Study of epigenetic regulation of virulence gene expression in *Plasmodium falciparum*

A thesis submitted to University of Hyderabad

for the award of Ph.D. degree in

Department of Biochemistry

By

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DECLARATION

I, **M. V. Shalu** hereby declare this thesis entitled "**Study of epigenetic regulation of virulence gene expression in *Plasmodium falciparum*"** submitted by me, under the supervision of **Dr. Mrinal Kanti Bahattacharyya**, is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this university or any other university or institute for the award of any degree or diploma.

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DECLARATION

I, **M. V. Shalu** hereby declare this thesis entitled "**Study of epigenetic regulation of virulence gene expression in *Plasmodium falciparum*"** submitted by me, under the supervision of **Dr. Mrinal Kanti Bahattacharyya**, **Associate Professor, Department of Biochemistry, University of Hyderabad,** is a bonafide research work which is also free from plagiarism. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma. I hereby agree that my thesis can be deposited in shodganga/INFLIBNET.

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This is to certify that this thesis entitled **"Study of epigenetic regulation of virulence gene expression in *Plasmodium falciparum*"** is a record of bona fide work done by **M.V. Shalu**, a research scholar for PhD programme in Department of Biochemistry, School of Life sciences, University of Hyderabad under my guidance and supervision. This thesis has not been submitted previously in part or in full to this or any University or Institute for the award of any degree or diploma.

Dr. Mrinal Kanti Bhattacharyya

Head of the Department **Principal Investigator**

Dean of the School

DEDICATED TO MY BELOVED PARENTS AND HUSBAND

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Shalu

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ABBREVIATIONS

Ade	Adenine
Amp	Ampere
ARP	Asparagine rich protein
bp	Base pair
$CaCl_2$	Calcium Chloride
cDNA	Complementary DNA
Da	Dalton
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
Etc	et cetera
EDTA	Ethylene diamine tetra acetic acid
iRBC	infectefed RBC
kDa	Kilo Dalton
LB	Luria-bertani broth
Leu	Leucine
LiOAC	Lithium Acitate
M	Molar
Mg	Milli gram
Min	Minute
mM	Milli molar
ml	Milli liter
Mwt	Molecular weight

NaOAc	Sodium Acitate
NaOH	Sodium hydroxide
Ng	nano gram
OD	Optical Density
ORF	Open reading frame
PBS	Phosphate buffered saline
PCIA	Phenyl Chloroform Isoamyl alcohol
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PVDF	Poly vinylidene Fluoride
Q-PCR	Quantitative PCR
RBC	Red Blood Cell
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	rotation per minute
RT	room temperature
RT-PCR	Reverse transcriptase - polymerase chain reaction
SC	synthetic complete
SDS	sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate Poly acryl amide gel electrophoresis
ssDNA	Single strand DNA
TBE	Tris Borate EDTA
TCA	Trichloroacetic acid

TE	Tris EDTA
Tric Cl	Tris Chloride
TRP	Tryptophan
Ura	Uracil
YPD	Yeast extract dextrose
μg	Microgram
μl	Microliter
17AAG	17-demethoxygeldanamycin

CHAPTER 1

INTRODUCTION

1.1 MALARIA

Malaria comes from the word "mal" and "aria" which means bad air as the people in ancient days; before the discovery of the malaria parasite use to think that the disease is caused by fowl air, and associated it with wet land and low lying swamps. Malaria is a very ancient disease known to be existing even before humans were evolved. It is the leading cause of morbidity and mortality, with an estimated 198 million infections and 584,000 deaths globally in the year 2013. The burden is heaviest in the WHO African regions where an estimated 90% of all the malaria deaths occur, 78% of which include children under the age of five [1]. The disease results from the multiplication of malaria parasites within red blood cells, causing symptoms that usually include fever and headache, in severe cases progressing to coma, and death. It is caused by a unicellular eukaryotic protozoan parasite of the genus *Plasmodium*. There are five species of *Plasmodium* that can naturally infect humans: *Plasmodium falciparum, Plasmodium vivax, Plasmodium malaria, Plasmodium ovale* and *Plasmodium knowlesi* among which *P. falciparum* is the most virulent form and is responsible for almost all malaria specific mortality. The disease spreads from one human to another by a female Anopheles mosquito.

P. falciparum exhibits a staggeringly complex life cycle. The human infection begins when an infected female Anopheles mosquito bites the human host injecting its saliva into the subcutaneous tissue in order to prevent the blood from clotting. Along with the saliva the mosquito injects sporozoites, the motile form of the parasite which rapidly invades the liver cells. Within the hepatocytes the parasite undergoes several rounds of cell division generating ten thousands of merozoites for each infective sporozoite that are released into the blood stream. Upon erythrocyte invasion, the parasite undergo additional round of schizogony and develop into ring, trophozoite and schizont stages. The mature schizont ruptures to release 6-32 daughter merozoites which invade fresh RBCs. Clinical symptoms such as fever, chills, impaired consciousness, etc occurs during this asexual blood stream cycle. In the erythrocytes, some parasites do not undergo schizogony but transform into male and female gametocytes. To complete the life cycle of the parasite these gametocytes has to be taken up by another mosquito where they further develop into female macro gamete and male micro gamete. The gametes then fuse to form ookinete, the motile form which migrates to the gut wall and transforms into oocyst where thousands of sporozoites are produced. These sporozoites eventually reach the salivary glands to infect another human host. With the exception

of blood stage parasites, the life cycle progresses in a linear fashion. The erythrocytic cycle is essential for the transmission of the malaria since it secures the supply of sexual precursors to complete the life cycle (Figure 1).

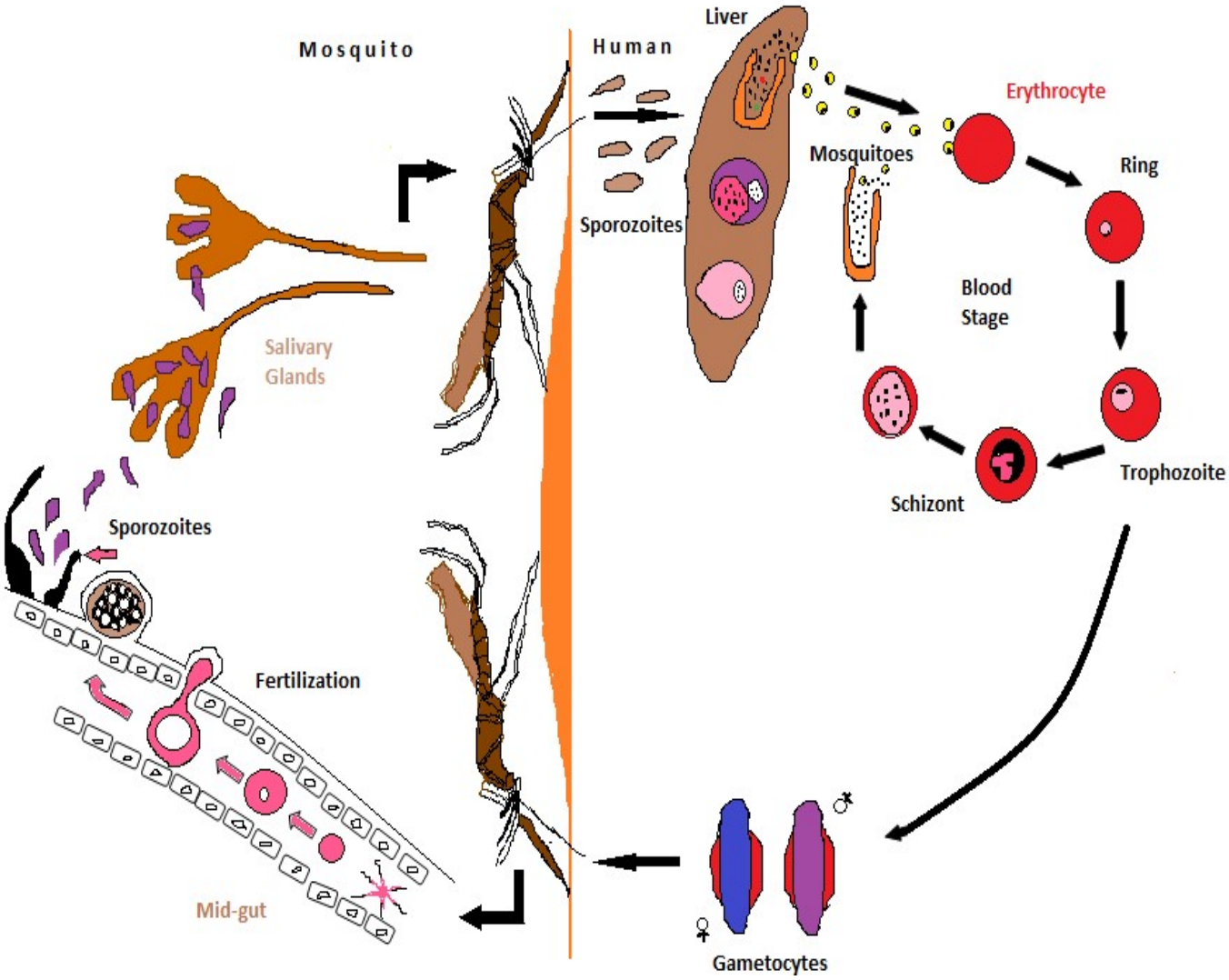


Figure 1: Schematic diagram of *P. falciparum* life cycle

1.2 ANTIGENIC VARIATION

The phenomenon where the parasite exhibits variable antigens on the surface of the infected RBCs in order to escape the host immune response and establish the chronic infection is called antigenic variation [2] . Cox for the first time identified antigenic variation in *Plasmodium* species by observing a relapsed parasite population in *P. berghei* infected mice [3] . Antigenic variation in human malaria species *P. falciparum* was shown for the first time experimentally using squirrel monkeys [4]. An important survival strategy of *P. falciparum* relies on its ability to evade the immune destruction by the host antibodies by changing the erythrocyte surface antigen. Antigens that are expressed on the surface of *P. falciparum* infected RBC in a strain specific manner and have biochemical property similar to schizont infected cell agglutination protein called *P. falciparum* erythrocyte membrane protein 1 (*Pf*Emp1) [5] . These proteins adhere to various receptors on the endothelial cell or on the other RBCs and thus mediate sequestration of infected RBCs in order to avoid splenic clearance leading to severe outcome of the disease such as organ specific damage or cerebral malaria [6]. Each parasite genome contains about 60 *var* gene that encode for *Pf*Emp1 protein [7], among which only one of the *var* gene is expressed on the surface of the trophozoite infected RBC at given a time [5] and the remaining are transcriptionally silenced. *P. falciparum* evades the host immune system by switching between these *var* genes. The rate of *var* gene switching can be as high as 2%, however, these rates change significantly between different variants [8] To this day, no gene has been identified that is involved in modifying rate of switching and no functional data is available on the molecular mechanism of switching. The parasites can change the expression of one *Pf*Emp1 to another on the surface of the iRBCs both *in vivo* and *in vitro* conditions. The reason why the intracellular parasites have evolved to express these antigens on the surface of the iRBCs is yet not clear. There are at least five possible reasons to why *P. falciparum* has evolved to display their proteins on the surface of iRBC. Firstly, it is frequently urged that this helps the sequestration of the parasite infected RBC in the deep tissue and thus preventing the splenic filtration where they could be recognized as foreign and removed. Second possible reason is assumed to be that it is important for the parasites to expose themselves to the host so that the parasite number is controlled by the host immune response. It is assumed that without the variable antigen the parasite might multiply too rapidly, which may lead to the death of the host before the transmission could occur. The third proposed alternative is that the parasite proteins are exposed on the surface of the erythrocyte in

order to protect the infected erythrocyte rather than targeting it for destruction. Naturally, there are many mechanisms that accelerate the destruction of the aged or the senescent RBC from the circulation and replacing them with fresh and healthy RBCs. Exporting the parasite protein to the surface might therefore be necessary to interfere with the natural clearance process. However, this hypothesis is totally speculative with no direct evidence. The fourth possible reason might be that the antigens expressed on the surface of iRBC inhibit the *in vitro* maturation and activation of dendritic cells and therefore mostly interfere *in vivo* with antigen presentation which may slow down the development of an effective host protective immune response and establish chronic infection. Finally, as it is necessary for the parasite to be transmitted between human host and the mosquito, it might be possible that antigenic variation, for some unknown reasons has been evolved to ensure the gametocyte survival. This is an attractive hypothesis as in *P. falciparum* gametocyte development is unusually prolonged to about 7 days [9] Experimental evidence has demonstrated that dynamic chromatin changes controls antigenic variation in the *P. falciparum* [10]. Unlike *trypanosoma brucei*,antigenic variation in *P. falciparum* does not require genetic rearrangement, thus epigenetic regulation plays a crucial role in *var* gene regulation. The transcriptionally active *var* locus is associated with histone markers such as histone 3 lysine 9 acetylation (H3K9ac), H3K4 di methylation (H3K4me2) and H3K4me3, whereas, the transcriptionally silent var genes are highly enriched with H3K9me3 heterochromatin marker [1]. However, transcriptional regulations of these var genes are still to be elucidated. Some studies suggest that the *var* gene expression are developmentally regulated, with most of the *var* genes being transcribed during the ring stage, but only one *Pf*Emp1 protein is expressed on the surface of the iRBC during the late trophozoite stage [11]. In the schizont stage, the *var* genes are not transcribed.

Var genes vary in size from 6 to 15 kb and are extremely divergent in sequence. These genes consist of two Exons, 5' Exon1 codes for a large, highly variable extra cellular domain which is exposed to the outer surface of the iRBC and a 3' Exon2 codes for semi conserved intra cellular domain connected by a single conserved intron [12]. *Pf*Emp1 proteins are large, ranging from 250 to 350 KDa. The structure of *Pf*Emp1 is partly conserved featuring an N-terminal segment (NTS), variable number of Duffy binding like domains (DBL), one-two cysteine rich domain (CIDR), a trans-membrane domain (TM), a C2 domain and a conserved intra-cellular acidic terminal segment (ATS) [13]. Though the basic structure of these proteins is similar, the amino acid sequence comparison

revealed the extreme diversity which is mainly in the DBL extracellular adhesion domains that are exposed to constant selection pressure by the host immune response [6].

Complete *P. falciparum* genome analysis confirmed the location of *var* genes to the highly polymorphic sub telomeric regions and few central to the chromosomes. Approximately 60% of the *var* genes are located around 40-50 kb from telomere [10]. Each of the 14 parasite chromosome ends with one to three *var* gene. *Var* genes exist at the telomere in one to three different directions, head to head orientation, head to tail orientation and tail to tail orientation, which is the most common. The central *var* genes are arranged in tandem array in head to tail orientation. The location and the orientation of the *var* genes are associated with 5' flanking region called *ups* [14]. The telomeric *var* genes that are transcribed towards the centromere are called *upsB* type and the telomeric *var* genes that are transcribed toward the telomere are called *upsA* type. Internal *var* genes are always associated with *upsC* type (Figure 2).

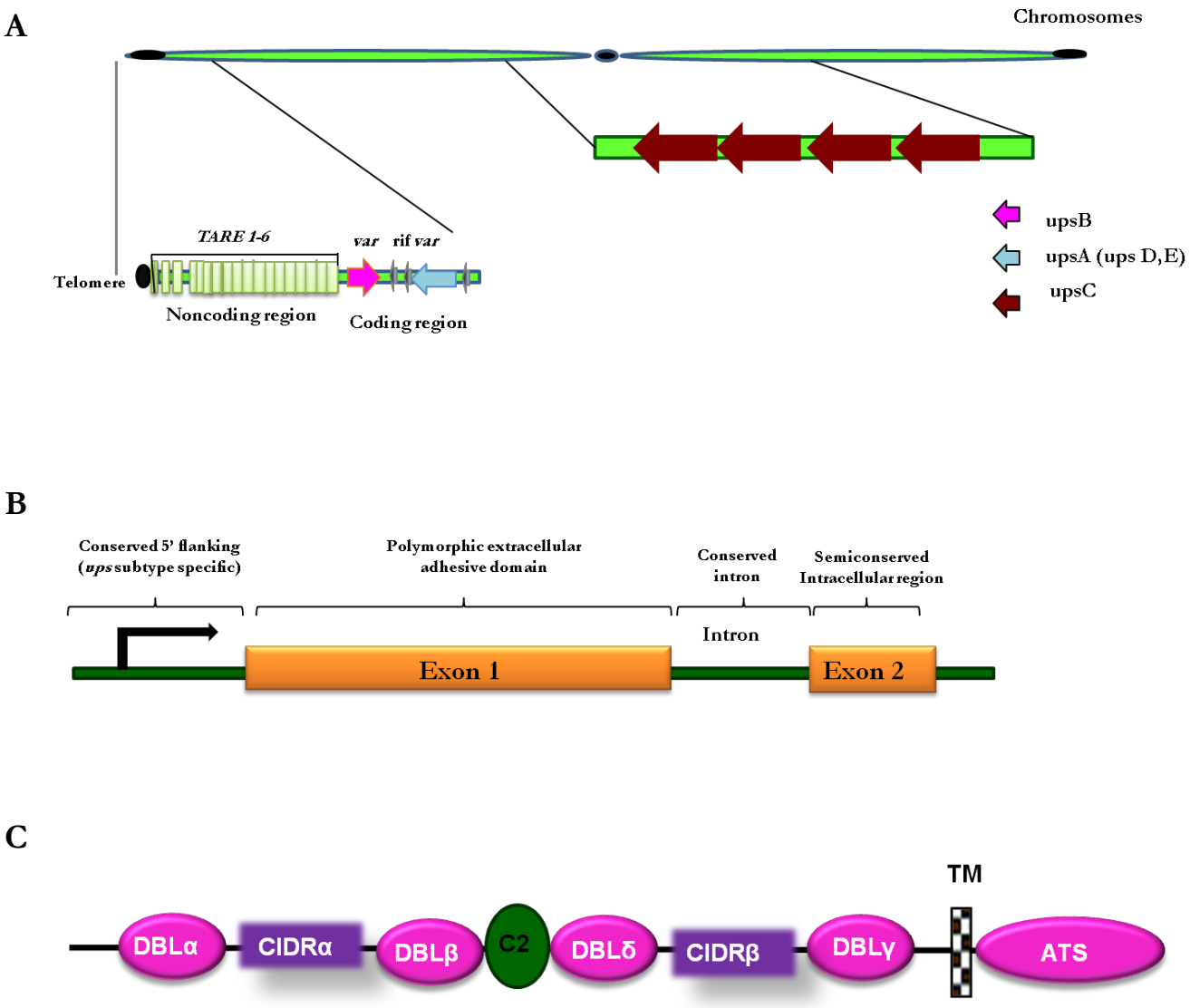


Figure 2: Genomic localization of *var* genes

A. Organization of *P. falciparum var* genes. B. *Var* gene features. C. *Pf*EMP1

1.3 GENE SILENCING MECHANISMS

In each cell type precise control mechanisms are required for cell differentiation that allows the expression of an appropriate subset of genes. The transcriptional silencing involves heritable modifications of the chromatin throughout the genome that helps to maintain or remember their gene expression program specified during embryonic development [15]. Gene silencing is a primary mechanism that contributes to the maintenance of programmed gene expression. Silencing is not specific to the promoter and can be referred to as a long range of repression wherein it is mediated by regulatory sites, known as silencers that act at some distance from their target genes. Most of the studies have revealed that majorly the sequence at 5' end of the gene and occasionally the sequences at 3' end play a crucial role in the expression or repression of the gene to which the regulatory proteins are bound [16]. Silencers flanks and represses certain genes with potential to control the mating type which led to the discovery of position effect in *Saccharomyces cerevisiae*. Silencing was originally defined in *S. cerevisiae* as a process that represses transcription of secondary copies of the mating type genes. Mating type is determined by the alternative alleles of a single mating type locus (*MAT*) which is located at the center of the chromosome III. Along with MAT locus, yeast contains two other mating type genes HML and HMR, which are located at the opposite ends of chromosome III and are transcriptionally repressed. Silencing involves the packaging of key key regulatory genes into a specialized chromatin structure which is inaccessible to DNA binding proteins leading to inactivation of chromosome domains. Multiple mechanisms have been proposed for gene silencing in *Saccharomyces cerevisiae* which is similar to heterochromatin formation in eukaryotes such as maize, flies and mammals. Heterochromatin is defined as the blocks of the genome in which structure of the chromatin is highly condensed throughout the cell cycle and lack gene expression [17]. They include telomeres, centromeres and the inactive 'X' chromosome in female mammalian cells. In 1920's Emil Heitz first identified heterochromatin in insects and plants based on its distinct behavior during the cell cycle [18]. The overall silencing mechanisms throughout phylogenetic members are very similar. Generally, there are two types of gene silencing mechanism which includes Sir2-containing silencing complex that is well studied in yeast and the *HP1* and Swi6 containing silencing complexes that are required for silencing in metazoans and fission yeast, respectively [15]. Identification of heterochromatin protein 1 (*HP1*) and Polycomb (Pc) in *Drosophila* gave the first molecular connection between gene silencing and heterochromatin [19]. The proteins encoded by *SIR* genes (silent information regulator) are responsible for silencing of repeated DNA sequence in

three genomic regions: two silent mating type loci, the telomere and rRNA encoding DNA (the rDNA) [20].

1.3.1 *SIR* mediated gene silencing mechanism:

Silencing at the *HM* loci and telomeres are mediated by a multiprotein nucleosome binding complex called the *SIR* complex (silent information regulator). The complex includes *SIR1, SIR2, SIR3* and *SIR4* genes which were identified while screening for mutants that derepressed *HML* and *HMR* loci [21-22]. The Sir proteins do not bind to the silencers directly, but are recruited via protein-protein interactions. Many results have suggested that the Sir proteins block the transcription by remodeling the chromatin into a repressed conformation [16]. *SIR2, SIR3* and *SIR4* genes are required for silencing at telomeres and mating type loci whereas for silencing at rDNA loci only *SIR2* is necessary, but not *SIR3* and *SIR4*. Sir2 is the most remarkable component of the *SIR* complex. For silencing of the chromatin the lysine residues at the amino terminal tail of Histone 3 and 4 are deacetylated and acetylated when it isactive. The deacetylated histone forms a more compact closed nucleosome structure [23] that is restrictive to transcription. Sir2 is a NAD^+ dependent Histone deacetylase and the only Sir protein that is conserved among the multicellular eukaryotes [24]. Sir2/Sir4 recruits Sir3 and binds to hypoacetylated N-terminal tails of histone H3 and H4 thus leading to spread of the deacetylated domain. Sir2 protein couples the deacetylation reaction to NAD^+ hydrolysis which is cleaved into ADP-ribose and nicotinamide per acetyl group removed.

$$\text{NAD + acetyl-K-histone} \xrightarrow{\text{Sir2}} \text{NAM + O-acetyl-ADP-ribose + K-histone}$$

Eukaryotic silencing is best studied in *S. cerevisiae* and occurs in a stepwise fashion. The assembly of Sir proteins in the chromatin silencing involves two steps, nucleation and spreading. The Rap1 protein along with yK70 and yK80 binds to the chromosome ends and recruits Sir2/Sir4 hetero dimer. This Sir2/Sir4 complex forms a firmly associated complex, both *in vitro* and *in vivo* which when bound to chromatin deacetylates the histones and further recruits Sir3 protein. This allows the recruitment of new Sir2/Sir4 complex and thus the cycle continues leading to the spreading of the *SIR* complex and silencing of the chromatin. Sir1 is also essential for silencing [25]and is a silencer associated protein that facilitates the assembly of other Sir proteins at the silencer (Figure 3). Unlike Sir2, Sir4 does not have any enzymatic activity and plays a structural role in chromatin silencing facilitating the association of Sir2 and Sir3 with

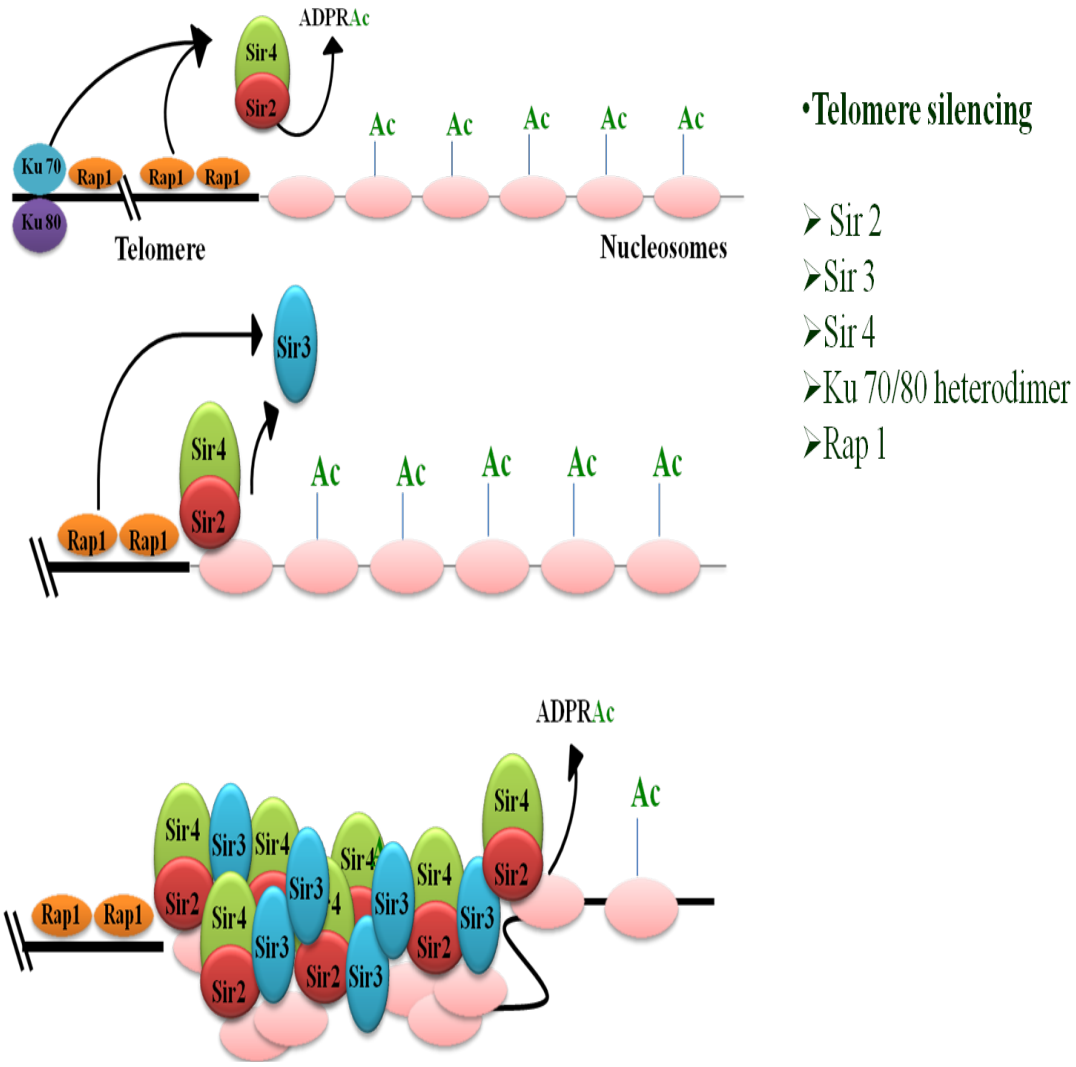


Figure 3: Schematic diagram *SIR* mediated gene silencing mechanism.

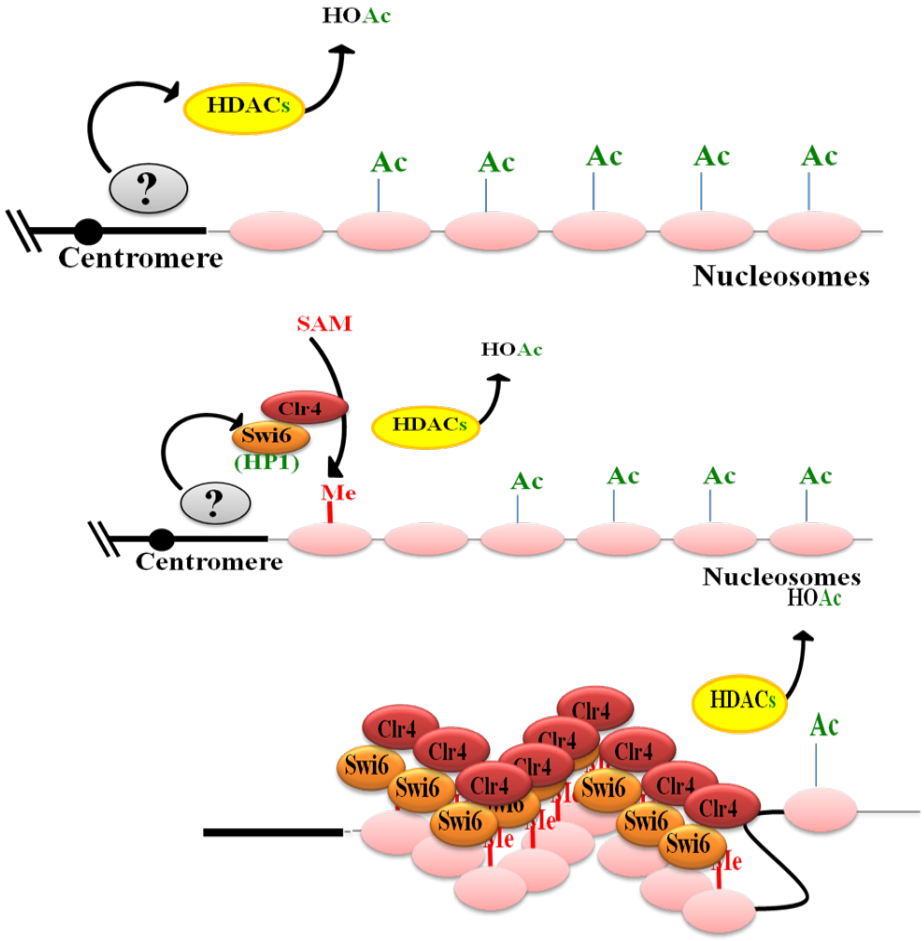


Figure 4: Schematic diagram of *HP1* and Swi6 mediated gene silencing mechanism.

the silencer or the telomeres. Sir3 similar to Sir4 does not have any enzymatic activity and plays a structural role in the formation of silenced chromatin. Sir3 is recruited at the silencers independent of Sir2/Sir4 complex as it has the ability to bind to both nucleosomes and DNA [26]. The first 214 amino acids of Sir3 share 50% identity with Orc1 which include the bromo adjacent homology (BAH) domain which is known to have histone binding activity [27]. *SIR3* has come from gene duplication of *ORC1*. Sir3 consists of a C- terminal AAA ATPase domain which is also similar to Orc1 and is involved in coupling ATP hydrolysis to conformational changes that drives the assembly and disassembly of protein complexes [28]. Both Sir3 and Sir4 are phosphorylated but the mechanism by which phosphorylation regulates their action is not clear. The most remarkable property of Sir3 is the ability to condense the chromatin even in the absences of Sir2/Sir4 complex [29]. Sir3 can bind to nucleosomes independent of DNA.

1.3.2 *HP1* and Swi6 mediated gene silencing mechanism:

Heterochromatin protein 1 was first identified in *Drosophila melanogaster* as a structural component of heterochromatin with a dose dependent effect on gene silencing. *HP1* is evolutionarily highly conserved ranging from *S.pombe* to humans [18]. *HP1* and Polycomb (Pc) share a cytologically identical protein motif called the chromo domain [30]. *HP1* and its homologues also share a related domain called the chromo shadow domain. The chromo domain (CD) and the chromo shadow domain (CSD) are separated by a less conserved hinge region of various lengths. Similar to *Drosophila,* yeast homologue of *HP1*, Swi6 plays a central role in chromatin silencing and is required for telomeric, mating type and centromeric silencing [31]. *HP1* chromo domain specifically recognizes and binds to di- or tri- methylated lysine 9 residue of histone 3 generating a distinct heterochromatin mark [32]. The chromo shadow domain has been known to be implicated in various protein-protein interaction and dimer formation. The hinge region is highly flexible and is subjected to post translational modification specially phosphorylation which influences the localization of *HP1* [33]. Along with playing an important role in telomere silencing, *HP1* is also involved in various processes such as transcriptional elongation, DNA repair, centromeric sister chromatide cohesion and telomere maintenance. *HP1a* one of the five paralogs in *Drosophila* promotes gene silencing by condensing the chromatin fiber. As the chromo shadow domain is involved in dimer formation and the chromo domain binds to the H3K9me3, *HP1a* could cross link the nucleosomes into a uniform array. In *S.pombe*, i that Swi6 efficiently binds to H3K9me3 through CD-

CD dimer formation leaving the two CSD domains to dimerize with two additional Swi6 molecules [34]. HP1β one of the mammalian homologs forms a stable complex with another conserved protein called SUV39H1. Similarly fission yeast and *Drosophila* also posses homologs of this protein named Clr4 and Su(var)3-9 respectively, which are essential for heterochromatic gene silencing. Recent studies have revealed that Clr4 and Su(var)3-9 possess intrinsic methyltransferase activity which is specific to lysine 9 of the histone 3 [35]. This methylation creates a binding site for *HP1* and Swi6 which binds to H3K9 through its conserved chromo domain. Deacetylation of histone 3 tail at lysine 9 by histone deacetylases (Rpd3 and Hda1) is proposed to precede the methylation step. Self association of Swi6/*HP1* to the nucleosomes and subsequent rounds of modifications and binding leads to the spreading of complex along the chromatin for several kilo bases resulting in heterochromatic silencing (Figure 4). These studies strongly suggest that the mechanism of heterochromatic gene silencing involved in budding yeast, fission yeast, *Drosophila* and mammalian cells are very similar to *SIR* mediated gene silencing which involves histone modifications.

1.3.3 Role of Orc1 in gene silencing:

Origin of replication complex (ORC) was first discovered in budding yeast consisting of six subunit proteins that promote DNA replication. Orc1 is the largest subunit of origin of replication complex and is found throughout eukaryotes and has orthologs in prokaryotes. Orc1 binds to origins of DNA replication throughout the cell cycle. A link between Orc1 and heterochromatin has been observed in a wide variety of species. In addition to the role of Orc1 in DNA replication, it also functions in transcriptional silencing. Sir3 has come from gene duplication of Orc1 [36] and sequence similarities have been observed between Orc1 and Sir3 with the N-terminal of Orc1 being highly related to Sir3. Orc1 acts like Sir3 by helping in spreading of silencing complex by binding to the nuclesomes. It has also been assumed that it acts as a landing pad for the silencing complex as in *S. cerevisiae.* *Sc*Orc1 stabilizes the *SIR* complex by interacting with ScSir1. Similar to Sir3, Orc1 has three conserved domain, the N-terminal bromo adjacent homology domain that binds to nucleosomes, AAA+ domain provides the energy for assembly and disassembly of macromolecules by ATP hydrolysis and the less conserved hinge region. *Kluyveromyces lactis* has orthologues of Sir2 and Sir4 but lacks Sir3. *Kl*Orc1 using it's BAH domain acts along with Sir2 and Sir4 similar to that of Sir3 to generate silenced chromatin structures at the telomeres and *HML.a.* In *Candida albicans,* Sir2 and Orc1 are the only conserved Sir proteins. In *P. falciparum,* Orc1 cooperates with Sir2 in telomeric silencing

and has been shown to possess Sir3 like silencing activity [37-38]. It has been established that the N-terminal of *Pf*Orc1 is targeted to the nuclear periphery *in vivo* and binds to the telomeric DNA *in vitro*. In *Pf*Sir2 deficient parasites the binding and propagation of *Pf*Orc1 to the telomere and sub-telomeres was extremely compromised leading to de-repression of some of the *var* genes, thus it features in contrary to yeast *Saccharomyceses cerevisiae* where Orc1 acts as a landing pad for Sir proteins. The role of amino terminal of Orc1 in chromatin silencing seems to be conserved among the eukaryotes since it is found to be interacting with heterochromatin protein 1 in *Xenopus* and *Drosophila,* which is similar to that of the interaction with Sir1.

1.4 SILENCING MECHANISM IN *P. FALCIPARUM*

Silencing of the genes located near the centromeres is called variegation effect (VE) and silencing of genes close to the telomeres is called telomeric position effect (TPE). TPE is caused by the spread of the heterochromatin across the telomeres in a Sir protein dependent manner. Telomeric position effect efficiently controls the silencing of the subtelomeric *var* genes which are involved in antigenic variation in the human malaria parasite *P. falciparum*. *Var* gene that encodes *Pf*Emp1 proteins which are expressed on the surface of the infected RBCs are the only genes known to be regulated by chromatin modeling in *P. falciparum* to date. There are about 60 copies of these *var* among which only one *var* gene is expressed at a particular time. The molecular mechanism by which the parasite manages to express only one *var* gene at a given time, transcriptionally silencing the remaining *var* gene and switching among these *var* genes remained a mystery. The first evidence of transcriptional silencing in *P. falciparum* was observed by studying the comparison of the transcription of HRP1 gene, which was 50 times down regulated upon re-localization from the telomeres to internal locus [39]. *Pf*Sir2 protiens is the only *SIR* protein that has been characterized in *P. falciparum*. There are two paralogues of *Pf*Sir2 which are *Pf*Sir2A and *Pf*Sir2B

1.4.1 *Pf*Sir2A

P. falciparum 'silent information regulator 2 a' is a member of class III histone deacetylase that has been known to be important for epigenetic regulation of virulent genes [40]. Disruption of *Pf*Sir2A leads to abolishment of mutually exclusive *var* genes expression and simultaneous upregulation of many of the *var* genes by chromatin modifications [41]. *Pf*Sir2A binds to the hypoacetylated histones forming a heterochromatic structure at the telomeric and the sub-telomeric regions. *Pf*Sir2A and acetylation of

histone in the chromosome ends are observed to be mutually exclusive by experiments such as FISH immunofluorescence (FISH-IF) and chromatin immunoprecipitation (ChIP) suggesting *Pf*Sir2A is the enzyme responsible for the relatively low acetylation of the histones at the chromosome ends. Direct role of *Pf*Sir2A in *var* gene regulation was observed as activation of *var* genes associated with telomeres lead to the removal of *Pf*Sir2A and acetylation of the promoter regions. Additionally *Pf*Sir2A can ADP ribosylate both itself and histone. Like all other members of eukaryotic Sir2 family, *Pf*Sir2A is a NAD+ dependent HDAC with an ability to act on histones. The protein was found to be co-fractionated with histones in the parasite nucleus. Moreover, *Pf*Sir2A could partially complement *Schizosaccharomyces pombe* species that lacked *sir2*. *Pf*Sir2A was found to bind to the promoter of the repressed *var* gene, but not to the active *var* gene while the acetylation status was complementary, with the silenced *var* gene being hypoacetylated and active one being hyperacetylated [42]. This was the first report which showed that the parasite uses post translational modification to regulate *var* gene expression. Thus *Pf*Sir2A possess all the required characteristics for the epigenetic control of *var* gene silencing. Apart from its role in telomeric silencing, *Pf*Sir2A is also involved in fine tuning the ribosomal RNA gene transcription [43]. Significant increase in all types of rRNA transcript was observed in parasite line mutant for *pfsri2A*. Also, the intra erythrocytes growth rate was increased on the disruption of *pfsir2A* [43]. *Pf*Sir2A plays an important role in the silencing of *var* genes controlled by UpsA, UpsC and UpsE [44].

1.4.2 *Pf*Sir2B

Blast search of PlasmoDB identified a protein that was similar to *Pf*Sir2A and other sirtuins, henceforth referred as *Pf*Sir2B. This homologue of *SIR2* is a large gene and is present on chromosome 14. The comparison of catalytic domains of *Pf*Sir2B with *Pf*Sir2A and other sirtuins revealed that *Pf*Sir2B has 26%/51% identity/similarity with *Pf*Sir2A, 29%/43% identity/similarity with yeast Sir2 (group I sirtuin) and 38%/53% identity/similarity with group IV sirtuins respectively [44-45]. *Pf*Sir2b shows greatest similarity with type IV sirtuins and is a NAD+ dependent HDAC similar to *Pf*Sir2A [44]. It has been demonstrated that both the Sir2 paralogues contributes to the telomeric silencing and antigenic variation but mechanistically and biochemically how this takes place is not clear. *Pf*Sir2B plays an important role in the silencing of non overlapping group of *var* genes controlled by UpsB promoter. This was determined by using *Pf*Sir2B disrupted parasite lines [44]. Experiments have shown that lose of *Pf*Sir2B function does not disrupt the ability of the parasite to activate specific silenced *var* gene. Both *Pf*Sir2A and *Pf*Sir2B

can affect the transcription of the same *var* gene, suggesting that they work cooperatively rather than exclusively [44]. *Pf*Sir2B, like *Pf*Sir2A regulate the *var* gene repression by directly acting on the conserved promoter and mediate the spreading of the silenced chromatin which is in contrast to yeast gene silencing. Both *Pf*Sir2A and *Pf*Sir2B are required for mutually exclusive expression of *var* genes since Δ*Pf*Sir2A and Δ*Pf*Sir2B parasites expresses multiple *var* genes and *Pf*Emp1 protein. Micro array data revealed that a proportion of *rif* gene family is activated in Δ*Pf*Sir2B parasites and these usually have close proximity with activated *var* genes. There is an increase in telomere length of about double the size when compared to the wild type in the parasites that lacked *Pf*Sir2A but not *Pf*Sir2B. It is unclear why the parasite requires two paralogues of Sir2 for the silencing *var* gene family [44].

1.4.3 *Pf*Hp1

*Pf*Hp1, an orthologue of heterochromatin protein 1, was the first member of a histone modification specific recognition *Plasmodium* protein to be identified. Amino acid sequence analysis revealed the presences of chromo domain and chromo shadow domain, the two characteristic domains of *HP1*. *Pf*Hp1 was shown to be localized to the perinuclear *foci*. Chromo domain binds to di or tri methylated lysine 9 residues of histone 3 but not to the unmodified or methylated histone 3 lysine 4. *Pf*Hp1 can interact with itself forming a homo dimer thus playing an important role in aggregating the nucleosomes for heteromatin formation. ChIP experiment has revealed that the protein is associated with the heterochromatin of subtelomeric non-coding repeat regions and mutually exclusive expression of *var* gene family. Since an active *var* gene devoid of *PfHP1*in its 5' UTR, data suggest that the protein is linked to epigenetically silenced *var* genes. These studies have shown that the machineries involved in *Plasmodium* silencing are more similar to metazoans than *S. cerevisiae*. ChIP assay analysis demonstrates that *Pf*Hp1 binds to *var* gene present both at the subtelomeric region and also at central to the chromosome. *In silico* analysis have revealed that a number of post translational modifications are possible in the hinge region such as sumylation, ubiquitination and phosphorylation and thus it is possible that *Pf*Hp1 has distinct function [46]. It has been shown that *Pf*Hp1 specifically binds to H3K9me3 but not to other repressive histone methyl marks. ChIP-on-chip revealed a striking occupancy of *Pf*Hp1 restricted to 425 genes, most of which are the members of *Plasmodium* exported virulence family. Data shows that *Pf*Hp1 is not associated with the centromeric region indicating the important differences with other eukaryotes. Furthermore, over expression of *Pf*Hp1 leads to down regulation of about 78 genes, which are majorly

located at the heterochromatic region. Nuclear fractionation results revealed that *Pf*Hp1 is responsible for chromosome end clustering through the interaction of chromo shadow domain and other structural components [47]. Conditional depletion of *Pf*Hp1 prevents mitotic proliferation of blood stage parasite and disrupts the antigenic variation and mutually exclusive expression of virulences gene factor *Pf*Emp1. *Pf*Hp1 is very crucial for sexual differentiation of *P. falciparum* and thus can serve as a transmission block against malaria [48]

1.4.4 *Pf*Orc1

P. falciparum Origin of replication complex 1 *(Pf*Orc1) has been known to be involved in DNA replication and *var* gene regulation. The C-terminal of *Pf*Orc1 is involved in the replication function and the N-terminal is involved in var gene regulation in a Sir2 dependent manner. The N-terminal of *Pf*Orc1 is targeted to the nuclear periphery and consists of leucine heptad repeats. In *Plasmodium*, *Pf*Orc1 and *Pf*Sir2 have been shown to co-localize during the developmental stages of the parasites which is not only at the telomeres but also at the nucleolus [37]. *Pf*Orc1 regulates var gene repression in Sir2 dependent manner as in *sir2* deficient back ground the binding and propagation of *Pf*Orc1 to telomeric and subtelomeric regions are severely compromised. *Pf*Orc1 cooperates with Sir2 in telomeric silencing and has been shown to possess Sir3 like silencing activity [37-38]. It has been established that the N-terminal of *Pf*Orc1 is targeted to the nuclear periphery *in vivo* and binds to the telomeric DNA *in vitro* [49]. In *Pf*Sir2 deficient parasites, the binding and propagation of *Pf*Orc1 to the telomere and sub-telomeres was extremely compromised leading to de-repression of some of the *var* genes. Thus it features in contrary to yeast *saccharomyces cerevisiae* where Orc1 acts as a landing pad for Sir proteins. The phosphorylation of N-termial domain by cyclin-dependent kinase (CDK) leads to abolishment in the DNA binding activity and changes in *var* gene silencing [50].

According to all the observed data we proposed a working model where *Pf*Sir2, a histone deacetylase is recruited to the chromatin which deacetylates H3K9 residue following the methylation step and *Pf*Orc1 binding. Methyl transferase methylates H3K9 residue leading to the binding of *PfHP1* to the H3K9 residues. As a consequence, *Pf*Hp1 dimerizes and interacts with *Pf*Orc1 leading to the silencing of virulences factor gene families (Figure 5).

WORKING MODEL

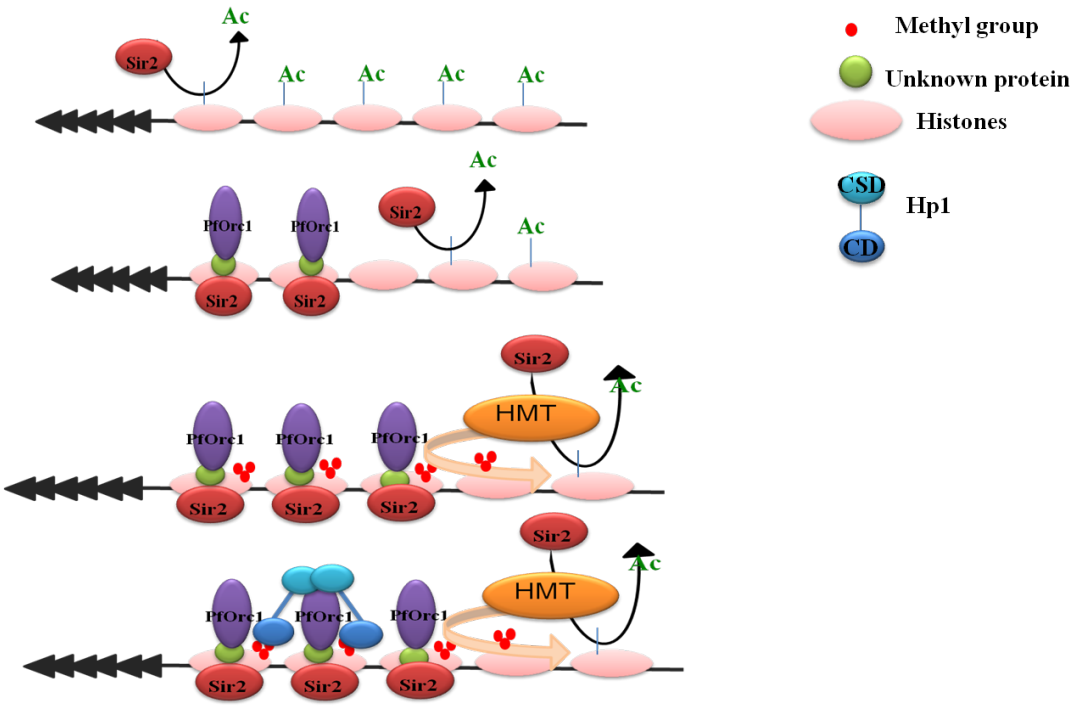


Figure 5: Schematic diagram of working model for gene silencing mechanism in *P. falciparum*

1.5 SIGNIFICANCES OF THE STUDY

P. falciparum uses antigenic variation to evade the host immune system by switching the expression of *Pf*Emp1 protein expressed on the surface of iRBCs and there by establishing chronic malaria infection. *Pf*Emp1 protein is encoded by approximately 60 *var* genes majority of which are located at the subtelomeric regions. Among these *var* genes there is mutulally exclusive expression of only one *var* gene and the rate of *var* gene switching is 2.4% per generation. The mechanism by which this repression and de-repression of the *var* gene occurs is still to be illucidated. In this study we have made an attempt to understand the epigenetic regulation of virulences gene expression in *P. falciparum*. Untill recently, Sir2 paralogues and *Pf*Hp1 were the only proteins known to be involved in *var* gene silencing in *P. falciparum*. Here, we for the first time, have shown using a yeast sarrogate system that *Pf*Orc1 has a Sir3 like silencing function which is Sir2 mediated. Together with earlier observation that *Pf*Orc1 and *Pf*Sir2 co-localize at the silent subtelomeric regions our data strongly suggests that *Pf*Orc1 plays a crucial role in silencing of the virulence gene factors. By constructing various chimera between *Pf*Orc1 and *Sc*Sir3 we demonstrated that the N-terminal domain of *Pf*Orc1 habours the silencing activity similar to the N-terminal domain of *Sc*Sir3. We have also shown by yeast two hybrid analyses that similar to *Xenopus* and *Drosophila Pf*Hp1also interacts with *Pf*Orc1.

We also wanted to investigate whether there is any key link betweeen the expression of the variable antigens and the physiological cues like heat shock or the febrile temperature. Recently it has been established that over expression of yHsp90, either by giving heat shock or by introducing yHsp90 overexpression plasmid yields reduced level of Sir2, with a consequential loss of telomere silencing. These result coinsides with the observation that during the rupture of the schizoints or the formation of ring stage *in vivo*, the parasites are exposed high febrile temperature and there is derepression of majority of the *var* gene. Thus we thought that understanding such mechanism will lead to a better insight of regulation of the virulence gene factors. Our findings clearly demonstrates that upon heat shock of two hours to the parasite there is down regulation of transcript level both the paralogues of *Pf*Sir2 which is only specific to the ring stage and this is *Pf*Hsp90 mediated. We also observed that a transcient heat shock leads to transgenerational down regulation of the transcript level both the paralogues of *Pf*Sir2. Thus our study provides very interesting link between fibril illness and the antigenic variation of the variable antigen in *P. falciparum*.

1.6 OBJECTIVES OF THE STUDY

P. falciparum is very clever organism as it diminishes the host immune clearences through mutually exclusive expression of virulence factors. The parasite uses antigenic variation to switch among these *var* genes and changes the structure of *Pf*Emp1 protein expressed on the surface of the infected RBC. Out of 60 *var* gene that encode the *Pf*Emp1 protein only one *var* is expressed and remaining are transcriptionally silenced. Our main objective was to understand the mechanism of the *var* gene silencing and how are these events regulated in response to physiological cues such as heat shock. *Pf*Sir2 paralogues are the only Sir proteins shown to be involved in telomeric silencing in *P. falciparum.* And it has been established that *SIR3* has come from gene duplication of *ORC1*. Since in *Plasmodium SIR3* is missing and reports have shown that *Pf*Sir2 and *Pf*Orc1 we wanted to investigate if *Pf*Orc1 has Sir3 like silencing function at the subtelomeric region.

In yeast over expression of Hsp90 yields reduced level of Sir2, with a consequential loss of telomere silencing. In *P.falciparum* during the ring stage when the Hsp90 levels are high it has been observed that there is de-repression of *var* gene transcript. Thus we wanted to investigate whether there is any correlation between *Pf*Hsp90 and *Pf*Sir2 similar to yeast. Therefore we hypothesized may be similar kind of mechanism occurs in *P. falciparum* where in over expression of Hsp90 yields reduced level of Sir2 transcript and hence de-repression of *var* genes expression. In this work we have also checked if this down regulation of Sir2 transcript due to heat shock is trans generational.

1.7 SPECIFIC AIMS

- Identification of the molecular players/events that regulate expression of variable antigens

  In this regard we wanted to investigate which protein is doing the Sir3 like function in *P. falciparum* and whether *Pf*Orc1 and *Pf*Hp1 interact with each other

- To study how are these events regulated in response to physiologic cues such as heat shock

  This can be achieved by investigating whether over expression of *Pf*Hsp90 by heat shock modulates telomere position effect by observing the transcript levels of the Sir2 paralogues.

CHAPTER 2

MATERIALS AND METHODS

2.1 MOLECULAR CELL BIOLOGY METHODS

2.1.1 Bacterial competent cell preparation

Overnight culture was inoculated with single bacterial colony containing appropriate antibiotics in 5 ml of LB at 200 rpm, 37°C. The next day, the secondary culture was started by inoculating 500 µl of overnight culture into 25 ml of LB medium with appropriate antibiotics. The culture was than incubated at 37°C at 200 rpm until the OD_{600} reached 0.5. The culture was then centrifuged at 8000 rpm for 8 minutes at 4°C. The supernatant was completely removed by inverting the tube on the paper towel. The pellet was then gently re-suspended in 12.5 ml of ice-cold 0.1 M $CaCl_2$ and centrifuged at 8000 rpm for 8 minutes at 4°C. The pellet was again re-suspended in 12.5 ml ice cold 0.1 M $CaCl_2$ and incubated on ice for 4-8 hours. The cells were then centrifuged at 8000 rpm for 8 min at 4° C. The pellet was gently re-suspended in 1070 µl of ice cold 0.1 M $CaCl_2$ and then 170 µl of 100% glycerol was added. The competent cells were then aliquoted into each of the pre-chilled micro centrifuge tubes and frozen immediately in liquid nitrogen. The tubes were then preserved by storing at -80°C.

2.1.2 Bacterial transformation

About 25-50 ng of DNA to be transformed was layered on the bacterial competent cells carefully and gently mixed with the tip. The competent cells were incubated at 4ºC for 30 minutes. After the incubation heat shock of 42ºC was given to the cells for the required period of time (about 30-90 seconds depending upon the type of the competent cells). Then the cells were immediately chilled on ice for 2 minutes. 900 µl of LB was added to the cells and incubated at 37ºC for 1 hour at 200 rpm. The samples were then centrifuged at 10,000 rpm for 1 minute. Most of the supernatant was discarded and the cells were re-suspended in the remaining supernatant and plated on LB agar plates containing appropriate antibiotic. The plates were then incubated at 37ºC for 12-16 hours.

2.1.3 Plasmid DNA isolation by alkaline lysis method

A single bacterial colony harboring the required plasmid DNA was inoculated in 5 ml of LB medium with appropriate antibiotics for overnight at 37ºC, 200 rpm. The next day culture was centrifuged at 4,500 rpm for 15 minutes at room temperature. The supernatant was discarded; the pellet was re-suspended in 200 µl of solution I (Tris 25 mM pH 8, EDTA 10 mM pH8) and mixed thoroughly by pipetting. The suspension was transferred to a 1.5 ml microfuge tube, 200 µl of solution II (0.2 M NaOH, 1% SDS) was added and the tube was mixed by inverting 4 to 5 times. The suspension was

incubated at room temperature for 5 minutes. The suspension turns clear and viscous upon incubation. To this 150 µl of solution III (3 M sodium acetate pH 5.2) was added and the tube was kept on ice for 5 minutes with intermittent mixing. A white precipitate was formed. The sample was then centrifuged at 13,000 rpm for 20 minutes at room temperature. The supernatant was gently transferred to a fresh microfuge tube, 2.2 volumes of 100% alcohol was added and kept for DNA precipitation at -20ºC for 45 minutes. The sample was then spun at 12,000 rpm for 30 minute at 4ºC. After which 70% wash was done by discarding the supernatant, adding 500 µl of 70% alcohol and spinning at 12,000 rpm for 5 minutes at 4ºC. The supernatant was discarded and the pellet was air dried for 5 min. Then the pellet was re-suspended in 50 µl of 1X TE (10 mM Tris pH 8.0, EDTA 1 mM pH 8.0). RNase treatment was done by adding 5 µl of RNase (10 mg/ml) to the sample and incubating in 37°C water bath for 30 minutes. The volume of the sample was made up to 400 µl by adding 1 X TE, and 400 µl of PCIA (Phenol: Chloroform: Isoamyl alcohol: 25: 24:1) was added to it. The sample was then vortexed for 3 minutes and spun at 12,000 rpm for 15 minutes. The top aqueous layered was transferred to a fresh microfuge tube, 1/10 volume of solution III and 2.2 volumes of 100% alcohol was added. The sample was precipitated at -80ºC for 2 hours. Then tube was centrifuged at 12,000 rpm for 30 minutes at 4ºC and washed with 70 % alcohol. The pellet was air dried for 5 minutes and re-suspended in 30 µl of 1 X TE.

2.1.4 RNA isolation from Yeast

The required yeast colony was inoculated in 5 ml of yeast growing medium and kept for overnight growth in 30ºC incubator at 200 rpm. The next day 10 ml of yeast growing medium was inoculated using the overnight culture in such a way that after two generation the OD_{600} reaches 1 which is the mid log phase. Then the cells were collected by centrifuging at 3000 rpm for 10 minutes. The cells were re-suspended in 500 µl of DEPC treated water and transferred to a fresh microfuge tube. The cells were then given a flash spin and the supernatant was discarded. The pellet was re-suspended in 400 µl of Tris-EDTA-SDS buffer (10 mM Tris HCl, 10 mM EDTA, 0.5% SDS), 400 µl of acid phenol (pre-equilibrated with water) was added and vortexed vigorously for 10 seconds. The sample was incubated at 65ºC for one hour with intermittent vortexing for every 15 minutes. The mixture was immediately chilled on ice for 10 minutes and then centrifuged at 14,000 rpm at 4ºC for 10 minutes. The top aqueous layer was collected to a fresh microfuge tube, 400 µl of chloroform was added, vortexed vigorously for 10 seconds and then centrifuged at 14,000 rpm, 4ºC, 10 minutes. The

aqueous layer was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.2 volumes of chilled 100% alcohol. The sample was kept at -80ºC for an hour or more. The RNA was pelleted by centrifuging at 14,000 rpm at 4ºC for 10 minutes. The pellet was then washed with 70% alcohol and was re-suspended in 50 µl of DEPC treated water. The concentration of the RNA was determined using spectrophotometer (JASCO spectrophotometer EMC-709) at 260 nm. About 15 µg of RNA was subjected to DNase treatment at room temperature for 30 minutes. The reaction was stopped by adding 3 µl of 25 mM EDTA and heat inactivating at 65ºC for 10 minutes. The sample was rapidly chilled on ice and stored at -80ºC.

2.1.5 Semi Quantitative PCR

About 10 µg of RNA was reverse transcribed with oligo dT (Sigma Aldrich) primer using the reverse transcriptase kit (Omniscript kit, Qiagen). RNase inhibitor was included in the reaction mixture. The resulting cDNA was diluted appropriately to normalize and subjected to PCR (27 cycles) to amplify the required gene with the specific primers. The PCR product was ran on 1.4% agarose gel stained with Ethidium Bromide (EtBr).

2.1.6 Real Time PCR

For the Real Time PCR cDNA was diluted appropriately and subjected to Real Time PCR using RT-PCR Kit (Roche/Takara). The instrument used for real time analysis was Applied Biosystems 7500 Fast Real Time PCR system. 10 µl volume reaction mixture was used which included 0.5 µl of each of the forward and reverse primers, 5 µl of SYBR Green, 2 µl of diluted cDNA and 2 µl of autoclaved milli Q water.

2.2 YEAST GENETIC METHODS

2.2.1 Yeast competent cell preparation

A single colony of the desired yeast strain was inoculated into the required yeast growth medium and kept in a 30ºC incubator for overnight at 200 rpm. Next day, OD_{600} of the overnight culture was measured by diluting the culture to 1:10 or 1:20. The volume of the overnight culture to be inoculated in 40 ml of the secondary culture was calculated using the following formula:

Volume of the inoculums = (Final volume of secondary culture × final OD)/ (Initial OD × 2^2 generation)

The cells were grown for about 3 hours at 30ºC, 200 rpm until the final OD reached 0.6-0.8. The

cells were harvested by centrifuging at 3,500 rpm for 5 minutes at 4ºC. The supernatant was discarded without disturbing the pellet; it was washed by re-suspending in 10 ml of autoclaved milli Q water and centrifuging at 3,500 rpm for 5 minutes at 4ºC. The cells were then re-suspended in 300 µl of Lithium Acetate solution (1 X Tris-EDTA, 1X Lithium acetate). 200 µl of the resulting competent cells were used for each transformation.

2.2.2 Yeast transformation

About 10 µg of sample DNA was mixed with 10 µg of carrier DNA (total volume being not more than 20 µl) and 10 µg of only carrier DNA (negative control) were taken in two fresh microfuge tubes. 200 µl of Yeast competent cells were added into each of the transformation tubes. 1.2 ml of freshly made PEG solution (For 5 ml, 4 ml 50% PEG2000, 500 µl 10X TE, 500 µl 10X Lithium acetate) was added into each tube and incubated at 30ºC, 200 rpm for 30 minutes. After the incubation heat shock was given to the cells at 42ºC for 15 minutes and immediately transferred onto ice for 2-3 minutes. The cells were pelleted by spinning for about 10 seconds at high speed and the supernatant was discarded. The pellet was re-suspended in 100 µl of 1X TE and the suspension was spread on synthetic deficient medium plates. The plates were incubated at 30ºC for 72 hours or more.

2.2.3 Genomic DNA isolation from yeast

A single colony of the desired yeast strain was inoculated into 10 ml of yeast growth medium for overnight at 30ºC, 200 rpm. The cells were harvested by centrifuging at 3000 rpm for 5 minutes, suspended in 500 µl of autoclaved milliQ water and transferred to a fresh 2 ml microfuge tube. The cells were given a flash spin and the supernatant was discarded. The pellet was disturbed by a brief vortex and re-suspended in 200 µl of breaking buffer (Triton X-100(2%), SDS (1 %,), NaCl(0.1 M)), 0.3 gm of glass beads and 200 µl PCIA (Phenol: Chloroform: Isoamyl alcohol: 25: 24:1). The suspension was vortexed for 5 minutes. 200 µl of 1 X TE (10 mM Tris-HCl, 1mM EDTA pH8) buffer was added followed by vortexing again and centrifuging at 12,000 rpm for 5 minutes. The top aqueous layer was transferred to a fresh microfuge tube and 1 ml of 100% alcohol was added to it. The sample was kept at -20ºC for precipitation for about 30 minutes and then centrifuged at 12,000 rpm for 5 minutes. The pellet was re-suspended in 100 µl 1X TE and 5 µl of RNase (10 mg/ml) was added. The sample was incubated at 37ºC for 30 minutes for RNase treatment. DNA was precipitated by adding 10 µl of 4 M NH_4OAc and 1 ml 100% alcohol. The suspension was kept at -

20°C for 30 minutes for precipitation. The sample was centrifuged at 12,000 rpm for 10 minutes followed by 70 % alcohol wash. The pellet was re-suspended in 30 µl of 1 X TE.

2.2.4 Gene Knock out

SIR2 gene knock out was done by homologous recombination method. Genomic DNA of the *sir2* null strain SLY12 (*sir2*::*KANMX*) was used as the template and pair of primers OMKB157 and OMKB158 were used for amplifying KANMX cassette with *SIR2* flanking regions. The PCR product was then integrated into Δ*sir3* yeast strain which was a kind gift from Arthur Lustig and selected on G418 containing plate. *SIR2* gene was knocked out by homologous recombination and Δ*sir3*Δ*sir2* strain was generated. In a similar way *SIR2* gene was knocked out from strains MVS2 (Δ*sir3* null bearing *Pf*ORC1 from a plasmid), MVS3 (Δ*sir3* null bearing chimera A from a plasmid) and MVS4 (Δ*sir3* null bearing chimera B from a plasmid) and the resultant knocked out strains were referred as MVS7 (Δ*sir3* null bearing *Pf*ORC1 from a plasmid in Δ*sir2* back ground), MVS8 (Δ*sir3* null bearing chimera A from a plasmid in Δ*sir2* background) and MVS9 (Δ*sir3* null bearing chimera B from a plasmid in Δ*sir2* background) respectively. The entire knock out phenotype was confirmed by PCR analysis. To confirm the knock out of the *sir2* gene OMKB 157 forward primer and KanB1 reverse primer was used to amplify the PCR product from the middle of the cassette.

2.2.5 Yeast two hybrid analysis

pJ694a yeast two hybrid strain was used to study the protein–protein interaction. Our gene of interest was cloned into bait vector pGBDUC1 which was in fusion with the binding domain and the other gene of interest was cloned into the prey vector pGADC1 where in it was fused with the activation domain of GAL4. Both the cloned plasmids were transformed into pJ694a yeast strain and selected on the double drop out plates Sc-Leu-Ura. The interaction of the proteins was confirmed by observing growth on triple drop out plates i.e. Sc-Leu-Ura-His and Sc-Leu-Ura-Ade.

The auto activation of the reporter gene was screened by checking the interaction between either of the empty vectors and either of the plasmids harboring the gene of interest. The Strong protein-protein interaction was identified by observing growth on Sc-Ura-Leu-Ade plates and also on Sc-Ura-Leu-His, whereas weak protein-protein interaction was identified by observing the growth only in Sc-Ura-Leu-His plates.

2.2.6 Spotting assay

Primary inoculums were given for the required yeast strains into 5 ml of yeast growth medium for

overnight at 30°C, 200 rpm. Next day, OD_{600} of the overnight culture was measured by diluting the culture to 1:10 or 1:20. The volume of the overnight culture to be inoculated in 5 ml of the secondary culture was calculated using the following formula:

Volume of the inoculums = (Final volume of secondary culture × final OD)/ (Initial OD × 2^2 generation)

The cells were grown for about 3 hours at 30ºC, 200 rpm until the final OD reached 0.5 (grown for two generation). OD_{600} of different strains were determined and normalized to the least OD. Each of the strains was serially diluted to 10^{-4}. About 5 µl was spotted directly on the required plates from lower to higher dilution. The plates were incubated at 30°C for about 3 days and the growth on the plates was recorded.

2.2.7 5-FOA assay for telomere position effect

5-Fluoroorotic acid (5-FOA) assay was used to measure the silencing of *URA3* marked chromosome VIIL. Different strains were grown in 5 ml of the Sc-trp (tryptophan drop out) broth for overnight at 30°C, 200 rpm. Next day, OD_{600} of the overnight culture was measured by diluting the culture to 1:10. The volume of the overnight culture to be inoculated in 5 ml of the secondary culture was calculated using the following formula:

Volume of the inoculums = (Final volume of secondary culture × 0.5)/ (Initial OD × 2^2 generation)

The cells were grown for about 3 hours at 30ºC, 200 rpm until the final OD_{600} reached 0.5 (grown for two generation). OD_{600} of different strains were determined and normalized to the least OD_{600}. Each of the strains was serially diluted to 10^{-4}. About 5 µl was spotted directly on the either Sc-trp plate (control) or Sc-trp plate containing 5-FOA followed by incubation at 30°C for 3 days or more.

2.3 BIOCHEMICAL METHODS

2.3.1 Western blotting

The proteins were isolated from Ring stage specific parasites (37°C) and heat shock treated Ring stage specific parasites (41°C heat shock for 2 hour). Appropriate amount protein sample was loaded onto 12% SDS gel polyacrylamide gel. The gel was transferred onto polyvinylidene difluoride (PVDF) membrane, which was pre treated with methanol for 20 seconds, double distilled water for 2 min and 1xtransfer buffer (5.86gm glycine, 11.64gm Tris base, 0.75gm SDS, 400ml methanol for 1L 2x transfer buffer) for 5 minutes. After the transfer the membrane was blocked with blocking

buffer (5 gm non fat dry milk dissolved in 1X TBS-T) for about 2 hours and incubated with the primary antibody on rocker for overnight at 4°C. The next day, the membrane was washed with large volumes of TBS-T (0.2M Tris base, 9% sodium chloride, pH 7.6, 0.1% Tween 20) for 3 times (each wash for 1 minute). The membrane was washed again with TBS-T for 15 minutes trice with intermittent double distilled water wash of one minute. The membrane was probed with secondary antibody conjugated with Horseradish peroxide for 2 hours and washed with TBS-T as described previously. Western blot was developed using chemiluminescent detecting system (Pierce). ImagJ software was used for estimating the band intensities. The protein levels of *Pf*Hsp90 in Ring stage specific parasites (37°C) and heat shock treated Ring stage specific parasites were detected by anti-Hsp90 antibody (Sigma Aldrich) which was used at 1:2,000 dilution and HRP- conjugated anti-mouse secondary antibody (Promega) at 1:10,000 dilution. The protein levels were normalized using human actin which was a kind gift from Dr. Ravi Gutti at 1:5,000 dilution raised in mouse and the secondary antibody anti-mouse antibody (Promega) at 1:10,000 dilutions.

2.4 METHODS IN *P. FALCIPARUM* CULTURE

2.4.1 Washing of RBCs

10 ml of blood was collected from a volunteer in a 15 ml centrifuge tube containing 1.4 ml CPDA, mixed thoroughly and kept in ice. The collected blood was centrifuged at 1,500 rpm for 15 minutes at 4°C. The upper pale yellow part which is the serum and the white buffy layer at the junction were aspirated carefully using Pasteur pipette attached to a vacuum pump. The red pellet contains the RBCs. An equal volume of RPMI 1640 incomplete medium was added to the RBC pellet and mixed gently. The blood sample was centrifuged at 2,500 rpm for 10 minutes at 4°C. The supernatant was aspirated out and the wash with incomplete medium was repeated two more times. Finally the washed RBC pellet was mixed gently with equal volume of incomplete medium to make 50% Hematocrit (Hct) and stored at 4°C. The tube was labeled with the blood group and date of blood wash.

2.4.2 Thawing of parasites from liquid Nitrogen

A vial of frozen parasite was taken out from liquid nitrogen tank and thawed in 37°C pre warmed milli Q water for 2-3 minutes. Care was taken so that the cryovial was not dipped completely in the water to avoid contamination. The vial was bathed with 70% alcohol, wiped properly and the

contents were transferred into 50 ml tube. For 1ml of thawed parasite culture 0.2 ml of solution –I (12% NaCl) was added drop wise (approximately 1 drop per second) and stirred gently to mix the contents. The sample was allowed to stand for 3-5 minutes. 10 ml of solution II (1.6% NaCl) per one ml of blood was added drop wise (1-2 drops/second) followed by gentle mixing by swirling the tube. The sample was centrifuged at 1,000 rpm for 10 minutes and the supernatant was discarded using the Pasteur pipette. The pellet was loosened by stirring the tube and 10 ml of solution III (0.9% NaCl, 0.2% glucose) per one ml of blood was added drop wise (approximately 1-2 drops per second). The tube was swirled gently to mix the contents. The sample was centrifuged at 1,000 rpm for 10 minutes and the supernatant was discarded. The pellet was re-suspended in 2ml of complete medium with 0.2 ml of washed blood (50% Hct) and transferred to culture plate well. The culture plate was kept in a candle jar in the 37°C incubator.

2.4.3 Maintenance of the parasite *in vitro* culture

3D7 *P. falciparum* strain in vitro culture was done by following candle jar method. Parasite culture was maintained in RPMI 1640 complete medium along with 1% Albumax II. 5% hematocrit RBC was used for maintenance and subculture. Every subculture was made when culture reached 3% or more than 3% parasitemia. The medium of the culture was changed with in every 24 hours. The RBCs settle to the bottom of the plate forming a thin layer. The plate was removed from the incubator and placed in the culture hood to maintain aseptic conditions for changing the medium. Care was taken so that the cellular layer was not disturbed. The old medium was carefully aspirated out using the Pasteur pipette and the required amount of pre-warmed complete medium was added. The culture plate was gently rotated horizontally to mix the culture. The plate was then transferred to a candle jar kept in 37°C incubator and a small candle was lit. The lid of the candle jar was closed with a small opening and was closed completely once the candle was extinguished.

For subculture, after the medium was changed 0.5 ml of the culture was transferred to the next well, 0.5 ml of washed blood and 4 ml of complete medium was added. The culture plate was gently mixed. Smear was regularly made of the culture to monitor the growth of the parasite.

2.4.4 Synchronization of parasites using 5% sorbitol

To synchronize the parasite culture 10 ml of culture (majorly Ring stage specific) was taken in a 15 ml centrifuge tube and centrifuged at 3,000 rpm at room temperature for 10 minutes. The supernatant was discarded using the Pasteur pipette and the pellet was carefully re-suspended in 1ml

of pre-warmed 5% sorbitol (kept at 37°C for one hour). The sample was incubated in 37°C water bath for 10 minutes with intermittent mixing. After the incubation the volume of the sample was made up to 10 ml by adding pre- warmed RPMI 1640 in-complete medium and centrifuged at 3,000 rpm at room temperature for 10 minutes. The supernatant was aspirated and the pellet was washed with the incomplete medium 2-3 times. Finally the pellet was re-suspended in 10 ml of complete medium and divided into two wells of the culture plate and kept in the candle jar in 37°C incubator.

2.4.5 Genomic DNA isolation from the parasite

About 15ml parasite culture with high percent parasitemia was centrifuged at 3,000 rpm at 4°C for 5 minutes. The pellet was washed with 15 ml of cold 1X PBS. The pellet was re-suspended in 750 µl of cold 1X PBS and transferred to a 2 ml centrifuge tube (500 µl + 250 µl). 255 µl of 0.15% saponin was added to the sample, inverted 8-10 times till it turned little dark and then centrifuged at 6,000 rpm at 4°C for 6 minutes. The supernatant was removed carefully with the pipette and discarded. The parasite was re-suspended in 75 µl of autoclaved milli Q water and 25 µl of lysis buffer (40mM Tris-HCl, pH 8.0; 80mMEDTA pH 8.0; 2% SDS and 0.1mg/ml proteinase K – just before the reaction) was added. The tube was tapped many times for proper mixing (1 µl of proteinase K 20 mg/ml was added to 100µl of lysis buffer). The sample was incubated in 37°C water bath for 3 hours and the tube was tapped vigorously for 1 minute for every 30 minutes. After the incubation 300 µl of autoclaved milli Q water and 400 µl of PCIA (Phenol: Chloroform: Isoamyl alcohol) was added and the tube mixed thoroughly by inverting the tube 6-7 times. The sample was centrifuged at 12,000 rpm for 15minutes at room temperature. The top aqueous layer about 500 µl was transferred into a fresh centrifuge tube and 50 µl of RNase was added for RNase treatment by incubating the sample at 37°C for 30 minutes. After the incubation the PCIA treatment was repeated as mentioned above. The top aqueous layer was carefully transferred in a fresh tube and genomic DNA was precipitated by adding 1/3 volume of solution III (3 M sodium acetate pH 5.2) and 2.2 volume of 100% alcohol. The sample was inverted 3-4 times and incubated at -80°C for 2 hours. The sample was centrifuged at 12,000 rpm at 4°C for 30 minutes and the pellet was washed with 500 µl of 70% alcohol. The supernatant was discarded and the pellet was air dried. Finally the pellet was re-suspended in 50 µl of autoclaved milli Q water. About 2 µl was run on 0.6% agarose gel to check the concentration of the genomic DNA.

2.4.6 RNA isolation from parasite culture (TRIZOL method)

10 ml of parasite culture with 10% parasitemia was centrifuged at 1,800 rpm for 4 minutes at room temperature. 5ml of pre-warmed TRIzol (incubated at 37°C for 15 minutes) was added to the pellet and the sample was shaken to dissolve the clumps. The sample was incubated at 37°C for 5 minutes. The sample was either stored at -80°C or continued for further process of RNA isolation. 0.2 TRIzol volumes of chloroform were added and the sample was shaken vigorously. The sample was allowed to stand for 2-3 minutes followed by spinning at 1,000 to 4,000 g at 4°C for 30 minutes. The aqueous layer was transferred to a new tube, avoiding the interface and 0.5 TRIzol volumes of 2-propanol was added. The tube was inverted many times for proper mixing. The sample was split into 1.5 ml snap-cap microfuge tube and precipitated for overnight at 4°C. The sample was centrifuged at 12,000 rpm for 30 minutes at 4°C followed by 75% ethanol wash. The supernatant was discarded and pellet was air dried for about 5 minutes. The pellet was re-suspended in 30 µl/50 µl autoclaved of DEPC water. The sample was stored at -80°C.

2.4.7 Method for parasite lysate preparation from asexual stages (for western blot analysis)

The packed cell volume of the parasite culture was estimated and 2 packed cell volumes of 0.15% saponin (prepared with RPMI 1640 incomplete medium) was added to the culture pellet. The sample was incubated in a 37°C water bath for 15 minutes with intermittent mixing by pipetting the content up and down. About more than five volumes of incomplete medium RPMI1640 was added to dilute the concentration of saponin and the contents was transferred to a 15 ml tube after mixing. The parasites were collected by centrifuging the sample at 4,000 rpm at 4°C for 10 minutes. The supernatant was discarded along with the top layer using the Pasteur pipette. The pellet was washed 3-4 times by centrifugation as mentioned above and the pellet was stored at -80°C. The parasite pellet was re-suspended in 2X Laemmli buffer for western blot analysis.

2.5 METHODS FOLLOWED IN BIOINFORMATICS TOOLS

2.5.1 Multiple sequence alignment

A BLAST search between the amino acid sequences of PfOrc1-N terminal and ScSir3-N terminal was done. The sequence alignment between the N-terminal & C-terminal of PfOrc1 and ScSir3 was done using MegAlign, DNA Star program.

2.5.2 Phylogenetic tree analysis

The Phylogenetic tree construction taking the N-terminal & C-terminal domains of Orc1 orthologs and ScSir3 was done by using MegAlign, DNA Star program.

2.5.3 Psipred tool to predict the secondary structure of the proteins

The required protein sequence was submitted to the online server tool Psipred and the secondary structure was generated. The primers were designed in the coil-coil region so that it did not disturb the secondary structure of the protein to generate various chimeras and deletion mutants.

2.5.4 Heat map analysis

List of genes and expression values were tabulated in the form of XML document and clustering analysis was done to generate .cdt file. The output file was viewed using JAVA TreeView tool. Up regulated genes were annotated with green color, down regulated genes with red and those with no change were annotated with black color.

TABLE 1: LIST OF YEAST STRAINS USED IN THIS STUDY

Strains	Genotype
ΔGK	*MATa rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP*
MVS30	*MATa rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTA ScSIR3:TRP1*
MVS27	*MATa rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTA PfORC1:TRP1*
MVS28	*MATa rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTA Pforc1NScsir3C:TRP1*
MVS29	*MATa rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTA Scsir3N Pforc1C:TRP1*
MVS5	*MATa rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTA Scsir3N:TRP1*
MVS31	*MATa rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTA Scsir3C:TRP1*
MVS32	*MATa rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTA Pforc1N(Δ70)Scsir3C:TRP1*
MVS33	*MATa rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTAPforc1N(Δ110)Scsir3C:TRP1*
MVS34	*MATa rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTAPforc1N(Δ210)Scsir3C:TRP1*
MVS7	*MATa rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 sir2::KAN^r VIIL::URA3 pRS313/RAP pTA PfORC1:TRP1*
MVS8	*MATa rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 sir2::KAN^r VIIL::URA3 pRS313/RAP pTA Pforc1NScsir3C:TRP1*
MVS9	*MATa rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 sir2::KANr VIIL::URA3 pRS313/RAP pTA Scsir3N Pforc1C:TRP1*
MVS39	*MATa rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3*

	pRS313/RAP pTA Sporc1NScsir3C:TRP1
PJ69-4A	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALl-HIS3 GAL2-ADE2, met2::GAL7-lacZ*
MVS45	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALl-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1, pGADC1*
MVS46	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALl-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/PfHP1, pGADC1*
MVS47	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALl-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1, pGADC1/ PfORC1*
MVS48	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALl-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/PfHP1, pGADC1/PfORC1*
MVS49	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALl-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/PfHP1ΔCD, pGADC1*
MVS50	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALl-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/PfHP1ΔCSD, pGADC1*
MVS55	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALl-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/PfHP1ΔCD, pGADC1/PfORC1*
MVS56	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALl-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/PfHP1ΔCSD, pGADC1/PfORC1*

TABLE 2: LIST OF PLASMIDS USED IN THIS STUDY

Name of the plasmid	Brief description
pTA	Yeast expression vector with GPD promoter and TRP marker, ampicillin resistance in bacteria.
pRS425-HA3	Yeast expression vector with GPD promoter, HA3 tag and LEU marker, ampicillin resistance in bacteria.
pGBDUC1	Yeast expression bait vector for yeast two hybrid assay, ampicillin resistance in bacteria
pGADC1	Yeast expression prey vector for yeast two hybrid assay, ampicillin resistance in bacteria.

TABLE 3: LIST OF PRIMERS USED IN THIS STUDY

Name of the primer	Primer sequence (5'-3')	Purpose
OMKB 199	ATTCATATGACTAGTATGACTCCTAAGAAAA AAATATTTC	Forward primer to amplify of *PfORC1* Nde1 and Spe1 overhang
OMKB 200	ATTGGATCCGTAAAAGTTTAATTTCTTTG	Reverse primer to amplify of *PfORC1* BamH1 overhang
OMKB 201	ATTACTAGTATGGCTAAAACATTGAAAGATT TGG	Forward primer to amplify of *ScSIR3* Spe1 overhang
OMKB 202	ATTGGATCCAATGCAGTCCATATTTTTGAAT TC	Reverse primer to amplify of *ScSIR3* BamH1 overhang
OMKB132	GCTTCTAGATGACAGAATGGTTTCTCTTCC	Reverse primer to amplify *ScSIR3N* Xba1 overhang
OMKB 203	ATTACTAGTATGTCAATGAAAAAAATTAAAA TCGAGC	Forward primer to amplify of *ScSIR3C* Spe1 overhang
OMKB130	GCTTCTAGATATATTTTTTTCATCATACTTAA TTC	Reverse primer to amplify *PfORC1*N Xba1 overhang
OMKB145	CGGTCTAGAAAAGCTCAAACAACAACAAATG	Forward primer to amplify *PfORC1*C Xba1 overhang
OMKB 204	ATTACTAGTATGAATAAAGAAATAGATGAG GTG	Forward primer to amplify Chi.A Δ70 Spe1 overhang
OMKB 205	ATTACTAGTATGAATGATAACAAGTTTACTC C	Forward primer to amplify Chi.A Δ110 Spe1 overhang
OMKB 206	ATTACTAGTATGAAAGAAATACATACCCTTC C	Forward primer to amplify Chi.A Δ210 Spe1 overhang
OMKB 207	TTACAAAAGGATTTTAGCAACGAC	Forward primer to amplify 200 bp of *YFR057w*
OMKB 208	TATGGCTTTGTTACGCTTGCAC	Reverse primer to amplify 200

		bp of *YFR057w*
OMKB 250	AAGCTGCGGTGTTTACAAGTC	Forward primer to amplify 200 bp of *YIR043c*
OMKB 251	ACTACCGGAAACAAGAAACGTG	Reverse primer to amplify 200 bp of *YIR043c*
OSB 14	TTAGAAACACTTGTGGTGAACG	Reverse primer to amplify 307 bp of *ACT1*
OSB 16	TGACCAAACTACTTACAACTCC	Forward primer to amplify 307 bp of *ACT1*
OMKB 256	GCT GGATCCATCCCTAGAAGAAAGTCA TTGAG	Forward primer to amplify of *SpORC1N* BamH1 overhang
OMKB257	GCT GGATCCAGACAAGCCCCTGTCTCT TC	Reverse primer to amplify *SpORC1N* BamH1 overhang
OMKB157	CTTTGTCCTCTTTGGCCG	Forward primer to amplify KANMX cassette with *ScSIR2* flanking regions
OMKB158	GCCTTGCGTCTTTAGCAG	Reverse primer to amplify KANMX cassette with *ScSIR2* flanking regions
OMKB147	GACGGATCCATGACAGGGTCAGATGAA	Forward primer to amplify of *PfHP1* with BamH1 overhang
OMKB148	GACGGATCCTTAAGCTGTACGGTATCTTA	Reverse primer to amplify of *PfHP1* BamH1 overhang
OMKB368	GACGGATCCTTATTCTTCTACCCA TTGTGG	Reverse primer to amplify of *PfHP1(ΔCSD)* BamH1 overhang
OMKB369	GACGGATCCGCTAATGAGACAAATGGTG	Forward primer to amplify of *PfHP1(ΔCD)* with BamH1 overhang
OSB94	CTGTAACACATAATAGATCCGAC	Forward primer to amplify 200 bp of *PfARP*

OSB95	TTAACCATCGTTATCATCATTATTTC	Reverse primer to amplify 200 bp of *PfARP*
OMKB294	GGAAATCCATAGACAAACAATG	Forward primer to amplify 200 bp of reference gene adenylosuccinate lyase (ASL)
OMKB295	TCCTGTGAGAAGTGCTCCAC	Forward primer to amplify 200 bp of reference gene adenylosuccinate lyase (ASL)
OMKB244	GTAATTGGCACATCGTCTACTG	Forward primer to amplify 200 bp of *PfSIR2A*
OMKB245	TATATATGTGCGTGTGAGCTAC	Reverse primer to amplify 200 bp of *PfSIR2A*
OMKB246	AAATACCAAAATATGTAAAGCCAC	Forward primer to amplify 200 bp of *PfSIR2B*
OMKB247	TTACATAATTTAGGATCCAATAAGG	Reverse primer to amplify 200 bp of *PfSIR2B*
OMKB360	GATACCTCTTTATTAACATCTGG	Forward primer to amplify 200 bp of *PfHSP86*
OMKB361	CGGTTGCATCTACAGTTTCTTC	Reverse primer to amplify 200 bp of *PfHSP86*
OMKB362	AAAAATTGACGATTTAGATCCATC	Forward primer to amplify 200 bp of *PfGRP94*
OMKB363	TTCGTCACTTTTATCATTTGATTC	Reverse primer to amplify 200 bp of *PfGRP94*
OMKB364	AGCTAGATATGCATACCATTATG	Forward primer to amplify 200 bp of *PfTRAP1(11)*
OMKB365	GTTGGATGACTGAGCTTCCTTC	Reverse primer to amplify 200 bp of *PfTRAP1(11)*
OMKB366	GGCGAAAATAGCATTGCTGAAG	Forward primer to amplify 200 bp of *PfTRAP1(14)*

OMKB367	TCAGAACCACTGAGCATATTAC	Reverse primer to amplify 200 bp of *PfTRAP1(14)*
OMKB236	TGGAAAGAACATGGACCTGA	Forward primer to amplify 300 bp of *VARA1* (PF3D7_0600200)
OMKB237	TTCCTCGAGGGAAGAATCAC	Reverse primer to amplify 200 bp of *VARA1*
OMKB238	ATGTGTGCGAGAAGGTGAAG	Forward primer to amplify 200 bp of *VARA2* (PF3D7_0632500)
OMKB239	TGCCTTCTAGGTGGCATACA	Reverse primer to amplify 200 bp of *VARA2*
OMKB240	CACAGGTATGGGAAGCAATG	Forward primer to amplify 200 bp of *VARA3* (PF3D7_1300300)
OMKB241	CCATACAGCCGTGACTGTTC	Reverse primer to amplify 200 bp of *VARA3*
OMKB280	ATATGGGAAGGGATGCTCTG	Forward primer to amplify 200 bp of *VARA4* (PF3D7_0400400)
OMKB281	TGAACCATCGAAGGAATTGA	Reverse primer to amplify 200 bp of *VARA4*
OMKB282	TGCTGAAGACCAAATTGAGC	Forward primer to amplify 200 bp of *VARA5* (PF3D7_1150400)
OMKB283	TTGTTGTGGTGGTTGTTGTG	Reverse primer to amplify 200 bp of *VARA5*
OMKB234	CAATCTGCGGCAATAGAGAC	Forward primer to amplify 300 bp of *VARB1*

		(PF3D7_0324900)
OMKB235	CCACTGTTGAGGGGTTTTCT	Reverse primer to amplify 300 bp of *VARB1*
OMKB242	CGGAATTAGTTGCCTTCACA	Forward primer to amplify 300 bp of *VARB2* (PF3D7_1373500)
OMKB243	CATTGGCCACCAAGTGTATC	Reverse primer to amplify 300 bp of *VARB2*
OMKB284	GGCACGAAGTTTTGCAGATA	Forward primer to amplify 300 bp of *VARB3* (PF3D7_1255200)
OMKB285	TTTGTGCGTCTTTCTTCGTC	Reverse primer to amplify 200 bp of *VARB3*
OMKB286	CGGAGGAGGAAAAACAAGAG	Forward primer to amplify 200 bp of *VARB4* (PF3D7_1200100)
OMKB287	TGCCGTATTTGAGACCACAT	Reverse primer to amplify 200 bp of *VARB4*
OMKB288	TGCAAACCACCAGAAGAAAG	Forward primer to amplify 200 bp of *VARB5* (PF3D7_0900100)
OMKB289	GTTCTCCGTGTTGTCCTCCT	Reverse primer to amplify 200 bp of *VARB5*

CHAPTER 3

INDENTIFICATION OF THE MOLECULAR PLAYERS THAT REGULATE EXPRESSION OF VARIABLE ANTIGENS IN *Plasmodium falciparum*

3.1 Introduction:

Malaria parasites employ antigenic variation to escape the immune clearance and increase the duration of infection in human host through switching expression of variant surface proteins encoded by the *var* gene family. Mutually exclusive expression of *var* genes is linked to dynamic remodeling of chromatin [10, 41]. *P. falciparum* erythrocyte membrane protein 1 (*PfEMP1*) is encoded by the members of *var* gene family and is the major determinant of antigenic variation in the parasite. There are sixty *var* genes that encode for *Pf*Emp1 proteins among which only one *var* gene is expressed at a given time and the remaining *var* genes are transcriptionally silenced [51]. Knock out studies have revealed that *var* genes are de-repressed in Sir2 (Silent Information Regulator) deficient *P. falciparum* lines [41]. Furthermore, Chromatin immunoprecipitaion (ChIP) experiments have shown that active *var* genes are associated with acetylated histone H3 and H4 whereas, histones associated with transcritionally silenced *var* genes are hypoacetylated [10]. In yeast *S. cerevisiae*, the Rap1 protein binds to multiple sites of telomere repeats and together with chromosome end binding proteins yKu70 and yK80 recruits SIR complex through interaction with the Sir3 and Sir4 proteins [15]. Sir2 deacetylates the histone protein and promotes Sir3 binding, which in turn recruits Sir2/Sir4 heterodimer. Thus the unidirectional Sir2 actions and spreading of deacetylated histone proteins along the length of chromatin is stabilized by Sir3/Sir4 interactions [52-53]. This spreading is greatly facilitated by over expressing Sir3 via an unknown mechanism. Physical interactions have been shown to occur between Sir2 and Sir4, between Sir3 and Sir4 and deacetylated histones (H3 and H4) with both Sir3 and Sir4. However, neither *in vivo* nor *in vitro* interactions have been observed between Sir2 and Sir3 [54].

Orc1, besides being the largest subunit of the origin of recognition complex, plays a crucial role in DNA replication and also interacts with Heterochromatin proteins in many organisms [55-57]. Heterochromatin protein 1 was first identified in *Drosophila melanogaster* as a structural component of heterochromatin with a dose dependent effect on gene silencing. *HP1* is evolutionarily highly conserved ranging from *S. pombe* to humans [18]. Hp1 gene consist of the chromo domain (CD) and the chromo shadow domain (CSD) which is separated by a less conserved hinge region of various lengths [30]. In *Drosophila* and *Xenopus* Orc1 also participates in the assembly of telomeric heterochromatin as it has been found that *HP1* interacts with the N-terminal domain of Orc1 [57-

58]. In Human cells also Orc1 interacts with Hp1 [59-61]. In budding yeast *S. cerevisiae* Orc1 plays a unique role only during the mating type silencing by recruiting Sir1 at the cryptic mating loci [62-63]. Also, tethered silencing assay have shown that *Sc*Orc1 has Sir3 like function at budding yeast telomeres [54]. Studies have demonstrated that Orc1 from another member of yeast family *Kluyveromyces lactis,* acts in conjugation with the deacetylase Sir2 and the histone-binding protein Sir4 to generate heterochromatin at telomeres and mating type locus, thus possessing a Sir3 like function [64]. The N-terminal BAH domain which binds to the nucleosomes plays an indispensable role during transcriptional silencing [64-66]. Sir3 has come from gene duplication of Orc1, with high degree of BAH domain homology among them [67]. In yeast subfunctionalization has been found after gene duplication event, where Orc1 retained its origin recognition function during DNA replication and Sir3 specialized in silencing function [27]. On the other hand since *K.lactis* has diverged from *S. cerevisiae* before duplication, it lacks Sir3 and both replication and silencing functions are performed by *Kl*Orc1 [64, 68].

Till date Sir2 is the only member of Sir family of proteins shown to be involved in the epigenetic transcriptional control of a large number of subtelomeric genes that are vital for virulence in *P. falciparum* [41]. *Pf*Orc1 has been characterized and its role during replication has been elucidated [69]. Super gel-shift and chromatinimmunoprecipitaton experiments have shown that *Pf*Orc1 physically associates with telomere repeats and that it cooperates with *Pf*Sir2 in telomeric silencing [37]. Furthermore, it has been shown that the unique N-terminal of *PfORC1* (*PfORC1*$N_{1\text{-}238}$) is targeted to the nuclear periphery *in vivo* and due to the presences of leucine heptad repeats this region binds to the telomeric DNA *in vitro*. Additionally, in *Pf*Sir2 deficient parasites the binding and propagation of *PfORC1* to telomeric and subtelomeric regions was severely compromised suggesting endogenous *PfORC1* requires *Pf*Sir2 for *var* gene regulation [49]. However, whether *PfORC1* plays any role in telomeric silencing has not been established. Notably, as the BAH domain in *PfORC1* is absent, its involvement in telomeric silencing is questionable because the BAH domain has been reported to be critical for the silencing function of Orc1 and Sir3 in other organism [70]. Furthermore, yeast two hybrid assays and GST pull down assay has shown no interaction between *Pf*Orc1 and *Pf*Sir2 [49, 71].

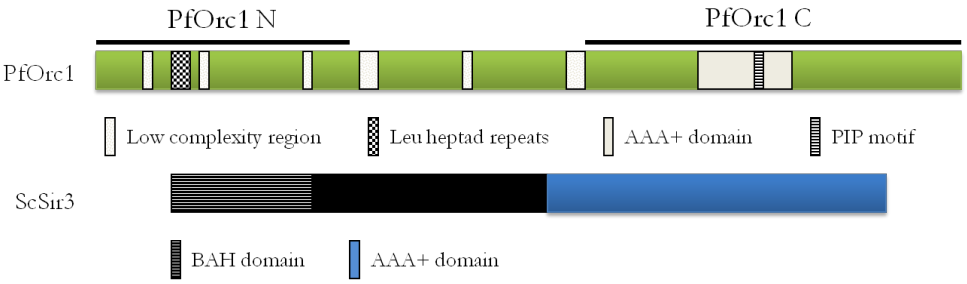
PfORC1 being an essential gene it is not possible to knock out *PfORC1* and look for its loss of silencing function. Generating separation of function mutant or conditional mutant in which only silencing function could be abrogated retaining the replication function is not feasible in *P. falciparum*. Thus in this objective we aim to test whether *Pf*Orc1 has any Sir3 like silencing activity using a yeast surrogate system through complementation assay. Here for the first time we report that *Pf*Orc1 posses Sir3 like silencing function and can complement yeast Sir3 which is Sir2 dependent. In the second part of this objective we have shown that *Pf*Orc1 interacts with *Pf*Hp1 for the silencing of telomeric genes in *P. falciparum* and both the chromo domain and chromo shadow domain is important for this interaction.

3.2 Results:

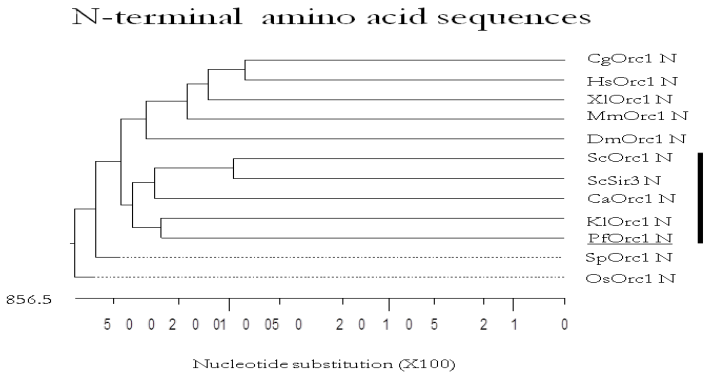
3.2.1 N terminal of *Pf*Orc1 bears homology with Sir3 and Orc1 of *Saccharomycotina*

*Pf*Orc1 has discrete sequence motifs at its N-terminal and C-terminal. In *P. falciparum* the C-terminal of Orc1 has a conserved DNA replication function where as the N-terminal is known to be associated with the "silencing complex". This N-terminal domain consists of a leucine heptad repeat region and several low complexity regions (lysine and asparagines rich). The N-terminal domains of other eukaryotic Orc1 orthologs and the N-terminal domain of Sir3 harbours a well defined Bromo-adjacent homology domain which is apparently absent in *Pf*Orc1 (Figure 6 A). We have observed an interesting relationship between the N-terminal domains of Orc1 orthogs and that of Sir3 by multiple sequence alignment and phylogenetic tree construction. N-terminal of *Pf*Orc1 was observed to be grouped with the members of the *Saccharomycotina* subphylum, but not with *Taprhinomycotina* (*Schizosaccharomyces pombe*), metazoans and plants (Figure 6 B). Interestingly, *S. pombe* and the metazoans lack Sir3. In these organisms, Orc1 along with *HP1* are involved in the silencing function. Since in *P. falciparum* Sir3 is apparently absent and *PfHP1* has been identified, we proposed that similar kind of Orc1-Hp1 mediated silencing mechanism might be involved like that of in the metazoans. It is thus very important to understand whether *Pf*Orc1N-terminal is actually closer to the *Saccharomycotina* Orc1/Sir3. We observed that N-terminal of *Pf*Orc1 shows more closeness to N-terminal *Kl*Orc1 than the post-genome duplicated *Sc*Orc1/*Sc*Sir3. It is already known that Sir3 has come from gene duplication of Orc1. However, both *P. falciparum* and *K. lactis* lacks Sir3 but posses Orc1. This lead us to investigate whether *P. falciparum* and *K. lactis* posses similar type of silencing mechanism. Unlike the N-terminal, the C-terminal sequence of *Pf*Orc1 did show any relatedness to the *Saccharomycotina* subphylum (Figure 6 C). A BLAST analysis revealed a relatively good similarity between the N-terminals of *Pf*Orc1 and *Sc*Sir3 with NCBI BLAST total score 48.1; query cover26%; E-value 0.36; maximum identity 45% and maximum positives 48% (Figure 6 D).

A


PfOrc1 N
PfOrc1 C
PfOrc1
Low complexity region
Leu heptad repeats
AAA+ domain
PIP motif
ScSir3
BAH domain
AAA+ domain

B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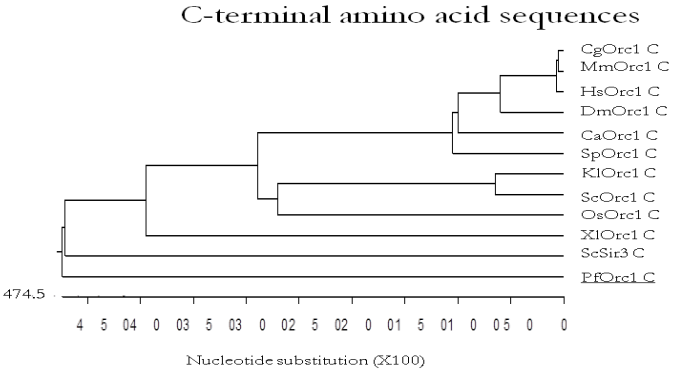
HsOrc1 N
XlOrc1 N
MmOrc1 N
DmOrc1 N
ScOrc1 N
ScSir3 N
CaOrc1 N
KlOrc1 N
PfOrc1 N
SpOrc1 N
OsOrc1 N
856.5
5 0 0 2 0 01 0 05 0 2 0 1 0 5 2 1 0
Nucleotide substitution (X100)

C


C-terminal amino acid sequences
CgOrc1 C
MmOrc1 C
HsOrc1 C
DmOrc1 C
CaOrc1 C
SpOrc1 C
KlOrc1 C
ScOrc1 C
OsOrc1 C
XlOrc1 C
ScSir3 C
PfOrc1 C
474.5
4 5 04 0 03 5 03 0 02 5 02 0 01 5 01 0 05 0 0
Nucleotide substitution (X100)

D

```
PfOrc1n         MTPKKKIFQNFQA----------NDNEILSPTKKGI-KLNVSKLN-ILNF
ScSir3n         MAKTLKDLDGWQVIITDDQGRVIDDNNRRRSRKRGGENVFLKRISDGLSF
                *: . * ::.:*.          :**:   . *:*  :: :.::.  *.*

PfOrc1n         E--NTIITKEKTNYEYKASLNKEIDEVLNNNNIINTHNNKKNNLNLYDYN
ScSir3n         GKGESVIFNDNVTETYSVYLIHEIRLN-TLNNVVEIWV--FSYLRWFEL-
                   :::* ::::.  *.. * :**    . **:::      . *. ::

PfOrc1n         NIKNSTHEFYIDLNEQNKQTIKYNDNKFTPINKKEKYNLDETSSSSISSS
ScSir3n         KPKLYYEQFRPDLIKEDHPLEFYKDKFFNEVNKSELYLTAELSEIWLKDF
                : *   .:*  ** ::::    *:*: *. :**.* *   * *.  :..

PfOrc1n         L-------------------------------------------------
ScSir3n         IAVGQILPESQWNDSSIDKIEDRDFLVRYACEPTAEKFVPIDIFQIIRRV
                :

PfOrc1n         ----------------TNISSSLTNISSSLTNISSSLSNSLDEKKKKKKI
ScSir3n         KEMEPKQSDEYLKRVSVPVSGQKTNRQVMHKMGVERSSKRLAKKPSMKKI
                               . :*.. ** .   .   .  *: * :* . ***

PfOrc1n         KKNNSTIINILNNNNNNSNIHH---------------------------
ScSir3n         KIEPSADDDV-NNGNIPSQRGTSTTHGSISPQEESVSPNISSASPSALTS
                * : *:  :: **.*  *:

PfOrc1n         -NNKHNIYNKYNISKKPQ-NKEIHTLPSNHQIKKKSNNTYNTCQQKMKKN
ScSir3n         PTDSSKILQKRSISKELIVSEEIPINSSEQESDYEPNNETSVLSSKPGSK
                 .:. :* :* .***:   .:**   .*::: . :.**  .. ..*  .:

PfOrc1n         ISKKNTHSIKNNQNDKNKEK---------NKEKDKNIKKDRDK---DIQT
ScSir3n         PEKTSTELVDGRENFVYANNPEVSDDGGLEEETDEVSSESSDEAIIPVNK
                 .*..*. :...:*    ::         ::*.*:  .:. *:    ::.

PfOrc1n         KRTSHQSQDQN----NHF--ERRILRSYTRNNDNVKN-NLKNNINNNNTL
ScSir3n         RRGAHGSELSSKIRKIHIQETQEFSKNYTTETDNEMNGNGKPGIPRGNTK
                :* :* *: ..     *:   :.: :.** :.**  * * * .* ..**

PfOrc1n         ----------KRSSQSV--------------------RIDSDLSSAHQNK
ScSir3n         IHSMNENPTPEKGNAKMIDFATLSKLKKKYQIILDRFAPDNQVTDSSQLN
                          ::.. .:                     *.::::: * :

PfOrc1n         RIKYD-----------------------EKN
ScSir3n         KLTDEQSSLDVAGLEDKFRKACSSSGRETILS
                ::. :                       .
```

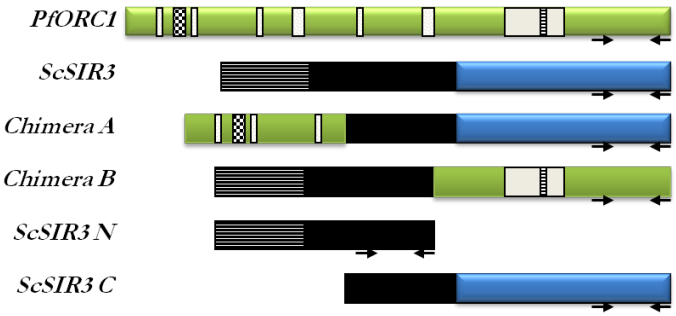
Figure 6. (A) Schematic representation of genes used in complementation experiments. *PfORC1*: full length *ORC1* gene from *P. falciparum*. *ScSIR3*: full length *SIR3* gene from yeast *S. cerevisiae*. (B) Phylogenetic tree construction taking the N-terminal domains of Orc1 orthologs and Sir3. (C) Phylogenetic tree construction taking the C-terminal domains of Orc1 orthologs and Sir3. (D) The sequence alignment between the N-terminal of *Pf*Orc1 and *Sc*Sir3 using T-Coffee,EMBL-EBI

3.2.2 *Pf*Orc1N terminal domain functionally complements *S. cerevisiae* Δ*sir3* null mutant

Next, we wanted to determine whether *Pf*Orc1 can complement *S. cerevisiae* Δ*sir3* null mutant. To this end, we generated several complementation vectors containing either full length *Pf*Orc1 or full length *Sc*Sir3 cloned in pTA-HA3 vector (a yeast expression vector with C-terminal HA3 tag, GPD promoter and *TRP1* as a selectable maker). We also generated chimera A consisting of the N-terminal region of *Pf*Orc1 (amino acids 1–350) and C-terminal region of *Sc*Sir3 (amino acids 243–979) and chimera B consisting of consisting of sequences corresponding to N-terminal region of *Sc*Sir3 (amino acids 1–527) and C-terminal region of *Pf*Orc1 (amino acids694–1190) in the same pTA-HA3 vector backbone (Figure 7A). We have selected these regions on the basis of homology analysis and previously generated information [69]. All these constructs were transformed into Δsir3 yeast strain (ΔGK strain: a kind gift from Arthur Lustig, Tulane University, USA) and the expression of genes were observed by real-time RT-PCR analysis (Figure 7B). However, we were unable to detect the proteins by western blot analysis using the anti-HA antibody probably due to low expressions of these proteins

To study the silencing activity of these constructs in Δ*sir3* yeast strain we assay for telomere position effect. In Δ*sir3* since Sir3 is absent all the sub-telomeric genes are de-repressed. Studies involving telomere position effect assays using marked telomeres such as *URA3* and *ADE2* is well established though these methods are less quantitative and unable to detect partial restoration of silencing. Thus we adopted qRT-PCR analysis method and carry out three different silencing assays for more sensitive and quantitative results (Figure 8A). In the first assay, we observed the silencing of the *URA3* genes present at sub-telomeric region of chromosome VII L. When the *URA3* gene is silenced the cells grow in presences of pro-drug FOA (5-Fluoroorotic acid), but when the *URA3* gene is de-repressed it converts FOA into a toxic product and becomes lethal to the cells. As in Δ*sir3* strain *URA3* gene is not silenced they are sensitive to FOA where as wild type strain which consists of *SIR3* are resistant to FOA. Using this assay we observed clearly that chimera A which has the N- terminal of *PfORC1*and C-terminal of *Sc*Sir3 were able to grow on FOA plates proving that chimera A has the silencing activity (Figure 8B).

A



B

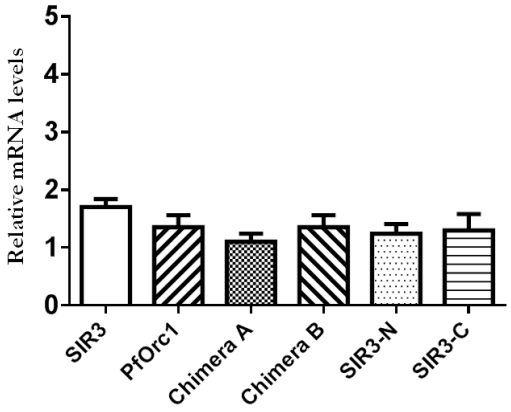


Figure 7.

(A) Schematic representation of genes or gene fragments used in complementation experiments. *ScSIR3*: full length *SIR3* gene from yeast *S. cerevisiae*. *PfORC1*: full length *ORC1* gene from *P.falciparum*. Chimera A: A fusion protein containing sequences corresponding to the N-terminal of *PfORC1* and the C-terminal of *ScSIR3*. Chimera B: A fusion protein containing DNA sequences corresponding to the N-terminal domain of *ScSIR3* and DNA sequences corresponding to the C-terminal domain of *PfORC1*. *ScSIR3N*: DNA sequences corresponding to the N-terminal of *ScSIR3*. *ScSIR3C*: DNA sequences corresponding to the C-terminal of *ScSIR3*. The arrows show the position of primer pairs used to amplify each transcript by RT-PCR. (B) Quantitative-PCR data showing similar levels of mRNA expression of each gene or gene fragments used in complementation experiments. In each case the mean value (±SD) from three independent experiments was normalized against the abundance of *ACT1* mRNA and plotted using Graph Pad prism 6 software.

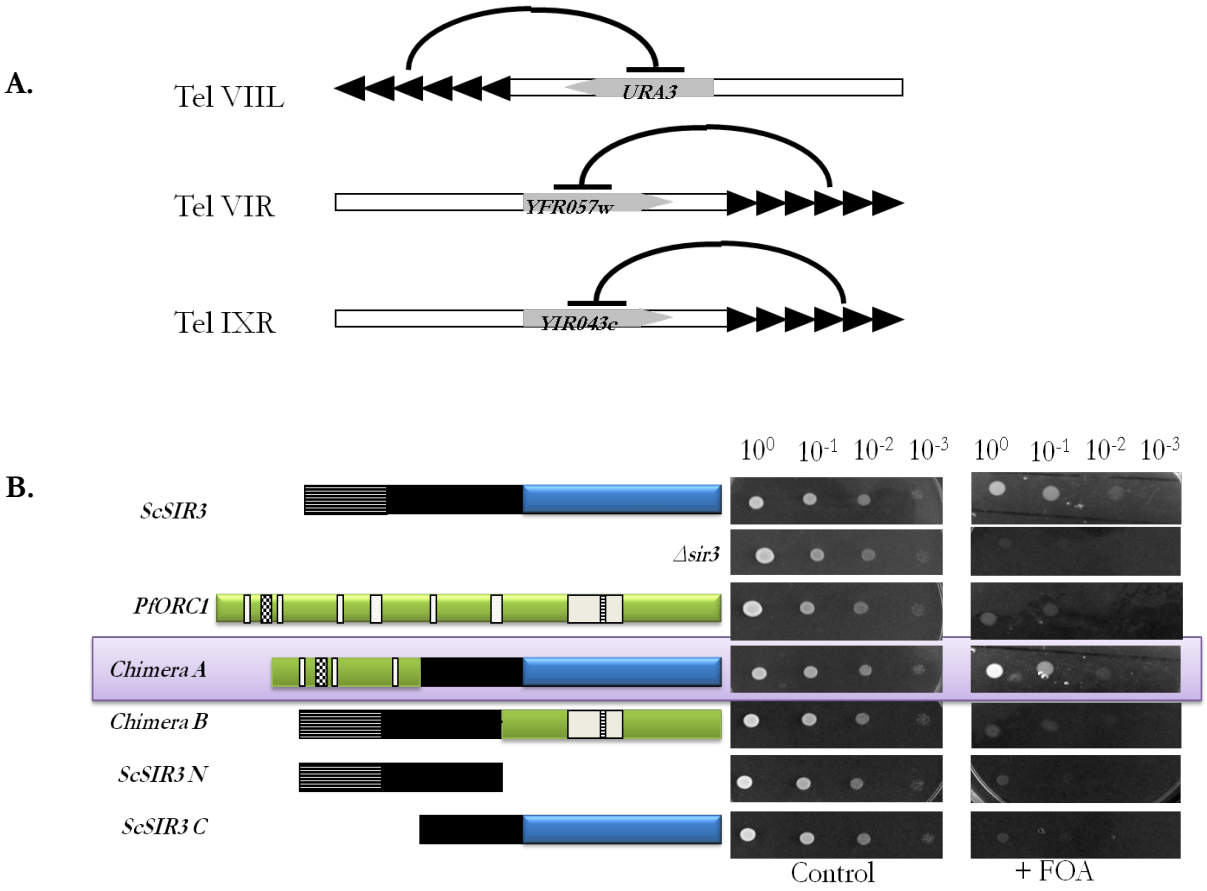
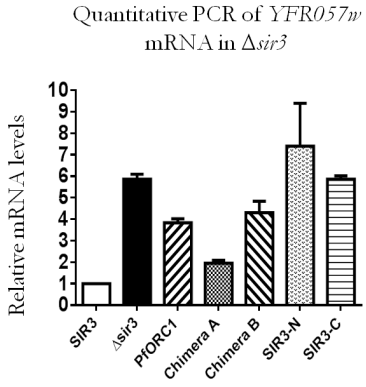


Figure 8. *Pf*Orc1 complements *Sc*Sir3 in telomere position effect. (A) Schematic representation of TPE assays. The telomeres and the corresponding sub-telomeric genes that were analyzed are indicated. (B) TPE assay of *URA3* marker gene is analyzed by 5-FOA sensitivity. When *URA3* gene is silenced the cells are resistant to 5-FOA. Similarly, when *URA3* gene is de-repressed the cells are sensitive to 5-FOA. The left panel shows growth of different cells (as indicated on the left) in non-selective medium and the right panels shows the growth of the cells on the 5-FOA plates.

We found that neither the cells expressing full length *Pf*Orc1 nor the cells expressing Chimera B were able to grow on FOA plate, suggesting that both these construct are unable to do the silencing function and hence fail to compliment in *S. cerevisiae* Δ*sir3* null mutant. Further, we performed more quantitative assay where in the mRNA levels of two naturally occurring sub-telomeric genes were quantified by qRT-PCR, which are *YFR057* and *YIR043c*. The *YFR057* gene is present on chromosome VIR and the *YIR043c* gene is located on chromosome IXR [72-73]. We observed that the transcript levels of *YFR057* and *YIR043c* in the cells expressing full length *Pf*Orc1 were 1.5 fold and 3 fold less when compared Δ*sir3* null cells respectively, clearly suggesting that *Pf*Orc1 could to some extent perform the silencing function in the Δ*sir3* null strain (Figure 9A and B). Telomere position effect involves many protein-protein interactions. Sir2 is a NAD^+ dependent Histone deacetylase and the only Sir protein that is conserved among the multicellular eukaryotes [24]. Sir2/Sir4 recruits Sir3 and binds to hypoacetylated N-terminal tails of histone H3 and H4 thus leading to spread of the deacetylated domain. The N-terminal- and the C-terminal-domains of Sir3 have different roles in this process. We reason that *Pf*Orc1 might not be able to interact with other yeast proteins involved in telomere silencing and thus is unable to exhibit complete silencing function. To further investigate this possibility we tested whether either chimera A or chimera B could complement SIR3 function in Δ*sir3* null strain. We observed that chimera A is able to complement Sir3 better than *Pf*Orc1 showing 3 fold down regulation of YFR057w transcript and 5 fold down regulation in *YIR043c* transcript (Figure 9A and B). To rule out the possibility that the silencing effect of chimera A is only because of the presences of C-terminal domain of *Sc*Sir3, we also tested the restoration of silencing due to of C-terminal domain of *Sc*Sir3 alone. We found no restoration of silencing by of C-terminal domain of *Sc*Sir3 alone proving chimera A to be genuine silencing protein. Chimera A thus, was able to restore silencing of all three different sub telomeric genes present on different chromosomes shown by three independent telomere position effect assays. Therefore, the restoration of telomere silencing by chimera A is a general phenomenon and is not restricted to any particular telomere. Unlike chimera A, chimera B is unable to restore the silencing function in Δ*sir3* null strain (Figure 8B, 9A and B). Our results shows that *Pf*Orc1 on its own also was able to exhibit limited silencing phenotype at two distinct telomeres when measured by quantitative RTPCR analysis, however it failed to effectively silence the sub telomeric *URA3* gene. This might be because 5-FOA sensitivity test is pretty harsh selection and unless the extent of

A



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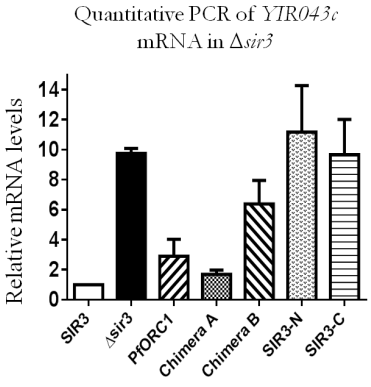


C

Figure 9. *Pf*Orc1 complements *Sc*Sir3 in telomere position effect. (A) TPE of *YFR057w* gene. The relative transcript levels of *YFR057w* gene as determined by Q-PCR from different strains (indicated on the X-axis) are plotted after normalization with *ACT1* mRNA. In each case the mean value (±SD) from three independent experiments with three independent harvests of cells is calculated and plotted using Graph Pad prism 6 software. (B) TPE of *YIR043c* gene. The relative levels of *YIR043c* mRNA are plotted as in A. (C) Schematic representation of telomere silencing in various cell as indicated. *ScSIR3*: full length *SIR3* gene from yeast *S. cerevisiae*, Δ*sir3, PfORC1*: full length *ORC1* gene from *P.falciparum*. Chimera A: A fusion protein containing sequences corresponding to the N-terminal of *PfORC1* and the C-terminal of *ScSIR3*. Chimera B: A fusion protein containing DNA sequences corresponding to the N-terminal domain of *ScSIR3* and DNA sequences corresponding to the C-terminal domain of *PfORC1*.

silencing is good enough the cells are unable to survive on 5-FOA plates. Constructs consisting of only *Sc*Sir3N or *Sc*Sir3C served as the internal controls that lacked silencing activity.

Furthermore we wanted to determine whether such heterologous complementation of *Sc*Sir3 is specific to N-terminal region of *Pf*Orc1. For this purpose we made another chimera that consisted of N-terminal domain of *Sp*Orc1 and C-terminal domain of *Sc*Sir3. Our data suggests that similar to *Pf*Orc1, N-terminal domain of *Sp*Orc1 also exhibits robust silencing activity by complementation assay (Figure 10).

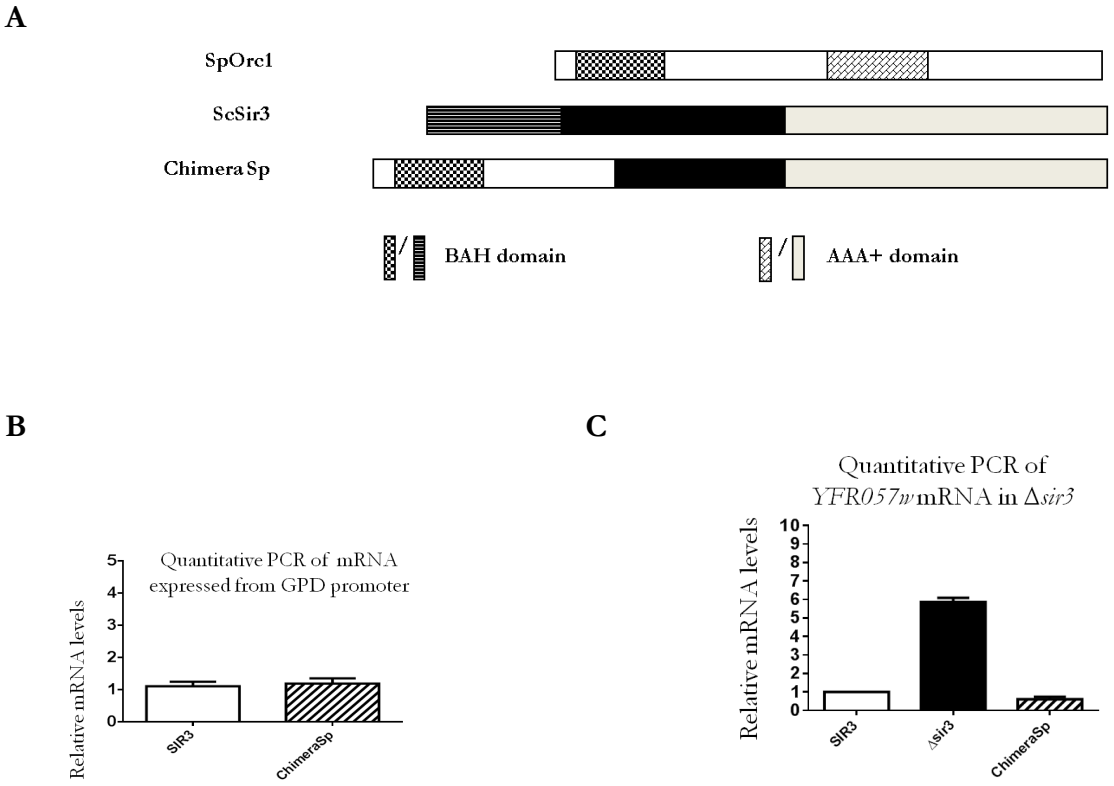


Figure 10. (A) Schematic representation of genes used in complementation experiments. *SpORC1*: full length *ORC1* gene from *Schizosaccharomyces pombe*, *ScSIR3*: full length *SIR3* gene from yeast *S. cerevisiae,* . Chimera Sp: A fusion protein containing sequences corresponding to the N-terminal of *SpORC1* and the C-terminal of *ScSIR3*. (B) Quantitative-PCR data showing similar levels of mRNA expression of *ScSIR3* gene or Chimera Sp gene used in complementation experiments. In each case the mean value (±SD) from three independent experiments was normalized against the abundance of ACT1 mRNA and plotted using Graph Pad prism 6 software. (C) TPE of *YFR057w* gene. The relative transcript levels of *YFR057w* gene as determined by Q-PCR from strains expressing either *ScSIR3* gene or Chimera Sp (indicated on the X-axis) are plotted after normalization with *ACT1* mRNA. In each case the mean value (±SD) from three independent experiments is calculated and plotted using Graph Pad prism 6 software.

3.2.3 Structure function analysis of *Pf*Orc1N terminal domain in TPE

*Pf*Orc1 N-terminal domain (NTD) lacks BAH domain. However, it contains one leucine heptad repeat (LHR) region and three low complexity regions which are lysine and asparagine rich. We have generated three deletion mutants of *Pf*Orc1 NTD to understand the importance of these regions and to map the functional domain. The first mutant chimera AΔ70 is devoid of the N-terminal extension of 70 amino acids, but retains all the three low complexity regions as well as the LHR. The second mutant chimera AΔ110 lacks the first 110 amino acids which includes the first low complexity region. The third mutant chimera AΔ220 was generated by deleting 210 amino acids and is devoid of the first two low complexity regions and the LHR region (Figure 11 A). We have shown that all the deletion mutants are expressed at similar transcript level by quantitative PCR (Figure 11B). In order to test the effect of these deletion mutants on the telomere position effect we quantified the transcript levels of *YFR057w* and *YIR043c* genes. Our data shows that the mRNA levels of *YFR057w* and *YIR043c* in chimera A and chimera AΔ70 are similar suggesting the N-terminal extension of chimera A are dispensable for the silencing function. On the other hand, chimera AΔ110 and chimera AΔ210 showed comparable levels of *YFR057w* and *YIR043c* transcripts similar to Δ*sir3* null mutant (Figure 11 C and D), proving that LHR and the three low complexity regions are important for telomere silencing.

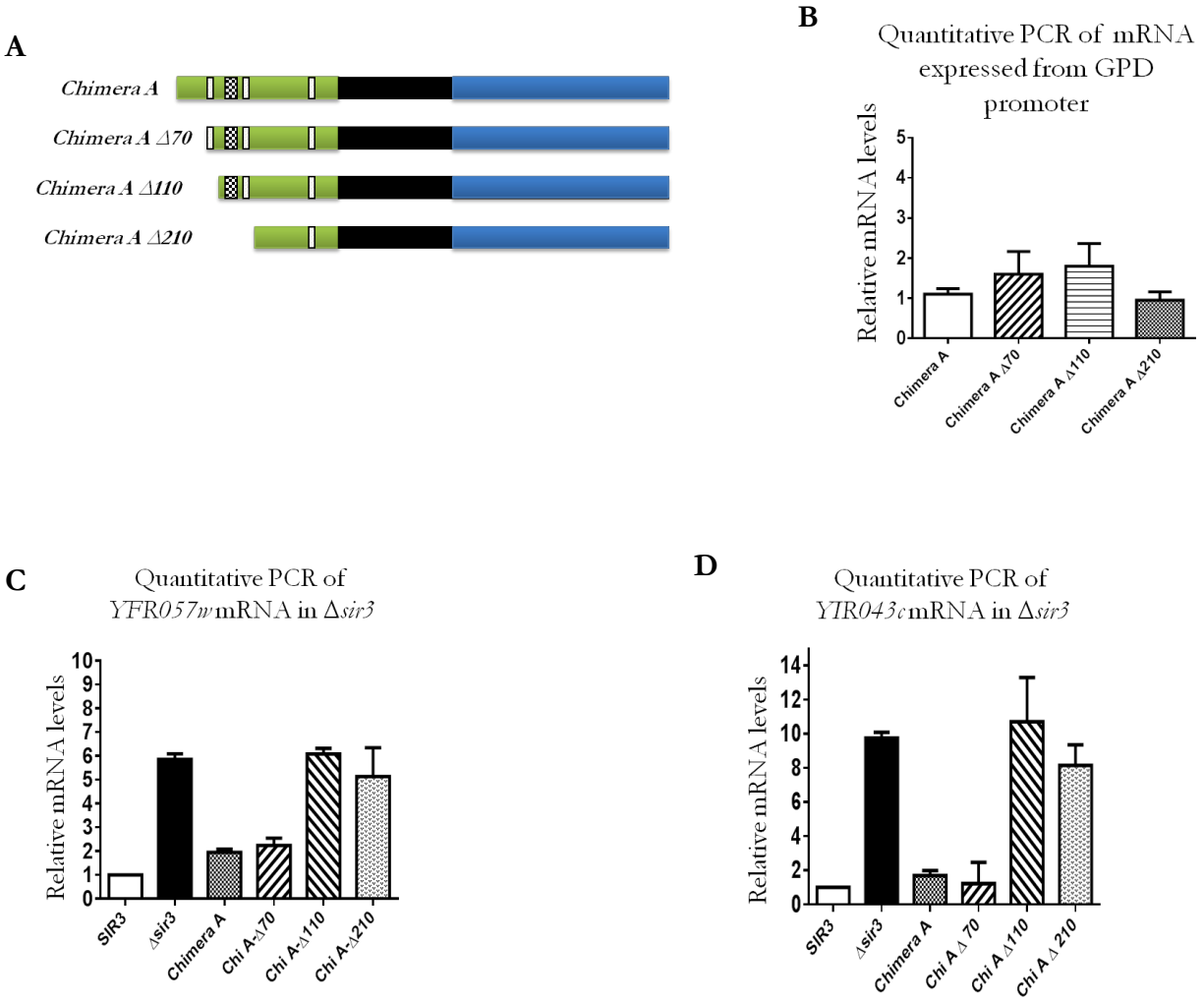
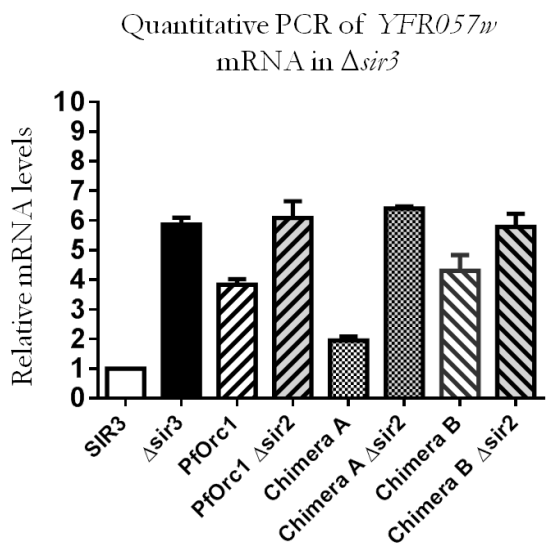


Figure 11. Structure function analysis of *Pf*Orc1 N-terminal in Telomere position effect. (A) Schematic representation of different deletions mutants. (B) Quantitative-PCR data showing similar abundance of mRNA from different mutants. (C) TPE of *YFR057w* gene expression. The relative abundances of mRNA from these two subtelomeric genes are plotted after normalization against *ACT1* mRNA. Each bar represents mean mRNA level (±SD) from three independent experiments. (D) TPE of *YIR043c* gene expression. The relative abundances of mRNA from these two subtelomeric genes are plotted after normalization against *ACT1* mRNA. Each bar represents mean mRNA level (±SD) from three independent experiments.

3.2.4 Functional complementation of *S. cerevisiae* Δ*sir3* null mutant by *Pf*Orc1N terminal domain is Sir2 dependent

Previous studies have shown that Sir complex assembles in a step wise fashion. Telomere binding proteins, the yku70/k80 heterodimer and Rap1 binds to the DNA and recruits the Sir2/Sir4 complex. Sir2 deacetylates the histone and further recruits Sir3 via interactions involving Rap1, Sir4 and histone tails. If *Pf*Orc1 functions like Sir3, it must work via Sir2 which has histone deacetylase activity. To address this possibility we have knock out the *SIR2* gene to create a strain which expresses chimera A in Δ*sir3,* Δ*sir2* background. Similarly we also created strains expressing *Pf*Orc1 and chimera B in Δ*sir3,* Δ*sir2* background. Our results demonstrate clearly that chimera A showed complete de-repression of *YFR057w* and *YIR043c* expressions in Δ*sir3,* Δ*sir2* background suggesting complementation of Sir3 function by chimera A is Sir2 dependent (Figure 12 A and B).

A



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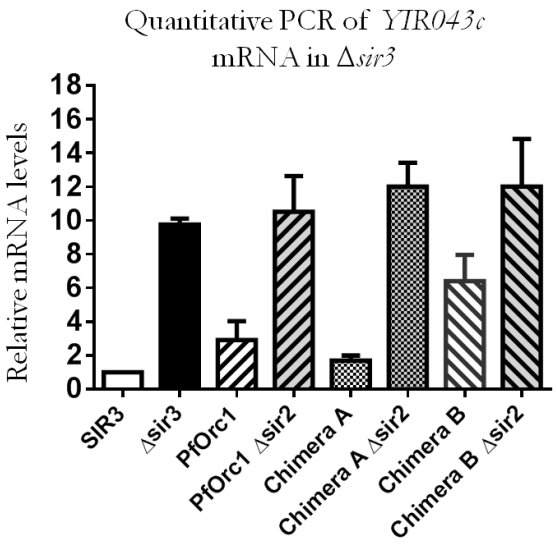
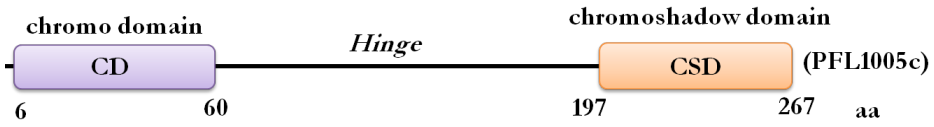


Figure 12. *Pf*Orc1 mediated silencing is Sir2 dependent. (A) Q-PCR data showing reversal of chimera A mediated silencing of *YFR057w* in Δ*sir2* cells. The relevant genotypes of each strain are indicated on the X-axis. The data from three independent experiments are normalized with respect to the abundance of *ACT1* mRNA. Each bar represents mean relative mRNA levels ±SD. (B) Q-PCR data showing reversal of chimera A mediated silencing of *YIR043c* in Δ*sir2* cells. The relevant genotypes of each strain are indicated on the X-axis. The data from three independent experiments are normalized with respect to the abundance of *ACT1* mRNA. Each bar represents mean relative mRNA levels ±SD.

3.2.5 *Pf*Orc1 interacts with *Pf*Hp1

One of the major roles of Hp1 protein is in the assembly and maintenance of heterochromatin which is a relatively condensed form of chromatin that promotes gene silencing. Hp1 protein consists of an N-terminal chromodomain (CD), flexible hinge region (H) and C-terminal chromoshadow domain [74] (Figure 13 A). It is found that N-terminal domain of Orc1 interacts with heterochromatin protein 1 in *Xenopus* and *Drosophila* leading to chromatin silencing [57]. Since in *P. falciparum Pf*Orc1 has Sir3 like function and *Pf*Hp1 is characterized as a component of subtelomeric chromatin [46], we hypothesized that there might be interaction between *Pf*Orc1 and *Pf*Hp1 similar to *Xenopus* and *Drosophila*. We have used yeast two-hybrid system to investigate if *Pf*Orc1 interacts with *Pf*Hp1 (Figure 13 B). Our results showed that indeed *Pf*Orc1 and *Pf*Hp1 interact (Figure 14 A). So the next question we asked was which of the domain of *Pf*Hp1 is important for its interaction with *Pf*Orc1. To answer this question we generated two deletion mutants in which either the chromo domain or the chromo shadow domain was deleted. According to our findings, we found that both the domains are indispensable for the interaction of *Pf*Orc1 and *Pf*Hp1 (Figure 14 B and C).

A



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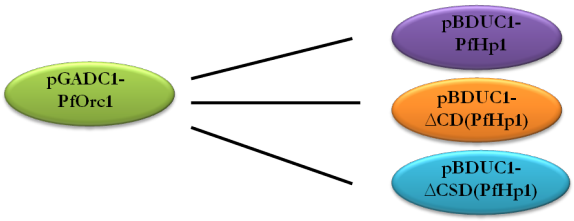


Figure 13. (A) Schematic representation of *Pf*Hp1 proteins. Hp1 protein consists of the chromodomain (CD) at the N terminus and the chromoshadow domain (CSD) at the C-terminus separated by the hinge region. Total length of protein is indicated and drawn to relative scale. (B) Schematic representation of yeast two hybrid system, to study the interaction between *Pf*Orc1 and *Pf*Hp1, *Pf*Orc1 and *Pf*Hp1ΔCD, *Pf*Orc1 and *Pf*Hp1ΔCSD respectively.

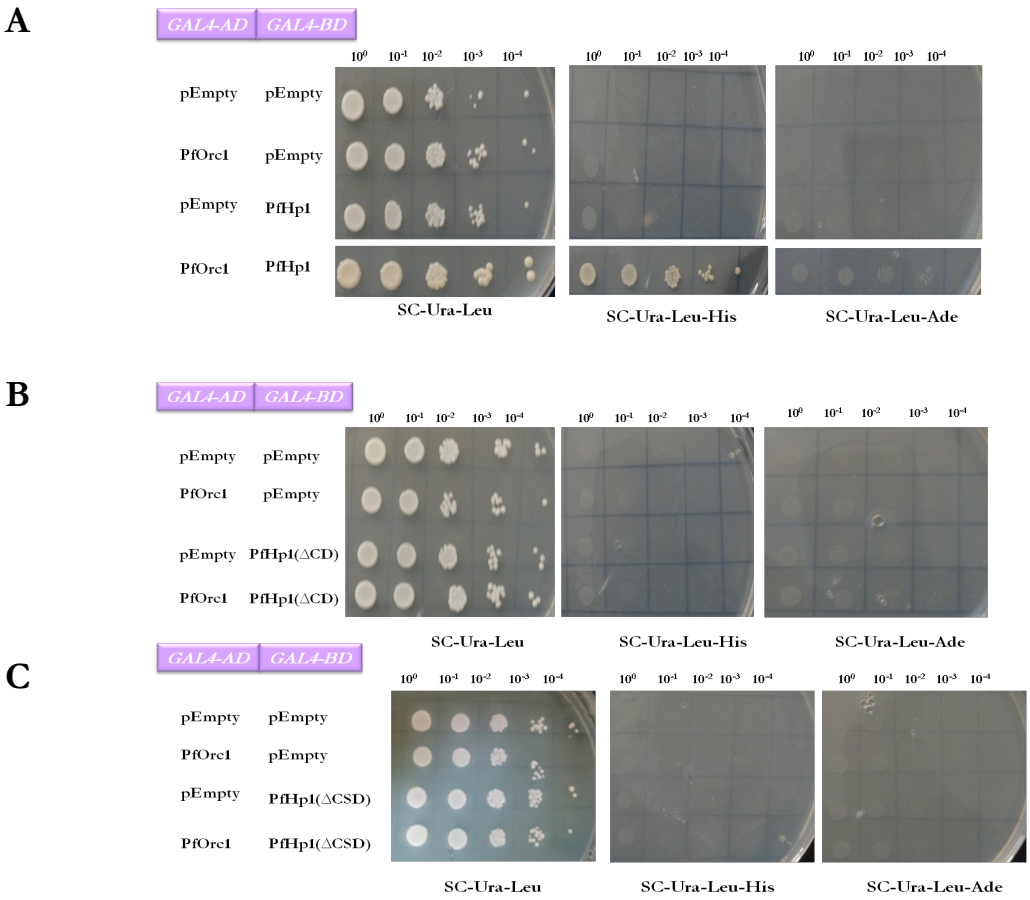


Figure 14. *Pf*Orc1 interacts with *Pf*Hp1. (A) Full length *Pf*Orc1 ORF was fused to GAL4 AD domains in pGADC1, similarly *Pf*Hp1 ORF was fused with GAL4 BD domain of pGBDUC1. Two-hybrid interactions were tested using yeast strain pJ694A, where ADE2 and HIS3 genes serve as reporter genes. All the strains were grown to same OD_{600} (1 OD_{600}) and serial dilutions of each strain was spotted on Sc-Ura-Leu plates lacking uracil and leucine to serve as control, and similarly samples were spotted on Sc-Ura-Leu-Ade and Sc-Ura-Leu-His plates laking either adenine or histidine, to test the protein interaction. (B) *Pf*Hp1ΔCD was fused with GAL4 BD domain of pGBDUC1 and the interaction between *Pf*Orc1 and *Pf*Hp1ΔCD was studied respective synthetic complete medium plates. (C) *Pf*Hp1ΔCSD was fused with GAL4 BD domain of pGBDUC1 and the interaction between *Pf*Orc1 and *Pf*Hp1ΔCD was studied respective synthetic complete medium plates.

3.3 Discussion

In *P. falciparum,* heterochromatin assembly at the telomere involves several players such as *PfHP1*, *PfSIR2* and *PfKMT1*, but the role of *PfORC1* in this process remained unknown. From the *P. falciparum* genome database analysis *SIR3* and *SIR4* have been found to be absent; instead, Orc1 (*Pf*Orc1), whose amino end is highly similar to that of the Sir3 protein, is identified. In this study we have shown for the first time that the N-terminal of *Pf*Orc1 possesses Sir3 like silencing function. Our result is supported by previous finding that *Pf*Orc1 and *Pf*Sir2 co-localize at silent subtelomeric regions including the *var* gene promoters [37]. We have demonstrated that in yeast strain Δ*sir3*, which lacks telomere silencing, expression of full length *Pf*Orc1 or chimera A (N-terminal region of *Pf*Orc1 and C-terminal region of *Sc*Sir3) restores back the silencing activity. Chimera A showed more silencing activity when compared to *Pf*Orc1. We have also shown chimera B which consists of N-terminal region of *Sc*Sir3 and C-terminal region of *Pf*Orc1 does not possess silencing activity. Additionally, our present study has shown that the N-terminal of *S .pombe* Orc1 is able to complement Sir3 function in a yeast surrogate system. Therefore, it is noticeable that the N-terminal region of Orc1 from different species possesses silencing activity. Interestingly, all the orthologs of Orc1 except for *Pf*Orc1 consist of a well defined BAH domain at their N-terminal which is known to be important for the silencing function. The ability of *Pf*Orc1 to possess the silencing function even in the absences of BAH domain is still to be elucidated. It could be possible that the three dimensional folding of N-terminal *Pf*Orc1 region mimics the BAH domain. Though N-terminal region of *Pf*Orc1 lacks BAH domain, it possess a leucine heptad repeat region and several low complexity regions (lysine and asparagines rich). A more efficient silencing of chimera A compared to *Pf*Orc1 can be explained by the specific contribution of the C-terminal domain of *Sc*Sir3 in telomere silencing. Although the N-terminal region of *Sc*Sir3 plays a crucial role in telomere silencing, the C-terminal region of *Sc*Sir3 consist of AAA+ domain that is required for its interaction with various other silencing proteins (Sir4 and histones) [70]. The AAA+ domain of *Sc*Sir3 has evolved much more rapidly when compared to the AAA+ domains of Orc1 proteins. More over the AAA+ domain of *Pf*Orc1 is very minimal than that of *Sc*Sir3. Thus, C-terminal of *Pf*Orc1 may not be able to interact with the other silencing proteins as efficiently as *Sc*Sir3 C-terminal. To identify important amino acid residues in the N-terminal of *Pf*Orc1 for its silencing function was difficult as it lacked BAH domain. By generating a series of deletion mutants we have found that these leucine

heptad repeat region and low complexity regions (lysine and asparagines rich) are crucial for its silencing function. Furthermore, we have shown the silencing function of *Pf*Orc1 is Sir2 mediated by knocking out *sir2* gene from the strains expressing *Pf*Orc1 and other chimeras in Δ*sir3*Δ*sir2* background which resulted in the loss of their silencing function. Since it is found that N-terminal domain of Orc1 interacts with heterochromatin protein 1 in *Xenopus* and *Drosophila* leading to chromatin silencing [57]. We also investigated the interaction between *Pf*Orc1 and *Pf*Hp1. Our yeast two hybrid data clearly shows that both *Pf*Orc1 and *Pf*Hp1 interact with each other and both chromo domain and chromo shadow domain are important for their interaction.

CHAPTER 4

THE ROLE OF HEAT SHOCK ON *var* GENE REGUATION IN *Plasmodium falciparum*

4.1 Introduction:

One of the most common generalized initial responses by humans to infectious agents, autoimmune diseases, cancer and other physical agents including injury is febrile illness. Pyrogens are the fever inducing agents that signals to the thermoregulatory regions of the brain to elevate the core body temperature thus causing fever. External stimuli (such as injuries or infection) or an internal stimulus (such as autoimmune diseases or cancer) leads to the release of these pyrogens that elicit the febrile response through a series of events [75]. However, malaria fever which often exceeds 40ºC in the acute illness is unique, as it has a cyclical and periodical nature which is different from other febrile manifestations and is indeed the most salient feature. The origin of malaria fever, its biological foundations and consequences are poorly understood [76]. Camillo Golgi was the first to discover in 1886 that malarial fever coincides with the release of merozoites through the rupture of the intraerythrocytic schizonts during a synchronous asexual blood stage infection [77]. This type of fever exist only for few hours and the frequency of the febrile episodes depends upon the parasite species, occurring every 48 hours for *P. falciparum*, *P. vivax* and *P. ovale*, and every 72 hours for *P. malariae*. The interaction between the toxins released during the rupture of schizonts and the phagocytic cells causes the malarial febrile episodes (Figure 15). The two major malaria toxins which are released during the rupture of schizonts are hemozoin and glycosylphosphatidylinositol (GPI) [78]. Several studies have demonstrated that elevated temperature adversely affects the growth of *P. falciparum* parasite culture [79-80]. A study shows that growth of the asynchronous *P. falciparum* culture was reduced by 23%, 66% and 100% following 2, 8 and 16 hours of cultivation at 41ºC respectively [81]. Conversely, a study has also shown that recurrent fever actually promotes intra-erythrocytic parasite development [82]. Furthermore, recurrent fever accelerated the parasite growth with four fold increase in the newly invaded ring stage parasites. Studies have demonstrated that exposing the parasite in erythrocytic culture to 40ºC inhibits its growth *in vitro* and that periodic fluctuation of temperature, similar to that of the natural infection, promotes synchronization of the asexual stage *P. falciparum* parasites in the culture. The data also suggest that rings are relatively temperature-resistant and that a synchronized culture can exhibit good growth despite periodic exposure to periodic fibril temperature [79]. The mechanism of synchronization, which is central to this phenomenon, is yet to be elucidated. These results suggest that the parasites

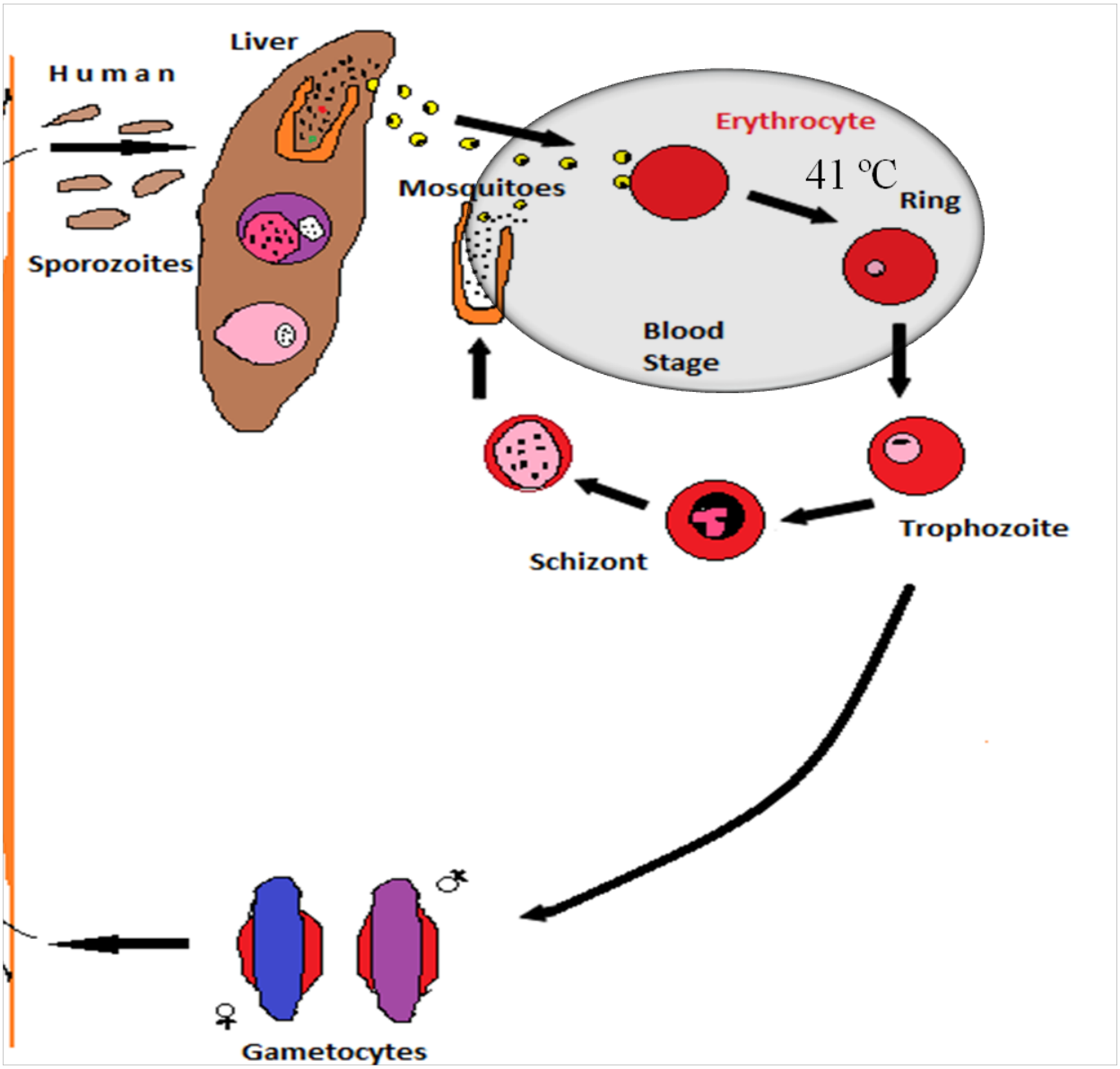


Figure 15: Schematic representation to show that ring stage parasites are exposed to febrile temperature

might have developed unique mechanism to respond to this febrile temperature. The ability of the parasite to sequester to the endothelial membrane of the host plays a crucial role in malaria virulence, and also helps in immune evasion and avoiding splenic filtration. The sequestration in *P. falciparum* is caused by the expression of the parasite protein *P. falciparum* erythrocyte membrane protein (*Pf*Emp1) on the surface of the infected RBCs, which are encoded by a family of genes called *var* genes. *In vitro* studies have shown that febrile temperature augments of the cytoadherence of the young parasites to the host receptors [83]. Thus we assume that the mammalian *Plasmodium* exploits the host febrile response to favor its own survival by some interesting unknown mechanism. Not surprisingly, Hsp90 and Hsp70, which have been implicated in heat shock response show increase in expression up on heat shock [81]. Laskar et al have demonstrated a very attractive result showing a link between environmental stress such as heat shock and the emerging phenotype due to alteration in gene expression. They have shown that over expression of yHsp90, either by exposing cells to heat shock or by introducing yHsp90 over expression plasmid yields reduced level of Sir2, with a consequential loss of telomere silencing [72]. And recently they have also established that heat shock or yHsp90 over expression causes up regulation of Cup9 that, in turn, represses *SIR2* transcription by binding to its upstream activator sequence [84]. These results made us to investigate whether similar kind of mechanism exist in *P. falciparum* as evidences indicate that *var* genes are highly transcribed (de-repressed) exclusively during the ring stage which is generally exposed to the febrile temperature and in the later stage of the parasite transcriptional activity is silent [85]. Thus in this objective we aimed to study if there is any mechanistic link between the physiological cues such as febrile temperature and the *var* gene regulation (Figure 16). To address these important outstanding questions we gave heat shock of two hours to the synchronized parasite culture and observed the transcript levels of both the paralogues of *P. falciparum* Silent information regulator 2 protein which are *PfSIR2A* and *PfSIR2B*. Interestingly our data demonstrates that similar to *Saccharomyces cerevisiae* a transient heat shock of two hours to the parasite culture leads to transcriptional down regulation of both the *SIR2* paralogues which is transgenerational. Our results also clearly demonstrate that the most of the *var* genes which are under the control of *Pf*Sir2A and *Pf*Sir2B are de-repressed upon a transient heat shock of two hours. These studies have shed new light on how malaria fever might be involved in the regulation of *var* gene silencing.

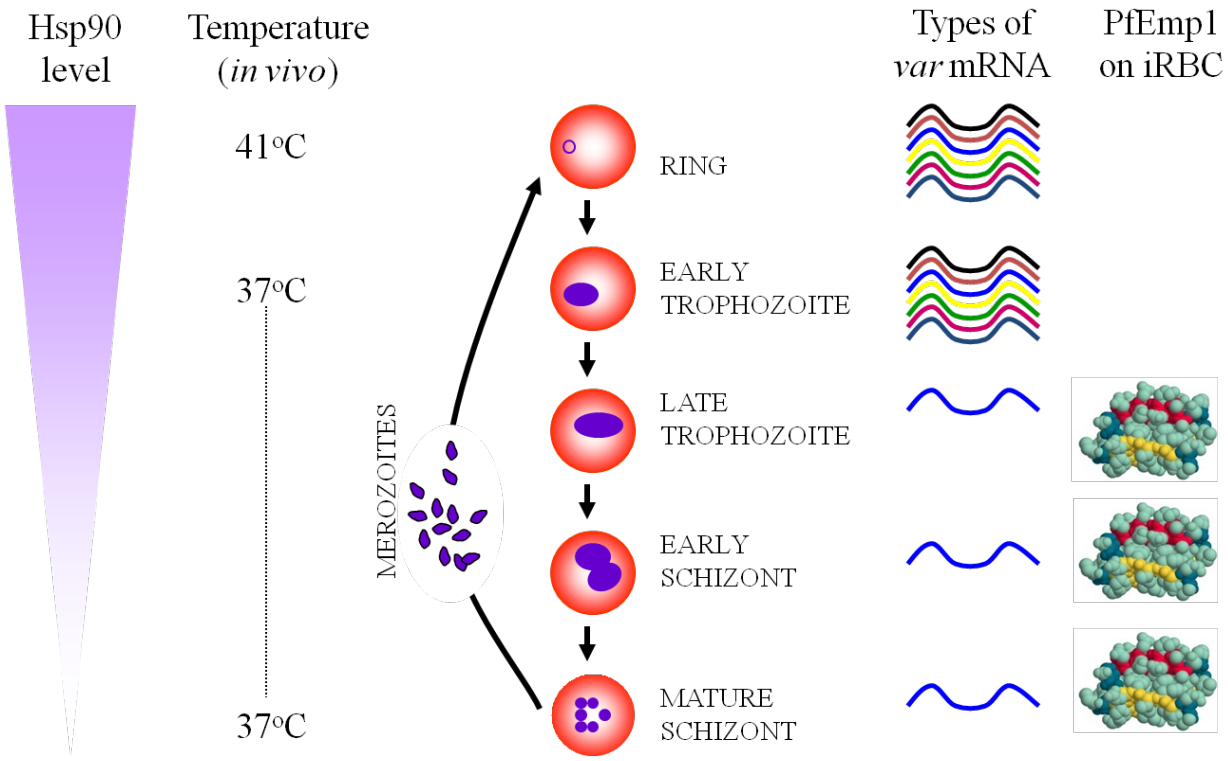


Figure 16: Schematic representation to show the connection between Hsp90 and *var* genes de-repression

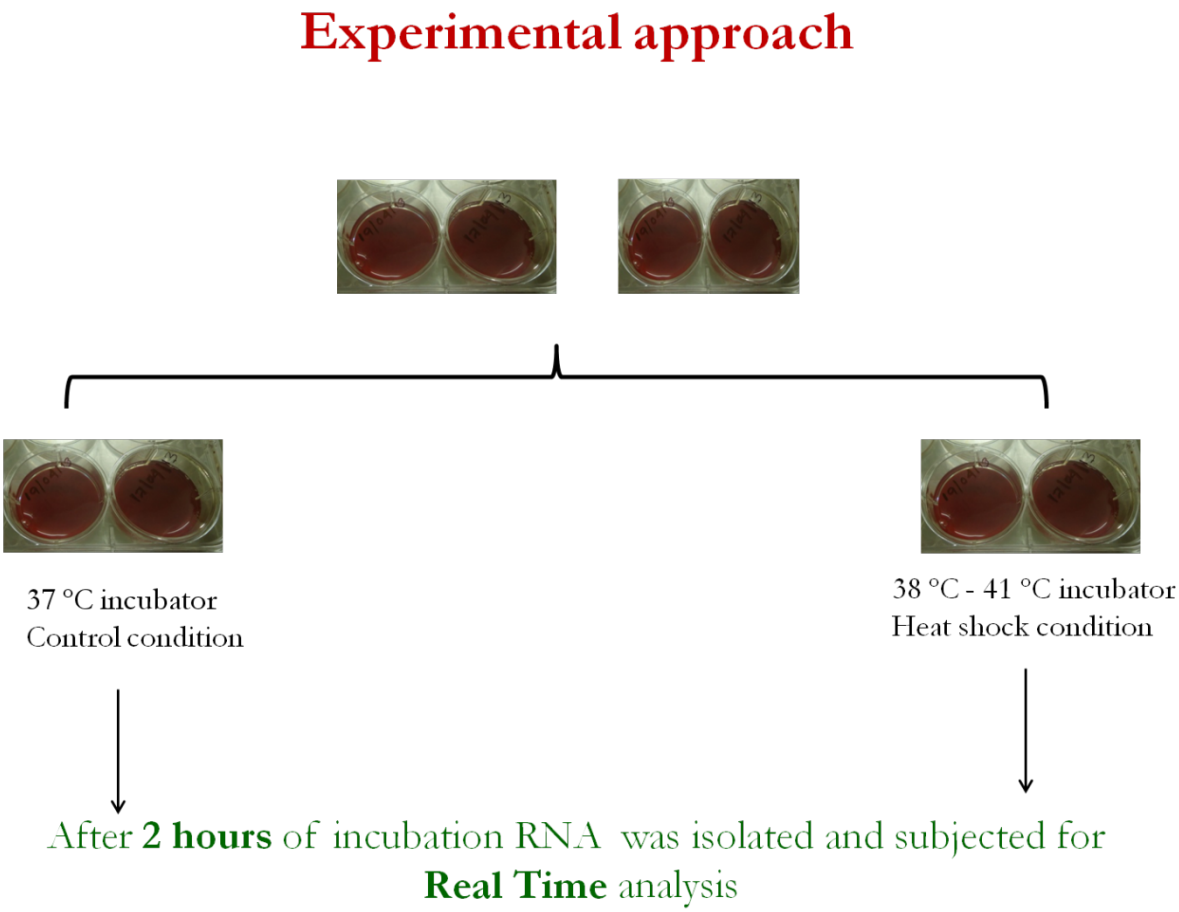


Figure 17: Illustration of the experimental approach to investigate temperature induced transcriptional regulation of *P. falciparum* genes.

4.2 Results:

4.2.1 Heat shock induces temperature dependent de-repression of telomere silencing in *P. falciparum* via down regulation of *PfSIR2* transcription:

It has already been established that in *S. cerevisiae* heat shock leads to de-repression of the telomeric gene by down regulating the Sir2 proteins [72]. Our fundamental question was whether heat shock or the febrile temperature has any role in the *var* gene regulation or if it leads to any alterations in the cellular pool of *Pf*Sir2 paralogues similar to that of *S. cerevisiae*. If yes, at what particular temperature is this effect observed as the body temperature ranging from 38ºC to 41ºC are commonly encountered during the febrile episodes in malaria [86]. The results presented here demonstrate that when synchronized ring stage parasite culture is exposed to heat shock for two hours, it leads to down regulation of transcript levels of both the *PfSIR2* paralogues i.e. *PfSIR2A* and *PfSIR2B* and this down regulation is temperature dependent. For this purpose ring stage synchronized parasite culture were divided into five parts: one part was taken as control which was grown at 37ºC and the remaining four parts were exposed to heat shock at 38ºC, 39ºC, 40ºC and 41ºC for two hours respectively. After the heat shock of two hours we isolated RNA from these cultures and subjected to Real Time RTPCR (Figure 17). The results demonstrate that there is a significant reduction in both *PfSIR2A* and *PfSIR2B* transcript level in heat stressed conditions compared to that culture growing at normal condition (37ºC). It was observed that this reduction in the transcript level of the *PfSIR2* paralogues was inversely proportional to the rise in temperature of the heat shock (Figure 18). This result establishes that heat shock leads to down regulation of *SIR2* transcript.

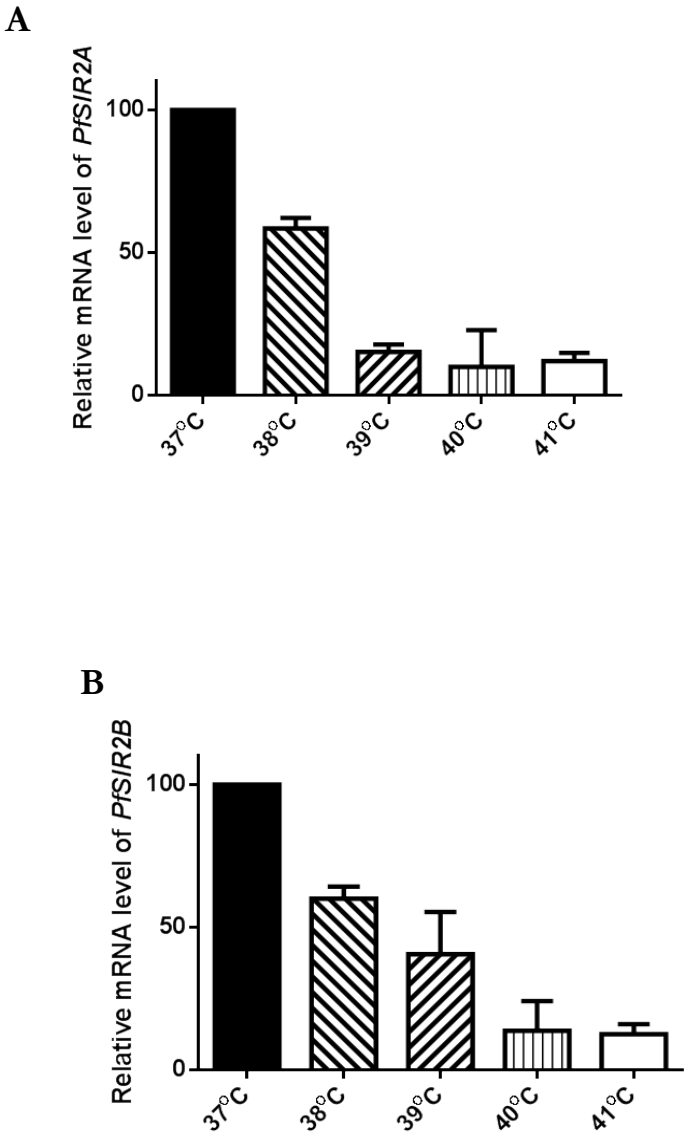


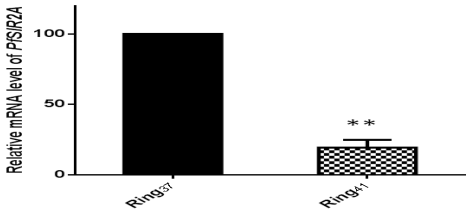
Figure 18: Temperature dependent down regulation of *PfSIR2* transcript.

(A) Synchronized ring stage parasite culture was divided into five parts: one part was grown at 37ºC and the remaining four parts were given a 2 hours heat shock of 38ºC, 39ºC, 40ºC and 41ºC temperature respectively. Real time RTPCR shows mRNA levels of *PfSIR2A* at different temperatures. Ref gene act as normalization control. Mean values (±SD) were taken from three individual experiments. (B) Real time RTPCR shows mRNA levels of *PfSIR2B* in the synchronized ring stage parasites at different temperatures.

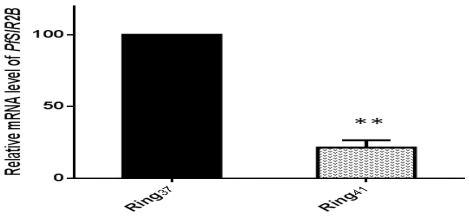
4.2.2 Heat shock induced down regulation of *PfSIR2* transcript is specific to only ring stage parasites:

Evidences indicate that the febrile temperature coincides with rupture of the schizonts and the release of the merozoites which infects fresh RBCs to form rings. Basically it is the young intra-erythrocytic parasites (ring stage) which are exposed to the febrile temperature during malaria. Since during the ring stage majority of the *var* genes are highly transcribed [85] strengthening our results which shows down-regulation of the *PfSIR2* transcript upon heat shock we wanted to investigate whether this down regulation is specific to only ring stage or the other stages of the parasite development. We synchronized the parasite culture, harvested it at different stages which are ring, trophozoite and schizonts and treated with heat shock of 41ºC for two hours. After which RNA was isolated and analyzed by Real Time RTPCR to observe the transcript levels of *PfSIR2A* and *PfSIR2B*. Interestingly we observe that the down regulation of both *PfSIR2A* and *PfSIR2B* transcript under heat stressed condition is specific to only ring stage. Our results clearly show that there is up to 5 fold down regulation of both the *SIR2* paralogues transcripts in *P. falciparum* culture under heat shock (Figure 19). Heat shock induced down regulation of the *SIR2* paralogues was not observed for trophozoites and schizonts.

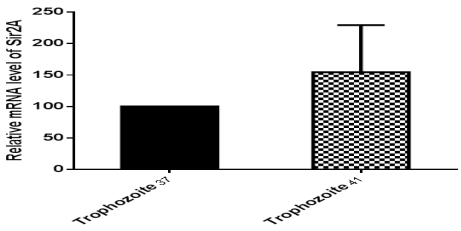
A


Relative mRNA level of PfSIR2A
100
50
0
* *
Ring37
Ring41

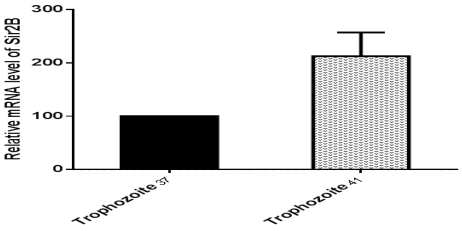
B


Relative mRNA level of PfSIR2B
100
50
0
* *
Ring37
Ring41

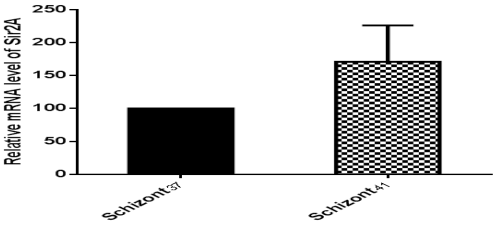
C


Relative mRNA level of Sir2A
250
200
150
100
50
0
Trophozoite37
Trophozoite41

D


Relative mRNA level of Sir2B
300
200
100
0
Trophozoite37
Trophozoite41

E


Relative mRNA level of Sir2A
250
200
150
100
50
0
Schizont37
Schizont41

F

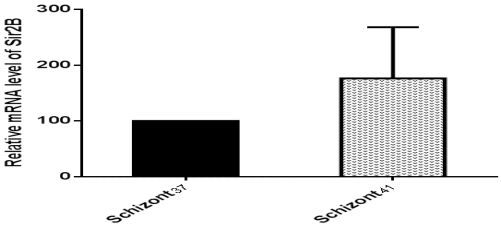

Relative mRNA level of Sir2B
300
200
100
0
Schizont37
Schizont41

Figure 19: Under heat shock condition of *P. falciparum* the down regulation of the transcript levels of *SIR2* is only specific for ring stage not for trophozoite and schizont stage.

(A) Real time RTPCR shows mRNA levels of *PfSIR2A* in the synchronized ring stage parasites subjected to heat shock of 41ºC temperature. (B) Real time RTPCR shows mRNA levels of *PfSIR2B* in the synchronized ring stage parasites subjected to heat shock of 41ºC temperature. (C) Real time RTPCR shows mRNA levels of *PfSIR2A* in the synchronized trophozoite stage parasites subjected to heat shock of 41ºC temperature. (D) Real time RTPCR shows mRNA levels of *PfSIR2B* in the synchronized trophozoite stage parasites subjected to heat shock of 41ºC temperature. (E) Real time RTPCR shows mRNA levels of *PfSIR2A* in the synchronized schizont stage parasites subjected to heat shock of 41ºC temperature. (F) Real time RTPCR shows mRNA levels of *PfSIR2B* in the synchronized schizont stage parasites subjected to heat shock of 41ºC temperature. For each experiment ref genes act as normalization control. Mean values (±SD) were taken from three individual experiments.

4.2.3 Down regulation of *PfSIR2A* and *PfSIR2B* transcripts upon heat shock is *Pf*Hsp90 mediated

4.2.3.1 Heat shock induces up regulation of *P. falciparum HSP90* family of genes

Our present study shows that there is down regulation of *PfSIR2* paralogues transcripts upon two hours of heat shock treatment to the synchronized ring stage parasite culture. In order to prove that this down regulation is mediated by *Pf*Hsp90 it was essential to show that *Pf*Hsp90 is over expressed in the heat shock treated parasite culture. Not surprisingly, Hsp90 and Hsp70, which have been implicated in heat shock response show increase in expression up on heat shock [81]. To confirm this we carried out Real Time RTPCR analysis by isolating RNA from control ring stage parasites which were grown at 37ºC and heat shocked induced (41ºC for two hours) ring stage parasite and observed the transcript levels of all the four family members of *PfHSP90* genes which include *PfHSP86, PfGRP94, PfTRRAP1-11* and *PfTRAP1-14*. As expected, our results showed the transcript levels of *PfHSP86, PfGRP94, PfTRRAP1-11* except *PfTRAP1-14* were significantly up regulated (Figure 20). To further confirm the over expression of *Pf*Hsp90 in the heat shock treated parasites we monitored its protein level by western blot analysis. Our results clearly demonstrate that there is at least 5 fold increases in the protein level of *Pf*Hsp90 in the control ring stage parasites compared to the heat treated ring stage parasites (Figure 21). *Pf*Actin served as the loading control.

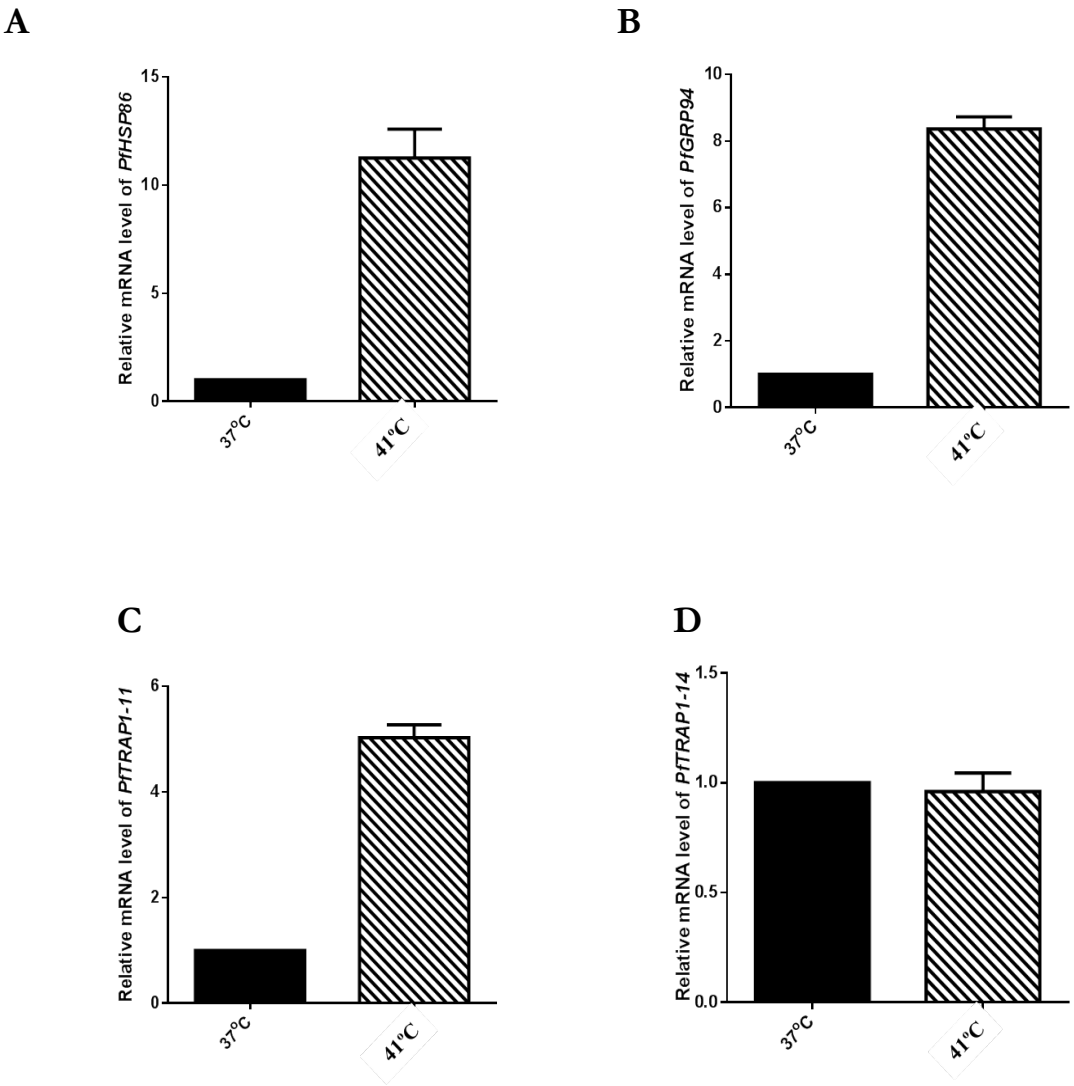


Figure 20: Heat shock induced up regulation of *P. falciparum HSP90* family of genes.

(A) Synchronized ring stage parasite culture was divided into two parts: one part was grown at 37ºC (control) and the other part was given a 2 hours heat shock at 41 º C temperatures. Real time RTPCR was done to quantify relative abundance of *PfHSP86* in ring stage parasites with out and with heat shock. Transcript levels were normalized by Ref gene transcript. Mean values (±SD) were taken from three individual experiments. (B) Similarly, Real time RTPCR was done to quantify relative abundance of *PfGRP94* in ring stage parasites with out and with heat shock. (C) Similarly, Real time RTPCR was done to quantify relative abundance of *PfTRAP1-11* in ring stage parasites with out and with heat shock. (D) Similarly, Real time RTPCR was done to quantify relative abundance of *PfTRAP1-14* in ring stage parasites with out and with heat shock.

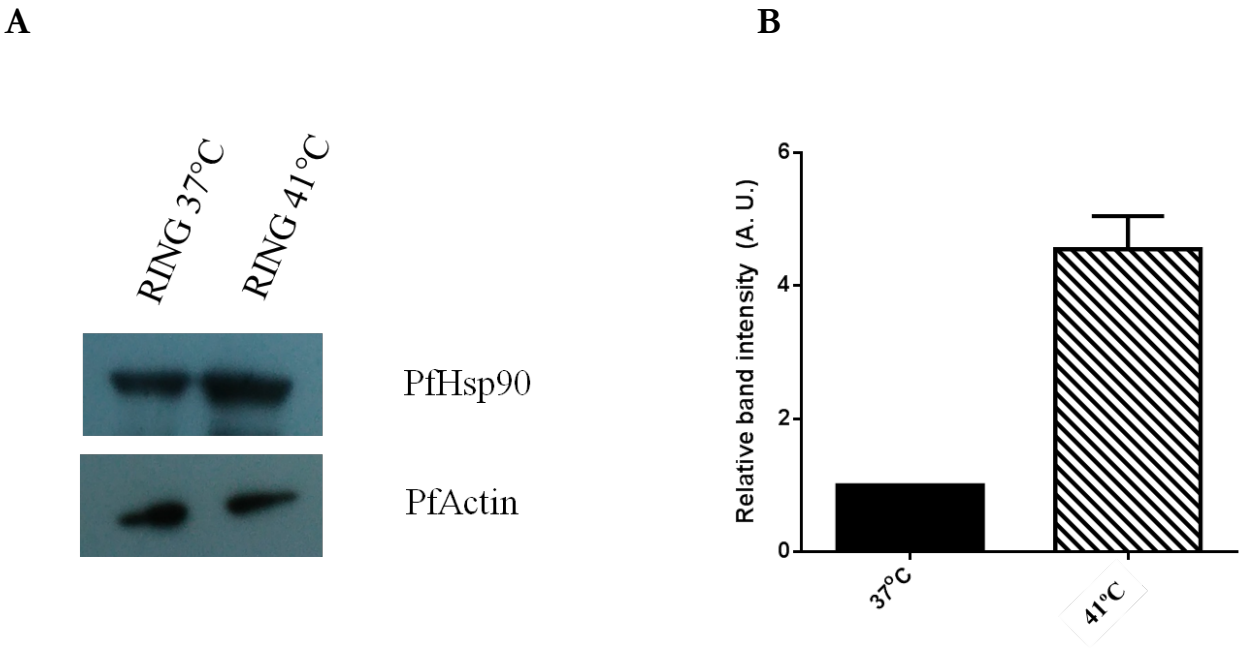


Figure 21: Under heat shock condition *Pf*Hsp90 (Hsp86) is over-expressed in *P.falciparum.*

(A) Western blot analysis to show *Pf*Hsp90 is over expressed in heat shock treated ring stage parasite. Actin was used as the loading control. The mean values ± SD provided from three individual expreriments.

(B) Quantification of western blot. Data was normalized against *Pf*Actin. *P* value was calculated using two tailed student's t- test.

4.2.3.2 Effect of 17AAG upon heat shock mediated down regulation of *PfSIR2A* and *PfSIR2B* transcripts

As heat shock leads to the down regulation of *PfSIR2A* and *PfSIR2B* transcripts, we monitored whether this down regulation is mediated by *Pf*Hsp90 by using Hsp90 inhibitor i.e. 17-(allylamino)-17-demethoxygeldanamycin (17 AAG). Hsp90 is the most abundant chaperon in the cell and is involved in protein folding, hence plays an essential role in functioning of a large number of proteins, especially those participating in cell cycle regulation and signal transduction. Geldanamycin (GA) and radicicol are natural antibiotics that have been demonstrated to compete with ATP for the binding to the N-terminal domain [87-88]. 17 AAG is a derivative of Geldanamycin [89]. Our findings clearly demonstrate that heat shock induced down regulation of the Sir2 paralogues is *Pf*Hsp90 mediated as we observe that upon 17 AAG treatment of the ring stage parasite culture there is no down regulation of *PfSIR2A* and *PfSIR2B* transcripts under heat shock conditions (Figure 22). We have used Real-time PCR to estimate the expression levels of *PfSIR2A* and *PfSIR2B* transcripts under different conditions. We have treated the synchronized ring stage parasite culture for 48 hours with 17 AAG by changing the medium for every 12 hours. The synchronized ring stage parasites were divided into four parts among which two parts were grown at 37ºC with and without 17 AAG treatment and the other two parts were given a heat shock of two hours with and without 17 AAG treatment respectively. After which the culture were harvested for RNA isolation and subjected to Real-time PCR analysis. Down-regulation of *PfSIR2A* and *PfSIR2B* transcripts were observed only in the heat shock induced culture without 17 AAG treatments. This result suggested that our finding is similar to that found in yeast where heat shock induced over-expression of Hsp90 leads to down regulation of *SIR2* transcript [72].

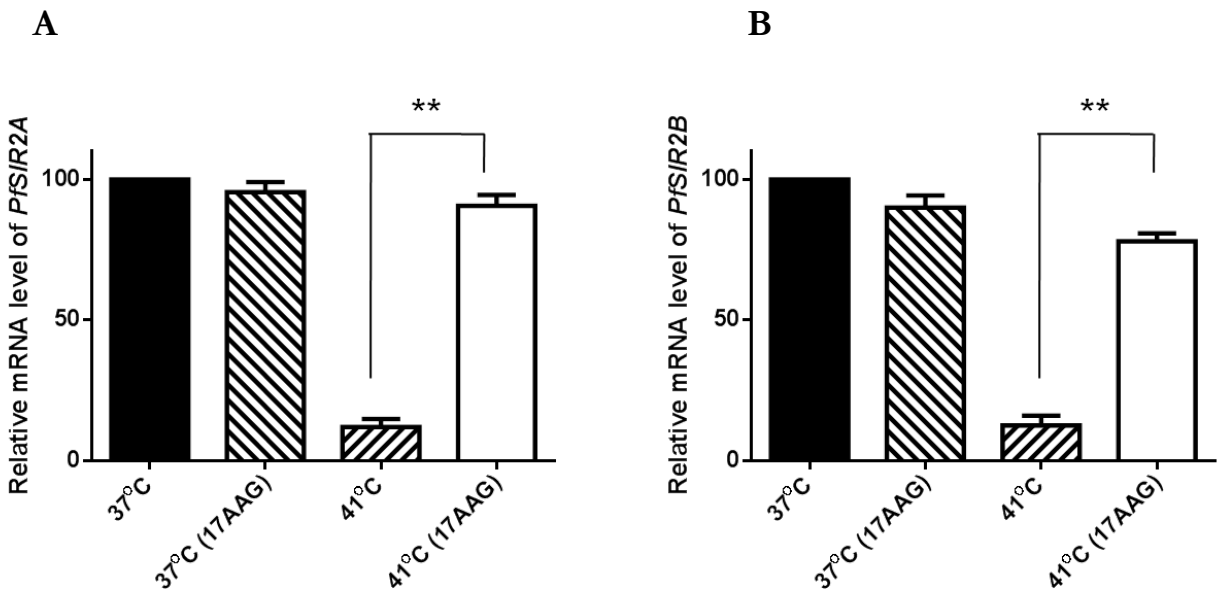


Figure 22: Down regulation of *PfSIR2A* and *PfSIR2B* transcripts upon heat shock is *Pf*Hsp90 mediated.

(A) Synchronized ring stage parasite culture was divided into four parts: one part was grown at 37ºC (control), the second part was treated with 17AAG for 48 hours and grown at 37ºC, the third part was given a 2 hours heat shock at 41ºC temperatures and the fourth part was treated with 17AAG for 48 hours and then subjected to a 2 hours heat shock at 41ºC temperatures. Real time RTPCR was done to quantify relative abundance of *PfSIR2A* transcript in ring stage parasites with above mentioned condition. Transcript levels were normalized by Ref gene transcript. Mean values (±SD) were taken from three individual experiments. (B) Similarly Real time RTPCR was done to quantify relative abundance of *PfSIR2B* transcript in ring stage parasites with above mentioned condition. (** means $P < 0.01$).

4.2.4 De-repression of majority of the *var* genes under heat shock condition

As our present study has already shown that heat shock treatment to the ring stage parasites leads to the down regulation of *SIR2* paralogues transcripts, we were further interested to investigate whether this heat shock induced down regulation of *PfSIR2* causes any change in its activity. In order to monitor silencing activity, we chose 10 *var* gene, 5 of which were under the control of *Pf*Sir2A (*VARA1, VARA2, VARA3, VARA4* & *VARA5*) and the other 5 under the control of *Pf*Sir2B (*VARB1, VARB2, VARB3, VARB4* & *VARB5*) respectively (Figure 23) and studied their transcription levels under heat shock condition. It was taken care that all the 10 *var* genes were selected mostly from different chromosomes in order to observe the silencing activity throughout the genome and that it's not restricted to a particular telomere. For this purpose we exposed synchronized ring stage parasite to 41ºC heat shock for 2 hours. We isolated RNA from these heat shock treated cells and the control cells and subjected to Real-time PCR to estimate the expression levels of the 10 *var* gene transcript. As expected we found that the silencing activity of both the *Pf*Sir2 paralogues are lost, since our results clearly indicate de-repression of all the 10 selected *var* genes (Figure 24 & 25). This result establishes that heat shock leads to lose of *Pf*Sir2 paralogues function at the telomeres indicating the possibility of its crucial role in *var* gene regulation. Finally we conclude that heat shock leads to up-regulation of the genes in the Hsp90 family, down-regulation of *PfSIR2* paralogues and de-repression of all the 10 selected *var* genes, suggesting that the febrile temperature of malaria may have some role in *var* gene regulation (Figure 26).

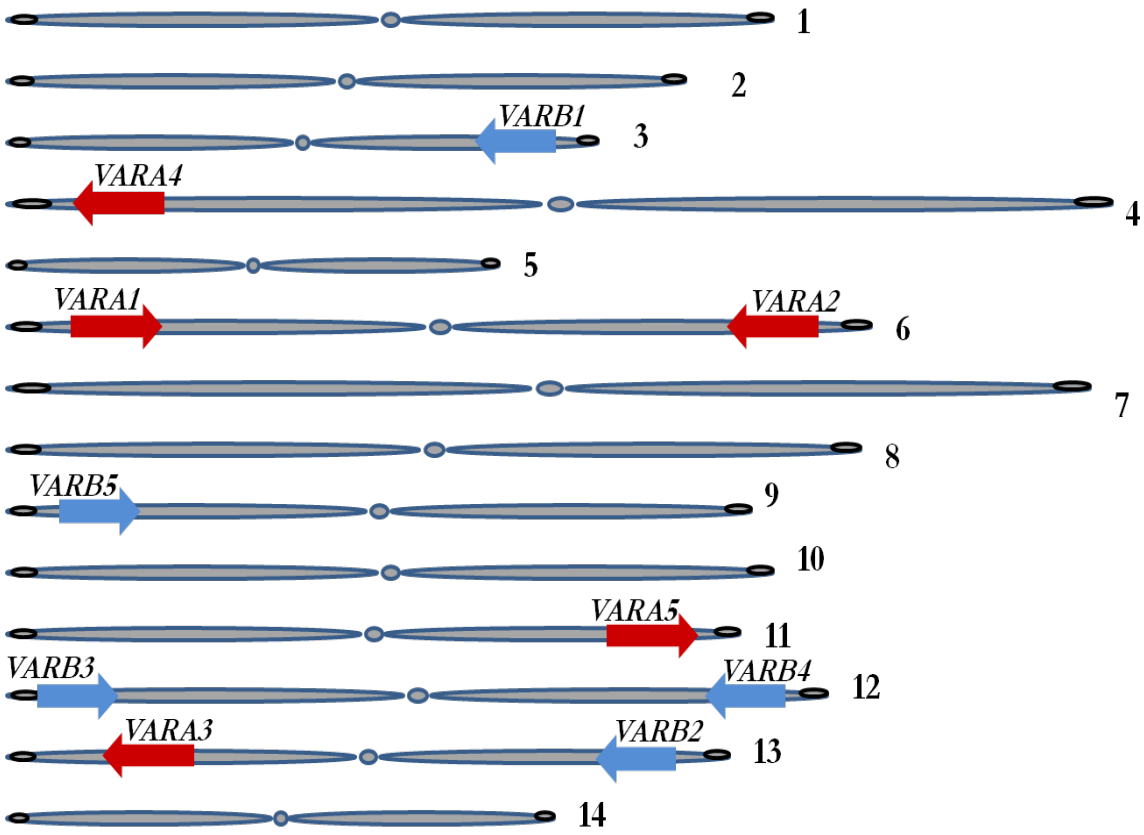
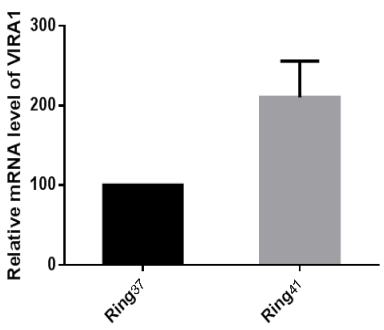
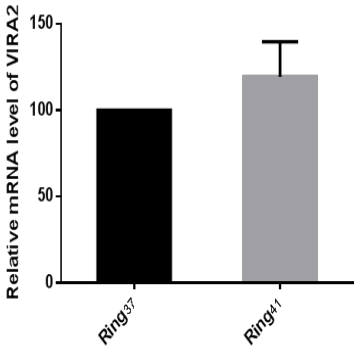


Figure 23: Schematic representation showing five selected *var* genes which are under the control of *Pf*Sir2A and *Pf*Sir2B

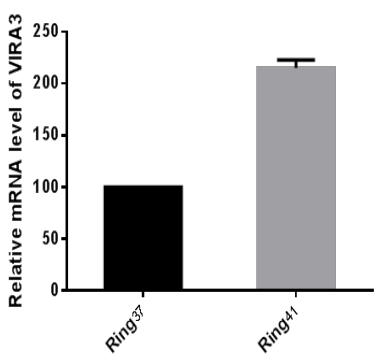
A


Relative mRNA level of VIRA1
300
200
100
0
Ring37
Ring41

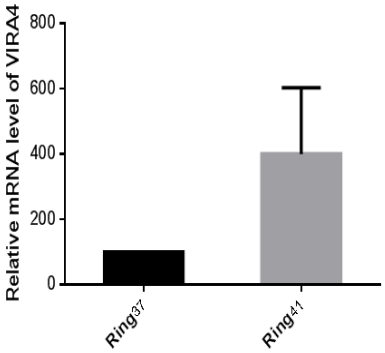
B


Relative mRNA level of VIRA2
150
100
50
0
Ring37
Ring41

C


Relative mRNA level of VIRA3
250
200
150
100
50
0
Ring37
Ring41

D


Relative mRNA level of VIRA4
800
600
400
200
0
Ring37
Ring41

E

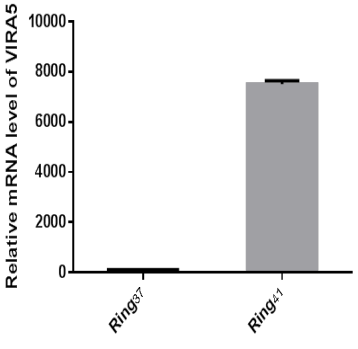
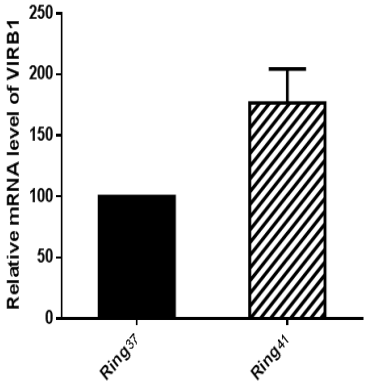

Relative mRNA level of VIRA5
10000
8000
6000
4000
2000
0
Ring37
Ring41

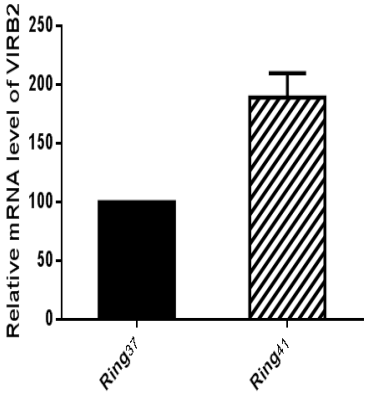
Figure 24: Heat shock induced up regulation of *P. falciparum VAR* family of genes that are under the control of *Pf*Sir2A.

(A) Synchronized ring stage parasite culture was divided into two parts: one part was grown at 37ºC (control) and the other part was given a 2 hours heat shock at 41 º C temperature. Real time RTPCR was done to quantify relative abundance of *VARA1* in ring stage parasites with out and with heat shock. Transcript levels were normalized by Ref gene transcript. Mean values (±SD) were taken from three individual experiments. (B) Similarly, Real time RTPCR was done to quantify relative abundance of *VARA2* in ring stage parasites with out and with heat shock. (C) Similarly, Real time RTPCR was done to quantify relative abundance of *VARA3* in ring stage parasites with out and with heat shock. (D) Similarly, Real time RTPCR was done to quantify relative abundance of *VARA4* in ring stage parasites with out and with heat shock. (E) Similarly, Real time RTPCR was done to quantify relative abundance of *VARA5* in ring stage parasites with out and with heat shock.

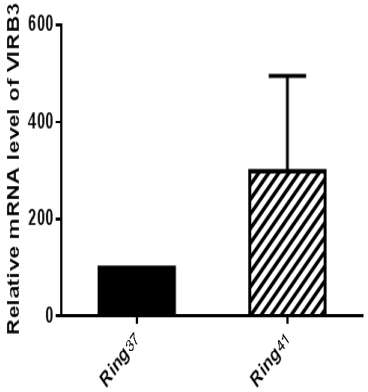
A


Relative mRNA level of VIRB1
250
200
150
100
50
0
Ring37
Ring41

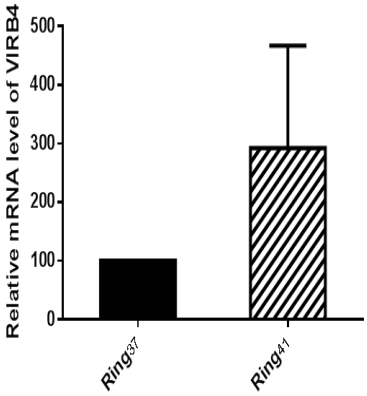
B


Relative mRNA level of VIRB2
250
200
150
100
50
0
Ring37
Ring41

C


Relative mRNA level of VIRB3
600
400
200
0
Ring37
Ring41

D


Relative mRNA level of VIRB4
500
400
300
200
100
0
Ring37
Ring41

E

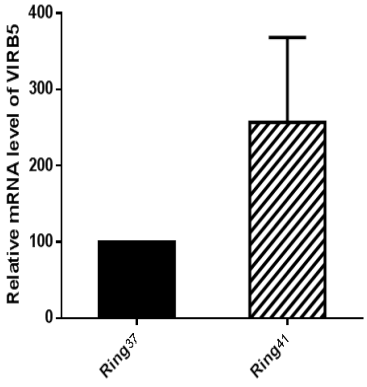

Relative mRNA level of VIRB5
400
300
200
100
0
Ring37
Ring41

Figure 25: Heat shock induced up regulation of *P. falciparum VAR* family of genes that are under the control of *Pf*Sir2B.

(A) Synchronized ring stage parasite culture was divided into two parts: one part was grown at 37ºC (control) and the other part was given a 2 hours heat shock at 41 º C temperature. Real time RTPCR was done to quantify relative abundance of *VARB1* in ring stage parasites with out and with heat shock. Transcript levels were normalized by Ref gene transcript. Mean values (±SD) were taken from three individual experiments. (B) Similarly, Real time RTPCR was done to quantify relative abundance of *VARB2* in ring stage parasites with out and with heat shock. (C) Similarly, Real time RTPCR was done to quantify relative abundance of *VARB3* in ring stage parasites with out and with heat shock. (D) Similarly, Real time RTPCR was done to quantify relative abundance of *VARB4* in ring stage parasites with out and with heat shock. (E) Similarly, Real time RTPCR was done to quantify relative abundance of *VARB5* in ring stage parasites with out and with heat shock.

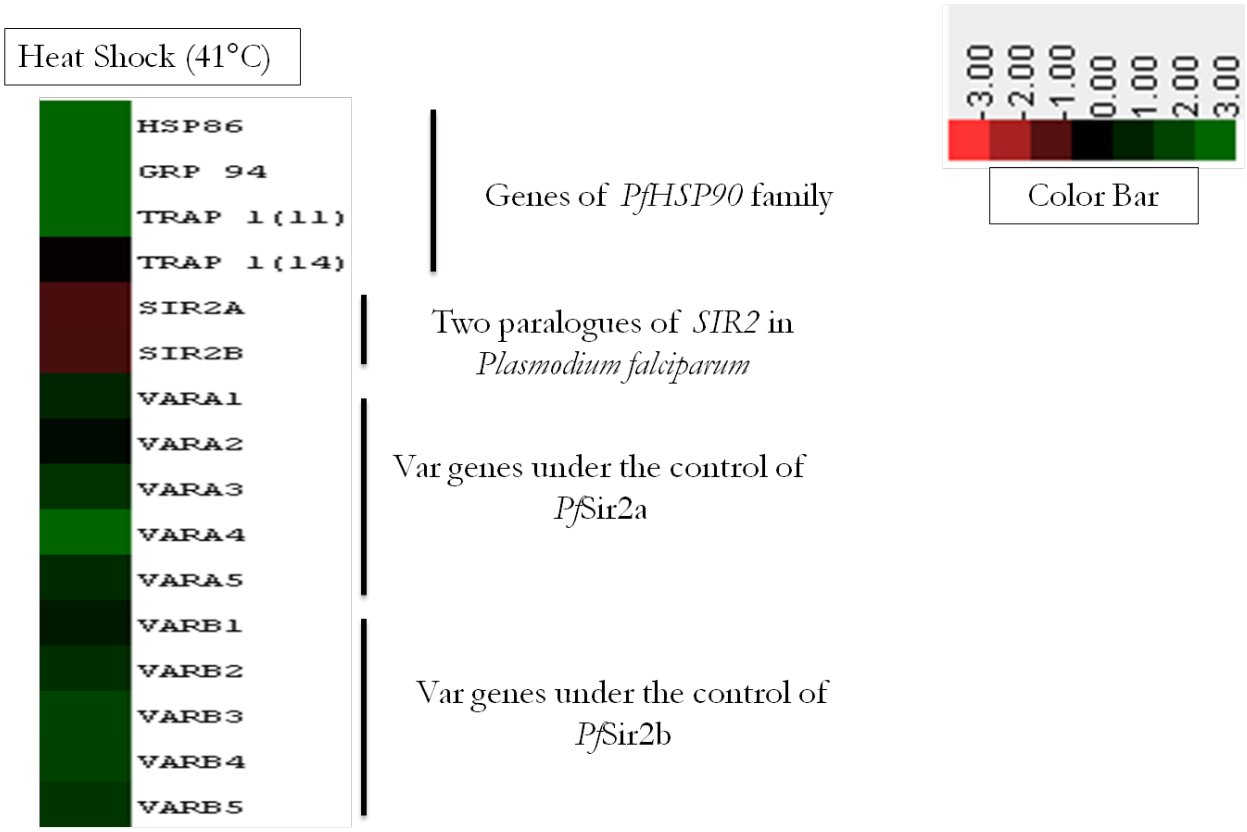


Figure 26: Heat-map representation of relative expression profiles of *P. falciparum* genes under heat shock condition (41°C for two hours).

4.2.5 Heat shock leads to trans-generational regulation of *PfSIR2A* and *PfSIR2B*

Previous studies have shown that changes in environmental temperature triggers epigenetic alterations in the individuals. Heat shock or osmotic pressure to the *Drosophila* leads to release of heterochromatin by phosphorylation of dATF-2 and this heterochromatic disruption was an epigenetic event that was trans-generational [90]. It was recently also reported that in yeast transient heat shock leads to trans-generational de-repression of sub-telomeric chromatin. Thus we focused on effects of heat shock over multi generations in *P. falciparum*. To this end, we exposed the synchronized ring stage parasite culture to heat stress at 41ºC for two hours and subsequently allowed them to grow at 37ºC. These heat stressed cells were maintained up to four generations with regular medium change for every 24 hours. In parallel, we maintained control parasite culture which was not subjected to any heat stress. We isolated RNA before the heat stress treated ring stage parasite culture (0^{th} hour), two hours post heat shock and thereafter at different intervals such as 10^{th} hour, 40^{th} hour (1^{st} generation), 50^{th} hour (2^{nd} generation), 100^{th} hour (3^{rd} generation) and 150^{th} (4^{th} hour generation). The RNA was used for cDNA preparation and subjected to Real Time RTPCR. The results are presented in Figure 27. Our study shows that heat shock mediated reduction in *PfSIR2A* transcript continued till the fourth generation where as for *PfSIR2B* continued till the second generation. The *PfSIR2A* transcript was observed to be reduced up to 10 folds, 5 folds and 2 folds in the 10^{th} hour, 3^{rd} generation and 4^{th} generation respectively, where as the *PfSIR2B* transcript was reduced up to 5 folds till the 2^{nd} generation and regained to the level comparable to that of unstressed culture in the 3^{rd} generation. It is important to note here that *PfSIR2A* transcript is unable to regain to the normal level when compared to the control parasite culture even in the fourth generation.

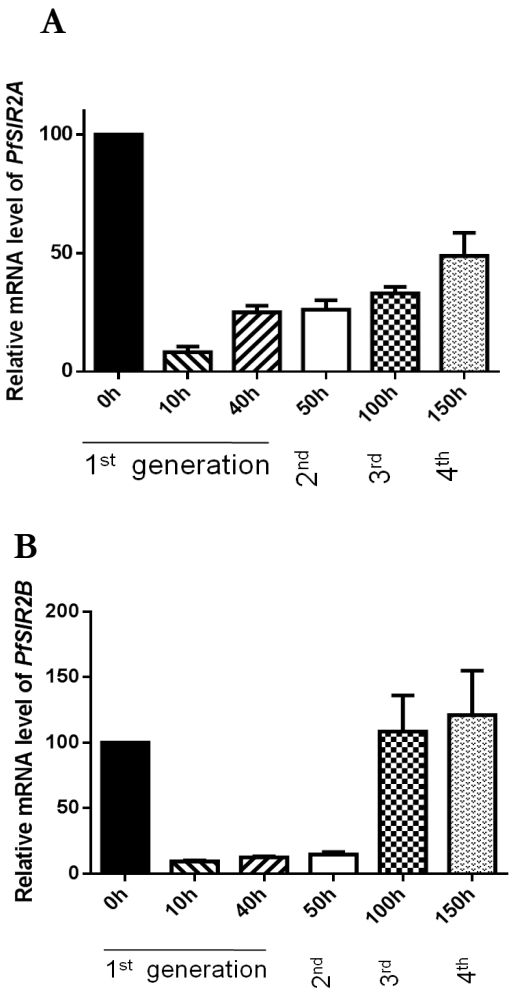


Figure 27: Transient heat shock leads to multi-generational transmission of disruption of heterochromatin in *P. falciparum*

(A)Relative mRNA levels of *PfSIR2A* in synchronized ring stage parasite culture grown at 37ºC (control) and synchronized ring stage parasite culture exposed to heat shock at (41 º C for 2 hours) at indicated time points are shown. Each bar represents the mean ±SD; n=3. (B)Relative mRNA levels of *PfSIR2B* in synchronized ring stage parasite culture grown at 37ºC (control) and synchronized ring stage parasite culture exposed to heat shock at (41 º C for 2 hours) at indicated time points are shown. Each bar represents the mean ±SD; n=3.

4.3 Discussion

Evolutionary evidence and laboratory data suggest that malaria fever has a profound significance that confers survival benefits for both parasite and human host. In this study we provide a very interesting link between the febrile temperature and immune evasion mechanism of *P. falciparum*. Firstly, our results clearly demonstrate that heat shock similar to that of malaria febrile temperature, to the synchronized ring stage parasite leads to down regulation of both *PfSIR2* paraogues at the transcript levels. We observe that there is a significant reduction in the transcript levels of both *PfSIR2A* and *PfSIR2B* when the ring stage parasites are exposed to temperature stress of 41ºC for two hours. We have also observed that as we increase the heat shock temperature from 38ºC to 41ºC there is decrement in the *SIR2* transcript levels. Malaria fever is initiated by the release of toxins due to the rupture of schizonts and the phagocytic cells; therefore it's the rings stage parasite which is exposed to the febrile temperature [76]. It is thus very important to understand whether the heat shock mediated down regulation of *PfSIR2* paraogues transcripts is specific to ring stage or other stage such as trophozoites and schizonts. According to our results, we observe that only in the ring stage there is reduction in *PfSIR2* paraogues transcripts under heat stress conditions and this effect is observed neither in trophozoites nor in schizonts. Our studies shows that under heat shock condition (41ºC), elevated levels of *PfHSP90* family genes leads to repression of *PfSIR2* transcription. To this end, we have shown both at transcription level and protein level that heat shock induces up regulation of *PfHSP90* family genes. It has been reported that during the malaria fever, in ring stage parasites the *Pf*Hsp90 levels are elevated [81] and there is de-repression of the *var* genes [85]. So the next question we asked was whether heat stress induced down regulation of *PfSIR2A* and *PfSIR2B* is mediated by *Pf*Hsp90. Using Hsp90 inhibitor 17 AAG, we have demonstrated clearly that Hsp90 over expression due to the heat stress results in reduced cellular pool of *SIR2* transcript, since upon 17 AAG treatment heat stress induced down regulation of *PfSIR* paralogues was not observed.

Secondly, our current data shows that heat stress induced down regulation of *SIR2* paralogues transcript leads to lose of its activity there by mimicking *sir2* knock out condition in *P. falciparum*. It has been reported earlier that two paralogues of Sir2 NAD+ dependent histone deacetylase family collaborate to achieve silencing of the whole *var* gene repertoire [44]. Here we report by Real Time RTPCR analysis that upon heat treatment of 41ºC for two hours to the synchronized ring stage

parasite majority of the *var* gene (under the control of both *Pf*Sir2A and *Pf*Sir2B) are significantly de-repressed. This is, to our knowledge first demonstration of heat stress mediated regulation of *var* gene repertoire.

Previously it has been reported in *Drosophila* that transient heat shock induces multi generational disruption of heterochromatin [90]. In another study, it was reported that transient heat shock to *S. cerevisiae* causes a drastic reduction in the *SIR2* transcript which results in sustained failure to initiate silencing for as long as 90 generations [84]. Thus it is important to understand whether transient heat stress leads to any trans-generational effect on cellular pool of *PfSIR2A* and *PfSIR2B* in *P. falciparum*. Our study shows that transient heat stress leads to trans-generational reduction of both the *SIR2* paralogues showing more severe effect on *PfSIR2A* similar to *Drosophila* and *S. cerevisiae*. Heat stress induced down regulation of *PfSIR2A* transcript was observed until the fourth generation where as *PfSIR2B* transcript regained to the normal levels similar to that of the control in the third generation. Taken together we propose a model that states that heat shock (due to fibril temperature) leads to up-regulation of Hsp90 protein, which in-turn results in the down-regulation of *Pf*Sir2A and *Pf*Sir2B with a concomitant de-repression of sub-telomeric *var* genes (Figure 28). Thus, our results provide a frame work for understanding how malaria parasite responds to environmental stress such as the febrile illness and might help in *var* gene regulation.

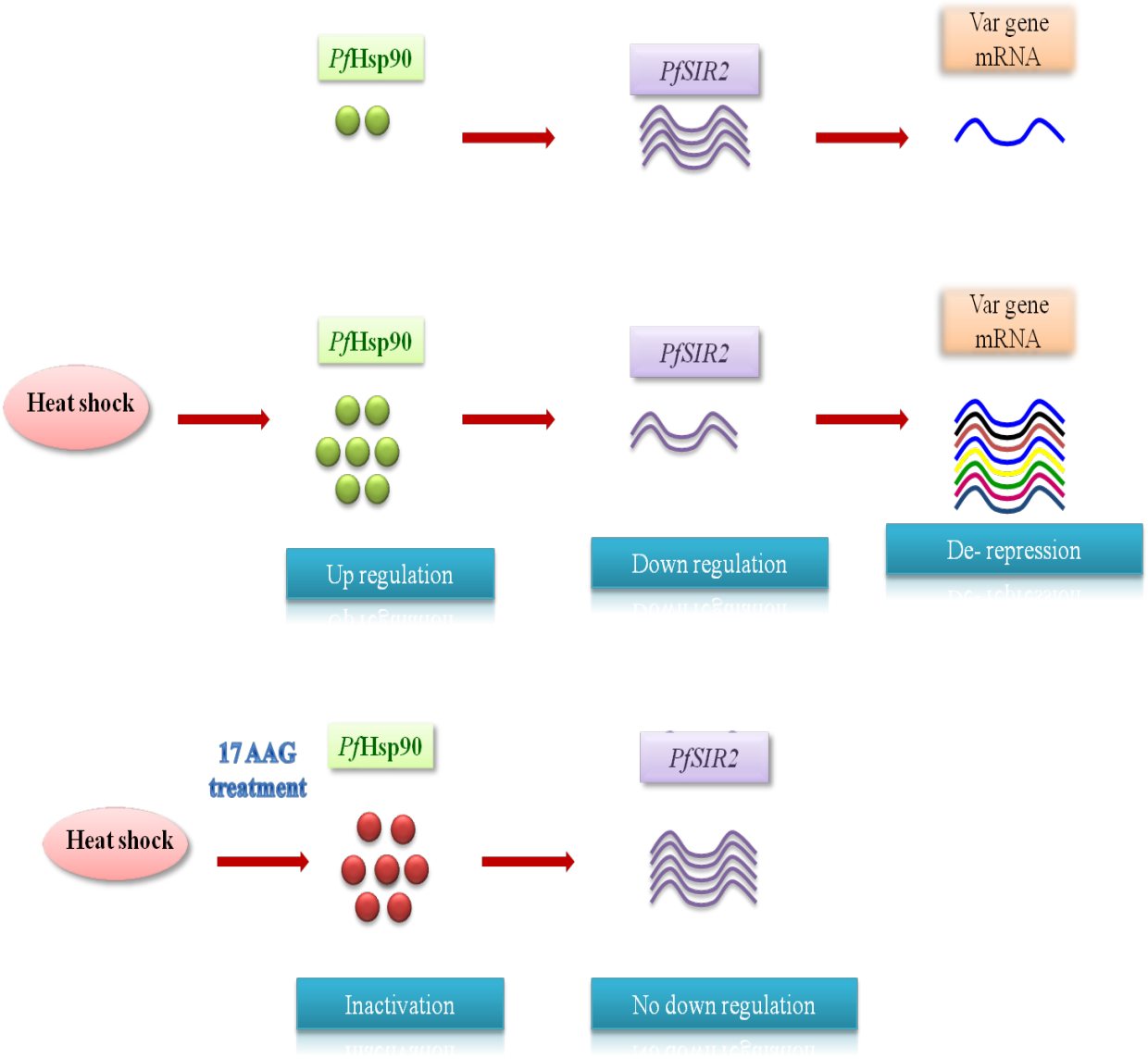


Figure 28: Schematic representation of a hypothetical model to show the connection between heat shock and *var* gene regulation

CHAPTER 5

DISCUSSION

6. Discussion:

In this study, we provide a mechanistic understanding of epigenetic regulation of *var* gene family, the most clinically relevant multicopy gene family, which encodes for the major surface antigen *Pf*EMP1 in *P. falciparum* [10, 41, 44, 91]. We have examined the molecular players involved in the sub-telomeric silencing in *P. falciparum* and the effect on it by environmental stress i.e. heat shock. So far, in *P. falciparum* several molecular players have been identified that are involved in the heterochromtin assembly such as *PfHP1, PfSIR2A, PfSir2B* and *PfKMT1*, but the role of *Pf*Orc1 was unclear. For the first time, our results provide direct evidence that *Pf*Orc1 has a Sir3/Orc1 like silencing function in a heterologous system. Our findings corroborates well with the previous studies which shows co-localization of *Pf*Orc1 and *Pf*Sir2 at silent sub-telomeric region [37]. Origin recognition complex subunit 1 (Orc1) is one of the largest subunit of replication origin recognition complex and plays crucial roles during replication initiation [92]. The C-terminal of *Pf*Orc1 has been shown to possess evolutionarily conserved replication function using yeast surrogate model [69]. It has been recently reported that *Pf*Orc1 and *Pf*Sir2 co-ordinate with each other and keeps an intricate balance for spreading heterochromatin like silencing zone leading to repression of sub-telomeric loci including *var* gene family [49]. Yet, the role of *Pf*Orc1 in sub-telomeric silencing remained cryptic. Firstly, if we consider *Pf*Orc1 to have a structural role in the sub-telomeric silencing, then over expression of its functional domain should lead to enhanced silencing activity (unless it has a dominant negative effect). Although, over expression of *Pf*Sir2 displayed increased silencing activity [43], it was not the same in case of *Pf*Orc1. Studies have shown that over expression of N-terminal domain of *Pf*Orc1 (1-238 amino acids) resulted in dominant negative effect by disrupting the complexes of heterochromatin proteins that are necessary for sub-telomeric silencing [49]. However, considering that appropriate amount of *Pf*Orc1 is required for the formation of silencing complex, this finding does not provide sufficient evidences for the role of *Pf*Orc1 in gene silencing. Secondly, some sub-telomeric loci are occupied by *Pf*Orc1 even in the absences of *Pf*Sir2 in Δ*sir2* knock out parasite line [43]; this confronts the notion that silencing function of *Pf*Orc1 is Sir2 mediated. Thirdly, either the full length or the N-terminal domain of *Pf*Orc1 does not show any physical interaction with *Pf*Sir2. We have used yeast surrogate system to study telomere silencing clinching many of these issues.

Our work gave compelling evidence that the N-terminal domain of *Pf*Orc1 possesses silencing activity. We have demonstrated that by expressing either full length *Pf*Orc1 or a fusion protein (chimera A) which consists of N-terminal domain of *Pf*Orc1 and C-terminal domain of *Sc*Sir3 in a Δ*sir3* knock out yeast strain, the sub telomeric silencing is restored back where the fusion protein was shown to be more efficient when compared to *Pf*Orc1.

It has been shown that in *S. cerevisiae* the N-terminal domain of Orc1 is involved in heterochromatin formation and the C-terminal domain is involved in replication. Despite the fact that *Sc*Orc1 and *Sc*Sir3 cannot complement each other functionally, a chimera generated by fussing N terminal domain of *Sc*Orc1 and *Sc*Sir3 is capable of performing the silencing function [70]. The N-terminal domain of *K. lactis* has also shown to be involved in silencing function. Also, our present study demonstrates that the N-terminal domain of *S. pombe* Orc1 is capable of performing Sir3 like silencing function. Therefore, it's clear that the N-terminal domain from a variety of organisms possess silencing function. On the contrary, all the orthologs of Orc1 possess a well defined BAH domain except for *Pf*Orc1, which is believed to be important for the silencing function. Despite lacking a canonical BAH domain how *Pf*Orc1 is able to perform the silencing function is not clear. We speculate that may be the three dimensional folding of the N-terminal domain mimics the BAH domain like structure.

According to our findings chimera A (consisting of N-terminal domain of *Pf*Orc1 and C-terminal domain of *Sc*Sir3) could silence the sub-telomeric region more efficiently compare to *Pf*Orc1. This can be explained by the specific contribution of C-terminal domain of *Sc*Sir3 in silencing which consist of AAA+ domain that is involved in engaging other silencing proteins (Sir4 and histones) [70]. The AAA+ domain of *Sc*Sir3 has evolved more rapidly than Orc1 proteins. Thus it could be possible that the C-terminal domain of *Pf*Orc1 is unable to interact with the other silencing proteins as that of C-terminal domain of *Sc*Sir3.

Though, *Pf*Orc1 lacks BAH domain, it consists of three unique low complexity regions (LC) and a leucine heptad repeat region (LHR) which are absent in other Orc1 orthologs. Our results showed a series of deletion mutants that lacked these low complexity regions (LC) and a leucine heptad repeat region (LHR) failed to complement Sir3 in Δ*sir3* knock out yeast strain suggesting that all these regions are necessary for its role in sub-telomeric silencing.

SIR3 is a gene duplication product of *ORC1* [67]. In yeast *K. lactis* Orc1 functions in conjunction with deacetylase Sir2 and the histone binding protein Sir4 to generate heterochromatin at the telomeres and mating type locus and it lacks Sir3 [64]. *Kl*Orc1 does both silencing and replication function. After Sir3 was duplicated from Orc1, Orc1 retained replication function and Sir3 specialized in silencing function in higher yeast such as *S. cerevisiae* [36]. *P. falciparum* diverged much before *K. lactis* and *S. cerevisiae,* and similar to *K. lactis* it lacks Sir3. Thus *P. falciparum* habours an ancient Orc1 which possess both replication and silencing function. We also established that complementation of Sir3 by chimera A or *Pf*Orc1 is Sir2 dependent.

*Pf*Hp1 was identified in *P. falciparum* that recognizes histone modifications [46]. It consists of Hp1 protein characteristic domain which is chromo-domain (CD) and chromo-shadow domain (CSD). Like in higher eukaryotes, CD of *Pf*Hp1 recognizes dimethylated or trimethylated H3K9 and CSD probably helps in homodimer formation [46]. Here, using yeast two hybrid system we found that *Pf*Hp1 and *Pf*Orc1 interact with each other similar to *Xenopus* and *Drosophila* and both CD and CSD are important for their interaction.

The hallmark of malaria fever is its characteristic periodical episodes of high temperature which is generally as high as 41ºC that last between two to six hours [81]. The molecular factors and biological pathways that trigger this temperature stress are unknown. We have studied the effect of this febrile temperature on the epigenetic regulation of the *var* gene silencing in *P. falciparum.* Studies have shown that heat shock as well as Hsp82 over expression in *S. cerevisiae* causes transcriptional down-regulation of *SIR2* [72]. We report that similar to *S. cerevisiae* in *P. falciparum* a transient heat shock for two hours at 41ºC to synchronized ring stage parasites leads to the down-regulation of both the paralogues of *PfSIR2* at the transcription level. Furthermore, we showed that heat shock mediated down-regulation of *PfSIR2A* and *PfSIR2B* transcripts are specific to only ring stage parasites and it is not observed in the trophozoite and schizoint stages. This coincides with the fact that the parasites are exposed to high febrile temperature during the rupture of schizoints or the formation of ring stage *in vivo* and there is derepression of majority of the *var* gene [79]. Our data establishes that the transcript level of *PfSIR2* paralogues is inversely proportional to the degree of rise in the heat shock temperature. It is worth noting that in *S. cerevisiae* the heat shock induced down-regulation of *SIR2* transcript is mediated by Hsp82 [72] and our data suggest that this might

also be the case for *P. falciparum* since we observed that by treating the synchronized ring stage parasites with Hsp90 inhibitor (17 AAG) there was no heat shock induced down-regulation of *PfSIR2* paralogues transcripts. Additionally we have shown that upon heat shock there is up-regulation of *Pf*Hsp90 family transcripts.

It is also interesting to note that the temperature stress affects the activity of *Pf*Sir2A and *Pf*Sir2B. Both the paralogues of *Pf*Sir2 NAD+ dependent histone deacetylases play a major role in *var* gene regulation and antigenic variation through mutually exclusive transcription of the full *var* gene repertoire [44]. *Pf*Sir2 paralogues have been shown to interact directly with the conserved *var* gene promoters [44]. Our study, by Real time PCR analysis revealed that temperature stress of 41ºC for two hours to synchronized ring stage parasites leads to significant de-repression of majority of the *var* gene transcripts that are under the control of *Pf*Sir2A or *Pf*Sir2B, suggesting loss of its activity. The present work provides valuable information on how the febrile temperature might benefit the parasite in *var* gene regulation and antigenic variation.

Transient heat shock has also shown to have transgenerational effect leading to disruption of heterochroatin in *Drosophila* as well as in *S. cerevisiae* [84, 90]. In *S. cerevisiae* transient heat shock causes a drastic reduction in the *SIR2* transcript which results in sustained failure to initiate silencing for as long as 90 generations [84]. Likewise, we report here that transient heat stress to *P. falciparum* leads to trans-generational effect on cellular pool of *PfSIR2A* and *PfSIR2B*. Our study showed that heat stress induced down regulation of *PfSIR2A* transcript was observed until the fourth generation where as *PfSIR2B* transcript regained to the normal levels similar to that of the control in the third generation. Further studies are required to understand as to how the parasites *in vivo* are able to replenish the *PfSIR2* paralogues transcript upon the febrile temperature stress. There might be various other factors, also involving host system that probably plays a crucial role in such mechanism. Thus the malaria fever might benefit the parasite survival in the host system.

In summary, our study sheds light on two major aspects in the *Plasmodium* biology. Firstly, we have shown that *Pf*Orc1 possess Sir3/Orc1 like silencing function which is Sir2 mediated and the N-terminal domain of *Pf*Orc1 is essential for its silencing activity. Our finding on *Pf*Hp1-*Pf*Oc1 physical interaction and *Pf*Orc1-*Pf*Sir2 genetic interaction predicts a step-wise assembly of *Plasmodium* silencing complex involving *Pf*Orc1, *PfHP1* and *Pf*Sir2. Secondly, we show that transient

heat shock leads to de-repression of the sub-telomeric *var* genes as there is down-regulation of *Pf*SIR2 transcription thereby mimicking *sir2* knockdown condition in *P. falciparum* which is *Pf*Hsp90 mediated. Thus our data suggests that malaria fever has a far reaching biological significance that confers survival benefits for the *Plasmodium* parasite.

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APPENDIX

SYNOPSIS

Malaria is one of the most deadly diseases caused by an obligate intracellular protozoan parasite of the genus *Plasmodium*, with an estimated 3.3 billion people at risk of the disease. There are five species of *Plasmodium* that can infect and be transmitted to humans, which are *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium knowlesi,* among which *P. falciparum* accounts for majority of the malaria morbidity and mortality (1). *P. falciparum* has a very complex life cycle where in it shuttles between two hosts i.e. warm blooded humans and cold blooded mosquito vector. In order to complete its life cycle, the parasite has to invade different types of cells and self propagate in distinct environment in both humans and mosquitoes. Each of these environments exerts distinct selective pressure on the parasite in order to fight the parasite, thus forcing it to exhibit differential gene expression (2-4). It has been observed by transcriptome analysis that the parasite displays tightly coordinated cascades of gene expression, although the molecular mechanism involved in these regulation are poorly understood (3-4). Searches also indicate a paucity of recognizable specific transcription factor in the proteome of the parasite suggesting that epigenetic regulation plays a crucial role in the control of gene expression (5). Many post translational modifications have been identified in malaria parasite among which phosphorylation, acetylation, methylation and lipidation are extensively studied. Phosphorylation and lipidation have shown to play a crucial role in host cell invasion, egress and transition to different developmental stages where as histone acetylation and methylation are involved in regulation of expression of *P. falciparum* Erythrocyte membrane protein 1 (*PfEMP1*) which is a major virulence factor (6). *PfEMP1* are encoded by a family of genes know as *var* genes and are expressed on the surface of the infected erythrocytes (7). There are 59 copies of *var* genes per haploid genome that encodes for *Pf*Emp1 protein with a molecular weight around 200 – 350 kDa (10, 11). These proteins adhere to the host endothelium receptors (12) leading to sequestration of the iRBC in the deep tissues (9) and are involved in evading the host immune system by varying the antigenic and adhesive character of parasitized erythrocyte, thus undergoing antigenic variation (8, 9). The phenotype of adherence have evolved in parasite to prevent the passage of the infected cell through spleen, where they would be recognized as foreign and eliminated from the circulation (9). The mechanism by which a single *var* gene is expressed at a time and the remaining ~60 *var* genes are maintained at transcriptionally silent state is still to be elucidated. Similar to other organisms the

highly variable gene families of the parasite are clustered towards the telomeres (13). Majorly there could be two reasons for the location of the *var* genes at the sub-telomeres one being that telomere silencing occurs at the sub telomeric region and the other could be the possibility of Recombination due to the clustering of the telomeres.

There are two types of eukaryotic gene silencing complex which include Sir2-containing gene silencing complexes that has been studied in budding yeast, and the Hp1 and Swi6 complexes that mediate silencing present in metazoans and fission yeast, respectively (14). The *var* gene silencing involves the Sir complex as *PfSIR2* gene disruption leads to de-repression of these genes (15). Studies have shown that conditional depletion of *Pf*Hp1disrupts mutually exclusive expression and antigenic variation of the major virulence factor *Pf*EMP1 (28).

In this work two major questions have been addressed. Firstly, what are the molecular players or events that regulate the expression of variable antigen and secondly how are these events regulated in response to physiological cues?

Orc1 besides playing an important role in replication also interacts with heterochromatin proteins in many organisms (16). In another member of yeast family *Kluyveromyces lactis* Orc1 plays a Sir3 like function during silencing at telomere and such activity is dependent on Sir2 and Sir4 (17). Interestingly, Sir3 has come from gene duplication of Orc1 (21).So far *Pf*Sir2 and *Pf*HP1 are the only proteins to be characterized which are known to be involved in heterochromatin formation in *Plasmodium* (18, 19). Different experiments like Immunofluorescence assays, EMSA and Chip have shown that *Pf*Orc1 co-localizes with *Pf*Sir2 and interacts with telomeric and sub-telomeric elements making *Pf*Orc1a strong candidate for contributing to telomeric silencing in *P. falciparum* (20). Thus to address the first objective we hypothesized that *Pf*Orc1 might be involved in Sir3 like silencing function in *Plasmodium* since it lacks Sir3 but has *Pf*Orc1. We generated different chimeras of *Pf*Orc1 and *St*Sir3 to study the complementation of *Pf*Orc1 protein using a yeast surrogate system that lacks *St*Sir3 and observed that Chimera A which has N-terminal *Pf*Orc1 and C-terminal *St*Sir3 is able to complement *St*Sir3 in *scsir3* null back ground where as Chimera B which has N-terminal *St*Sir3 and C-terminal *Pf*Orc1 is unable to do so. We also observed that the silencing activity of Chimera A was Sir2 mediated since *sir2* knock out leads to de-repression of subtelomeric genes in Chimera A background indicating lose of silencing activity of Chimera A. *Pf*Orc1 N terminal domain lacks BAH

domain, but contains one leucine heptad repeat (LHR) and three low complexity regions which are rich in lysine and asparagines. We generated three different deletion mutants of Chimera A to carry out the structure function analysis of *Pf*Orc1N terminal domain. First deletion mutant is devoid of the N-terminal extension of 70 amino acids, but retains all the three low complexity regions as well as the LHR, the second mutant lacks 110 amino acids where in the first low complexity region is lost and the third mutant is devoid of the first two low complexity regions and the LHR (Δ210 amino acid). By telomere position effect assay we observed that Chimera A Δ70 is fully capable of restoring the silencing activity in Δ*sir3* strain where as chimera A Δ110 and chimera A Δ210 is unable to silence the subtelomeric genes proving that the N-terminal extension is dispensable for the silencing function while the low complexity regions and the LHR are important. Thus our data strongly support that *Pf*Orc1 plays a crucial role in telomeric silencing in *P. falciparum.*

In the second part of the first objective, we wanted to investigate the inter play between *Pf*Hp1 and *Pf*Orc1 mediated gene silencing by studying the interaction between *Pf*Hp1 and *Pf*Orc1. *Pf*Hp1 is known to control antigenic variation in *P. falciparum* by heterochromatin formation of subtelomeric non-coding repeat regions and mono allelic expression of the major virulence *var* gene family (19). It has been shown that in *Drosophila* and *Xenopus* Hp1 interacts with N-terminal domain of Orc1 and also in human cells Orc1 interacts with Hp1. Thus we wanted to know if *Pf*Orc1 and *Pf*Hp1 interact. The interaction between the proteins was studied by using yeast two hybrid system where in pJ694a strain was used. We cloned *Pf*Orc1 in the prey vector and *Pf*Hp1 in the bait vector and carried out the yeast two hybrid assays. We observed that *Pf*Orc1 and *Pf*Hp1 were able to interact with each other similar to *Drosophila, Xenopus* and humans. *Pf*Hp1 amino acid sequence analysis revealed the presences of two characteristic HP1 domains: Chromo domain and Chromo shadow domain. Chromo domain binds to di- and tri-methylated lysine 9 from histone 3 and chromoshadow domain is known to be involved in homo dimmer formation (19). To investigate which of these domains are important for interaction of *Pf*Hp1 and *Pf*Orc1, we generated two deletion mutants lacking either of the chromo domain or the chromo shadow domain. Our data suggest that both the domains are important for the interaction of *Pf*Hp1 and *Pf*Orc1 as both Δchromo domain *Pf*Hp1 and Δchromo shadow domain *Pf*Hp1 were unable to interact with *Pf*Orc1.

In the second objective we wanted to investigate how are the expressions of the variable antigen regulated in response to physiological cues like heat shock. Sue Kyes et al have shown that there is a clear stage specific regulation of the *var* genes. Northern blot analysis proved the presence of high steady state levels of the *var* gene transcripts during the ring stage, which rapidly declines to undetectable levels in trophozoite and schizont stages (22). Though the *var* gene transcript is detected during the early intraerythrocytic cycle of the parasite, *Pf*Emp1 protein is detected on the surface of the RBC only until the late trophozoite and the schizont stage (23). Studies on the nature and the structure of the *var* gene transcription so far has been limited. The most common feature of malaria is the cyclic fever with unique pattern which coincides with the rupture of schizoint and the ring stage (24). We proposed that there might be a link between the high temperature and the *var* gene de-repression which is ring stage specific. A clue came from S Laskar et al work where in it has been shown that over-expression of yHsp90, either by exposing cells to heat shock or by introducing yHsp90 over-expression plasmid yields reduced level of Sir2, with a consequential loss of telomere silencing (25). Later they showed that heat shock or yHsp90 over-expression causes up-regulation of a specific transcriptional repressor Cup9 that, in turn, represses *SIR2* transcription by binding to its upstream activator sequence (26). Thus we hypothesized that similar kind of mechanism could also be occurring in *P. falciparum* where in over-expression of Hsp90 or heat shock might yield reduced level of *SIR2* and there by leading to *var* gene de-repression. We have observed that similar to yeast there is down-regulation of the transcript levels of both the *SIR2* paralogues of *P. falciparum* i.e *PfSIR2A* and *PfSIR2B* when the parasites are given a transcient heat shock of 41°C for 2 hours which is specific to only ring stage. Real time PCR analysis shows about 5 fold reduction in the transcript levels of both *PfSIR2A* and *PfSIR2B* under the mentioned condition when compared to the wild control un-treated parasites. To study the effect of heat shock on the activity of the Sir2 paralogues we observed the transcript levels of *var* genes that are under the control of *Pf*Sir2A and *Pf*Sir2B. Our results suggest that upon heat shock there is de-repression of all the 10 *var* genes which are under the regulation of *Pf*Sir2A and *Pf*Sir2B. Using both western blot analysis and Real time PCR we have observed that there is up regulation of *Pf*Hsp90 upon heat shock of 41°C for two hours to the parasites. To understand if the down regulation of *PfSIR2A* and *PfSIR2B* transcript level is mediated by *Pf*Hsp90 over expression we treated the parasites with Hsp90 inhibitor 17AAG and than observed the effect of heat shock. Our data clearly suggests that the

down regulation *SIR2* transcripts is *Pf*Hsp90 mediated as no down regulation upon heat shock was observed when treated with 17AAG. Most interestingly, it was observed that transient heat shock mediated down regulation of *SIR* transcript was trans-generational. Our data suggest that the trans-generational effect was more on *PfSIR2A* transcript when compared to *PfSIR2B*. Our study shows that transient heat shock (41°C for 2 hours) mediated down regulation of *PfSIR2A* transcript is maintained even till the fourth generation with gradual increase in the transcript level from 5 fold down regulation to 2 fold down regulations where as *PfSIR2B* transcript is down regulated up to the 2nd generation and returns back to the normal state by the 3rd generation.

In summary, studies from our first objective provides a direct evidence that the N-terminal of *Pf*Orc1 is able to perform the Sir3 like silencing function in Δ*sir3* yeast strain and showed that there is more effective complementation of the fusion protein chimera A (N-terminal *Pf*Orc1 and C-terminal *Sc*Sir3) than *Pf*Orc1 itself (27). Additionally, our yeast two hybrid data shows that *Pf*Orc1 is likely to silence the subtelomeric gene by interacting with *Pf*Hp1. Studies from our second objective shows a very interesting link between the environmental stress (heat stress) and the *var* gene regulation. These results help us to understand to some extent the role of the fibril temperature in modulating antigenic variation of the variable antigens in *P. falciparum.* As understanding antigenic variation is critical in fight against malaria parasite.

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Plasmodium falciparum origin recognition complex subunit 1 (PfOrc1) functionally complements Δ*sir3* mutant of *Saccharomyces cerevisiae*

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ABSTRACT

Telomere position effect efficiently controls silencing of subtelomeric *var* genes, which are involved in antigenic variation in human malaria parasite *Plasmodium falciparum*. Although, PfOrc1 has been found to be associated with PfSir2 in the silencing complex, its function in telomere silencing remained uncertain especially due to an apparent lack of BAH domain at its amino-terminal region. Here we report that PfOrc1 possesses a Sir3/Orc1 like silencing activity. Using yeast as a surrogate organism we have shown that PfOrc1 could complement yeast Sir3 activity during telomere silencing in a Sir2 dependent manner. By constructing a series of chimera between PfOrc1 and ScSir3 we have observed that the amino-terminal domain of PfOrc1 harbors silencing activity similar to that present in the amino-terminal domain of ScSir3. We further generated several amino-terminal deletion mutants to dissect out such silencing activity and found that the first seventy amino acids at the amino-terminal domain are dispensable for its activity. Thus our results strongly supports that PfOrc1 may have a role in telomere silencing in this parasite. This finding will help to decipher the mechanism of telomere position effect in *P. falciparum*.

1. Introduction

The human malaria parasite *Plasmodium falciparum* diminishes the immune clearance by the host through mutually exclusive expression of variant surface molecules. It has been speculated that such allelic exclusion of gene expression helps in the establishment of prolonged chronic infections and is likely to be controlled by telomere silencing mechanism [1,2]. However, the underlying molecular mechanism of such telomere position effect (TPE) is only poorly understood in this protozoan parasite. *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is a major determinant of antigenic *var*iation in this parasite. PfEMP1 is encoded by the members of *var* gene family. Out of the sixty *var* genes that encode PfEMP1 proteins, only one of them is expressed at a given time and the rest of them remain transcriptionally silent [3]. Sir proteins (Silent Information Regulators) mediated heterochromatinization plays a central role in such regional silencing of sub-telomeric genes. Knockout studies have shown that subtelomeric *var* genes are de-repressed in Sir2 deficient *P. falciparum* line [1]. Furthermore, chromatin immunoprecipitation (ChIP) experiments have revealed that the upstream regions of active *var* genes are associated with acetylation of histone H3 and H4, whereas the histones associated with silenced *var* genes are hypoacetylated [2].

In the budding yeast *Saccharomyces cerevisiae* transcriptional silencing at the telomere-adjacent region (also known as TPE) initiates at specific "silencer" sequences and consequently spreads over nearby sequences through intimate interactions between silencer proteins and specifically modified histone proteins. TPE is a stepwise orderly phenomenon that begins with the recruitment of Sir2/Sir4 structural complex by telomere binding protein Rap1 (or the end binding hetero-dimeric proteins yKu70/80) [4]. Deacetylation of neighboring histone proteins by Sir2 promotes Sir3 binding, which in turn recruits additional Sir2/Sir4 complex. Thus the unidirectional (away from chromosomal ends) Sir2 action and spreading of deacetylated histone proteins along the length of chromatin fiber is stabilized by Sir3–Sir4 interaction [4,5]. Such spreading is greatly facilitated by over expression of Sir3 protein via a poorly understood mechanism [6,7]. Sir complex mediated silencing is also observed at the cryptic mating loci and at the rDNA locus in budding yeast [8]. Physical interactions have been observed between Sir2 and Sir4, between Sir3 and Sir4, and of deacetylated histones (H3 and H4) with both Sir3 and Sir4. However, neither in vivo nor in vitro interaction has been found between Sir2 and Sir3 in any context [9].

Besides defining the origins of replication in eukaryotes, the largest member of origin recognition complex (Orc1) also interacts with heterochromatin proteins in many organisms [10–12].

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Orc1 is also part of the protein complex that participates in the assembly of telomeric heterochromatin in *Drosophila* and *Xenopus* [12,13]. Additionally, telomeric and pericentromeric heterochromatin occupancy was reported for human Orc1, which also bind to HP1 (heterochromatin protein 1) [14–16]. In budding yeast *S. cerevisiae* Orc1 has limited but distinct role only during mating type silencing. It is involved in recruitment of Sir1 at the cryptic mating loci [17,18]. Furthermore, it has been demonstrated through tethered silencing assay that ScOrc1 is capable of doing a Sir3 like function at budding yeast telomeres [9]. In another member of yeast family *Kluyveromyces lactis* Orc1 plays a Sir3 like role during silencing at telomere as well as mating type loci and such activity is dependent on Sir2 and Sir4 [19]. The N-terminally located BAH domain of Orc1 that binds to nucleosomes plays an indispensable role during transcriptional silencing [19–21]. Sir3 has arisen from Orc1 after gene duplication and the BAH domain of Sir3 and Orc1 share high degree of homology [22]. In yeast subfunctionalization of Orc1/Sir3 has been observed after gene duplication event, where Orc1 retained the origin recognition function during replication and Sir3 specialized in silencing function [23]. On the other hand, in *K. lactis*, which diverged from *S. cerevisiae* before the duplication and thus lacks Sir3, both replication and silencing functions are performed by KlOrc1 [19,24].

So far Sir2 is the only member of the Sir family of proteins whose function in telomere silencing has been characterized in *P. falciparum* [25–28]. PfOrc1 has also been identified and its role during replication has been elucidated [29]. Immunofluorescence assays have shown that PfOrc1 co-localizes with PfSir2 at telomeric clusters [30]. Also, EMSA and ChIP assays have been used to demonstrate that PfOrc1 can specifically interact with telomeres and subtelomeric elements similar to PfSir2, making PfOrc1 a strong candidate for contributing to telomeric silencing in *P. falciparum* [30]. Furthermore, it has been shown that the N-terminal 238 amino acids of PfOrc1 are necessary and sufficient for its co-localization with PfSir2 as well as occupancy at distinct subtelomeric loci [31]. However, whether or not PfOrc1 plays any role in TPE has not been established. Notably, the apparent absence of a BAH domain in PfOrc1 questions its involvement in telomere silencing, because the BAH domain has been reported to be critical for the silencing function of Sir3 and Orc1 in other organisms [32]. Notably no interaction between PfOrc1 and PfSir2 is observed in yeast two-hybrid assays ([33], our unpublished data) and in GST-pull down assay [31].

PfORC1 being an essential gene (indispensable for *Plasmodium* replication) it is not possible to knockout *PfORC1* and then look for loss of silencing function. Experiments involving genetic manipulation leading to the generation of separation of functions mutant, in which only the silencing function could be abrogated retaining the replication function, is technically difficult to carry out in *P. falciparum*. We propose that complementing a known *SIR3* function by *PfORC1* could be a feasible way to test whether PfOrc1 has any Sir3 like silencing activity. Here we report for the first time that PfOrc1 possesses silencing function and can complement yeast Sir3. We also show that such complementation is dependent on Sir2 function.

2. Materials and methods

2.1. Yeast strains

The yeast strains used in this work are tabulated in Table 1. The yeast expression vectors harboring PfORC1, Chimera A, Chimera B, ScSIR3, ScSIR3N, ScSir3C, Chimera A Δ70, Chimera A Δ110, Chimera A Δ210 and Chimera Sp were transformed into ΔGK pRS314 Δ*sir3* to generate MVS27, MVS28, MVS29, MVS30, MVS5, MVS31, MVS32, MVS33, MVS 34 and MVS39 respectively. *SIR2* gene was deleted from cells expressing either PfORC1 or chimera A or chimers B to create Δ*sir3*Δ*sir2* strain background.

2.2. Plasmids

From pRS425-HA3 vector the GPD promoter and HA3 tag flanking the multiple cloning site was excised by Sal I and Sac I double digestions and cloned into pTA vector [34] giving rise to pTA-HA3 vector. The open reading frame encoding PfORC1 was amplified from genomic DNA of 3D7 strain using OMKB119 and OMKB200 primers with the help of hot start Kapa Hifi DNA polymerase (Kapa Biosystems) as described by the manufacturer. The amplified product was then digested with Nde I and BamH I and subcloned into the Nde I and Bgl II site of pTA-HA3 vector. Similarly Chimera A (FP-OMKB199 & RP-OMKB202), Chimera AΔ70 (FP-OMKB204 & RP-OMKB202), Chimera AΔ110 (FP-OMKB205 & RP-OMKB202) and Chimera AΔ210 (FP-OMKB206 & RP-OMKB202) were amplified, digested with Nde I and BamHI and cloned into the Nde I and Bgl II sites of pTA-HA3 vector. On the other hand Chimera B (FP-OMKB201 & RP-OMKB200), ScSIR3 (FP-OMKB201 & RP-OMKB202), ScSir3C (FP-OMKB203 & RP-OMKB202) were amplified, digested with Spe I and Bam HI and cloned into the Spe1 and Bgl II sites of pTA-HA3 vector. To construct chimera Sp the open reading frame corresponding to the N-terminal of SpOrc1 was amplified from *S. pombe* genomic DNA using primer pair OMKB256 and OMKB257, digested with BamHI and cloned into the BamHi site of chimera containing pTA-HA3 vector replacing PfORC1N fragment. The correct orientation was confirmed by HindIII digestion. The sequences of each primer are shown in Supplementary Table S1.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2013.08.004.

2.3. 5-FOA assay for TPE

5-Fluoroorotic acid (5-FOA) assays to measure the silencing of a URA3 marked chromosome VIIL was performed as described elsewhere [35]. Briefly, different strains grown over night on Sc-trp (tryptophan dropout) broth were first normalized to the same OD_{600} and then serially diluted. The diluted cells were spotted either on SC-trp plate (control) or Sc-trp plate containing 5-FOA followed by incubation at 30 °C.

2.4. RNA isolation and real-time RT-PCR

Total RNA was isolated from different strains after growing them at 30 °C, by using acid phenol method as described [34]. Equal amount of RNA measured by spectroscopic analysis (JASCO spectrophotometer EMC-709) was subjected to DNase I (Fermentas) digestion to remove contaminating genomic DNA. The absence of genomic DNA was evaluated by PCR reaction prior to reverse transcription reaction (Supplementary Fig. S1). Synthesis of cDNA was performed according to previously described method [34]. Briefly, 10 μg of total RNA from each sample was reverse transcribed with oligo dT primer (Sigma Aldrich) using reverse transcriptase (Omniscript kit, Qiagen). Resulting cDNA was diluted (1:50) and used for PCR using Roche RT-PCR kit. The Real-time analysis was done using Applied Biosystems 7500 Fast Real Time PCR system. Primers pairs used for amplification of *ACT1*, *YFR057w* and *YIR043c* transcripts are tabulated in Table S1. The threshold cycle (C_T) value of *ACT1* transcript of each sample was used to normalize the corresponding C_T values of *YFR057w* or *YIR043c* transcripts. The normalized C_T values of *YFR057w* (or of *YIR043c*) from different samples were compared to obtain ΔC_T values. The relative levels of mRNA was deduced from the formula (change in mRNA level = $2^{\Delta CT}$). The mean values (±SD)

Table 1
Yeast strains used in this study.

Strains	Description	Genotype	Reference
ΔGK	Δ*sir3* null	*MATα rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP*	Liaw and Lustig [6]
MVS30	Δ*sir3* null bearing *ScSIR3* from a plasmid	*MATα rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTA ScSIR3:TRP1*	This study
MVS27	Δ*sir3* null bearing *PfORC1* from a plasmid	*MATα rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTA PfORC1:TRP1*	This study
MVS28	Δ*sir3* null bearing chimera A from a plasmid	*MATα rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTA Pforc1NScsir3C:TRP1*	This study
MVS29	Δ*sir3* null bearing chimera B from a plasmid	*MATα rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTA Scsir3N Pforc1C:TRP1*	This study
MVS5	Δ*sir3* null bearing ScSIR3N from a plasmid	*MATα rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTA Scsir3N:TRP1*	This study
MVS31	Δ*sir3* null bearing ScSIR3C from a plasmid	*MATα rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTA Scsir3C:TRP1*	This study
MVS32	Δ*sir3* null bearing chimera A without the first 70 amino acids	*MATα rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTA Pforc1N(Δ70)Scsir3C:TRP1*	This study
MVS33	Δ*sir3* null bearing chimera A without the first 110 amino acids	*MATα rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTAPforc1N(Δ110)Scsir3C:TRP1*	This study
MVS34	Δ*sir3* null bearing chimera A without the first 210 amino acids	*MATα rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTAPforc1N(Δ210)Scsir3C:TRP1*	This study
MVS7	Δ*sir3* null bearing *PfORC1* from a plasmid in Δ*sir2* background	*MATα rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 sir2::KAN^r VIIL::URA3 pRS313/RAP pTA PfORC1:TRP1*	This study
MVS8	Δ*sir3* null bearing chimera A from a plasmid in Δ*sir2* background	*MATα rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 sir2::KAN^r VIIL::URA3 pRS313/RAP pTA Pforc1NScsir3C:TRP1*	This study
MVS9	Δ*sir3 null bearing chimera B from a plasmid in Δsir2 background*	*MATα rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 sir2::KANr VIIL::URA3 pRS313/RAP pTA Scsir3N Pforc1C:TRP1*	This study
MVS39	Δ*sir3* null bearing chimera Sp from a plasmid	*MATα rap1::LEU2 leu2-3,112 ade2-1 his3 trp1 ura3-1 sir3::ADE2 VIIL::URA3 pRS313/RAP pTA Sporc1NScsir3C:TRP1*	This study

from three independent experiments were plotted using GraphPad prism 6 software.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2013.08.004.

3. Results

3.1. PfOrc1N terminal bears homology with Sir3 and Orc1 of Saccharomycotina

PfOrc1 has many distinct sequence motifs at its N-terminal and C-terminal domains. While the C-terminal domain has conserved function during parasite DNA replication, the N-terminal domain has been found to be associated with the "silencing complex" in *P. falciparim*. This domain harbors a leucine heptad repeat region and several low complexity regions (lysine and asparagine rich). The N-terminal domains of other eukaryotic Orc1 orthologs and the N-terminal domain of Sir3 possess a well defined BAH (Bromo-adjacent homology) domain. A canonical BAH domain is apparently absent in PfOrc1 (Supplementary Fig. S2A). Multiple sequence alignment and subsequent phylogenetic tree construction taking the N-terminal domains of Orc1 orthologs and that of Sir3 revealed an interesting relationship. PfOrc1 N-terminal was found to be grouped with the members of the *Saccharomycotina* subphylum (Supplementary Fig. S2B). It was not grouped with *Taprhinomycotina* (*Schizosaccharomyces pombe*), metazoans and plants. Interestingly, *S. pombe* and the metazoans do not possess Sir3. It is the Orc1 along with HP1 (heterochromatin protein 1) that constitutes the core "silencing complex" in these organisms. Identification of PfHP1 and the apparent absence of Sir3 protein proposed a similar metazoan like Orc1-HP1 silencing mechanism in *P. falciparum* too. Thus, it is extremely interesting to observe that PfOrc1 N-terminal is actually closer to the *Saccharomycotina* Orc1/Sir3 than to metazoan Orc1, given that *Saccharomyces* does not possess HP1 like proteins. Among the *Saccharomycotina* PfOrc1 N-terminal is closer to *Saccharomycetecae* (*K. lactis*) than the CTG clade (*Candida albicans*). KlOrc1 shows more closeness to PfOrc1 than the post-genome duplicated ScOrc1/ScSir3. *SIR3* emerged from gene duplication of *ORC1* and gained a specialized silencing function after the genome duplication in the budding yeasts. On the other hand, like *P. falciparum*, *K. lactis* also possesses only one copy of Orc1 and lacks Sir3. Thus, it could be possible that *P. falciparum* and *K. lactis* share similar silencing mechanism. As opposed to the N-terminal, when the C-terminal sequence of PfOrc1 was analyzed, it did not show any relatedness to the *Saccharomycotina* subphylum (Supplementary Fig. S2C). A BLAST search between the amino acid sequences of PfOrc1-N terminal and ScSir3-N terminal showed fairly good similarity between the N-terminal of these two proteins (NCBI BLAST total score 48.1; query cover 26%; *E*-value 0.36; maximum identity 45% and maximum positives 48%). The sequence alignment between the N-terminal of PfOrc1 and ScSir3 is shown in Supplementary Fig. S3 (T-Coffee, EMBL-EBI).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2013.08.004.

3.2. Functional complementation of S. cerevisiae Δsir3 null mutant by PfOrc1N terminal domain

To determine whether PfOrc1 can complement Sir3 deficiency in telomere silencing, we have constructed several complementation vectors (Fig. 1A). Two of the complementation vectors containing either full length *PfORC1* or full length *ScSIR3* were cloned in pTA-HA3 vector (a yeast expression vector with C-terminal

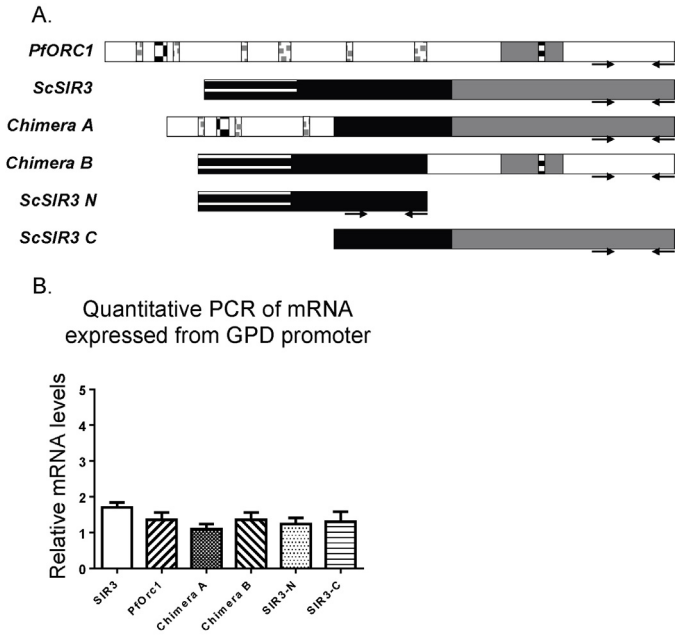

Fig. 1. (A) Schematic representation of genes or gene fragments used in complementation experiments. *ScSIR3*: full length *SIR3* gene from yeast *S. cerevisiae*. *PfORC1*: full length *ORC1* gene from *P. falciparum*. Chimera A: Chimeric gene containing sequences corresponding to the N-terminal of *PfORC1* and fused to the C-terminal of *ScSIR3*. Chimera B: Chmeric gene containing DNA sequences corresponding to the N-terminal domain of *ScSIR3* and fused to the DNA sequences corresponding to the C-terminal domain of *PfORC1*. *ScSIR3*N: DNA sequences corresponding to the N-terminal of ScSIR3. *ScSIR3*C: DNA sequences corresponding to the C-terminal of *ScSIR3*. The arrows show the position of primer pairs used to amplify each transcript by RT-PCR. (B) Q-PCR data showing similar levels of mRNA expression of each gene or gene fragments used in complementation assay. In each case the mean value (±SD) from three independent experiments with three independent harvests of cells is normalized against the abundance of *ACT1* mRNA and plotted using Graph Pad prism 6 software.

HA3 tag having GPD promoter and *TRP1* as a selectable marker). Another two complementation vectors having chimera A and chimera B as inserts (Fig. 1A) were also created in pTA-HA3 vector backbone. Chimera A consists of sequences corresponding to the N-terminal region of PfOrc1 (amino acids 1–350) and C-terminal region of ScSir3 (amino acids 243–979), whereas, chimera B consists of sequences corresponding to N-terminal region of ScSir3 (amino acids 1–527) and C-terminal region of PfOrc1 (amino acids 694–1190). These regions of *PfORC1* and *ScSIR3* genes have been chosen on the basis of homology analysis and information gathered from previous literature [29]. These chimeras were constructed in order to get an insight whether a particular domain of *PfORC1* in conjunction with the rest of the *ScSIR3* gene can compensate for the function of full length ScSir3 in a Δ*sir3* background. All the constructs were transformed into Δ*sir3* yeast strain (ΔGK strain: a kind gift from Arthur Lustig, Tulane University, USA) and the expression of genes from these constructs were tested by real-time RT-PCR analysis. Similar levels of mRNA were detected by qRT-PCR analysis (Fig. 1B). However the protein could not be detected by Western blot analysis using anti-HA antibody probably due to low expression of these proteins.

The Δ*sir3* strains harboring different expression plasmids were assayed for telomere silencing activity. In Δ*sir3* strain telomere silencing is de-repressed leading to expressions of sub-telomeric genes. Investigation of TPE in yeast using marked telomere (placing an auxotrophic marker gene such as *URA3* or *ADE2* at subtelomeric regions) is well established. However, these methods are less quantitative and unable to detect partial restoration of silencing. In order to circumvent this problem we have also adopted more sensitive and quantitative methods which are based on qRT-PCR analysis. Thus, we have performed three different assays for silencing (Fig. 2A). In the first assay, silencing of sub-telomeric *URA3* gene (situated at chromosome VIIL) allows the cells to grow in presence of a pro-drug FOA (5-Fluoroorotic acid), where as de-repression of *URA3* expression converts FOA into a toxic product, killing the cells. Thus, Δ*sir3* cells are sensitive to FOA and *SIR3* cells are resistant to FOA. We found that cells expressing chimera A (PfORC1N terminal region fused with ScSIR3C terminal region) are able to grow on FOA plate, suggesting efficient silencing of URA3 gene expression (Fig. 2B).

Neither full length PfORC1 nor chimera B expressing cells were able to grow on FOA plate, suggesting failure to complement the silencing defect of Δ*sir3* null mutant (Fig. 2B). The second and the third assays were more quantitative where the mRNA expressions of two naturally present sub-telomeric yeast genes (*YFR057w* and *YIR043c*) were measured by qRT-PCR. The genes *YFR057w* and *YIR043c* are present adjacent to telomere on chromosome VIR and IXR respectively [34,36]. The mRNA levels of *YFR057w* and *YIR043c* were found to be 1.5 fold and 3 fold less in PfOrc1 expressing cells than in Δ*sir3* strain (Fig. 2C and D). This result suggests that full length *PfORC1* was able to partially restore back the lack of silencing resulted from deletion of *SIR3*. TPE is a multi factorial process involving many protein–protein interactions. Detailed work in yeast have shown that once Sir3 is recruited at the de-acetylated histones (a result of Sir2 action), it recruits more Sir2–Sir4 complex via the interaction with Sir4, and thereby helps in spreading of the Sir complex. The N-terminal- and the C-terminal-domains of Sir3 have distinct roles in this process. We reason that PfOrc1 may not been able to effectively engage itself with other yeast proteins involved in initiation, spreading or maintenance of TPE and thus exhibits partial restoration of silencing. To address this possibility we have tested whether either of chimera A or chimera B could complement SIR3 function. We observed that the chimera A results in 3 fold down regulation of *YFR057w* transcript and 5 fold down regulation in *YIR043c* transcript (Fig. 2C and D). Thus, chimera A is able to complement the silencing defect in Δ*sir3* mutant. Since this chimera contains the C-terminal domain of ScSir3, we have also tested the possibility that the restoration of silencing is not due to the C-terminal domain of ScSir3 alone. When only the C-terminal of ScSir3 was expressed, we found no restoration of silencing. As opposed to chimera A, the chimera B was unable to complement the Sir3 function (Fig. 2B–D). Thus, in all three independent TPE assays on three different sub-telomeric loci (*URA3* on left arm of chromosome VII, *YFR057w* on right arm of chromosome VI and *YIR043c* on right arm of chromosome IX) chimera A was able to complement Δ*sir3* mutation. Thus, the restoration of telomere silencing conferred by chimera A is a general phenomenon and is not restricted to any particular telomere. Interestingly, PfOrc1 on its own was also able to exhibit partial silencing phenotype at two distinct telomeres when measured by quantitative RTPCR analysis, but failed to effectively silence the sub-temomeric *URA3* gene as evident from 5-FOA sensitivity measurement. We reasoned that 5-FOA sensitivity test is very harsh selection and unless the extent of silencing is good enough the cells are not resistant to the drug. Nonetheless, the partial restoration of silencing by PfOrc1 is consistent at two independent chromosomal loci and the clear-cut lack of silencing by ScSir3N or ScSir3C, which actually acted as internal controls, argued for a silencing function of PfOrc1. In order to find out whether such heterologous complementation of ScSir3 function is specific to the N-terminal region of PfOrc1, we made another chimera fusing the N-terminal domain of SpOrc1 (*Schizocaccharomyces pombe*) and C-terminal domain of ScSir3. When tested on complementation assay the N-terminal of SpOrc1 exhibited robust silencing activity (Supplementary Fig. S4).

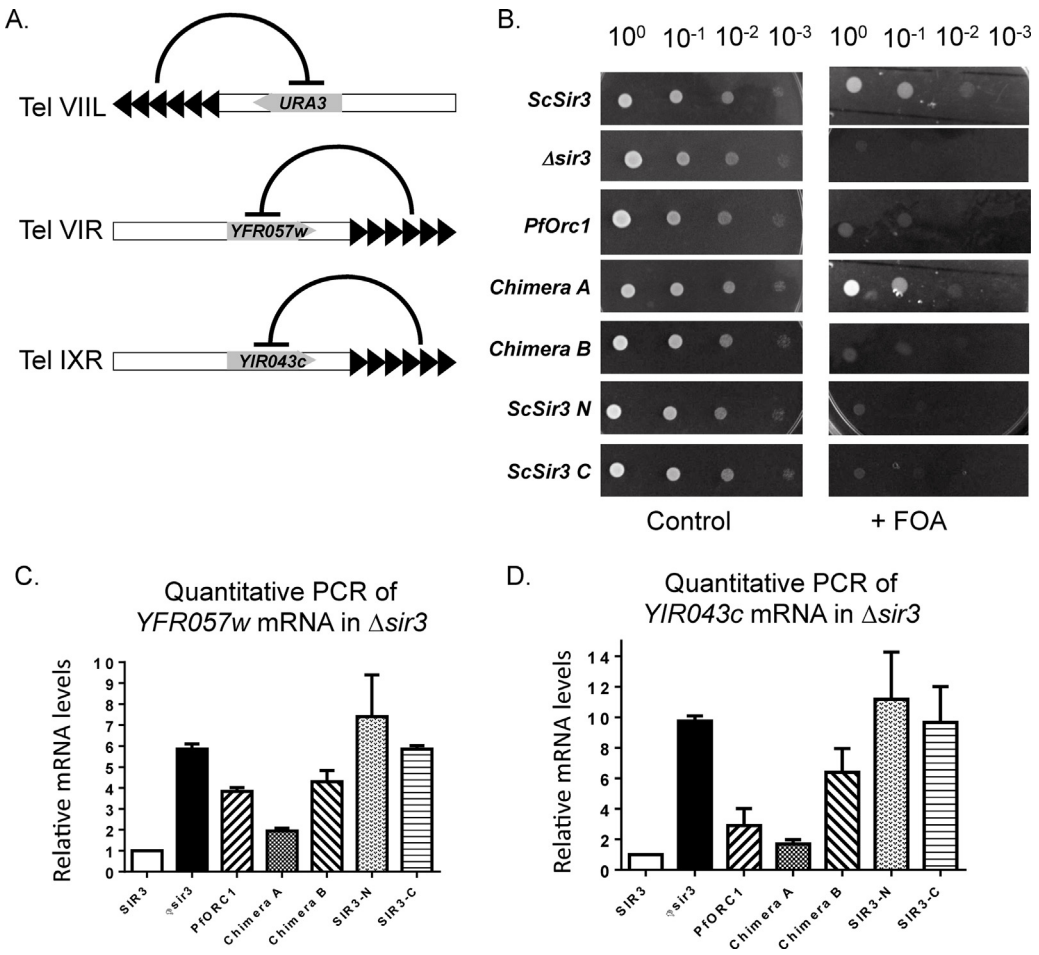

Fig. 2. PfOrc1 complements ScSir3 in TPE. (A) Scheme of TPE assays. The telomeres and the corresponding sub-telomeric genes analyzed are indicated. (B) TPE of *URA3* marker gene is analyzed by 5-FOA sensitivity. When *URA3* gene is repressed the cells are resistant to 5-FOA. Similarly, when *URA3* gene is de-repressed the cells are sensitive to 5-FOA. The left panel shows growth of different cells (as indicated on the left) in non-selective medium and the right panels shows the growth in presence of 5-FOA. (C) TPE of *YFR057w* gene. The relative levels of *YFR057w* mRNA as determined by Q-PCR from different strains (indicated on the *X*-axis) are plotted after normalization with *ACT1* mRNA. In each case the mean value (±SD) from three independent experiments with three independent harvests of cells is calculated and plotted using Graph Pad prism 6 software. (D) TPE of *YIR043c* gene. The relative levels of *YIR043c* mRNA are plotted as in C.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2013.08.004.

3.3. Structure function analysis of PfOrc1N terminal domain:

PfOrc1 N-terminal domain (NTD) does not contain a BAH domain. However, it contains one leucine heptad repeat (LHR) region and three low complexity regions (rich in lysine and asparagine). In order to know the importance of these regions and to map the functional domain, we have generated three deletions mutants of PfOrc1 NTD. The first one (chimera A Δ70) is devoid of the N-terminal extension of 70 amino acids, but retains all the three low complexity regions as well as the LHR. The second one (chimera A Δ110) does not contain the first low complexity region. The third deletion mutant (chimera A Δ210) is devoid of the first two low complexity regions and the LHR region (Fig. 3A). Fig. 3B shows similar levels of expression of all the mutant genes at the transcription level. We have quantified the mRNA levels of *YFR057w* and *YIR043c* genes in order to test the effect of these deletion mutants on repression/de-repression at these chromosomal loci. We found that the relative levels of *YFR057w* and *YIR043c* mRNAs are comparable between chimera A and chimera A Δ70. On the contrary, chimera A Δ110 and chimera A Δ210 showed similar levels of *YFR057w* and *YIR043c* transcripts as in Δ*sir3* strain (Fig. 3C and D) Thus, chimera A Δ70 is fully capable of restore silencing in Δ*sir3* strain, whereas chimera A Δ110 and chimera A Δ210 results in complete de-repression. These results suggest that the N-terminal extension (the first 70 amino acids) are dispensable for silencing function, while the region containing the LHR and the three low complexity regions are important.

3.4. Complementation of Δsir3 null mutant by PfOrc1N terminal domain is dependent on Sir2

If such complementation of Sir3 function by PfOrc1 is genuine, it must work via Sir2, which has histone deacetylase activity. In order to investigate this we have knocked out the *SIR2* gene to create MVS8 strain. Thus, MVS8 strain expresses chimera A in Δ*sir3*, Δ*sir2* background. Similarly Δ*sir3*, Δ*sir2* strains were created expressing either PfOrc1 or chimera B. When tested for TPE, chimera A showed complete de-repression of *YFR057w* and *YIR043c* expressions in Δ*sir3*, Δ*sir2* background (Fig. 4A and B). The de-repression is comparable to those found in Δ*sir3* strain. This result suggests that the complementation of Sir3 function by chimera A is Sir2 dependent.

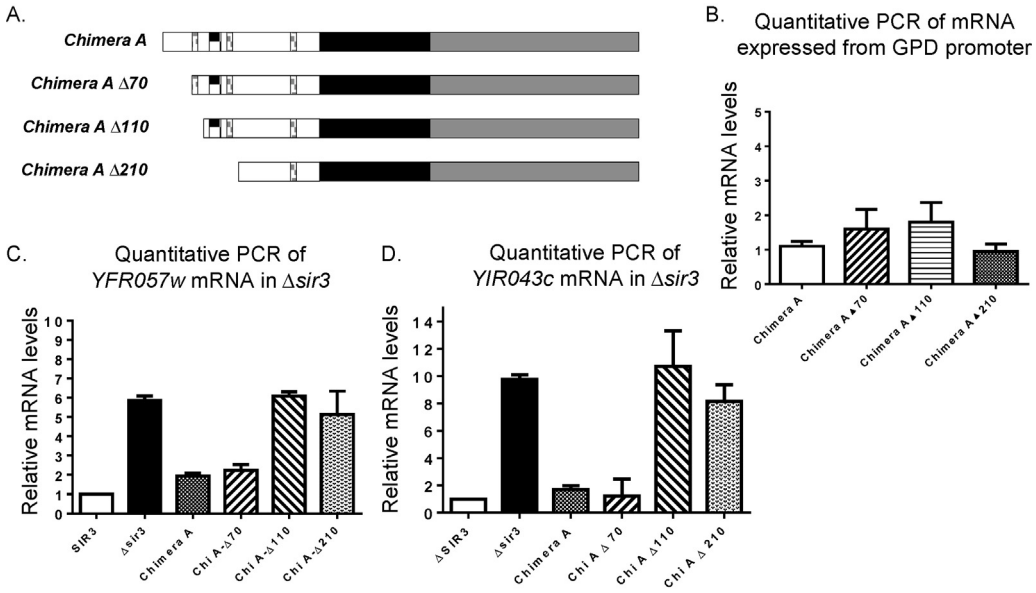

Fig. 3. Structure function analysis of PfOrc1 N-terminal in TPE. (A) Schematic diagram of different deletions mutants. (B) Q-PCR data showing more or less comparable abundance of mRNA from different mutants. (C) and (D) TPE of *YFR057w* and *YIT043c* gene expression respectively. The relative abundances of mRNA from these two subtelomeric genes are plotted after normalization against *ACT1* mRNA. Each bar represents mean mRNA level (±SD) from three independent experiments.

4. Discussions

Our results provide direct proof that PfOrc1 has a Sir3/Orc1 like silencing function in a heterologous system. Together with the earlier observation that PfOrc1 and PfSir2 co-localize at silent sub-telomeric regions, our finding suggests that PfOrc1 is likely to have silencing function during TPE in *Plasmodium*. Origin recognition complex subunit 1 (Orc1) is an integral part of the replication origin recognition complex and plays crucial roles during replication initiation [37]. Using yeast surrogate model it has been found that the C-terminal of PfOrc1 has evolutionary conserved replication function [29]. In a recent study it has been shown that the N-terminal region of PfOrc1 co-localizes with PfSir2 at the nuclear periphery within silencing complexes formed at distinct sub-telomeric loci including *var* gene promoters [31]. However, its direct involvement in gene silencing remained ambiguous. Firstly, for PfOrc1 to be considered to have a structural role during the establishment of silencing, over expression of its functional domain is expected to have a positive effect on silencing (unless, over-expression causes a dominant negative effect). While, over expression of PfSir2 exhibited enhanced silencing [38], an exactly opposite result was obtained in case of PfOrc1. Over-expression of an N-terminal polypeptide ($PfOrc1N_{1-238}$) resulted in de-repression of sub-telomeric genes and destabilization of the silencing complex [31]. Although, it is not experimentally tested whether over expression of PfOrc1 creates a dominant negative effect, such possibility cannot be ruled out. Furthermore, considering the possibility that "right amount" of PfOrc1 is required for the formation of silencing complex, this finding does not necessarily negate a possible role of PfOrc1 in gene silencing. Nevertheless, it fails to provide any direct evidence for PfOrc1 involvement in silencing. Secondly, lack of co-occupancy of PfSir2 and PfOrc1 at

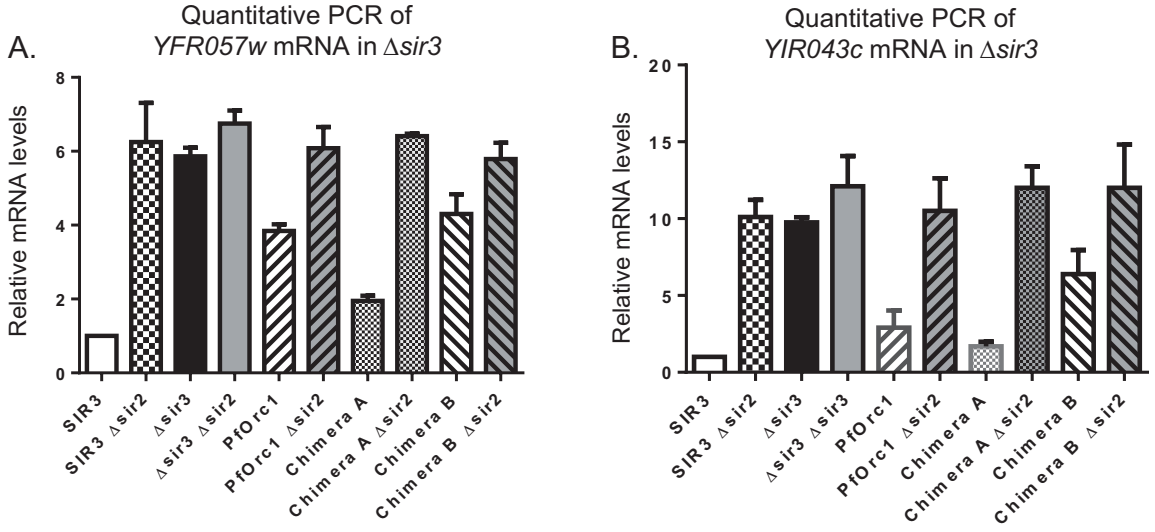

Fig. 4. PfOrc1 mediated silencing is dependent on Sir2. Q-PCR data showing reversal of chimera A mediated silencing of *YFR057w* (A) and *YIR043c* (B) in Δ*sir2* cells. The relevant genotypes of each strain are indicated on the *X*-axis. The data from three independent experiments are normalized with respect to the abundance of *ACT1* mRNA. Each bar represents mean relative mRNA levels ±SD.

certain sub-telomeric loci (especially at *var* promoters of *upsE* and *upsC* types) challenges the notion that recruitment of PfOrc1 at silenced *var* promoters is PfSir2 dependent. These promoter regions were occupied by PfOrc1 even in the absence of PfSir2 in Δ*sir2* knock-out parasite lines [31]. Thirdly, no physical interaction was found between PfSir2 and PfOrc1 (either with full length or with the N-terminal fragments of PfOrc1 alone). Our study taking yeast model to study telomere silencing clinches many of these issues.

In this study we provide the first direct evidence that the N-terminal of PfOrc1 possesses silencing activity. We show that in a Δ*sir3* yeast strain, which is incapable of telomere silencing, expression of PfOrc1 or a fusion between PfOrc1-N terminal and ScSir3-C terminal restore back silencing at multiple telomeric loci. The fusion protein showed more effective complementation than PfOrc1 itself.

Our result is consistent with the domain activities of Orc1 as observed in other organisms. It has been demonstrated earlier that the N-terminal domain of Orc1 is involved in the formation of heterochromatin at the *S. cerevisiae* mating locus, while the C-terminal domain is involved in replication [23]. Although ScOrc1 and ScSir3 cannot complement each other's function, a chimera between the N-terminal region of ScOrc1 and C-terminal region of ScSir3 is capable of performing Sir3 function [23]. The N-terminal region of *K. lactis* Orc1 has also been implicated for silencing function. Additionally, our present study shows that the N-terminal region of *S. pombe* Orc1 is capable of complementing Sir3 function. Thus, it appears that the N-terminal region of Orc1 from a variety of species possess silencing activity. Interestingly, all the Orc1 orthologs except for PfOrc1 possess a well defined BAH domain at their N-terminal domain, which is believed to play a pivotal role in silencing. It is unclear how despite lacking a canonical BAH domain PfOrc1 N-terminal domain is also equipped with silencing activity. It could be possible that the three-dimensional fold of PfOrc1 N-terminal region might mimic a BAH domain structure.

A more efficient functional complementation of Δ*sir3* by chimera A (fusion between PfOrc1 N-terminal and ScSir3 C-terminal) compared to full length PfOrc1, can be explained by the specific contribution of the C-terminal domain of Sir3 in silencing. Although Sir3 N-terminal region plays the key role in silencing, the C-terminal AAA+ domain is involved in engaging other silencing proteins (Sir4 and histones) [32]. The AAA+ domain of ScSir3 has evolved much more rapidly than the AAA+ domains of Orc1 proteins. In fact the AAA+ domain of PfOrc1 is very minimal compared to that of ScSir3. Thus, it could be possible that PfOrc1 C-terminal is unable to interact with ScSir4 as efficiently as ScSir3 C-terminal.

Since, PfOrc1 does not have a defined BAH domain, it was not possible to identify critical amino acids in the N-terminal domain that are important for silencing function. Interestingly, PfOrc1 N-terminal domain contains three unique low complexity (LC) regions that are absent in other Orc1 orthologs. Analyzing a series of deletion mutants we found that these regions are functionally important. Earlier work has suggested that the leucine heptad repeat (LHR) region is involved in PfOrc1 dimerization and thus likely to be important for the silencing function. We observed that an immediately upstream LC region is also critical for the silencing function.

SIR3 has emerged from gene duplication of *ORC1* gene [22]. Orc1 from the yeast *K. lactis*, which diverged from *S. cerevisiae* before the gene duplication event, acts in conjunction with the deacetylase Sir2 and the histone-binding protein Sir4 to generate heterochromatin at telomeres and at mating-type locus [19]. *K. lactis* does not possess Sir3 and KlOrc1 plays both replication as well as silencing functions. After the emergence of *SIR3* from gene duplication, Orc1 retained the replication function and Sir3 emerged as a specialized silencing protein in higher yeasts such as *S. cerevisiae* [39]. The protozoan parasite *P. falciparum* diverged very early in the evolution and thus harbors an ancient *ORC1* having both replication and silencing activities. This is consistent with the fact that *Plasmodium* lacks *SIR3*. At present it is unclear whether analogous to the Sir3 function PfOrc1 acts at the step of initiation and/or spreading of silencing complex along the heterochromatin and being involved in indirect recruitment of PfSir2 to the telomeric repeats. Identification of a functional ortholog of Sir4 also remains elusive. In conclusion, we for the first time show that PfOrc1 plays a Sir3/Orc1 like role in telomere silencing. Investigation of the interplay between PfSir2, PfOrc1 and PfHP1 and discovering additional members of *Plasmodium* silencing complex would be necessary to understand the molecular mechanism of telomere silencing in general and var gene silencing in particular.

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