry.* **16**, 5254-5265.

193. Elbadawi, M. A. A., Awadalla, M. K. A., Hamid, M. M. A., Mohamed, M. A. & Awad, T. A. (2015) Valproic acid as a potential inhibitor of Plasmodium falciparum histone deacetylase 1 (PfHDAC1): an in silico approach, *International Journal of Molecular Sciences.* **16**, 3915-3931.

194. Andrews, K., Tran, T., Lucke, A., Kahnberg, P., Le, G., Boyle, G., Gardiner, D., Skinner-Adams, T. & Fairlie, D. (2008) Potent antimalarial activity of histone deacetylase inhibitor analogues, *Antimicrobial Agents Chemotherapy.* **52**, 1454-1461.

195. Marfurt, J., Chalfein, F., Prayoga, P., Wabiser, F., Kenangalem, E., Piera, K. A., Fairlie, D. P., Tjitra, E., Anstey, N. M. & Andrews, K. T. (2011) Ex vivo activity of histone deacetylase

inhibitors against multidrug-resistant clinical isolates of Plasmodium falciparum and P. vivax, *Antimicrobial Agents Chemotherapy.* **55**, 961-966.

196. Engel, J. A., Jones, A. J., Avery, V. M., Sumanadasa, S. D., Ng, S. S., Fairlie, D. P., Adams, T. S. & Andrews, K. T. (2015) Profiling the anti-protozoal activity of anti-cancer HDAC inhibitors against Plasmodium and Trypanosoma parasites, *International Journal for Parasitology: Drugs Drug Resistance.* **5**, 117-126.

197. Rawat, M., Kanyal, A., Sahasrabudhe, A., Vembar, S. S., Lopez-Rubio, J.-J. & Karmodiya, K. (2021) Histone acetyltransferase PfGCN5 regulates stress responsive and artemisinin resistance related genes in Plasmodium falciparum, *Scientific Reports.* **11**, 1-13.

198. Josling, G. A. & Llinás, M. (2015) Sexual development in Plasmodium parasites: knowing when it's time to commit, *Nature Reviews Microbiology.* **13**, 573-587.

199. Crowther, G. J., Napuli, A. J., Gilligan, J. H., Gagaring, K., Borboa, R., Francek, C., Chen, Z., Dagostino, E. F., Stockmyer, J. B. & Wang, Y. J. M. (2011) Identification of inhibitors for putative malaria drug targets among novel antimalarial compounds, *Molecular Biochemical Parasitology.* **175**, 21-29.

200. Coetzee, N., von Grüning, H., Opperman, D., van der Watt, M., Reader, J. & Birkholtz, L.-M. (2020) Epigenetic inhibitors target multiple stages of Plasmodium falciparum parasites, *Scientific Reports.* **10**, 1-11.

201. Vanheer, L. N., Zhang, H., Lin, G. & Kafsack, B. F. (2020) Activity of epigenetic inhibitors against Plasmodium falciparum asexual and sexual blood stages, *Antimicrobial Agents Chemotherapy.* **64**, e02523-19.

202. Joshi, M. B., Lin, D. T., Chiang, P. H., Goldman, N. D., Fujioka, H., Aikawa, M. & Syin, C. (1999) Molecular cloning and nuclear localization of a histone deacetylase homologue in Plasmodium falciparum, *Molecular Biochemical Parasitology.* **99**, 11-19.

203. Bushell, E., Gomes, A. R., Sanderson, T., Anar, B., Girling, G., Herd, C., Metcalf, T., Modrzynska, K., Schwach, F. & Martin, R. E. (2017) Functional profiling of a Plasmodium genome reveals an abundance of essential genes, *Cell.* **170**, 260-272. e8.

204. Zhang, M., Wang, C., Otto, T. D., Oberstaller, J., Liao, X., Adapa, S. R., Udenze, K., Bronner, I. F., Casandra, D. & Mayho, M. (2018) Uncovering the essential genes of the human malaria parasite Plasmodium falciparum by saturation mutagenesis, *Science.* **360**.

205. Hansen, F. K., Sumanadasa, S. D., Stenzel, K., Duffy, S., Meister, S., Marek, L., Schmetter, R., Kuna, K., Hamacher, A. & Mordmüller, B. (2014) Discovery of HDAC inhibitors with potent activity against multiple malaria parasite life cycle stages, *European Journal of Medicinal Chemistry.* **82**, 204-213.

206. Wheatley, N. C., Andrews, K. T., Tran, T. L., Lucke, A. J., Reid, R. C. & Fairlie, D. P. (2010) Antimalarial histone deacetylase inhibitors containing cinnamate or NSAID components, *Bioorganic Medicinal Chemistry Letters.* **20**, 7080-7084.

207. Huang, Z., Li, R., Tang, T., Ling, D., Wang, M., Xu, D., Sun, M., Zheng, L., Zhu, F. & Min, H. (2020) A novel multistage antiplasmodial inhibitor targeting Plasmodium falciparum histone deacetylase 1, *Cell Discovery.* **6**, 1-15.

208. Segré, C. V. & Chiocca, S. (2010) Regulating the regulators: the post-translational code of class I HDAC1 and HDAC2, *Journal of Biomedicine Biotechnology.* **2011**.

209. Puri, P. L., Iezzi, S., Stiegler, P., Chen, T., Schiltz, R. L., Muscat, G. E., Giordano, A., Kedes, L., Wang, J. Y. & Sartorelli, V. (2001) Class I histone deacetylases sequentially interact with MyoD and pRb during skeletal myogenesis, *Molecular Cell.* **8**, 885-897.

210. Jacob, C., Christen, C. N., Pereira, J. A., Somandin, C., Baggiolini, A., Lötscher, P., Özçelik, M., Tricaud, N., Meijer, D. & Yamaguchi, T. (2011) HDAC1 and HDAC2 control the transcriptional program of myelination and the survival of Schwann cells, *Nature Neuroscience.* **14**, 429-436.

211. Montgomery, R. L., Davis, C. A., Potthoff, M. J., Haberland, M., Fielitz, J., Qi, X., Hill, J. A., Richardson, J. A., Olson, E. N. & development (2007) Histone deacetylases 1 and 2 redundantly regulate cardiac morphogenesis, growth, and contractility, *Genes.* **21**, 1790-1802.

212. Dovey, O. M., Foster, C. T. & Cowley, S. M. (2010) Histone deacetylase 1 (HDAC1), but not HDAC2, controls embryonic stem cell differentiation, *Proceedings of the National Academy of Sciences.* **107**, 8242-8247.

213. Cacan, E., Ali, M. W., Boyd, N. H., Hooks, S. B. & Greer, S. F. (2014) Inhibition of HDAC1 and DNMT1 modulate RGS10 expression and decrease ovarian cancer chemoresistance, *PLoS One.* **9**, e87455.

214. Wang, X., Bai, H., Li, S., Sun, H., Min, L., Tao, B., Zhong, J. & Li, B. (2017) Knockdown of HDAC1 expression suppresses invasion and induces apoptosis in glioma cells, *Oncotarget.* **8**, 48027.

215. Thurn, K. T., Thomas, S., Raha, P., Qureshi, I. & Munster, P. N. (2013) Histone deacetylase regulation of ATM-mediated DNA damage signaling, *Molecular Cancer Therapeutics.* **12**, 2078-2087.

216. Milutinovic, S., Zhuang, Q. & Szyf, M. (2002) Proliferating cell nuclear antigen associates with histone deacetylase activity, integrating DNA replication and chromatin modification, *Journal of Biological Chemistry.* **277**, 20974-20978.

217. Li, Z. & Zhu, W.-G. (2014) Targeting histone deacetylases for cancer therapy: from molecular mechanisms to clinical implications, *International Journal of Biological Sciences.* **10**, 757.

218. Radfar, A., Méndez, D., Moneriz, C., Linares, M., Marín-García, P., Puyet, A., Diez, A. & Bautista, J. M. (2009) Synchronous culture of Plasmodium falciparum at high parasitemia levels, *Nature Protocols.* **4**, 1899-1915.

219. Treeck, M., Sanders, J. L., Elias, J. E. & Boothroyd, J. C. (2011) The phosphoproteomes of Plasmodium falciparum and Toxoplasma gondii reveal unusual adaptations within and beyond the parasites' boundaries, *Cell Host Microbe.* **10**, 410-419.

220. Pflum, M. K. H., Tong, J. K., Lane, W. S. & Schreiber, S. L. (2001) Histone deacetylase 1 phosphorylation promotes enzymatic activity and complex formation, *Journal of Biological Chemistry.* **276**, 47733-47741.

221. Khan, D. H., He, S., Yu, J., Winter, S., Cao, W., Seiser, C. & Davie, J. R. (2013) Protein kinase CK2 regulates the dimerization of histone deacetylase 1 (HDAC1) and HDAC2 during mitosis, *Journal of Biological Chemistry.* **288**, 16518-16528.

222. Kim, E., H Bisson, W., V Lohr, C., E Williams, D., Ho, E., H Dashwood, R. & Rajendran, P. (2016) Histone and non-histone targets of dietary deacetylase inhibitors, *Current Topics in Medicinal Chemistry.* **16**, 714-731.

223. Bazou, D., Ng, M. R., Song, J. W., Chin, S. M., Maimon, N. & Munn, L. L. (2016) Flow-induced HDAC1 phosphorylation and nuclear export in angiogenic sprouting, *Scientific Reports.* **6**, 1-11.

224. Karmodiya, K., Krebs, A. R., Oulad-Abdelghani, M., Kimura, H. & Tora, L. (2012) H3K9 and H3K14 acetylation co-occur at many gene regulatory elements, while H3K14ac marks a subset of inactive inducible promoters in mouse embryonic stem cells, *BMC Genomics.* **13**, 1-18.

225. Lai, Q., He, Y., Peng, X., Zhou, X., Liang, D. & Wang, L. (2019) Histone deacetylase 1 induced by neddylation inhibition contributes to drug resistance in acute myelogenous leukemia, *Cell Communication Signaling.* **17**, 1-14.

226. Prommana, P., Uthaipibull, C., Wongsombat, C., Kamchonwongpaisan, S., Yuthavong, Y., Knuepfer, E., Holder, A. A. & Shaw, P. J. (2013) Inducible knockdown of Plasmodium gene expression using the glmS ribozyme, *PloS One.* **8**, e73783.

227. Greer, C. B., Tanaka, Y., Kim, Y. J., Xie, P., Zhang, M. Q., Park, I.-H. & Kim, T. H. (2015) Histone deacetylases positively regulate transcription through the elongation machinery, *Cell Reports.* **13**, 1444-1455.

228. Morimoto, R. I., Selkoe, D. J. & Kelly, J. W. (2011) *Protein homeostasis*, Cold Spring Harbor Laboratory Press.

229. Hott, A., Casandra, D., Sparks, K. N., Morton, L. C., Castanares, G.-G., Rutter, A. & Kyle, D. E. (2015) Artemisinin-resistant Plasmodium falciparum parasites exhibit altered patterns of development in infected erythrocytes, *Antimicrobial Agents Chemotherapy.* **59**, 3156-3167.

230. Klonis, N., Crespo-Ortiz, M. P., Bottova, I., Abu-Bakar, N., Kenny, S., Rosenthal, P. J. & Tilley, L. (2011) Artemisinin activity against Plasmodium falciparum requires hemoglobin uptake and digestion, *Proceedings of the National Academy of Sciences.* **108**, 11405-11410.

231. Mita, T., Tanabe, K. & Kita, K. (2009) Spread and evolution of Plasmodium falciparum drug resistance, *Parasitology International.* **58**, 201-209.

232. Jeffares, D. C., Pain, A., Berry, A., Cox, A. V., Stalker, J., Ingle, C. E., Thomas, A., Quail, M. A., Siebenthall, K. & Uhlemann, A.-C. (2007) Genome variation and evolution of the malaria parasite Plasmodium falciparum, *Nature Genetics.* **39**, 120-125.

233. Payne, D. (1987) Spread of chloroquine resistance in Plasmodium falciparum, *Parasitology Today.* **3**, 241-246.

234. Sibley, C. H., Hyde, J. E., Sims, P. F., Plowe, C. V., Kublin, J. G., Mberu, E. K., Cowman, A. F., Winstanley, P. A., Watkins, W. M. & Nzila, A. M. (2001) Pyrimethamine–sulfadoxine resistance in Plasmodium falciparum: what next?, *Trends in Parasitology.* **17**, 582-588.

235. Haldar, K., Bhattacharjee, S. & Safeukui, I. (2018) Drug resistance in Plasmodium, *Nature Reviews Microbiology.* **16**, 156-170.

236. Escalante, A. A., Smith, D. L. & Kim, Y. (2009) The dynamics of mutations associated with anti-malarial drug resistance in Plasmodium falciparum, *Trends in Parasitology.* **25**, 557-563.

237. WHO (2010) Global report on antimalarial drug efficacy and drug resistance: 2000-2010 in

238. Lin, J. T., Juliano, J. J. & Wongsrichanalai, C. (2010) Drug-resistant malaria: the era of ACT, *Current Infectious Disease Reports.* **12**, 165-173.

239. Nájera, J. A., González-Silva, M. & Alonso, P. L. (2011) Some lessons for the future from the Global Malaria Eradication Programme (1955–1969), *PLoS Medicine.* **8**, e1000412.

240. Verdrager, J. (1995) Localized permanent epidemics: the genesis of chloroquine resistance in Plasmodium falciparum, *The Southeast Asian Journal of Tropical Medicine Public Health.* **26**, 23-28.

241. Trape, J.-F. (2001) The public health impact of chloroquine resistance in Africa, *The American Journal of Tropical Medicine and Hygiene*.

242. Vinayak, S., Alam, M. T., Mixson-Hayden, T., McCollum, A. M., Sem, R., Shah, N. K., Lim, P., Muth, S., Rogers, W. O. & Fandeur, T. (2010) Origin and evolution of sulfadoxine resistant Plasmodium falciparum, *PLoS Pathogens.* **6**, e1000830.

243. Cui, L. & Su, X. (2009) Discovery, mechanisms of action and combination therapy of artemisinin, *Expert Review of Anti-Infective Therapy.* **7**, 999-1013.

244. D'alessandro, U. & Buttiens, H. (2001) History and importance of antimalarial drug resistance, *Tropical Medicine International Health.* **6**, 845-848.

245. Roberts, D. J. & Williams, T. N. (2003) Haemoglobinopathies and resistance to malaria, *Redox Report.* **8**, 304-310.

246. Meshnick, S. R. (2002) Artemisinin: mechanisms of action, resistance and toxicity, *International Journal for Parasitology.* **32**, 1655-1660.

247. Nosten, F. & White, N. J. (2007) Artemisinin-based combination treatment of falciparum malaria, *The American Journal of Tropical Medicine Hygiene*.

248. Fairhurst, R. M. & Dondorp, A. M. (2016) Artemisinin-resistant Plasmodium falciparum malaria, *Microbiology Spectrum.* **4**, 4.3. 36.

249. Park, D. J., Lukens, A. K., Neafsey, D. E., Schaffner, S. F., Chang, H.-H., Valim, C., Ribacke, U., Van Tyne, D., Galinsky, K. & Galligan, M. (2012) Sequence-based association and selection scans identify drug resistance loci in the Plasmodium falciparum malaria parasite, *Proceedings of the National Academy of Sciences.* **109**, 13052-13057.

250. Yuan, J., Cheng, K. C.-C., Johnson, R. L., Huang, R., Pattaradilokrat, S., Liu, A., Guha, R., Fidock, D. A., Inglese, J. & Wellems, T. E. (2011) Chemical genomic profiling for antimalarial therapies, response signatures, and molecular targets, *Science.* **333**, 724-729.

251. Mu, J., Myers, R. A., Jiang, H., Liu, S., Ricklefs, S., Waisberg, M., Chotivanich, K., Wilairatana, P., Krudsood, S. & White, N. J. (2010) Plasmodium falciparum genome-wide scans for positive selection, recombination hot spots and resistance to antimalarial drugs, *Nature Genetics.* **42**, 268-271.

252. Okombo, J., Kanai, M., Deni, I. & Fidock, D. A. (2021) Genomic and Genetic Approaches to Studying Antimalarial Drug Resistance and Plasmodium Biology, *Trends in Parasitology*.

253. Cheeseman, I. H., Miller, B. A., Nair, S., Nkhoma, S., Tan, A., Tan, J. C., Al Saai, S., Phyo, A. P., Moo, C. L. & Lwin, K. M. (2012) A major genome region underlying artemisinin resistance in malaria, *Science.* **336**, 79-82.

254. Takala-Harrison, S., Clark, T. G., Jacob, C. G., Cummings, M. P., Miotto, O., Dondorp, A. M., Fukuda, M. M., Nosten, F., Noedl, H. & Imwong, M. (2013) Genetic loci associated with delayed clearance of Plasmodium falciparum following artemisinin treatment in Southeast Asia, *Proceedings of the National Academy of Sciences.* **110**, 240-245.

255. Miotto, O., Almagro-Garcia, J., Manske, M., MacInnis, B., Campino, S., Rockett, K. A., Amaratunga, C., Lim, P., Suon, S. & Sreng, S. (2013) Multiple populations of artemisinin-resistant Plasmodium falciparum in Cambodia, *Nature Genetics.* **45**, 648-655.

256. Ghorbal, M., Gorman, M., Macpherson, C. R., Martins, R. M., Scherf, A. & Lopez-Rubio, J.-J. (2014) Genome editing in the human malaria parasite Plasmodium falciparum using the CRISPR-Cas9 system, *Nature Biotechnology.* **32**, 819-821.

257. Ismail, H. M., Barton, V., Phanchana, M., Charoensutthivarakul, S., Wong, M. H., Hemingway, J., Biagini, G. A., O'Neill, P. M. & Ward, S. A. (2016) Artemisinin activity-based probes identify multiple molecular targets within the asexual stage of the malaria parasites Plasmodium falciparum 3D7, *Proceedings of the National Academy of Sciences.* **113**, 2080-2085.

258. Bridgford, J. L., Xie, S. C., Cobbold, S. A., Pasaje, C. F. A., Herrmann, S., Yang, T., Gillett, D. L., Dick, L. R., Ralph, S. A. & Dogovski, C. (2018) Artemisinin kills malaria parasites by damaging proteins and inhibiting the proteasome, *Nature Communications.* **9**, 1-9.

259. Dogovski, C., Xie, S. C., Burgio, G., Bridgford, J., Mok, S., McCaw, J. M., Chotivanich, K., Kenny, S., Gnädig, N. & Straimer, J. (2015) Targeting the cell stress response of Plasmodium falciparum to overcome artemisinin resistance, *PLoS Biology.* **13**, e1002132.

260. Mbengue, A., Bhattacharjee, S., Pandharkar, T., Liu, H., Estiu, G., Stahelin, R. V., Rizk, S. S., Njimoh, D. L., Ryan, Y. & Chotivanich, K. (2015) A molecular mechanism of artemisinin resistance in Plasmodium falciparum malaria, *Nature.* **520**, 683-687.

261. Bhattacharjee, S., Coppens, I., Mbengue, A., Suresh, N., Ghorbal, M., Slouka, Z., Safeukui, I., Tang, H.-Y., Speicher, D. W. & Stahelin, R. V. (2018) Remodeling of the malaria parasite and host human red cell by vesicle amplification that induces artemisinin resistance, *Blood, The Journal of the American Society of Hematology.* **131**, 1234-1247.

262. Birnbaum, J., Scharf, S., Schmidt, S., Jonscher, E., Hoeijmakers, W. A. M., Flemming, S., Toenhake, C. G., Schmitt, M., Sabitzki, R. & Bergmann, B. (2020) A Kelch13-defined endocytosis pathway mediates artemisinin resistance in malaria parasites, *Science.* **367**, 51-59.

263. Lu, F., He, X.-L., Richard, C. & Cao, J. (2019) A brief history of artemisinin: Modes of action and mechanisms of resistance, *Chin J Nat Med.* **17**, 331-336.

264. Yang, T., Yeoh, L. M., Tutor, M. V., Dixon, M. W., McMillan, P. J., Xie, S. C., Bridgford, J. L., Gillett, D. L., Duffy, M. F. & Ralph, S. A. (2019) Decreased K13 abundance reduces hemoglobin catabolism and proteotoxic stress, underpinning artemisinin resistance, *Cell Reports.* **29**, 2917-2928. e5.

265. Rocamora, F., Zhu, L., Liong, K. Y., Dondorp, A., Miotto, O., Mok, S. & Bozdech, Z. (2018) Oxidative stress and protein damage responses mediate artemisinin resistance in malaria parasites, *PLoS Pathogens.* **14**, e1006930.

266. Straimer, J., Gnädig, N. F., Witkowski, B., Amaratunga, C., Duru, V., Ramadani, A. P., Dacheux, M., Khim, N., Zhang, L. & Lam, S. (2015) K13-propeller mutations confer artemisinin resistance in Plasmodium falciparum clinical isolates, *Science.* **347**, 428-431.

267. Witkowski, B., Amaratunga, C., Khim, N., Sreng, S., Chim, P., Kim, S., Lim, P., Mao, S., Sopha, C. & Sam, B. (2013) Novel phenotypic assays for the detection of artemisinin-resistant Plasmodium falciparum malaria in Cambodia: in-vitro and ex-vivo drug-response studies, *The Lancet Infectious Diseases.* **13**, 1043-1049.

268. Niaré, K., Paloque, L., Ménard, S., Tor, P., Ramadani, A. P., Augereau, J.-M., Dara, A., Berry, A., Benoit-Vical, F. & Doumbo, O. K. (2018) Multiple phenotypic and genotypic artemisinin sensitivity evaluation of Malian Plasmodium falciparum isolates, *The American Journal of Tropical Medicine Hygiene.* **98**, 1123.

269. Mok, S., Stokes, B. H., Gnädig, N. F., Ross, L. S., Yeo, T., Amaratunga, C., Allman, E., Solyakov, L., Bottrill, A. R. & Tripathi, J. (2021) Artemisinin-resistant K13 mutations rewire Plasmodium falciparum's intra-erythrocytic metabolic program to enhance survival, *Nature Communications.* **12**, 1-15.

270. Zhu, L., Tripathi, J., Rocamora, F. M., Miotto, O., van der Pluijm, R., Voss, T. S., Mok, S., Kwiatkowski, D. P., Nosten, F. & Day, N. P. (2018) The origins of malaria artemisinin resistance defined by a genetic and transcriptomic background, *Nature Communications.* **9**, 1-13.

271. Yang, H., Salz, T., Zajac-Kaye, M., Liao, D., Huang, S. & Qiu, Y. (2014) Overexpression of histone deacetylases in cancer cells is controlled by interplay of transcription factors and epigenetic modulators, *The FASEB Journal.* **28**, 4265-4279.

272. Kato, T., Shimono, Y., Hasegawa, M., Jijiwa, M., Enomoto, A., Asai, N., Murakumo, Y. & Takahashi, M. (2009) Characterization of the HDAC1 complex that regulates the sensitivity of cancer cells to oxidative stress, *Cancer Research.* **69**, 3597-3604.

273. Olliaro, P. & therapeutics (2001) Mode of action and mechanisms of resistance for antimalarial drugs, *Pharmacology.* **89**, 207-219.

274. Ecker, A., Lehane, A. M., Clain, J. & Fidock, D. A. (2012) PfCRT and its role in antimalarial drug resistance, *Trends in Parasitology.* **28**, 504-514.
275. Zhu, L., van der Pluijm, R. W., Kucharski, M., Nayak, S., Tripathi, J., Nosten, F., Faiz, A., Amaratunga, C., Lek, D. & Ashley, E. A. (2021) The mechanism of artemisinin resistance of Plasmodium falciparum malaria parasites originates in their initial transcriptional response, *bioRxiv*.
276. Awandare, G., Amato, R., Miotto, O., Woodrow, C., Almagro-Garcia, J., Sinha, I., Campino, S. & Mead, D. (2016) Genomic epidemiology of artemisinin resistant malaria, *ELife*.
277. Stokes, B. H., Rubiano, K., Dhingra, S. K., Mok, S., Straimer, J., Gnadig, N. F., Bath, J. R., Deni, I., Ward, K. E. & Striepen, J. (2021) P. falciparum K13 mutations present varying degrees of artemisinin resistance and reduced fitness in African parasites, *bioRxiv*.
278. Singh, G. P., Goel, P. & Sharma, A. (2016) Structural mapping of Kelch13 mutations associated with artemisinin resistance in malaria, *Journal of Structural Functional Genomics.* **17**, 51-56.
279. Xie, S. C., Ralph, S. A. & Tilley, L. (2020) K13, the cytostome, and artemisinin resistance, *Trends in Parasitology.* **36**, 533-544.
280. Stokes, B. H., Dhingra, S. K., Rubiano, K., Mok, S., Straimer, J., Gnadig, N. F., Deni, I., Schindler, K. A., Bath, J. R. & Ward, K. E. (2021) Plasmodium falciparum K13 mutations in Africa and Asia impact artemisinin resistance and parasite fitness, *ELife.* **10**, e66277.
281. Miotto, O., Amato, R., Ashley, E. A., MacInnis, B., Almagro-Garcia, J., Amaratunga, C., Lim, P., Mead, D., Oyola, S. O. & Dhorda, M. (2015) Genetic architecture of artemisinin-resistant Plasmodium falciparum, *Nature Genetics.* **47**, 226-234.
282. Das, S., Kar, A., Manna, S., Mandal, S., Mandal, S., Das, S., Saha, B. & Hati, A. K. (2021) Artemisinin combination therapy fails even in the absence of Plasmodium falciparum kelch13 gene polymorphism in Central India, *Scientific Reports.* **11**, 1-12.
283. Birnbaum, J., Flemming, S., Reichard, N., Soares, A. B., Mesén-Ramírez, P., Jonscher, E., Bergmann, B. & Spielmann, T. (2017) A genetic system to study Plasmodium falciparum protein function, *Nature Methods.* **14**, 450-456.
284. Portugaliza, H. P., Miyazaki, S., Geurten, F. J., Pell, C., Rosanas-Urgell, A., Janse, C. J. & Cortés, A. (2020) Artemisinin exposure at the ring or trophozoite stage impacts Plasmodium falciparum sexual conversion differently, *ELife.* **9**, e60058.
285. Witmer, K., Dahalan, F. A., Delves, M. J., Yahiya, S., Watson, O. J., Straschil, U., Chiwcharoen, D., Sornboon, B., Pukrittayakamee, S. & Pearson, R. D. (2020) Artemisinin-

resistant malaria parasites show enhanced transmission to mosquitoes under drug pressure, *bioRxiv*.

286. Gelband, H., Panosian, C. B. & Arrow, K. J. (2004) *Saving lives, buying time: economics of malaria drugs in an age of resistance*.

287. Ouattara, A., Barry, A. E., Dutta, S., Remarque, E. J., Beeson, J. G. & Plowe, C. V. (2015) Designing malaria vaccines to circumvent antigen variability, *Vaccine*. **33**, 7506-7512.

288. Hollin, T. & Le Roch, K. G. (2020) From Genes to Transcripts, a Tightly Regulated Journey in Plasmodium, *Frontiers in Cellular Infection Microbiology*. **10**, 801.