# Investigating the putative phosphorylation of PfHDAC1 by PfCKII and its possible role in gene regulation

A Thesis

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by

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# Certificate

This is to certify that this dissertation entitled 'Investigating the putative phosphorylation of PfHDAC1 by PfCKII and its possible role in gene regulation' towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents work carried out by Dilsha Farheen P M at Indian Institute of Science Education and Research under the supervision of Dr. Krishanpal Karmodiya, Assistant Professor, Department of Biology, during the academic year 2020-2021.

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(Supervisor)

Date : 01.08.2021

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*This thesis is dedicated to my Ummachi*

# Declaration

I hereby declare that the matter embodied in the report entitled ‘Investigating the putative phosphorylation of PfHDAC1 by PfCKII and its possible role in gene regulation’ are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Krishanpal Karmodiya and the same has not been submitted elsewhere for any other degree.

Dilsha Farheen P M

Date:01.08.2021

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# ABSTRACT

Even though artemisinin based combination therapies have made remarkable progress in reducing the number of malaria infections globally, the acquisition of resistance against these drugs by the parasite *Plasmodium falciparum* is also getting reported with time. Histone deacetylase 1 (HDAC 1) belonging to Class I HDACs is a potential regulator of artemisinin resistance phenotype and phosphorylation status of HDAC1 plays an important role in controlling its activity. Casein Kinase II (CKII) is a regulator of phosphorylation of Class I HDACs in higher eukaryotes. In *Plasmodium*, CKII is present in the interactome of artemisinin. In this study, we show that PfHDAC1 gets phosphorylated by PfCKII through in-vitro assays. We also demonstrate that deacetylase activity of PfHDAC1 is enhanced by PfCKII phosphorylation.

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# 1.Introduction

## 1.1 Malaria Occurrence

Malaria is a deadly disease caused by the protozoan parasites of the *Plasmodium* genus. Female anopheles mosquitoes act as vectors for the spread of the disease. Malaria still causes the greatest number of deaths around the world [45]. It is mostly reported in Africa and some Asian subcontinents while its spread in the developed countries traces its origin from endemic areas. Diagnosis in the early stages and timely treatment can control the disease to a greater extent. Thus, a large number of programmes aimed at eradicating malaria including betterment of primary health care, early diagnosis, immediate treatment,prevention methods etc. are carried out by the World Health Organisation but the recent reports show an increase in malaria cases around the world [45]. According to the World Health Organization (WHO) 2018 report around 219 million malaria cases were recorded globally (Fig 1)[50].

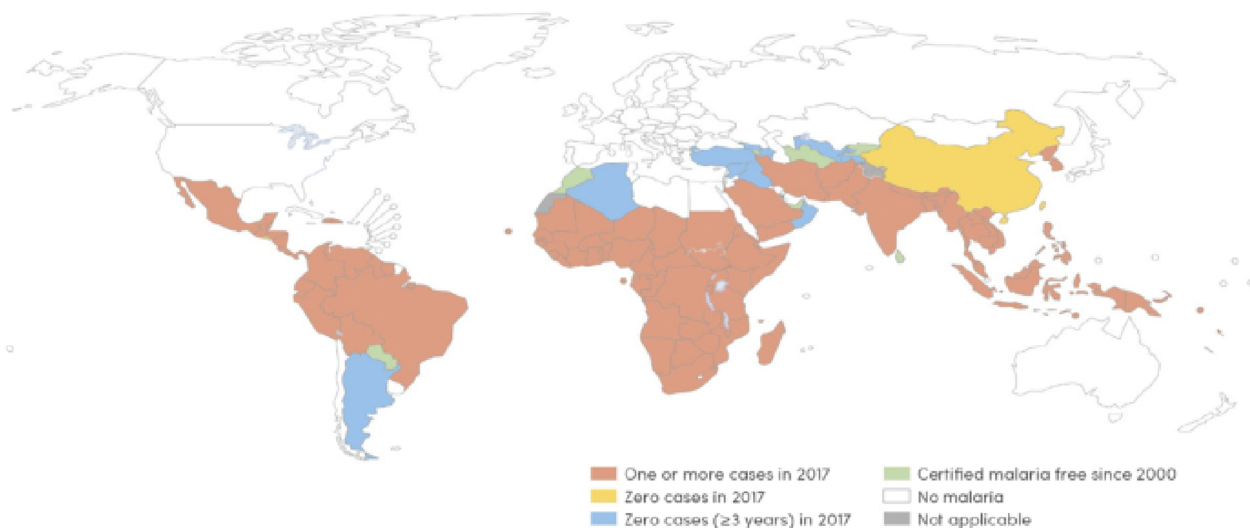



***Fig 1. Geographical distribution of falciparum malaria in the world,*** adapted from *WHO report 2018*

## 1.2 Malaria in India

India contributes to 77% of the malaria burden in southeast Asia. About 2 million malaria infections and 1000 deaths are recorded annually in India. Around 1.3 billion people are at the verge of getting infected with the disease[53]. Malaria is the main reason for the infant,child and

adult death rate in India. 70 % of malaria cases in India are reported from Odisha ,Jharkhand , Chhattisgarh ,Maharashtra and Madhya Pradesh[50]. States occupied with ethnic tribal communities have the most number of cases of stable malaria particularly those caused by *Plasmodium falciparum. P. falciparum* and *P. vivax* cause the most number of cases in India although *P.ovale* and *P.malariae* have also been reported from other parts of the country. *P. vivax* occurrences are dominant in the plains whereas *P. falciparum* prevalence is highly in forested areas. Malaria infection in India reports to create the most complications in pregnancy including abortions,still births and mortality of the mother[53].

## 1.3 *Plasmodium falciparum* life cycle

*Plasmodium falciparum* life cycle mainly demands two hosts,human and mosquito (Fig 2)[54]. The infection starts when a mosquito carrying *Plasmodium* parasites bites a human being. In the process, mosquitoes release parasites in the form of sporozoites into the bloodstream. It reaches the liver cells, undergoes mitosis, develops into liver schizont and further reproduces asexually. After about seven days, hepatocytes bursts releasing the merozoites into the peripheral bloodstream which go and infect the red blood cells continuing their asexual lifecycle in the RBCs[54]. Over the course of the 48 hour life cycle parasites progress

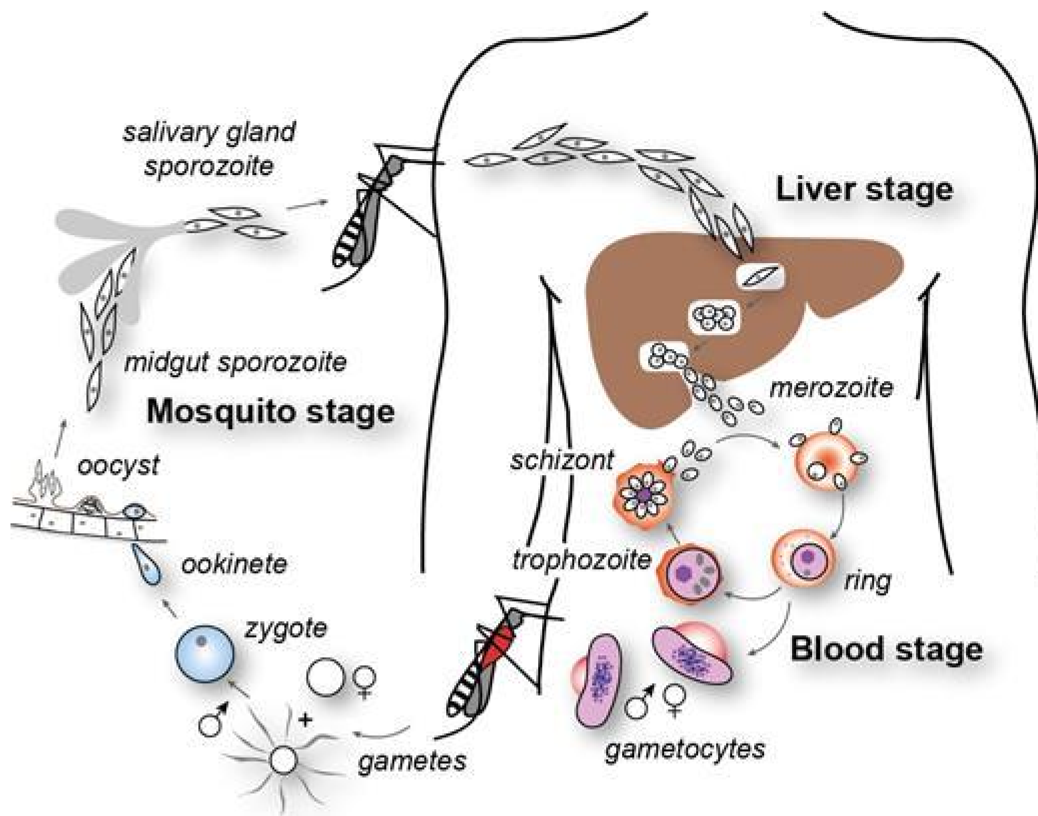



***Fig 2 .A schematic representation of the life cycle of Plasmodium falciparum,*** *Adapted from Cowman et al,2012*

through ring, trophozoite and schizont stages which eventually burst releasing merozoites into the bloodstream infecting new RBCs. A subset of them undergoes gametogenesis which could later be taken by the mosquitoes in their blood meal. The development of these gametocytes into mature gametes happen in the mosquito's midgut and the fused product of male and female gametes, the zygote, would develop as ookinete. The further growth of these ookinete into oocysts proceeds in the mosquito's salivary glands. The oocyte undergoes mitosis to form merozoites which would be released when this mosquito bites another individual and the cycle continues[3].

## 1.4 *Plasmodium* Genome

*Plasmodium* genome sequencing was undertaken in 2002. The *Plasmodium falciparum* genome is made up of 22.8 Megabases and is present in 14 chromosomes[19]. This genome encodes

about 5300 genes and has 80.6% AT composition which is one of the highest AT rich genomes. *Plasmodium* also possesses a linear mitochondrial genome which was found to be 6 kb [19].

## 1.5 Transcriptional Regulation in *Plasmodium*

Several studies in the past have helped in deciphering the transcriptional regulation in *Plasmodium* [11] . Bozdech et al in 2003 showed that the transcription regulation involves most genes getting transcribed once every cycle and that there is a correlation between gene expression and the developmental cycle of the organism [7]. The efficient translational and post translational regulation of gene expression is believed to play an important role in the pathogenicity of the *Plasmodium falciparum* [11,14]. The asexual life cycle in RBCs is also the result of the fine regulation of this gene expression [29].

## 1.6 Epigenetic Regulation in *Plasmodium*

The gene regulation can take place at different levels including transcriptional, translational and epigenetic mechanisms [29]. Heritable changes which do not change the sequence of DNA are named as the epigenetic regulation. These epigenetic regulation involve DNA modification, histone post-translational modification and the changes in chromatin organisation [62,63]. Epigenetic mechanisms in *Plasmodium* are involved in invasion, gametogenesis, antigenic variation etc [64,65]. The N terminal of the histone proteins are the substrates for the post translational modifications like methylation,acetylation, ubiquitination and phosphorylation. These processes are catalysed by a number of enzymes like histone deacetylases(HDACs), histone acetyltransferases(HATs), demethylases and histone methyltransferases which thereby take part in gene regulation. *Plasmodium* has a significant amount of these enzymes, the targeted proteins and chromatin remodelers [1,14]. The resulting modifications at various points in the genome determine the chromatin structure and thereby the expression of the gene. The epigenetic modifications are indispensable in regulating the packaging of genomes . They change the affinity of DNA to histones and thereby determine the expression and suppression of genes [65]. Enhancing the affinity causes tight packaging creating heterochromatin which causes gene silencing and loose affinity results in open chromatin named as euchromatin which promotes gene activation by making it accessible for transcription [67].

## 1.7 Histone Acetyl transferases and Histone Deacetylases

Histone acetylation regulated by Histone Acetyl transferases (HATs) and Histone Deacetylases (HDACs) are one of the most studied histone modifications which are called the 'writers' and 'erasers' respectively [15]. Acetylation occurs dominantly on lysine residues and are catalysed by histone acetyltransferases. There are five families of HATs; MYST, Gcn5- related N-acetyltransferases (GNATs),nuclear receptor coactivator, p300/CBP and other transcription associated families all of which are classified on the grounds of their protein sequence and domain organisation [1,26]. Histone deacetylases (HDACs) are distributed in five classes by virtue of their similarity with yeast HDACs [1]. They have different nucleo cytoplasmic localisation; Class1 is in the nucleus while Class2 and class III shuttles between cytoplasm and nucleus [11]. HDAC 1 in *Plasmodium* belongs to class1 HDACs. These HDACs use $Zn2+$ as a cofactor for their activity and are reported to play key roles in gametocytogenesis, schizogony and hepatocyte invasion [9].

## 1.8 Antimalarial Drugs

Antimalarials are the drugs used to treat malaria infection. Currently there is no authorized malaria vaccine obtainable in the market to fight against malarial infection. Hence, antimalarial drugs are considered to be indispensable in the control of malarial infection. Despite several drugs available in the market, elimination of malaria is still not possible due to the inherent ability of *Plasmodium* to generate resistance against most of the drugs [55]. Currently most of the antimalarial drugs broadly fall into three categories broadly according to their chemical structure: 1) Aryl amino alcohol compounds: quinine, chloroquine, mefloquine, amodiaquine, piperaquine, lumefantrine, tafenoquine 2) Antifolate compounds: pyrimethamine, proguanil 3) Artemisinin compounds: artemisinin, dihydroartemisinin, artemether, artesunate [56,57].

## 1.9 Artemisinin

Artemisinin is a sesquiterpene lactone used as an antimalarial for the cure of *falciparum* malaria with powerful action against almost all blood stages of *Plasmodium falciparum* parasites[58]. These involve asexual stages (rings, trophozoites and schizonts), which give rise to the clinical symptoms of malaria, and sexual stages, which develops to the mature gametocytes that spread

infection to other humans through mosquitoes. These blood stages are prone to artemisinins because they intently digest haemoglobin as they mature within RBCs. It is reported that the heme-associated iron liberated from this action rives the endoperoxide component of artemisinins, thereby creating the reactive oxygen species that attack nucleophilic groups in parasite lipids and proteins [60]. Artemisinin combination therapies (ACTs) are combination of a rapid-acting, effective artemisinin and a slow-acting, less-effective ally drug (e.g.,piperaquine, mefloquine, lumefantrine) that are given orally for three days and are now the proposed first-line cure for uncomplicated *P.falciparum* malaria globally [59]. The basis of ACTs is that the artemisinin moiety kills the major portion of parasites over several days by one method, and the ally drug destroys remaining parasites over several weeks by an alternate method.

### 1.10 Artemisinin Resistance and PfHDAC1

Recent reports show evidence of resistance of *Plasmodium* for Artemisinin based Combination Therapy (ACT). According to WHO, artemisinin resistance has been interpreted as a parasite removal half-life ≥ five hours following administration of artesunate monotherapy or an ACT. It was first marked as a hundred fold deduction in parasite removal rate in Pailin, Western Cambodia, in 2009[59]. While investigating mechanisms of artemisinin resistance, transcriptomics studies have revealed the deregulation of redox stress response and proteostasis associated pathways, mainly upregulation of these pathways[24] . *Plasmodium falciparum* HDAC is believed to be a potential regulator of artemisinin resistance phenotype as its down-regulation is observed in artemisinin resistant parasites [7]. PfHDAC1 is essential for parasite survival and it is relatively understudied in *Plasmodium falciparum*.

Also,artemisinin in *P.falciparum* is found to target a lot of biological pathways like glycolysis pathways,protein biosynthesis pathways etc which thereby affects the signalling cascade in the cells[61].

### 1.11 HDAC as a potential antimalarial target

Histone acetyltransferases and HDACs play a very important role in a composite web of biological processes encompassing DNA replication, gene expression, cell cycle advancement, differentiation and cell death making them an appropriate target for developing a remedy for several diseases [1,14]. Multiple HAT/HDAC inhibitors have been analysed for their

antiplasmodial action over the time. HDAC inhibitors were observed to bring about extensive generic impairment of the parasite transcriptome and were found to hinder parasite development [68]. Apicidin,a HDAC inhibitor, was found to inhibit parasite growth and induce hyper-acetylation of histones through inhibition of HDAC function [68]. Several in-silico studies have been conducted to unravel the molecular constitution and anchoring properties of HDAC1 and was found that it had a significant amount of toxicity against *Plasmodium* than human HDAC1 [69]. Several HDAC inhibitors like suberoylanilide hydroxamic acid (SAHA), romidepsin and belinostat were found to have strong antimalarial activity [70] .

### 1.12 Phosphorylation of HDACs in higher eukaryotes

Mammalian HDACs are known to have paramount importance in balancing gene expression, survival, proliferation and cell growth. Their activities are controlled by several mechanisms including recruitment into different corepressor complexes, protein protein interactions, post translational modifications such as sumoylation and phosphorylation [18,49]. Every mammalian HDACs have prospective phosphorylation sites and are phosphorylated in vitro and in vivo. Phosphorylation regulates subcellular localisation and biological activities in certain HDACs [18,31,49]. The phosphorylation of N-terminal serine residues in HDACs are responsible for the regulation of the localisation and downstream signalling. The phosphorylated residues bind with chaperone proteins and thereby get escorted from nucleus to cytoplasm [49].Various kinases including protein kinases and casein kinases transmit signals to HDAC via this regulatory phosphorylation in response to different external stimuli in cells [31].

### 1.13 Casein Kinase II(CKII) and HDACs

Casein Kinase II (CKII) is reported to be a key regulator for all Class I HDACs. CKII is an enzyme studied to be actively encompassed in cell cycle regulation, survival, proliferation, transcription-based chromatin remodelling etc. and is distributed both in the nucleus and cytoplasm of eukaryotic cells [13]. There is a minimum of two serine residues at identical positions (S423/S424 and S421/S422) that are phosphorylated by CK2 in HDAC1 and HDAC2 in the Human genome [18,71]. Studies show that phosphoserine mutations in HDAC1 and HDAC2 are detrimental for its enzymatic activity and also that it disturbs the connections with endogenous associated proteins. Thus, it can be said that phosphorylation perhaps improves the

affinity of HDACs for crucial interacting proteins, which then promote their enzymatic function. HDAC1 and HDAC2 are reported to contain functional CK2 in their immunocomplexes. Class I and class II HDACs by their interaction with different enzymes are reported to control important cellular processes by targeting them to their specific substrates. In this way, there are two different regulatory mechanisms of interaction of HDAC with the kinase enzymes, one based on transporting the protein kinase to fixed targets and the other one associated with direct phosphorylation of the HDAC within the complex [18,]. By probing studies, Casein kinase was found to be included in the artemisinin interactome also [61].

Taken together, PfHDAC1 is a very potent antimalarial target and since its phosphorylation has the major role in regulating its activity, it would be important to investigate the effect of phosphorylation post translational modification on its activity and nucleo-cytoplasmic localization in *Plasmodium falciparum*. CKII is a major regulator of phosphorylation modification in higher eukaryotes. It is also found to be a part of the interactome in artemisinin probing studies. Thus it would be intriguing to investigate the effect of PfCKII on PfHDAC1. In this study we have investigated the phosphorylation changes brought about by PfCKII on PfHDAC1, the effect it has on HDAC1 activity and localization.

# 2. Materials and methods

### 2.1. *Plasmodium* in-vitro culture

Parasites were cultured in RPMI1640 medium added with 0.5 % AlbuMAX I, 25 mM HEPES, 100 µM hypoxanthine, 1.77 mM sodium bicarbonate and 12.5 µg ml−1 gentamicin sulfate at 37 °C. Cultures were split and expanded after every two days to sustain parasitemia around 5%. Hematocrit was kept at 1 -1.5% by supplementing newly washed human RBCs. Parasitemia was tracked using Giemsa staining of blood smear.

### 2.2 Harvesting of parasite culture

*Plasmodium falciparum* 3D7 culture was spun down at 4000rpm in 4degC. Washes were given with PBS at 3000rpm. It was then treated with 0.15% saponin for 15minutes at 37℃. More PBS are added to it after incubation and then centrifuged for 12minutes. After three more washes with PBS,pellets were collected.

### 2.3 Immunoprecipitation of PfHDAC1 using PfHDAC1 antibody

The parasite pellets were resuspended in same volume of lysis buffer (20M TrisCL pH8,150M NaCl,0.5% NP40,0.1mM EDTA,1.5mM MgCl2,20% Sodium deoxycholate,1mM PIC,1mM PMSF) and were given three rounds of freeze-thawing to enable complete lysis of the cells. The lysate was then centrifuged at 12000 rpm for 30 mins and the supernatant was collected. Concentration estimation of the protein was done using bradford assay. The lysates were then precleared using 1/50th the volume of sepharose recombinant G beads for 1 hr. Anti-PfHDAC1 antibody (concentration of 1:100) was added to the supernatant collected after preclearing and incubated overnight. 1/20th volume of sepharose recombinant beads were added to this and was incubated for 4hrs. The beads were spun down and were given four washes with lysis buffer for 5 mins and were then given three washes with Ip100(25mM Tris pH 7.9, 5mM MgCl2, 10% glycerol, 0.1%NP40, 0.3mM DTT,100mM KCl). The elutions were collected in 0.5M glycine pH2.5. The elutions were then pH corrected using Tris.HCl pH 8.8. SDS samples were made

from the elutions by heating with 6xSDS dye.These were loaded onto 10% SDS gel and ran at 80V till the samples properly stacks and then ran at 120V till the diaphragm reached the bottom. It was then transferred to a PVDF membrane at 600mA and blocking of the membrane was done with 5% skimmed milk. It was then probed with the primary antibody which was anti-HDAC1 for 12hrs at 1:1000 dilution in milk. Later four washes were given with TBST,15 minutes each and probed the membrane with a secondary antibody which is anti-rabbit. TBST washes were given again and the membrane was developed using western ECL substrate.

### 2.4. CIP Treatment for Immunoprecipitated Fractions

Immunoprecipitated fractions of PfHDAC1 prepared as mentioned above were given CIP treatment at different concentrations and were loaded onto 10% SDS gel and western blotting was done using anti-HDAC1.

### 2.5. Mass Spectrometry of Immunoprecipitated HDAC1 bands

Immunoprecipitation was performed using anti-PfHDAC1 antibody and samples were loaded onto 10% SDS PAGE gel. The bands after Coomassie staining and destaining were cut from sizes 51 kDa and 65 kDa. Bands were chopped into pieces and did destaining with 40% acetonitrile in 50mM TEABC.It was proceeded with reduction using 10mM DTT and alkylation using 20mM IAA.100% acetonitrile was used for dehydrating the gel pieces and it was followed with overnight trypsin digestion at a concentration of 10ng/microL. The gel pieces after spinning were then incubated with 30%,40% and 100% acetonitrile in a water bath sonicator. The supernatant was vacuum dried and desalting was done using Zip-tips made from C18 membrane. The samples were then eluted in 50microl of 50%acetonitrile in 0.1% TFA and vacuum dried. These were sent for in-gel Mass Spectrometry. The results were then analysed using MaxQuant software and was compared with the genomic sequence of *Plasmodium falciparum* 3D7 HDAC1.

## 2.6. Co-Transformation of GST-CKIIalpha and GST-HDAC1 into BL21DE3 *E.coli* cells

GST tagged *Plasmodium falciparum* Casein Kinase II-alpha and GST tagged *Plasmodium falciparum* HDAC1 (both already cloned) were co-transformed into *E.coli* BL21DE3 cells. Colonies were inoculated onto 5ml cultures and induced with IPTG when the OD reached 6. It was then incubated in 18degC for 16hrs. Cultures were spun down and lysates were prepared in sonication buffer(50mM TrisCl,150mM NaCl, 1mM PMSF,10% glycerol, 2mM DTT, 0.1% TritonX100, 1mM PIC) .Sonication was done onto the lysates made from each colony that was picked up. SDS samples were made from the colony lysates and were screened for co-transformation by doing a western blotting using anti-GFP antibody. Later, co-transformed colony lysates were analysed for post translational modification by probing with anti-serine phospho antibody in Western blotting with HDAC1 and CKII controls. It was then stripped and reprobed with anti-GST antibody as loading control.

## 2.7. Overexpression and Purification of GST tagged HDAC1

GST tagged HDAC1 clone was transformed into BL21 DE3 *E.coli* competent cells. Colony was inoculated to a primary culture of 4ml and then transferred to a secondary culture of 400ml culture after 12 hrs. It was induced with 1M IPTG when OD reached 0.6. It was spun down after 12hr incubation in 18degC. The cell lysate made in sonication buffer was sonicated and the supernatant after centrifugation at 12000 rpm was incubated with glutathione beads for 3hrs.The beads were then spun down and five washes were given with sonication buffer. Elutions were then collected at 10,20 and 30mM concentration of glutathione. The elutions were loaded onto a 10% SDS PAGE gel for confirming the purification. Dialysis for 2hrs was done later to the eluted fractions.

### 2.8. Overexpression and Purification of GST tagged CKII

GST tagged CKII alpha clone was transformed into BL21 DE3 *E.coli* competent cells. Colony was inoculated to a primary culture of 4ml and then transferred to a secondary culture of 400ml culture after 12 hrs. It was induced with 1M IPTG when OD reached 0.6. It was spun down after 12hr incubation in 18degC. The cell lysate made in sonication buffer was sonicated and the supernatant after centrifugation at 12000 rpm was incubated with glutathione beads for 3hrs.The beads were then spun down and washes were given with sonication buffer. Elutions were then collected at 10,20 and 30mM concentration of glutathione. The elutions were loaded onto 10% SDS PAGE gel for confirming the purification. Dialysis for 2hrs was done later to the eluted proteins.

### 2.9 Purification of Histones

Histones were purified from *Plasmodium falciparum* using urea-salt lysis and acid extraction method. The *P.falciparum* 3D7 harvested pellet was resuspended in 2V of Hb removal buffer (1 mM EDTA, 25mM Tris-HCl pH 7.8, 0.2% NP40) to remove contaminating membrane and Hb. Spin was given at 12000 rpm and the pellet was washed again withHb 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 8V 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 12000 rpm was washed with acetone and was dissolved in dH2O+0.1M beta mercaptoethanol [47]. Histones proteins were then visualised by loading onto a 15% SDS PAGE gel.

### 2.10. Histone deacetylation Assay of Recombinant PfHDAC1

Histone deacetylase activity assay was performed in 40μl reaction in HDAC assay buffer (137mM NaCl, 25mM Tris HCl,pH8, 1mM MgCl2, 2.7mM KCl, 0.1mM ZnCl2) with histones as substrate and HDAC1 in varying concentrations as enzyme. The reactions were incubated in

37°C for 1 hr. SDS samples were made from the reactions and then loaded onto 15% SDS-PAGE gel and western blotting was performed with anti-H3K9 acetylation antibody [18,51].

| | control | test1 | test2 | test3 |
|---|---|---|---|---|
| 10X Buffer | 4 | 4 | 4 | 4 |
| Histones | 5 | 5 | 5 | 5 |
| PfHDAC1 | 0 | 20 | 25 | 30 |
| NFW | 31 | 11 | 6 | 1 |

*Table 1: Table contains the volume of each component (in microlitre) added to each reaction in the deacetylation assay*

### 2.11. Interaction Assay of Recombinant PfHDAC1 and Recombinant PfCKII

Equal concentrations of PfHDAC1 and PfCKII were mixed and kept for incubation on a rotor(reaction A). After one hr,two other reactions were set up, RecG beads were kept for incubation with Anti-HDAC1 antibody(reaction B) and RecG beads were kept for incubation with IgG antibody(Reaction C). After 4 hrs of completion of incubation of reaction A, it was kept for preclearing with RecG beads. After another hr, reaction B and reaction C which by now has completed 4hrs of incubation were taken out. The supernatant after centrifugation of reaction A was split into two equal halves and was incubated with both reaction B and reaction C. This was kept for a duration of 4 hrs after which the beads were spun down and washed with GST buffer. This was followed by elution with glycine pH 2.5. Eluted samples were loaded onto 10% SDS PAGE gel.

### 2.12. Kinase Assay of Recombinant PfHDAC1 and Recombinant PfCKII

Kinase assay was performed in a 50µl reaction in kinase assay buffer (20 mM Tris HCl pH 7.5, 2 mM MnCl2, 20 mM MgCl2, 0.1mM PMSF ) containing 10µM ATP with PfHDAC1 as substrate(0.6µg) and PfCKII as enzyme in varying concentrations(0.36g,0.72 µg,1.08 µg). Reactions were also setup with CKII inhibitor TBB ( IC50 and 2*IC50) and also with artemisinin. Reactions were set in 30°C for 30 minutes and were loaded onto a 10% SDS PAGE gel [8]. Western blotting was done using anti-GST antibody and anti-serine phospho antibody.

| | HDAC1<br>Non incubated control | HDAC1<br>Incubated control | CKII<br>control | T1 | T2 | T3 | I1 | I2 | A1 |
|---|---|---|---|---|---|---|---|---|---|
| PfHDAC1 | 20 | 20 | - | 20 | 20 | 20 | 20 | 20 | 20 |
| PfCKII | - | - | 23 | 6 | 12 | 23 | 23 | 23 | 23 |
| Kinase Buffer+ATP | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| CKII Inhibitor (600microM) | - | - | - | - | - | - | 0.5 | 1 | - |
| Artemisinin (20microM) | - | - | - | - | - | - | - | - | 1 |
| NFW | 24 | 24 | 21 | 18 | 12 | 1 | 0.5 | 0 | 0 |

*Table 2: shows the volume of components(in microlitre) added to each reaction in the kinase assay*

## 2.13. Deacetylation Assay after Phosphorylating PfHDAC1 using PfCKII from Kinase assay

Kinase assay as mentioned above was set up to phosphorylated PfHDAC1 and was then used to set up the deacetylase activity assay with histones. Another reaction in kinase assay with PfCKII was also used for the activity assay. The reactions from kinase assay were incubated with histones purified from *Plasmodium falciparum* (as mentioned above) in deacetylase activity buffer for 1hr in 37℃. Samples made from the respective reactions were loaded onto a 15% SDS PAGE gel and western blotting was done by probing with anti-acetyl H3K9 antibody.

| | KA 2 *2 | KA 3 |
|---|---|---|
| PfHDAC1 | 20 | 20 |
| PfCKII | 23 | 23 |
| Buffer+ATP | 6 | 6 |
| CKII Inhibitor (600microM) | - | 1 |
| NFW | 1 | 0 |

*Table 3: The volume of components of reaction(in microlitre) sets for kinase assay for usage in deacetylation assay*

| | Histone control | Test1 | Test2 | Test3 |
|---|---|---|---|---|
| Histones | 5 | 5 | 5 | 5 |
| KA 2 | - | 40 | - | 40 |
| KA 3 | - | - | 40 | - |
| Buffer | 5 | 5 | 5 | 5 |
| Entinostat (2.5mM) | - | - | - | 1 |
| NFW | 30 | 0 | 0 | 0 |

*Table 4:The volume of each component added in the reaction sets for the deacetylation assay*

### 2.14. Nuclear fractionation of *Plasmodium falciparum* 3D7 treated with artemisinin

*Plasmodium falciparum* 3D7 culture was given treatment with artemisinin at different concentrations for 16 hours. Parasites were harvested afterwards and proceeded with nucleocytoplasmic fractionation. Pellets were resuspended in five times the volume of lysis buffer (10mM KCl, 10mM HEPES, 0.1mM EDTA, 0.65% NP40, 0.1mM EGTA, 1mM DTT, 1X PIC, 2mM PMSF) incubated on ice with mixing at regular intervals. A spin was given at 13,000rpm for 20mins at 4degC. Supernatant would be the cytoplasmic fraction. Resultant pellet was washed three times with lysis buffer by resuspending and incubating it on ice for 5 mins and then giving spin at 13,000rpm for 10mins. Three washes were given then with a PIC-PBS mix by incubating and spinning in the same manner. Then three times the volume of the extraction buffer ( 200mM HEPES, 1mM EDTA, 400mM NaCl, 1mM DTT, 1mM EGTA) was added to that and properly resuspended. It was set on ice for 20 mins with intermittent mixing and spin was given at the same speed for 20 mins. The supernatant was collected as a nuclear fraction. Fractions were loaded onto 12.5% SDS gel and western blotting was done probing with HDAC1 antibody. Anti-histone H3 antibody was used for loading control.

# 3.RESULTS

### 3.1 Identification and validation of high molecular band of PfHDAC1

While post translational modifications of PfHDAC1 and the role of phosphorylation in regulating its enzymatic activity is known in higher eukaryotes [18,31,49] ,studies have not been done in *Plasmodium falciparum.* So one of our aims was to characterise PfHDAC1 and its post translational modifications in *Plasmodium falciparum*. Parasites were cultured in laboratory conditions and the lysate prepared from the harvested parasite pellets was used for doing an immunoprecipitation of PfHDAC1 using anti-PfHDAC1 antibody. The immunoprecipitated fractions were loaded onto SDS-PAGE gel and the PfHDAC1 status was investigated using a western blotting with anti-PfHDAC1 antibody (Fig 3).

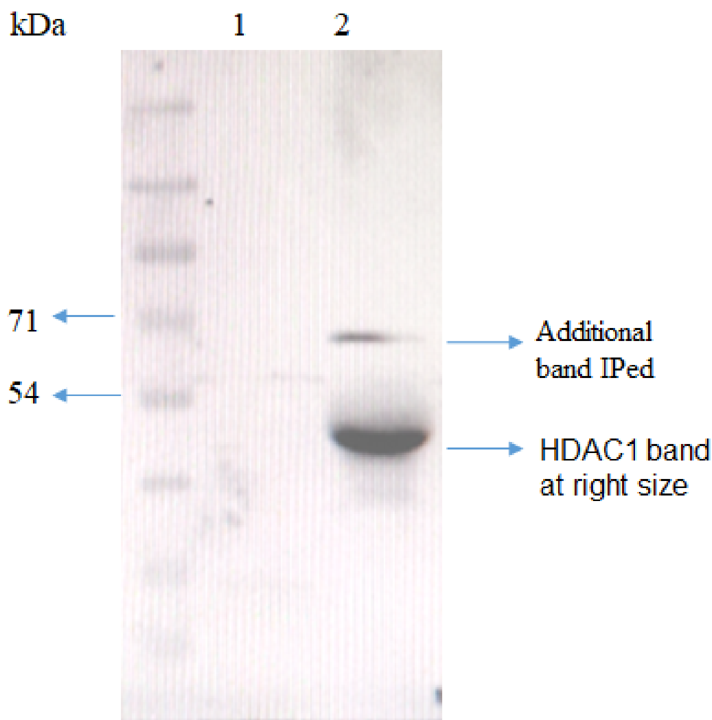



**Fig 3.** ***Western Blot for the immunoprecipitation of HDAC1 using HDAC1 antibody,*** *Western blot probed with anti-PfHDAC1 antibody showed that an additional band at about 65kDa appears above the expected size of 51kDa in the lane loaded with immunoprecipitated fraction using HDAC1(lane 2) and nothing appears in the lane loaded with IgG pulled down fraction(lane1).*

The further investigation on the additional band was done by treating with a phosphatase such as CIP and the treated samples were used for a western blot with anti-PfHDAC1 antibody(Fig 4).

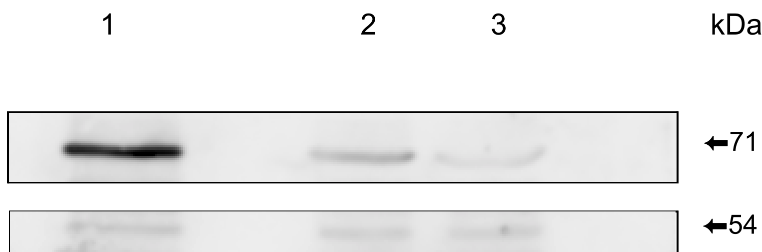



***Fig 4. Western blot for the CIP treatment,*** *Western blot probed with anti-HDAC1 antibody for the CIP treated samples where lanes represent 1)control 2)10U CIP treated 3)20U CIP treated. It showed the depletion of the additional band below 71kDa from control as compared to the CIP treated samples.*

The depletion of additional band in a concentration dependent fashion upon CIP treatment indicates that it can be a phosphorylated form of the PfHDAC1 which gets depleted on treatment

with a phosphatase. To further validate this band, we performed mass spectrometry of sliced additional band from the SDS PAGE gel (Fig 5).

a)

| Protein IDs | Majority protein IDs | Peptide counts (all) | Mol. weight | Sequence length | Q-value | Score | Intensity | MS/MS count |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| tr\|Q9XYC7\|Q9XYC7_PLAFA | tr\|Q9XYC7\|Q9XYC7_PLAFA | 8 | 51.375 | 449 | 0 | 53.324 | 5989.5 | 13 |

80 90
890123456789012345678
ENYREFTYQLKRFNVGEATDC

170 180 190 200
5678901234567890123456789012345678901234567890123
HARVMYIDIDVHHGDGVEEAFYVTHRVMTVSFHKFGDY

240 250 260 270 280
901234567890123456789012345678901234567890123
FKVVIDKCVQTYRPGAIIIQCGADSLTGDRLGRFNLTIKGHARCV

300 310 320
9012345678901234567890123456
GGYTIRNVSRCWAYETGVVLNKHHEMPD

360 370 380 390 400 410 420
90123456789012345678901234567890123456789012345678901234567890123456789012345
LSRIKMKIAENLRHIEHAPGVQFSYVPPDFFNSDIDDESDKNQYELKDDSGGGRAPGTRAKEHSTT

440
90123456789
LSDRDQSIVPY

←amino acid position

←mapped peptide

b)

| Protein IDs | Majority protein IDs | Peptide counts (all) | Mol. weight [kDa] | Sequence length | Q-value | Score | Intensity | MS/MS count |
|---|---|---|---|---|---|---|---|---|
| tr\|Q9XYC7\|Q9XYC7_PLAFA | tr\|Q9XYC7\|Q9XYC7_PLAFA | 7 | 51.375 | 449 | 0 | 45.293 | 3379.8 | 11 |

```
         10        20        30        40        50        60
123456789012345678901234567890123456789012345678901234567890
MSNRKKVAYFHDPDIGSYYYCAGHPMKPQRIRMTHSLIVSYNLYKYMEVYRPHKSDVNELTI

     90       100       110       120
678901234567890123456789012345678901234567890
QLKRFNVGEATDCPVFDGLFQFQQSCAGASIDGASKLNHHCADIG

     190       200       210       220       230
5678901234567890123456789012345678901234567890
FYVTHRVMTVSFHKFGDYFPGTGDITDVGVNHGKYYSVNVPLNDGMTI

   280       290       300              400       410       420       430
78901234567890123456789012345678901234   45678901234567890123456789012345678901234567
KGHARCVEHVRSYNIPLLVLGGGGYTIRNVSRC        DDESDKNQYELKDDSGGGRAPGTRAKEHSTTHHLRRKNYDDD
```

←amino acid position

←mapped peptide

***Fig 5. Peptides mapped in mass spectrometry,*** *a) and b)show the peptides mapped with the genome sequence of Plasmodium falciparum HDAC1 from additional band and HDAC1 band respectively using Maxquant software. 8 and 7 peptides were detected from the additional band and HDAC1 band respectively.*

The mass spectrometry results analysed by genome mapping using the maxquant software confirmed that the additional band picked up in the immunoprecipitated fraction is indeed a post-translationally modified version of PfHDAC1.

### 3.2 Kinases were detected as interacting partners in mass spectrometry

The mass spectrometry analysis was done using the maxquant software to identify the interacting partners of PfHDAC1 (Fig 6).

***Fig 6. Schematic representation of the PfHDAC1 interacting partners,*** *Mass spectrometry analysis identified 81 proteins as potent interactors of HDAC1 out of which two were kinases.*

Two kinases namely Ser-THr Kinase CLK1 (PF3D7-1445400) and cAMP dependent Kinase PKAr (PF3D7-1223100) were detected as interacting partners out of the 81 protein interactors identified in mass spectrometry.

### 3.3 Identification of the interaction between PfHDAC1 and PfCKII

Casein Kinase II (CKII) is widely quoted in literature as a potent interactor of HDAC1 in higher eukaryotes including zebrafish and human cells [13,18,71]. This led us to investigate the interaction between CKII and HDAC1 in the *Plasmodium falciparum*. For that PfCKII and PfHDAC1 were cloned.

A cotransformation experiment was first attempted to check the interaction between PfHDAC1 and PfCKII. Both of the clones were transformed into *E.coli* cells and the lysates were examined in a western blot to see any shift or change in the co-transformed colony lysates compared to the controls. Since both of the clones had the same antibody for selection an analysis at the protein level was needed to check for co-transformation (Fig 7).

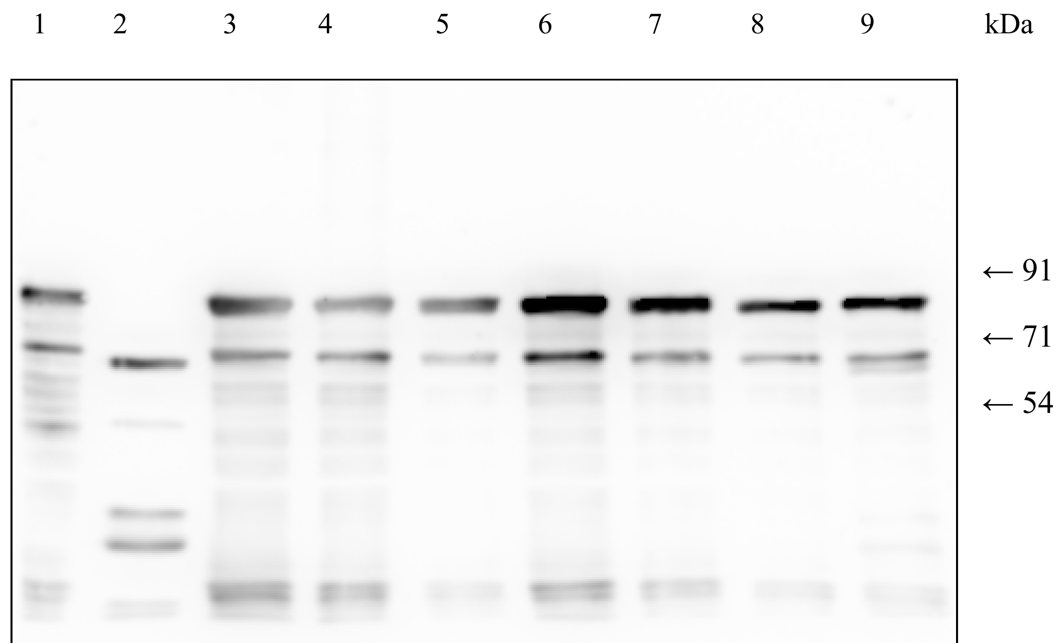



***Fig 7. Western blot of cotransformation,*** *Western blot probed with anti-GST for screening colonies after cotransformation, 1st lane and 2nd lane represents PfHDAC1 and PfCKII controls respectively and 8-9 lanes are the co-transformed colony lysates. The 9th lane showed bands corresponding to both the controls and hence was confirmed to be a lysate from a co-transformed colony.*

The confirmed colony lysates screened this way were then used for identification of changes in phosphorylation by probing with anti-serine phospho-antibody (Fig 8).

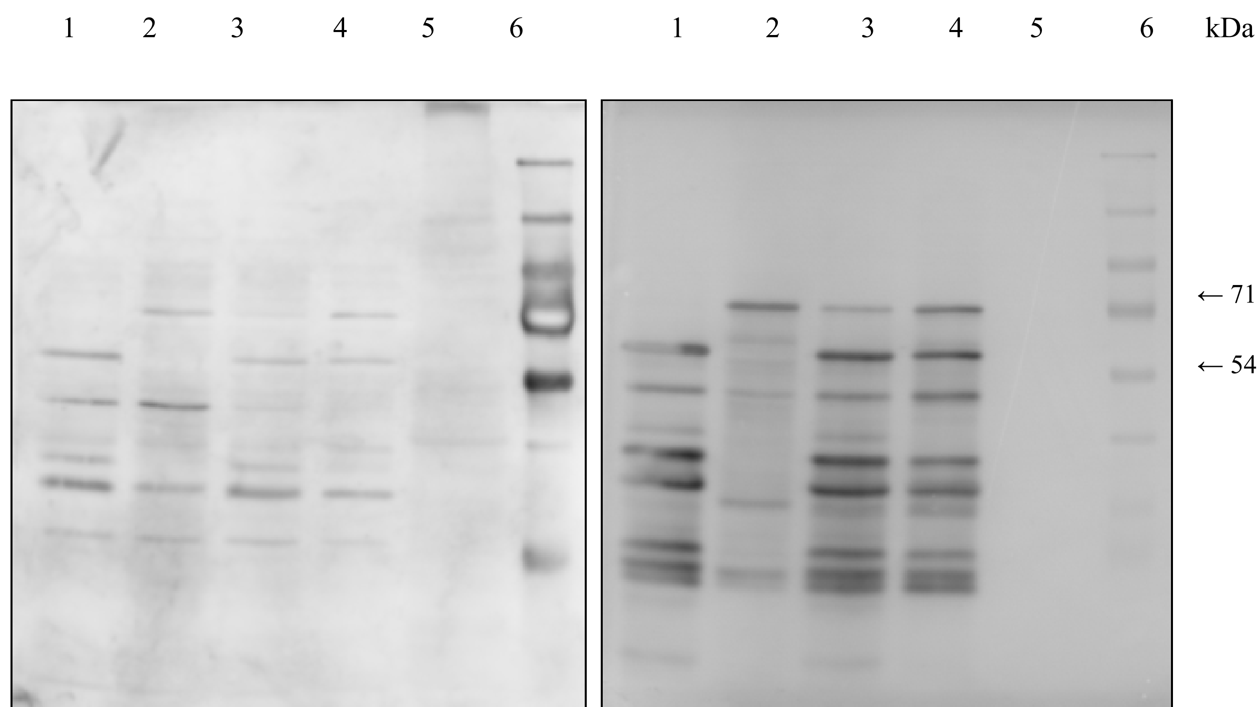
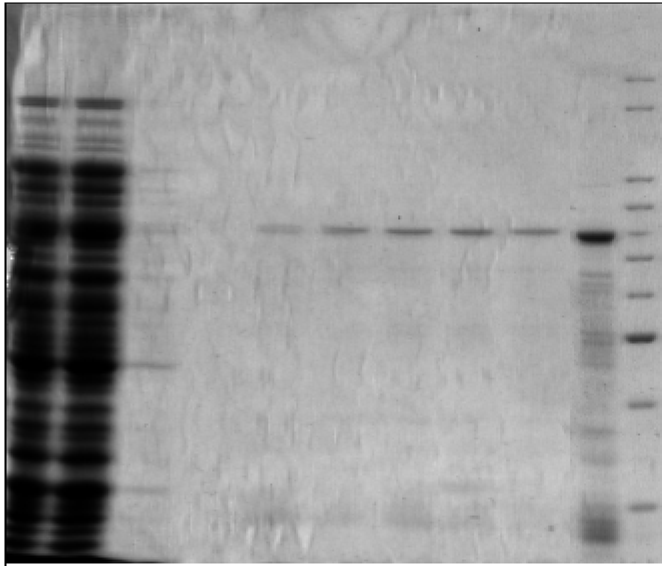



***Fig 8. Western blot for investigation of phosphorylation changes in co-transformed colonies,****Western blot probed with anti-Serine Phospho for the co-transformed colonies (lanes 3 and 4) with the PfCKII and PfHDAC1 controls(lanes 1 and 2 respectively). The co-transformed colonies do not show any shift or changes compared to the Fig c; the same western blot re-probed with anti-GST antibody for the loading confirmation.*

The absence of any shift or changes in the co-transformed colony lysates didn't give any conclusion or confirmation of the interaction between PfHDAC1 and PfCKII. So the next plan was to purify recombinant PfHDAC1 and PfCKII by transforming clones to *E.coli* and to check their interaction *in-vitro*. So the purification of GST tagged PfCKII and GST tagged PfHDAC1 was done using GST affinity chromatography (Fig 9, Fig 10, Fig 11).

1 2 3 4 5 6 7 8 9 10 11 kDa



←71

***Fig 9. Overexpression and Purification of GST tagged PfHDAC1****, 10% SDS-PAGE gel loaded with samples after GST affinity purification of GST tagged PfHDAC1, 1)Input, 2)flowthrough, 3)wash1, 4)wash3, 5)2nd 10mM elution, 6)3rd 10mM elution, 7)2nd 20mM elution, 8)3rd 20mM elution, 9)2nd 30mM elution, 10)GST beads, 11)ladder . The HDAC1 at the fusion product size of GST and HDAC1 appeared at around 75kDa in the elutions.*

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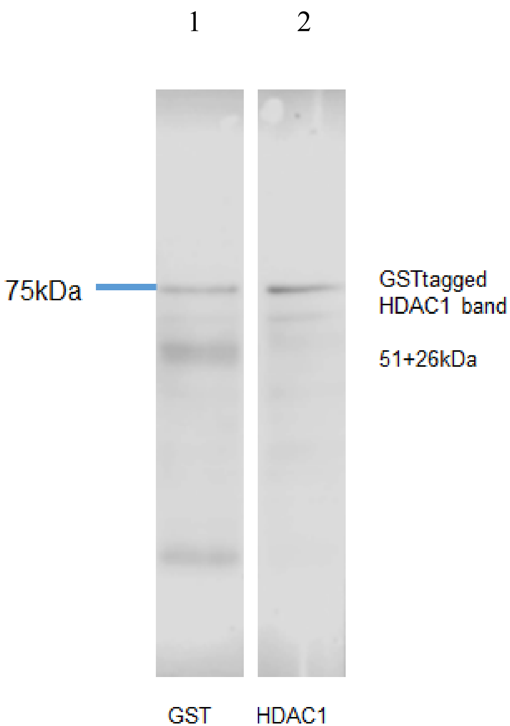
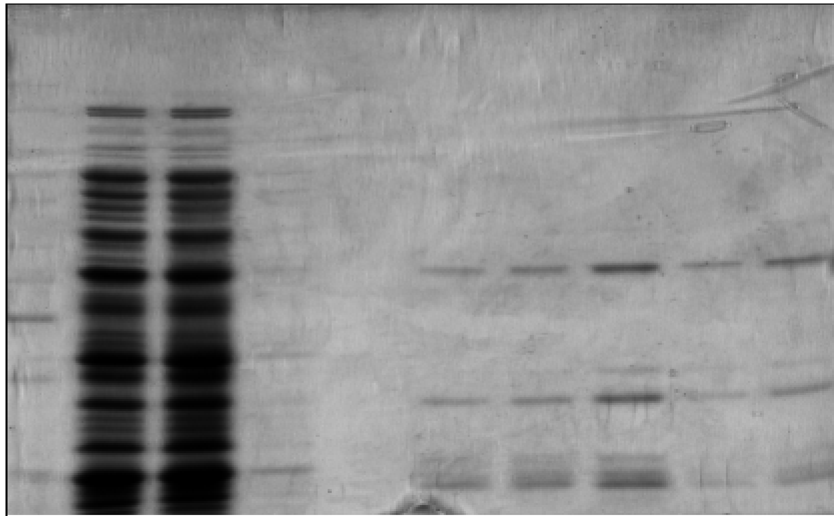



***Fig 10. Western blot showing Recombinant purified PfHDAC1,*** *Western blot probed with anti-GST in first lane and anti-PfHDAC1 in second lane. The GST tagged fusion product appears at the size of 75 kDa and it confirms the purification.*

1 2 3 4 5 6 7 8 9 10 kDa

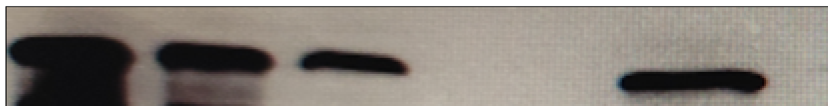


←65

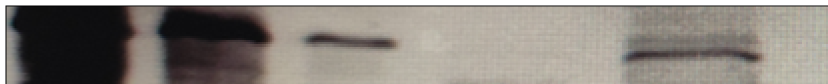
***Fig 11. Overexpression and Purification of GST tagged PfCKII,*** *10%SDS gel loaded with samples after GST affinity purification of GST tagged CKII alpha 1)ladder, 2)Input , 3)flowthrough, 4)wash1, 5)wash3, 6)2nd 10mM elution, 7)3rd 10mM elution, 8)2nd 20mM elution, 9)3rd 20mM elution, 10)2nd 30mM elution. The GST tagged PfCKII eluted at the fusion product size of 65kDa in all the elutions.*

An interaction assay was set up in vitro to check the interaction between PfCKII and PfHDAC1 by incubating both of them together and then incubating with beads bound with anti-PfHDAC1 antibody and another reaction with beads bound with IgG as negative control (Fig 12).

1 2 3 4 5 kDa



←71



←54

***Fig 12.Interaction Assay blot of recombinant PfHDAC1 and recombinant PfCKII,*** *Western blot probed with anti-GFP,anti-rabbit conformation specific and anti-mouse for the samples of interaction assay;1)Input, 2) Flowthrough IgG, 3) Flowthrough HDAC1, 4) Elution1 IgG, Elution1 HDAC1. CkII eluted from beads incubated with anti-HDAC1 and not with beads probed with IgG.*

Elutions from the beads incubated with anti-PfHDAC1 antibody showed PfCKII at its fusion product size of 65kDa whereas the IgG control did not. This in turn indicates that the HDAC1 which is bound to the anti-PfHDAC1 antibody is interacting with PfCKII. Thus, the interaction between PfHDAC1 and PfCKII was confirmed.

## 3.4 Confirmation of PfHDAC1 phosphorylation by PfCKII

A kinase assay was performed to investigate the functional consequence of interaction between PfHDAC1 and PfCKII where both were incubated in a kinase assay buffer in presence of ATP. Also CKII inhibitor (TBB) and artemisinin were added in some reactions to see the effect it has on the phosphorylation (Fig 13).

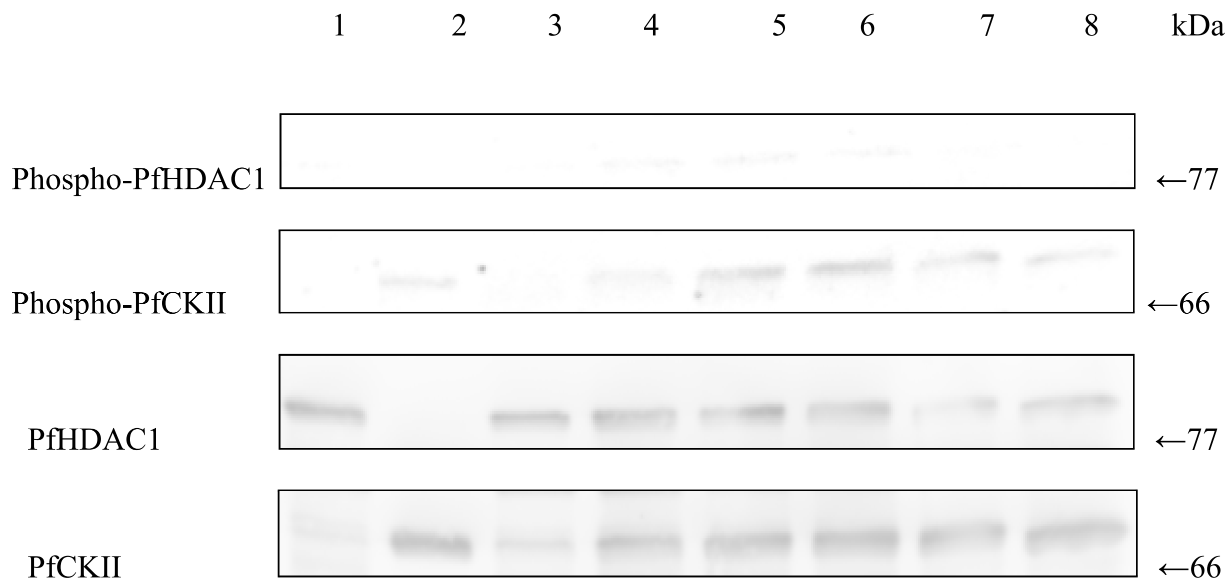


***Fig 13. Kinase Assay blots of recombinant PfHDAC1 and Recombinant PfCKII,*** *First two rows are western blot probed with anti-serine phospho antibody and next two are loading controls for the kinase assay reactions 1)HDAC1 control, 2)CKII control, 3)HDAC1+ xCKII 4)HDAC1+2xCKII, 5)HDAC1+3xCKII, 6)HDAC1+3xCKII+yCKII inhibitor, 7)HDAC1+3xCKII+2yCKII inhibitor, 8)HDAC1+3xCKII+ artemisinin. The PfHDAC1 phosphorylation is showing a concentration dependent increase on treatment with CKII. It also gets depleted on treatment with CKII inhibitor in a concentration dependent manner. Same trend is shown by CKII phosphorylation. Interestingly, PfCKII and PfHDAC1 phosphorylation depleted upon artemisinin treatment.*

The kinase assay gave evidence that PfHDAC1 is getting phosphorylated in presence of PfCKII. The concentration dependent increase in phosphorylation on CKII treatment and depletion in presence of CKII inhibitor gives more confidence to the data.

## 3.5 Deacetylation activity of PfHDAC1 occurs in its phosphorylated form

The functional consequence of PfHDAC1 phosphorylation was tested upon its activity by a deacetylation assay using purified histones from *Plasmodium falciparum*. Histone purification was done using salt-lysis and acid extraction (Fig 14).

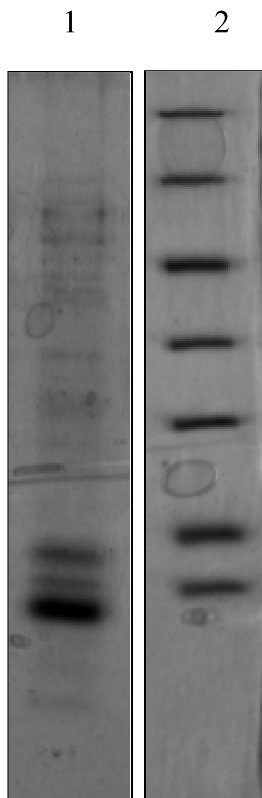



***Fig 14. SDS PAGE showing purified histones,*** *15% SDS-PAGE gel loaded with elutions from histone purification using salt-urea lysis method,1) histone eluted 2)ladder*

←15 kDa

The histones were purified in the right size of 12-14kDa. With these purified histones,the assay was conducted with recombinant PfHDAC1 to check its deacetylation activity (Fig 15).

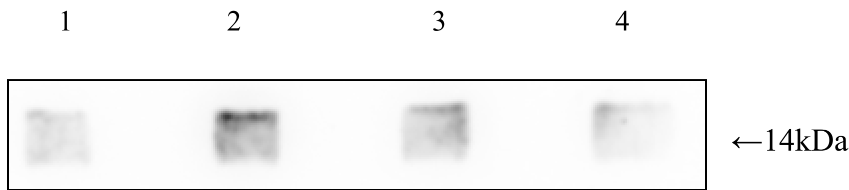



***Fig 15. Deacetylation assay blot  for non-phosphorylated HDAC1,*** *Western blot probed with anti-acetyl H3K9 antibody, 1)histone control 2) histone with x amount of rec PfHDAC1 3)histone with 2x amount of PfHDAC1 4)histone with 3x amount of PfHDAC1. The addition of recombinant HDAC1 does not show a decrease in acetylation signal of the histones.*

The deacetylation activity of HDAC1 was not visible in its recombinant form as it did not show a decrease in the acetylation signal in histones. So the assay was repeated with phosphorylated form of HDAC1 made by CKII treatment in presence of ATP (Fig 16).

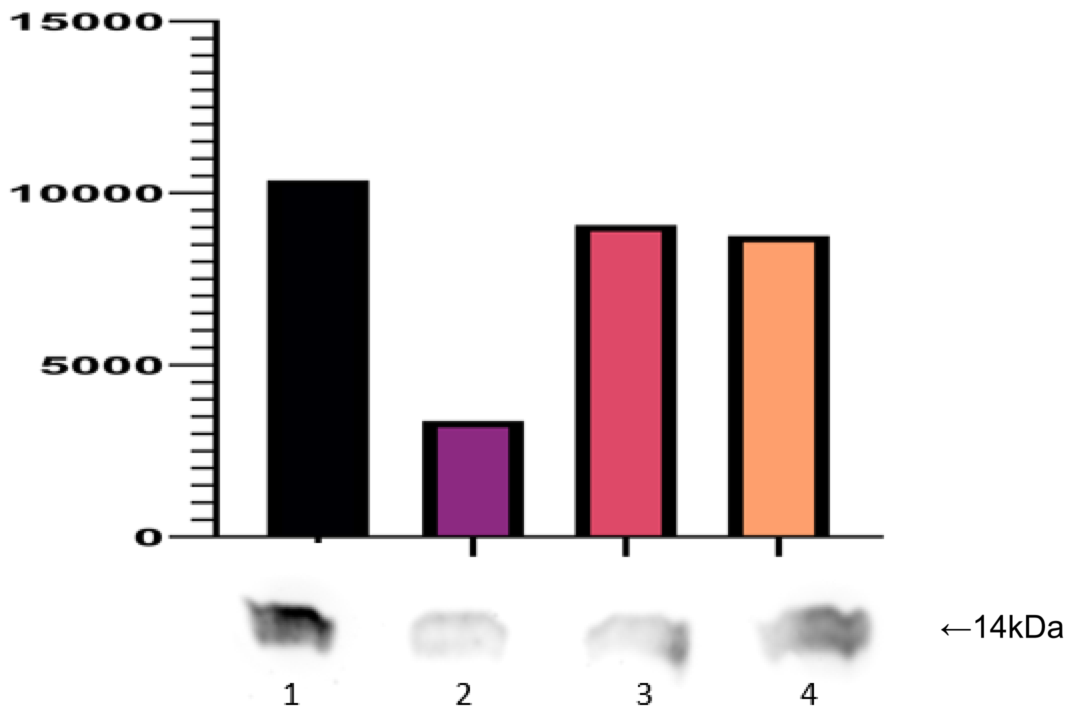


***Fig 16.Schematic representation of densitometry measurements of the acetylation signal,*** *The densitometric measurements of the acetylation signal for the deacetylation assay reactions in western blot probed with H3K9 acetylation antibody where the lanes are 1) Histone only control, 2) histone with HDAC1 phosphorylated in kinase assay , 3) histone with kinase assay reaction having PfHDAC1, PfCKII and CKII inhibitor 4)histone with phosphorylated PfHDAC1 from kinase assay and HDAC1 inhibitor Entinostat. The graph shows that acetylation of the histone decreases on treatment with phosphorylated PfHDAC1. It also shows the regain of the signal in presence of CKII inhibitor and entinostat.*

The deacetylation assay showed that PfHDAC1 is active upon phosphorylation by PfCKII. The acetylation signal was regained in reaction with CKII inhibitor which shows that PfCKII and thereby the phosphorylation plays an important role in the deacetylase activity of PfHDAC1.
The deacetylase activity in presence of phosphorylation and its absence in the native form of PfHDAC1 shows that phosphorylation is essential for PfHDAC1 to perform its activity.

### 3.6 Artemisinin has effect on nuclear localisation of phosphorylated PfHDAC1

The depletion of phosphorylation signal in presence of artemisinin in kinase assay gave a thread that artemisinin treatment might have an effect on PfHDAC1 phosphorylation and in turn the activity of PfHDAC1. So the treatment was extended in-vivo. The parasite culture was given artemisinin doses and the nuclear fractions prepared from the lysates were analysed in western blot (Fig 17).

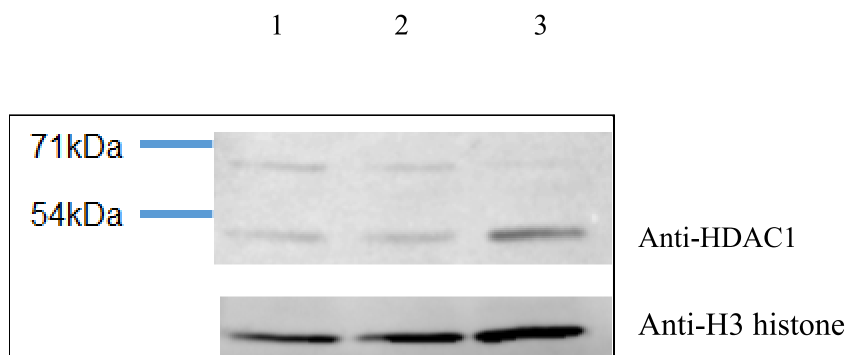



***Fig 17. Western blot of nuclear fractions from artemisinin treated parasite culture lysates,***

*Western blot probed with anti-HDAC1 for the nuclear fractions of artemisinin treated P.falciparum culture, 1)control treatment, 2) test1 treated with x amount of artemisinin, 3) test2 treated with 2x amount of artemisinin*

The presence of phosphorylated HDAC1 in the nuclear fraction seems to decrease while the native HDAC1 increases with increased doses of artemisinin treatment.

# 4. DISCUSSION

Malaria is a life threatening disease and besides all the precautions made and the treatments available, the number of infections are high around the globe [45,50]. Emergence and spread of artemisinin resistance cases are also being highly reported [24]. Since PfHDAC1 being an important target which is downregulated in artemisinin resistant parasites and PfHDAC1 inhibitors are identified to be detrimental to the parasites, it clearly can be a potent antimalarial target [14,68]. The characterisation of PfHDAC1 and its phosphorylation being very important for its enzymatic and transcriptional activity gains importance this way. Thus this study aims to investigate the phosphorylation of PfHDAC1, the kinases that play a role in this process and its functional consequences.

Western blot of 3D7 *Plasmodium falciparum* lysate with purified anti-PfHDAC1 antibody showed two bands . One at expected size of 51kDa and the other at a molecular weight higher than expected which led us to speculate if this was a post translationally modified version of the protein. This possible phosphorylated version of PfHDAC1 when detected in SDS PAGE was found to have different electrophilic mobility than the unmodified PfHDAC1. We thus checked for CIP treatment and found the additional band to disappear. To confirm this, the additional band was cut and sent for mass spectrometric analysis. The analysis with the 3D7 HDAC1 sequence picked up 8 peptides for the higher molecular weight band and 6 peptides for the 51kDa band showing that the additional band picked up is indeed a modification of the PfHDAC1 band.

Interactome study of PfHDAC1 was carried out using protein IP and a number of interacting kinases were identified to be pulled down with PfHDAC1 hinting that a number of these kinases

could be responsible for differential phosphorylation of PfHDAC1. Each of these kinases could have specific residues which they phosphorylate and these marks could modulate the characteristics of PfHDAC1 differently. Thus PfHDAC1 seems to possess a kinome which can greatly dictate its functions in the cell in a phospho modification specific manner. Additionally since the IP was done on mixed stage culture of parasites, it is possible that these kinases may have stage specific phosphorylation roles for PfHDAC1. All this demands further studies of the interactions of these individual kinases to understand the roles.

The literature on higher eukaryotes actively showed the influence of Casein Kinase II(CKII) on HDAC1 phosphorylation and thereby regulating its activity such as localisation and complex formation [13,18,71]. Thus the experiments were directed in finding an interaction if any between PfCKII and PfHDAC1. A co-transformation experiment was performed where the clones prepared for both of these were transformed into *E.Coli* cells. Western blot probed with anti-serine phospho antibody for the co-transformed colonies was used to check whether there is an additional band or any shift in the PfHDAC1 band appearing in them compared to the PfHDAC1 control. It didn't show any shift or an additional band but was almost similar to the control PfHDAC1. One possible reason behind this experiment not showing the expected phosphorylation of PfHDAC1 is that the level of phosphorylation in this instance may be very low. Another reason could be that the cellular conditions in the bacterial environment may not be conducive for the phosphorylation to proceed.

To check whether there is a phosphorylation that can be brought on PfHDAC1 by PfCKII in-vitro, protein purification of them was conducted. GST tagged PfHDAC1 was overexpressed and purified using GST affinity chromatography. GST tagged PfCKII was also overexpressed and purified similarly.An interaction assay was performed then to see whether there is an interaction between purified PfCKII alpha and PfHDAC1 in-vitro. A pulldown experiment was performed using anti-PfHDAC1 ab and IgG antibody control to see if IP of PfHDAC1 could also co IP PfCKII. We finally observed that in the IgG control I, PfCKII was not present while it was pulled down along with PfHDAC1 using anti-PfHDAC1 ab. This confirms the *in vitro* interaction of the two proteins.

The functional consequence of this interaction was analysed using a kinase assay. It was performed to see if the interaction of the two proteins has functional consequences in the form of PfHDAC1 getting phosphorylated.

Our kinase assay demonstrates that PfHDAC1 can be phosphorylated by PfCKII in a dose dependent fashion. Furthermore the kinase activity was found to be specific to PfCKII since use of CKII specific inhibitor TBB could suppress the extent of phosphorylation in a dose dependent fashion. Furthermore, PfCKII was seen to undergo autophosphorylation validated by depletion of phospho PfCKII by TBB treatment. Finally artemisinin treatment could also suppress the kinase activity of PfCKII.

Autophosphorylation of CKII is reported to be very essential for its kinase activity and the presence of autophosphorylation is considered as evidence for the presence of active CKII.

The inhibition of the PfCKII specific kinase activity by TBB inhibitor reaffirms the possibility of its usage in in vitro settings to investigate the effect of suppression of CKII specific kinase activity on various aspects of the parasite biology and biochemistry.

We observed a robust phosphorylation of PfHDAC1 in the artemisinin null reaction. However, a dose-dependent depletion of PfHDAC1 phosphorylation was recorded in reactions with artemisinin coincubation.

The phosphorylation of HDACs is reported to regulate catalytic activity as well as protein protein interactions. 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 [49]. For HDACs the phospho 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 and in turn lead to a reduction in it's deacetylase activity. 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 phospho 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). This could again dramatically affect the catalytic state and interactome of PfHDAC1 and thus its gene regulatory functions in artemisinin resistant parasites.

The next aim was to check the activity of this phosphorylated PfHDAC1 from kinase assay. For that the reaction samples from kinase assay were used in performing a deacetylation activity assay using *Plasmodium falciparum* histones. The western blot probed with anti-acetyl H3K9 antibody showed that the acetylation decreased in the reaction set which had phosphorylated HDAC1.The acetylation then increased with reaction having kinase assay sample treated with CKII inhibitor and also increased in reaction having HDAC1 inhibitor entinostat.The densitogram for these bands clearly showed that there is a clear deacetylation activity observed with phosphorylated HDAC1 which gets inhibited with CKII inhibitor and also with entinostat.

Next aim was to extend the experiments in-vivo and then to functionally characterise the results. On that basis *Plasmodium falciparum* 3D7 culture was given treatment with artemisinin and nuclear fractionation of the cells were done. Western blot probed with anti-HDAC1 showed that there is a depletion of the phosphorylated HDAC1 in the nuclear fraction of artemisinin treated samples in a dose dependent manner.

Post translational modifications of HDAC1 like phosphorylation have already been reported to be really important for its enzymatic activity and complex formation in higher eukaryotes including human cells. The depletion of this phosphorylation by artemisinin in nuclear fraction can thus be characterised more on how this regulation is happening.The phosphorylation of

HDAC1 had been found to be really important for its enzymatic activity and thereby maintaining the transcriptional status of genes. On these lines, further investigation on the phosphorylation regulation can really help in finding ways to effectively deal with the parasite's genome functioning and its ability to cause infection.

Differential phosphorylation of HDAC1 and its association with kinases has been observed to affect the localisation of kinases and its signalling in several biological systems. In this regard,the interaction between CKII and HDAC1 found here in-vitro can be extended to *Plasmodium falciparum in-vivo*. Functional consequences of this interaction like signalling can be worth investigating as the signalling is of paramount importance in helping cells to deal with the stress. The study of regulation or deregulation of functions through this interaction such as signalling might thus give more knowledge on how the parasites cope up with different forms of stresses. This knowledge may lead to finding ways for controlling parasite's stress response and thereby its infection.

It is also worth investigating whether the kinase abundance in the cell can have any effect on PfHDAC1 and thereby its transcription. Kinases are common modes of signalling in cells and with external stimuli it can vary inside the cell. So it would be fascinating to check whether such external cues affecting kinase abundance can in turn regulate PfHDAC1 and thereby transcription and gene expression of *Plasmodium falciparum.* If so, we would be able to get more insight on how parasites deal with external cues and stresses.

Mass spectrometry can also be performed on phosphorylated and non-phosphorylated PfHDAC1 to identify the interactome specifically associated with their post translationally modified status. It would also be interesting to find out the effect that a mutation in phosphorylated sites can bring out on the activity of PfHDAC1 and thereby its localization and also the gene expression in the cell.

It can be further checked whether there is a crosstalk in the interaction between PfHDAC1 and PfCKII where PfHDAC1 can regulate or influence PfCKII. If so,then its effect on the kinase signalling cascade can be investigated. Also it can be investigated whether the phosphorylated

state of PfHDAC1 regulates target selection in the cell as there are reports that post translationally modified HDACs target kinases to their substrates around the cell.

As a whole, this work by finding the effects that PfCKII can bring about on the activity of PfHDAC1 through its phosphorylation modification can further help in more studies in finding the genome regulation of *Plasmodium falciparum ,* its stress management etc thereby creating different angles for drug development and in-turn control of the infection.

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