Characterizing the role of putative prohibitins of *Plasmodium falciparum*

Thesis submitted to

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For the award of the degree of

Doctor of Philosophy (Ph.D)

In the Faculty of Science

By

Ms. Savitha C M.Sc.,
(Registration No: KLEU/Ph.D./2014-2015/DO1214025)

Under the Guidance of

Dr. Subarna Roy,
Director, Scientist-G
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Belagavi, Karnataka
India.

November 2021

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*I hereby declare that the thesis entitled "**Characterizing the role of putative prohibitins of Plasmodium falciparum**" is a bonafide and original research carried out by me under the guidance of **Dr. Subarna Roy, Director, Scientist-G, Indian Council for Medical Research, National Institute of Traditional Medicine (ICMR-NITM), Belagavi, Karnataka, India.** The thesis or any part thereof has not formed the basis for the award of any degree/fellowship or similar title to any candidate of any University.*

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With all my heart, I thank GOD Almighty for keeping me going

Finally, I dedicate this thesis to **my mom and dad** for always believing in me, for their continuous love and their supports.

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Ms. Savitha C **M.Sc.,**
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LIST OF ABBREVIATIONS

Abbreviation	Definition
%	Percentage
>	greater than
~	approximate
°C	Degree Celsius
µF	microfarad
µl	microliter
µm	Micrometre
AAA proteases	ATPase associated with diverse cellular activities proteases
Ä	Angstrom
ATc	Anhydrotetracycline
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
Cas9	**C**RISPR **as**sociated protein **9**
CDD	Conserved Domain Database
cm	Centimeter
cMCM	complete malaria culture medium
CO_2	Carbon dioxide
COG	clusters of orthologous groups
CPD	citrate phosphate dextrose
CRISPR	Clustered, regularly interspaced, short palindromic repeat
DD	destabilization domain
DDD	DHFR destabilizing domain
DHFR	Dihydrofolate reductase
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide Triphosphate
dCas9	dead Cas9/ nuclease-deficent Cas9
DSM1	5-Methyl-N-(2-naphthyl)1,2,4triazolo1,5-apyrimidin-7-amine
EDTA	Ethylene Diamine Tetra Acetic Acid

ETC	Electron Transport Chain
EuPaGDT	Eukaryotic Pathogen CRISPR gRNA Design Tool
EUROSCARF	European Saccharomyces Cerevisiae ARchive for Functional analysis
ExPASy	Expert Protein Analysis System
Fe^{3+}	Ferric ion
Fig	Figure
FKBP	FK506 binding protein
GlcN	glucosamine – 6 – phosphate
H_2O_2	Hydrogen peroxide
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HMM	Hidden Markov model
iTOL	Interactive Tree of Life
KDa	kilodaltons
kV	kilovolt
L	Litre
LB medium	Luria broth medium
MAFFT	Multiple Alignment using Fast Fourier Transform
MDa	Megadalton
ml	Milliliter
mM	Millimolar
MR4	Malaria Research and Reference Reagent Resource Center
N_2	Nitrogen
NCBI	National Center for Biotechnology Information
nm	nanometre
nt	nucleotides
O_2	Oxygen
OD	optical density
OPA1	optic atrophy 1
P. falciparum	*Plasmodium falciparum*
PAM	Protospacer Adjacent Motif
PDB	Protein Data Bank
PFAM	Protein Families (database)

PHBL	Prohibitin-like protein
Phyre2	Protein Homology/AnalogY Recognition Engine
PSI-BLAST	Position-Specific Iterative -BLAST
RBC	Red Blood corpuscles
RNAi	RNA interference
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
RPMI 1640	Roswell Park Memorial Institute medium 1640
SCOP	Structural Classification of Protein
sgRNA	single guide RNA
Shy	SURF Homolog of Yeast
SNP	Single Nucleotide Polymorphism
SPFH	Stomatin, Prohibitin, Flotillin and HflK/C
ssDNA	single-stranded DNA
ssRNA	single-stranded RNA
STOML	Stomatin-like protein
TAE	Tris-Acetate EDTA
TALENs	Transcription activator-like effector nucleases
TCA cycle	Tricarboxylic acid cycle
TE buffer	Tris EDTA Buffer
TRAD	Transcriptional transactivation domain
–Ura-Leu -	Synthetic Complete medium without Uracil and Leucine
–Ura-Leu-Ade -	Synthetic Complete medium without Uracil, Leucine and Adenine
UTR	Untranslated region
V	Volts
WHO	World Health Organization
WR99210	1,6-Dihydro-6,6-dimethyl-1-3-(2,4,5-trichlorophenoxy) propoxy-1,3,5-triazine-2,4-diamine
YNB	Yeast Nitrogen Base
ZFNs	Zinc-finger nucleases

ABSTRACT

BACKGROUND

Understanding the malaria pathogen, *Plasmodium falciparum* at the molecular level is quite challenging. As the cellular machinery is highly dynamic, any biological system requires an array of interactions among its molecular partners. Protein – protein interactions and complementation studies of putative proteins and proteins of unknown function are essential techniques in deciphering and assessing its functionality.

OBJECTIVE

Protein – protein interactions regulates all the living processes in the cells. Exploring the interacting proteins and its networks finds a significant impact on identifying the biological role, understanding the disease and for drug discovery. Moreover, assembly of complexes in the biological systems determines the efficient functioning. Cytochrome c oxidase (complex IV) of respiratory chain is assembled in mitochondria. Shy1, an assembly factor, promotes the complex IV biogenesis through association with different protein modules. Surf1 is the human homologue of Shy1. Mutation in Surf1 leads to severe human disorders.

METHDOLOGY

We demonstrate the insilico identification and complementation of PfSurf1 with Shy1 of *Saccharromyces cerevisiae*. Prohibitins (PHB) initially identified as inhibitor of cell proliferation. This SPFH (Stomatin, Prohibitin, flotillin, and HflK/C) domain containing protein is widely distributed in the cells of microorganism, yeast, plants and mammals. Prohibitins are multifunctional proteins, involves in regulation of proliferation, apoptosis, transcription, cell signalling, mitochondrial protein folding. These proteins also have a role in disease pathogenesis which includes oxidative stress, mitochondrial dysfunction, etc. Cellular functions of these proteins are not known in *Plasmodium falciparum*. We explain the probable essentialities of these proteins using CRISPR –Cas 9 knockout system and Aptamer and GlmS based knock down approaches. We have analysed the interaction of putative Prohibitins using yeast two hybrid system.

RESULTS

Putative prohibitins of *Plasmodium falciparum* shown to have essential function in mitochondria. Homology modelling and yeast two-hybrid analysis, we show that putative *Plasmodium* PHBs (PfPHB1 and PfPHB2) interact with each other, which suggests that they could form super complexes of heterodimers in *Plasmodium*, the functional form required for optimum mitochondrial function. Yeast complementation studies along with phylogenetic analysis explains the mitochondrial role of putative PfSurf1.

CONCLUSION

Putative prohibitins of apicomplexans forms cluster and phylogenetically related to higher eukaryotes. Both the putative proteins sequences of PfPHB1 and PfPHB2 found to have transmembrane domain and interactive matrix domain similar to prohibitins of other eukaryotes in Phyre2 predicted 3D model PfPHB1 and PfPHB2 proteins have strong interaction in yeast two hybrid system. PfSURF1 full length gene is partially complementing the ScSHY1 full length gene in growth assays with non-fermentable carbon sources.

KEYWORDS

Plasmodium falciparum, prohibitins, SURF1, protein – protein interaction, Mitochondrial proteins, Yeast two hybrid.

1.0 INTRODUCTION

1.1 Disease Malaria

Malaria, a major mosquito-borne health problem worldwide. World Health Organization (WHO) shows 229 million new malaria infection cases in 2019 (1). In humans, this parasitic disease is caused by five *Plasmodium Species: Plasmodium Vivax, Plasmodium falciparum, Plasmodium Ovule, Plasmodium malariae,* and *Plasmodium knowlesi*. The two major species that are responsible for malaria infections throughout the world are *Plasmodium vivax* and *Plasmodium falciparum*, and the utmost drastic and lethal form of malaria is caused by *Plasmodium falciparum.* In addition to the above *Plasmodium* spp., many other species of *Plasmodium* also responsible for this disease in vertebrates. Non-human primates are infected by other plasmodium species and few examples are: *Plasmodium cynomolgi* in macaques and *Plasmodium reichenowi* in chimpanzees, *Plasmodium berghei* and *Plasmodium yoelli* in rodents, *Plasmodium gallinaceum, Plasmodium relictum*, and *Plasmodium elongatum* in avians (2). As of now, there are no vaccines approved for use, however, there are several antimalarial drugs available in the market. Unfortunately, resistance to most drugs has already been reported (3, 4).

Malaria control programs throughout the World are also focusing on controlling and eliminating anopheline vectors through vector control strategies such as insecticide spraying, long-lasting insecticide nets, entomopathogenic fungi, larvivorous fish, bacteria agents, etc. Insecticide resistance in vector populations is posing a grave challenge to malaria elimination efforts. Malaria research throughout the world is focused on developing new drugs and identifying drug targets to control and eliminate malaria (5).

1.2 *Plasmodium*

The *Plasmodium* parasite is currently classified on a molecular basis under Kingdom Protozoa, Subkingdom Biciliata, Infrakingdom Alveolata, Phylum Myzozoa, Subphylum Apicomplexa, Class Aconoidasida, Order Haemosporina and Genus *Plasmodium* (6). There are 5000 species in the Subphylum Apicomplexa, which includes all medically and economically important parasites; the most important ones are *Plasmodium*, *Babesia*, *Toxoplasma*, *Cryptosporidium*, *Therileria*, *Eimeria*, and *Isospora*. All of them lack motile cellular appendages like cilia and flagella, excluding microgametes in the sexual cycle. Apicomplexans have a characteristic form of gliding locomotion. All members of this subphylum owns organelles in particular rhoptries, micronemes, and polar rings in the apical region of its structure, which comforts the process of invasion and hence they are called Apicomplexan. The cellular organelle includes one or more double membrane bound mitochondria, and an elongated membranous non-photosynthetic plastid organelle called apicoplast (7). The members of this genus can infect a wide variety organisms ranging from mammals, to reptiles and are transmitted through the bite of the female mosquitoes. But Transmitters are different in mammals (*Anopheles)* and in birds and lizards (*Culex)*. Antelopes, lemurs, bats, rodents, and primates are the mammals identified with malaria, but this disease is not reported in felids, canids, equids, and bovids for reasons unknown (7).

There are multitude of *Plasmodium* species exist, and are identified by many parasitology and molecular studies (8). Molecular clock data demonstrates the divergence of *Plasmodium falciparum* and *Plasmodium reichenowi* around 6-8 million years back, during the evolutionary lines of humans and chimpanzees

diverged. Interesting DNA studies show that *Hepatosis* referred to the mammalian group of malaria, which infects bats (8). Genomic data suggest the lateral transfer from monkeys to humans in Asia gave rise to *Plasmodium vivax* (9). Human genetic variations clearly show the evolution of the human race to be closely associated with malaria, and genetic variations have given some measure of protection against the disease. Few examples of genetic variations include hemoglobin C, Thalassaemias, sickle cell anemia, a array of blood group variants – Group O, Duffy-negative, Immune system polymorphism, Glucose6 phosphate dehydrogenase deficiency, hereditary ovalocytosis (10), and several others that could be attributed to *Plasmodium* (7).

1.3 Molecular Biology of *Plasmodium falciparum*

In 2002, the genome of *Plasmodium falciparum* was sequenced. The nuclear genome is composed of 23.6Mb distributed in 14 chromosomes. Interestingly, the genome is made up of 80% AT-rich sequences in the coding and ~90% AT in the non-coding regions (11). The apicoplast harbors a 35Kb genome and codes for 30 proteins (12). The mitochondrial genome of *Plasmodium* is small, linear and measures ~6Kb and encodes three proteins: Cytochrome b, Cytochrome oxidase I, and Cytochrome oxidase III, and a fragmented rRNA gene (13-16). It is one of the smallest mitochondrial genome that has been sequenced so far. Even though the mitochondrial genome of *plasmodium* is minimalistic in nature, the mitochondrion is critical for the survival of the parasite (17). The *P. falciparum* in the process of endosymbiosis gave up many of its genes to its nuclear genome that are frequently found in the mitochondrial genome of other organisms (18, 19). The genome sequencing of *Cryptosporidium* reveals the absence of mitochondrial DNA. But this

apicomplexan appears to maintain a vestigial organelle resembling a mitochondrion for some essential metabolic process (20-22).

Similar to other apicomplexans, the *Plasmodium* parasite also contains numerous intracellular organelles. These intercellular organelles perform an influential performance in the development of the parasites. Cowman and Crabb have shown that the secretory organelles of the parasite namely rhoptries and micronemes are involved in the invasion process and they appear late in the cell cycle (23). Also, the food vacuoles become evident only during the transition between the ring and trophozoites stages of development (24). All stages of the parasites have a single endoplasmic reticulum, mitochondrion, plastid (apicoplast), and an unstacked Golgi (25-27). The role of these organelles in parasite biology has been an intense area of research. The mitochondrion and apicoplast are important drug targets as the parasites heavily rely on several key metabolic processes in these organelles for their survival (28-31).

The single mitochondrion in the asexual stage parasite is essential for its survival. The size, morphology, and number vary between the two life cycles (sexual and asexual) of the parasite. The mitochondrion of *P. falciparum* lacks well-defined cristae that are present in the bird malarial parasites (32). There is a tight association between the mitochondrion and the plastid organelle apicoplast throughout the asexual stages of the parasites development (33, 34).

The asexual life cycle of *P. falciparum* shows, the rod-shaped mitochondrion in the ring stage, elongates in the trophozoite stage and branches out in the schizont stage. At the end of the schizont stage, the mitochondrion and apicoplast divide and form an organellar pair before segregating into the daughter merozoites (35). The

electron microscopic imaging suggests that the movement of the organellar pair is either posterior or lateral to the nucleus in the early daughter merozoites. The molecular mechanism of mitochondrial division in *P. falciparum* is not clear (33, 34).

The complex cycle of *Plasmodium* parasites also involves the proliferative liver stage and sexual stages in the vector. Very limited information is established around the physiology of mitochondria in the further developmental stages of the parasite. Krungkrai et al., 2000 have shown multiple rounded mitochondria in gametocytes that have distinct tubular cristae (36). Oocysts of *Plasmodium* that happens in the midgut of mosquito also accommodate multiple mitochondria (37).

Similar to the other two organelles, endoplasmic reticulum also forms a highly complex organelle by schizogony. The endoplasmic reticulum in *P. falciparum* is involved in many processes: phospholipid biosynthesis (38, 39), calcium storage (40, 41), glycosylphosphatidylinositol biosynthesis (42), and trafficking proteins to different cellular destinations.

The dynamic actions of mitochondrion and apicoplast in the biology of the parasite make them favorable targets for a range of drugs (28-30, 43). Most eukaryotic cells depend on the mitochondrial electron transport chain for the generation of proton motive force which in turn is necessary for energy metabolism (44). However, the function of mitochondria in blood-stage malaria parasites, and also in other parasites is reduced to that of a consumer than as an energy producer (45, 46). A better understanding of different organelles and their interaction will facilitate not only the identification and characterization of new targets but will also increase our knowledge of drug resistance. The capability to genetically utilize the haploid genome

of *plasmodium* to assess the gene function is the central feature of advanced engineering technologies.

1.4 Current Molecular tools in Malaria

Transient transfection in *Plasmodium gallinaceum*, a bird malaria parasite was the noted landmark transfection two decades back (47). After few years, development of stable transfection in *Plasmodium falciparum* (48-50) and *Plasmodium berghei* was described (51, 52). The development of genetic tools has expanded the genetic manipulations in some *Plasmodium* species by stable transfections from rodent malaria parasites to human parasites including *Plasmodium yoelii*, (53, 54) *Plasmodium chabaudi* (55) and *Plasmodium vivax* (56, 57). Plasmid DNA can be introduced into the blood-stage malaria parasite using electroporation. Initially, plasmid DNA replicates episomally and form large concatamers which enable the expression of the transgenes (52, 58, 59). In contrast, to introduce mutations, knock out a gene, or replace homologous sequence, allelic exchange technology is used to drive the integration of the sequence of interest in the genome. To facilitate single cross-over integration, the transfectants are cycled 'on' and 'off' of drug (WR99210 or BSD) to achieve stable integration of the plasmid and also to eliminate episomal plasmids (58, 60). A negative selectable marker such as Yeast cytosine deaminase is also used in the double crossover recombination strategies (61, 62). This process of allelic recombination in *Plasmodium falciparum* is highly inefficient. In *Plasmodium berghei*, linear DNA is targeted for single as well as double cross-over recombination in the genome. Compared to *P. falciparum* the recombination frequency is four folds higher in *P. berghei* (63). Hence *P. berghei* can be easily manipulated to dissect the gene function all through its life cycle, inclusive of the sexual and liver stages of

development. Also, well-established assays for *in vivo* and *in vitro* studies are available for phenotyping *P. berghei*. Whereas, such assays were less efficient or completely lacking in *Plasmodium falciparum*. As a result, conditional gene expression systems were developed a decade back for *Plasmodium falciparum* to study the genes in the blood stages (64). The conditional gene expression systems are applicable for both *Plasmodium* falciparum and *Plasmodium* berghei but conflict with their mode of action.

Conditional expression of either RNA or Protein in *Plasmodium falciparum* is not an easy task. The lack of such promising technologies is due to poor knowledge of naturally occurring endogenous inducible systems. For example, RNAi machinery is absent in the genome of *Plasmodium* (65). Dissimilarities in *Plasmodium* spp at the genetic level with other organisms have led to the development of several systems. One such system that conditionally controls transcription is Tet operator of *Toxoplasma gondii* and *P.falciparum* Api AP2 (64, 66). However, it does not appear to be functionally useful for a wide range of proteins.

Post-translationally, functional protein levels are modified in the blood stages of *P. falciparum* by introducing domains that are stabilized by small ligands or molecules. The small molecule confers the dose-dependent control of protein attached and reversibility (67). Armstrong and Goldberg demonstrated FK506 binding protein (FKBP) based inducible stabilization domain that is fused to the target and degrades the protein in the absence of ligand Shield 1 (68). A similar method was developed using *Escherichia coli* DHFR destabilizing domain with folate analog trimethoprim as the ligand. Naturally, the drug trimethoprim is toxic to the developing malaria parasites and a human DHFR cassette is a must in its genome to perform the

knockdown experiments (69). Both the systems are useful to knock down the level of protein at a particular point of the cycle. Fusion of large FKBP domain in the N or C terminus of the protein may hinder protein localization. As the degradation machinery is cytosolic, the domain is non-functional with compartmental proteins.

Conditional knockdown at mRNA level can be achieved using RNA- mediated interference. Since *P. falciparum* lacks the machinery, another conditional system was developed using the "Tet-Off" system. Multiple tetracycline operator sites in the promoter sequence of the gene of interest (GOI) converts it to a weak promoter. ATc turns off the transcription of GOI and its expression is induced by TRAD (Transcriptional transactivation domain) when it binds to the 'Tet O' site and recruits transcriptional factor. The addition of ATc inhibits the binding of TRADs to the 'Tet O' site (64, 70). This system is useful to express transgenes from multi-copy episomes. However, the system is not efficient with conditional gene knockouts as TRAD is inefficient in the recruitment process of transcriptional factors.

Riboswitch is one more technique which also post-transcriptionally regulate the genes of *P. falciparum*. The ribozymes are RNA molecules capable of catalysing the cis-cleavage of mRNA once integrated. The mRNA degradation happens with the removal of UTR and leads to reduced protein levels. By placing the glmS ribozyme downstream to the stop codon of the genes in *Plasmodium falciparum* presence of inducer glucosamine – 6 – phosphate successfully reduce the expression of mRNA and concomitant protein (71). The riboswitch is an attractive system as it can be easily targeted to the genome by simple 3’ substitution. An alternative approach is achieved in *P. falciparum* based on the protein-RNA interaction by ATc. The Tet R- aptamer interaction represses the translation of protein that is fused with the aptamer. The

addition of ATc breaks the Tet R- aptamer interaction and results in protein expression (72). The repression of translation induced by this system is rapid, stable, and homogeneous.

1.4.1 Genome editing in *Plasmodium falciparum*:

Spontaneous recombination (single or double crossover) using a plasmid with a sequence homologous to the target is the common approach to modify the chromosomal loci in *Plasmodium falciparum*. This classical genomic approach is time-consuming, laborious and the selection of stable transfection is extremely difficult. Zinc finger nucleases have been used for knockouts and allelic replacements in *Plasmodium falciparum* (73). But, these nucleases are not easy to make and are not always efficient (74, 75). Recently, a very efficient site-specific gene editing technique has been demonstrated in several organisms (76-80) including *Plasmodium falciparum* (81, 82) using Cas9 nucleases. Clustered, regularly interspaced, short palindromic repeat (CRISPR) and CRISPR associated protein (Cas) complex is a natural genome editing system in bacteria. This prokaryotic adaptive immune system works against infecting viruses (83). The type II Streptococcus pyogenes CRISPR–Cas9 system works on a single guide RNA (sgRNA) that instruct the Cas9 endonucleases to mediate a specific break on both the strands of the target DNA sites (84). This system is shown to be effective not only in *P. falciparum* but also in *P. yoelii* (85).

The cells attempt to restore the double-strand break created by the Cas9 endonuclease. During the process the available new information gets incorporated by homologous recombination using donor DNA. This system works in combination with heterogeneously expressed Cas9 bearing the nuclear localization signal which is

guided by sgRNA. The Cas9-sgRNA complex pairs with the complementary DNA in the genomic locus to induce a very specific break on both the strands. CRISPR-Cas9 system is adopted to establish a propeller mutation in the kelch gene (PF3D7_1343700) to understand the slow clearing parasites with artemisinin resistance (82). Therefore, in the future, it is likely that CRISPR-Cas 9 system will put back the conventional gene targeting approaches to create gene knockouts and mutations.

2.0 JUSTIFICATION OF THE STUDY

Over the years, varying degrees of drug resistance has emerged against almost all drugs used in the treatment of malaria. Hence the present plot is a race against time to find a new treatment to control the disease. To achieve this, understanding the parasite biology by characterizing the genes and their functions is necessary to target the drugs towards the genes responsible for pathogenesis. Despite the sequence availability for various genomes of *Plasmodium* parasites, many genes have unknown functions.

"Omics" approaches are very crucial in understanding many features of parasite biology. Omics studies identify the candidate genes putatively and these genes require functional validation. Hence merging of "omics" techniques with genetic manipulation strategies will be highly helpful in explaining the function of these genes and the related structures they govern.

Drug resistance in *Plasmodium falciparum* is one of the major problems associated with disease treatment. Current malaria control and elimination rely on efficacious antimalarial drugs. Determination of anti-malarial drug resistance markers is useful in drug efficacy surveillance of malaria. Parasite genetic factors like Single nucleotide polymorphisms (SNPs) and copy number variations (CNV) gains a main role in drug resistance. Genes which stands with less or no functional annotation and are unique to *Plasmodium falciparum* are essentially important in uncapping the potential explorations for unique vaccine candidates and innovative drug targets.

Analysis of functional "omics" data from all the stages of *Plasmodium falciparum* life cycle updates and enlightens the understandings of parasite physiology. This comprehensive fundamental parasite biology is the base for drug targets and vaccine candidate identification, and in addition, it contributes to mechanism of drug action and effectiveness of developing drug resistance.

3.0 OBJECTIVES

1. Cellular localization of PHB1 and PHB2 in *Plasmodium falciparum*
2. Essentiality of PHB1 and PHB2 in *Plasmodium* biology
3. Identification of interacting partners of PfPHB1 and PfPHB2

4.0 REVIEW OF LITERATURE

4.1 The Unusual Mitochondria

Mitochondria are an organelle from alpha proteobacterium by genesis and was engulfed by a eukaryotic progenitor (86). The organelle has maintained the double membrane structure and the core function of ATP production. But their form and overall composition have been drastically altered by evolutionary forces (87). In vertebrates, each of this energy-making organelle includes two membranes which are functionally distinct named as outer and inner membranes that creates cristae with its circular mitochondrial genome called the mitochondrial DNA (mtDNA). Normally, vertebrate mtDNA is approximately 16Kb, and codes for genes including two of the ribosomal RNAs (rRNAs), 13 different proteins, and 22 tRNAs (88). The mtDNA displays enormous diversity in different evolutionary lineages. Alveolates provide the most significant example of mtDNA diversity and mitochondria in these organisms have tubular cristae. Ciliates, dinoflagellates, and apicomplexans are the three major alveolates that have dramatically diverged in the organization and gene contents of mitochondria. Among alveolates, apicomplexan mtDNA encodes only three ORFs. The first apicomplexan mtDNA was identified from *Plasmodium yoelii*, a rodent malaria parasite (89).

Rudzinska showed cristae in bird malaria species and noted the lack of obvious cristae in asexual stages of the mammalian malaria species (32). The structure of *Plasmodium* mitochondrion is tubular (90) with 6Kb organelle DNA encoding only three genes: subunit1(cox1) and subunit3 (cox3) of cytochrome c oxidase complex (complex IV), and cytb (cob) a member of cytochrome bc1complex, and in addition also contain the highly fragmented rRNA genes of the large subunit

(LSU) and small subunit(SSU) (16, 91, 92). The organization of mitochondrial genome is highly conserved in 23 *plasmodium* species. There are 27 small rRNA fragments (15 LSU rRNAs and 12 SSU rRNAs) ranging from 23 to 190 nt (93). Transfer RNA – tRNA is imported from the cytoplasmic tRNA pool, because of its absence in Plasmodium mitochondria (94). The mtDNA of malaria parasites replicate by a rolling circle mechanism similar to some bacteriophages and plasmids (95).

Mitochondrion of plasmodium is tightly associated with a multi membrane-bound organelle, speculated as storage organelle of the mitochondrion (33). Later the organelle was identified as plastid of *Plasmodium*, the apicoplast (25, 96, 97). Association of mitochondria and apicoplast is seen throughout the asexual life cycle (35, 98).

4.2 Sexual and asexual life cycle of *Plasmodium falciparum*

The life process of the malaria parasite is extremely complex and it requires two different hosts for its development. The sporozoites enters the human bloodstream all along the blood meal of an infected mosquito. These sporozoites moves to the liver and occupy the parenchymal cells and undergo asexual development. The *P. vivax* and *P. ovale*, sporozoites may enter the dormant stage in the liver for weeks or months. These dormant uninucleate parasites are hypnozoites. Multiplication of sporozoitestakes place in the liver, tens of thousands of merozoites rupture the hepatic cells and enter the bloodstream (99). In the bloodstream, merozoites enter the red blood cells (RBCs) and go through asexual reproduction termed the Intraerythrocytic cycle. During this cycle, the first stage of the parasite is called the ring stage. to The host cell modifications begins in the ring-stage parasites by using remodeling factors and variable antigens. The abundant hemoglobin of the

host erythrocyte is swallowed by the endocytic process. The toxic form of haem is polymerized within the food vacuoles of the trophozoites and these are parasites in the second stage of development (33). In this stage of the cycle, trophozoites develop and occupy most of the erythrocyte. Finally, trophozoites undergo a process of nuclear divisions named schizogony, not coupled with cytokinesis to form multinucleated schizonts. The multinucleated schizont undergoes cytokinesis resulting in the segregation of the nucleus into individual daughter merozoites. At the end of schizogony, schizonts rupture and release 16-32 merozoites into the bloodstream. These merozoites are ready for the next round of replication in fresh red blood cells (23). Apart from undergoing the asexual cycle, a very minor segment develops into gametocytes which determines the transmission of the infection through *Anopheles* mosquitoes.

Fig. 1 Life cycle of *Plasmodium falciparum* in Human and mosquitoes

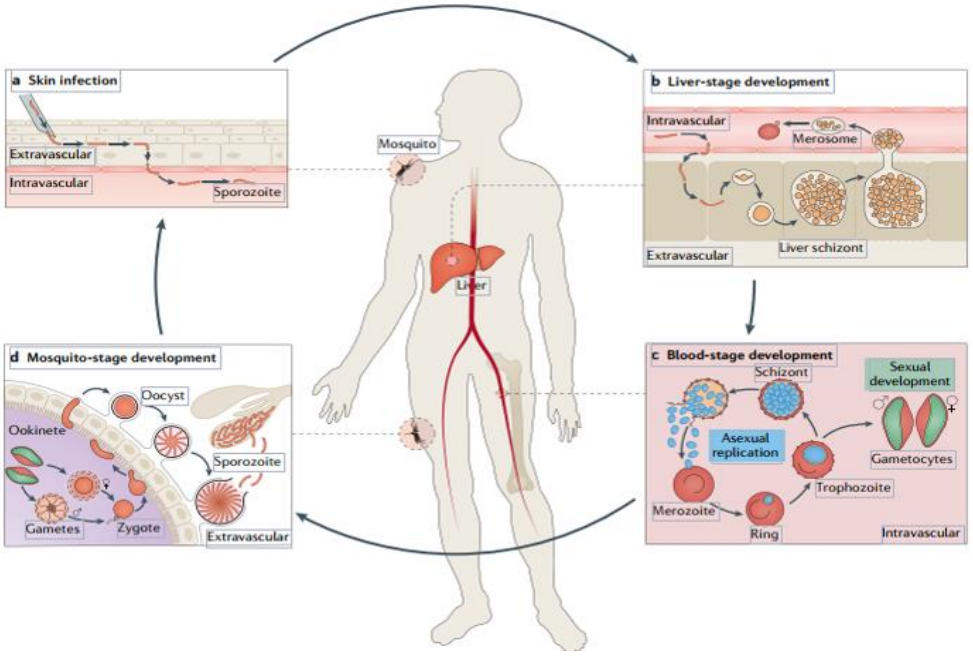


Figure adapted from Venugopal, Hentzschel, Velkiunas and Marti., 2020 (2)

The invasion of RBCs by merozoites has been a major focus of research in many labs around the world. The receptors involved in parasite invasion have been characterized in great detail. Parasite invasion involves apical reorientation, junction formation, and signaling. Three parasite organelles rhoptries, microneme, and dense granules play a key role in the parasite invasion pathways (23).

The sexual reproduction cycle takes place in mosquitoes when they ingest the gametocytes while taking a blood meal from an affected individual. The male gametocyte and female gametocyte fuse and establish a diploid zygote, leads to the development of ookinetes in the midgut of the mosquito. These Ookinetes penetrate the mosquito midgut where they transform into oocysts. Sporozoites develop in the oocysts and when the oocysts rupture, sporozoites are released. The released sporozoites travel to the salivary glands, and when the mosquito bites a human, they are released into the bloodstream (100).

Fig. 2 Transmission electron micrographs of major blood stages of *Plasmodium falciparum*

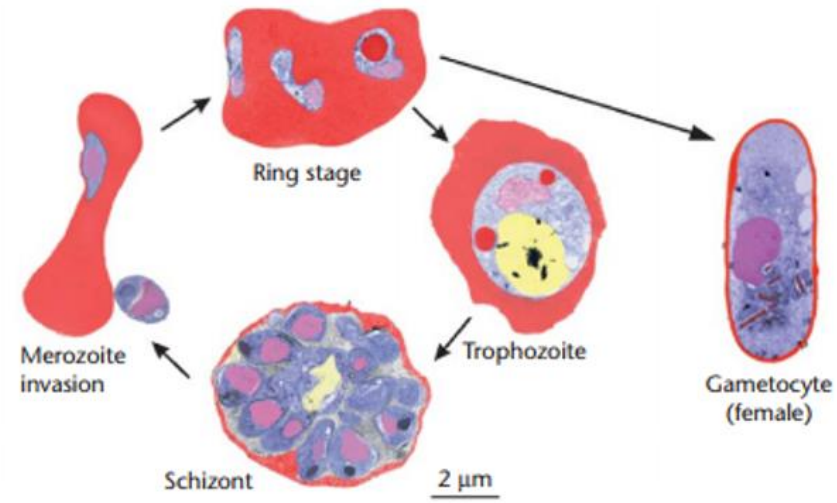


Figure adapted from Bannister and Sherman 2009 (7)

Upon infection, it may take several days to months for the symptoms to show up depending on the species. Usually, *P. falciparum* takes 9-14 days, while *P. vivax* infection manifestation may take 12-18 days or even up to 6-12 months to appear (101). Pathogenesis of malaria develops during the different stages of asexual life cycle. The nonspecific symptoms of malarial disease are fever, muscle pain, headache, chills, fatigue, and anorexia. These symptoms often appears in synchronous with the parasitic cycle (23, 102, 103). Severe disease manifestation of the disease is seen in *P. falciparum,* and cerebral malaria and renal failure are possible outcomes if not diagnosed and treated early. Cerebral malaria is a serious form of the disease and is accompanied by headache, confusion, coma, and eventually death (104).

4.2.1 Cellular developments in sexual and asexual stages of *Plasmodium*

A. Merozoites

The merozoites, ring, trophozoite, and schizont are the sequential stages in the asexual blood stages of development. Merozoites are the invasive forms and are the smallest stage in the life cycle. They are oval and their anterior end is sustained with three cytoskeletal rings also called polar rings, anchors by two sets of secretory organelles: micronemes and rhoptries. There are two pear-shaped rhoptries, whereas micronemes are numerous in number and much smaller in size. The narrow end of these organelles converges at the apical region of the merozoites of secretion (105). The minimal organelle development for the next intracellular stages is also seen in this stage. The organelles are solitary nucleus, a mitochondrion, an apicoplast, and some ribosomes. A combination of molecules comprises adhesive proteins (adhesins), proteases, and membrane-altering agents are secreted from micronemes and rhoptries

which facilitates erythrocyte capturing, establishing juncture, parasitophorous vacuole genesis, merozoite coat expulsion, and final entry of the parasites (106).

Fig. 3 Transmission electron microscopy of merozoite

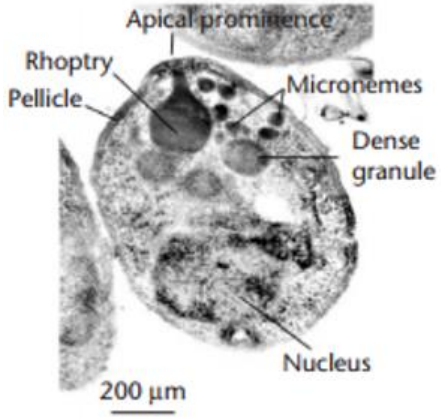


Figure adapted from Bannister and Sherman 2009 (7)

Fig. 4 Blood stages of *Plasmodium falciparum* light microscopy

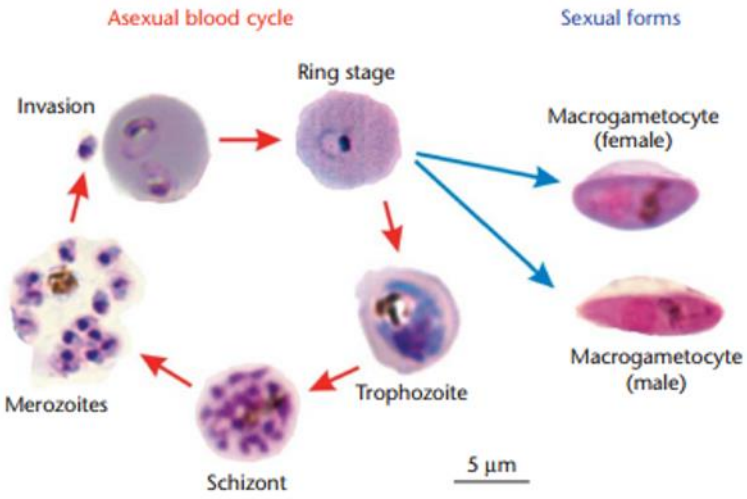


Figure adapted from Bannister and Sherman 2009 (7)

B. Ring and Trophozoite

Merozoites lose the invasive organelles after entering erythrocytes. The invaded parasite becomes a disc- or cup-shaped structure under a light microscope, and hence is called 'ring'. It starts ingesting the erythrocyte cytoplasm by hemoglobin proteolysis within the small vacuoles. The parasite gradually increases in size to form larger trophozoites and continues feeding. Parallel to feeding, parasites export protein to the surface of the erythrocytes to prepare the membrane permeable to nutrients from the surrounding blood plasma by transforming the erythrocyte surface (107).

C. Schizont

The division of the parasite nucleus and its final number depends on the species. In *Plasmodium falciparum*, mature trophozoites develop into schizont, during the process nuclear and cytoplasm undergoes 3-6 rounds of division contribute to 8-36 merozoites in each schizont of development (108, 109). Each differentiated nuclei enters the merozoite bud and other organelles assemble nearby. The mature merozoite dissociate from the parent schizont leaving behind very less cytoplasm and the residual body hemozoin. Eventually, the protease from secretory vesicles triggers a series of reactions that leads to the release of merozoites from the surrounding erythrocytes (110).

D. Gametocytes

In the process for the sexual phase, a fraction of intracellular parasites foregoes mitosis and progresses to gametocytogenesis. The regulators involved in this process are not completely known. But the process build upon parasite density, genetic variations and is mediated by many signaling pathways (111). The male and

female gametocytes develop and possess a sets of characteristic structures and chemistry, though they hold identical chromosomal contents. In *Plasmodium falciparum*, mature gametocytes both male and female are long and curved but differ from each other in their cellular details. Osmiophilic bodies aids in the departure from host cell, which is a secretory vesicles found in all matured gametocytes (112, 113). After ingestion into the mosquitoes, the gametocytes escape from erythrocytes. This escape is triggered by a temperature drop and chemicals from the mosquito. Several male gametes develop from the rapid division of male gametocytes which also sprout long motile flagella.

E. Ookinete

The fused zygote undergoes fused meiotic division and elongates to form Ookinete. Ookinete penetrates the gut epithelium and forms the oocyst. Several rounds of DNA replication followed by repeated internal mitotic division takes place within the oocyst. The group of chromosomes separate and form finger-like projections, generate new organelles and detach as a sporozoite (100).

F. Sporozoite

Sporozoites are elongated and slightly curved cells ~ 10µm long and tapering at the ends. The cellular structures are the central nucleus and apical organelles comprising a polar ring, and a set of rhoptries. At the posterior location, a single mitochondrion and apicoplast can be seen (114). Like merozoites, apical organelles of sporozoites contain invasive proteins which enable the attachment to glycosaminoglycan of the host cell.

4.3 Mitochondrial Cell Biology

This organelle exists as a single mitochondrion during the asexual life cycle (115, 116). This single mitochondrion elongates, branches, and divides with the progress of the life cycle (35). This highly branched structure undergoes numerous rounds of nuclear division which is uncoupled with cytokinesis results in the formation of schizont cell with multinucleated structures, with up to 30 nuclei. Now, the schizont cell undergoes cytokinesis to give daughter merozoites with a properly segregated nucleus. The process of cell division is known as schizogony. While segregating into daughter merozoites, divided mitochondria associates with divided apicoplast as a paired organelle (35). Electron microscopic images show the movement of paired organelles in the daughter merozoite cells, suggesting a posterior or lateral move to the nucleus (33, 34). The mitochondrion frequently appears to have interacting points with the plasma membrane. Elongated, as well as branched mitochondrion, contain loop regions where the fusion of this organelle takes place by itself (35).

The complex life cycle of *Plasmodium* parasites involves additional proliferative stages succeed in the liver and in the mosquito midgut. In these stages, very limited information is noted about the general aspects of mitochondria. Each round of the asexual cycle leaves behind a meager section of merozoites to form gametocyte cells. These gametocytes differentiate into gametes and sexual reproduction takes place when mosquitoes take up the gametocytes. Even though branched or clustered mitochondria are seen, gametocyte most often contains multiple rounded structures of mitochondria (36). Oocysts also have multiple mitochondria, which is a propagative stage of *Plasmodium* occurs in the mosquito midgut (37).

In comparison with apicoplast which is morphologically static, the mitochondrion undergoes spectacular morphological development with gametocyte maturation. There are five morphological development stages of gametocytes. The stage II shows the elongating and branching mitochondrion somewhat similar to asexual schizont cells. Now the mitochondrion appears like an elongated organelle encasing the functionally active apicoplast (117). Mitochondrion has a close organization with apicoplast throughout the whole gametocyte development process (118). The absence of mitochondria and apicoplast in male gametes is evidence for maternal inheritance (119).

Mitochondrion elongates longitudinally as the gametocyte elongates during stage III of development. Mitochondrion folds along with elongated apicoplast in these elongated cells (118). The expanding organelle is highly comparable with the activation of mitochondrial metabolism in gametocytes. The asexual cycle eschews the mitochondrial tricarboxylic acid cycle and relies exclusively on cytosolic glycolysis. But these parasites produce ATP in their mitochondria during the mosquito stages (120). Painter *et al*., show that a mitochondrial electron transport chain is only required for the reduction of dihydroorotate dehydrogenase (17). The parasites of erythrocyte stages depend on glycolysis with the end product of lactate and pyruvate (121).

The parasite's genome was found to encode all the enzymes responsible for the TCA cycle (11) in asexual development (122). Studies show eight enzymes concerned with TCA cycle is localized to the mitochondria (123-126). Isotopic labelling studies show that glucose and glutamine are the main carbon sources for TCA reactions (127, 128). Recent studies show six out of eight enzymes of the TCA cycle can be knocked

out. Knock-out lines are found to grow normally in asexual blood stages but do not progress to oocysts in mosquitoes. These mutant lines show that parasites have significant flexibility to alter the exchange of substrates between cytosol and mitochondrial pool (129). The acetyl-CoA for mitochondrial TCA is produced from pyruvate by branched-chain keto acid dehydrogenase (BCKDH) (130).

4.4 The Oxidative Phosphorylation

The tricarboxylic acid cycle acts as the primary component of mitochondrial metabolism which is followed by the electron transport chain happen in the inner mitochondrial membrane. This electron transport chain (ETC) acts as a biochemical motor in driving countless mitochondrial and cellular functions. The *plasmodium* ETC is a well-studied system and the for antimalarial drug atovaquone targeted to this system (131-134). The mitochondrial electron transport complexes from genomic data suggest a simpler unit composition than complexes from humans and yeast (11, 135). The electron transport chain of the malaria parasite contains five dehydrogenases: NADH: ubiquinone oxidoreductase (PfNDH2) (136), succinate: ubiquinone oxidoreductase (Complex II or PfSQR) (137), and the malate quinone oxidoreductase (PfMQO) (136). Glycerol-3-phosphate dehydrogenase (PfG3PDH) is involved in redox homeostasis (138) and dihydroorotate dehydrogenase (PfDHODH) serves as an electron sink. The functional contribution of these enzymes are not fully understood. These dehydrogenases contribute electrons to the downstream complexes. All the dehydrogenase involved in ETC are displayed to magnify oxygen consumption during the asexual blood stages of parasites (138). There are three protein complexes specifically named as ubiquinol: cytochrome c oxidoreductase

(Complex III /cytochrome bc_1), cytochrome c oxidase (complex IV) with ubiquinone (also known as coenzyme Q), and ATP synthase (Complex V) (139-141).

Each enzyme is responsible for shuttling electrons from upstream reaction avenue to the ubiquinone pool. The electrons are utilized by the proton – pump bc1 complex (complex III or quinol: cytochrome *c* reductase) and cytochrome *c* oxidase (complex IV) to create electrochemical gradient for ATP synthesis by ATP synthase (complex V) (142).

4.4.1 NADH: ubiquinone oxidoreductase (PfNDH2)

The functional role of NADH: ubiquinone oxidoreductase (PfNDH2) in mitochondrial ETC is not thoroughly understood. The absence of the transmembrane domain shows that this enzyme may not be associate in proton pumping. However, the activity of this enzyme may incidental support to the formation of electrochemical transmembrane potential (140, 143).

4.4.2Dihydroorotate dehydrogenase (PfDHODH)

Dihydroorotate dehydrogenase (PfDHODH) is a fundamental fourth enzyme involved in the pyrimidine synthesis of DNA, RNA, glycoproteins, and phospholipids (17, 141). PfDHODH catalyzes the reaction of oxidation of dihydroorotate to orotate (144, 145).

4.4.3 Succinate: ubiquinone oxidoreductase (Complex II or PfSQR)

PfSQR is considered to be inessential during these lifecycle stages (137). However, along with $PfNDH_2$, it is considered to be crucial for the parasite development in mosquitoes (146, 147). Similar to most eukaryotes, *Plasmodium* SQR is comprised of four polypeptides: one flavoprotein (Fp) subunit, one iron-sulfur (Ip)

subunit, and two cytochrome *b* (cyt*b*) subunits namely CybL and CybS (148). The catalytic portion or succinate dehydrogenase of the complex is formed by Fp and Ip subunits. This catalytic portion is responsible for the oxidation of succinate by water-soluble electron acceptors of PfSQR in the matrix periphery of the inner mitochondrial membrane. CybL along with CybS subunits form the membrane anchoring proteins (124, 149, 150).

4.4.4 The malate quinone oxidoreductase (PfMQO)

Sequence analysis of *Plasmodium* MQO classifies this reductase under group II. This suggests that apicomplexan parasites acquire MQOs by lateral gene transfer from ε-proteobacteria (151). PfMQO is required in ETC, TCA cycle, and fumarate cycle (129). PfMQO is proven to be necessary for protein synthesis, purine salvage pathway, and pyrimidine de novo biosynthesis (152).

4.4.5 Glycerol-3-phosphate dehydrogenase(PfG3PDH)

The *Plasmodium falciparum* genome has both cytoplasmic and mitochondrial homologs of glycerol 3 phosphate dehydrogenase (134, 136, 153). The NAD-dependent glycerol 3 phosphate dehydrogenase (G3PDH-1) is a cytosolic enzyme that converts dihydroxyacetone phosphate to glycerol 3 phosphate coupled with NADH oxidation. G3PDH-1 enzyme is required in the lipid biosynthesis pathway. This enzyme also has an vital role in the transport of reduced equivalents from the cytosol to the mitochondria (154). The FAD-dependent glycerol 3 phosphate dehydrogenase (G3PDH-2) re-oxidizes glycerol 3 phosphates to dihydroxyacetone in the outer mitochondrial membrane. This shuttle is responsible for the transport of cytosolic NADH to the mitochondrial quinone (155).

4.4.6 *Plasmodium falciparum* cytochrome bc_1 complex (Complex III)

The cytochrome bc_1 complex plays a main part in oxidative phosphorylation in various organisms (156). Eukaryotic bc_1 is a dimeric enzyme complex in the mitochondrial inner membrane and is formed of 11 non-redundant subunits (157-159). Crystal structure of bc_1 have been established for yeast, chicken, and bovine but the complex remains unsolved in *Plasmodium* spp (157, 158, 160). So far, seven subunits are found to involve in the formation of a complex in *Plasmodium* spp. including cytochrome b encoded by mitochondrial DNA of the parasite (161). The catalytic core which is responsible for the electron transfer pathway is built of three subunits: cytochrome b, cytochrome $c_1$and Rieske iron-sulfur protein (162). The bc_1complex catalyzes the conduct of electrons from ubiquinol to cytochrome c which is joined with the vertical translocation of protons along with the generation of transmembrane electrochemical gradient ($\Delta\Psi$m). The bc_1 complex catalysis the mechanism of proton transfer referred to as Q cycle which is first postulated by Mitchell (163).

4.4.7 ATP Synthase (Complex V)

Plasmodium ATP synthase is suggested to act as a proton leak for the electron transport chain and has no role in the generating of ATP (139, 141). The respiratory stimulant almitrine has activity against PfATP synthase at its cellular level (164). Recent genetic studies show that ATP synthase is dispensable in *Plasmodium berghei* blood stages but essential in mosquito stages (165).

4.5 Cytochrome c Oxidase complex (Complex IV)

The enzyme cytochrome c oxidase catalyses the last oxidation step in the mitochondrial respiratory chain. The electrons are received from cytochrome c pass to oxygen along with two **a** type of hemes namely **a** and $\mathbf{a_3}$, and two copper centers called **CuA** and **CuB**. Similar to many eukaryotes, subunits I and III of cytochrome c oxidase are encoded by the mitochondrial genome of *Plasmodium* spp. but subunit II is coded by split genes in the nucleus genome particularly: COXIIa and COXIIb. COX-II subunit is highly hydrophobic and the split may help in the import of this subunit into mitochondria (155). Cytochrome c oxidase discharges four protons from the matrix and operates as a true proton pump. Essential amino acid residues engaged in proton pumping in yeast and bacterial cytochrome c oxidase are conserved in *Plasmodium* enzymes. Cytochrome c oxidase in malaria parasites has been proven in different stages of *Plasmodium berghei* (166-168), *Plasmodium falciparum* (169), and *Plasmodium yoelii yoelii* (134). From *Plasmodium berghei*, partially purified and characterized of this enzyme has been shown (170). The enzyme is sensitive to cyanide inhibition has also been characterized in *Plasmodium falciparum* mitochondria (171, 172). *Plasmodium falciparum* also has a nuclear-encoded subunit COX Vb which regulates the flow of electrons between the different reaction centers of the enzyme (173).

4.6 Assembly of cytochrome c oxidase complex

The cytochrome c oxidase complex is gathered from 13 subunits in humans whereas 11 subunits in *Saccharomyces cerevisiae*. In all respiring organisms, the three core highly conserved subunits named as Cox1, Cox2, and Cox3 are coded by mitochondrial genome. The additional subunits are coded by the nucleus and are

imported to mitochondria. The structure analysis of bovine cytochrome c oxidase discloses that redox cofactors that is haem and copper are embedded within the core enzymes of Cox1 and Cox2 (174). The Cox1 subunit carries the two haem molecule and a copper ion in CuB site and the Cox2 subunit harbor two copper ions in CuA site. There is only limited information available with respect to the enzymatic functions of Cox3. A generous number of assembly factors are suggested in the assembly of cytochrome c oxidase from both sides of the mitochondrial membrane (175).

The mitochondrially synthesized subunits of cytochrome oxidase are Cox1, Cox2, and Cox 3 have specific translational regulators and assembly factors, which have been well defined in yeast. Cytochrome c oxidase assembly 1 (Coa1) and cytochrome c oxidase assembly 3(Coa3) are the assembly factors which turns on or off the translation. Precursor Cox2 (pCox2) is translated with 15 amino acid extensions and is imported into the inner mitochondrial membrane. Membrane peptidase 1 located the inner mitochondrial membrane process the precursor and Cox 2 binds to Cox 20 to maintain the assembly competency (176). Cox5 and Cox6 are the nuclear-encoded subunits associated with Cox1. Insertion of copper in Cox2 is the prerequisite for it to bind the Cox1-Cox5-Cox6 complex (177). The organization of Cox2 and Cox3 with this complex is not clear. However, the subunits Cox7, Cox8, and Cox9 bind to form a complex ahead of joining the above-mentioned complex. The formation of the complex is complete with the joining of Cox12 and Cox13, even though they are inessential for the enzymatic action of the cytochrome c oxidase complex (178, 179). The correct assembly of all subunits is essential for its function and defects in the biogenesis process leads to several respiratory deficiencies such as encephalomyopathy, hypertrophic cardiomyopathy, and Leigh syndrome (180, 181).

Many patients have been shown to display genetic variations in the genes encoding assembly factors required in copper metabolism like Cox 11, Cox17, SCO1, and SCO2 (182-185) or with heme biosynthesis (Cox10 and Cox15) (186-188). The utmost frequent mitochondrial disorder in infants is Leigh syndrome characterized by cytochrome c oxidase and mutations in the *SURF1* gene (189-191).

Fig. 5 Heme a synthase Cox15 and Shy1 complexes associates with cytochrome c oxidase assembly intermediates.

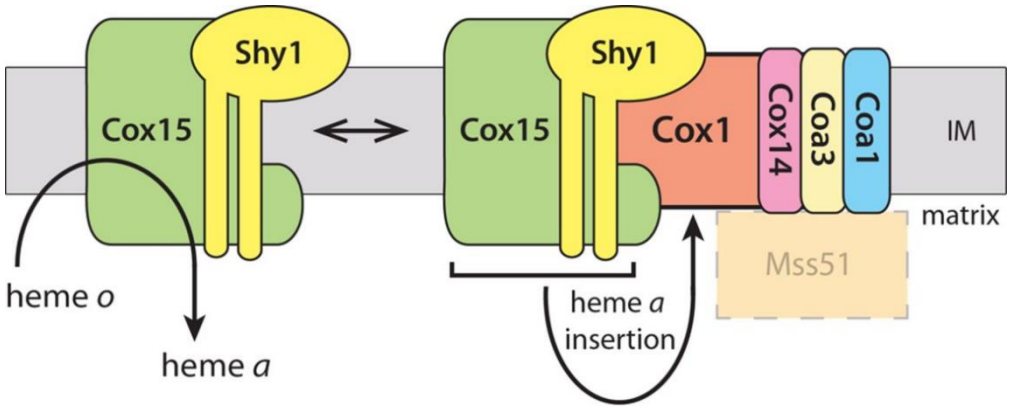


Figure adapted from Berath *et al*., 2013

4.7 Significance of Surf1 in mitochondria

SURF1, a well sustained assembly factor of cytochrome c oxidase from respiring bacteria to humans (192-194). SURF1 acts as heme insertase and also shows chaperonic activity by assisting in heme a_3 insertion (194-198). The SURF1 homolog in *Saccharomyces cerevisiae* is named Shy1 (stands for SURF Homolog of Yeast). Shy1 in yeast serves as a model for the analysis of molecular functioning and assembly of cytochrome c oxidase (195, 197). Shy1 localizes to the inner mitochondrial membrane. Protease protection analysis explains SURF1/Shy1 retains a broad intermembrane space domain which get inserted in the inner membrane with

the help of N- and C- terminal helices (192). In *shy1*Δ cells, lacks shy1 protein greatly reduces the assemblage of cytochrome c oxidase which has a residual activity of ~30% inadequate for respiratory growth (199). Shy1 is essential for Cox1 expression, and in Shy1 mutants, COX assembly will be affected (200). Protein interaction studies reveal that Shy1 shows physical interaction with translational factors of Cox1 inclusive of Coa1, Coa3, Cox14, and Mss51 (201). In yeast, Shy1 is shown to be associated with the mature complex of cytochrome c oxidase (195). In *Rhodobacter sphaeroides* lacking Surf1, cytochrome c oxidase activity reduced to 35% and 50% of the enzyme complex assembled lacked heme a_3. *In vitro* studies with *Paracoccus denitrificans* show the binding of Surf1 with heme a (196, 202). The molecular mechanism of heme insertion is not known but heme is inserted into Cox1 by a Shy1 dependent step (198, 203).

Yeast based molecular and biochemical studies in displays Shy1 act as a lead protein in recognition of various new factors necessitate the electron transport chain complex assembly and function of mitochondria. A relevant health-connected goal is to annotate the complete function of factors involved in the control mechanism that operates in the mitochondria of organisms (175). The study of such candidate genes would be advantageous in the exploration and understanding of the molecular biology of the organelles.

4.8 Prohibitins: Pleiotropic proteins

Proteins having the stomatin, prohibitin, flotillin, and HflK/C domains are called SPFH proteins. These are found almost across all prokaryotic and eukaryotic organisms. The protein family comprises stromatin, stromatin like protein, prohibitin, flotillins, podocin, erlins, and many bacterial membrane proteins (204).

The eukaryotic prohibitin family contains two vastly homologous proteins specifically prohibitin 1 (PHB1) and prohibitin 2 (PHB2). PHB1, a tumor suppressor protein was first identified in mammalian with antiproliferative activity, hence named as prohibitins (205). PHB2 was isolated together with PHB1 which is bound to the receptors of IgM antigen. Hence these proteins are named as B-Cell receptor complex associated protein (BAP32 and BAP37) (206). PHB2 also act as a repressor of the nuclear estrogen receptor also referred to as REA based on its action. as (207). Both the prohibitins are pleiotropic proteins with multiple functions (208).

Both PHB1 and PHB2 proteins are multifunction proteins responsible for diverse functions. Prohibitins have been shown to regulate transcription (209, 210), cellular signaling (211), apoptosis (212), biogenesis of mitochondria, and maintenance of mitochondrial DNA (213). PHB2 is involved in the regulation of sister chromatid cohesion (214).

4.9 The mitochondrial prohibitin complex

The native structures of PHB1 and PHB2 analysis in yeast, nematodes, and mammals show that PHBs form large complexes with excessive molecular weight. This complex occupy the inner mitochondrial membrane and measures ~1.2MDa (215-218). Co-immunoprecipitation experiments of yeast and mammalian cells show both PHB1 and PHB2 are physically interacting (218-220). The function of each subunit is independent in various organisms (215, 221-223).

Fig. 6 Schematic assembly of PHB1 and PHB2 subunits, ring-shaped prohibitin complex and its topology in the inner mitochondrial membrane

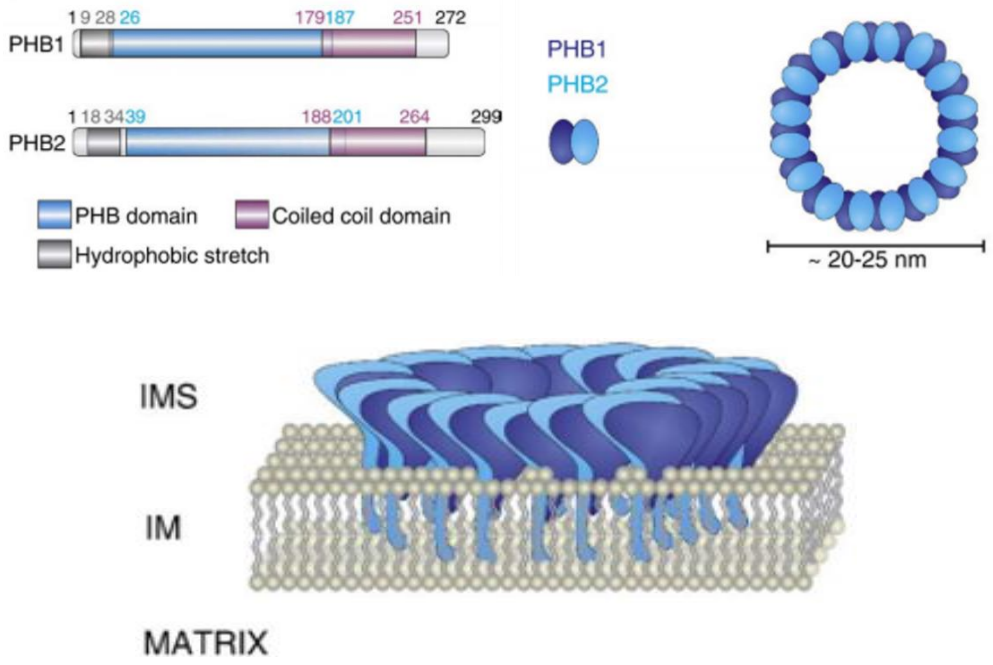


Figure adapted from Merkwirth and Langer., 2009 (222)

Electron microscopic analysis of purified yeast PHB complex declares a ring-like complex with an outer diameter of 200-250Å (217). Cross-linking experiments show the interaction between PHB1 and PHB2 subunits and absence of homodimers formation. This suggests that the complex is made of both PHB1 and PHB2 subunits that alternate with each other (224). The PHB complex assembly depends on both subunits. Deficiency of one of two (PHB1 or PHB 2) subunit results in lack of complex formation, thus proves the interdependency between the proteins (215, 219, 221, 222). The absence of either of prohibitin does not influence the expression rather results in degradation. Thus the mitochondrial complex of prohibitins form a physiologically active structure that stabilize each other (225). PHB1 and PHB2 are lead to mitochondria by N terminal signal sequences. Insertion of N terminal sequence in the inner mitochondrial membrane is resolved by Tim23 translocase. This

insertion is also promoted by the Tim8-Tim13 chaperonic complex in the intermembrane space of the mitochondria. The C terminal of PHB1 and PHB2 forms huge domains with coiled-coil structures in the intermembrane space. These domains subsequently undergo oligomerization to form a large complex. Tetrameric PHB1 and PHB2 form the intermediate complex of ~120KDa and are the building blocks for the large ring complex (224).

4.10 Molecular functions of prohibitin

The prohibitin supercomplex in mitochondria maintains the structure of mitochondria and regulates the organelle functions (217). The newly imported proteins are protected by PHBs in mitochondria from degradation by m-AAA proteases; these are mitochondrial ATPases involved in diverse cellular activities (218). These proteins are also found to involve in promoting mitochondrial protein synthesis (219) and help in maintaining the organization and copy number of mitochondrial DNA (213). Nijtmans *etal* show the chaperonic activity of PHBs for the newly synthesized proteins of mitochondria (216, 226), and also in GTPase optic atrophy 1(Opa1) during mitochondrial fission and morphogenesis (227).

Fig. 7 Mitochondrial functions of prohibitin

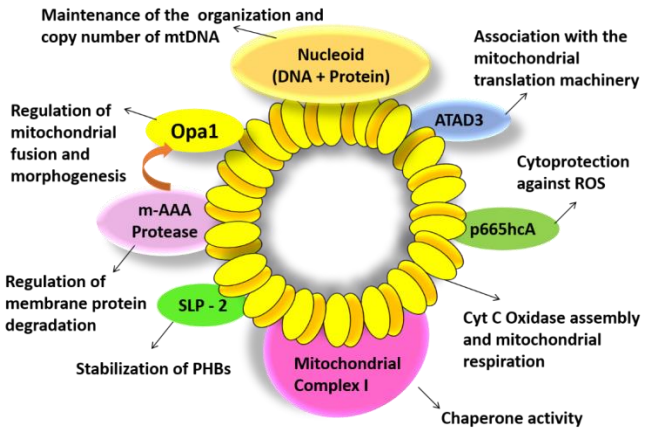


Figure adapted from Thuaud, Ribeiro, Nebigil, and Desaubry., 2013 (208)

The PHB complex also appears to stabilize the mitochondrial genome (213, 228, 229). PHB1 interacts with p66Shc, an adaptor protein. This stress-induced adaptor protein gets activated by UVC, H_2O_2, and reactive oxygen species (ROS). which in turn primes to apoptosis (230). Sphingosine 1 phosphate (S1P) binds to PHB2 with high affinity and maintains the integrity of mitochondrial respiration (231). Not limited to mitochondria, PHBs are found to have multiple roles in cell physiology. Other organelles where PHBs are shown to localize include the endoplasmic reticulum, nucleus, plasma membrane, and macrophage phagosomes (232). The wide role of these proteins is extremely complex and is highly regulated by post-translational modifications of several tyrosine and serine phosphorylations, palmitoylation, tyrosine nitrosylation, transamidations and O-Glc*N*Ac modifications (233).

Mitochondria is a central organelle in iron homeostasis. This organelle forms the unique site for synthesis of iron and a major site for biosynthesis of iron-sulfur cluster (234). PHB1 binds to iron and is upregulated during oxidative stress; Tyr114 binds to heme (235). Also, the Tyr114 residue of prohibitin has a unique function in each cellular compartments (236).

4.10.1 Prohibitins in Oxidative Phosphorylation System

The foremost functional attributed to PHBs in mitochondria is mitochondrial protein quality control. the respiratory complexes are instable in the absence of PHB1 protein in the cell (216, 218, 237). Overexpression of PHBs in yeast has shown the stability of newly synthesized proteins of mitochondria, explaining holdase/unfoldase chaperone-like activity of prohibitins in yeast (216). The proteolysis process of unassembled inner membrane proteins of oxidative

phosphorylation system is regulated by PHB complex by forming complexes with m-AAA proteases, whereas the process of proteolysis is increased in phb null yeast cells (218). In yeast study, it is shown that the PHB complex interacts with Pim1, a Lon-like protease that stabilizes the unassembled Atp7 subunit of the F_1-F_0-ATP synthase during biogenesis (237). Several studies have examined the silencing of either of these proteins (PHB1 or PHB2), and results show silencing of PHBs lead to diminished activity of the respiratory chain complexes (238-242). Similarly, over expression of PHB1 enhances the function of complex I – dependent oxidative phosphorylation (243), and also improves ATP formation (243, 244). Physical interaction of prohibitins with different subunits of complex IV has been shown in yeast cells (216), and also with subunits of complex I proteins of mammals (245). Moreover, PHBs have a direct role in mitochondrial protein translation. Reduced expression of either one of the protein these proteins reduces the mitochondrial protein translation (246). STAT 3 indicates Signal transducer and activator of transcription 3, transcription factor that control the cell growth and reacts to cellular stress. PHB1 interacts with the STAT3 pool of mitochondria, and in turn interacts with complex I and II to regulate mitochondrial respiration (247, 248). In addition to the establishment of equilibrium, proteolysis process, and protein translation of mitochondrial subunits, some data suggest the role of these proteins in the assemblage of oxidative phosphorylation complexes (249) and the formation of supercomplexes (238, 250). The F_1F_0- ATP complex assembly is unstable to the loss of prohibitins (249).

4.10.2 Prohibitins in Ultrastructure and Dynamics of Mitochondria

The highly potent structures of mitochondria undergo continuous fusion and fission to adjust to the shape and cellular distribution, and to match the energy requirements of different cells. Many proteins centralized in the inner and outer l membranes of mitochondria are linked to fusion and fission of the organelle. In *C. elegans*, loss of PHBs affects the mitochondrial morphology, and depletion of PHBs induces fragmentation and disorganized mitochondria (215). OPA1 is associated with remodeling of cristae structure, fusion of the organelle, and also go through changeover from long-form represented as L-OPA1 (-uncleaved form) to short form implies S-OPA1 (- cleaved form) (248). The proportion within these two forms determine the cell fate (251-253). Proteases m-AAA, OMA1, and YMEL1 are involved in the conversion of OPA1 (254, 255). OPA1 stability depends on the PHB complex potency in the inner mitochondrial membrane (227). Also if the interaction of PHB1 and m-AAA protease is disturbed the conversion of OPA1 towards the formation of S-OPA1 is increased, and m-AAA proteases trigger the cleavage (256).

Mitochondrial shape and ultra-structure are highly connected to the lipid content of mitochondria. The maturation process of cardiolipins are highly influenced by PHBs (257), a phospholipid of inner mitochondrial membrane which performs a fundamental role in OPA1 mediated mitochondrial fusion (258). Mdm33 interacts with PHB1 and PHB2, which is a protein identified in yeast -regulates the homeostasis phospholipids of mitochondria like phosphatidylethanolamine and cardiolipin (259). The above-described functions of PHB1 and PHB2 in mitochondria, especially in organelle function, oxidative phosphorylation, assembly of complex proteins, translational machinery, and protein quality control emphasize their

significance. Furthermore, the highlighted central role of these twin proteins in mitochondrial biology suggest the possibility of a potential drug target (260) in infectious organisms whose PHBs have low similarity with human.

4.11 Prohibitins of apicomplexan

The mitochondria of apicomplexans have extensively adapted to a wide niche and nutrition availabilities during evolution (261). The cumulative function of the prohibitin protein complex in various molecular platforms for a multitude of cellular functions have been well demonstrated (262). The current understanding of PHBs function comes from studies in worms, yeast and mouse. The primary function of these ubiquitous proteins are explored in very few apicomplexans and parasitic pathogens (263). RNA interference studies show that PHB1 is essential in *Trypanosoma brucei*. Both PHB1 and PHB2 are indispensable for mitochondrial translation. The study shows that the absence of PHB1 and PHB2 decreases the mitochondrial membrane potential without affecting reactive oxygen species. Flagellates lacking PHB1 and PHB2 show morphological changes but no change in mitochondrial DNA and cristae (263).

In a recent study, PHBs of *Leishmania major* shows both the proteins PHB1 and PHB2 changed their cellular location in the presence of H_2O_2 which explains the protective action of PHBs against reactive oxygen species (ROS) (264). The same research also suggests that the prevention of DNA damage by prohibitins is directly linked to affinity for Fe^{3+} ions (264). Jain *et al*., 2010 show that prohibitins concentrate on the surface of flagellar and aflagellar poles of *Leishmania donovani,* and the attachment is through GPI (Glycosylphosphatidylinositol) anchor resulting in higher infectivity (265).

Gene targeting studies of PHB1, PHB2, and STOML shows the developmental essentiality of these protein in *Plasmodium berghei* in the asexual blood stages. Conserved roles of these proteins in mitochondrial morphogenesis could be the reason behind unsuccessful genetic disruption. Compared to the other three proteins PHBL expresses in very low levels, and has a specific function in the regulation of mitochondrial membrane polarity but is not involved in mitochondrial morphology and segregation. In addition, PHBL deficient malaria parasites display non dividing gametocytes and ookinetes in the sexual life cycle (266).

4.12 Genome engineering techniques in malaria

The complex life cycle of *Plasmodium* parasites show a precise spatiotemporal gene regulation (267). Genome sequencing of *Plasmodium falciparum* has accelerated gene functional studies to understand the underlying molecular mechanism of pathogenesis process, transmission between vector and host, and development of drug resistance. Understanding the basic biology of gene function and molecular mechanism of *Plasmodium* growth and development is crucial to eradicate the global risk of malaria (268). Despite enormous research, several genes in the genome remain as hypothetical or putative proteins in all *Plasmodium* species. It is critical to figure out the genetic functions and essentialities of these putative and hypothetical proteins to explore novel drugs and vaccine targets (269). Among all *Plasmodium* species of human malaria, blood stages of *Plasmodium falciparum* are cultured in vitro using suitable supplements, and exogenous DNA is introduced into synchronous ring stages by means of transfection (270, 271). Electroporation is the only effective way of transfection of plasmid DNA in this parasite, but the transfection efficiency is very low at 10^{-6}. Whereas, *P.berghei,* rodent malaria species has much higher transfection

efficiency and is extensively used in targeted gene knockout studies. Ultimately, the haploid genomic structure of the parasite in the blood stages limits gene manipulation studies in understanding molecular function (269).

Transient transfection in *Plasmodium gallinaceum,* avian malaria species was the first reported transfection (47). Later, transient and stable transfections were developed and demonstrated in *Plasmodium falciparum* (48-50) and *Plasmodium berghei* (51, 272). Genetic manipulation has been widen to include rodent malaria species like *Plasmodium yoelii* (53, 54), *Plasmodium chabaudi* (273), simian parasites *Plasmodium cynomolgi* (274) and *Plasmodium knowlesi* (275-277). Plasmid DNA transfected by electroporation initially replicates episomally forming large concatamers (58, 59, 272). To introduce, mutate, knockout, or replace an appropriate gene, a homologous targeting sequence is incorporated in the plasmid DNA to trigger the integration into the genome locus. The process of integration within the locus is remarkably ineffective in *Plasmodium falciparum*. Generally, transfected parasites are rotationally maintained with on and off drug selection pressure to get steady integrated plasmids by means of cross-over recombination (58, 60). On the other hand, episomes are eliminated by negative selection (61, 62). From this viewpoint, significant progress is made in expanding genetic techniques to study *Plasmodium spp*., and that are well reported in malaria publications as core tools.

4.12.1 CRISPR/Cas9 System

Genetic manipulation tools include a range of strategies from conventional homologous crossover to zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR) with Cas9. Between all, CRISPR/Cas9 makes genome editing more

easy, convenient, and efficient. CRISPR/Cas9 based genome editing tool is derivational of microbial adaptive immune system where it protects the bacteria from foreign genetic elements (84). CRISPR/Cas9 system is grouped into two classes and six types based on the effector modules. Class 1 system of CRISPR has three types (Type I, III, and IV) and utilizes multi-proteins complexes, similarly, Class 2 has three types (Type II, V, and VI) which make use of effector protein with single-component like Type II – A Cas9 or Type V – A Cpf1 (278, 279).

Ghorbal et al., (82) and Wagner et al., (280) reported the CRISPR tools for *Plasmodium falciparum* using two plasmid systems: one to deliver Cas9 and another to bring in the guide RNA (gRNA) and donor template. Unlike ZFNs and TALENs nucleases, the Cas9 system doesn't require modification to alter the target specificity. The genomic target can be identified using potential gRNAs with 20 nucleotide sequences followed by NGG, the protospacer adjacent motif (PAM). Guide RNA can be designed through many freely available tools including protospacer (281), CHOPCHOP (282), and EuPaGDT (283). All of which score gRNAs based on the number and position of mismatches at potential off-target sites in the genome. In addition to this, on-target scores are also provided by some gRNA prediction tools that aim to predict gRNA activity. A recent study recommends on – target scores to be predictive of success in *Plasmodium falciparum* CRISPR/Cas9 experiments, and shows the annotated report of 662,795 gRNA sites in the parasite genome (284). The secondary challenge in CRISPR – mediated gene regulation of *Plasmodium* species is the difficulty in identifying unique gRNA binding sites with canonical (-NGG) PAM in the AT- content rich genomes. Interestingly, a wide range of Cas9 variants (285) with altered PAM specificity is in development with new CRISPR nucleases. Among many emerging nucleases, one well suited for AT-rich genomes is Cpf1 (also known

as Cas12a) with (TTTN-) PAM (286). Another fascinating development is RNA targeting nucleases like Cas13 which allows post-transcriptional regulation by RNA degradation and lacks non-specific RNAse activity (287, 288).

Fig. 8 Cas 9 endonuclease is directed to a genomic locus sgRNA

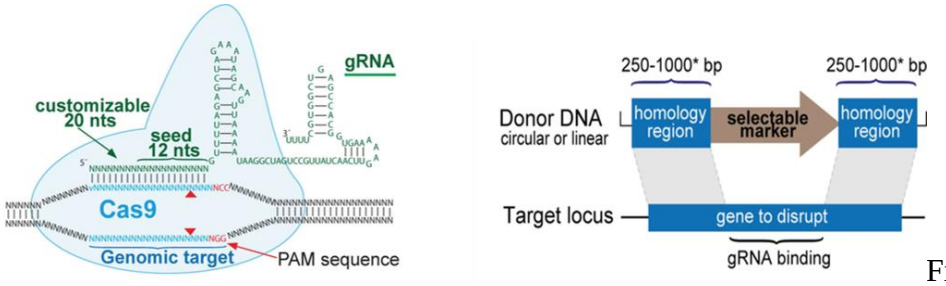


Figure adapted from Lee, Linder, Lopez-Rubio and Llinas., 2019 (288)

Parasites are cotransfected with two plasmids: one having Cas9 nuclease gene and second with DNA fragment encoding sgRNA and the donor DNA sequence. With successful transfection of both the plasmids and positive drug selection, the complex is formed between Cas9 nuclease protein and sgRNA. Cleavage in the double-stranded target DNA takes place by the binding of the Cas9-sgRNA complex. Homologous recombination in the target genomic DNA and the donor sequence in the plasmid causes the insertion of donor plasmid in the locus. Based on this engineering technique, both directional knockout and gene editing can be successfully achieved (81, 85, 289).

Fig. 9 CRISPR – Cas9 genome editing depends on Cas9 endonuclease and the single guide RNA (sgRNA)

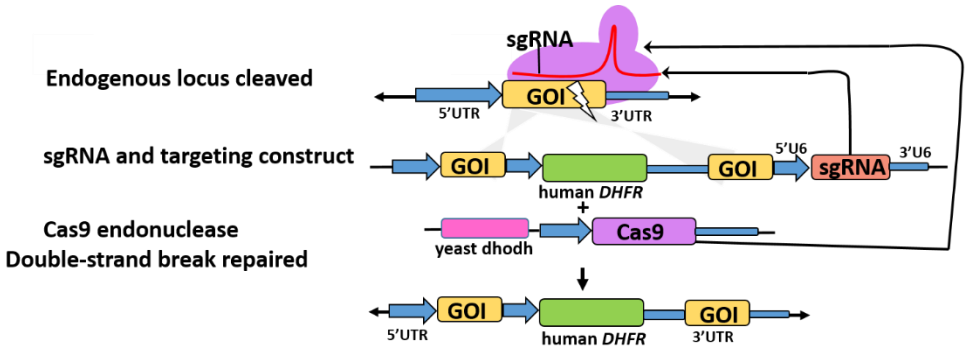


Figure adapted from de-Koning, Gilson and Crabb., 2015 (290)

This revolutionary technique is used in many advanced studies to understand the underlying mechanism of resistance to antimalarial drugs also the action of drug candidates. The CRISPR-Cas9 technique is used to identifying gene modifications in Pfmdr1 which causes resistance against a catagory of piperazine incorporated compounds in blood stages of *Plasmodium falciparum* (291). This same engineered technique is used to examine the resistance developed across drug candidate DSM265, suggesting the need for combinational therapy (292). Being faster, accurate, and cheaper than conventional homologous methods, CRISPR-Cas9 technology also has its limitations. The homologous recombination mechanism in malaria parasites depends on the co-existence of two plasmids, a limiting factor by reason of efficiency low transfections in *Plasmodium falciparum*. Recently, *the Plasmodium falciparum* cell line with internal expression of Cas9 nuclease in a centromeric plasmid (PfCas9) was established, but stable genomic integrants may overcome these limitations (293).

4.12.2 Conditional and inducible tools

Knocking out of any constitutive genes is not enough to understand or analyze the functionality of target genes in different stages of development (294). Gene expression can be controlled temporally and spatially using conditional and inducible approaches (295). Like any other eukaryotes, the coding region cannot be directly and inducible degraded by RNA medicated interference because *Plasmodium* spp., lacks the necessary machinery (296). The destabilization domain (DD)/*Escherichia coli* DHFR destabilization domain (DDD), Tet-Off, riboswitch, and Cre/FLP recombinases systems are generated for conditional knockouts/knockdowns in *Plasmodium falciparum* (297).

A. The DD/DDD domains

Conditional knockdown in protein level are achieved by integrating FK506 – binding protein (FKBP) destabilization domain (DD) (67) or with *Escherichia coli* DHFR destabilizing domain (DDD) (68). By fusing on with the target leads the protein to ubiquitylation and degradation of the target by degradation machinery of *Plasmodium*. The addition of stabilizing compounds reverses the Instability which allows controlled protein expression. Even though powerful, destabilization domain is checked by protein location and secretory proteins which don't follow degradation mechanism of the parasites and hence the technique cannot be used (290).

B. The Tet-Off System

Tet-Off System is a transcriptional level inducible knockdown using anhydrotetracycline (ATc) with conditional transgene expression in a defined stage and time (64). In this technique, the gene of interest is tagged with TRAD strands for

transcriptional transactivator domain (TRAD) and is located under the transcriptional control of the target gene promoter via homologous recombination. Here, tetracycline is the operator (TetO), and binding of expressed TRAD to TetO induces the gene expression of target. The addition of anhydrotetracycline (ATc) inactivates TetO which inhibits the binding to TRAD and halts transcription of the target gene. However, the tet-Off system is not perfectly functional in *P.falciparum* conditional knockouts. The major reason is the inefficiency of TRAD in recruiting transcriptional factors in this parasite (64, 290). However, this TetR domain, a part of TRAD is well used in the development of another conditional knockdown technique by linking with the ATc (inducible system) and RNA aptamers (72, 298). Aptamer-based knockdown is explained in detail later in this chapter.

C. Riboswitch System

This inducible gene knockdown approach makes use of post-transcriptional regulation by tagging with self–cleaving GlmS ribozymes (71, 299). Being with an inducer, glucosamine – 6 – phosphate (GlcN) cis-cleavage of the mRNA takes place, thereby reducing protein levels (71, 299, 300).

Fig. 10 Schematic of the glmS ribozyme reverse genetic tool

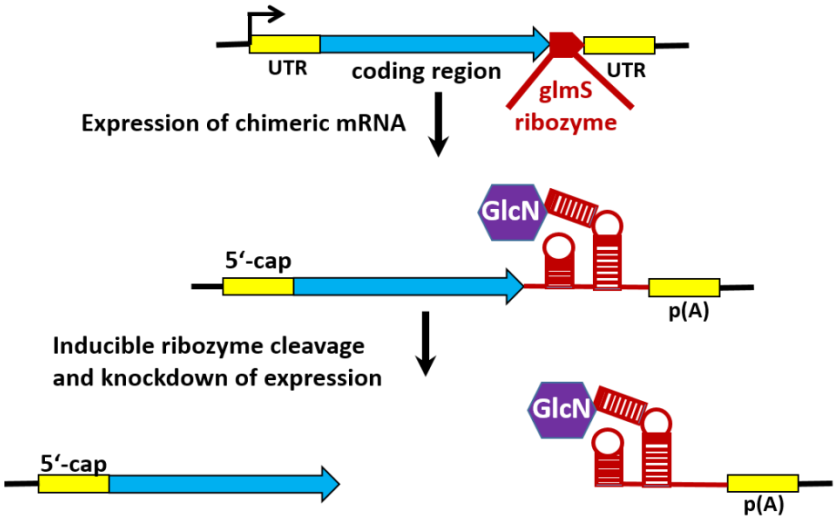


Figure adapted from Prommana et al., 2013 (71)

The assessment of multiple genes in *Plasmodium falciparum* has been made successful with this approach and is the current technique of choice for an inductive knockdown strategy in malaria. In a recent study, conditional disruption of cyclic AMP signaling pathway components, adenylyl cyclase beta (ACβ) and cAMP-dependent protein kinase (PKA) using riboswitch system was found to be very critical for RBC invasion of parasites (301). Even though this approach is highly promising, exposure to high doses of GlcN causes cytotoxicity in the parasites which is a drawback of the technique (299).

4.12.3 Proteomic approaches

Post-translational variations of many gene products determine the functionality of the proteins. Proteomic approved tools are very essential and ideal in the identification of drug-target-specific proteins. These tools are more advantageous in malarial parasite studies, especially on essential proteins (302).

A. Aptamers

Aptamers are oligonucleotides. Single-stranded RNA (ssRNA) or single-stranded DNA (ssDNA) forming exclusive three-dimensional structures that facilitate the interaction with specific targets (303). The Tet-DOZI-aptamer system combines with RNA aptamer and enables depending control of many target genes in a very effective manner (72, 298). Compared to monoclonal antibodies, aptamers bind specifically to the targets with no limitations to highly immunogenic targets.

Fig. 11 Tet-R – aptamer interaction post-transcriptionally regulates protein synthesis

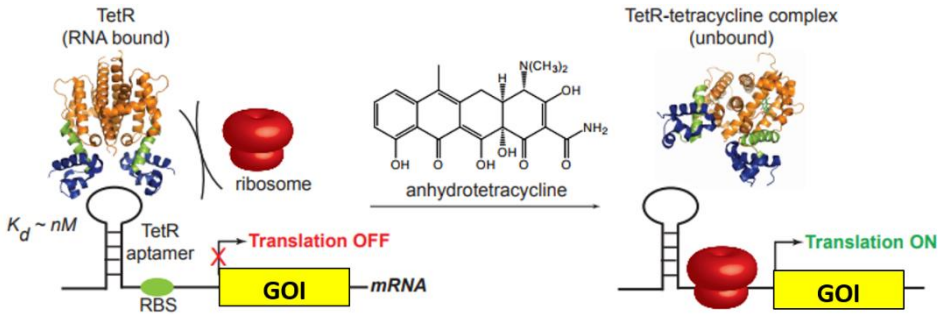


Figure adapted from Belmont and Niles., 2010 (304)

Because of their small size, aptamers can reach smaller compartments of the cells where the antibodies have penetration limits. Aptamers are highly susceptible to nucleases, which is a major disadvantage in the development of stable transfectants. To minimize the obstacles by nucleases, chemical modifications and molecules like biotin or polyethylene glycol (PEG) can be used to prevent the process of degradation (305).

4.13.0 Choosing a system for Gene of interest

Many criteria determine the choice of conditional protein expression in *Plasmodium falciparum*. The first and foremost consideration should be the putative function of the gene, especially if it has enzymatic activity (306-309). The enzymatic proteins need a higher degree of knockdown to show observable phenotypic change. In these cases, the system needs to downregulate the protein expression by >95% (310). Also, the site of action of the gene of interest and the putative biological process that is linked to the gene is also an important consideration. It is very important to match the kinetics of the knockdown system besides the function of the gene of interest to better understand the phenotypic changes during a conditional knockdown. Proteins targeted to membranes or having transmembrane domains in their structures may mislocalize when proteasome-based knockdown systems (DD/DDD systems) are used. For these proteins, conditional GlmS ribozyme or tet R aptamer knock-down systems are the best options. On the other hand, a large proportion of *Plasmodium falciparum* genes have unknown functions, (310) either in insect or blood stages development.

4.14 Yeast in deciphering the gene functions

A broad - knockout study in yeast genome is easy to carry out because of the ease of introducing exogenous DNA, and high ability of homologous recombination. An extensive number of phenotypic information is currently accessible for each gene knockout of yeast (311).

4.14.1 Yeast Functional Orthologues

There are thousands of recognizable orthologous genes identified in yeast and human (312), and these organisms are separated a billion years ago (313). Interspecies swaps to identify the homologs of equivalent functions have been carried out for decades (314, 315); the course of evolution has retained some characteristics of orthologous genes in different organisms. Even though there are many known exceptions of 'ortholog – function – conjecture', orthologous gene studies remain a valuable and widely used method for predicting gene functions (312). Hundreds of genes from one species can functionally replace or complement their orthologous genes in another species (316). Functional complementation of genes between species which are evolutionarily distant can help identify conserved function and also open up further molecular characterization of the gene of interest in the heterologous species. Some of the systems that could be studied include metabolic pathways, regulatory cascades, identifying interacting partners and mutant phenotypes (317).

Orthology and paralogy are two fundamental catagories of homologous relationships among genes are classified based on the mode of descent from the common ancestor (318, 319). 'Ortho' means 'exact', orthologues genes are evolved through speciation and 'para' means 'besides' or 'next to' (320). Orthology studies help in the assigning of functional information from experimentally known genes to unidentified newly sequenced genes of another organism (320-322). The relationship inference of orthology and paralogy from anonymous sequence data is a topic of intense research. The methodological aspects of inference of either orthology or paralogy and its methods are extensively studied (323, 324). The key implementations in orthologous and paralogous relationships between any genes are derived from their

sequence, structural and functional similarities (325). In any functional annotation, the use of orthologue is mainly by domain shuffling, similarities of a domain, lineage-specific gene resemblance, and gene loss (326). The process of accurate orthologue detection is an essential step in comparative genomics research (327), which helps in the functional annotation of genes in various organisms (328). Computational tools used in this study to identify the orthologs are discussed later in this chapter.

4.14.2 Yeast Two-hybrid interaction system

Protein-protein interaction has a vital role in life processes, and aberrations in these interactions are associated with diseases like cancer, infectious diseases, and neurodegenerative diseases. Recent investigations have started exploring the potential of protein-protein interactions in drug discovery to find novel targets for diseases with highly unmet medical needs (329).

Biochemical techniques like crosslinking, co-immunoprecipitation, and co-fractionation by chromatography are used in protein-protein interaction between any two proteins. GAL4 of Saccharomyces cerevisiae is a transcriptional activator required for the expression of the gene involved in galactose utilization (330). This protein has two parted functional domains: N-terminal domains bind to exact DNA sequence and C terminal domain to activate transcription (331, 332). A system of two hybrid proteins was created by merging the GAL4 DNA-binding domain to a protein 'A' and a GAL4 activation domain to protein 'B'. The protein-protein complex interactions of ‘A’ and ‘B’ reconstitute the GAL4 domain, and results in transcription of the gene under its regulation or control (333, 334). The DNA-binding domain fusion was defined as 'bait' and the name 'prey' is given to the activation

domain. Many two-hybrid yeast strains have multiple reporter genes with different promoter regions for a wider range of sensitivity and selectivity (335).

Fig. 12 Yeast two-hybrid System

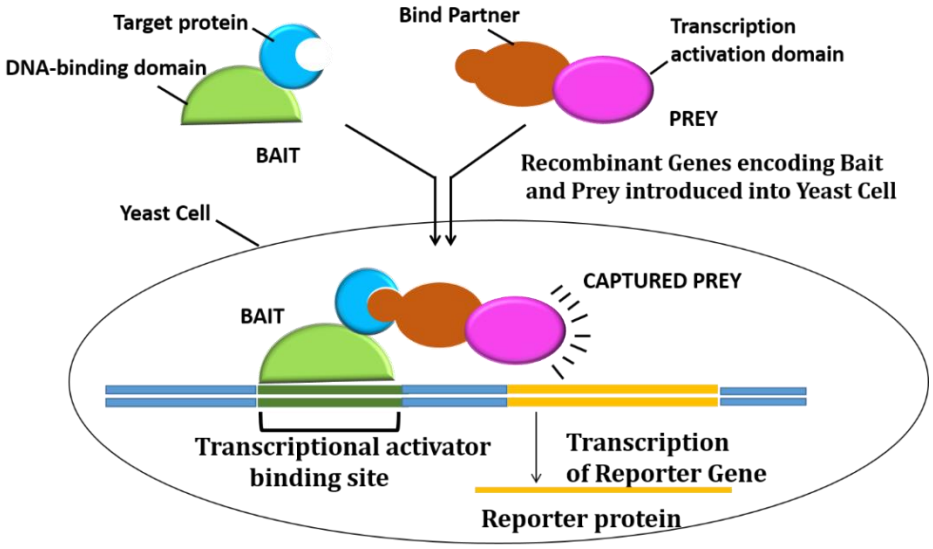


Figure adapted from Bio-Resources., 2014 (336)

A large-scale two-hybrid screening was first done in *S. cerevisiae* in 2000 (337). A high throughput *P. falciparum* protein-protein interaction was done to identify 2,846 unique interactions (338). These assays offer additional information on the structure of the interaction network and the evolvability of interactomes. Developments in two-hybrid techniques in higher eukaryotes are very attractive alternatives to the traditional yeast system (339, 340).

4.15.0 Computational tools in Orthologue analysis

Evolution is not simply conserving the function of proteins, it also generates or includes new functions (326), many proteins described in this chapter are known examples. Phylogenomic studies have been used in the inference of protein molecular functions of individual protein families (341, 342), comparative genomics (343, 344),

and in whole-genome analysis (345, 346). The rapid computer algorithm for clustering the homologous proteins are BLAST and PSI-BLAST (347, 348). The accuracy of multiple sequence alignment is very crucial as it determines the phylogenetic signal in tree construction (349). Among many tools for multiple sequence alignment, ClustalW is a widely used computational tool (350). The other tools include MAFFT (351) and T-Coffee (352). Phylogenetic trees are constructed using two main methods: one is distance-based [examples are neighbor-joining (353)], and another is character-based [examples are maximum parsimony, maximum likelihood, and Bayesian approaches]. Two main basic approaches are used in differentiating the orthologs and homologs. The first is the clusters of orthologous groups database at NCBI abbreviated as COG (354) which uses top BLAST hits (bi-directional) across the genomes databases to form clusters, and is a dynamic tool for search of genome (355). The ing approach is followed by phylogenetic tree construction and investigation (326).

The combination of structure prediction and sequence analysis together forms **structural phylogenomics,** which contributes understanding on protein molecular evolution. The Structural Classification of Protein (SCOP) database clusters the protein's hierarchy on structural and functional similarity (356). Structure prediction and investigation reveals the structural domains in a protein family. Protein domain under evolutionary constraints along with Phylogenomic analysis established on the integrated protein fold provides proof to the functional role of proteins under study (326). PFAM (357) and NCBI Conserved Domain Database (358) are very impressive resources; both combine structural and functional domains. Conserved Domain Database (CDD) site annotations provide a wide range of information including an extensive figures of active sites, chemical binding, protein-protein interaction sites,

and to some extent experimentally or computationally generated site annotations from SwissProt data set (359). Structural motifs provide more precise annotations on sequences under study like short structural repeats acting as beta-propeller, coiled – coils, transmembrane segments. They also provide short functional motifs, for example, DNA-binding zinc fingers (358). It is a major challenge in understanding evolution of any superfamily of proteins, investigations are indispensable to bring out the ancestry facts.

5.0 MATERIALS AND METHODS

5.1 Reagents and Microorganisms

5.1.1 Chemicals and enzymes

Standard chemicals in molecular grade quality are obtained and used from Sigma, MPBio, Invitrogen, Lonza, Gibco. Restriction enzymes were ordered from New England Biolabs. Primers and Oligos are ordered from integrated DNA technologies. Special chemicals and enzymes are tabulated in table 3.1. Kit systems were used based on the manufacturer's instructions. Kit systems used are shown in table 3.2. Primers designed and used in this study are recorded in Table 3.3.

5.1.2 Microorganisms and Cell lines

Escherichia coli (*E. coli*) strain XL10-Gold cells (Stratagene) are used in molecular cloning. BL21(DE3) RIL strain for protein expression was purchased from Stratagene. Knockout strains are purchased from EUROSCARF. of *Saccharomyces cerevisiae* (*S. cerevisiae*) genotypes and strains used in this study are represented in Table 3.5. *Plasmodium falciparum* 3D7, *Plasmodium falciparum* NF54, *Plasmodium falciparum* Dd2 attB strains are purchased from MR4 (Malaria Research and Reference Reagent Resource Center) listed in table 3.6.

5.1.3 Plasmids

Plasmids, constructed and used in this study are explained in Table 3.7. Plasmids are constructed using the method described in 3.5.5 and propagated in XL10 Gold cells.

5.2 Bacterial culture

Escherichia coli cells are grown using a ready-made Luria broth (LB) medium. For solid medium 1.5% bacteriological agar is added before autoclaving. For a selection of plasmids with antibiotic markers, respective antibiotics were added to the medium based on the recommended concentration (100 µg/ml of ampicillin, 50µg/ml of Kanamycin, 25µg/ml of chloramphenicol). Liquid cultures were inoculated from a solid media with antibiotics and incubated at 37 °C and 200rpm on a shaker. The growth of bacterial *cultures* was checked by OD600 measurements. Glycerol stocks of E. *coli* strains are prepared and stored at –80 °C as cryostocks for future use

5.3 Cultivation of Yeast

S. cerevisiae strains are grown in a rich medium, supplemented with 2% glucose (YPD). The synthetic YNB (Yeast nitrogen Base) medium was used for the selection of genetic markers. All components of the yeast media used in this study are entered in Table 3.9. solid medium was made by adding 2% agar to the medium before sterilization.

Liquid yeast cultures were prepared from a plate or 1:20 to 1:50 to get an appropriate cell density. Cell density was checked by OD600 measurements (OD600 of 1 ~ 107 cells per ml). Cultures are incubated at 30 °C with speed of 140 rpm.

5.4 Bioinformatic tools

5.4.1 Sequencing retrieving and analysis

Putative prohibitin(PF3D7_1014700, PF3D7_0829200) and putative prohibitin-like (PF3D7_0416600) protein sequences were retrieved from PlasmoDB (360, 361). BLAST analysis was done to identify the orthologs with other organisms. Domain search was done to identify the similarity between the sequences.

SHY1 protein sequence (Swiss-Prot: P53266.1) from *Saccharomyces cerevisiae* and Surfeit locus protein 1 (Swiss-Prot: Q15526.1) of Homo sapiens are used in the BLAST tool of PlasmoDB to identify its ortholog. Domain search was done to identify the conserved domain in the sequence (362).

5.4.2 Multiple sequence analysis

Multiple sequence alignment was done using prohibitin1 and prohibitin 2 sequences from *Plasmodium falciparum*, *Saccharomyces cerevisiae* (GenBank: AAA53144.1 and GenBank: EDV10030.1), Homo sapiens (GenBank: AAB21614.1 and GenBank: EAW88699.1), *Caenorhabditis elegans*: mitochondrial prohibitin complex protein 1 (GenBank: CCD73430.1), mitochondrial prohibitin complex protein-2 (GenBank:CCD66149.1), *Arabidopsis thaliana*: PHB1 (GenBank:AEE85496.1), PHB2 (GenBank:AEE27625.1), Rattus norvegicus: prohibitin (NCBI Reference Sequence: NP_114039.1), prohibitin-2 (GenBank: EDM01946.1), putative prohibitins of *Cyclospora cayetanensis* (GenBank: OEH75360.1 and GenBank: OEH73968.1), putative prohibitins of Toxoplasma gondii (GenBank: KYF40998.1 and NCBI Reference Sequence: XP_002368169.1), putative prohibitins of *Cryptosporidium parvum* (NCBI Reference Sequence: XP_628578.1

and NCBI Reference Sequence: XP_626169.1) separately using Clustal X 2.1 (363). Percentage similarity was identified between aligned sequences using ExPASy (364).

Surf1 orthologs from different organisms were retrieved from NCBI (Homo sapiens, surf1: Q15526; *Drosophila melanogaster* surfeit 1: NP_524758; *Caenorhabditis elegans* SURF1-like protein: CCD72250; *Monosiga brevicollis* MX1 predicted protein: EDQ90553; *Saccharomyces cerevisiae* SHY1:KZV11341; *Schizosaccharomyces pombe* SURF-family protein Shy1 (predicted): CAB50922; *Arabidopsis thaliana* Surfeit locus 1 cytochrome c oxidase biogenesis protein: NP_566592; *Dictyostelium discoideum*, surf1 family protein: XP_644359; *Chlamydomonas reinhardtii*, cytochrome c oxidase assembly protein: XP_001701449; *Babesia microti*, uncharacterized protein: XP_012648526; *Toxoplasma gondii*, SURF1 family protein: KFH02210; *Trypanosoma brucei gambiense* DAL972hypothetical protein, conserved: XP_011779678; *Plasmodium falciparum*, conserved *Plasmodium* protein unknown function: XP_001351864). Multiple sequence alignment was done using Clustal X 2.1 followed Jalview for conserved domain analysis.

5.4.3 Phylogenetic tree

The phylogenetic tree was constructed using the multiple sequence alignment made from protein sequences of prohibitins and Surf1 of different orthologs mentioned in 3.4.1.iTOL was used for tree construction and analysis (365).

5.4.4 Identification of guide sequence for CRISPR design

Guide sequence for PfPHB1 and PfPHB2 was identified using various online tools to find a unique sequence. The tools are ZiFit Targeter version 4.2 (80) CHOPCHOP (282) and Eukaryotic Pathogen CRISPR gRNA Design Tool (283).

5.4.5 Homology modeling using PHYRE2

Phyre2 adopts Hidden Markov Method (HMM) to bring out alignments of the query protein sequence against proteins of familiar structures. The alignments are used to predict the protein structures of the query sequence (366).

5.5 Molecular Biology methods

5.5.1 Estimation of nucleic acid concentration

DNA and RNA concentrations was determined using the NanoDrop2000 UV-Vis spectrophotometer (ThermoScientific) with an assumption of 50 μg/ml DNA or 40 μg/ml RNA solutions correlates to OD260 of 1.

5.5.2 DNA electrophoresis

Plasmids and DNA fragments are separated in 1% agarose gel supplemented with 1 μg/ml ethidium bromide using 1xTAE (40 mMTris/acetate, pH 8.0, 2 mM EDTA) electrophoresis buffer. DNA samples are mixed with loading dye (6x stock: 30% glycerol, 0.25% Bromophenol blue) before loading and electrophoresis was performed in Mini-Sub Cell GT chambers (Biorad) for 60 min at 80 V. As a standard, GeneRuler DNA Ladder Mix (Fermentas) was used to estimate the size of DNA fragments. DNA bands were documented using a gel documentation system (syngene) or cut out are made to purify the fragments using a long UV-transilluminator.

5.5.3 Purification of Plasmids

Plasmids are isolated from an overnight liquid culture of E. *coli* using the QIAprep Spin Miniprep Kit and Qiagen Plasmid Maxi kit based on the requirements and instructions from manufacturer's.

5.5.4 PCR amplification of DNA fragments

DNA fragments for respective experiments are amplified for cloning from genomic DNA of *Plasmodium falciparum* 3D7 or *S. cerevisiae* by PCR using Prime STAR GXL DNA Polymerase (Takara) according to the recommendations of the manufacturer. Standardized PCR reactions were performed on a 50 µl scale. Templates for amplification are based on the experimental designs, either 25 ng plasmid DNA or 200 ng of genomic DNA from yeast or *Plasmodium falciparum* 3D7(purified using the innuPREP Blood DNA Kit, Analytik Jena).

Bacterial colony PCR are performed to identify the positive clones using Emerald Amp GT PCR Master Mix from Takara. Liquid colony suspension in LB broth is used as a template.

5.5.5 Molecular cloning

Using genomic DNA as a template, PCR was used to amplify inserts and introduce restriction sites for cloning. The PCR products are verified by agarose gel electrophoresis and purified using the Wizard SV Gel and PCR CleanUp System (Promega). Intermediate cloning was done using the StrataClone PCR cloning Kit. Intermediate clones were subjected to sequencing and verified before final clone construction.

Restriction analysis of vector backbones and insert DNA fragments from intermediate clones was performed using High fidelity restriction endonucleases (New England Biolabs) and fragments are again purified. Subsequently, the fragments were ligated with the T4DNA ligase enzyme (Takara) and the ligated plasmid

constructs are transformed *in E. coli* competent cells. The clones were verified by restriction digestion analysis.

In-Fusion HD cloning kit from Clonetech is used for critical insert with many internal restriction sites and oligos and homology sequence cloning in pL6 plasmids. The manufacturer's instruction was followed for cloning and transformed into XL10 Gold competent cells for large and stable plasmid constructs.

5.5.6 Restriction analysis

After cloning, all the clones were verified using restriction analysis. Final constructs are confirmed by cross verifying the restriction analysis with respective plasmid maps.

5.5.7 Sequence analysis

The final plasmid constructs are sequenced. Sequencing of DNA was executed using the Sanger method of DNA sequencing by outsourcing the samples. The sequence along with the chromatogram was analyzed for its correctness using finchTV 1.4.0 (367).

5.5.8 Transformation of *E. coli*

XL10 GOLD Competent *E. coli* (Stratagene) cells are used for the transformation of ligation products based on the manufacturer's instructions. Colonies were selected with appropriate antibiotics for the respective plasmids.

5.5.9 Transformation of *S. cerevisiae*

Transformation of plasmids into yeast Knock-out strains are done using the lithium acetate method for complementation assay (368). Transformants are selected in the respective dropout YNB plates.

5.5.10 Protein expression and analysis

BL21(DE3) RIL strain from Stratagene is used for protein expression of pET28a plasmid cloned genes using an IPTG as inducer. Expression of proteins was analyzed using denaturing protein electrophoresis (369) standard methods: Protein samples were resuspended in SDS-sample-buffer, incubated at 95 °C for5 min, and subsequently loaded on a polyacrylamide gel. the gels are made using 0.1% SDS and 4% acrylamide for the stacking gel or 12 – 14% acrylamide for the resolving gel. Acrylamide stock solutions are prepared using 30% acrylamide/bisacrylamide (37.5: 1). Electrophoresis was done using the MINI-ProteanII (Biorad) system (200 V, 25 mA per gel) or custom-made midi gel systems (230 V, 30 mA per gel). As a marker, the SDS-PAGE standard Broad Range ladders (Biorad) are used to identify the protein size.

SDS-sample-buffer:10% glycerol, 2% SDS, 0.01% Bromphenol blue, 0.5% 2-mercaptoethanol, 60 mM Tris, pH 6.8

SDS-running-buffer: 25 mM Tris, 191 mM glycine, 0.1% SDS

Stacking-gel-buffer (10x): 0.8 M Tris/HCl, pH 6.8

Resolving-gel-buffer (5x):1.875 M Tris/HCl, pH 8.8

5.5.11 Genomic DNA isolation from *Plasmodium falciparum*

Genomic DNA is isolated from the saponin lysis of *Plasmodium falciparum* in vitro culture using innuPREP Blood DNA Kit, Analytik Jena.

5.6 In vitro culture of *Plasmodium falciparum*

5.6.1 Reviving the frozen stocks of *Plasmodium falciparum*

The method used to thaw the glycerolyte frozen parasites was done using NaCl as described by Hodan Ahmed Ismail (370).

5.6.2 Erythrocytic asexual culturing of *Plasmodium falciparum*

The candle jar technique of Trager-Jensen is followed (371). Parasites were cultured in a complete malaria culture medium (cMCM) which includes RPMI1640 media supplemented with 5g/L albumax II, 2g/L $NaHCO_3$, 25mM HEPES-K pH 7.4, 1mM hypoxanthine, and 50mg/L gentamicin. Human blood is collected from healthy volunteers using CPD as an anti-coagulant every 3 weeks and stored at 4°C. Washed human erythrocytes are added to cMCM to achieve 5% hematocrit.

5.6.3 Giemsa staining of thin blood films:

Thin blood films are made from the culture and stained using Giemsa staining protocol as explained in the methods in malaria research (372). All the slides were individually counted to determine the percent parasitemia. Dilutions were done to maintain the culture not to exceed 6-7% parasitemia.

5.6.4 Cryo stock *Plasmodium falciparum*

The cryoprotective solution was used for freezing ring-stage parasites by the protocol described by Hodan Ahmed Ismail (373). Ring stage parasites with >5% parasitemia are used for cryo stocking.

5.6.5 Synchronization of *Plasmodium falciparum*

Synchronization of *Plasmodium falciparum*-infected erythrocytes was done using sorbitol to maintain the uniform stages of the parasites. Kirsten Moll's protocol was followed as explained in the MR4 malaria research manual (374).

5.7 Transfection of *Plasmodium falciparum*

5.7.1 Direct electroporation of ring-stage parasites

Direct electroporation of *Plasmodium* parasites in the human red blood cells was performed using the protocol given by Yimin Wu (375). 50 - 100µg plasmid was used for the transfection which is dissolved in incomplete cytomix buffer (120 mMKCl, 0.15 mM $CaCl_2$,2 mM EGTA,5 mM $MgCl_2$,10 mM K_2HPO_4/KH2PO_4,25 mM HEPES). Electroporation was performed using Biorad gene pulsar at 0.31kV/960µF in 0.2 cm gap cuvette.

5.7.2 Drug selection

Drug selection was given to the transfected parasites from the third day of transfection. The selection of transfectants depends on the plasmid design and the choice of selectable markers in the plasmid. There are markers established for stable episomal transfections and selection of *Plasmodium falciparum* transfectants are human dihydrofolate reductase (dhfr), blasticidin S deaminase (from *Aspergillus terrus*), and neomycin phosphotransferase II (transposon Tn*5*). The markers in the plasmids is responsible for resistance to drugs like WR99210, blasticidin S HCl, and geneticin G418 respectively. 5mM WR99210 stock was prepared in DMSO and stored at-80°C. 5nm WR99210 in RPMI media was prepared to select the transfectants. Blasticidin Hydrochloride S was dissolved in water and stored at -80°C. 2.5ug/ml of blasticidin S HCL was used in RPMI to select the plasmid with

blasticidin S deaminase marker. 250µg/mL G418 is used in RPMI for the selection of neomycin phosphotransferase II marker (376).

5.7.3 Drug cycle

For the integration to take place the respective drug on and off-cycle was repeated once in 2 weeks.

5.7.4 PCR analysis of transfectants

Genomic DNA was isolated from the saponin lysate of transfected parasites and used for PCR analysis to differentiate the episomal and integrated plasmids. Amplicons were analysed in agarose gel to identify the integrants.

5.8 Yeast experiments

5.8.1 Yeast complementation assay

Yeast complementation assay for PfSURF1 was performed using Shy1 knock-out yeast strain (YGR112w) in fermentable and non-fermentable medium and incubated at 30°C, 24°C, and 19°C (377).

5.8.2 Yeast two-hybrid assay

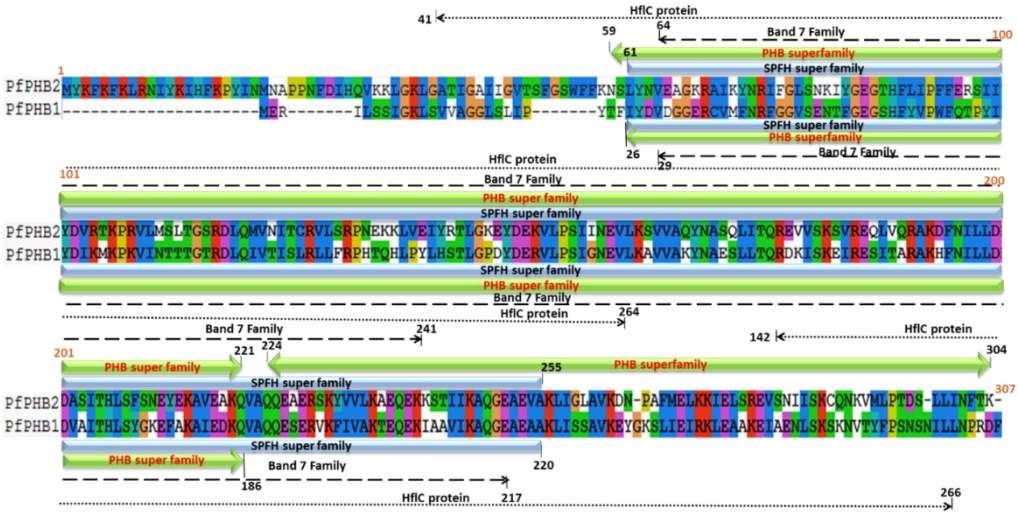
The interacting efficiency of PfPHB1 and PfPHB2was experimented using yeast two-hybrid system as described by James *et al* (335).

6.0 RESULTS AND DISCUSSION

6.1 Sequence annotation and domain analysis of prohibitins

Protein domains are conserved functional units that can fold independently with a distinct function. *Plasmodium falciparum's* prohibitins share the conserved domains of the SPFH (**S**tomatin, **P**rohibitin, **F**lotillin, **H**flK/C) superfamily, prohibitin homologs, HflC domain, and Band7. Regulators of protease HflC stomatin/prohibitin superfamily are involved in the posttranslational modification, protein turnover, and chaperonic function.

Fig. 13 Protein sequence alignment of PfPHB1 and PfPHB2 with Domains



The diverse family of SPFH proteins are associated with the scaffolding of detergent-resistant microdomains. Based on the sequence and structure studies, a division of 12 subfamilies are observed. Out of which conserved operon structure are found in SPFH1, SPFH2, and SPFH5. The complex pattern of occurrences confirms the lateral gene transfer. The organisms that does not hold SPFH proteins are simply endoparasites or endosymbionts (378). Band 7 protein family of SPFH superfamily forms oligomers and regulates the assembly and function of super-molecular

complexes in various cellular locations (204). The function of prohibitin in mitochondrial cristae formation is well documented (227). In animal cells, flotillins are lipid rafts active in trafficking events (379). HflC/K type proteins regulate the action of membrane-bound proteases in prokaryotes (380). Based on these annotations, Prohibitins of *Plasmodium* may also have similar molecular actions of known eukaryotes as they share similar domains in their sequence. Fig. 13 shows the marked domains on the protein sequence.

6.2 Multiple sequence analysis of prohibitins

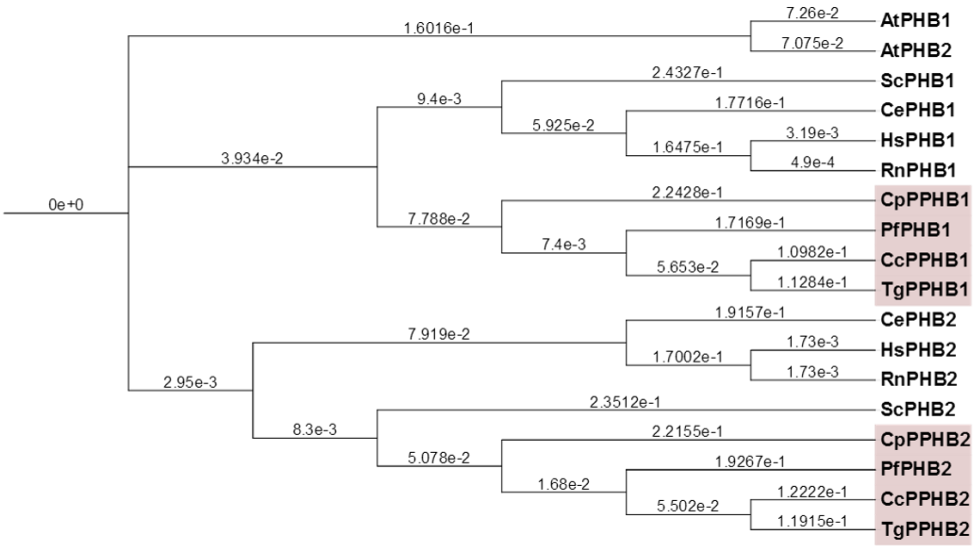
As the first step of the phylogenomic analysis, multiple sequence alignment was executed using the protein sequence of prohibitin1 and prohibitin 2 of selected organisms including model organisms like *Arabidopsis*, yeast, *C.elegans*, and so on. Both homologous protein sequences show conserved sequences represented in different shades of blue in Appendix I using Jalview. Prohibitin 1 of *Plasmodium falciparum* labelled as PfPHB1 shares 47.9% identity with prohibitin 1 of Homo sapiens, and also 51.0% identity with *Saccharomyces cerevisiae* prohibitin 1 labelled as ScPHB1. Prohibitin 2 of *Plasmodium falciparum* (PfPHB2) shares 54.5% identity with prohibitin 2 of *Homo sapiens* (HsPHB2) and 58.2% sequence identity with *Saccharomyces cerevisiae* prohibitin 2 (ScPHB2). The multiple sequence alignment provides important information on the conserved regions of the protein family which are crucial for structural and functional analysis. Shaded residues of the alignment were found to highly correlate with the functional domains that are represented in Fig. 13 of PHBs. These alignments also show the shared evolutionary relationship with respect to structural and functional identity. The sequence alignment of conserved

regions (blue shaded residues) is used to determine equivalent changes or replacement of similar residues.

Alternatively, variations between the sequences can be inferred using phylogeny. These are graphs, commonly known as evolutionary trees, and consists of nodes and branches, mathematically called vertices and edges. Here, the rooted tree constructed from the protein sequence of Prohibitin 1 and Prohibitin 2 of selected species is represented in Fig. 15. The rooted tree of prohibitin represents the directionality of the evolution of these proteins in different organisms. In this phylogram, the vertical distance is used for separation and clarity, whereas the horizontal distance represents the evolutionary changes encountered by the organisms under study.

The horizontal distance measures either the actual time or 'time' on the molecular clock. The tree represents 'ancestral' and 'derived' character state or sequence variations. The phylogenetic tree below shows a clear 'derived' sequence variation of selected apicomplexans: *Plasmodium falciparum*, *Toxoplasma gondii*, *Cyclospora cayetanensis*, and *Cryptosporidium parvum*. The selected apicomplexans form a separate cluster from the remaining higher eukaryotes in the group. More interestingly, Prohibitin1 and Prohibitin 2 show a clear separate 'derived' sequence variations. A step-by-step 'derived' character states are well noted in the tree with horizontal branches. The only plant model *Arabidopsis thaliana* in this group shows a clear segregation from the ancestral node. Homologs are two identical character states derived from a common ancestral in multiple terminal nodes. Hence, apicomplexans in the group are examples of orthologs (381).

Fig. 14 Phylogenetic analysis of PHB1 and PHB2



6.3 Conserved domain analysis of SURF1

As described before, it is necessary to compare a sequence of interest with multiple related sequences to obtain the best-detailed alignment. We compared the PfSurf1 protein sequence with other related proteins including Shy1, Shy1 like proteins to find the common domains they share. Fig. 16 shows the multiple sequence alignment with many other organisms like *Drosophila melanogaster*, *Caenorhabditis elegans*, *Monosiga brevicollis*, *Saccharomyces cerevisiae*,*Schizosaccharomyces pombe*,*Arabidopsis thaliana*,*Dictyostelium discoideum*, *Chlamydomonas reinhardtii*, *Babesia microti*, *Toxoplasma gondii*, *Trypanosoma brucei gambiense*, along with *Plasmodium falciparum*. The blue color shaded region shows the conserved regions of the Surf1 domain superfamily. The orange bar above the alignment represents the transmembrane region of the protein.

SURF1 superfamily of Surf1 or Shy1 (**S**URF1 **h**omolog of **y**east) performs a remarkable role in the posttranslational processes of the mitochondrially-coded and translated Cox1 subunit of cytochrome c oxidase (complex IV). The multiple

sequence alignment shows hot spots in the sequence, among which region between Pro[70]and Lys[87](numbers based on Human protein sequence) is the region between the first hydrophobic domain and α-helix, and is found in all species except mycobacteria (193). Tree analysis followed by sequence alignment shows stepwise sequence variations in the SURF1 protein sequence of the selected organisms. The four apicomplexans in the group are: *Plasmodium falciparum, Trypanosome brucei, Babesia microti, and Toxoplasma gondii*. All these organisms form a separate cluster, and share a common ancestry with *Chlamydomonas reinhardtii,* leaving higher eukaryotes to branch out separately with relevant evolutionary timescale. The horizontal scales are informative and highly relevant to the 'molecular clock' of evolution.

Fig. 15 Multiple Sequence analysis of Surf1 /Shy1 like proteins

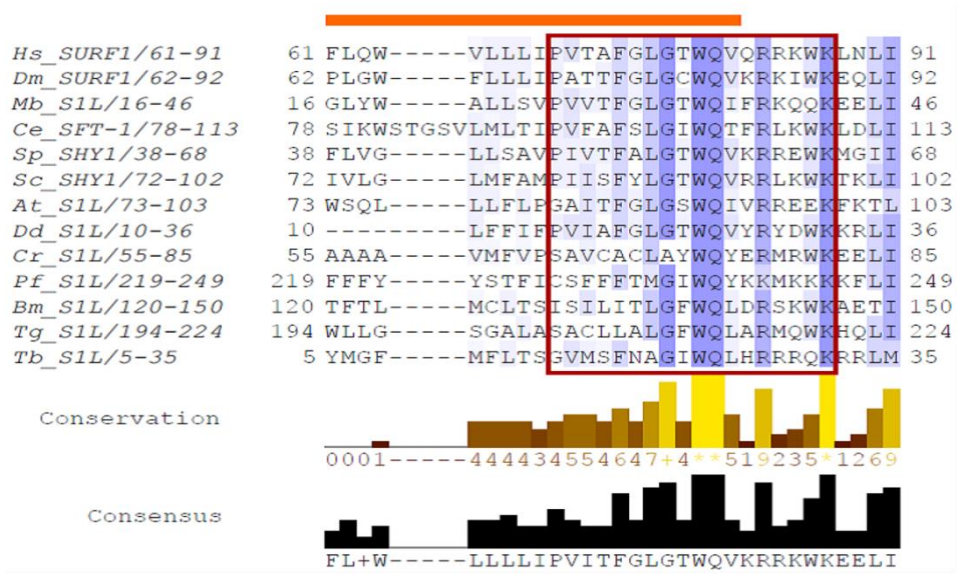
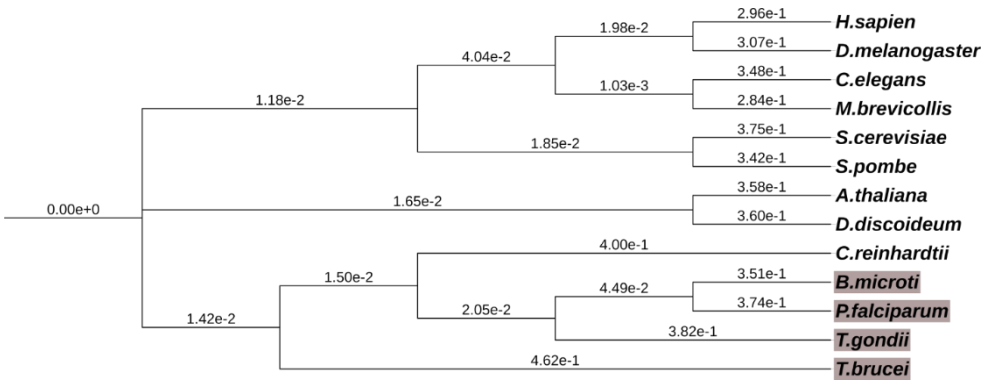


Fig. 16 Phylogenetic analysis of PfSURF1, ScShy1, and Shy1 like proteins



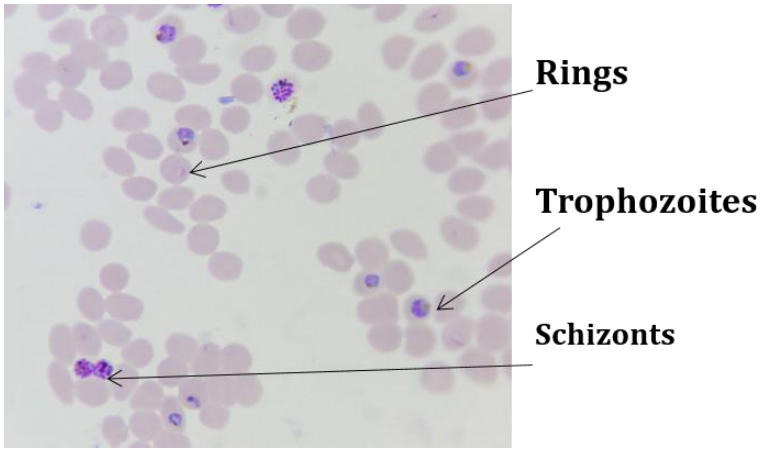
6.4 *Plasmodium falciparum* Culture

In vitro culturing of *Plasmodium falciparum* form basic requirement in malaria research where the primary objective is to use viable parasites of the life cycle. The ingredients used in *in vitro* cultures of *Plasmodium* parasite include human erythrocytes, a lipid source, in synthetic media which supplies suitable ionic concentrations and soluble nutrients for growth. The RPMI 1640 contains cations and anions matching the physiological levels of human plasma. Glucose, amino acids, and key vitamins are mainly supplied from this synthetic media which is supplemented with hypoxanthine a purine source. This purine has a significant impact in the propagation of *Plasmodium falciparum in vitro*. The various ions concentrations (Na^+, K^+, Cl^-, Ca^{++}, Mg^{++}, and H^+) in this media closely resembles the physiological values of human plasma. These standardized culture conditions result in parasite propagation and facilitate the comparison of results from different studies (382).

The cultures are incubated at 37°C using a candle jar – a desiccator glass chamber with candlelit which self-extinguish when oxygen is combusted. Daily media change is required to maintain the pH and parasite growth. The candle jar technique shows a decrease in oxygen to 16.1% and a rise in carbon dioxide levels to 3.5%.

Research shows candle jar maintains a gas measure of 80% N_2, 3% CO_2, 17% O_2 throughout the incubation period (371, 383).

Fig. 17 Giemsa staining of *Plasmodium falciparum*



Thin blood films were daily analyzed to understand the growth of the parasites and different asexual stages in the development. Eosin and methylene blue (azure) are the components of Giemsa staining solution. Eosin dyes the parasite nucleus red, at the same time methylene blue colors the cytoplasm blue. The addition of 0.01% Triton X- 100 facilitates the visualization of clear and distinct morphological details. Fig. 18 shows the microscopic examination of Giemsa stained asexual stages of *Plasmodium falciparum*. The ring stage appears between 6–22 hours of invasion into a fresh RBC. A signet ring-shaped structure in the RBC is called as the ring stage. Trophozoites appear between 22–38 hours of the asexual cycle. During the development, trophozoites enlarge in their size with irregular bulges in their surface with deep tubular invaginations. By the mid trophozoite stage, the surface of the parasites is accumulated with dense materials within the RBC cytosol. Repetitive nuclear replication takes place during the schizont stage of development. The schizont stage appears between 38-48 hours in the asexually developing parasites. During the last nuclear division, a series of merozoite forming foci are formed around the

circumference of the parasite at regular intervals. This merozoite formation occurs during the last hour (48 hours) of the asexual development stages.

Synchronized uniform culture stages are very crucial for the transfection to be performed. Synchronized erythrocyte stages of *Plasmodium falciparum* cultures are accomplished by suspending the culture in 5% D-sorbitol solution. After treating with sorbitol only single or multiple ring staged parasites survive for further uniform development and multiplication (384). Electroporation of the infecting RBC is the method of choice to introduce the Plasmid DNA into *Plasmodium falciparum*. Among the different methods of electroporation, spontaneous uptake of DNA from erythrocytes is found to be a very effective and promising method. This is done using 0.2cm chilled cuvette pulsed at 0.31kV/ 960µf using Bio-Rad gene pulsar as described in the methods chapter.

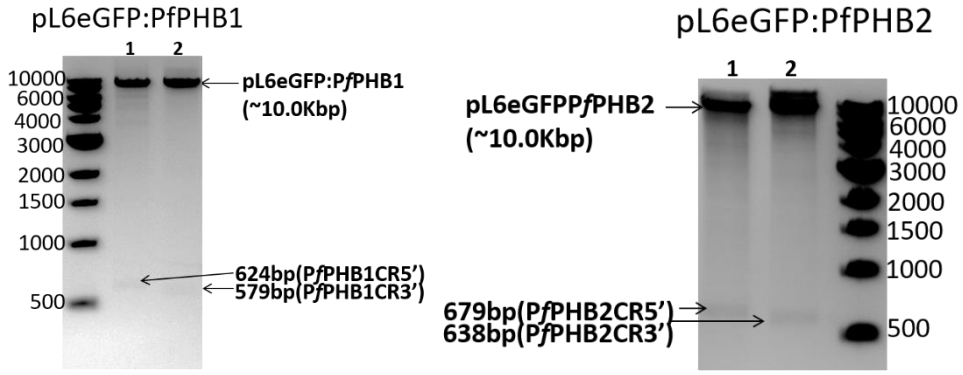
6.5 CRISPR Cas 9 knock out of PfPHB1 and PfPHB2

The introduction of the CRISPR/Cas9 system in *Plasmodium falciparum* has increased the efficiency as well as decreased the amount of time involved for parasite genome modifications, in comparison with any previously available conventional methods. As described in the review, this engineering strategy is used for tagging genes, gene knockout experiments, and introducing point mutations. The two separate PfPHB1 and PfPHB2 constructs for CRISPR/Cas9 knockout experiments are represented in Fig. 19 and 20.

The early crucial step is to determine the gRNA sequence that is very unique to the gene of interest. The gRNA sequence is selected using different online tools like CHOPCHOP, EuPaGDT, and so on. The EuPaGDT tool provides additional information on the characterized sgRNA sequences, like off-target hits, potential

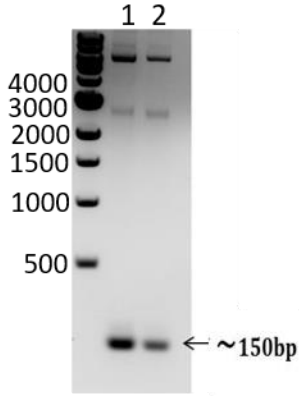
issues which hinder the transcription of gRNA. The respective guide sequence designed for PfPHB1 and PfPHB2 is listed in table 4. The cloning of gRNA in the CRISPR plasmid was confirmed by PCR amplification and is represented in Fig. 19. Homologous sequences for the respective prohibitins genes were cloned. and Fig. 18 represents the restriction digestion of CRISPR/Cas9 final plasmids of PfPHB1 and PfPHB2. The gel analysis also shows the length of 5' and 3' homologous regions along with the size markers. The complete plasmids maps of pL6eGFP: PfPHB1 and pL6eGFP: PfPHB2 labelled with guide sequences and homologous regions on either side of the drug cassette are shown in Fig. 20. The Plasmid map of pUF-Cas9 is represented in Fig. 20.

Fig. 18 Restriction Analysis of pL6eGFP: PfPHB1 and pL6eGFP: PfPHB2



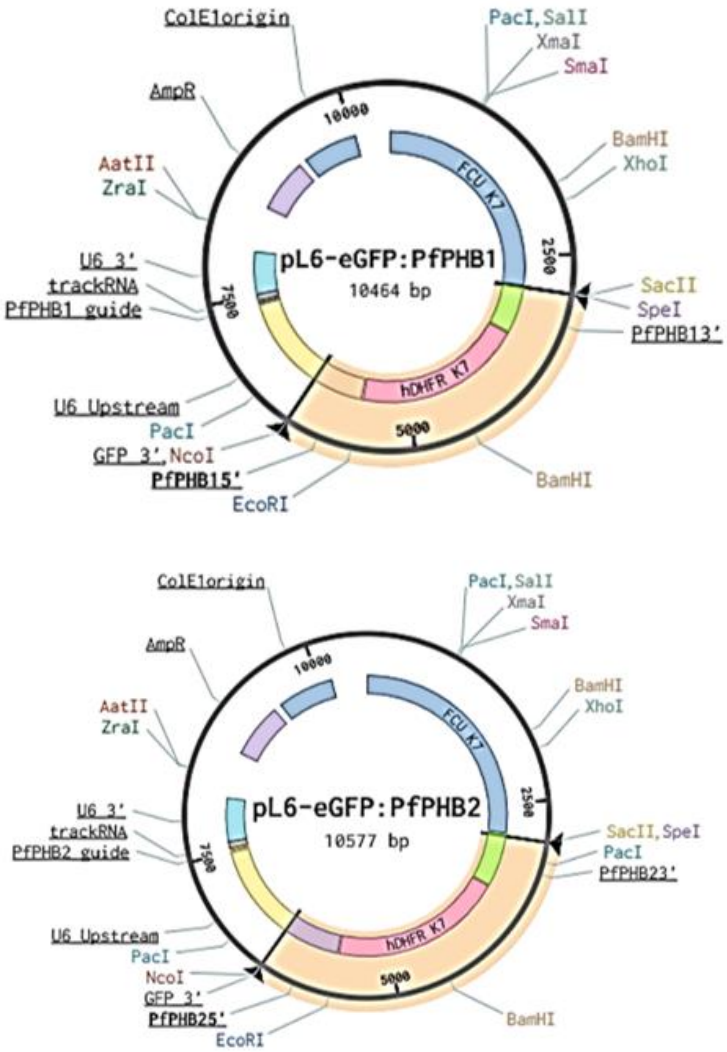
Lane1: Restriction analysis of pL6eGFP: P*f*PHB1 and pL6eGFP: PfPHB2plasmids using EcoRI and NcoI
Lane2: Restriction analysis of pL6eGFP: P*f*PHB1 and pL6eGFP: PfPHB2 plasmids using AflII and SpeI

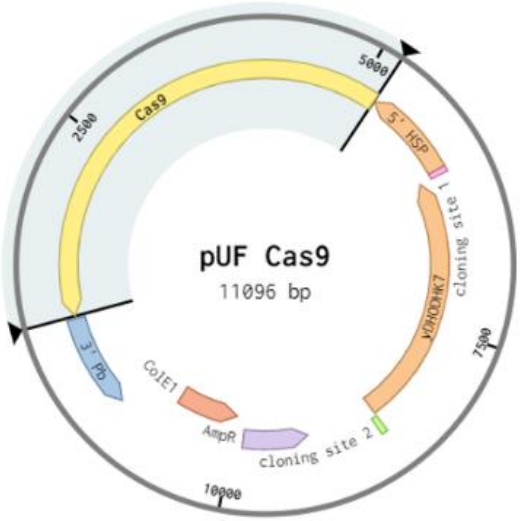
Fig. 19 Cloning confirmation of guide sequences of PfPHB1 and PfPHB2



Lane 1: Guide sequence check of PfPHB1
Lane 2: Guide sequence check of PfPHB2

Fig. 20 Plasmid map of pL6eGFP:PfPHB1, pL6eGFP:PfPHB2 and pUF Cas9





Both the plasmids were individually mixed with pUF-Cas9 plasmid and transfected into ring staged parasites. Depending on the construct used, the choice of drug pressure varies. The Cas-9 expressing plasmids were selected using DSM1 until parasites reappear. The plasmids with the human dihydrofolate reductase gene were selected using WR99210. The transfectants were maintained in the continuous culture and aliquots were made for saponin lysis followed by genomic DNA isolation. The genomic DNA from the transfectants were PCR analyzed with selective primers, and are shown Fig. 21. The primers AB is used to identify the presence of plasmids in the parasite lines. Primer CD and CE are used to find the variation in the genomic locus and integration of knockout plasmids in its specific gene locus. The PCR amplification of respective transfectants are shown in Fig. 22. The transfectants were maintained for 45-50 days and PCR analysis was done with an interval of 4 days. No integration is seen during this period for both the genes. The transfections were repeated three times and the knockouts were not successful. The PCR amplification indicates that PfPHB1 and PfPHB2 are not knocked out using the designed gRNA sequence with the CRISPR/Cas9 strategy.

Fig. 21 Schematic representation of specific primers used in integration analysis of PfPHB1 and PfPHB2.

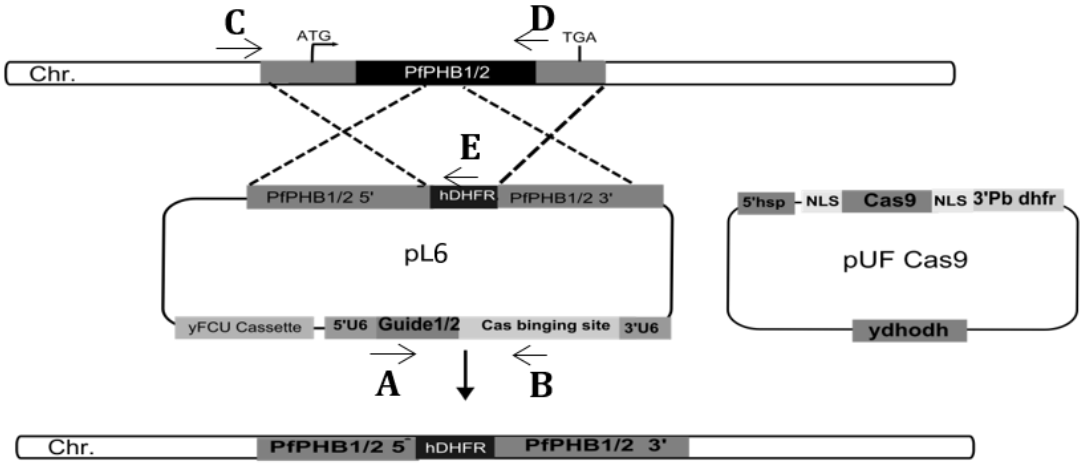
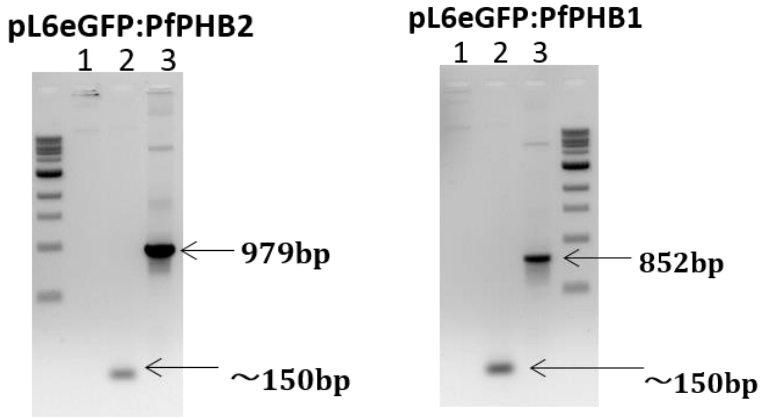


Fig. 22 Integration analysis PCR amplification of pL6eGFP: PfPHB1 and pL6eGFP: PfPHB2



Lane 1: Amplification using CE primers of respective PfPHB1 and PfPHB2
Lane 2: AB primers used to check the guide sequence
Lane 3: CD primers are used to check the locus of respective primers

6.6 Glms ribozyme knockdown of PfPHB1 and PfPHB2

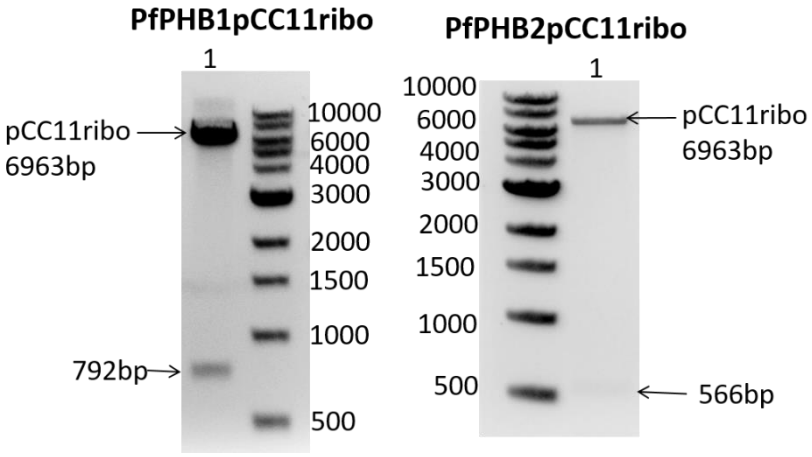
Inducible protein knockout by targeting the translation of mRNA is a very effective strategy in studying the protein function in various organisms. This is achieved by RNAi (RNA interference) methods, which are widely used in a few

protozoans. Unfortunately, *Plasmodium* lacks a functional RNAi pathway. RNA-based knockout systems hold a lot of promise in functional studies (296). The glmS ribozyme is one such tool developed and is introduced into the gene sequence understudy after the stop codon, and is switched on by glucosamine -6- phosphate leading to the instability and degradation as described in the review.

This technique is used in dissecting the diversified aspects of *Plasmodium* asexual biology including nutrient uptake, protein trafficking and export, plastid function, egress, invasion, and much more (310). Unfortunately, tagging of PfPHB1 and PfPHB2 after the stop codon with the GlmS tag was unsuccessful. The GlmS tagging was targeted after and without stop codon using conventional homologous recombination with individual plasmids represented in Fig. 24. Two-third of the coding sequences of the respective genes were taken for recombination and restriction analysis of final plasmids are shown in Fig. 23. After transfection, the transfectants were identified and maintained with WR99210 drug media. Drug on and off selections were done for a duration of 2 months to get the stable integration in the genomic locus. Genomic locus was frequently checked with respective PCR primers to identify the integration (Gel images not shown).

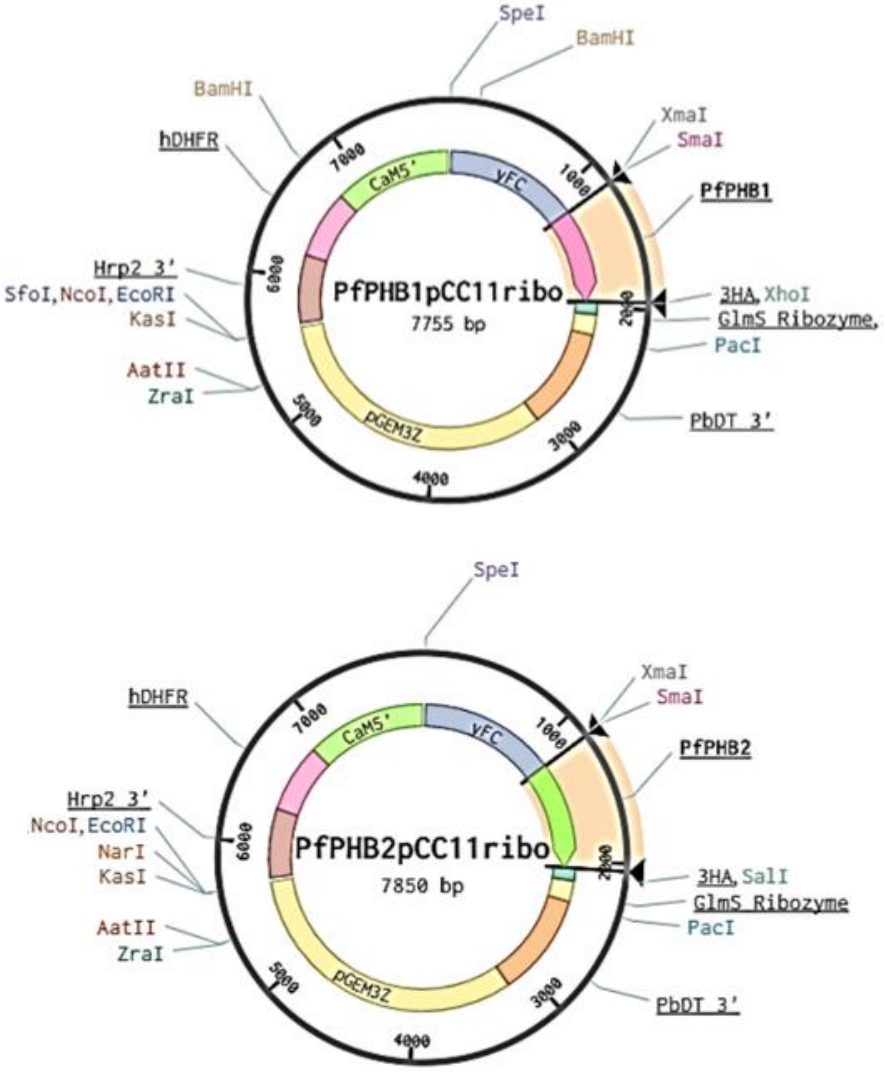
Transfections were repeated three times, and the integration event did not happen. Integration of the plasmid and in turn tagging of GlmS was unsuccessful to execute the knockdown experiment.

Fig. 23 Restriction Analysis of PfPHB1pCC11ribo and PfPHB2pCC11ribo



Lane 1: PfPHB1pCC11ribo and P*f*PHB2pCC11ribo digested with KpnI and Xho I

Fig. 24 Plasmid map of PfPHB1pCC11ribo and PfPHB2pCC11ribo

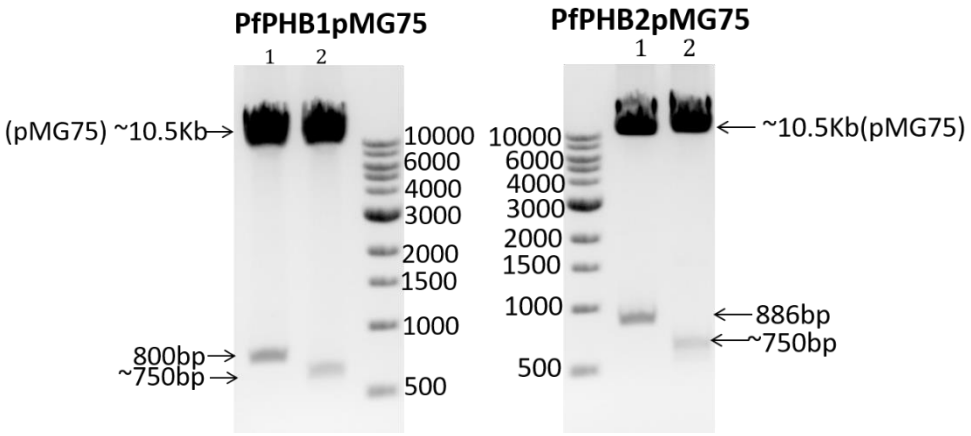


6.7 Aptamer knockdown of PfPHB1 and PfPHB2

The tetR aptamer is another tool to control the expression of the gene of interest post-transcriptionally by controlling the RNA binding proteins and translation. To apply the tet-R binding aptamer as a reverse genetic tool to the gene of interest, the aptameric sequence must be inserted as tags in the sequence of interest as explained in the introduction and review chapters. TetR protein binding to the aptamer in the mRNA inhibits translation and ATc reverses the binding and unblocks translation. The advantages of this approach include tunability, reversibility, robustness, and lack of ligand-based drawbacks. The main requirement of this strategy is to place the aptamer close to the translation initiation site. This addition is more difficult to achieve and typically needs genetic modification tools to insert the aptamer sequence in the translation initiation sites.

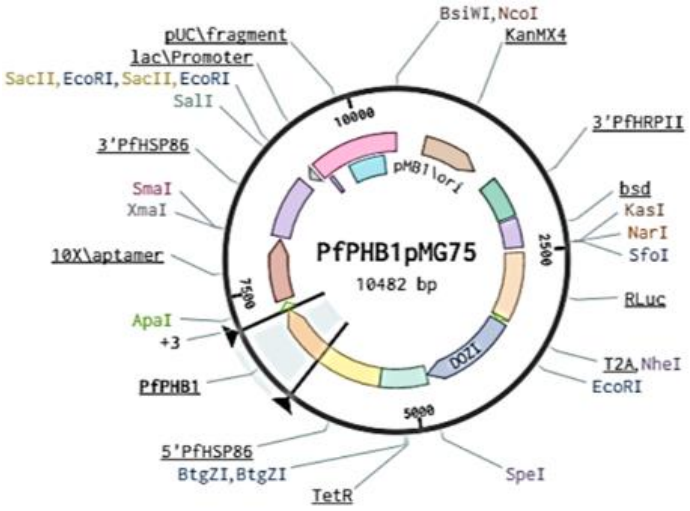
The addition of aptamer sequence before the stop codon was tried in PfPHB1 and PfPHB2. Fig. 26 shows the aptamer plasmid maps of PfPHB1and PfPHB2. The two-third sequence of coding regions of respective genes was taken to introduce the aptamer tag to the gene. But again the tagging was not successful. Fig. 25 shows the restriction analysis of the aptamer plasmids of PfPHB1 and PfPHB2. After transfection of these plasmids, blasticidin (BSD) media was used to select the transfectants. Drug on and off-cycle was maintained for a period of 2 months to achieve the integration of aptamer sequence in the genomic locus. Similar to GlmS tagging, aptamer tagging was also unsuccessful. These findings of unsuccessful tagging have been reported in prohibitin genes of *Plasmodium berghei* (266).

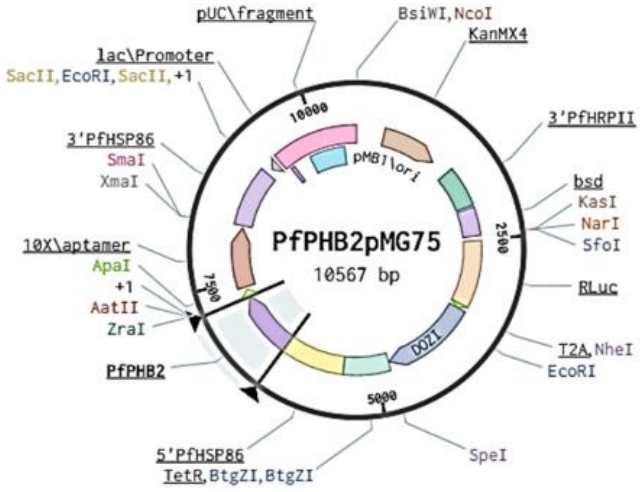
Fig. 25 Restriction Analysis of Aptamer plasmids of PfPHB1 and PfPHB2



Lane1: Restriction analysis of PfPHB1pMG75and PfPHB2pMG75 plasmid using AflII and BstEII
Lane2: Restriction analysis of PfPHB1pMG75and PfPHB2pMG75 plasmid using BstEII and XmaI

Fig. 26 Plasmid map of PfPHB1pMG75 and PfPHB2pMG75





In a recent study, PHB1 tagging was not successful in *Plasmodium berghei* which strongly suggests the disturbance to the locus may be lethal to the parasites. But tagging of PHB2, STOML, and PHBL proteins were successful and expression of PHBL appears to be very low than PHB2 and STOML proteins. Fluorescent microscopic imaging of tagged lines of PHB2, STOML, and PHBL demonstrates ubiquitous expression of these proteins in the development of parasites in vertebrates and mosquito hosts (266). These investigations suggest the importance of PHB1 and PHB2 in the development of parasites in vertebrates and vectors. These proteins implicate the mitochondrial functions, and their essentiality is vital for parasite survival.

6.8 Yeast two-hybrid analysis of PfPHB1 and PfPHB2

Because of its modularity and ease to use, yeast two-hybrid is well known to understand the interactions of complex multi-protein systems. Many biological activities are regulated by protein-protein interactions including the prohibitin proteins as described in the review. These interactions in the cell form a complicated network termed as "interactome". Protein-protein interactions have great potential as targets for novel treatments, also their regulation has shown a promising strategy in drug discovery (385-387).

Yeast two-hybrid screening was performed by cloning PfPHB1 in the activation domain and PfPHB2 in the DNA binding domain. Fig. 26 shows the complete plasmid maps of the yeast two-hybrid experiment. The cell lines with empty plasmids act as control whereas the cell line with PfPHB1 alone inactivation domain with blank binding domain acts as binding control in the experiments. The interaction of PfPHB1 and PfPHB2 was studied using drop-out yeast media based on

the markers available in the respective plasmids. The plates with –Ura-Leu acts as a control plate and –Ura-Leu-Ade plate to check the strength of interaction. Serial dilution of the clones was done to check the growth rate, in turn, the interaction of these proteins. Interaction of PfPHB1 and PfPHB2 was depicted in Fig. 30. Growth of yeast clones in –Ura-Leu-Ade plates suggest the strong interaction between the proteins.

Fig. 27 Plasmid map of PfPHB1pGAD-C1 and PfPHB2pGBDU-C1

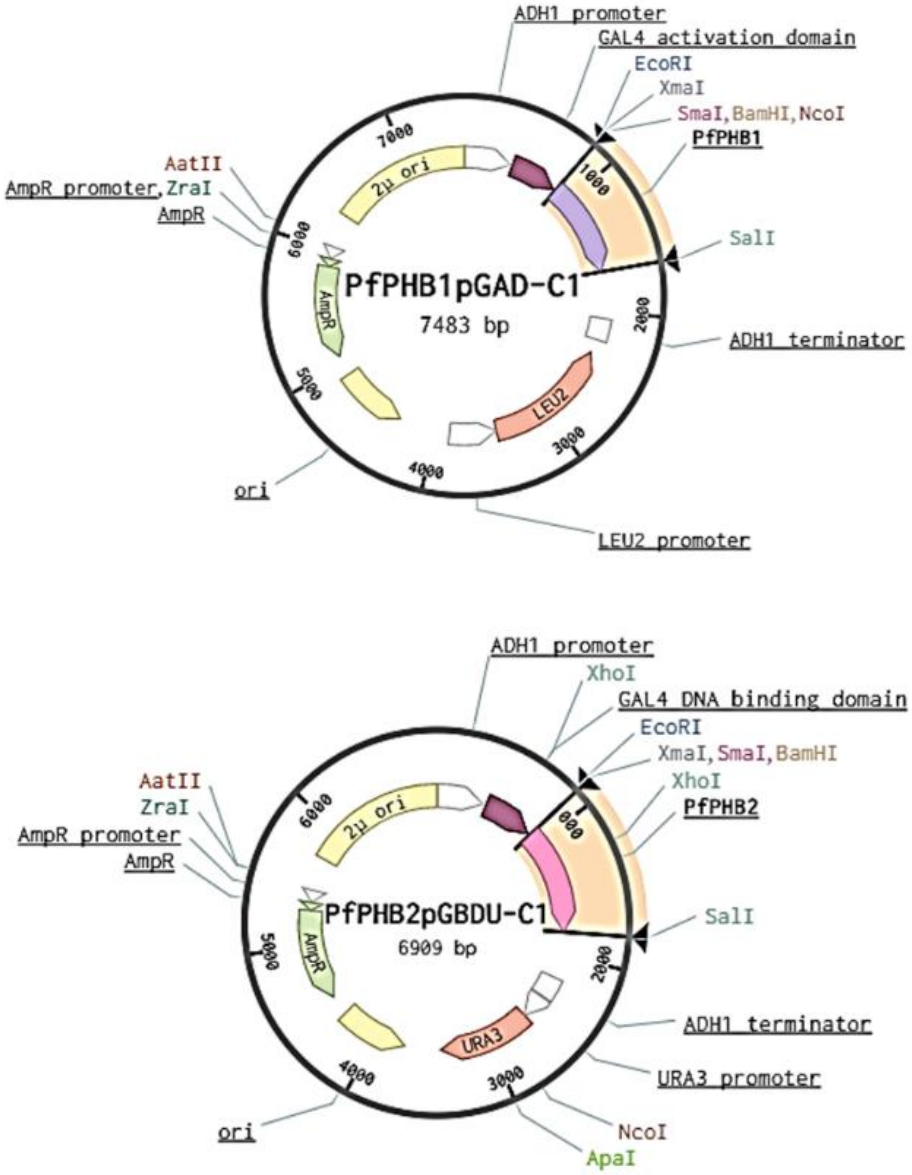
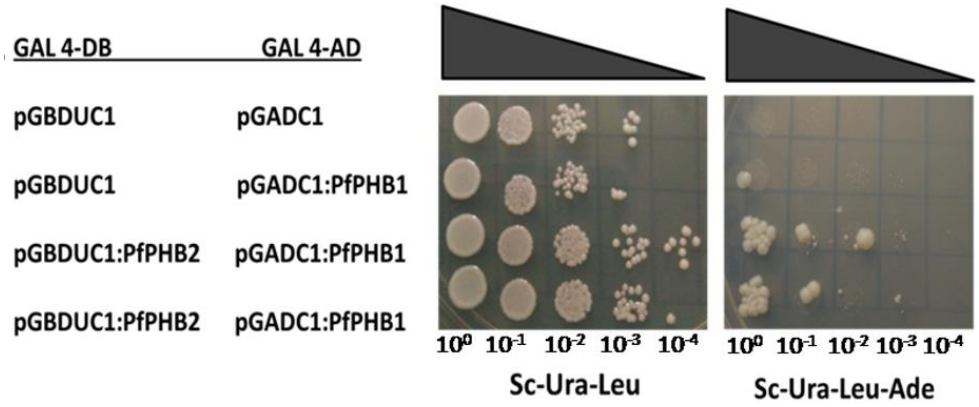


Fig. 28 Yeast Two-Hybrid analysis of PfPHB1 and PfPHB2

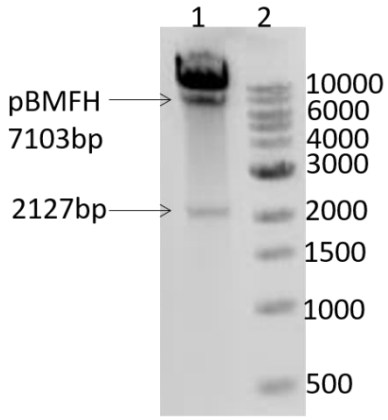


6.9 Yeast complementation analysis of PfSURF1

Unlike other microorganisms, Saccharomyces cerevisiae strains have steady haploid and diploid states. Hence, recessive mutations are comfortably isolated and manifested in haploid strains. DNA transformation in yeast drives gene cloning and genetic engineering techniques. Genes are introduced to analyze the genetic traits virtually through plasmids to identify the complementing genes (388). Using cross-species complementation in model organisms like budding yeast clues the functional role of putative genes from other organisms. Cross-species complementation experiments are evaluated by any measurable phenotypic rescue. The most straightforward phenotype to assay and quantify is to rescue the growth defect caused by the mutations in the native gene (389). As already described in the review, Yeast lacking Shy1 retains the residual cytochrome c oxidase activity. But these mutant cells compromised on respiratory, nonfermentable carbon sources (200). Fig. 27 shows the restriction analysis of the full-length PfSurf1 gene in the yeast expression plasmid pBMFH. The Δshy1 mutant yeast strains were transformed with ScShy1 full-length genes using the same pBMFH plasmid. The growth of these transformed mutant cells was assayed in 2% glucose and 2% glycerol-containing media with

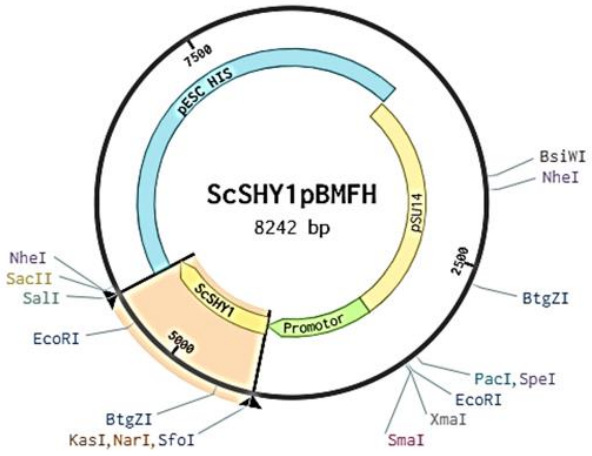
W303α wild strain as control and pBMFH as a negative control. The growth assay is represented in Fig. 31. The plasmid maps of respective genes are shown in Fig. 30.

Fig. 29 Restriction Analysis of PfSurf1pBMFH



Lane 1: PfSurf1pBMFH digested with BamHI and SalI

Fig. 30 Plasmid map of PfPHB1pGAD-C1 and PfPHB2pGBDU-C1



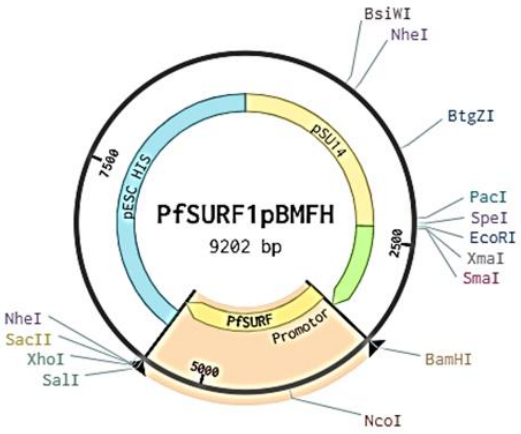
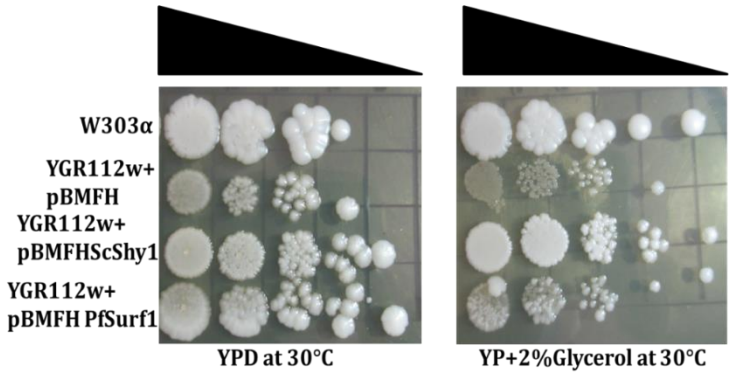


Fig. 31 Complementation Assay of PfSURF1 and ScShy1 using different carbon sources



W303α: control,
YGR112w+ pBMFH : ScSHY1 knock out + empty vector,
YGR112w+pBMFHScShy1:ScSHY1 knockout +ScShy1 full length gene in pBMFH,
YGR112w+pBMFHPfSurf1: ScSHY1 knockout +PfSurf1 full length gene in pBMFH

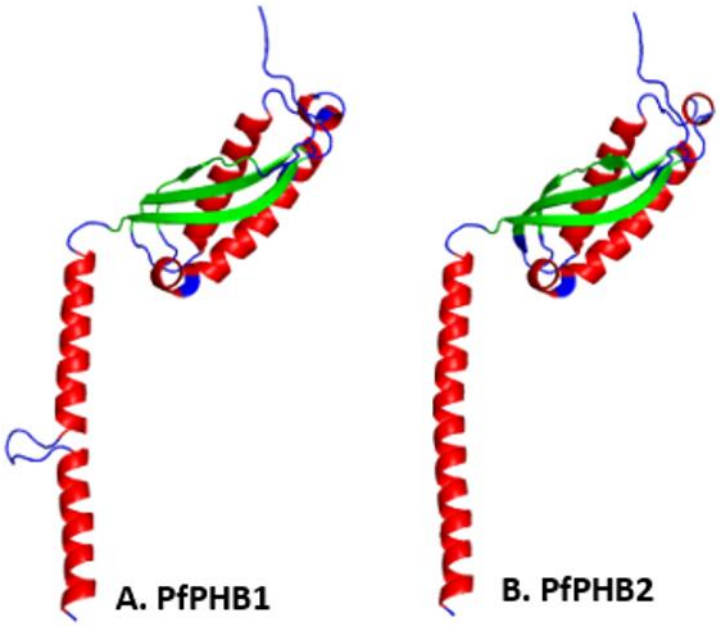
Yeast cells propagate well on the glycerol-lactate plate at 30°C when cytochrome c oxidase activity is >40% of wild type (377). The mutant strain with plasmid alone shows a very poor growth in glycerol media explaining the deficit function of cytochrome c oxidase in the cells. Whereas the transformed mutant strains show improved growth in glycerol. Serial dilution was done to check the growth variations.

6.10 Structural analysis of PfPHBs

Protein domains are elementary units of protein structure, folding, evolution, function, and design. Homologous domains are those sequences encoded by different genes which remain intact at sequence level by the process of evolution. Homology modeling also called comparative modeling, a type of template-based modeling, where the template is homologous to the target protein. The procedure involves fitting in the sequence of a target protein into a known structure which acts as a template.

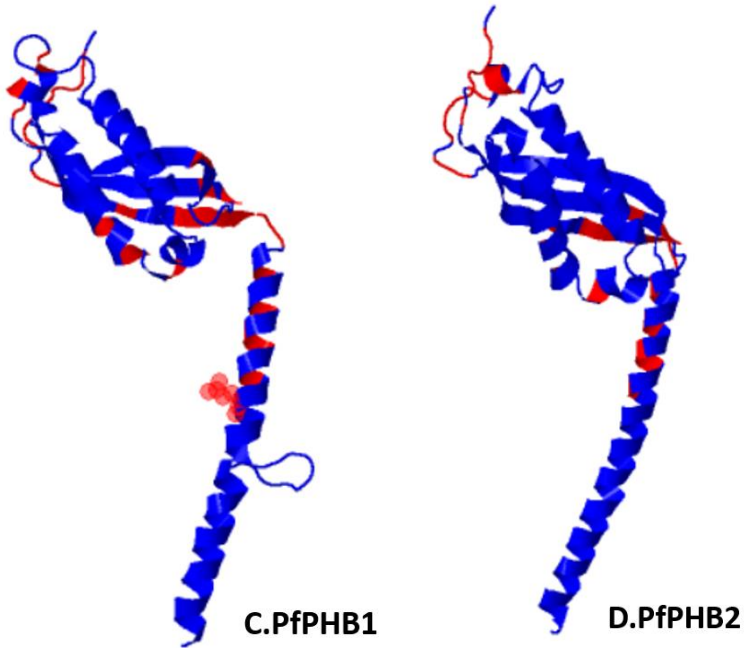
Phyre2 relies on *HHsearch* searches, which examines the query sequence against a library HMMs and gives a list of the output of structural matches with query-template matches. HH suites are positioned on the pairwise alignment of profile Hidden Markov models (HMMs), which show the multiple sequence alignment of homologous proteins. In addition to the provisional modeling pipeline, to model queries, *Phyre2* allows multiple - template and ab initio approaches. *Phyre2* has a good ranking overall for 20 years and has been applied to generate large to very large libraries of models. Hence *Phyre2*, a portal provides a submission interface to modeling servers for any queries with no currently available structure models (390). Both PfPHB1 and PfPHB2 protein sequences were modeled against the crystal structure of the important domains of stomatin of *Pyrococcus harikoshii*, a hyperthermophilic anaerobic archaeon.

Fig. 32 Predicted structures of PfPHB1 and PfPHB2



From the total aminoacid sequence submitted for modeling, 156 residues of PfPHB1 (57% of total amino acids) have been modeled with 99.9% confidence against the C chain of stomatin of *Pyrococcus horikoshii*. Similar to PfPHB1, 156 residues of PfPHB2 protein sequence (51% of total amino acids) are modeled against the same chain C of the important domain of stomatin from *Pyrococcus horikoshii*. The predicted structures of PfPHB1and PfPHB2 are shown in Fig. 31. The secondary structures of the predicted protein are represented in different colors as follows, helix in red; sheets in green, and coils in blue. Both structures are similar with very few dissimilarities. The stomatin structures in *P. horikoshii* are thought to act as a scaffold protein to support the cell membranes. Along with STOP (Stomatin Operon pattern protein), they may involve in regulatory functions in the cell (391). The alpha – helical segments of stomatin protein flexibly move with the membrane surface. Lipid raft clustering takes place during the formation of high order homo-oligomers of SPFH domain proteins which led to movement and membrane bending (392).

Fig. 33 Protein-protein interaction interfaces of PfPHB1 and PfPHB2



Protein-protein interactions happen with several amino acids, having a key role in the interaction. The regions of amino acids that contribute to binding free energy to form the protein-protein interaction interface are named "hot-spots" (393-395). Compared to other amino acids, tryptophan, arginine, and tyrosine are the most likely to appear in the hot spot regions of interactions (386, 394).

Arginine is abundant in the protein-protein interface also in its core. Interface arginine is an essential residue to the biological function. Furthermore, arginine has a role in DNA-Protein recognition. Related to the protein surface, the regions in the interface are depleted in lysine, Aspartate, and glutamate. In analysing the homodimer interfaces of proteins, the amino acid compositions are different, likely Alanine, Valine, and Leucine are most abundant. Again Arginine and Leucine are major contributors to the formation of the homodimer interface core (394).

Based on the Phyre2 investigations, probable residues in the protein-protein interaction interfaces were identified based on the PiSite, a database for protein interaction sites (396). Protein-protein interaction sites are represented in red are based on the template residues shown in Fig. 32. Two continuous interface regions are seen in PfPHB1 and PfPHB2, and the sequences are FQTPYIY-IK/HLSYGK-A and FERSIIY-VR/HLSFSN-E, respectively. As discussed before, these sequences hold a mixture of residues involves in interaction within themselves or with other proteins.

7.0 SUMMARY

Lack of vaccines and emergence of drug resistance to almost all existing drugs including artemisinin shows the importance of exploratory research like ours, where specific proteins suspected to play key roles in the parasite biology are assessed. Even though mitochondria of *Plasmodium* have one of the smallest genome sequenced, and has considerably reduced its metabolic functions, it is still an essential organelle. Atovaquone targeting the mtETC is a justified drug target, and several other molecules are evaluated in various laboratories around the world as potential therapeutics. Here, our work on Prohibitins of *Plasmodium* sheds important insight in the importance of these proteins in the biology of the parasite.

Domain analysis of these proteins indicate their divergence from the ancestral origin. Conserved domains and their sequence explain the functional importance of these proteins in the mitochondrial cascade of processes. Prohibitin super families have a functional association between prokaryotic SPFH proteins and eukaryotic prohibitins in the design of interaction with proteases and their topology. The unsuccessful gene targeting experiments using the CRISPR/Cas9 knockout system shows the essentiality of PfPHB1 and PfPHB2 in the asexual stages of development of *Plasmodium falciparum*. Notably tagging of PfPHB1 and PfPHB2 with non-distributive tagging strategies failed, and is highly likely the tagging may interfere in the protein function.

Protein-protein interactions are an extensive complex network of reactions between proteins counting for important regulations of most biological processes. This study reports the interaction of PfPHB1 and PfPHB2 using classical Yeast-Two hybrid analysis. Both the proteins have shown a possible high degree of interactions

in the selection dropout plates. These interactions suggest the possible formation of homodimer structures in the parasites. Hot spots are the interface residues contributing to the interaction. The information of hot spot residues is very important to understand the biological mechanism, rationalizing the conformational transitions, interpreting the functional role of mutations in the residues, and also in the identification of inhibitors of interactions. *In silico* identification of hot-spot residues in PfPHB1 and PfPHB2 interaction shows few continuous sequences of amino acids as described in the discussion. Targeting protein-protein interactions form a new direction in treating the disease and is a crucial strategy for the advancement of new drugs. Small molecule drugs need to act on these hot-spot interfaces to intervene in these interactions.

Homology modeling using computational structure prediction methods from the sequence of amino acids using 3D structures (PDB) of closely related proteins is highly reliable. The structure of proteins is highly conserved and changes happen at a relatively very slow rate compared to the amino acid changes in the sequence during evolution. Protein structures from modeling have a wide range of applications like drug designing, mutational experiments, ligand hunting and designing, protein-protein docking experiments, substrate specificity experiments, analysis of binding mechanisms of proteins and other ligands, simulation studies, molecular replacement experiments in structural refinement studies and many more. Modeling studies of PfPHB1 and PfPHB2 reveals high confidence structures as detailed in the discussion. The secondary structure analysis predicts the possible transmembrane regions in the structures which are highly informative.

Many assembly factors have emerged to perform various steps in the mitochondria in addition to insertions of proteins in the inner mitochondrial membrane, maturation of polypeptide, prosthetic group, and co-factors incorporation, and other regulatory processes of the organelles. PfSURF1 is one such protein with well-conserved domains in its functional sequence. As discussed in the review, among many interacting partners of the prohibitin complex, PfSURF1 could be directly or indirectly regulated (based on yeast experiments). Hence we initiated the complementation studies with **S**urf **h**omolog of **y**east (Shy) to understand its function. An appreciable recovery of mutant with PfSURF1 indicated the mitochondrial function of the protein.

8.0 CONCLUSION AND FURTHER DIRECTIONS

Functional genomics helps us to understand the gene function by creating gene mutations, gene knockouts and knockdowns. Genetic manipulation tools towards evaluating the performance of any protein are a requisite in drug discovery. Recent advancements in CRISPR-based tools of particular interest in *Plasmodium* are dead Cas 9 (dCas9). This dCas9 physically occupies the target and prevents its transcription. The RNA targeting Cas13 RNA nucleases which has similar action of RNAi for knocking down the gene of interest is a promising tool in the field of *Plasmodium* biology. These cutting-edge genetic technologies can conditionally control the functions of essential genes. These indispensable tools will open up our perception on the functionality of essential genes in every stage of *Plasmodium* life cycle.

Prohibitins as described in the review are heterodimers in the mitochondrial membrane. Many cellular functions are coordinated because of their interaction with each other or with other proteins. Mutational studies in the interface residues of PfPHB1 and PfPHB2 may explain geometrical and physiochemical changes in the interaction sites, interface stability, interface conformational dynamics, and specific conformational states of the protein molecules. Studying the Interaction of these proteins with other protein partners including PfOXA1, Pfm-AAA proteases, PfSurf1 will shed more light on its role in the parasite life cycle.

Genetic manipulations of PfSurf1 gene, especially mutations will enhance our understanding of the importance of this protein in the mitochondrial physiology of *Plasmodium falciparum*. With the help of ever-evolving CRISPR-based tools, extra research is needed to study the importance of many mitochondrial proteins in the asexual and sexual stages.

9.0 TABLES

Table 1: Special chemicals, enzymes and consumables

Product	Manufacturer	Product	Manufacturer
RPMI1640	Lonza	DNARuler Marker	Thermoscientific
Albumax	Invitrogen	Protein ladder	New England Biolabs
Hypoxanthine	Sigma	Kanamycin	MPBio
Gentamicin	Sigma	Ampicillin	MPBio
Sodium bicarbonate	Invitrogen	Agarose	MPBio
Giemsa	Sigma	T4 DNA ligase	Takara
WR99210	Sigma	Alkaline phosphatase	Takara
Blasticidin	MPBio	Chloramphenicol	Amresco
Geneticin G418	Gibco	0.2 cm Electroporation cuvette	Biorad
Isopropanol	Sigma	EGTA	Sigma
Sodium lactate	Sigma	HEPES free acid	Sigma

Table 2: Kit systems

Kit systems	Manufacturer
Plasmid MiniPrep Kit	Qiagen
Plasmid MaxiPrep Kit	Qiagen
XL10 Gold Competent cells	Stratagene
In-Fusion HD cloning Kit	Takara
Wizard SV gel and PCR cleanup system	Promega
Innuprep Blood DNA Mini Kit	Analytik Jena

Table 3: Primers used in this study

Primer name	Primer Sequence
PfPHB1glmSfwd	ATGGTACCGGAAAATTAAGTGTTGTAGCAGGGGGGCTAAGTTTAATTC
PfPHB1glmSrev	ATCTCGAGAAAATCCCTAGGATTTAACAAAATATTCGAATTGGATGG
PfPHB2glmSfwd	TGTGTTTAACCCGGGGGTACC*GG*AACATTTACAAAATTCATTTCAAGCC
PfPHB2glmSrev	AACATCATATGGATACTCGAGTTTTGTAAAATTTATCAACAACGAATCCGTAGGTAACATAACC
pCC11ribo vec rev	CCATTTGCATAGTCAGGAACATC
PfPHB1 Apt fwd	CCTTTCCGGGCGCGCCTTAAGCTTCTATAGGAAAATTAAGTGTTGTAGCAGG
PfPHB1 Apt rev	AATCAGTTTCTGTTCGGTAACCGCAAAATCCCTAGGATTTAACAAAATATTCGAATTG
PfPHB2 Apt fwd	CCTTTCCGGGCGCGCCTTAAGAACATTTACAAAATTCATTTCAAGCCTTATATTAAC
PfPHB2 Apt rev	AATCAGTTTCTGTTCGGTAACCGCTTTTGTAAAATTTATCAACAACGAATCCGTAGG
pMG74 vec rev	GTATATTGGGGTGATGATAAAATGAAAG

PfPHB1CR 5'fwd	ATTATTTTTACCGTTCCATGGCATAATAAATATAATTTTATAGAAATACTAACATTAAAG
PfPHB1CR 5' rev	TATTTATTAAATCTAGAATTCGTTGTGTATGAGGTCTAAATAATAATCTCAAACTTATTG
PfPHB1CR3'fwd	TTTTTTTACAAAATGCTTAAGGATGATGTAGCTATTACACATTTAAGTTATGG
PfPHB1CR 3' rev	CTTTCCGCGGGGAGGACTAGTCCAAAAATATTTTCAAATAATGATTGACTACTTGTC
PfPHB2CR 5'fwd	ATTATTTTTACCGTTCCATGGCTTTATTTTTATTTATTTTTCCAATATATTTGTC
PfPHB2CR 5' rev	TATTTATTAAATCTAGAATTCCATTAATACTCTAGGTTTTGTTCTTACATC
PfPHB2CR3'fwd	TTTTTTTACAAAATGCTTAAGGTACGTGAACAATTAGTACAAAGAGCAAAAG
PfPHB2CR 3' rev	CTTTCCGCGGGGAGGACTAGTCAAAAAAAAGTATAACATCTTATTTAAGAAC
pL6 vec rev	TAGGAAATAATAAAAAAGCACC
PfPHB1YC fwd	GAACTTAGTTTCGACGGATCCATGGAAAGAATATTATCTTCTATAGGAAAATTAAGTG
PfPHB1YC rev	CAACTTCTGTTCCATGTCGACAAAATCCCTAGGATTTAACAAAATATTCGAATTG
PfPHB2YC fwd	GAACTTAGTTTCGACGGATCCATGTATAAATTTAAATTTAAATTAAGGAACATTTAC
PfPHB2YC rev	CAACTTCTGTTCCATGTCGACTTTTGTAAAATTTATCAACAACGAATCCGTAGG
PfSurf1YCfwd	GAACTTAGTTTCGACGGATCCATGCCCTTATTTAAGTATGATCTTAAGGCAAAG
PfSurf1YC rev	CAACTTCTGTTCCATGTCGACAAAAACCCACCTTTTGAATTGTAC
ScShy1fwd	GAACTTAGTTTCGACGGATCCATGTCTCTACTAGGCGCCAGGTC
Scshy1rev	CAACTTCTGTTCCATGTCGACCATATATTTCCTTGAATGCTTCAG

Table 4: Guide and Oligos sequences

Oligos/Guide name	Oligos sequence
PfPHB1CROligo1	CATATTAAGTATATAATATTCCATATTTACATAGTACCCTGTTTTAGAGCTAGAAATAGC
PfPHB1CR Oligo2	GCTATTTCTAGCTCTAAAACAGGGTACTATGTAAATATGGAATATTATATACTTAATATG
PfPHB1 guide	CCATATTTACATAGTACCCT
PfPHB2CR Oligo1	CATATTAAGTATATAATATTGTAGAAATATATAGAACCTTGTTTTAGAGCTAGAAATAGC
PfPHB2CR Oligo2	GCTATTTCTAGCTCTAAAACAAGGTTCTATATATTTCTACAATATTATATACTTAATATG
PfPHB2 guide	GTAGAAATATATAGAACCTT

Table 5: E. coli strains used in this study

Strains	Manufacturer
DH5α competent cells	New England Biolabs
XL10 GOLD competent cells	Stratagene

Table 6: Yeast strains used in this study

S.cerevisiae strain	Genotype	Reference
YGR112w	BY4741;MAT a;his3Δ1;leu2Δ0;met15Δ0;ura3Δ0;YGR112w:: kanMX4	EUROSCARF

Table 7: List of *Plasmodium falciparum* strains used in this study

Strain name	Source
Plasmodium falciparum 3D7	MR4
Plasmodium falciparum NF54	

Table 8: List of Plasmids used in this study

Plasmid	Backbone	Insert	Reference
PfPHB1 pCC11ribo	pCC11ribo	PfPHB1[#]	This study
PfPHB2 pCC11ribo	PCC11ribo	PfPHB2[#]	This study
PfPHB1 pMG75	pMG75	PfPHB1[#]	This study
PfPHB2 pMG75	pMG75	PfPHB2[#]	This study
PfPHB1pL7	pL6	PfPHB1[#]	This study
PfPHB2pL7	pL6	PfPHB2[#]	This study
pUF Cas9		-	(82)
pBMFH		-	This study
PfPHB1pGATC	pGATC	PfPHB1*	This study
PfPHB2pGBDU	pGBDU	PfPHB2*	This study
PfSurf1pBMFH	pBMFH	PfSurf1*	This study
ScShy1pBMFH	pBMFH	ScShy1*	This study

[#]two third of the open reading frame

*****complete open reading frame

Table 9: Yeast media and Components

Medium	Components
YPD	1% yeast extract, 2% peptone, 2% glucose
YPG	1% yeast extract, 2% peptone, 3% glycerol
YPE	1% yeast extract, 2% peptone, 3% ethanol
SC-His	0.67% Yeast nitrogen base,1%leucine,0.25%uracil, 1% methionine
SC-Ura	0.67% Yeast nitrogen base,1%leucine, 1%Histidine, 1% methionine,1% Arginine,1% lysine,0.5% tryptophan,0.1% adenine
SC-Leu-Ura	0.67% Yeast nitrogen base,1%Histidine, 1% methionine, 1% Arginine,1% lysine,0.5% tryptophan,0.1% adenine
SC-Ura-Leu-His	0.67% Yeast nitrogen base, % methionine,1% Arginine, 1% lysine,0.5% tryptophan,0.1% adenine
SC-Ura-Leu-Ade	0.67% Yeast nitrogen base, 1% methionine, 1% Arginine, 1% lysine,0.5% tryptophan.

10.0 BIBLIOGRAPHY

1. WHO. World Malaria Report 2020,20 years of global progress & challenges 2021 [Available from: https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2020 (Accessed on 28th March 2021).
2. Venugopal K, Hentzschel F, Valkiunas G, Marti M. Plasmodium asexual growth and sexual development in the haematopoietic niche of the host. Nature reviews Microbiology. 2020;18(3):177-89.
3. Greenwood BM, Bojang K, Whitty CJ, Targett GA. Malaria. Lancet. 2005;365(9469):1487-98.
4. White NJ. Artemisinin resistance--the clock is ticking. Lancet. 2010;376(9758):2051-2.
5. Hoffman SL, Subramanian GM, Collins FH, Venter JC. Plasmodium, human and Anopheles genomics and malaria. Nature. 2002;415(6872):702-9.
6. Cavalier-Smith T. Protist phylogeny and the high-level classification of Protozoa. European Journal of Protistology. 2003;39(4):338-48.
7. Bannister LH, Sherman IW. Plasmodium. eLS2009.
8. Martinsen ES, Perkins SL, Schall JJ. A three-genome phylogeny of malaria parasites (Plasmodium and closely related genera): evolution of life-history traits and host switches. Mol Phylogenet Evol. 2008;47(1):261-73.
9. Cornejo OE, Escalante AA. The origin and age of Plasmodium vivax. Trends in parasitology. 2006;22(12):558-63.
10. Kwiatkowski DP. How malaria has affected the human genome and what human genetics can teach us about malaria. American journal of human genetics. 2005;77(2):171-92.
11. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, et al. Genome sequence of the human malaria parasite Plasmodium falciparum. Nature. 2002;419(6906):498-511.
12. Wilson RJ, Denny PW, Preiser PR, Rangachari K, Roberts K, Roy A, et al. Complete gene map of the plastid-like DNA of the malaria parasite Plasmodium falciparum. Journal of molecular biology. 1996;261(2):155-72.
13. Aldritt SM, Joseph JT, Wirth DF. Sequence identification of cytochrome b in Plasmodium gallinaceum. Molecular and cellular biology. 1989;9(9):3614-20.
14. Vaidya AB, Akella R, Suplick K. Sequences similar to genes for two mitochondrial proteins and portions of ribosomal RNA in tandemly arrayed 6-kilobase-pair DNA of a malarial parasite. Molecular and biochemical parasitology. 1989;35(2):97-107.
15. Feagin JE. The 6-kb element of Plasmodium falciparum encodes mitochondrial cytochrome genes. Molecular and biochemical parasitology. 1992;52(1):145-8.
16. Feagin JE, Mericle BL, Werner E, Morris M. Identification of additional rRNA fragments encoded by the Plasmodium falciparum 6 kb element. Nucleic acids research. 1997;25(2):438-46.
17. Painter HJ, Morrisey JM, Mather MW, Vaidya AB. Specific role of mitochondrial electron transport in blood-stage Plasmodium falciparum. Nature. 2007;446(7131):88-91.
18. McFadden GI. Plastids and protein targeting. The Journal of eukaryotic microbiology. 1999;46(4):339-46.
19. Gabaldon T, Huynen MA. Shaping the mitochondrial proteome. Biochimica et biophysica acta. 2004;1659(2-3):212-20.

20. LaGier MJ, Tachezy J, Stejskal F, Kutisova K, Keithly JS. Mitochondrial-type iron-sulfur cluster biosynthesis genes (IscS and IscU) in the apicomplexan Cryptosporidium parvum. Microbiology. 2003;149(Pt 12):3519-30.
21. Riordan CE, Ault JG, Langreth SG, Keithly JS. Cryptosporidium parvum Cpn60 targets a relict organelle. Current genetics. 2003;44(3):138-47.
22. Roberts CW, Roberts F, Henriquez FL, Akiyoshi D, Samuel BU, Richards TA, et al. Evidence for mitochondrial-derived alternative oxidase in the apicomplexan parasite Cryptosporidium parvum: a potential anti-microbial agent target. International journal for parasitology. 2004;34(3):297-308.
23. Cowman AF, Crabb BS. The Plasmodium falciparum genome--a blueprint for erythrocyte invasion. Science. 2002;298(5591):126-8.
24. Francis SE, Sullivan DJ, Jr., Goldberg DE. Hemoglobin metabolism in the malaria parasite Plasmodium falciparum. Annual review of microbiology. 1997;51:97-123.
25. McFadden GI, Reith ME, Munholland J, Lang-Unnasch N. Plastid in human parasites. Nature. 1996;381(6582):482.
26. Van Wye J, Ghori N, Webster P, Mitschler RR, Elmendorf HG, Haldar K. Identification and localization of rab6, separation of rab6 from ERD2 and implications for an 'unstacked' Golgi, in Plasmodium falciparum. Molecular and biochemical parasitology. 1996;83(1):107-20.
27. Bannister LH, Hopkins JM, Fowler RE, Krishna S, Mitchell GH. A brief illustrated guide to the ultrastructure of Plasmodium falciparum asexual blood stages. Parasitology today. 2000;16(10):427-33.
28. Jomaa H, Wiesner J, Sanderbrand S, Altincicek B, Weidemeyer C, Hintz M, et al. Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. Science. 1999;285(5433):1573-6.
29. Srivastava IK, Morrisey JM, Darrouzet E, Daldal F, Vaidya AB. Resistance mutations reveal the atovaquone-binding domain of cytochrome b in malaria parasites. Molecular microbiology. 1999;33(4):704-11.
30. Surolia N, Surolia A. Triclosan offers protection against blood stages of malaria by inhibiting enoyl-ACP reductase of Plasmodium falciparum. Nature medicine. 2001;7(2):167-73.
31. Waller RF, Ralph SA, Reed MB, Su V, Douglas JD, Minnikin DE, et al. A type II pathway for fatty acid biosynthesis presents drug targets in Plasmodium falciparum. Antimicrobial agents and chemotherapy. 2003;47(1):297-301.
32. Rudzinska MA. The fine structure of malaria parasites. International review of cytology. 1969;25:161-99.
33. Aikawa M. The fine structure of the erythrocytic stages of three avian malarial parasites, Plasmodium fallax, P. lophurae, and P. cathemerium. The American journal of tropical medicine and hygiene. 1966;15(4):449-71.
34. Hepler PK, Huff CG, Sprinz H. The fine structure of the exoerythrocytic stages of Plasmodium fallax. The Journal of cell biology. 1966;30(2):333-58.
35. van Dooren GG, Marti M, Tonkin CJ, Stimmler LM, Cowman AF, McFadden GI. Development of the endoplasmic reticulum, mitochondrion and apicoplast during the asexual life cycle of Plasmodium falciparum. Molecular microbiology. 2005;57(2):405-19.
36. Krungkrai J, Prapunwattana P, Krungkrai SR. Ultrastructure and function of mitochondria in gametocytic stage of Plasmodium falciparum. Parasite. 2000;7(1):19-26.

37. Aikawa M. Parasitological review. Plasmodium: the fine structure of malarial parasites. Experimental parasitology. 1971;30(2):284-320.
38. Baunaure F, Eldin P, Cathiard AM, Vial H. Characterization of a non-mitochondrial type I phosphatidylserine decarboxylase in Plasmodium falciparum. Molecular microbiology. 2004;51(1):33-46.
39. Santiago TC, Zufferey R, Mehra RS, Coleman RA, Mamoun CB. The Plasmodium falciparum PfGatp is an endoplasmic reticulum membrane protein important for the initial step of malarial glycerolipid synthesis. The Journal of biological chemistry. 2004;279(10):9222-32.
40. Alleva LM, Kirk K. Calcium regulation in the intraerythrocytic malaria parasite Plasmodium falciparum. Molecular and biochemical parasitology. 2001;117(2):121-8.
41. Eckstein-Ludwig U, Webb RJ, Van Goethem ID, East JM, Lee AG, Kimura M, et al. Artemisinins target the SERCA of Plasmodium falciparum. Nature. 2003;424(6951):957-61.
42. Delorenzi M, Sexton A, Shams-Eldin H, Schwarz RT, Speed T, Schofield L. Genes for glycosylphosphatidylinositol toxin biosynthesis in Plasmodium falciparum. Infection and immunity. 2002;70(8):4510-22.
43. Waller RF, Keeling PJ, Donald RG, Striepen B, Handman E, Lang-Unnasch N, et al. Nuclear-encoded proteins target to the plastid in Toxoplasma gondii and Plasmodium falciparum. Proceedings of the National Academy of Sciences of the United States of America. 1998;95(21):12352-7.
44. Saraste M. Oxidative phosphorylation at the fin de siecle. Science. 1999;283(5407):1488-93.
45. Vertommen D, Van Roy J, Szikora JP, Rider MH, Michels PA, Opperdoes FR. Differential expression of glycosomal and mitochondrial proteins in the two major life-cycle stages of Trypanosoma brucei. Molecular and biochemical parasitology. 2008;158(2):189-201.
46. Michelotti EF, Hajduk SL. Developmental regulation of trypanosome mitochondrial gene expression. The Journal of biological chemistry. 1987;262(2):927-32.
47. Goonewardene R, Daily J, Kaslow D, Sullivan TJ, Duffy P, Carter R, et al. Transfection of the malaria parasite and expression of firefly luciferase. Proceedings of the National Academy of Sciences of the United States of America. 1993;90(11):5234-6.
48. Wu Y, Sifri CD, Lei HH, Su XZ, Wellems TE. Transfection of Plasmodium falciparum within human red blood cells. Proceedings of the National Academy of Sciences of the United States of America. 1995;92(4):973-7.
49. Wu Y, Kirkman LA, Wellems TE. Transformation of Plasmodium falciparum malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. Proceedings of the National Academy of Sciences of the United States of America. 1996;93(3):1130-4.
50. Crabb BS, Cowman AF. Characterization of promoters and stable transfection by homologous and nonhomologous recombination in Plasmodium falciparum. Proceedings of the National Academy of Sciences of the United States of America. 1996;93(14):7289-94.
51. van Dijk MR, Waters AP, Janse CJ. Stable transfection of malaria parasite blood stages. Science. 1995;268(5215):1358-62.

52. van Dijk MR, Janse CJ, Waters AP. Expression of a Plasmodium gene introduced into subtelomeric regions of Plasmodium berghei chromosomes. Science. 1996;271(5249):662-5.
53. Mota MM, Thathy V, Nussenzweig RS, Nussenzweig V. Gene targeting in the rodent malaria parasite Plasmodium yoelii. Molecular and biochemical parasitology. 2001;113(2):271-8.
54. Jongco AM, Ting LM, Thathy V, Mota MM, Kim K. Improved transfection and new selectable markers for the rodent malaria parasite Plasmodium yoelii. Molecular and biochemical parasitology. 2006;146(2):242-50.
55. Spence PJ, Cunningham D, Jarra W, Lawton J, Langhorne J, Thompson J. Transformation of the rodent malaria parasite Plasmodium chabaudi. Nature protocols. 2011;6(4):553-61.
56. Sanchez CP, Pfahler J, Del Portillo HA, Lanzer M. Transient transfection of Plasmodium vivax blood-stage parasites. Methods in molecular biology. 2013;923:151-9.
57. Moraes Barros RR, Straimer J, Sa JM, Salzman RE, Melendez-Muniz VA, Mu J, et al. Editing the Plasmodium vivax genome, using zinc-finger nucleases. The Journal of infectious diseases. 2015;211(1):125-9.
58. Crabb BS, Cooke BM, Reeder JC, Waller RF, Caruana SR, Davern KM, et al. Targeted gene disruption shows that knobs enable malaria-infected red cells to cytoadhere under physiological shear stress. Cell. 1997;89(2):287-96.
59. O'Donnell RA, Freitas-Junior LH, Preiser PR, Williamson DH, Duraisingh M, McElwain TF, et al. A genetic screen for improved plasmid segregation reveals a role for Rep20 in the interaction of Plasmodium falciparum chromosomes. The EMBO journal. 2002;21(5):1231-9.
60. Crabb BS, Rug M, Gilberger TW, Thompson JK, Triglia T, Maier AG, et al. Transfection of the human malaria parasite Plasmodium falciparum. Methods in molecular biology. 2004;270:263-76.
61. Braks JA, Franke-Fayard B, Kroeze H, Janse CJ, Waters AP. Development and application of a positive-negative selectable marker system for use in reverse genetics in Plasmodium. Nucleic acids research. 2006;34(5):e39.
62. Maier AG, Braks JA, Waters AP, Cowman AF. Negative selection using yeast cytosine deaminase/uracil phosphoribosyl transferase in Plasmodium falciparum for targeted gene deletion by double crossover recombination. Molecular and biochemical parasitology. 2006;150(1):118-21.
63. Janse CJ, Ramesar J, Waters AP. High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite Plasmodium berghei. Nature protocols. 2006;1(1):346-56.
64. Meissner M, Krejany E, Gilson PR, de Koning-Ward TF, Soldati D, Crabb BS. Tetracycline analogue-regulated transgene expression in Plasmodium falciparum blood stages using Toxoplasma gondii transactivators. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(8):2980-5.
65. Ullu E, Tschudi C, Chakraborty T. RNA interference in protozoan parasites. Cellular microbiology. 2004;6(6):509-19.
66. Pino P, Sebastian S, Kim EA, Bush E, Brochet M, Volkmann K, et al. A tetracycline-repressible transactivator system to study essential genes in malaria parasites. Cell host & microbe. 2012;12(6):824-34.

67. Banaszynski LA, Chen LC, Maynard-Smith LA, Ooi AG, Wandless TJ. A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. Cell. 2006;126(5):995-1004.
68. Armstrong CM, Goldberg DE. An FKBP destabilization domain modulates protein levels in Plasmodium falciparum. Nature methods. 2007;4(12):1007-9.
69. Muralidharan V, Oksman A, Iwamoto M, Wandless TJ, Goldberg DE. Asparagine repeat function in a Plasmodium falciparum protein assessed via a regulatable fluorescent affinity tag. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(11):4411-6.
70. Gilson PR, O'Donnell RA, Nebl T, Sanders PR, Wickham ME, McElwain TF, et al. MSP1(19) miniproteins can serve as targets for invasion inhibitory antibodies in Plasmodium falciparum provided they contain the correct domains for cell surface trafficking. Molecular microbiology. 2008;68(1):124-38.
71. Prommana P, Uthaipibull C, Wongsombat C, Kamchonwongpaisan S, Yuthavong Y, Knuepfer E, et al. Inducible knockdown of Plasmodium gene expression using the glmS ribozyme. PloS one. 2013;8(8):e73783.
72. Goldfless SJ, Wagner JC, Niles JC. Versatile control of Plasmodium falciparum gene expression with an inducible protein-RNA interaction. Nature communications. 2014;5:5329.
73. Straimer J, Lee MC, Lee AH, Zeitler B, Williams AE, Pearl JR, et al. Site-specific genome editing in Plasmodium falciparum using engineered zinc-finger nucleases. Nature methods. 2012;9(10):993-8.
74. Maeder ML, Thibodeau-Beganny S, Sander JD, Voytas DF, Joung JK. Oligomerized pool engineering (OPEN): an 'open-source' protocol for making customized zinc-finger arrays. Nature protocols. 2009;4(10):1471-501.
75. Sander JD, Dahlborg EJ, Goodwin MJ, Cade L, Zhang F, Cifuentes D, et al. Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). Nature methods. 2011;8(1):67-9.
76. Bassett AR, Tibbit C, Ponting CP, Liu JL. Highly efficient targeted mutagenesis of Drosophila with the CRISPR/Cas9 system. Cell reports. 2013;4(1):220-8.
77. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, et al. Multiplex genome engineering using CRISPR/Cas systems. Science. 2013;339(6121):819-23.
78. Dickinson DJ, Ward JD, Reiner DJ, Goldstein B. Engineering the Caenorhabditis elegans genome using Cas9-triggered homologous recombination. Nature methods. 2013;10(10):1028-34.
79. Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, et al. Efficient genome editing in zebrafish using a CRISPR-Cas system. Nature biotechnology. 2013;31(3):227-9.
80. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, et al. RNA-guided human genome engineering via Cas9. Science. 2013;339(6121):823-6.
81. Wagner JC, Platt RJ, Goldfless SJ, Zhang F, Niles JC. Efficient CRISPR-Cas9-mediated genome editing in Plasmodium falciparum. Nature methods. 2014;11(9):915-8.
82. Ghorbal M, Gorman M, Macpherson CR, Martins RM, Scherf A, Lopez-Rubio JJ. Genome editing in the human malaria parasite Plasmodium falciparum using the CRISPR-Cas9 system. Nature biotechnology. 2014;32(8):819-21.
83. Bhaya D, Davison M, Barrangou R. CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. Annual review of genetics. 2011;45:273-97.

84. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012;337(6096):816-21.
85. Zhang C, Xiao B, Jiang Y, Zhao Y, Li Z, Gao H, et al. Efficient editing of malaria parasite genome using the CRISPR/Cas9 system. mBio. 2014;5(4):e01414-14.
86. Lane N, Martin W. The energetics of genome complexity. Nature. 2010;467(7318):929-34.
87. Friedman JR, Nunnari J. Mitochondrial form and function. Nature. 2014;505(7483):335-43.
88. Boore JL. Animal mitochondrial genomes. Nucleic acids research. 1999;27(8):1767-80.
89. Vaidya AB, Mather MW. Mitochondrial evolution and functions in malaria parasites. Annual review of microbiology. 2009;63:249-67.
90. Das A, Syin C, Fujioka H, Zheng H, Goldman N, Aikawa M, et al. Molecular characterization and ultrastructural localization of Plasmodium falciparum Hsp 60. Molecular and biochemical parasitology. 1997;88(1-2):95-104.
91. Feagin JE, Gardner MJ, Williamson DH, Wilson RJ. The putative mitochondrial genome of Plasmodium falciparum. The Journal of protozoology. 1991;38(3):243-5.
92. Feagin JE, Werner E, Gardner MJ, Williamson DH, Wilson RJ. Homologies between the contiguous and fragmented rRNAs of the two Plasmodium falciparum extrachromosomal DNAs are limited to core sequences. Nucleic acids research. 1992;20(4):879-87.
93. Feagin JE, Harrell MI, Lee JC, Coe KJ, Sands BH, Cannone JJ, et al. The fragmented mitochondrial ribosomal RNAs of Plasmodium falciparum. PloS one. 2012;7(6):e38320.
94. Sharma A, Sharma A. Plasmodium falciparum mitochondria import tRNAs along with an active phenylalanyl-tRNA synthetase. The Biochemical journal. 2015;465(3):459-69.
95. Preiser PR, Wilson RJ, Moore PW, McCready S, Hajibagheri MA, Blight KJ, et al. Recombination associated with replication of malarial mitochondrial DNA. The EMBO journal. 1996;15(3):684-93.
96. Kilejian A. Spherical bodies. Parasitology today. 1991;7(11):309; author reply
97. Kohler S, Delwiche CF, Denny PW, Tilney LG, Webster P, Wilson RJ, et al. A plastid of probable green algal origin in Apicomplexan parasites. Science. 1997;275(5305):1485-9.
98. Hopkins J, Fowler R, Krishna S, Wilson I, Mitchell G, Bannister L. The plastid in Plasmodium falciparum asexual blood stages: a three-dimensional ultrastructural analysis. Protist. 1999;150(3):283-95.
99. John DT, Petri WA. Markell and Voge's Medical Parasitology: Elsevier Health Sciences; 2013.
100. Baker DA. Malaria gametocytogenesis. Molecular and biochemical parasitology. 2010;172(2):57-65.
101. Heymann DL. NPHIs as focal points for leadership in prevention and control of infectious diseases. Journal of public health policy. 2008;29(3):374-6.
102. Morrissette NS, Sibley LD. Cytoskeleton of apicomplexan parasites. Microbiology and molecular biology reviews : MMBR. 2002;66(1):21-38; table of contents.

103. Soldati D, Meissner M. Toxoplasma as a novel system for motility. Current opinion in cell biology. 2004;16(1):32-40.
104. Nelson KE, Williams C. Infectious Disease Epidemiology: Theory and Practice: Jones & Bartlett Learning; 2007.
105. Bannister LH, Hopkins JM, Fowler RE, Krishna S, Mitchell GH. Ultrastructure of rhoptry development in Plasmodium falciparum erythrocytic schizonts. Parasitology. 2000;121 (Pt 3):273-87.
106. Cowman AF, Tonkin CJ, Tham WH, Duraisingh MT. The Molecular Basis of Erythrocyte Invasion by Malaria Parasites. Cell host & microbe. 2017;22(2):232-45.
107. Ito D, Schureck MA, Desai SA. An essential dual-function complex mediates erythrocyte invasion and channel-mediated nutrient uptake in malaria parasites. eLife. 2017;6.
108. Cowman AF, Crabb BS. Invasion of red blood cells by malaria parasites. Cell. 2006;124(4):755-66.
109. Singh S, Chitnis CE. Signalling mechanisms involved in apical organelle discharge during host cell invasion by apicomplexan parasites. Microbes and infection. 2012;14(10):820-4.
110. Yeoh S, O'Donnell RA, Koussis K, Dluzewski AR, Ansell KH, Osborne SA, et al. Subcellular discharge of a serine protease mediates release of invasive malaria parasites from host erythrocytes. Cell. 2007;131(6):1072-83.
111. Bousema T, Drakeley C. Epidemiology and infectivity of Plasmodium falciparum and Plasmodium vivax gametocytes in relation to malaria control and elimination. Clinical microbiology reviews. 2011;24(2):377-410.
112. Alano P. Plasmodium falciparum gametocytes: still many secrets of a hidden life. Molecular microbiology. 2007;66(2):291-302.
113. Khan SM, Franke-Fayard B, Mair GR, Lasonder E, Janse CJ, Mann M, et al. Proteome analysis of separated male and female gametocytes reveals novel sex-specific Plasmodium biology. Cell. 2005;121(5):675-87.
114. Amino R, Thiberge S, Martin B, Celli S, Shorte S, Frischknecht F, et al. Quantitative imaging of Plasmodium transmission from mosquito to mammal. Nature medicine. 2006;12(2):220-4.
115. Divo AA, Geary TG, Jensen JB, Ginsburg H. The mitochondrion of Plasmodium falciparum visualized by rhodamine 123 fluorescence. The Journal of protozoology. 1985;32(3):442-6.
116. Slomianny C, Prensier G. Application of the serial sectioning and tridimensional reconstruction techniques to the morphological study of the Plasmodium falciparum mitochondrion. The Journal of parasitology. 1986;72(4):595-8.
117. Sullivan M, Li J, Kumar S, Rogers MJ, McCutchan TF. Effects of interruption of apicoplast function on malaria infection, development, and transmission. Molecular and biochemical parasitology. 2000;109(1):17-23.
118. Okamoto N, Spurck TP, Goodman CD, McFadden GI. Apicoplast and mitochondrion in gametocytogenesis of Plasmodium falciparum. Eukaryotic cell. 2009;8(1):128-32.
119. Creasey A, Mendis K, Carlton J, Williamson D, Wilson I, Carter R. Maternal inheritance of extrachromosomal DNA in malaria parasites. Molecular and biochemical parasitology. 1994;65(1):95-8.
120. Hall N, Karras M, Raine JD, Carlton JM, Kooij TW, Berriman M, et al. A comprehensive survey of the Plasmodium life cycle by genomic, transcriptomic, and proteomic analyses. Science. 2005;307(5706):82-6.

121. Jensen MD, Conley M, Helstowski LD. Culture of Plasmodium falciparum: the role of pH, glucose, and lactate. The Journal of parasitology. 1983;69(6):1060-7.
122. Bozdech Z, Llinas M, Pulliam BL, Wong ED, Zhu J, DeRisi JL. The transcriptome of the intraerythrocytic developmental cycle of Plasmodium falciparum. PLoS biology. 2003;1(1):E5.
123. Tonkin CJ, van Dooren GG, Spurck TP, Struck NS, Good RT, Handman E, et al. Localization of organellar proteins in Plasmodium falciparum using a novel set of transfection vectors and a new immunofluorescence fixation method. Molecular and biochemical parasitology. 2004;137(1):13-21.
124. Takeo S, Kokaze A, Ng CS, Mizuchi D, Watanabe JI, Tanabe K, et al. Succinate dehydrogenase in Plasmodium falciparum mitochondria: molecular characterization of the SDHA and SDHB genes for the catalytic subunits, the flavoprotein (Fp) and iron-sulfur (Ip) subunits. Molecular and biochemical parasitology. 2000;107(2):191-205.
125. Hodges M, Yikilmaz E, Patterson G, Kasvosve I, Rouault TA, Gordeuk VR, et al. An iron regulatory-like protein expressed in Plasmodium falciparum displays aconitase activity. Molecular and biochemical parasitology. 2005;143(1):29-38.
126. Gunther S, McMillan PJ, Wallace LJ, Muller S. Plasmodium falciparum possesses organelle-specific alpha-keto acid dehydrogenase complexes and lipoylation pathways. Biochemical Society transactions. 2005;33(Pt 5):977-80.
127. Cobbold SA, Vaughan AM, Lewis IA, Painter HJ, Camargo N, Perlman DH, et al. Kinetic flux profiling elucidates two independent acetyl-CoA biosynthetic pathways in Plasmodium falciparum. The Journal of biological chemistry. 2013;288(51):36338-50.
128. MacRae JI, Dixon MW, Dearnley MK, Chua HH, Chambers JM, Kenny S, et al. Mitochondrial metabolism of sexual and asexual blood stages of the malaria parasite Plasmodium falciparum. BMC biology. 2013;11:67.
129. Ke H, Lewis IA, Morrisey JM, McLean KJ, Ganesan SM, Painter HJ, et al. Genetic investigation of tricarboxylic acid metabolism during the Plasmodium falciparum life cycle. Cell reports. 2015;11(1):164-74.
130. Oppenheim RD, Creek DJ, Macrae JI, Modrzynska KK, Pino P, Limenitakis J, et al. BCKDH: the missing link in apicomplexan mitochondrial metabolism is required for full virulence of Toxoplasma gondii and Plasmodium berghei. PLoS pathogens. 2014;10(7):e1004263.
131. Krungkrai J. The multiple roles of the mitochondrion of the malarial parasite. Parasitology. 2004;129(Pt 5):511-24.
132. Mi-Ichi F, Takeo S, Takashima E, Kobayashi T, Kim HS, Wataya Y, et al. Unique properties of respiratory chain in Plasmodium falciparum mitochondria. Advances in experimental medicine and biology. 2003;531:117-33.
133. Vaidya AB. Mitochondrial and plastid functions as antimalarial drug targets. Current drug targets Infectious disorders. 2004;4(1):11-23.
134. Fry M, Beesley JE. Mitochondria of mammalian Plasmodium spp. Parasitology. 1991;102 Pt 1:17-26.
135. Mather MW, Henry KW, Vaidya AB. Mitochondrial drug targets in apicomplexan parasites. Current drug targets. 2007;8(1):49-60.
136. Uyemura SA, Luo S, Vieira M, Moreno SN, Docampo R. Oxidative phosphorylation and rotenone-insensitive malate- and NADH-quinone oxidoreductases in Plasmodium yoelii yoelii mitochondria in situ. The Journal of biological chemistry. 2004;279(1):385-93.

137. Tanaka TQ, Hirai M, Watanabe Y, Kita K. Toward understanding the role of mitochondrial complex II in the intraerythrocytic stages of Plasmodium falciparum: gene targeting of the Fp subunit. Parasitology international. 2012;61(4):726-8.
138. Sakata-Kato T, Wirth DF. A Novel Methodology for Bioenergetic Analysis of Plasmodium falciparum Reveals a Glucose-Regulated Metabolic Shift and Enables Mode of Action Analyses of Mitochondrial Inhibitors. ACS infectious diseases. 2016;2(12):903-16.
139. Balabaskaran Nina P, Morrisey JM, Ganesan SM, Ke H, Pershing AM, Mather MW, et al. ATP synthase complex of *Plasmodium falciparum*: dimeric assembly in mitochondrial membranes and resistance to genetic disruption. The Journal of biological chemistry. 2011;286(48):41312-22.
140. Fisher N, Bray PG, Ward SA, Biagini GA. The malaria parasite type II NADH:quinone oxidoreductase: an alternative enzyme for an alternative lifestyle. Trends in parasitology. 2007;23(7):305-10.
141. Fry M, Webb E, Pudney M. Effect of mitochondrial inhibitors on adenosinetriphosphate levels in Plasmodium falciparum. Comparative biochemistry and physiology B, Comparative biochemistry. 1990;96(4):775-82.
142. K.K. Kenji Hikosaka SS, Kiyoshi Kita. Mitochondria of malaria parasites as a drug target. A Samie (Ed), An Overview of Tropical Diseases, InTech. 2015.
143. Biagini GA, Viriyavejakul P, O'Neill P M, Bray PG, Ward SA. Functional characterization and target validation of alternative complex I of Plasmodium falciparum mitochondria. Antimicrobial agents and chemotherapy. 2006;50(5):1841-51.
144. Baldwin J, Farajallah AM, Malmquist NA, Rathod PK, Phillips MA. Malarial dihydroorotate dehydrogenase. Substrate and inhibitor specificity. The Journal of biological chemistry. 2002;277(44):41827-34.
145. Gutteridge WE, Dave D, Richards WH. Conversion of dihydroorotate to orotate in parasitic protozoa. Biochimica et biophysica acta. 1979;582(3):390-401.
146. Boysen KE, Matuschewski K. Arrested oocyst maturation in Plasmodium parasites lacking type II NADH:ubiquinone dehydrogenase. The Journal of biological chemistry. 2011;286(37):32661-71.
147. Hino A, Hirai M, Tanaka TQ, Watanabe Y, Matsuoka H, Kita K. Critical roles of the mitochondrial complex II in oocyst formation of rodent malaria parasite Plasmodium berghei. Journal of biochemistry. 2012;152(3):259-68.
148. Kawahara K, Mogi T, Tanaka TQ, Hata M, Miyoshi H, Kita K. Mitochondrial dehydrogenases in the aerobic respiratory chain of the rodent malaria parasite Plasmodium yoelii yoelii. Journal of biochemistry. 2009;145(2):229-37.
149. Takashima E, Takamiya S, Takeo S, Mi-ichi F, Amino H, Kita K. Isolation of mitochondria from Plasmodium falciparum showing dihydroorotate dependent respiration. Parasitology international. 2001;50(4):273-8.
150. Suraveratum N, Krungkrai SR, Leangaramgul P, Prapunwattana P, Krungkrai J. Purification and characterization of Plasmodium falciparum succinate dehydrogenase. Molecular and biochemical parasitology. 2000;105(2):215-22.
151. Mogi T, Kita K. Diversity in mitochondrial metabolic pathways in parasitic protists Plasmodium and Cryptosporidium. Parasitology international. 2010;59(3):305-12.
152. Niikura M, Komatsuya K, Inoue SI, Matsuda R, Asahi H, Inaoka DK, et al. Suppression of experimental cerebral malaria by disruption of malate:quinone oxidoreductase. Malaria journal. 2017;16(1):247.

153. Uyemura SA, Luo S, Moreno SN, Docampo R. Oxidative phosphorylation, Ca(2+) transport, and fatty acid-induced uncoupling in malaria parasites mitochondria. The Journal of biological chemistry. 2000;275(13):9709-15.
154. Mitamura T, Palacpac NM. Lipid metabolism in Plasmodium falciparum-infected erythrocytes: possible new targets for malaria chemotherapy. Microbes and infection. 2003;5(6):545-52.
155. van Dooren GG, Stimmler LM, McFadden GI. Metabolic maps and functions of the Plasmodium mitochondrion. FEMS microbiology reviews. 2006;30(4):596-630.
156. Rich PR. The molecular machinery of Keilin's respiratory chain. Biochemical Society transactions. 2003;31(Pt 6):1095-105.
157. Iwata S, Lee JW, Okada K, Lee JK, Iwata M, Rasmussen B, et al. Complete structure of the 11-subunit bovine mitochondrial cytochrome bc1 complex. Science. 1998;281(5373):64-71.
158. Lange C, Hunte C. Crystal structure of the yeast cytochrome bc1 complex with its bound substrate cytochrome c. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(5):2800-5.
159. Xia D, Yu CA, Kim H, Xia JZ, Kachurin AM, Zhang L, et al. Crystal structure of the cytochrome bc1 complex from bovine heart mitochondria. Science. 1997;277(5322):60-6.
160. Zhang Z, Huang L, Shulmeister VM, Chi YI, Kim KK, Hung LW, et al. Electron transfer by domain movement in cytochrome bc1. Nature. 1998;392(6677):677-84.
161. Petmitr S, Krungkrai J. Mitochondrial cytochrome b gene in two developmental stages of human malarial parasite Plasmodium falciparum. The Southeast Asian journal of tropical medicine and public health. 1995;26(4):600-5.
162. Zara V, Conte L, Trumpower BL. Evidence that the assembly of the yeast cytochrome bc1 complex involves the formation of a large core structure in the inner mitochondrial membrane. The FEBS journal. 2009;276(7):1900-14.
163. Mitchell P. The protonmotive Q cycle: a general formulation. FEBS letters. 1975;59(2):137-9.
164. Basco LK, Le Bras J. In vitro activity of mitochondrial ATP synthetase inhibitors against Plasmodium falciparum. The Journal of eukaryotic microbiology. 1994;41(3):179-83.
165. Sturm A, Mollard V, Cozijnsen A, Goodman CD, McFadden GI. Mitochondrial ATP synthase is dispensable in blood-stage Plasmodium berghei rodent malaria but essential in the mosquito phase. Proceedings of the National Academy of Sciences of the United States of America. 2015;112(33):10216-23.
166. Howells RE. Cytochrome oxidase activity in a normal and some drug-resistant strains of Plasmodium berghei--a cytochemical study. II. Sporogonic stages of a drug-sensitive srain. Annals of tropical medicine and parasitology. 1970;64(2):223-5.
167. Howells RE, Peters W, Fullard J. Cytochrome oxidase activity in a normal and some drug-resistant strains of Plasmodium berghei--a cytochemical study. I. Asexual erythrocytic stages. Military medicine. 1969;134(10):893-915.
168. Theakston RD, Howells RE, Fletcher KA, Peters W, Fullard J, Moore GA. The ultrastructural distribution of cytochrome oxidase activity in Plasmodium Berghei and P. Gallinaceum. Life sciences. 1969;8(10):521-9.

169. Scheibel LW, Pflaum WK. Cytochrome oxidase activity in platelet-free preparations of Plasmodium falciparum. The Journal of parasitology. 1970;56(6):1054.
170. Krungkrai J, Krungkrai SR, Bhumiratana A. Plasmodium berghei: partial purification and characterization of the mitochondrial cytochrome c oxidase. Experimental parasitology. 1993;77(2):136-46.
171. Krungkrai J, Burat D, Kudan S, Krungkrai S, Prapunwattana P. Mitochondrial oxygen consumption in asexual and sexual blood stages of the human malarial parasite, Plasmodium falciparum. The Southeast Asian journal of tropical medicine and public health. 1999;30(4):636-42.
172. Krungkrai J, Krungkrai SR, Suraveratum N, Prapunwattana P. Mitochondrial ubiquinol-cytochrome c reductase and cytochrome c oxidase: chemotherapeutic targets in malarial parasites. Biochemistry and molecular biology international. 1997;42(5):1007-14.
173. Burke PV, Poyton RO. Structure/function of oxygen-regulated isoforms in cytochrome c oxidase. The Journal of experimental biology. 1998;201(Pt 8):1163-75.
174. Tsukihara T, Aoyama H, Yamashita E, Tomizaki T, Yamaguchi H, Shinzawa-Itoh K, et al. The whole structure of the 13-subunit oxidized cytochrome c oxidase at 2.8 A. Science. 1996;272(5265):1136-44.
175. Mick DU, Fox TD, Rehling P. Inventory control: cytochrome c oxidase assembly regulates mitochondrial translation. Nature reviews Molecular cell biology. 2011;12(1):14-20.
176. Hell K, Tzagoloff A, Neupert W, Stuart RA. Identification of Cox20p, a novel protein involved in the maturation and assembly of cytochrome oxidase subunit 2. The Journal of biological chemistry. 2000;275(7):4571-8.
177. Williams SL, Valnot I, Rustin P, Taanman JW. Cytochrome c oxidase subassemblies in fibroblast cultures from patients carrying mutations in COX10, SCO1, or SURF1. The Journal of biological chemistry. 2004;279(9):7462-9.
178. Taanman JW, Capaldi RA. Subunit VIa of yeast cytochrome c oxidase is not necessary for assembly of the enzyme complex but modulates the enzyme activity. Isolation and characterization of the nuclear-coded gene. The Journal of biological chemistry. 1993;268(25):18754-61.
179. Massa V, Fernandez-Vizarra E, Alshahwan S, Bakhsh E, Goffrini P, Ferrero I, et al. Severe infantile encephalomyopathy caused by a mutation in COX6B1, a nucleus-encoded subunit of cytochrome c oxidase. American journal of human genetics. 2008;82(6):1281-9.
180. Shoubridge EA. Cytochrome c oxidase deficiency. American journal of medical genetics. 2001;106(1):46-52.
181. Robinson BH. Human cytochrome oxidase deficiency. Pediatric research. 2000;48(5):581-5.
182. Tzagoloff A, Capitanio N, Nobrega MP, Gatti D. Cytochrome oxidase assembly in yeast requires the product of COX11, a homolog of the P. denitrificans protein encoded by ORF3. The EMBO journal. 1990;9(9):2759-64.
183. Glerum DM, Shtanko A, Tzagoloff A. Characterization of COX17, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase. The Journal of biological chemistry. 1996;271(24):14504-9.
184. Papadopoulou LC, Sue CM, Davidson MM, Tanji K, Nishino I, Sadlock JE, et al. Fatal infantile cardioencephalomyopathy with COX deficiency and mutations in SCO2, a COX assembly gene. Nature genetics. 1999;23(3):333-7.

185. Leary SC, Cobine PA, Kaufman BA, Guercin GH, Mattman A, Palaty J, et al. The human cytochrome c oxidase assembly factors SCO1 and SCO2 have regulatory roles in the maintenance of cellular copper homeostasis. Cell metabolism. 2007;5(1):9-20.
186. Valnot I, von Kleist-Retzow JC, Barrientos A, Gorbatyuk M, Taanman JW, Mehaye B, et al. A mutation in the human heme A:farnesyltransferase gene (COX10) causes cytochrome c oxidase deficiency. Human molecular genetics. 2000;9(8):1245-9.
187. Antonicka H, Leary SC, Guercin GH, Agar JN, Horvath R, Kennaway NG, et al. Mutations in COX10 result in a defect in mitochondrial heme A biosynthesis and account for multiple, early-onset clinical phenotypes associated with isolated COX deficiency. Human molecular genetics. 2003;12(20):2693-702.
188. Diaz F, Thomas CK, Garcia S, Hernandez D, Moraes CT. Mice lacking COX10 in skeletal muscle recapitulate the phenotype of progressive mitochondrial myopathies associated with cytochrome c oxidase deficiency. Human molecular genetics. 2005;14(18):2737-48.
189. Tiranti V, Hoertnagel K, Carrozzo R, Galimberti C, Munaro M, Granatiero M, et al. Mutations of SURF-1 in Leigh disease associated with cytochrome c oxidase deficiency. American journal of human genetics. 1998;63(6):1609-21.
190. Zhu Z, Yao J, Johns T, Fu K, De Bie I, Macmillan C, et al. SURF1, encoding a factor involved in the biogenesis of cytochrome c oxidase, is mutated in Leigh syndrome. Nature genetics. 1998;20(4):337-43.
191. Leigh D. Subacute necrotizing encephalomyelopathy in an infant. Journal of neurology, neurosurgery, and psychiatry. 1951;14(3):216-21.
192. Mashkevich G, Repetto B, Glerum DM, Jin C, Tzagoloff A. SHY1, the yeast homolog of the mammalian SURF-1 gene, encodes a mitochondrial protein required for respiration. The Journal of biological chemistry. 1997;272(22):14356-64.
193. Poyau A, Buchet K, Godinot C. Sequence conservation from human to prokaryotes of Surf1, a protein involved in cytochrome c oxidase assembly, deficient in Leigh syndrome. FEBS letters. 1999;462(3):416-20.
194. Smith D, Gray J, Mitchell L, Antholine WE, Hosler JP. Assembly of cytochrome-c oxidase in the absence of assembly protein Surf1p leads to loss of the active site heme. The Journal of biological chemistry. 2005;280(18):17652-6.
195. Mick DU, Wagner K, van der Laan M, Frazier AE, Perschil I, Pawlas M, et al. Shy1 couples Cox1 translational regulation to cytochrome c oxidase assembly. The EMBO journal. 2007;26(20):4347-58.
196. Bundschuh FA, Hannappel A, Anderka O, Ludwig B. Surf1, associated with Leigh syndrome in humans, is a heme-binding protein in bacterial oxidase biogenesis. The Journal of biological chemistry. 2009;284(38):25735-41.
197. Pierrel F, Bestwick ML, Cobine PA, Khalimonchuk O, Cricco JA, Winge DR. Coa1 links the Mss51 post-translational function to Cox1 cofactor insertion in cytochrome c oxidase assembly. The EMBO journal. 2007;26(20):4335-46.
198. Khalimonchuk O, Bestwick M, Meunier B, Watts TC, Winge DR. Formation of the redox cofactor centers during Cox1 maturation in yeast cytochrome oxidase. Molecular and cellular biology. 2010;30(4):1004-17.
199. Nijtmans LG, Artal Sanz M, Bucko M, Farhoud MH, Feenstra M, Hakkaart GA, et al. Shy1p occurs in a high molecular weight complex and is required for efficient assembly of cytochrome c oxidase in yeast. FEBS letters. 2001;498(1):46-51.

200. Barrientos A, Korr D, Tzagoloff A. Shy1p is necessary for full expression of mitochondrial COX1 in the yeast model of Leigh's syndrome. The EMBO journal. 2002;21(1-2):43-52.
201. Mick DU, Vukotic M, Piechura H, Meyer HE, Warscheid B, Deckers M, et al. Coa3 and Cox14 are essential for negative feedback regulation of COX1 translation in mitochondria. The Journal of cell biology. 2010;191(1):141-54.
202. Bundschuh FA, Hoffmeier K, Ludwig B. Two variants of the assembly factor Surf1 target specific terminal oxidases in Paracoccus denitrificans. Biochimica et biophysica acta. 2008;1777(10):1336-43.
203. Khalimonchuk O, Bird A, Winge DR. Evidence for a pro-oxidant intermediate in the assembly of cytochrome oxidase. The Journal of biological chemistry. 2007;282(24):17442-9.
204. Browman DT, Hoegg MB, Robbins SM. The SPFH domain-containing proteins: more than lipid raft markers. Trends in cell biology. 2007;17(8):394-402.
205. McClung JK, Danner DB, Stewart DA, Smith JR, Schneider EL, Lumpkin CK, et al. Isolation of a cDNA that hybrid selects antiproliferative mRNA from rat liver. Biochemical and biophysical research communications. 1989;164(3):1316-22.
206. Terashima M, Kim KM, Adachi T, Nielsen PJ, Reth M, Kohler G, et al. The IgM antigen receptor of B lymphocytes is associated with prohibitin and a prohibitin-related protein. The EMBO journal. 1994;13(16):3782-92.
207. Montano MM, Ekena K, Delage-Mourroux R, Chang W, Martini P, Katzenellenbogen BS. An estrogen receptor-selective coregulator that potentiates the effectiveness of antiestrogens and represses the activity of estrogens. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(12):6947-52.
208. Thuaud F, Ribeiro N, Nebigil CG, Desaubry L. Prohibitin ligands in cell death and survival: mode of action and therapeutic potential. Chemistry & biology. 2013;20(3):316-31.
209. Wang S, Fusaro G, Padmanabhan J, Chellappan SP. Prohibitin co-localizes with Rb in the nucleus and recruits N-CoR and HDAC1 for transcriptional repression. Oncogene. 2002;21(55):8388-96.
210. Kurtev V, Margueron R, Kroboth K, Ogris E, Cavailles V, Seiser C. Transcriptional regulation by the repressor of estrogen receptor activity via recruitment of histone deacetylases. The Journal of biological chemistry. 2004;279(23):24834-43.
211. Rajalingam K, Wunder C, Brinkmann V, Churin Y, Hekman M, Sievers C, et al. Prohibitin is required for Ras-induced Raf-MEK-ERK activation and epithelial cell migration. Nature cell biology. 2005;7(8):837-43.
212. Fusaro G, Dasgupta P, Rastogi S, Joshi B, Chellappan S. Prohibitin induces the transcriptional activity of p53 and is exported from the nucleus upon apoptotic signaling. The Journal of biological chemistry. 2003;278(48):47853-61.
213. Kasashima K, Sumitani M, Satoh M, Endo H. Human prohibitin 1 maintains the organization and stability of the mitochondrial nucleoids. Experimental cell research. 2008;314(5):988-96.
214. Takata H, Matsunaga S, Morimoto A, Ma N, Kurihara D, Ono-Maniwa R, et al. PHB2 protects sister-chromatid cohesion in mitosis. Current biology : CB. 2007;17(15):1356-61.
215. Artal-Sanz M, Tsang WY, Willems EM, Grivell LA, Lemire BD, van der Spek H, et al. The mitochondrial prohibitin complex is essential for embryonic

viability and germline function in Caenorhabditis elegans. The Journal of biological chemistry. 2003;278(34):32091-9.
216. Nijtmans LG, de Jong L, Artal Sanz M, Coates PJ, Berden JA, Back JW, et al. Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins. The EMBO journal. 2000;19(11):2444-51.
217. Tatsuta T, Model K, Langer T. Formation of membrane-bound ring complexes by prohibitins in mitochondria. Molecular biology of the cell. 2005;16(1):248-59.
218. Steglich G, Neupert W, Langer T. Prohibitins regulate membrane protein degradation by the m-AAA protease in mitochondria. Molecular and cellular biology. 1999;19(5):3435-42.
219. He B, Feng Q, Mukherjee A, Lonard DM, DeMayo FJ, Katzenellenbogen BS, et al. A repressive role for prohibitin in estrogen signaling. Molecular endocrinology. 2008;22(2):344-60.
220. Ross JA, Nagy ZS, Kirken RA. The PHB1/2 phosphocomplex is required for mitochondrial homeostasis and survival of human T cells. The Journal of biological chemistry. 2008;283(8):4699-713.
221. Berger KH, Yaffe MP. Prohibitin family members interact genetically with mitochondrial inheritance components in Saccharomyces cerevisiae. Molecular and cellular biology. 1998;18(7):4043-52.
222. Merkwirth C, Langer T. Prohibitin function within mitochondria: essential roles for cell proliferation and cristae morphogenesis. Biochimica et biophysica acta. 2009;1793(1):27-32.
223. Kasashima K, Ohta E, Kagawa Y, Endo H. Mitochondrial functions and estrogen receptor-dependent nuclear translocation of pleiotropic human prohibitin 2. The Journal of biological chemistry. 2006;281(47):36401-10.
224. Back JW, Sanz MA, De Jong L, De Koning LJ, Nijtmans LG, De Koster CG, et al. A structure for the yeast prohibitin complex: Structure prediction and evidence from chemical crosslinking and mass spectrometry. Protein science : a publication of the Protein Society. 2002;11(10):2471-8.
225. Coates PJ, Jamieson DJ, Smart K, Prescott AR, Hall PA. The prohibitin family of mitochondrial proteins regulate replicative lifespan. Current biology : CB. 1997;7(8):607-10.
226. Nijtmans LG, Artal SM, Grivell LA, Coates PJ. The mitochondrial PHB complex: roles in mitochondrial respiratory complex assembly, ageing and degenerative disease. Cellular and molecular life sciences : CMLS. 2002;59(1):143-55.
227. Merkwirth C, Dargazanli S, Tatsuta T, Geimer S, Lower B, Wunderlich FT, et al. Prohibitins control cell proliferation and apoptosis by regulating OPA1-dependent cristae morphogenesis in mitochondria. Genes & development. 2008;22(4):476-88.
228. Bogenhagen DF, Rousseau D, Burke S. The layered structure of human mitochondrial DNA nucleoids. The Journal of biological chemistry. 2008;283(6):3665-75.
229. Wang Y, Bogenhagen DF. Human mitochondrial DNA nucleoids are linked to protein folding machinery and metabolic enzymes at the mitochondrial inner membrane. The Journal of biological chemistry. 2006;281(35):25791-802.
230. Gertz M, Fischer F, Wolters D, Steegborn C. Activation of the lifespan regulator p66Shc through reversible disulfide bond formation. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(15):5705-9.

231. Strub GM, Paillard M, Liang J, Gomez L, Allegood JC, Hait NC, et al. Sphingosine-1-phosphate produced by sphingosine kinase 2 in mitochondria interacts with prohibitin 2 to regulate complex IV assembly and respiration. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2011;25(2):600-12.
232. Garin J, Diez R, Kieffer S, Dermine JF, Duclos S, Gagnon E, et al. The phagosome proteome: insight into phagosome functions. The Journal of cell biology. 2001;152(1):165-80.
233. Mishra S, Ande SR, Nyomba BL. The role of prohibitin in cell signaling. The FEBS journal. 2010;277(19):3937-46.
234. Hentze MW, Muckenthaler MU, Andrews NC. Balancing acts: molecular control of mammalian iron metabolism. Cell. 2004;117(3):285-97.
235. Ande SR, Mishra S. Nuclear coded mitochondrial protein prohibitin is an iron regulated iron binding protein. Mitochondrion. 2011;11(1):40-7.
236. Zhou TB, Qin YH, Lei FY, Huang WF, Drummen GP. Prohibitin is associated with antioxidative protection in hypoxia/reoxygenation-induced renal tubular epithelial cell injury. Scientific reports. 2013;3:3123.
237. Bayot A, Gareil M, Rogowska-Wrzesinska A, Roepstorff P, Friguet B, Bulteau AL. Identification of novel oxidized protein substrates and physiological partners of the mitochondrial ATP-dependent Lon-like protease Pim1. The Journal of biological chemistry. 2010;285(15):11445-57.
238. Anderson CJ, Kahl A, Qian L, Stepanova A, Starkov A, Manfredi G, et al. Prohibitin is a positive modulator of mitochondrial function in PC12 cells under oxidative stress. J Neurochem. 2018;146(3):235-50.
239. Merkwirth C, Martinelli P, Korwitz A, Morbin M, Bronneke HS, Jordan SD, et al. Loss of prohibitin membrane scaffolds impairs mitochondrial architecture and leads to tau hyperphosphorylation and neurodegeneration. PLoS genetics. 2012;8(11):e1003021.
240. Supale S, Thorel F, Merkwirth C, Gjinovci A, Herrera PL, Scorrano L, et al. Loss of prohibitin induces mitochondrial damages altering beta-cell function and survival and is responsible for gradual diabetes development. Diabetes. 2013;62(10):3488-99.
241. Schleicher M, Shepherd BR, Suarez Y, Fernandez-Hernando C, Yu J, Pan Y, et al. Prohibitin-1 maintains the angiogenic capacity of endothelial cells by regulating mitochondrial function and senescence. The Journal of cell biology. 2008;180(1):101-12.
242. Zheng H, Lu GM. Reduction of prohibitin expression contributes to left ventricular hypertrophy via enhancement of mitochondrial reactive oxygen species formation in spontaneous hypertensive rats. Free Radic Res. 2015;49(2):164-74.
243. Li L, Guo JD, Wang HD, Shi YM, Yuan YL, Hou SX. Prohibitin 1 gene delivery promotes functional recovery in rats with spinal cord injury. Neuroscience. 2015;286:27-36.
244. Liu X, Ren Z, Zhan R, Wang X, Wang X, Zhang Z, et al. Prohibitin protects against oxidative stress-induced cell injury in cultured neonatal cardiomyocyte. Cell Stress Chaperones. 2009;14(3):311-9.
245. Bourges I, Ramus C, Mousson de Camaret B, Beugnot R, Remacle C, Cardol P, et al. Structural organization of mitochondrial human complex I: role of the ND4 and ND5 mitochondria-encoded subunits and interaction with prohibitin. The Biochemical journal. 2004;383(Pt. 3):491-9.

246. He J, Cooper HM, Reyes A, Di Re M, Sembongi H, Litwin TR, et al. Mitochondrial nucleoid interacting proteins support mitochondrial protein synthesis. Nucleic acids research. 2012;40(13):6109-21.
247. Wegrzyn J, Potla R, Chwae YJ, Sepuri NB, Zhang Q, Koeck T, et al. Function of mitochondrial Stat3 in cellular respiration. Science. 2009;323(5915):793-7.
248. Szczepanek K, Chen Q, Larner AC, Lesnefsky EJ. Cytoprotection by the modulation of mitochondrial electron transport chain: the emerging role of mitochondrial STAT3. Mitochondrion. 2012;12(2):180-9.
249. Osman C, Wilmes C, Tatsuta T, Langer T. Prohibitins interact genetically with Atp23, a novel processing peptidase and chaperone for the F1Fo-ATP synthase. Molecular biology of the cell. 2007;18(2):627-35.
250. Jian C, Xu F, Hou T, Sun T, Li J, Cheng H, et al. Deficiency of PHB complex impairs respiratory supercomplex formation and activates mitochondrial flashes. Journal of cell science. 2017;130(15):2620-30.
251. Pernas L, Scorrano L. Mito-Morphosis: Mitochondrial Fusion, Fission, and Cristae Remodeling as Key Mediators of Cellular Function. Annu Rev Physiol. 2016;78:505-31.
252. Wang K, Liu CY, Zhang XJ, Feng C, Zhou LY, Zhao Y, et al. miR-361-regulated prohibitin inhibits mitochondrial fission and apoptosis and protects heart from ischemia injury. Cell Death Differ. 2015;22(6):1058-68.
253. Signorile A, Santeramo A, Tamma G, Pellegrino T, D'Oria S, Lattanzio P, et al. Mitochondrial cAMP prevents apoptosis modulating Sirt3 protein level and OPA1 processing in cardiac myoblast cells. Biochim Biophys Acta Mol Cell Res. 2017;1864(2):355-66.
254. Anand R, Wai T, Baker MJ, Kladt N, Schauss AC, Rugarli E, et al. The i-AAA protease YME1L and OMA1 cleave OPA1 to balance mitochondrial fusion and fission. The Journal of cell biology. 2014;204(6):919-29.
255. Rainbolt TK, Lebeau J, Puchades C, Wiseman RL. Reciprocal Degradation of YME1L and OMA1 Adapts Mitochondrial Proteolytic Activity during Stress. Cell reports. 2016;14(9):2041-9.
256. Sato S, Murata A, Orihara T, Shirakawa T, Suenaga K, Kigoshi H, et al. Marine natural product aurilide activates the OPA1-mediated apoptosis by binding to prohibitin. Chemistry & biology. 2011;18(1):131-9.
257. Richter-Dennerlein R, Korwitz A, Haag M, Tatsuta T, Dargazanli S, Baker M, et al. DNAJC19, a mitochondrial cochaperone associated with cardiomyopathy, forms a complex with prohibitins to regulate cardiolipin remodeling. Cell metabolism. 2014;20(1):158-71.
258. Ban T, Ishihara T, Kohno H, Saita S, Ichimura A, Maenaka K, et al. Molecular basis of selective mitochondrial fusion by heterotypic action between OPA1 and cardiolipin. Nature cell biology. 2017;19(7):856-63.
259. Klecker T, Wemmer M, Haag M, Weig A, Bockler S, Langer T, et al. Interaction of MDM33 with mitochondrial inner membrane homeostasis pathways in yeast. Scientific reports. 2015;5:18344.
260. Signorile A, Sgaramella G, Bellomo F, De Rasmo D. Prohibitins: A Critical Role in Mitochondrial Functions and Implication in Diseases. Cells. 2019;8(1).
261. Seeber F, Limenitakis J, Soldati-Favre D. Apicomplexan mitochondrial metabolism: a story of gains, losses and retentions. Trends in parasitology. 2008;24(10):468-78.

262. Langhorst MF, Reuter A, Stuermer CA. Scaffolding microdomains and beyond: the function of reggie/flotillin proteins. Cellular and molecular life sciences : CMLS. 2005;62(19-20):2228-40.
263. Tyc J, Faktorova D, Kriegova E, Jirku M, Vavrova Z, Maslov DA, et al. Probing for primary functions of prohibitin in Trypanosoma brucei. International journal for parasitology. 2010;40(1):73-83.
264. Cruz-Bustos T, Ibarrola-Vannucci AK, Diaz-Lozano I, Ramirez JL, Osuna A. Characterization and functionality of two members of the SPFH protein superfamily, prohibitin 1 and 2 in Leishmania major. Parasites & vectors. 2018;11(1):622.
265. Jain R, Ghoshal A, Mandal C, Shaha C. Leishmania cell surface prohibitin: role in host-parasite interaction. Cellular microbiology. 2010;12(4):432-52.
266. Matz JM, Goosmann C, Matuschewski K, Kooij TWA. An Unusual Prohibitin Regulates Malaria Parasite Mitochondrial Membrane Potential. Cell reports. 2018;23(3):756-67.
267. Philip N, Waters AP. Conditional Degradation of Plasmodium Calcineurin Reveals Functions in Parasite Colonization of both Host and Vector. Cell host & microbe. 2015;18(1):122-31.
268. Zhao Y, Wang F, Wang C, Zhang X, Jiang C, Ding F, et al. Optimization of CRISPR/Cas System for Improving Genome Editing Efficiency in Plasmodium falciparum. Frontiers in microbiology. 2020;11:625862.
269. Balu B. Moving “Forward” in Plasmodium Genetics through a Transposon-Based Approach. Journal of Tropical Medicine. 2012;2012:829210.
270. Deitsch K, Driskill C, Wellems T. Transformation of malaria parasites by the spontaneous uptake and expression of DNA from human erythrocytes. Nucleic acids research. 2001;29(3):850-3.
271. Fidock DA, Wellems TE. Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(20):10931-6.
272. van Dijk MR, Janse CJ, Waters AP. Expression of a Plasmodium Gene Introduced into Subtelomeric Regions of Plasmodium berghei Chromosomes. Science. 1996;271(5249):662.
273. Spence PJ, Cunningham D, Jarra W, Lawton J, Langhorne J, Thompson J. Transformation of the rodent malaria parasite Plasmodium chabaudi. Nature protocols. 2011;6(4):553-61.
274. Kocken CH, van der Wel A, Thomas AW. Plasmodium cynomolgi: transfection of blood-stage parasites using heterologous DNA constructs. Experimental parasitology. 1999;93(1):58-60.
275. van der Wel AM, Tomas AM, Kocken CH, Malhotra P, Janse CJ, Waters AP, et al. Transfection of the primate malaria parasite Plasmodium knowlesi using entirely heterologous constructs. J Exp Med. 1997;185(8):1499-503.
276. Kocken CH, Ozwara H, van der Wel A, Beetsma AL, Mwenda JM, Thomas AW. Plasmodium knowlesi provides a rapid in vitro and in vivo transfection system that enables double-crossover gene knockout studies. Infection and immunity. 2002;70(2):655-60.
277. Moon RW, Hall J, Rangkuti F, Ho YS, Almond N, Mitchell GH, et al. Adaptation of the genetically tractable malaria pathogen Plasmodium knowlesi to continuous culture in human erythrocytes. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(2):531-6.

278. van der Oost J, Westra ER, Jackson RN, Wiedenheft B. Unravelling the structural and mechanistic basis of CRISPR-Cas systems. Nature reviews Microbiology. 2014;12(7):479-92.
279. Wright AV, Nunez JK, Doudna JA. Biology and Applications of CRISPR Systems: Harnessing Nature's Toolbox for Genome Engineering. Cell. 2016;164(1-2):29-44.
280. Wagner JC, Goldfless SJ, Ganesan SM, Lee MC, Fidock DA, Niles JC. An integrated strategy for efficient vector construction and multi-gene expression in Plasmodium falciparum. Malaria journal. 2013;12:373.
281. MacPherson CR, Scherf A. Flexible guide-RNA design for CRISPR applications using Protospacer Workbench. Nature biotechnology. 2015;33(8):805-6.
282. Montague TG, Cruz JM, Gagnon JA, Church GM, Valen E. CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. Nucleic acids research. 2014;42(Web Server issue):W401-7.
283. Peng DT, R. EuPaGDT: a web tool tailored to design CRISPR guide RNAs for eukaryotic pathogens. Microb Genom. 2015;1(4):e000033.
284. Ribeiro JM, Garriga M, Potchen N, Crater AK, Gupta A, Ito D, et al. Guide RNA selection for CRISPR-Cas9 transfections in Plasmodium falciparum. International journal for parasitology. 2018;48(11):825-32.
285. Adli M. The CRISPR tool kit for genome editing and beyond. Nature communications. 2018;9(1):1911.
286. Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, et al. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell. 2015;163(3):759-71.
287. Terns MP. CRISPR-Based Technologies: Impact of RNA-Targeting Systems. Molecular cell. 2018;72(3):404-12.
288. Lee MCS, Lindner SE, Lopez-Rubio JJ, Llinas M. Cutting back malaria: CRISPR/Cas9 genome editing of Plasmodium. Brief Funct Genomics. 2019;18(5):281-9.
289. Kuang D, Qiao J, Li Z, Wang W, Xia H, Jiang L, et al. Tagging to endogenous genes of Plasmodium falciparum using CRISPR/Cas9. Parasites & vectors. 2017;10(1):595.
290. de Koning-Ward TF, Gilson PR, Crabb BS. Advances in molecular genetic systems in malaria. Nature reviews Microbiology. 2015;13(6):373-87.
291. Ng CL, Siciliano G, Lee MC, de Almeida MJ, Corey VC, Bopp SE, et al. CRISPR-Cas9-modified pfmdr1 protects Plasmodium falciparum asexual blood stages and gametocytes against a class of piperazine-containing compounds but potentiates artemisinin-based combination therapy partner drugs. Molecular microbiology. 2016;101(3):381-93.
292. White J, Dhingra SK, Deng X, El Mazouni F, Lee MCS, Afanador GA, et al. Identification and Mechanistic Understanding of Dihydroorotate Dehydrogenase Point Mutations in Plasmodium falciparum that Confer in Vitro Resistance to the Clinical Candidate DSM265. ACS infectious diseases. 2019;5(1):90-101.
293. Payungwoung T, Shinzawa N, Hino A, Nishi T, Murata Y, Yuda M, et al. CRISPR/Cas9 system in Plasmodium falciparum using the centromere plasmid. Parasitology international. 2018;67(5):605-8.
294. Jones LH. An industry perspective on drug target validation. Expert Opin Drug Discov. 2016;11(7):623-5.
295. Grindley ND, Whiteson KL, Rice PA. Mechanisms of site-specific recombination. Annual review of biochemistry. 2006;75:567-605.

296. Baum J, Papenfuss AT, Mair GR, Janse CJ, Vlachou D, Waters AP, et al. Molecular genetics and comparative genomics reveal RNAi is not functional in malaria parasites. Nucleic acids research. 2009;37(11):3788-98.
297. Batista FA, Gyau B, Vilacha JF, Bosch SS, Lunev S, Wrenger C, et al. New directions in antimalarial target validation. Expert Opin Drug Discov. 2020;15(2):189-202.
298. Ganesan SM, Falla A, Goldfless SJ, Nasamu AS, Niles JC. Synthetic RNA-protein modules integrated with native translation mechanisms to control gene expression in malaria parasites. Nature communications. 2016;7:10727.
299. Naik RS, Krishnegowda G, Gowda DC. Glucosamine inhibits inositol acylation of the glycosylphosphatidylinositol anchors in intraerythrocytic Plasmodium falciparum. The Journal of biological chemistry. 2003;278(3):2036-42.
300. Elsworth B, Matthews K, Nie CQ, Kalanon M, Charnaud SC, Sanders PR, et al. PTEX is an essential nexus for protein export in malaria parasites. Nature. 2014;511(7511):587-91.
301. Patel A, Perrin AJ, Flynn HR, Bisson C, Withers-Martinez C, Treeck M, et al. Cyclic AMP signalling controls key components of malaria parasite host cell invasion machinery. PLoS biology. 2019;17(5):e3000264.
302. Wang S, Sim TB, Kim YS, Chang YT. Tools for target identification and validation. Curr Opin Chem Biol. 2004;8(4):371-7.
303. Ospina-Villa JD, Lopez-Camarillo C, Castanon-Sanchez CA, Soto-Sanchez J, Ramirez-Moreno E, Marchat LA. Advances on Aptamers against Protozoan Parasites. Genes (Basel). 2018;9(12).
304. Belmont BJ, Niles JC. Engineering a direct and inducible protein-RNA interaction to regulate RNA biology. ACS Chem Biol. 2010;5(9):851-61.
305. Lakhin AV, Tarantul VZ, Gening LV. Aptamers: problems, solutions and prospects. Acta Naturae. 2013;5(4):34-43.
306. Boonyalai N, Collins CR, Hackett F, Withers-Martinez C, Blackman MJ. Essentiality of Plasmodium falciparum plasmepsin V. PloS one. 2018;13(12):e0207621.
307. Florentin A, Stephens DR, Brooks CF, Baptista RP, Muralidharan V. Plastid biogenesis in malaria parasites requires the interactions and catalytic activity of the Clp proteolytic system. Proceedings of the National Academy of Sciences of the United States of America. 2020;117(24):13719-29.
308. Florentin A, Cobb DW, Fishburn JD, Cipriano MJ, Kim PS, Fierro MA, et al. PfClpC Is an Essential Clp Chaperone Required for Plastid Integrity and Clp Protease Stability in Plasmodium falciparum. Cell reports. 2017;21(7):1746-56.
309. Polino AJ, Nasamu AS, Niles JC, Goldberg DE. Assessment of Biological Role and Insight into Druggability of the Plasmodium falciparum Protease Plasmepsin V. ACS infectious diseases. 2020;6(4):738-46.
310. Kudyba HM, Cobb DW, Vega-Rodriguez J, Muralidharan V. Some conditions apply: Systems for studying Plasmodium falciparum protein function. PLoS pathogens. 2021;17(4):e1009442.
311. Skrzypek MS, Hirschman J. Using the Saccharomyces Genome Database (SGD) for Analysis of Genomic Information. 2011;35(1):1.20.1-1..3.
312. Gabaldon T, Koonin EV. Functional and evolutionary implications of gene orthology. Nature reviews Genetics. 2013;14(5):360-6.
313. Douzery EJ, Snell EA, Bapteste E, Delsuc F, Philippe H. The timing of eukaryotic evolution: does a relaxed molecular clock reconcile proteins and

fossils? Proceedings of the National Academy of Sciences of the United States of America. 2004;101(43):15386-91.
314. Elledge SJ, Spottswood MR. A new human p34 protein kinase, CDK2, identified by complementation of a cdc28 mutation in Saccharomyces cerevisiae, is a homolog of Xenopus Eg1. The EMBO journal. 1991;10(9):2653-9.
315. Lee MG, Nurse P. Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2. Nature. 1987;327(6117):31-5.
316. Kachroo AH, Laurent JM, Yellman CM, Meyer AG, Wilke CO, Marcotte EM. Evolution. Systematic humanization of yeast genes reveals conserved functions and genetic modularity. Science. 2015;348(6237):921-5.
317. Sun S, Yang F, Tan G, Costanzo M, Oughtred R, Hirschman J, et al. An extended set of yeast-based functional assays accurately identifies human disease mutations. Genome research. 2016;26(5):670-80.
318. Fitch WM. Distinguishing homologous from analogous proteins. Syst Zool. 1970;19(2):99-113.
319. Fitch WM. Homology a personal view on some of the problems. Trends Genet. 2000;16(5):227-31.
320. Koonin EV. Obituary: Walter Fitch and the orthology paradigm. Brief Bioinform. 2011;12(5):377-8.
321. Dolinski K, Botstein D. Orthology and functional conservation in eukaryotes. Annual review of genetics. 2007;41:465-507.
322. Sonnhammer EL, Koonin EV. Orthology, paralogy and proposed classification for paralog subtypes. Trends Genet. 2002;18(12):619-20.
323. Kuzniar A, van Ham RC, Pongor S, Leunissen JA. The quest for orthologs: finding the corresponding gene across genomes. Trends Genet. 2008;24(11):539-51.
324. Kristensen DM, Wolf YI, Mushegian AR, Koonin EV. Computational methods for Gene Orthology inference. Brief Bioinform. 2011;12(5):379-91.
325. Nehrt NL, Clark WT, Radivojac P, Hahn MW. Testing the ortholog conjecture with comparative functional genomic data from mammals. PLoS Comput Biol. 2011;7(6):e1002073.
326. Sjolander K. Phylogenomic inference of protein molecular function: advances and challenges. Bioinformatics. 2004;20(2):170-9.
327. Petersen M, Meusemann K, Donath A, Dowling D, Liu S, Peters RS, et al. Orthograph: a versatile tool for mapping coding nucleotide sequences to clusters of orthologous genes. BMC bioinformatics. 2017;18(1):111.
328. Kim K, Kim W, Kim S. ReMark: an automatic program for clustering orthologs flexibly combining a Recursive and a Markov clustering algorithms. Bioinformatics. 2011;27(12):1731-3.
329. Petta I, Lievens S, Libert C, Tavernier J, De Bosscher K. Modulation of Protein-Protein Interactions for the Development of Novel Therapeutics. Mol Ther. 2016;24(4):707-18.
330. Johnston M. A model fungal gene regulatory mechanism: the GAL genes of Saccharomyces cerevisiae. Microbiol Rev. 1987;51(4):458-76.
331. Ma J, Ptashne M. Deletion analysis of GAL4 defines two transcriptional activating segments. Cell. 1987;48(5):847-53.
332. Keegan L, Gill G, Ptashne M. Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. Science. 1986;231(4739):699.

333. Fields S, Song O. A novel genetic system to detect protein-protein interactions. Nature. 1989;340(6230):245-6.
334. Lin JS, Lai EM. Protein-Protein Interactions: Yeast Two-Hybrid System. Methods in molecular biology. 2017;1615:177-87.
335. James P, Halladay J, Craig EA. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics. 1996;144(4):1425-36.
336. Yeast two hyrid System for protein-protein interaction Studies: Bio-Resource; 2014 [Available from: http://technologyinscience.blogspot.com/2014/07/yeast-two-hybrid-system-for-protein.html#.YLNKwqgzbIU.
337. Uetz P, Giot L, Cagney G, Mansfield TA, Judson RS, Knight JR, et al. A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. Nature. 2000;403(6770):623-7.
338. LaCount DJ, Vignali M, Chettier R, Phansalkar A, Bell R, Hesselberth JR, et al. A protein interaction network of the malaria parasite Plasmodium falciparum. Nature. 2005;438(7064):103-7.
339. Fiebitz A, Nyarsik L, Haendler B, Hu YH, Wagner F, Thamm S, et al. High-throughput mammalian two-hybrid screening for protein-protein interactions using transfected cell arrays. BMC Genomics. 2008;9:68.
340. Lievens S, Lemmens I, Tavernier J. Mammalian two-hybrids come of age. Trends in biochemical sciences. 2009;34(11):579-88.
341. Citerne HL, Luo D, Pennington RT, Coen E, Cronk QC. A phylogenomic investigation of CYCLOIDEA-like TCP genes in the Leguminosae. Plant Physiol. 2003;131(3):1042-53.
342. Gadelle D, Filee J, Buhler C, Forterre P. Phylogenomics of type II DNA topoisomerases. Bioessays. 2003;25(3):232-42.
343. Daubin V, Gouy M, Perriere G. A phylogenomic approach to bacterial phylogeny: evidence of a core of genes sharing a common history. Genome research. 2002;12(7):1080-90.
344. Sicheritz-Ponten T, Andersson SG. A phylogenomic approach to microbial evolution. Nucleic acids research. 2001;29(2):545-52.
345. Eisen JA, Nelson KE, Paulsen IT, Heidelberg JF, Wu M, Dodson RJ, et al. The complete genome sequence of Chlorobium tepidum TLS, a photosynthetic, anaerobic, green-sulfur bacterium. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(14):9509-14.
346. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, et al. The sequence of the human genome. Science. 2001;291(5507):1304-51.
347. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. Journal of molecular biology. 1990;215(3):403-10.
348. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic acids research. 1997;25(17):3389-402.
349. Thompson JD, Plewniak F, Poch O. A comprehensive comparison of multiple sequence alignment programs. Nucleic acids research. 1999;27(13):2682-90.
350. Higgins DG, Thompson JD, Gibson TJ. Using CLUSTAL for multiple sequence alignments. Methods in enzymology. 1996;266:383-402.
351. Katoh K, Misawa K, Kuma K, Miyata T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic acids research. 2002;30(14):3059-66.

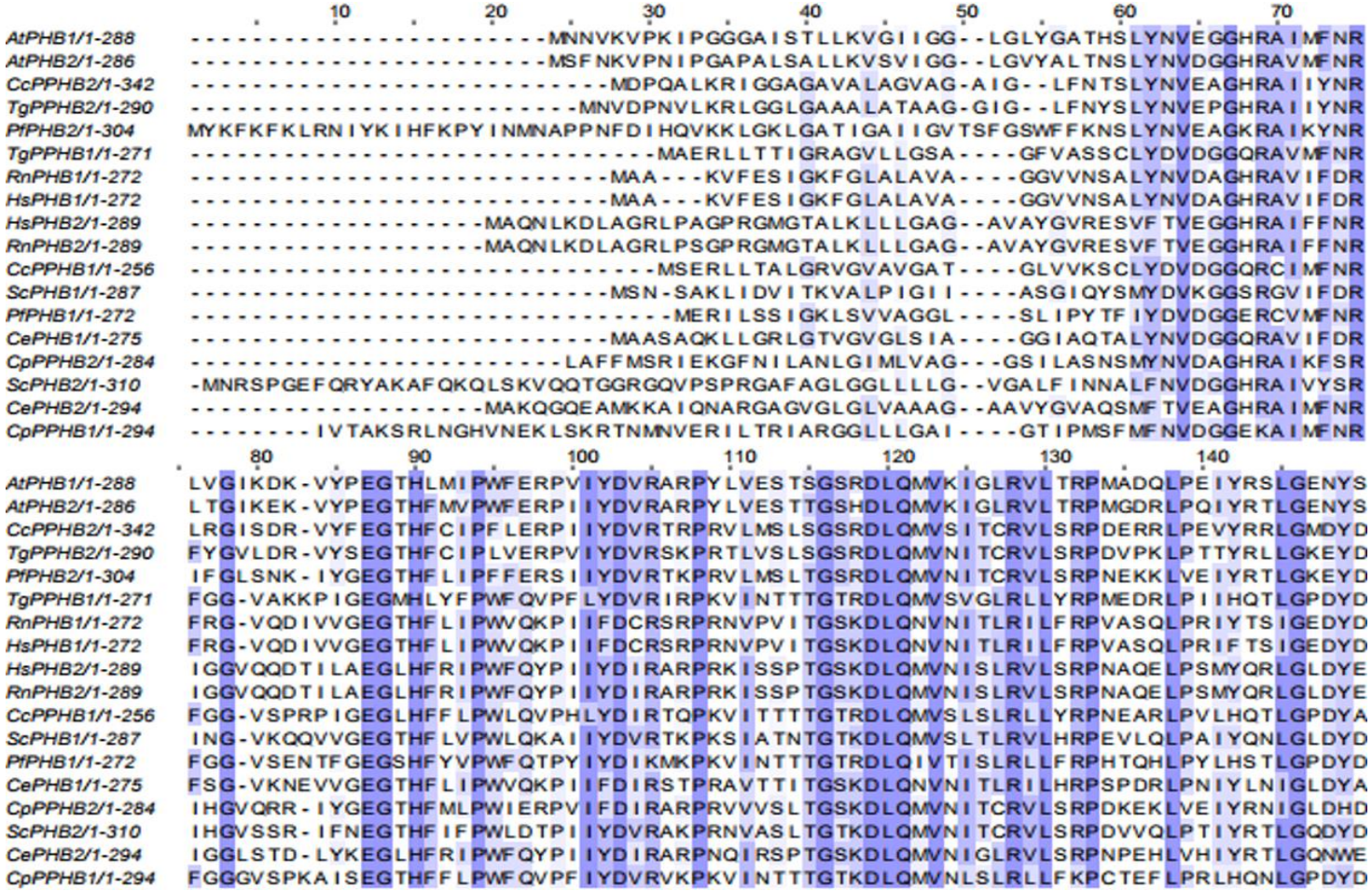
352. Notredame C, Higgins DG, Heringa J. T-Coffee: A novel method for fast and accurate multiple sequence alignment. Journal of molecular biology. 2000;302(1):205-17.
353. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular biology and evolution. 1987;4(4):406-25.
354. Tatusov RL, Galperin MY, Natale DA, Koonin EV. The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic acids research. 2000;28(1):33-6.
355. Natale DA, Galperin MY, Tatusov RL, Koonin EV. Using the COG database to improve gene recognition in complete genomes. Genetica. 2000;108(1):9-17.
356. Lo Conte L, Ailey B, Hubbard TJ, Brenner SE, Murzin AG, Chothia C. SCOP: a structural classification of proteins database. Nucleic acids research. 2000;28(1):257-9.
357. Bateman A, Birney E, Cerruti L, Durbin R, Etwiller L, Eddy SR, et al. The Pfam protein families database. Nucleic acids research. 2002;30(1):276-80.
358. Marchler-Bauer A, Derbyshire MK, Gonzales NR, Lu S, Chitsaz F, Geer LY, et al. CDD: NCBI's conserved domain database. Nucleic acids research. 2015;43(Database issue):D222-6.
359. Derbyshire MK, Lanczycki CJ, Bryant SH, Marchler-Bauer A. Annotation of functional sites with the Conserved Domain Database. Database. 2012;2012.
360. PlasmoDB. http://plasmodborg/plasmo/.
361. Aurrecoechea C, Brestelli J, Brunk BP, Dommer J, Fischer S, Gajria B, et al. PlasmoDB: a functional genomic database for malaria parasites. Nucleic acids research. 2009;37(Database issue):D539-43.
362. Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ. Jalview Version 2--a multiple sequence alignment editor and analysis workbench. Bioinformatics. 2009;25(9):1189-91.
363. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. Bioinformatics. 2007;23(21):2947-8.
364. Swiss Bioinformatics resource portal: Swiss Institute of Bioinformatics (SIB); [Available from: https://www.expasy.org/.
365. Letunic I, Bork P. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic acids research. 2016;44(W1):W242-5.
366. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ. The Phyre2 web portal for protein modeling, prediction and analysis. Nature protocols. 2015;10(6):845-58.
367. Geospiza, Inc.; Seattle, WA, USA [Available from: http://www.geospiza.com.
368. Ito H, Fukuda Y, Murata K, Kimura A. Transformation of intact yeast cells treated with alkali cations. Journal of bacteriology. 1983;153(1):163-8.
369. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227(5259):680-5.
370. Ismail HA. Thawing of glycerolyte-frozen parasites with NaCl. Methods in Malaria Research. 2013.
371. Trager W, Jensen JB. Human malaria parasites in continuous culture. Science. 1976;193(4254):673-5.

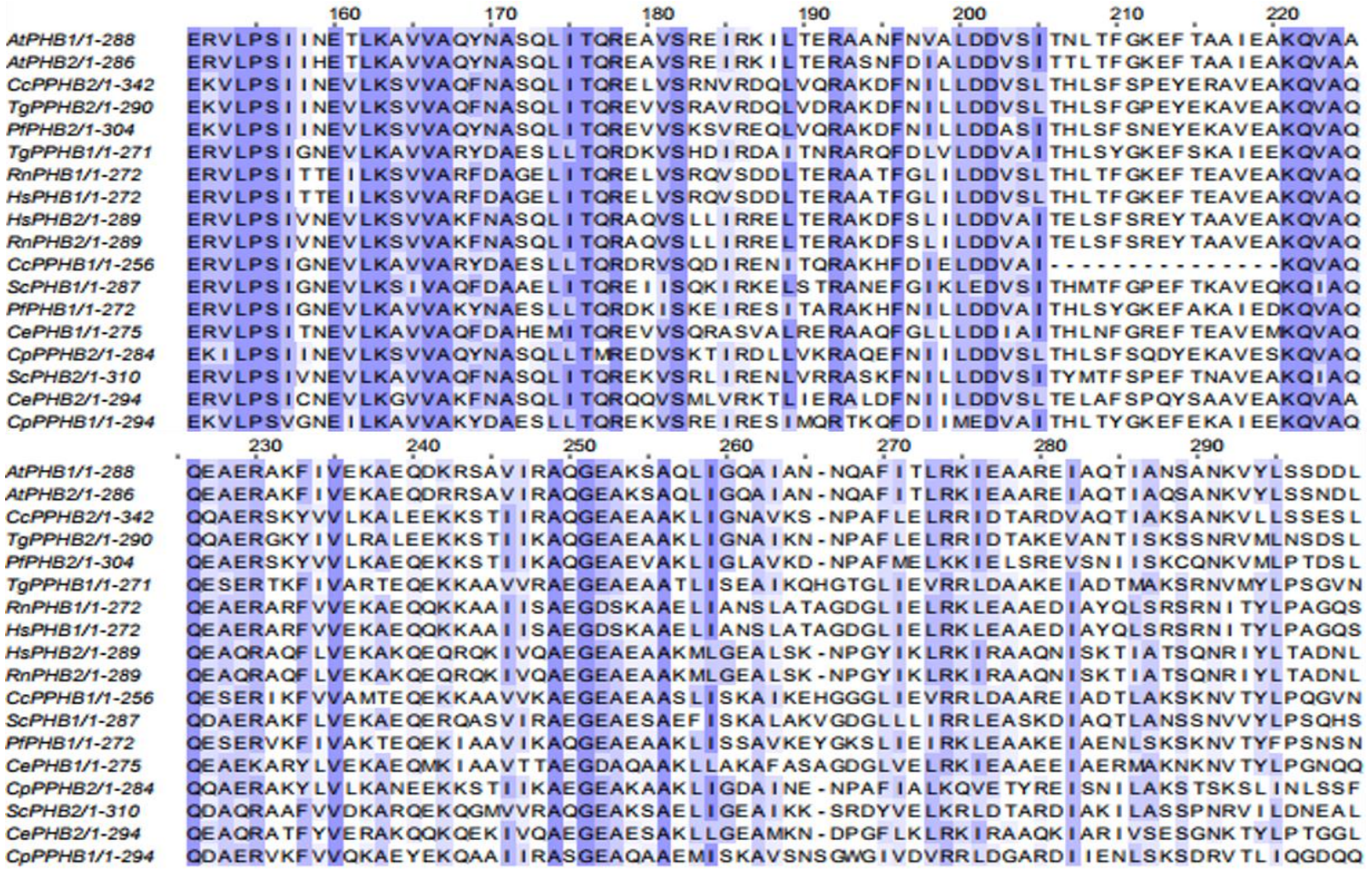
372. Schmidt BA. Giemsa staining of thick or thin blood films Manassas, VA, USA: MR4/ATCC,; 2013 [Available from: https://www.beiresources.org/portals/2/MR4/Methods_In_Malaria_Research-6th_edition.pdf.
373. Ismail HA. Freezing of patient isolates and strains with glycerolyte. Methods in Malaria Research. 2013.
374. Moll K. Sorbitol-synchronization of Plasmodium falciparum-infected erythrocytes 2013 [6th edition:[Available from: https://www.beiresources.org/portals/2/MR4/Methods_In_Malaria_Research-6th_edition.pdf.
375. Wu Y. Transfection of Plasmodium falciparum within human red blood cells MR4/ATCC, Manassas, VA, USA,: EVIMalaR Glasgow, UK, 2013; 2013 [Available from: https://www.beiresources.org/portals/2/MR4/Methods_In_Malaria_Research-6th_edition.pdf.
376. Mamoun CB, Gluzman IY, Goyard S, Beverley SM, Goldberg DE. A set of independent selectable markers for transfection of the human malaria parasite Plasmodium falciparum. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(15):8716-20.
377. Bestwick M, Jeong MY, Khalimonchuk O, Kim H, Winge DR. Analysis of Leigh syndrome mutations in the yeast SURF1 homolog reveals a new member of the cytochrome oxidase assembly factor family. Molecular and cellular biology. 2010;30(18):4480-91.
378. Hinderhofer M, Walker CA, Friemel A, Stuermer CA, Moller HM, Reuter A. Evolution of prokaryotic SPFH proteins. BMC Evol Biol. 2009;9:10.
379. Glebov OO, Bright NA, Nichols BJ. Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. Nature cell biology. 2006;8(1):46-54.
380. Kihara A, Akiyama Y, Ito K. A protease complex in the Escherichia coli plasma membrane: HflKC (HflA) forms a complex with FtsH (HflB), regulating its proteolytic activity against SecY. The EMBO journal. 1996;15(22):6122-31.
381. Robinson SW, Afzal AM, Leader DP. Chapter 13 - Bioinformatics: Concepts, Methods, and Data. In: Padmanabhan S, editor. Handbook of Pharmacogenomics and Stratified Medicine. San Diego: Academic Press; 2014. p. 259-87.
382. Desai SA. Insights Gained from P. falciparum Cultivation in Modified Media. The Scientific World Journal. 2013;2013:363505.
383. Bei AK, Patel SD, Volkman SK, Ahouidi AD, Ndiaye D, Mboup S, et al. An adjustable gas-mixing device to increase feasibility of in vitro culture of Plasmodium falciparum parasites in the field. PloS one. 2014;9(3):e90928.
384. Lambros C, Vanderberg JP. Synchronization of Plasmodium falciparum erythrocytic stages in culture. The Journal of parasitology. 1979;65(3):418-20.
385. Lu H, Zhou Q, He J, Jiang Z, Peng C, Tong R, et al. Recent advances in the development of protein-protein interactions modulators: mechanisms and clinical trials. Signal Transduct Target Ther. 2020;5(1):213.
386. Scott DE, Bayly AR, Abell C, Skidmore J. Small molecules, big targets: drug discovery faces the protein-protein interaction challenge. Nat Rev Drug Discov. 2016;15(8):533-50.
387. Wells JA, McClendon CL. Reaching for high-hanging fruit in drug discovery at protein-protein interfaces. Nature. 2007;450(7172):1001-9.
388. Schneiter R, editor Genetics, Molecular and Cell Biology of Yeast2004.

389. Hamza A, Tammpere E, Kofoed M, Keong C, Chiang J, Giaever G, et al. Complementation of Yeast Genes with Human Genes as an Experimental Platform for Functional Testing of Human Genetic Variants. Genetics. 2015;201(3):1263.
390. Lam SD, Das S, Sillitoe I, Orengo C. An overview of comparative modelling and resources dedicated to large-scale modelling of genome sequences. Acta Crystallogr D Struct Biol. 2017;73(Pt 8):628-40.
391. Yokoyama H, Suzuki K, Hara K, Matsui I, Hashimoto H. Inactive dimeric structure of the protease domain of stomatin operon partner protein. Acta Crystallogr D Struct Biol. 2020;76(Pt 6):515-20.
392. Yokoyama H, Matsui I. The lipid raft markers stomatin, prohibitin, flotillin, and HflK/C (SPFH)-domain proteins form an operon with NfeD proteins and function with apolar polyisoprenoid lipids. Crit Rev Microbiol. 2020;46(1):38-48.
393. Geppert T, Hoy B, Wessler S, Schneider G. Context-based identification of protein-protein interfaces and "hot-spot" residues. Chemistry & biology. 2011;18(3):344-53.
394. Janin J, Bahadur RP, Chakrabarti P. Protein-protein interaction and quaternary structure. Q Rev Biophys. 2008;41(2):133-80.
395. Moreira IS, Fernandes PA, Ramos MJ. Hot spots--a review of the protein-protein interface determinant amino-acid residues. Proteins. 2007;68(4):803-12.
396. Higurashi M, Ishida T, Kinoshita K. PiSite: a database of protein interaction sites using multiple binding states in the PDB. Nucleic acids research. 2009;37(Database issue):D360-4.

ANNEXURE

I. Multiple sequence alignment of PHB1 and PHB2




160 170 180 190 200 210 220
AtPHB1/1-288 ERVLPSIINETLKAVVAQYNASQLITQREAVSREIRKILTERAANFNVALDDVSITNLTFGKEFTAAIEAKQVAA
AtPHB2/1-286 ERVLPSIIHETLKAVVAQYNASQLITQREAVSREIRKILTERASNFDIALDDVSITTLTFGKEFTAAIEAKQVAA
CcPPHB2/1-342 EKVLPSIINEVLKSVVAQFNASQLITQRELVSRNVRDQLVQRAKDFNILLDDVSLTHLSFSPEYERAVEAKQVAQ
TgPPHB2/1-290 EKVLPSIINEVLKSVVAQFNASQLITQREVVSRAVRDQLVDRAKDFNILLDDVSLTHLSFGPEYEKAVEAKQVAQ
PfPHB2/1-304 EKVLPSIINEVLKSVVAQYNASQLITQREVVSKSVREQLVQRAKDFNILLDDASITHLSFSNEYEKAVEAKQVAQ
TgPPHB1/1-271 ERVLPSIGNEVLKAVVARYDAESLLTQRDKVSHDIRDAITNRARQFDLVLDDVAITHLSYGKEFSKAIEEKQVAQ
RnPHB1/1-272 ERVLPSITTEILKSVVARFDAGELITQRELVSRQVSDDLTERAATFGLILDDVSLTHLTFGKEFTEAVEAKQVAQ
HsPHB1/1-272 ERVLPSITTEILKSVVARFDAGELITQRELVSRQVSDDLTERAATFGLILDDVSLTHLTFGKEFTEAVEAKQVAQ
HsPHB2/1-289 ERVLPSIVNEVLKSVVAKFNASQLITQRAQVSLLIRRELTERAKDFSLILDDVAITELSFSREYTAAVEAKQVAQ
RnPHB2/1-289 ERVLPSIVNEVLKSVVAKFNASQLITQRAQVSLLIRRELTERAKDFSLILDDVAITELSFSREYTAAVEAKQVAQ
CcPPHB1/1-256 ERVLPSIGNEVLKAVVARYDAESLLTQRDRVSQDIRENITQRAKHFDIELDDVAI----------KQVAQ
ScPHB1/1-287 ERVLPSIGNEVLKSIVAQFDAAELITQREIISQKIRKELSTRANEFGIKLEDVSITHMTFGPEFTKAVEQKQIAQ
PfPHB1/1-272 ERVLPSIGNEVLKAVVAKYNAESLLTQRDKISKEIRESITARAKHFNILLDDVAITHLSYGKEFAKAIEDKQVAQ
CePHB1/1-275 ERVLPSITNEVLKAVVAQFDAHEMITQREVVSQRASVALRERAAQFGLLLDDIAITHLNFGREFTEAVEMKQVAQ
CpPPHB2/1-284 EKILPSIINEVLKSVVAQYNASQLLTMREDVSKTIRDLLVKRAQEFNIILDDVSLTHLSFSQDYEKAVESKQVAQ
ScPHB2/1-310 ERVLPSIVNEVLKAVVAQFNASQLITQREKVSRLIRENLVRRASKFNILLDDVSITYMTFSPEFTNAVEAKQIAQ
CePHB2/1-294 ERVLPSICNEVLKGVVAKFNASQLITQRQQVSMLVRKTLIERALDFNIILDDVSLTELAFSPQYSAAVEAKQVAA
CpPPHB1/1-294 EKVLPSVGNEILKAVVAKYDAESLLTQREKVSREIRESIMQRTKQFDIIMEDVAITHLTYGKEFEKAIEEKQVAQ
230 240 250 260 270 280 290
AtPHB1/1-288 QEAERAKFIVEKAEQDKRSAVIRAQGEAKSAQLIGQAIAN-NQAFITLRKIEAAREIAQTIANSANKVYLSSDDL
AtPHB2/1-286 QEAERAKFIVEKAEQDRRSAVIRAQGEAKSAQLIGQAIAN-NQAFITLRKIEAAREIAQTIAQSANKVYLSSNDL
CcPPHB2/1-342 QQAERSKYVVLKALEEKKSTIIRAQGEAEAAKLIGNAVKS-NPAFLELRRIDTARDVAQTIAKSANKVLLSSESL
TgPPHB2/1-290 QQAERGKYIVLRALEEKKSTIIKAQGEAEAAKLIGNAIKN-NPAFLELRRIDTAKEVANTISKSSNRVMLNSDSL
PfPHB2/1-304 QEAERSKYVVLKAEQEKKSTIIKAQGEAEVAKLIGLAVKD-NPAFMELKKIELSREVSNIISKCQNKVMLPTDSL
TgPPHB1/1-271 QESERTKFIVARTEQEKKAAVVRAEGEAEAATLISEAIKQHGTGLIEVRRLDAAKEIADTMAKSRNVMYLPSGVN
RnPHB1/1-272 QEAERARFVVEKAEQQKKAAIISAEGDSKAAELIANSLATAGDGLIELRKLEAAEDIAYQLSRSRNITYLPAGQS
HsPHB1/1-272 QEAERARFVVEKAEQQKKAAIISAEGDSKAAELIANSLATAGDGLIELRKLEAAEDIAYQLSRSRNITYLPAGQS
HsPHB2/1-289 QEAQRAQFLVEKAKQEQRQKIVQAEGEAEAAKMLGEALSK-NPGYIKLRKIRAAQNISKTIATSQNRIYLTADNL
RnPHB2/1-289 QEAQRAQFLVEKAKQEQRQKIVQAEGEAEAAKMLGEALSK-NPGYIKLRKIRAAQNISKTIATSQNRIYLTADNL
CcPPHB1/1-256 QESERIKFVVAMTEQEKKAAVVKAEGEAEAASLISKAIKEHGGGLIEVRRLDAAREIADTLAKSKNVTYLPQGVN
ScPHB1/1-287 QDAERAKFLVEKAEQERQASVIRAEGEAESAEFISKALAKVGDGLLLIRRLEASKDIAQTLANSSNVVYLPSQHS
PfPHB1/1-272 QESERVKFIVAKTEQEKIAAVIKAQGEAEAAKLISSAVKEYGKSLIEIRKLEAAKEIAENLSKSKNVTYFPSNSN
CePHB1/1-275 QEAEKARYLVEKAEQMKIAAVTTAEGDAQAAKLLAKAFASAGDGLVELRKIEAAEEIAERMAKNKNVTYLPGNQQ
CpPPHB2/1-284 QQAERAKYLVLKANEEKKSTIIKAEGEAKAAKLIGDAINE-NPAFIALKQVETYREISNILAKSTSKSLINLSSF
ScPHB2/1-310 QDAQRAAFVVDKARQEKQGMVVRAQGEAKSAELIGEAIKK-SRDYVELKRLDTARDIAKILASSPNRVILDNEAL
CePHB2/1-294 QEAQRATFYVERAKQQKQEKIVQAEGEAESAKLLGEAMKN-DPGFLKLRKIRAAQKIARIVSESGNKTYLPTGGL
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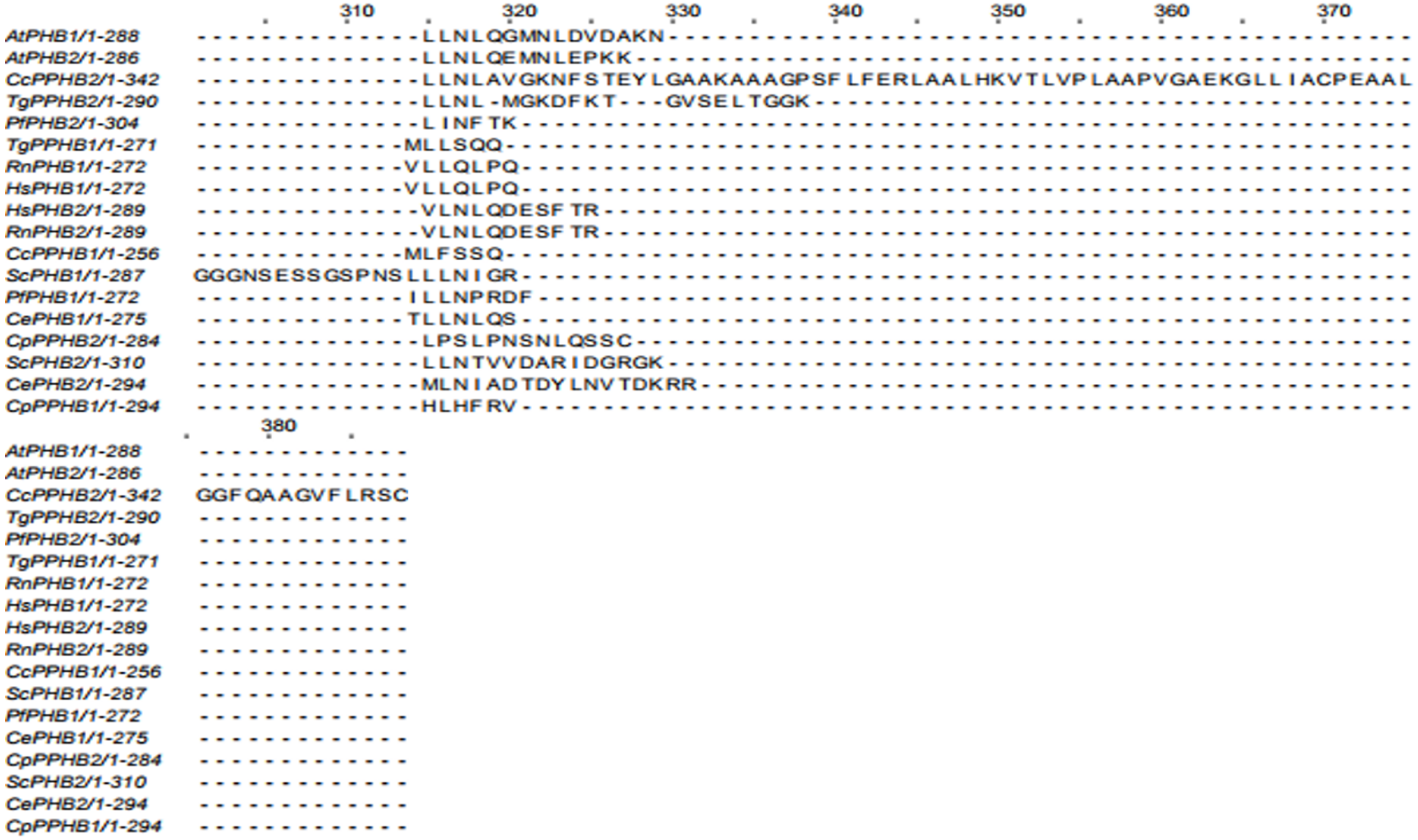
310 320 330 340 350 360 370
AtPHB1/1-288 ----------LLNLQGMNLDVDAKN----------
AtPHB2/1-286 ----------LLNLQEMNLEPKK----------
CcPPHB2/1-342 ----------LLNLAVGKNFSTEYLGAAKAAAGPSFLFERLAALHKVTLVPLAAPVGAEKGLLIACPEAAL
TgPPHB2/1-290 ----------LLNL-MGKDFKT---GVSELTGGK----------
PfPHB2/1-304 ----------LINFTK----------
TgPPHB1/1-271 ----------MLLSQQ----------
RnPHB1/1-272 ----------VLLQLPQ----------
HsPHB1/1-272 ----------VLLQLPQ----------
HsPHB2/1-289 ----------VLNLQDESFTR----------
RnPHB2/1-289 ----------VLNLQDESFTR----------
CcPPHB1/1-256 ----------MLFSSQ----------
ScPHB1/1-287 GGGNSESSGSPNSLLLNIGR----------
PfPHB1/1-272 ----------ILLNPRDF----------
CePHB1/1-275 ----------TLLNLQS----------
CpPPHB2/1-284 ----------LPSLPNSNLQSSC----------
ScPHB2/1-310 ----------LLNTVVDARIDGRGK----------
CePHB2/1-294 ----------MLNIADTDYLNVTDKRR----------
CpPPHB1/1-294 ----------HLHFRV----------
380
AtPHB1/1-288 ----------
AtPHB2/1-286 ----------
CcPPHB2/1-342 GGFQAAGVFLRSC
TgPPHB2/1-290 ----------
PfPHB2/1-304 ----------
TgPPHB1/1-271 ----------
RnPHB1/1-272 ----------
HsPHB1/1-272 ----------
HsPHB2/1-289 ----------
RnPHB2/1-289 ----------
CcPPHB1/1-256 ----------
ScPHB1/1-287 ----------
PfPHB1/1-272 ----------
CePHB1/1-275 ----------
CpPPHB2/1-284 ----------
ScPHB2/1-310 ----------
CePHB2/1-294 ----------
CpPPHB1/1-294 ----------

II. DST –Women Scientist Award

No.SR/WOS-A/LS-47/2014 (G)
Government of India
Ministry of Science & Technology
Department of Science & Technology

Technology Bhavan
New Mehrauli Road
New Delhi-110 016
Dated 20.01.2015

ORDER

Sub: Financial approval of the project under Women Scientist Scheme A (WOS-A) entitled "Characterizing the role of putative prohibitins in Plasmodium falciparum."

PI Ms C Savitha, Regional Medical Research Centre (ICMR), Nehru Nagar, Belgaum-590010, Karnataka.

Sanction of the President is hereby accorded to the above mentioned project at a total cost of ₹ **17,90,000/-** (Rupees Seventeen Lac Ninety Thousand only) for a duration of three years. The items of expenditure for which the total allocation of ₹ **17,90,000/- (recurring)** has been approved for three years are given below:

Sl. No.	Heads	1st Year	2nd Year	3rd Year	Total
A.	***Non-Recurring* (Capital Items)**				
	Equipments: Nil	----------	----------	----------	----------
B.	***Recurring*(General)**				
	Fellowship @ ₹ 20,000/-	2,40,000/-	2,40,000/-	2,40,000/-	7,20,000/-
	Consumables	3,00,000/-	3,00,000/-	2,00,000/-	8,00,000/-
	Contingencies	20,000/-	20,000/-	20,000/-	60,000/-
	Travel	20,000/-	20,000/-	20,000/-	60,000/-
C.	**Overhead**	50,000/-	50,000/-	50,000/-	1,50,000/-
D.	**Total of Recurring Grant (B+C)**	**6,30,000/-**	**6,30,000/-**	**5,30,000/-**	**17,90,000/-**
E.	**GRAND TOTAL (A+D)**	**6,30,000/-**	**6,30,000/-**	**5,30,000/-**	**17,90,000/-**

2. Overhead expenses are meant for the host institute towards the cost for providing infrastructure Facilities and benefits to the staff engaged in the project, etc.

3. Sanction of the grant is subject to the conditions as detailed in website www.online-wosa.gov.in

4. Sanction of the President is accorded to the payment of ₹ **6,30,000/-** (Rupees Six Lac Thirty Thousand only) as first installment of **recurring grant** as per following budget heads during the year 2014-2015:

Demand No.86 Department of Science & Technology
3425 Other Scientific Research (Major Head)
60 Others (Sub-Major Head)
60.200 Assistance to other Scientific Bodies (Minor Head)
55 Disha Programme for Women in Science
55.01 Disha Programme for Women in Science
55.01.31 **Grants-in-aid General** for the year 2014-2015 **(Plan Expenditure-General)**
This release is being made under the **Disha Programme for Women in Science**.

Contd..p/- 2

5. The Sanction has been issued under the powers delegated to the Ministries and with the concurrence of IF Division of Department of Science & Technology vide their Concurrence Diary Number C/**4366**/IFD/2014-15 dated 05.11.2014.

6. The institute will furnish to the DST, Utilization certificate and an audited statement of accounts pertaining to the grant immediately after the end of the each financial year. As this is the first grant being released for the project, no previous U/C is required.

7. The Institute will maintain separate audited accounts for the project; If it is found expedient to keep a part or whole of the grant in a bank account earning interest, the interest earned should be reported to DST. The interest thus earned will be treated as a credit to the institute to be adjusted towards further installment of the grant.

8. The amount of **₹ 6,30,000/-** (Rupees Six Lac Thirty Thousand only) as recurring grant will be Disbursed to the **Officer-in-Charge, Regional Medical Research Centre (ICMR), Nehru Nagar, Belgaum-590010, Karnataka** by means of electronic transfer as per the details given below:

Institute name	:	Regional Medical Research Centre, Belgaum.
Bank Name	:	Canara Bank.
Account No.	:	0505201010915
Branch	:	Khade Bazar, Belgaum.
IFSC code	:	CNRB0000505

9. As per Rule 211(1) of GFRs, the accounts of the project shall be open to inspection by the sanctioning authority/audit whenever the institute is called upon to do so.

10. There is no due pending UC from the Institute for this Scheme/Project.

11. This sanction has been entered SI. No……..in the Register of Grants (2014-15).

(HB SINGH)
Scientist-E

Copy forwarded for information and necessary action to :-

1. The Director of Audit (CW & M-II), AGCR Building, IP Estate, New Delhi-110 002.
2. Copy with two spare copies of the sanction to the Drawing & Disbursing Officer, DST, Cash Section.
3. The Officer-in-Charge, Regional Medical Research Centre (ICMR), Nehru Nagar, Belgaum-590010, Karnataka.
4. Dr. Praven Balabashkaram Nina, Regional Medical Research Centre (ICMR), Nehru Nagar, Belgaum-590010, Karnataka.
5. Ms C Savitha, Regional Medical Research Centre (ICMR), Nehru Nagar, Belgaum-590010, Karnataka.
6. Pay & Accounts Officer, DST, New Delhi
7. Accounts Section, DST, New Delhi
8. Head, SERC Division
9. Sanction Folder.
10. COA, DST, New Delhi
11. IFD DST, New Delhi
12. SERC Secretariat
13. FICCI Cell, DST

(HB SINGH)
Scientist-E

III. Institutional Biosafety

REGIONAL MEDICAL RESEARCH CENTRE
(Indian Council of Medical Research)
Nehru Nagar, Belgaum- 590 010
Institutional Biosafety Committee
No. BT/BS/17/609/2015-PID

RMRC/ICMR/BGM/15-16/158-IBSC/188 09-04-2015

CERTIFICATE

This is to certify that the project proposed by **Ms. Savitha C.** entitled **Characterizing the role of putative prohibitins in Plasmodium falciparum** has been approved by the IBSC, RMRC, Belgaum, which met on 09.04.2015.

The Investigator must report the outcome/ progress of the experiment to IBSC. Any change in the protocol/procedures should be brought to the notice of IBSC. This approval is valid up to one year from the date of approval or till the end of the proposed project, whichever is earlier.

Dr. Harsha Hegde
IBSC Members Secretary

Dr. Subarna Roy
for IBSC Chairperson

IV. PUBLICATIONS

Brief Communication

Functional Studies of *Plasmodium falciparum's* Prohibitin1 and Prohibitin 2 in Yeast

Savitha Chellappan[1], Subarna Roy[1], Jyoti M. Nagmoti[2], Wahida Tabassum[3], S. L. Hoti[1], Mrinal Kanti Bhattacharyya[3], Praveen Balabaskaran Nina[1,4]

[1]Indian Council of Medical Research - National Institute of Traditional Medicine, [2]Department of Microbiology, K.L.E. University, Belagavi, Karnataka, [3]Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad, Telangana, [4]Department of Epidemiology and Public Health, Central University of Tamil Nadu, Thiruvarur, Tamil Nadu, India

Abstract

Prohibitins (PHBs) are evolutionarily conserved mitochondrial integral membrane proteins, shown to regulate mitochondrial structure and function, and can be classified into PHB1 and PHB2. PHB1 and PHB2 have been shown to interact with each other, and form heterodimers in mitochondrial inner membrane. *Plasmodium falciparum* has orthologues of PHB1 and PHB2 in its genome, and their role is unclear. Here, by homology modelling and yeast two-hybrid analysis, we show that putative *Plasmodium* PHBs (P*f*PHB1 and P*f*PHB2) interact with each other, which suggests that they could form supercomplexes of heterodimers in *Plasmodium*, the functional form required for optimum mitochondrial function.

Keywords: Mitochondria, *Plasmodium falciparum*, prohibitins, supercomplex, yeast two-hybrid analysis

Introduction

Prohibitins (PHBs) cluster into PHB1 and PHB2, and these proteins share >50% similarity, and are evolutionarily conserved across all phyla.[1] PHBs, along with stomatin, flotillin and HflK/C, belong to the SPFH family.[2] Alternating blocks of PHB1 and PHB2 form high-molecular-weight complexes (~1.2 MDa) in the mitochondrial inner membrane.[3] PHBs are made up of an N-terminal transmembrane domain, a conserved PHB domain and a C-terminal coiled-coil domain through which the two PHBs interact with each other.[4] Even though PHBs are considered to be pleiotropic proteins with diverse cellular functions, their role in mitochondrial function has been of considerable interest.[1,5] In mitochondria, PHBs maintain the copy number and organisation of mitochondrial DNA, support mitochondrial protein synthesis, act as membrane-bound chaperones in assisting respiratory complex assembly, maintain structural integrity, regulate respiratory complex assembly and respiration and are thought to serve as protein and lipid scaffolds in mitochondria.[1,5]

Mitochondrial electron transport chain (mtETC) in *Plasmodium falciparum (Pf)* is an established drug target.[6] Atovaquone, an inhibitor of the bc1 complex of the mtETC, is already in clinical use, and drug discovery efforts have led to the advancement of ELQ-300, a mtETC inhibitor as a pre-clinical drug candidate.[7] Given the importance of mtETC as an attractive drug target, it is a high priority that we understand the structure and functional importance of the mitochondrial membrane proteins in *Plasmodium*. P*f* contains two PHBs: P*f*PHB1 and P*f*PHB2. In addition, stomatin-like protein and unusual PHB-like protein are also present in the *Plasmodium* genome. PHB1 and PHB2 of *Plasmodium berghei* (Pb) localise to the mitochondria, and are suggested to be essential.[8] Here, based on homology modelling, and yeast two-hybrid studies, we show that P*f*PHB1 and P*f*PHB2 are structurally similar to other PHBs, and physically interact with each other, presumably to form heterodimers, required for regulating the structure and function of mitochondria.

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Received: 23-01-2020 **Revised:** 23-05-2020
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How to cite this article: Chellappan S, Roy S, Nagmoti JM, Tabassum W, Hoti SL, Bhattacharyya MK, *et al*. Functional studies of *Plasmodium falciparum's* prohibitin1 and prohibitin 2 in yeast. Indian J Med Microbiol 2020;38:213-5.

Methods

Homology modelling

Phyre2 (Protein Homology/Analog Y Recognition Engine)[9] server was used to predict the protein structure of PfPHB1 and P*f*PHB2 (http://www.sbg.bio.ic.ac.uk/phyre2). The output of Phyre2 was analysed using RasMol as shown in Figure 1. After Phyre2 prediction, P*f*PHB1 and P*f*PHB2 were modelled based on the crystal structure of a core stomatin domain (chain c) of c3bk6c from *Pyrococcus horikoshii*. The confidence rate of prediction is >99.9%, with 57% and 51% sequence coverage for P*f*PHB1 and P*f*PHB2, respectively.

Interacting sites in PfPHB1 and PfPHB2 modelled protein structures were determined in the Phyre2 web portal for protein modelling, prediction and analysis,[9] and the interacting residues are shown in red for P*f*PHB1 and P*f*PHB2 [Figure 1c and d]. Two continuous interface regions are seen in P*f*PHB1 and P*f*PHB2, and the sequences are FQTPYIY-IK/HLSYGK-A and FERSIIY-VR/HLSFSN-E, respectively. These amino acids could facilitate interaction with one another and with other proteins.

Yeast two-hybrid analysis

Yeast two-hybrid experiments were carried out to identify the interaction between P*f*PHB1 and P*f*PHB2. Yeast two-hybrid assay was done as described by James *et al*.[10] using the pGADC1 and pGBDUC1 plasmids encoding GAL4 activation domain and DNA-binding domain, respectively.

The constructs, pGADC1:P*f*PHB1 and pGBDUC1:P*f*PHB2, were transformed into PJ69-4A yeast cells using the lithium acetate method.[11] The cells transformed with empty pGADC1 and pGBDUC1 were used as negative control. Two-hybrid interactions were tested with the yeast strain PJ69-4A which has the ADE2 reporter gene. The transformed strains were grown in SC-Ura-Leu medium till log phase at 30°C. Tenfold serial dilutions were prepared starting with an equal optical density of 0.5 OD ml^{-1} and spotted on the control plate (Sc-Ura-Leu) and experimental plate (Sc-Ura-Leu-Ade) to test the strength of the protein–protein interaction. The plates were incubated at 30°C for 72 h. The colonies on the control plate were observed from the 2nd day of incubation. The colonies in the experimental plate (Sc-Ura-Leu-Ade) were observed after 3 days, and their growth was slow compared to that of the control plate (Sc-Ura-Leu). Growth was measured in liquid media, and the number of cells in the media was equalised before spotting. Transformation efficiency and expression of URA3 and LEU2 genes in the plasmids were assessed by the growth of colonies in the SC-Ura-Leu plates. Two random clones were taken to test the strength of protein–protein interaction, and both the clones responded equally. Both the clones grew till the third dilution (10^{-3}) on SC-Ura-Leu-Ade plates, which suggests a strong interaction between P*f*PHB1 and P*f*PHB2, as shown in Figure 1e.

Results and Discussion

PHB1 and PHB2 have been found to be integral membrane

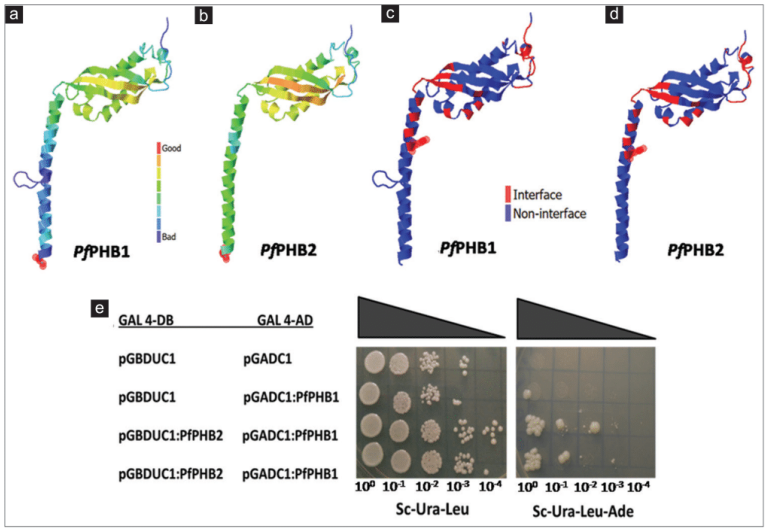

Figure 1: Phyre2 predicted structures of P*f*PHB1 (a) and P*f*PHB2 (b). The colour bars represent the quality of prediction. ProQ2 quality assessment algorithm was used to predict the local and global quality of the protein model. The quality (bad to good) of P*f*PHB1 and P*f*PHB2 models is represented by the colour scale (a and b). Protein–protein interface residues of PfPHB1 (c) and PfPHB2 (d) interface (red) and non-interface (blue) residues are represented in different colours. (e) Yeast two-hybrid analysis of P*f*PHB1 and P*f*PHB2. pGDBUC1 and pGADC1 represent empty plasmids. pGADC1:P*f*PHB1 represents full-length P*f*PHB1 in pGADC1 plasmid. pGDBUC1:P*f*PHB2 indicates full-length P*f*PHB2 in pGDBUC1 plasmid. The control plate: Sc-Ura-Leu and experimental plate: Sc-Ura-Leu-Ade shows spots with increasing order of dilution

proteins of the mitochondrial inner membrane.[3] PHB1 and PHB2 physically interact, and the PHB complex is considered to be the physiologically active form as loss of one subunit leads to the degradation of the other.[12] Yeast has been shown to be an excellent heterologous model system to study the function of *Plasmodium* proteins.[13] Homology modelling predicts the presence of putative interacting sites in P*f*PHBs. Yeast two-hybrid experiments show that P*f*PHB1 and P*f*PHB2 strongly interact with each other. These experiments strongly suggest that P*f*PHBs could make similar physiological interactions in the *Plasmodium* mitochondrion. Native page experiments on isolated *Plasmodium* mitochondria will help us identify the size of the PHB complex.

Knockdown of PHBs in other organisms has been shown to disrupt the mitochondrial morphology.[14,15] Processes regulating mitochondrial morphology and mitochondrial DNA organisation and maintaining copy number in *Plasmodium* are poorly understood, and PfPHBs may play an important role. Attempts to knockout PHB1 and PHB2 in Pb have been unsuccessful.[8] Whole-genome saturation screen in P*f* suggests that PHB2 is non-mutable in the coding sequence.[16] Overall, we provide an important mechanical insight into the functioning of P*f*PHB1 and P*f*PHB2 by showing that they physically interact with each other. Future studies should focus on understanding the role of PHB1 and PHB2 in Pf biology.

Acknowledgement for financial support

We thank Department of Science and Technology (DST) for financial support.

Financial support and sponsorship

This study was financially supported by DST through DSTWOS A funds to Ms.Savitha Chellappan and Inspire faculty research funds to Dr. Praveen Balabaskaran Nina.

Conflicts of interest

There are no conflicts of interest.

References

1. Osman C, Merkwirth C, Langer T. Prohibitins and the functional compartmentalization of mitochondrial membranes. J Cell Sci 2009;122:3823-30.
2. Browman DT, Hoegg MB, Robbins SM. The SPFH domain-containing proteins: More than lipid raft markers. Trends Cell Biol 2007;17:394-402.
3. Tatsuta T, Model K, Langer T. Formation of membrane-bound ring complexes by prohibitins in mitochondria. Mol Biol Cell 2005;16:248-59.
4. Winter A, Kämäräinen O, Hofmann A. Molecular modeling of prohibitin domains. Proteins 2007;68:353-62.
5. Thuaud F, Ribeiro N, Nebigil CG, Désaubry L. Prohibitin ligands in cell death and survival: Mode of action and therapeutic potential. Chem Biol 2013;20:316-31.
6. Sheiner L, Vaidya AB, McFadden GI. The metabolic roles of the endosymbiotic organelles of Toxoplasma and Plasmodium spp. Curr Opin Microbiol 2013;16:452-8.
7. Frueh L, Li Y, Mather MW, Li Q, Pou S, Nilsen A, *et al*. Alkoxycarbonate ester prodrugs of preclinical drug candidate ELQ-300 for prophylaxis and treatment of malaria. ACS Infect Dis 2017;3:728-35.
8. Matz JM, Goosmann C, Matuschewski K, Kooij TW. An unusual prohibitin regulates malaria parasite mitochondrial membrane potential. Cell Rep 2018;23:756-67.
9. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ. The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protoc 2015;10:845-58.
10. James P, Halladay J, Craig EA. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics 1996;144:1425-36.
11. Ito H, Fukuda Y, Murata K, Kimura A. Transformation of intact yeast cells treated with alkali cations. J Bacteriol 1983;153:163-8.
12. Coates PJ, Nenutil R, McGregor A, Picksley SM, Crouch DH, Hall PA, *et al*. Mammalian prohibitin proteins respond to mitochondrial stress and decrease during cellular senescence. Exp Cell Res 2001;265:262-73.
13. Bergman LW, Kaiser K, Fujioka H, Coppens I, Daly TM, Fox S, *et al*. Myosin A tail domain interacting protein (MTIP) localizes to the inner membrane complex of Plasmodium sporozoites. J Cell Sci 2003;116:39-49.
14. Artal-Sanz M, Tsang WY, Willems EM, Grivell LA, Lemire BD, van der Spek H, *et al*. The mitochondrial prohibitin complex is essential for embryonic viability and germline function in Caenorhabditis elegans. J Biol Chem 2003;278:32091-9.
15. Osman C, Haag M, Potting C, Rodenfels J, Dip PV, Wieland FT, *et al*. The genetic interactome of prohibitins: Coordinated control of cardiolipin and phosphatidylethanolamine by conserved regulators in mitochondria. J Cell Biol 2009;184:583-96.
16. Zhang M, Wang C, Otto TD, Oberstaller J, Liao X, Adapa SR, *et al*. Uncovering the essential genes of the human malaria parasite *Plasmodium falciparum* by saturation mutagenesis. Science 2018;360:eaap7847.

J Vector Borne Dis 57, December 2020, pp. 325–330

Functional studies of *Plasmodium falciparum* putative SURF1 in *Saccharomyces cerevisiae*

Savitha Chellappan[1], Subarna Roy[1], Jyoti M Nagmoti[2], Wahida Tabassum[3], Raja Vukanti[4], SL Hoti[1], Mrinal Kanti Bhattacharyya[3], Praveen Balabaskaran Nina[1, 5]

[1]*Indian Council of Medical Research-National Institute of Traditional Medicine, Belagavi, Karnataka, India;* [2]*Department of Microbiology, KLE Academy for higher education, Jawaharlal Nehru Medical college, Belagavi, Karnataka, India;* [3]*Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad, Telangana State, India;* [4]*Department of Microbiology, Central University of Tamil Nadu, Thiruvarur, Tamil Nadu, India;* [5]*Department of Epidemiology and Public Health, Central University of Tamil Nadu, Thiruvarur, Tamil Nadu, India*

ABSTRACT

Background and objectives: The mitochondrial electron transport chain (mtETC) of *Plasmodium falciparum* is an important drug target. Identification and functional validation of putative mitochondrial proteins of the mtETC is critical for drug development. Many of the regulatory subunits and assembly factors of cytochrome c oxidase readily identifiable in humans and yeast are missing in *P. falciparum*. Here, we describe our efforts to identify and validate the function of putative *Pfsurf1*, a key assembly factor of complex IV of the mtETC.

Methods: Multiple sequence alignment of SURF 1/Shy 1 was carried out in Clustal X 2.1. Phylogenetic tree was constructed using "Draw tree" option in Clustal X, and was analyzed using interactive Tree of Life software. To identify the conserved sequences, domain search was done using Jalview version 2.8.2 (BLOSUM 62 scoring). The haploid *Saccharomyces cerevisiae* strain (BY4741) containing the null allele *shy1* (Orf: YGR112w) (*shy1*::*Kan*) was complemented with putative *Pfsurf1* to study its ability to rescue the growth defect.

Results: Similarity searches of *Pf*SURF1-like protein in the Pfam shows statistically significant E = 4.7e-10 match to SURF1 family. Sequence alignment of *Pf*SURF1 with other SURF1-like proteins reveals the conservation of transmembrane domains, α-helices and β-pleated sheets. Phylogenetic analysis clusters putative *Pf*SURF1 with apicomplexan SURF1-like proteins. Yeast complementation studies show that *Pfsurf1* can partially rescue the yeast *shy1* mutant, YGR112w.

Interpretation & conclusion: Bioinformatics and complementation studies in yeast show that *P. falciparum*'s SURF1 is the functional ortholog of human SURF1 and yeast Shy1.

Key words *Plasmodium falciparum*, SURF1, Yeast complementation

INTRODUCTION

Cytochrome c oxidase (COX) or complex IV is a multisubunit catalytic enzyme of the electron transport chain that participates in energy conservation under aerobic conditions. COX is located in the inner mitochondrial membrane and is made up of 14 structural subunits in human and 11 in yeast[1-3]. The core subunits: COX1, COX2 and COX3 are mitochondrially encoded and are highly conserved across organisms, while the rest of the subunits are encoded in the nucleus and are imported from the cytosol[4]. COX1 and COX2 are mechanistically important subunits that contain CuB (two haem molecules and a copper ion) and CuA (two copper ions) sites respectively; these sites along with the haem a_3 molecule form the active centre of the enzyme[1].

COX being a multi-protein and multi-cofactor enzyme, requires many assembly factors to help in its assembly. In *Saccharomyces cerevisiae*, more than 25 assembly factors that help in Cox maturation have been described[4–6]. Human COX does not have all the yeast homologs, but more than 15 proteins involved in COX biogenesis have been identified[4, 7–8]. One of the well-studied assembly factors in *Saccharomyces cerevisiae* Cox is Shy1. Disruption of Shy1 leads to drastic reduction of Cox and mutants show growth defect in non-fermentable carbon sources[9]. Biochemical and genetic studies of Shy1 have helped identify several key proteins (Coa1- Coa4) involved in Cox assembly[10–14]. Surfeit locus protein1 (SURF1), the human homologue of Shy1, was used as the starting bait to identify other assembly factors such as MITRAC12, TIM21, MITRAC15 and CMC1[7]. Shy1 and its homologues have been suggested to play an important role in the maturation of Cox1 by direct incorporation of haem *a3* or by acting as a chaperone[12, 15–17]. Shy1 has been suggested to play a role in the release of Mss51 from Cox1

assembly intermediate[11–12, 15]. Mutations in SURF1 lead to COX deficiency and patients exhibit the phenotype of the Leigh syndrome[18–19].

The 6 kb mitochondrial genome of *Plasmodium* encodes just 3 proteins – Cytochrome *b*, COX1 and COX3[20]. Intriguingly, COX2 is split into COX2a and COX2b, and are coded in the nucleus[21]. In addition to these core subunits, *Plasmodium* database PlasmoDB (*http://www.plasmodb.org/*) has yeast and human orthologs of COX4, COX5B, COX6B and COX7A. Many of the putative assembly factors (COX10, COX11, COX14, COX15, COX17, COX19, SCO1, OXA1, PET117, PET191, and SURF1-domain containing protein) of COX have been annotated in PlasmoDB.

The role of assembly factors in *Plasmodium* COX biogenesis has not been investigated so far. This study details the bioinformatics analysis and functional complementation of putative *Pfsurf1* in a yeast *shyl* mutant.

MATERIAL AND METHODS

Multiple sequence alignment and domain analysis

The Shy1 and SURF1 protein sequences were retrieved from NCBI (*https://www.ncbi.nlm.nih.gov/protein*) and used in multiple sequence alignment and domain analysis. The protein sequences include: *Homo sapiens* SURF1 (Swiss-Prot: Q15526.1), *Drosophila melanogaster* Surfeit 1 (NCBI reference sequence: NP_524758), *Caenorhabditis elegans* SURF1-like protein (GenBank: CCD72250.1), *Monosiga brevicollis* MX1 predicted protein (GenBank: EDQ90553.1), *Saccharomyces cerevisiae* Shy1 (GenBank: KZV11341.1), *Schizosaccharomyces pombe* SURF-family protein Shy1 (predicted) (GenBank: CAB50922.1), *Arabidopsis thaliana* Surfeit locus 1 cytochrome c oxidase biogenesis protein (NCBI reference sequence: NP_566592.1), *Dictyostelium discoideum* SURF1 family protein (NCBI reference sequence: XP_644359.2), *Chlamydomonas reinhardtii* cytochrome c oxidase assembly protein (NCBI reference sequence: XP_001701449), *Babesia microti* SURF1 family (NCBI reference sequence: XP_012648526), *Toxoplasma gondii* SURF1 family protein (GenBank: KFH02210.1), *Trypanosoma brucei gambiense* DAL972 hypothetical protein, conserved (NCBI reference sequence: XP_011779678.1), *Plasmodium falciparum* conserved *Plasmodium* protein with unknown function (NCBI reference sequence: XP_001351864.1). Multiple sequence analysis was performed in Clustal X 2.1 using complete sequences from the selected prokaryotic and eukaryotic species[22]. Phylogenetic tree was constructed using "Draw tree" option in Clustal X using Neighbour-Joining algorithm with default settings (Gap opening:10, Gap extension:0.2, bootstrap number:1000), and the output PHYLIP file was analyzed using interactive Tree of Life (iTOL) software for scientific representation[23]. Domain search was done to identify the conserved sequences in the selected sequences using Jalview version 2.8.2 (BLOSUM 62 scoring)[24].

Plasmids

Full length gene of *Pfsurf1* (2127 bp) and *ScSHY1*(1167 bp) were cloned in the pBMFH plasmid between BamHI and SalI sites, and is under the control of the bi-directional GPD promoter. The plasmids were sequence verified before transformation. The pBMFH plasmid is a fusion plasmid containing the GPD promoter from pBEVY-T, and selection marker (HIS3) and tag from pESC-His plasmid (Stratagene, La Jolla, USA).

Yeast transformation and complementation studies

A haploid *Saccharomyces cerevisiae* strain (BY4741) containing the null allele *shy1* (Orf: YGR112w) (*shy1*::*Kan*) was purchased from European *Saccharomyces cerevisiae* Archive For Functional Analysis (EUROSCARF); referred to as YGR112w. YGR112w was transformed with either full length *Pfsurf1* or *ScSHY1* genes using lithium acetate procedure[25]. Transformants were selected by their growth in synthetic medium lacking histidine and containing glucose as carbon source. Approximately 10^5 yeast cells, and their subsequent 10 fold serial dilutions were spotted on yeast extract, peptone (YEP) plates containing either 2% glucose or 2% glycerol as carbon source. With glucose as carbon source, plates were incubated at 30 °C for three days and with glycerol as carbon source, plates were incubated at 30 °C for five days.

Fluctuation assay

To estimate the phenotypic complementation rate, fluctuation assay[26] was performed for YGR112w+ pBMFH*ScSHY1*, YGR112w+pBMFH*Pfsurf1* and YGR112w-pBMFH. A single clone of each transformant was grown overnight to saturation in a synthetic medium lacking histidine with glucose as carbon source. The cells were grown till uniform cell density was reached. Equal number of cells (~500 cells) were spread on YEP (2% glycerol) plates at 30 °C, and individual colonies were counted after 3–4 days of incubation, and their growth is shown as percentage survival. The percentage complementation of *Pf*SURF1 and *ScSHY1* in YGR112w was calculated by Rosche and Foster method[27].

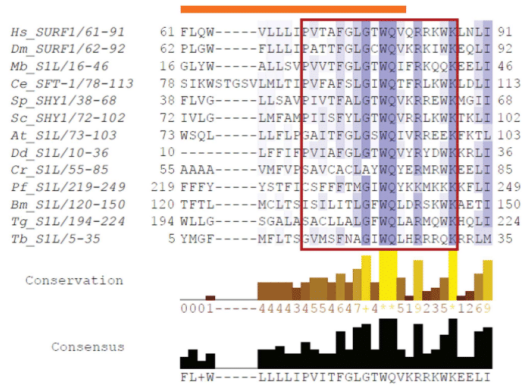

Fig. 1:(a) Bioinformatics analysis of *Pf*SURF1. Conserved domain in *SURF1/Shy1* proteins from different organisms using Jalview as listed below (numbers represent the amino acid residues of domains coverage in each organism). Conservation bars represents the degree of conservation in the amino acid residues in numbers.

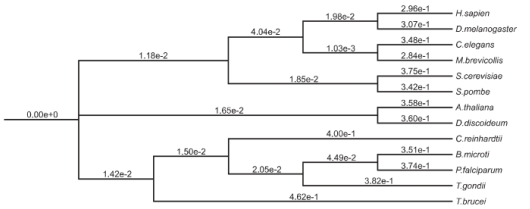

Fig. 1:(b) Phylogenetic analysis of putative PfSURF1. *H. sapien*: *Homo sapien*; *D. melanogaster*: *Drosophila melanogaster*, *C. elegans*: *Caenorhabditis elegans*; *M. brevicollis*: *Monosiga brevicollis*; *S. cerevisiae*: *Saccharomyces cerevisiae*; *S. pombe*: *Schizosaccharomyces pombe*; *A. thaliana*: *Arabidopsis thaliana*; *D. discoideum*: *Dictyostelium discoideum*; *C. reinhardtii*: *Chlamydomonas reinhardtii*; *B. microti*: *Babesia microti*; *P. falciparum*: *Plasmodium falciparum*; *T. gondii*: *Toxoplasma gondii*; *T. brucei*: *Trypanosoma brucei*. **Apicomplexans* are highlighted in grey.

RESULTS

*Multiple sequence alignment and phylogenetic analysis of Pf*SURF1

*Pf*SURF1 is listed as a putative SURF1 domain containing protein (PF3D7_0531000) in PlasmoDB, and has not been characterized yet. SURF1-like proteins are conserved and are seen in evolutionarily disparate groups such as mammals and bacteria. Multiple sequence alignment (Fig. 1a) shows that the putative *Pf*SURF1 shares both the conserved transmembrane domains with SURF1-like proteins from evolutionarily disparate spp. Fig. 1a shows the conservation of one of the transmembrane domains across all aligned SURF1-like proteins. The α-helices and β-sheets of *Pf*SURF1 align at a similar conserved region of other SURF-1 like proteins (Supplementary Fig. 1, Supplementary Table 1). Similarity searches of *Pf*SURF1-like protein in the Pfam shows statistically significant E = 4.7e-10 match to SURF1 family. In NCBI CD databases, it is a E = 8.82e-09 match to CDD cd06662 SURF1 superfamily. Phylogenetic analysis of putative *Pf*SURF1 indicates that it clusters with SURF-1 like proteins of other apicomplexans (Fig. 1b).

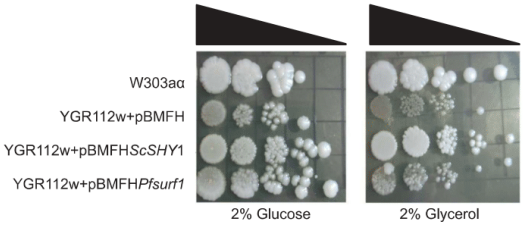

Fig. 2:(a) Growth of different yeast strains on YEP agar plates containing either 2% glucose or 2% glycerol. Changes in the amount of growth among different yeast strains after their spot inoculation with equal number of cells on YEP agar containing 2% glycerol. Different yeast strains were: wild type (W303a); YGR112w+pBMFH (mutant with Shy1 deletion and the empty plasmid [pBMFH]); YGR112w+pBMFH*ScShy1* (mutant complemented with *ScShy1*); YGR112w+pBMFH*Pfsurf1* (mutant complemented with *Pfsurf1*). *Sc = Saccharomyces cerevisiae. Pf = Plasmodium falciparum*.

Yeast complementation studies

To assess the extent of growth of the colony, wild type and transformed yeasts were uniformly spotted on YEP plates containing 2% glucose or 2% glycerol as carbon source. The inoculated plates were grown at three different temperatures-30 °C, 25 °C and 19 °C. As the growth of YGR112w with empty plasmid pBMFH was very slow at 25 °C and 19 °C (data not shown), growth was compared at 30 °C. With glucose as carbon sugar, growth (morphology of the colony) of wild type yeast (W303a), transformed yeasts (YGR112w+pBMFH, YGR112w+pBMFH*ScShy*1, and YGR112w+pBMFH*Pfsurf1*) were similar (Fig. 2a). With glycerol as carbon source, the growth of YGR112w+pBMFH is slower and lesser when compared to either W303a or YGR112w+pBMFH*ScShy*1 (Fig. 2a). However, the growth of YGR112w+pBMFH*Pfsurf1* is more than the growth of YGR112w+pBMFH and lesser than W303a. To better understand the growth phenotype, a fluctuation assay was performed. While 57±1% cells of YGR112w+pBMFH seeded cells

formed colonies, 68±0.7% and 68±0.1% cells, respectively of YGR112w+pBMFH*ScShy*1 and YGR112w+pBMFH*Pfsurf1*, survived and formed colonies (Fig. 2b). The number of survived cells were significantly higher for YGR112w+pBMFH*ScShy*1 and YGR112w+pBMFH*Pfsurf1*, when compared to those of YGR112w+pBMFH ($p < 0.001$). The difference in % survival between YGR112w+pBMFH*ScShy1* and YGR112w+pBMFH*Pfsurf1* was not significant.

DISCUSSION

The SURF1 proteins are evolutionarily conserved across eukaryotes and prokaryotes[28]. Sequence alignment shows that *Pf*SURF1 contains the two conserved hydrophobic transmembrane domains found in all SURF1 sequences. The α-helices and β-sheets of the putative *Pf*SURF1 align at the similar predicted location of SURF1-like proteins. Phylogenetic analysis clusters putative *Pf*SURF1 with apicomplexan SURF1-like proteins. Bioinformatics analysis strongly suggest that the *Pf*SURF1 is the ortholog of human SURF1 and yeast Shy1.

Saccharomyces cerevisiae is an excellent model organism to understand the mechanism of cellular and biochemical maintenance of respiration. In yeast, even under aerobic conditions, ATPs are primarily generated by fermentation and glycolysis[29].Yeast cells grown on non-fermentable carbon sources like glycerol or ethanol require fully active and functional mitochondria for respiration[30]. The *shy1*Δ mutants are defective in oxidative capacity; their size is smaller and their doubling time is much longer than the wild type[31]. A similar slow growth rate and small colonies were observed when the *shy1*Δ mutant was grown in glycerol, and when complemented with *Pfsurf1* or *ScSHY1*, the growth rate and colony morphology could be partially restored to that of the wild type. The fluctuation assay showed a significant difference in the number of colonies between the YGR112w (*shy1*Δ) and YGR112w complemented with *Pfsurf1* or *ScSHY1*, strongly indicating a growth defect in the YGR112w mutant that is rescued by complementing with *Pfsurf1* or *ScSHY1*. Rescue of YGR112w was more efficient with *ScSHY1* when compared to *Pfsurf1*, and this is not surprising as the tertiary structure of *Pf*SURF1 may not be the same as that of Shy1, and the slight differences in conformation could affect protein function.

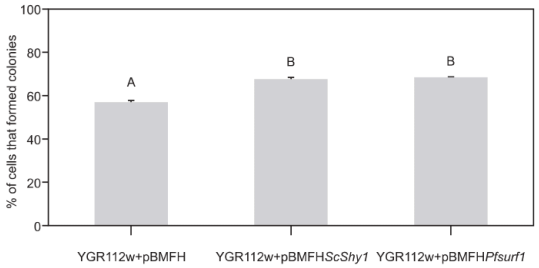

Fig. 2:(b) Fluctuation assay was done by inoculating 500 cells of three yeast strains (YGR112w+ pBMFH, YGR112w+ pBMFH*ScShy1*, and YGR112w+ pBMFH*Pfsurf1*) onto the YEP agar containing 2% glycerol and counting the colonies after 3 days at 30 °C. Values (% of cells that formed colonies) are means (n = 3) along with ±1 standard error of the mean. The bars connected by different letters are significantly different ($F = 85.55$; $P < 0.001$). The bars connected by the same letter are not statistically different (one-way ANOVA along with Tukey's post hoc tests for multiple comparisons; [SPSS v20; IBM Inc.]).

The minimalistic mitochondrion of *Plasmodium* is essential for the parasite survival, and hosts several pathways involved in the production of molecules essential for nucleic acid metabolism, DNA transcription, replication and other key processes[20, 32]. A recent saturation mutagenesis study in *P. falciparum* has shown that many proteins and assembly factors of the mtETC are essential for parasite survival[33]. The mutagenesis index score and mutant fitness score based on the saturation mutagenesis study are 0.12 and -3.156 respectively for *Pfsurf1*, indicating that it is highly essential for the parasite lifecycle[33]. Similarly, a CRISPR screen in the related apicomplexan *Toxoplasma gondii* shows the putative *Tgsurf1* to be indispensable for its life cycle[34]. The mitochondrial electron transport chain of *P. falciparum* is an established target, and several drug molecules targeting the mtETC are constantly being evaluated by the drug development programs[35]. Validation of the function of *Pfsurf1* is an important first step for future studies that could use *Pf*SURF1 as a bait to pull down Complex IV, as done in humans and yeast[12, 36], and characterize the novel subunits of *Pf*COX complex, some of them could be attractive drug targets.

CONCLUSION

Bioinformatics and complementation studies in yeast show that *P. falciparum*'s SURF1 is the functional ortholog of human SURF1 and yeast Shy1. In yeast and humans, using Shy1/SURF1, many of the assembly factors and assembly intermediates of Complex IV have been characterized. Efforts have been initiated to pull down the *Pf*COX complex using *Pf*SURF1 as the bait.

Conflict of Interest: None

ACKNOWLEDGEMENTS

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REFERENCES

1. Tsukihara T, Aoyama H, Yamashita E, Tomizaki T, Yamaguchi H, Shinzawa-Itoh K, *et al*. The whole structure of the 13-sub-unit oxidized cytochrome c oxidase at 2.8 A. *Science* 1996; *272*(5265): 1136–1144.
2. Balsa E, Marco R, Perales-Clemente E, Szklarczyk R, Calvo E, Landazuri MO, *et al*. NDUFA4 is a subunit of complex IV of the mammalian electron transport chain. *Cell Metab* 2012; *16*(3): 378–386.
3. Pitceathly RD, Rahman S, Wedatilake Y, Polke JM, Cirak S, Foley AR, *et al.* NDUFA4 mutations underlie dysfunction of a cytochrome c oxidase subunit linked to human neurological disease. *Cell Rep* 2013; *3*(6): 1795–1805.
4. Mick DU, Fox TD, Rehling P. Inventory control: cytochrome c oxidase assembly regulates mitochondrial translation. *Nat Rev Mol Cell Biol* 2011; *12*(1): 14–20.
5. Barrientos A, Gouget K, Horn D, Soto IC, Fontanesi F. Suppression mechanisms of COX assembly defects in yeast and human: insights into the COX assembly process. *Biochim Biophys Acta* 2009; *1793*(1): 97–107.
6. Kim HJ, Khalimonchuk O, Smith PM, Winge DR. Structure, function, and assembly of heme centers in mitochondrial respiratory complexes. *Biochim Biophys Acta* 2012; *1823*(9): 1604–1616.
7. Mick DU, Dennerlein S, Wiese H, Reinhold R, Pacheu-Grau D, Lorenzi I, *et al.* MITRAC links mitochondrial protein translocation to respiratory-chain assembly and translational regulation. *Cell* 2012; *151*(7): 1528– 1541.
8. Dennerlein S, Oeljeklaus S, Jans D, Hellwig C, Bareth B, Jakobs S, *et al.* MITRAC7 Acts as a COX1-Specific Chaperone and Reveals a Checkpoint during Cytochrome c Oxidase Assembly. *Cell Rep* 2015; *12*(10): 1644–1655.
9. Mashkevich G, Repetto B, Glerum DM, Jin C, Tzagoloff A. SHY1, the yeast homolog of the mammalian SURF-1 gene, encodes a mitochondrial protein required for respiration. *J Biol Chem* 1997; *272*(22): 14356–14364.
10. Pierrel F, Khalimonchuk O, Cobine PA, Bestwick M, Winge DR. Coa2 is an assembly factor for yeast cytochrome c oxidase biogenesis that facilitates the maturation of Cox1. *Mol Cell Biol* 2008; *28*(16): 4927–4939.
11. Pierrel F, Bestwick ML, Cobine PA, Khalimonchuk O, Cricco JA, Winge DR. Coa1 links the Mss51 post-translational function to Cox1 cofactor insertion in cytochrome c oxidase assembly. *EMBO J* 2007; *26*(20): 4335–4346.
12. Mick DU, Wagner K, van der Laan M, Frazier AE, Perschil I, Pawlas M, *et al*. Shy1 couples Cox1 translational regulation to cytochrome c oxidase assembly. *EMBO J* 2007; *26*(20): 4347–4358.
13. Bestwick M, Jeong MY, Khalimonchuk O, Kim H, Winge DR. Analysis of Leigh syndrome mutations in the yeast SURF1 homolog reveals a new member of the cytochrome oxidase assembly factor family. *Mol Cell Biol* 2010; *30*(18): 4480–4491.
14. Mick DU, Vukotic M, Piechura H, Meyer HE, Warscheid B, Deckers M, *et al*. Coa3 and Cox14 are essential for negative feedback regulation of COX1 translation in mitochondria. *J Cell Biol* 2010; *191*(1): 141–154.
15. Khalimonchuk O, Bestwick M, Meunier B, Watts TC, Winge DR. Formation of the redox cofactor centers during Cox1 maturation in yeast cytochrome oxidase. *Mol Cell Biol* 2010; *30*(4): 1004–1017.
16. Bundschuh FA, Hannappel A, Anderka O, Ludwig B. Surf1, associated with Leigh syndrome in humans, is a heme-binding protein in bacterial oxidase biogenesis. *J Biol Chem* 2009; *284*(38): 25735–25741.
17. Smith D, Gray J, Mitchell L, Antholine WE, Hosler JP. Assembly of cytochrome-c oxidase in the absence of assembly protein Surf1p leads to loss of the active site heme. *J Biol Chem* 2005; *280*(18): 17652–17656.
18. Zhu Z, Yao J, Johns T, Fu K, De Bie I, Macmillan C, *et al*. SURF1, encoding a factor involved in the biogenesis of cytochrome c oxidase, is mutated in Leigh syndrome. *Nat Genet* 1998; *20*(4): 337–343.
19. Tiranti V, Hoertnagel K, Carrozzo R, Galimberti C, Munaro M, Granatiero M, *et al*. Mutations of SURF-1 in Leigh disease associated with cytochrome c oxidase deficiency. *Am J Hum Genet* 1998; *63*(6): 1609–1621.
20. Vaidya AB, Mather MW. Mitochondrial evolution and functions in malaria parasites. *Annu Rev Microbiol* 2009; *63*: 249–267.
21. Funes S, Davidson E, Reyes-Prieto A, Magallon S, Herion P, King MP, *et al.* A green algal apicoplast ancestor. *Science* 2002; *298*(5601): 2155.
22. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, *et al*. Clustal W and Clustal X version 2.0. *Bioinformatics* 2007; *23*(21): 2947–2948.
23. Letunic I, Bork P. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Res* 2016; *44*(W1): W242–245.
24. Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ. Jalview Version 2–a multiple sequence alignment editor and analysis workbench. *Bioinformatics* 2009; *25*(9): 1189–1191.
25. Gietz RD, Woods RA. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol* 2002; *350*: 87–96.
26. Luria SE, Delbruck M. Mutations of Bacteria from Virus Sensitivity to Virus Resistance. *Genetics* 1943; *28*(6): 491–511.
27. Rosche WA, Foster PL. Determining mutation rates in bacterial populations. *Methods* 2000; *20*(1): 4–17.
28. Poyau A, Buchet K, Godinot C. Sequence conservation from human to prokaryotes of Surf1, a protein involved in cytochrome c oxidase assembly, deficient in Leigh syndrome. *FEBS Lett* 1999; *462*(3): 416–420.
29. Gancedo JM. Yeast carbon catabolite repression. *Microbiol Mol Biol Rev* 1998; *62*(2): 334–361.

30. Altmann K, Durr M, Westermann B. Saccharomyces cerevisiae as a model organism to study mitochondrial biology: general considerations and basic procedures. *Methods Mol Biol* 2007; *372*: 81–90.
31. Barrientos A, Korr D, Tzagoloff A. Shy1p is necessary for full expression of mitochondrial COX1 in the yeast model of Leigh's syndrome. *EMBO J* 2002; *21*(1-2): 43–52.
32. Sheiner L, Vaidya AB, McFadden GI. The metabolic roles of the endosymbiotic organelles of Toxoplasma and Plasmodium spp. *Curr Opin Microbiol* 2013; *16*(4): 452–458.
33. Zhang M, Wang C, Otto TD, Oberstaller J, Liao X, Adapa SR, *et al.* Uncovering the essential genes of the human malaria parasite Plasmodium falciparum by saturation mutagenesis. *Science* 2018; *360*(6388): eaap7847.
34. Sidik SM, Huet D, Ganesan SM, Huynh MH, Wang T, Nasamu AS, *et al*. A Genome-wide CRISPR Screen in Toxoplasma Identifies Essential Apicomplexan Genes. *Cell* 2016; *166*(6): 1423–35 e12.
35. Nixon GL, Pidathala C, Shone AE, Antoine T, Fisher N, O'Neill PM, *et al.* Targeting the mitochondrial electron transport chain of Plasmodium falciparum: new strategies towards the development of improved antimalarials for the elimination era. *Future Med Chem* 2013, *5*(13): 1573–1591.
36. Fontanesi F, Clemente P, Barrientos A. Cox25 teams up with Mss51, Ssc1, and Cox14 to regulate mitochondrial cytochrome c oxidase subunit 1 expression and assembly in Saccharomyces cerevisiae. *J Biol Chem* 2011, *286*(1): 555–566.

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V. Annexure

No. JITMM 2017 /SC327

12 October 2017

Mrs. Savitha Chellappan
Email: savithachellappan@gmail.com

Results of Travel Award (JITMM 2017)

Dear Mrs. Savitha Chellappan,

Congratulations, you have been selected to receive a travel award for JITMM2017!

The annual Joint International Tropical Medicine Meeting (JITMM) will be held 6 – 8 December 2017 at the Amari Watergate, in Bangkok. JITMM is an opportunity for leading researchers in the field of Tropical Medicine to share their findings and ideas and to build relationships with colleagues from around the world.

The Meeting theme this year is **"Tropical Medicine 4.0: Effective Collaboration for an Impact on Global Health"**. We chose this theme to highlight the need for researchers and practitioners to think and act innovatively in to order to solve Global Health problems. Many of the sessions this year will focus on the benefits of collaboration and inclusion and the importance of turning research into sustainable solutions. Through this 4.0 lens we will cover topics, breakthroughs, and updates that are relevant across the tropical world.

Based on your abstract the Travel Award and Scientific Committees have chosen you to give a poster presentation* (**Scientific Committee is currently reviewing your abstract whether you will give a poster presentation **and** a turbo talk. We will confirm thru email not later 31 October if you are also going to present for a turbo talk and include the instructions)*.

The rectangular poster-board surface area is 90 cm wide x 180 cm high. An appropriate poster size, therefore, is **85 cm wide x 150 cm high**. All poster presentations at the JITMM2017 will be considered for the Poster Presentation Award.

Please let us know if you will be attending JITMM2017 before October 20th 2017.

As a travel award winner you are entitled to –
1. Complimentary JITMM2017 registration
2. Reimbursement of your actual costs of airfare and 4 nights' accommodation costs up to **11,100 THB**

Name	Transportation (Economy-class airfare)	Accommodation	Total
Mrs. Savitha Chellappan	**Actual cost that not exceed your proposed of 6,800.00**	**Actual cost that not exceed your proposed of 4,300.00**	**Actual cost that not exceed your proposed of 11,100.00**

Please be sure to read the notes and instructions at the end of this letter.

JITMM2017

Joint International Tropical Medicine Meeting 2017
at Amari Watergate, Bangkok, Thailand, 6-8 December 2017

For more information about the event, please visit www.jitmm.com . If you have any questions about your travel award, feel free to get in touch by email.

We look forward to welcoming you to Bangkok this December.

Yours sincerely

Prof. Dr. Srivicha Krudsood
Chair, Scientific Committee Meeting, JITMM2017
Tel: +66 (0) 2354 9100-4, ext 1524 & 1525; Fax: +66 (0) 2306 9125
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Instructions for reimbursement:
1. On 7th December, go to the Registration Desk between 8.30am and 12pm.
2. Hand in the following documents –
- photocopy of your passport
- economy-class airfare receipt
- airfare ticket or e-ticket
- your boarding pass to Bangkok
- receipt for payment of your accommodation *(receipt must have your name on it, should you share a room with someone, your names should be on the receipt)*

3. You will receive reimbursement in Thai Baht up to your awarded amount.

Visas
- You may require a visa to enter Thailand
- The JITMM Secretariat will help you obtain a visa by providing a Letter of Invitation
- If you are not able to obtain a visa, you are no longer eligible for the Travel Award

ANNEXURE –VI

28th NATIONAL CONGRESS OF PARASITOLOGY

FEBRUARY 22nd - 24th, 2018

"Challenges and Innovations in Controlling Parasitic Diseases"

ICMR-NITM & KAHER Belagavi, Karnataka 590010, India

EMPOWERING PROFESSIONALS

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH Deemed-to-be-University

Certificate

This is to certify that

Ms. /Mr. /Dr. Savitha Chellappan *is awarded* ~~1st~~ / 2nd / ~~3rd~~ *prize for* ~~poster~~*/Oral Presentation at 28th 'National Congress of Parasitology' on the theme 'Challenges and Innovations in 'Controlling Parasitic Diseases' from February 22nd to 24th, 2018 held at ICMR- National Institute of Traditional Medicine & KLE Academy of Higher Education and Research, Belagavi, Karnataka.*

Prof. Sukhbir Kaur	Dr. J K Saxena	Dr. S L Hoti
President ISP	Secretary ISP	Convener

VII. Glossary

- **"omics"-** collection of analytical techniques used in systems biology that includes genomics, transcriptomics, proteomics, and metabolomics
- **Alpha helix and beta sheet** - secondary protein structures
- **Anopheles**-A genus of mosquitoes that includes all mosquitoes that transmit malaria to humans.
- **Artemisinins**- A class of drugs used for the treatment (not prevention) of malaria usually as a part of a combination therapy, derived from the sweet wormwood or Qinghao plant (Artemisia annua).
- **Atovaquone**- A drug used against malaria. It is found in the combination atovaquone-proguanil which can be used for both prevention and treatment.
- **Deletion**- A mutation involving the removal of one or more base pairs
- **DNA ligase**- An enzyme that catalyses a reaction that links two DNA molecules via the formation of a phospho-diester bond between the 3'hydroxyl and 5' phosphate of adjacent nucleotides.
- **Domain-** A segment of a protein that has a discrete function or conformation. At the protein level, a domain can be as small as a few amino acid residues or as large as half of the entire protein.
- **Drug resistance -**a heritable change allowing a pathogen to survive in the presence of a drug at concentrations that will normally kill it.
- **Electron transport chain -**series of protein complexes and small molecules used to transfer electrons between them to capture energy from sugars, via oxidative phosphorylation, or light, via photosynthesis.
- **Electrophoresis-** A technique that separates charged molecules – such as DNA, RNA or protein – on the basis of relative migration in an appropriate matrix
- **Electroporation-** An electrical treatment of cells that induces transient pores, through which DNA can enter the cell.
- **Genetic manipulation -** the modification of genomic sequences via the introduction of DNA sequences into cells, the results of which include permanent disruption of genes ("knock-out") and conditional disruption of genes/proteins ("knock-down")

- **Homology modelling-** the use of similar protein sequences with known structures in protein modelling
- **Homology** relation by descent, inferred by similarity. Often used synonymously with 'similarity'
- **Hydrophilic-** "water loving". Hydrophilic compounds dissolve easily in water, and are usually polar.
- **Hydrophobic-** "water fearing". Hydrophobic compounds do not dissolve easily in water, and are usually non-polar. In DNA sequence. Large deletions are visible as the lack of chromosomal segments.
- **Metabolomics -** the characterization of all small molecules ("metabolites") in a particular biological system
- **Molecular evolution-** the study of the evolution of DNA, RNA, or proteins
- **Multiple-sequence alignment-** an alignment of several sequences, often used in phylogenetics and protein modeling
- **Orthology-** homology where the sequences have deviated by speciation
- **Paralogy-** homology where the sequences have deviated by gene duplication
- **Parasitemia-** The presence of parasites in the blood. The term can also be used to express the quantity of parasites in the blood
- **PDB file-** a file listing coordinates for the atoms of a protein
- **Phylogenetics-** the identification of evolutionary relationships between organisms or between genes
- **Protein modelling-** the estimation of a protein's structure using its sequence
- **Proteomics -** the characterization of all proteins in a particular biological system
- **Protozoan-** Single-celled organism that can perform all necessary functions of metabolism and reproduction.
- **Putative gene -** a gene that has a predicted function
- **Quinine-** A drug used against malaria, obtained from the bark of the cinchona tree. Quinine is used for treatment but not prevention of malaria.
- **Resistance-** The ability of an organism to develop ways to be impervious to specific threats to their existence.

- **RNA interference (RNAi)-**RNAi is a biological process in which RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules.
- **Transcriptomics -** the characterization of all mRNA transcripts in a particular biological system
- **Transfection-** The transfer of DNA to a eukaryotic cell.
- **Transformant-** In prokaryotes, a cell that has been genetically altered through the uptake of foreign DNA.
- **Uncharacterized gene -** a gene with an annotation of "unknown function", sometimes referred to as a "hypothetical" gene
- **Vector-borne-** Transmitted from one host to another by a vector.