Details of the research work duly signed by the applicant, for which the Sun Pharma Science Foundation Research Award is claimed, including illustrations (not to exceed 6000 words)

The following are the details of the two major research work that I am submitting for Sun Pharma Science Foundation Research Award in Pharmaceutical Sciences under the category of **"New Drug Discovery and Development"**. All these data have been published in Nature Communications as two independent manuscripts (Chandana et al., 2022 and Ghosh et al., 2023). Both the work fit into the sub-categories of 'Design and development of new drugs' and 'Identification of new drug targets'.

1) Griseofulvin as an adjunct drug to prevent the mortality due to cerebral and severe malaria

Chandana et al. Malaria parasite heme biosynthesis promotes and griseofulvin protects against cerebral malaria in mice (2022). *Nature Communications*, *13*, 4028.

Before describing the scientific details, I would like to highlight the novelty of this work.

Background:

Heme synthesis in malaria parasite has remained enigmatic and as a topic of debate for more than two decades. The parasite pathway is dispensable for asexual stages, but essential for the development of sporozoites in mosquitoes and pre-erythrocytic stages in liver. Although non-essential and the blood stage knockout (KO) parasites can acquire heme/porphyrin intermediates/host enzymes for their survival, it is extremely surprising that the parasite synthesizes heme. Therefore, the mystery of why RBC-stage parasites expresse heme pathway enzymes and synthesize heme in RBC stages despite having numerous back-up mechanisms has remained as an unresolved long-standing question[1,2]. Further, malaria mortality occurs despite treating the infected individuals with artemisinin-based combination therapies (ACTs). The last resort is intravenous administration of artemisinin or quinine (antimalarials) with supportive therapies. The fatality rate due to cerebral malaria remains high and there is no specific adjunct drug available to prevent malaria mortality.

Novelty:

1) For the first time, this study offers new functional insights into “dispensable” heme pathway in RBC stages and explains the dichotomy of parasite heme pathway and host heme acquisition pathways in RBC stages by showing the association of parasite heme pathway with disease virulence and pathogenesis. Thus, it provides an answer to the long-standing question on role of *de novo* heme in the blood stages.

2) This study has provided evidences for the role of *de novo* heme in cerebral malaria and disease severity. In comparison with massive amounts of host heme derived from hemoglobin degradation, *de novo* heme synthesized by the parasite could be miniscule. Interestingly, it is the parasite *de novo* heme that decides the fate of host heme detoxification and determines the outcome of disease pathogenesis by influencing the levels of hemozoin and free heme - a key PAMP and DAMP associated with cerebral and severe malaria.

3) This study demonstrates the unexpected role of *de novo* heme in influencing the detoxification of imported hemoglobin-heme into hemozoin by modulating food vacuole integrity in the asexual stages. The findings on its specific role in oleic acid synthesis that can influence lipid unsaturation without altering the total phospholipids and neutral lipids is also expected to have a broader impact beyond food vacuole integrity and hemozoin formation, and it opens a new avenue of research areas for future investigations.

4) This study shows that the genetic ablation of parasite heme pathway does not affect the function of artemisinin *in vivo*. Even with a 50% decrease in parasite cytosolic free heme observed for KO parasites, artemisinin can still be effective.

5) A unique approach to mitigate parasite virulence by targeting hemozoin through dispensable *de novo* heme pathway that may exert less selection pressure on the parasites has been identified.

6) Importantly, this study demonstrates the translational potential of griseofulvin - an already existing FDA-approved antifungal drug that can be repurposed to tackle parasite virulence and disease severity along with ACTs. This assumes significance in the context of malaria deaths that occur despite ACT treatment and the decreasing efficacy of ACTs that lead to the increased parasite load and delayed parasite clearance during the course of treatment. Until now,

parenteral administration of artemisinin or quinine is the only option for cerebral and severe malaria, along with supportive therapies. This study raises the interesting option that griseofulvin can be used as an adjunct therapy with ACTs to prevent and treat cerebral and severe malaria. This merits for evaluation in clinical trials and serves an unmet need to prevent malaria mortality. We are now initiating preclinical studies followed by Phase I trial in collaboration with Ipca Laboratories Ltd., Mumbai and Prado Pvt Ltd., Pune. The first phase of BIRAC funding is in the final stage of approval.

Scientific Details with Illustrations:

Since the present study has a huge amount of data, selected findings are provided below. The entire data along with the supplementary information are available in the attached publication (Chandana et al., Nature communications 2022. https://doi.org/10.1038/s41467-022-31431-z).

Heme pathway KO parasite-infected mice are protected from ECM

Experimental cerebral malaria (ECM) studies performed in C57BL/6 mice infected with *P. berghei* (*Pb*) wild-type (WT) and heme-pathway knockout (KO) parasites suggested that the mice infected with KO parasites were completely protected from ECM. While 80% of the WT-infected mice succumbed to ECM within day 10, KO-infected mice did not undergo ECM and the death due to anemia was also delayed by ~10 days. The rapid murine coma and behavioral scale (RMCBS) score of WT parasite-infected mice that succumbed to ECM was below 5 on day 7 whereas, RMCBS score of KO parasite-infected mice was around 17 and 14 on day 7 and 14, respectively. (Figure 1). These results were verified with another set of independent *Pb*KO^{Luc} parasite lines wherein, *ALAS* and *FC* genes were replaced with luciferase. For control, *c-ssurRNA* locus in the WT parasite was replaced with luciferase (*Pb*Control^{Luc}). *In vivo* bioluminescence and *ex vivo* studies showed accumulation of pRBCs in the brain of *Pb*Control^{Luc}-infected mice, but not in the *Pb*KO^{Luc}-infected mice. To rule out any off-target effect, we performed genetic complementation in *Pb*FCKO^{Luc} by reintroducing *FC* (*Pb*FCKO^{+FC}) expressed under its native promoter. *Pb*FCKO^{+FC} parasites behaved like WT parasites and caused ECM in the infected mice (Figure 2). These data suggested that mice infected with heme pathway KO parasites are completely protected from ECM.

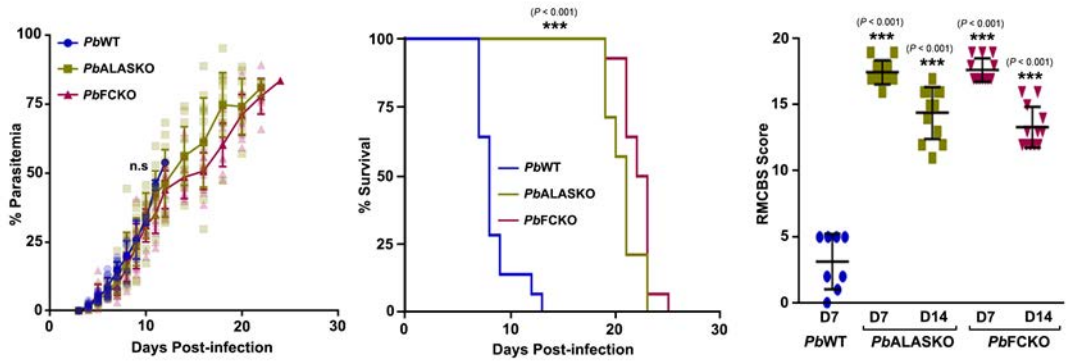

Figure 1: CM protection in heme pathway KO parasite-infected mice.

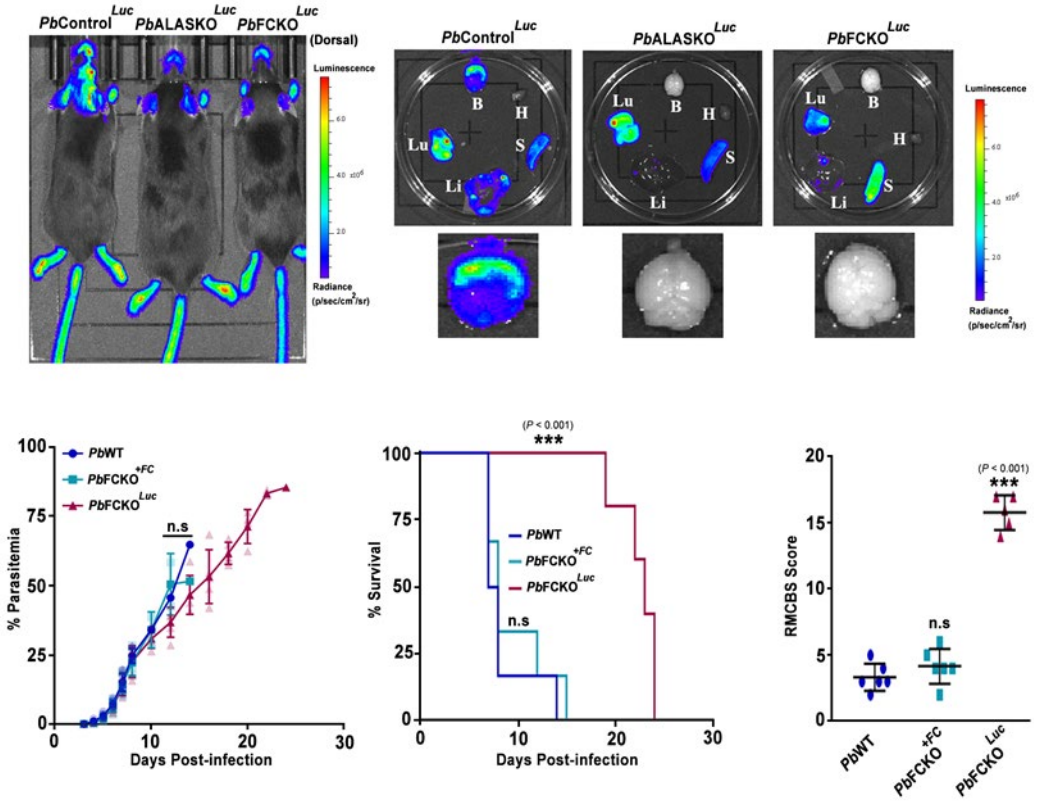

Figure 2: *In vivo* bioluminescence imaging of mice infected with Luc-expressing heme pathway KO parasites and genetic complementation.

To evaluate the integrity of blood-brain barrier (BBB) and assess vascular leakage, Evans blue extravasation analyses were carried out. While the brain collected from WT-infected mice on day 7/8 stained intensely with Evans blue, the extravasation of Evans blue into the brain was barely detectable in KO-infected mice on day 7 and 14. Histopathological assessment performed for the brain sections of KO-infected mice showed the absence of hallmark features of cerebral malaria such as intracerebral hemorrhages, extravasation of erythrocytes into the

perivascular space, petechial hemorrhages, thrombosed and leukocyte-packed vessels, gross demyelination and myelin pallor.

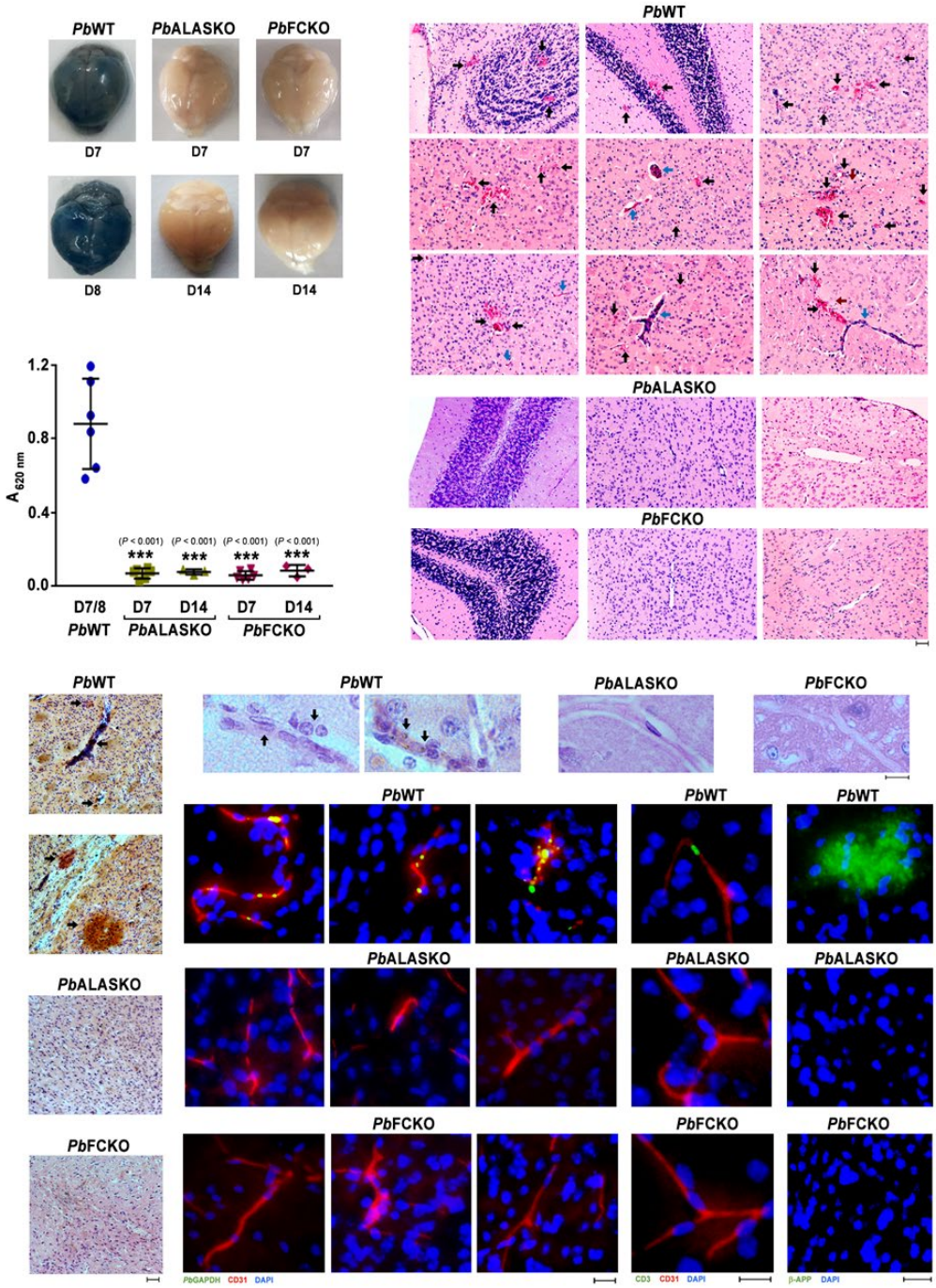

Figure 3: Assessment of cerebral pathology in heme pathway KO parasite-infected mice.

This was also true for immunohistochemical and immunofluorescence analyses on the extravasation of IgG in cerebral parenchyma, luminal and abluminal leukocytes, parasite-derived Hz, accumulation of parasites and $CD3^+$ T cells in $CD31^+$ vasculature, extravascular

parasites in the hemorrhages and axonal injury. These data suggested the absence of ECM-associated brain lesions in mice infected with heme pathway KO parasites (Figure 3).

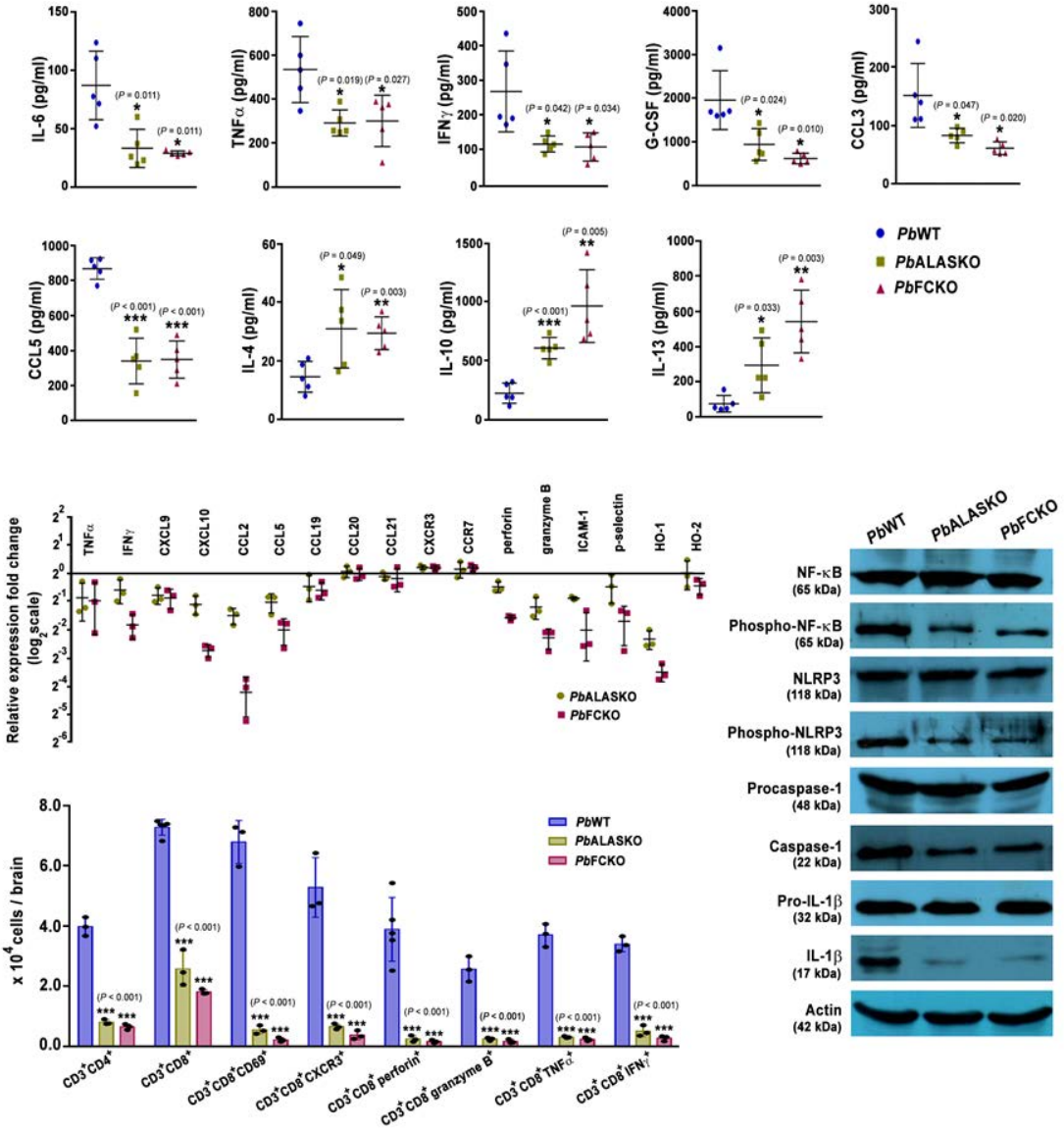

Figure 4: Assessment of systemic and neuronal inflammtion in heme pathway KO parasite-infected mice.

Mice infected with KO parasites showed a significant decrease in the plasma levels of proinflammatory cytokines and chemokines, and a significant increase in anti-inflammatory cytokines. There was a substantial reduction in the brain transcript levels of TNFα, IFNγ, CXCL9, CXCL10, CCL2 (MCP-1), CCL5, CCL19, perforin, granzyme B, ICAM-1, p-selectin and HO-1. T-cell infiltration in brain was drastically reduced and Western analyses for the brain homogenates showed reduction in phospho-NLRP3, phospho-NF-κB, cleaved caspase-1

and IL-1β (Figure 4). These data suggested an overall decrease in systemic and neuronal inflammation, and T cell infiltration in the brain milieu of KO parasites.

De novo heme is essential for the functional integrity of FV and Hz formation

Giemsa-stained peripheral blood smears, paraformaldehyde-fixed pRBCs and hemozoin (Hz) dynamics in live pRBCs suggested a substantial decrease in the Hz formation. There was also a significant decrease in parasite free heme, and plasma free heme and heme/hemopexin ratio of the KO-infected mice, and the quantification of Hz load in the spleen and liver showed 40-50% decrease (Figure 5).

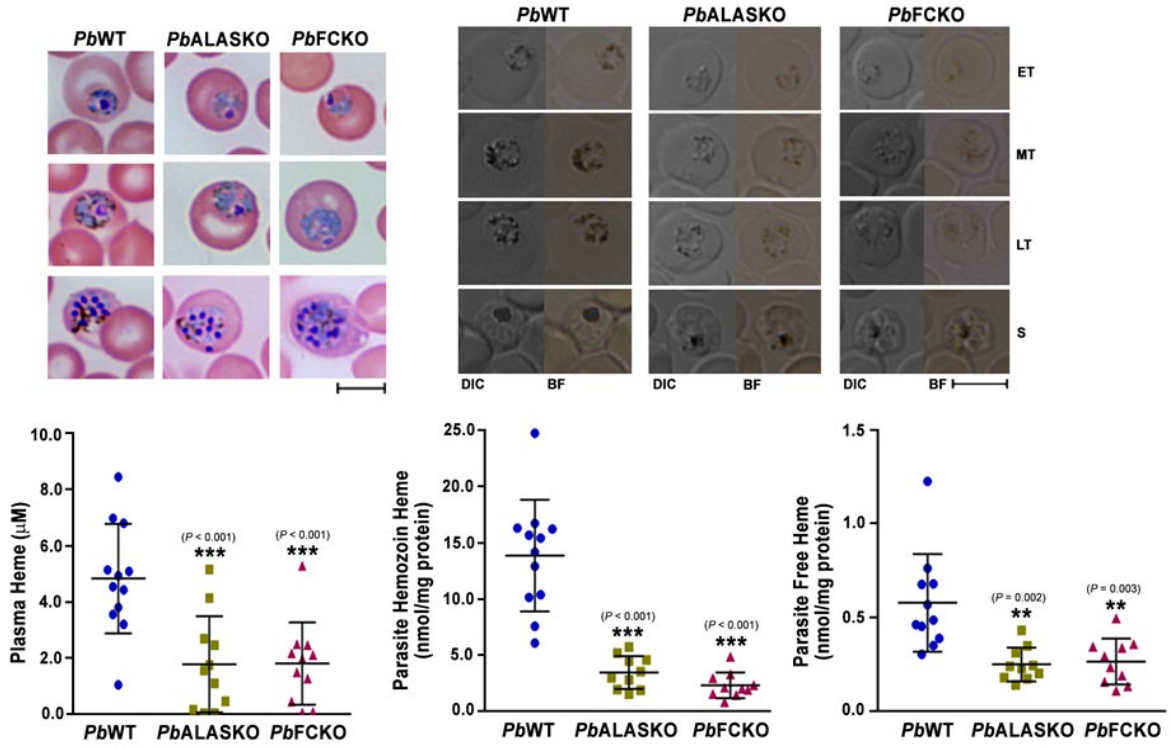

Figure 5: Hemozoin and heme levels in heme pathway KO parasites.

These results suggested an overall decrease in the Hz synthesis of KO parasites. This was further verified by the restoration of Hz and free heme levels in *Pb*FCKO^{+FC} parasites and the presence of cerebral pathogenesis in the *Pb*FCKO^{+FC}-infected mice. To understand the molecular mechanisms underlying decreased Hz synthesis, we assessed pH, lipids and protein content of the food vacuoles (FVs) in FCKO parasites and compared with WT parasites. LysoTracker Deep Red uptake indicated less fluorescence in the FCKO parasites, suggesting that the pH of the FCKO parasite FVs is compromised. *In vitro* radiolabelling studies together

with BODIPY 493/503 and Nile Red staining showed no significant differences in phospholipids and neutral lipids (Figure 6).

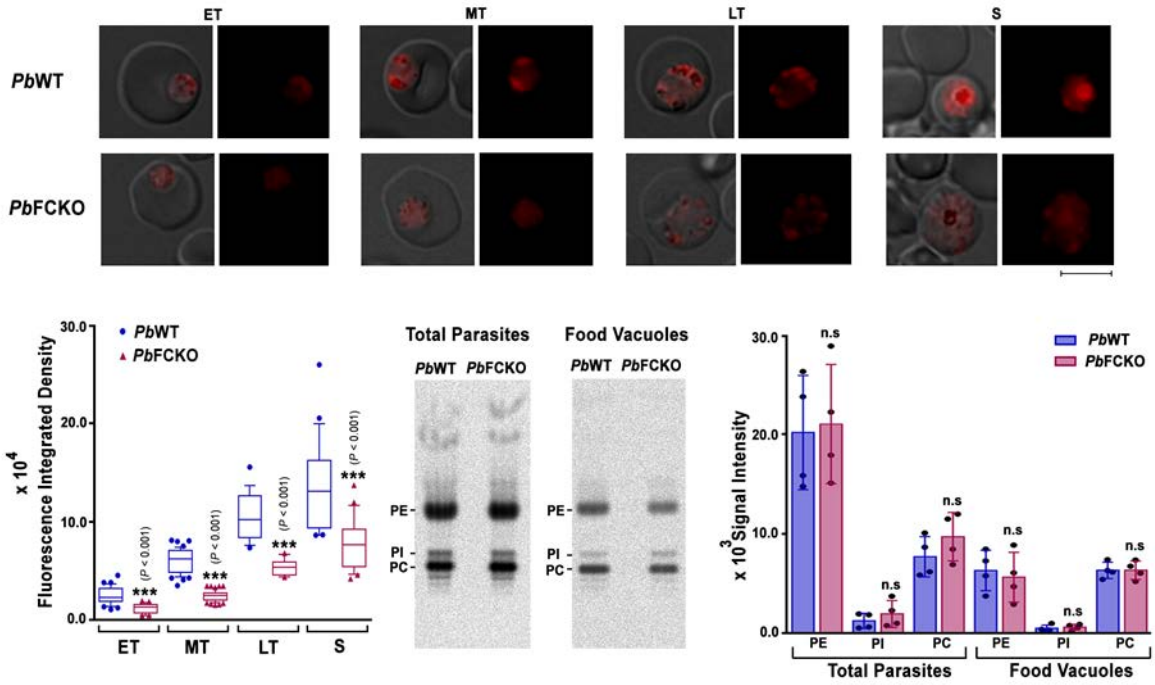

Figure 6: Assessment of food vacuole pH, phospholipids and neutral lipids in FCKO parasites.

Malaria parasite scavenges stearic acid (SA) from plasma and converts it into oleic acid (OA) by ER-localized Δ9-desaturase (stearoyl-CoA 9-desaturase). This requires heme since the electrons transferred by cytochrome b5 from NADH cytochrome b5 reductase are utilized[3,4]. Lipids associated with parasite Hz are reported to be abundant in monohydroxy derivatives of polyenoic fatty acids and GC-MS analysis of fatty acid methyl esters (FAMEs) prepared from the FVs of *Pb* parasites indicated OA as a major unsaturated fatty acid. *In vitro* ^{14}C-SA radiolabelling of KO parasites suggested a drastic decrease in the synthesis of OA. FV proteomics suggested that none of the subunits of V-type H^+ATPase - a proton pump maintaining the acidic pH of FV[5] could be detected in FCKO FVs suggesting the lower abundance of these proteins. In WT FV preparations, A, B and G subunits of V-type H^+ATPase could be detected. While plasmepsin IV, the only *Pb* aspartic protease involved in Hb degradation could be detected, berghepain-2 - a cysteine protease involved in Hb degradation[6,7] could not be detected in FCKO FVs. Western analysis for V-type H^+ATPase subunits B and G showed a significant decrease in FCKO FVs with not much change in the total parasites (Figure 7). In agreement with less Hz formation, FCKO FVs appeared paler in comparison to WT. There was ~3-4 -fold increase in hemoglobin (Hb) α and β chains in FCKO FVs as quantified

by iTRAQ and similar to the mutants of Hb proteolysis[6], translucent vesicles were also observed. These data suggested that the functional integrity of FCKO FVs is compromised.

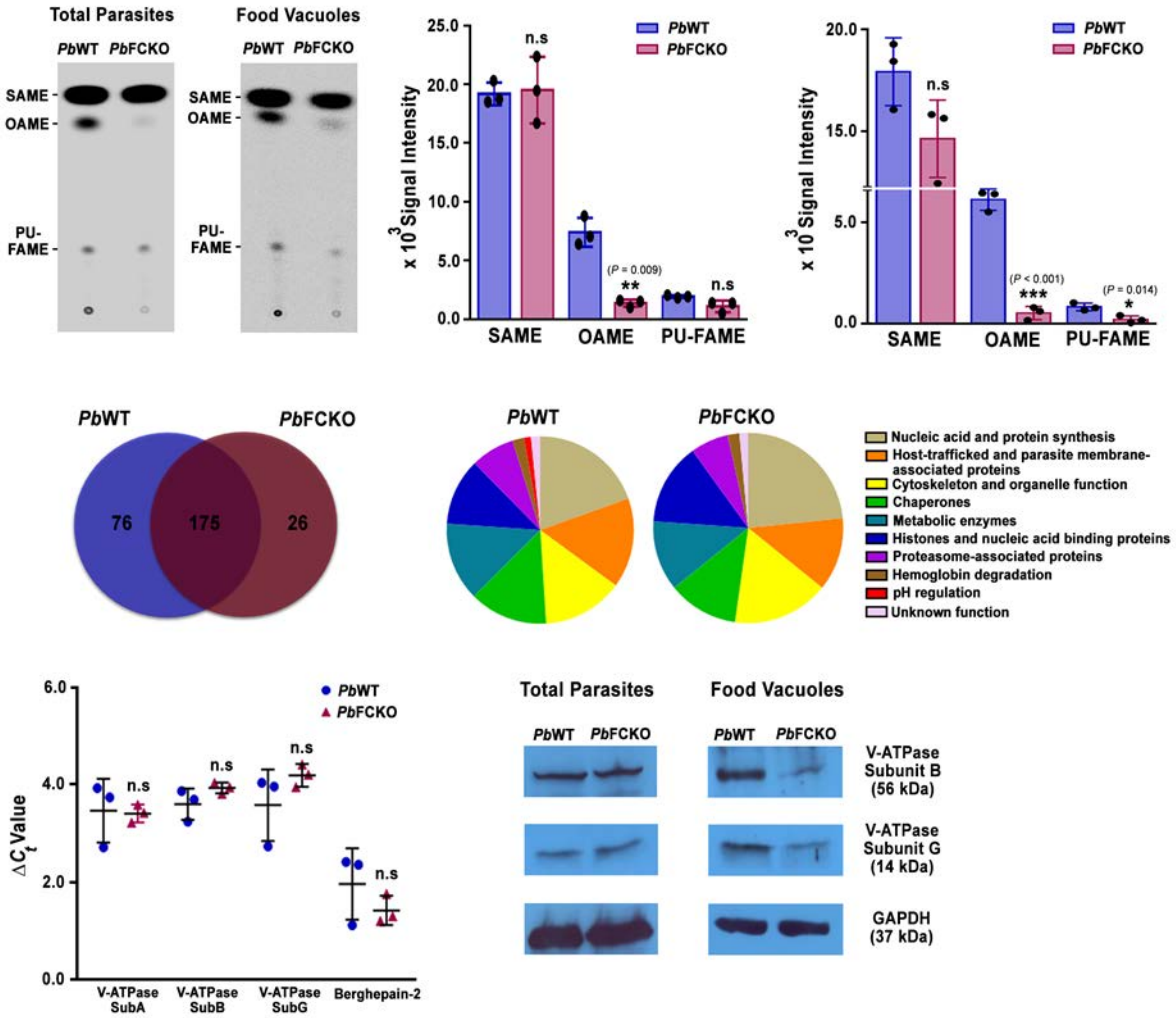

Figure 7: Evaluation of oleic acid synthesis and food vacuole proteomics for WT and FCKO parasites.

Griseofulvin treatment protects cerebral malaria

Griseofulvin, isolated from *Penicillium griseofulvum*, is a FDA-approved antifungal drug used to cure tinea infections. It interacts with fungal microtubules and disrupts spindle assembly leading to mitotic arrest. In humans, griseofulvin dosage is given to the extent of 1000 mg/day in adults and 10 mg/kg/day in children for several weeks[8,9,10]. It can also inhibit FC by generating N-methyl protoporphyrin IX (NMPP) through the action of cytochrome P450 enzymes[11]. Therefore, the potential of griseofulvin in preventing ECM was evaluated by treating WT-infected C57BL/6 mice from day 4 when the blood parasitemia was around 2%. A single dose of 2 mg/day (comparable with the dosage of humans) administered from day 4

and continued until day 8 showed the best protection with more than 80% of the treated mice were protected from ECM. Interestingly, the growth curves of WT parasites in treated and untreated mice were very much comparable. *In vitro* ^{14}C-ALA radiolabelling studies with griseofulvin-treated WT parasites suggested a decrease in parasite heme synthesis. Further, griseofulvin-treated mice showed less Evans blue extravasation in the brain, with the absence of intracerebral hemorrhages, lack of accumulation of parasites and $CD3^+$ T cells in the cerebral vasculature and undetectable axonal injury. As observed for KO parasites, the total Hz content, and plasma and parasite free heme levels in the griseofulvin-treated parasites was ~60% less when compared with untreated parasites (Figure 8). These results suggested that griseofulvin can prevent ECM through inhibition of parasite heme synthesis.

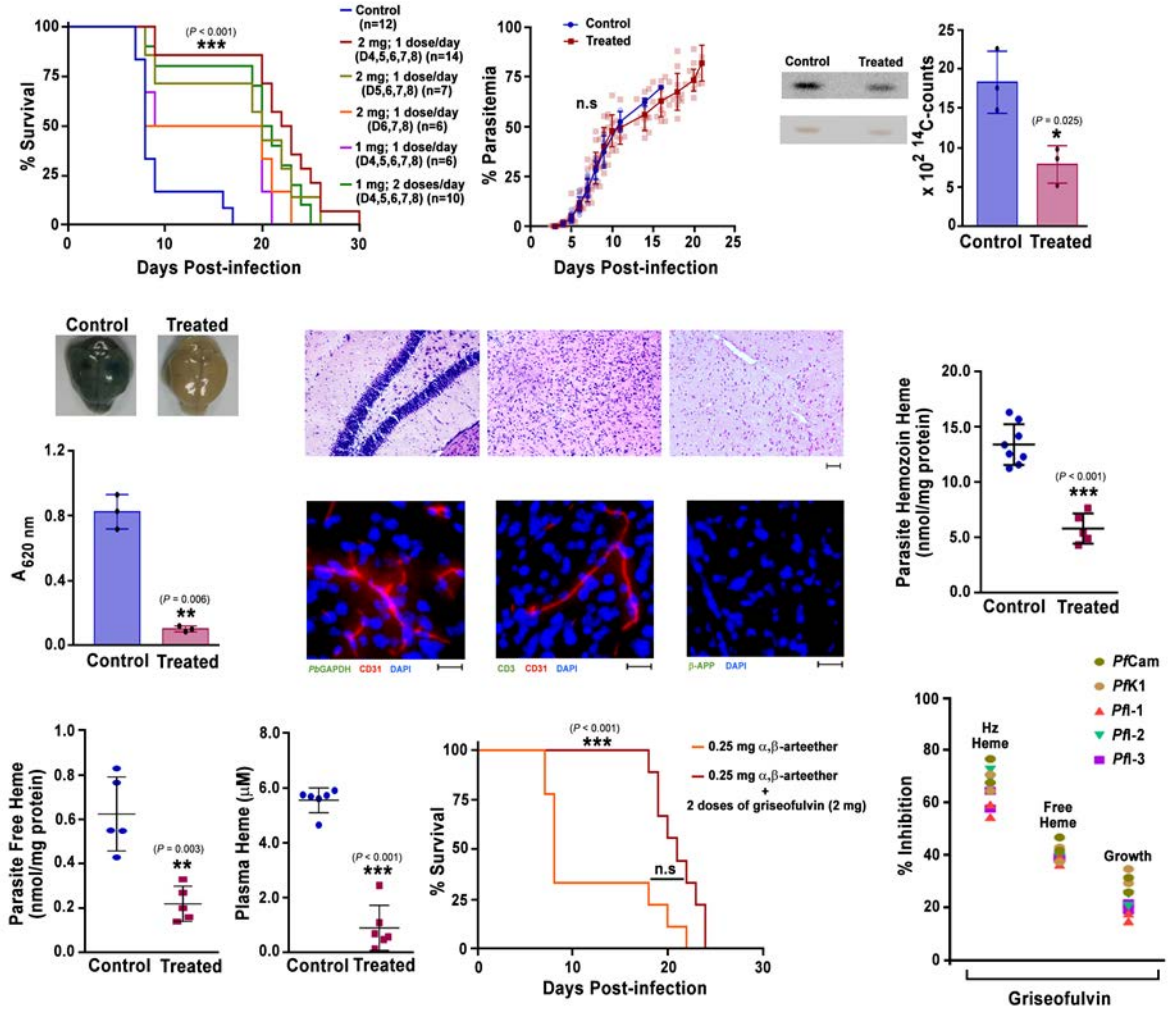

Figure 8: Effect of griseofulvin treatment on cerebral malaria pathogenesis.

We then examined the potential of griseofulvin as an adjunct drug with α,β-arteether (primary artemisinin component of ACTs) in preventing ECM. The dose optimization studies performed

with α,β-arteether suggested that a single intramuscular dose of 0.25 mg/mouse on day 6 when there was onset of neurological symptoms and blood parasitemia was around 10%, led to ECM in ~60-70% of WT-infected mice. ECM occurred despite the clearance of peripheral parasitemia as observed in the blood smears. However, the inclusion of two doses of 2 mg griseofulvin at 24 h interval on day 6 and 7, post-α,β-arteether treatment, led to a complete protection of ECM (Figure 8). To examine the relevance of our findings in human parasite, we assessed the ability of griseofulvin to inhibit Hz formation in *Pf*3D7 strain, artemisinin-resistant *Pf* clinical isolate from Cambodia (*Pf*Cam), multidrug-resistant *Pf* clinical isolate from Thailand (*Pf*K1) and three *Pf* clinical isolates from India (*Pf*I-1,-2 and -3). Griseofulvin did not inhibit parasite growth, a pattern that was consistent with the dispensable nature of parasite heme pathway and similar to that of succinylacetone - another heme pathway inhibitor[1]. Interestingly, ^{14}C-ALA radiolabelling studies showed that girseofulvin could inhibit *de novo* heme synthesis and hemozoin formation in *Pf*3D7 cultures. There was also a significant decrease in free heme levels. All these data suggested that griseofulvin has the potential to serve as an adjunct drug for cerebral and severe malaria in humans that needs to be evaluated in clinical trials.

Summary:

Hemozoin is a parasite molecule associated with inflammation, aberrant host-immune responses, disease severity and cerebral pathogenesis. Heme synthesized in asexual stages promotes cerebral pathogenesis by enhancing hemozoin formation. Heme pathway KO parasites synthesize less hemozoin, and mice infected with KO parasites are completely protected from cerebral malaria. Biosynthetic heme regulates FV integrity and FVs from KO parasites are compromised in pH, lipid unsaturation and proteins, essential for Hz formation. It is known that acidic pH of FV is critical for Hb digestion by proteases and subsequent Hz formation. Our results also suggest the lower abundance of V-type H^+ATPase subunits and berghepain-2 in FCKO FVs. V-type H^+ATPase is a proton pump responsible for maintaining the acidic pH of FV and berghepain-2 seems to be involved in Hb digestion[6,7]. There is also a reduction in OA synthesis of KO parasites suggesting an alteration in the degree of lipid unsaturation that can affect Hz formation. The levels of unsaturated fatty acids are shown to stimulate the function of V-type H^+ATPase in plants[12]. The decrease in OA synthesis and lipid unsaturation can affect other cellular processes such as membrane homeostasis, protein/lipid trafficking, protein folding, cell signalling etc[13]. Although host Hb-heme acquisition starts from

late ring stages, it is possible that FV integrity and maturation depend on *de novo* heme. Our results provide functional insights on *de novo* heme that could be a miniscule in comparison with massive amounts of heme derived from host Hb. Intriguingly, we show it is the *de novo* heme that influences the detoxification of host heme into Hz and disease pathogenesis. Despite the ability of blood stage parasites to acquire host Hb-heme for survival, lack of *de novo* heme synthesis renders them less virulent (Figure 9).

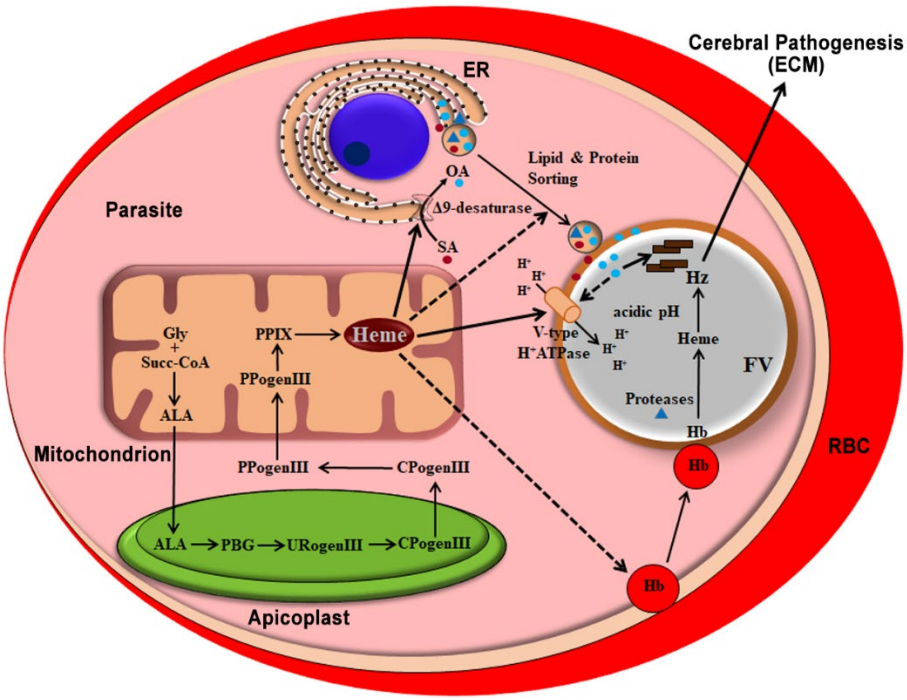

Figure 9: Model depicting the role of *de novo* heme pathway of malaria parasite in hemozoin formation and disease pathogenesis

The fatality rates in malaria do not correlate with parasite clearance and therefore, targeting parasite virulence becomes important. The malaria deaths that occur despite treating the infected individuals with ACTs and the decreasing efficacy of ACTs that lead to delayed parasite clearance underscore the need of an adjunct therapy in the initial stages of ACT treatment. Until now, the attempts to develop adjunct therapies with candidates acting mainly on host responses did not succeed in clinical trials[14,15]. We show that *de novo* heme in the blood stages can serve as a target for malaria pathogenesis. Griseofulvin - a FDA-approved antifungal drug prevents ECM and delays death due to anemia by inhibiting parasite heme synthesis. The ability of griseofulvin to prevent disease pathogenesis is observed despite the absence of any antimalarial treatment. Griseofulvin treatment does not affect the parasite growth suggesting

that ECM protection is not because of the inhibition of parasite mitosis. In summary, targeting parasite heme synthesis by griseofulvin - a FDA-approved antifungal drug, prevents cerebral malaria and disease severity in mice, and provides an adjunct therapeutic option to prevent the mortality due to cerebral and severe malaria.

2) Glutamine synthetase as a new target to combat *falciparum* malaria and artemisinin resistance

Ghosh et al. Distinct evolution of type I glutamine synthetase in *Plasmodium* and its species-specific requirement (2023). Nature Communications, 14, 4216.

Background:

Malaria parasites have lost *de novo* pathways for amino acid biosynthesis. The asexual stage parasites acquire host hemoglobin (Hb) and degrade it in the food vacuole (FV)[16]. Hb degradation caters to amino acid requirements and provides space for the parasites to grow. The only amino acid that is absent in human Hb is isoleucine. *In vitro* cultures of *P. falciparum* (*Pf*) can be maintained continuously by providing isoleucine as the sole amino acid, suggesting that other amino acids from Hb degradation are adequate to support asexual growth[17]. Unlike asexual stages, sexual stage development in mosquitoes is extracellular and they depend primarily on extracellular sources of amino acids in the hemolymph. The liver stages may have to depend on host hepatocyte reserves and/or extracellular sources for amino acids[18]. Further, sexual and liver stages do not have FV and therefore, lack the host protein degradation machinery[19].

Glutamine synthetase (GS) is an ancient ubiquitous enzyme and considered as a 'molecular clock' that evolved prior to the divergence of prokaryotes and eukaryotes. The existence of three different GS (I, II and III) with diverged primary sequences, structural conformations and distinct regulatory mechanisms exemplifies the extent of evolutionary adaptations. Glutamine is the most abundant amino acid in human, mosquito hemolymph and liver[20,21]. Despite its abundance and the ability of parasites to access Hb-derived and extracellular glutamine, a putative gene for GS is retained. In this study, an extensive characterization of *Plasmodium* GS has been performed providing new insights on the evolution of GS, its species-specific essentiality and the potential of GS as a new drug target for *P. falciparum* (*Pf*) and artemisinin resistance.

Novelty:

1) It is shown that *Plasmodium* GS has undergone an extensive evolution as a unique type I enzyme with distinct structural and regulatory features to adapt to the asexual stage niches.

2) Parasite GS defies the known classical regulatory mechanisms of GS from other organisms. It lacks feedback inhibition by amino acids for sustaining its activity in the asexual stages wherein, massive Hb degradation in FV releases surplus amino acids in millimolar concentrations. It is also known that the malaria parasite shows extensive adaptations to febrile temperatures. Interestingly, parasite GS has evolved with a compact dodecamer channel that offers thermal stability at febrile temperatures. Similarly, malaria parasite derives ATP through glycolysis in the asexual stages with minimal TCA cycle activity. Parasite GS has also lost its regulation by adenylylation that is controlled by α-ketoglutarate - a TCA cycle intermediate.

3) *Plasmodium* GS is inhibited by the transition state analogues, methionine sulfoximine (MSO) and phosphinothricin (PPT), providing a new target for therapeutic intervention.

4) There is an unexpected species-specific essentiality despite the fact that GS is abundantly expressed in all the life cycle stages of *Plasmodia*. GS is essential for *P. falciparum*, but non-essential for *P. berghei* (*Pb*) and *P. vivax* (*Pv*).

5) Glutamine serves as an amide donor for asparagine biosynthesis, and *P. falciparum* proteins are asparagine-rich with extensive asparagine repeats. The species-specific essentiality arises due to the asparagine-rich proteome of *Pf*. Targeting GS in *Pf* alters asparagine levels and drastically affects protein synthesis, but not in *Pb*. MSO and PPT can inhibit the growth of *Pf* clinical isolates, but not of *Pv*.

6) Importantly, GS can be a potential target for artemisinin-resistance in *P. falciparum* - a major concern. Glutamine serves as a nitrogen source that helps artemisinin-resistant parasites to recover from artemisinin-induced starvation. The combination of GS inhibitors with artemisinin renders artemisinin-resistant parasites sensitive to artemisinin.

Scientific Details with Illustrations:

The selected findings are provided below and the entire data along with the supplementary information are available in the attached publication (Ghosh et al., Nature communications 2023. https://doi.org/10.1038/s41467-023-39670-4).

Unique features of *Plasmodium* GS

GS can be classified into three types: I, II and III based on the sequence and structural conformation. *Plasmodium* GS is a type I enzyme with sequence length ~60–70 amino acids more than a typical GS I. This can be attributed to two different peptide inserts spanning across the regions that are not directly associated with the substrate-binding and catalytic sites. Further, GS I can be classified into two subtypes based on the regulatory mechanisms – Iα regulated through the feedback inhibition by glutamine, AMP and other amino acids like serine, alanine and glycine, and Iβ that is additionally inhibited by adenylylation of a tyrosine residue near the active site and not feedback-inhibited by glutamine[22,23,24,25]. The characteristic features of GS Iβ are the presence of a specific ~25 amino acid insertion and an adenylylation site that are absent in GS Iα. Interestingly, the sequence corresponding to 25-amino acid insertion in *Plasmodia* GS is diverged from other organisms and it is flanked by the first peptide insert. Further, *Plasmodia* GS lack a tyrosine residue in the corresponding position of adenylylated tyrosine in *Salmonella* and *Mycobacterium* GS, flanked by the second peptide insert towards the C-terminus. While the first peptide insert comes in proximity to the pore of dodecamer channel, the second peptide insert forms a long loop that folds down in the opposite direction from the active site (Figure 1). These evidences suggest the unique structural features of parasite GS that are absent in other GS I.

Inhibition and regulation of *Plasmodium* GS activity

L-Methionine sulfoximine (MSO) is a potent irreversible inhibitor of GS. MSO-phosphate generated by GS serves as a transition state analogue that binds non-covalently and stabilizes the flexible loop of GS active site preventing glutamate entry. Methyl group of MSO-phosphate occupies the ammonium binding site and prevents further reaction.

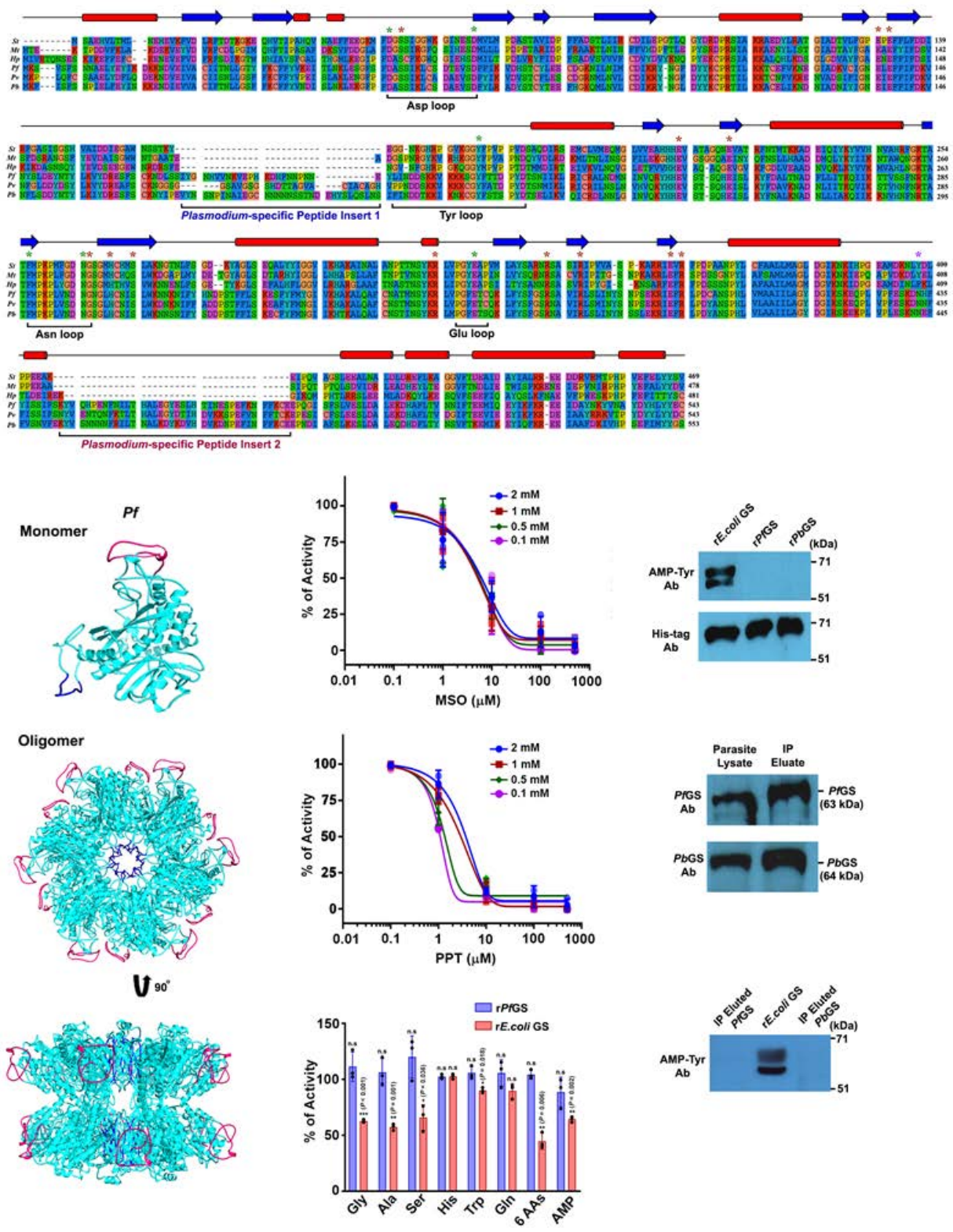

Figure 1: Unique features of *Plasmodium* GS and its inhibition and regulation.

Kinetic studies showed that parasite GS is sensitive to MSO inhibition. The K_i values of r*Pf* and *Pb*GS are ~10^2 times lower when compared with GS II present in humans.

Phosphinothricin (PPT) is another potent irreversible inhibitor and parasite GS is equally sensitive to PPT (Figure 1). Enzyme assays performed with glycine, serine, alanine, tryptophan, histidine, glutamine and AMP suggested that none of the amino acids either tested individually or in a cumulative manner could inhibit r*Pf*GS activity. Another salient feature of type Iβ GS is its regulation by adenylylation[26,27]. Interestingly, Western analysis using anti-AMP-tyrosine antibody showed that both r*Pf* and r*Pb* GS do not undergo adenylylation. This was also confirmed by performing immunoprecipitation for parasite lysates (Figure 1). These results suggest that parasite GS is unique lacking the characteristic regulatory mechanisms of GS 1α or 1β, and it is inhibited by transition state analogs.

Functional significance of *Plasmodium*-specific peptide inserts

Independent deletions were performed for the first (ΔI1r*Pf*GS) and second peptide inserts (ΔI2r*Pf*GS). ΔI1r*Pf*GS behaved like r*Pf*GS in terms of specific activity, inhibition by MSO and PPT, and lack of feedback inhibition by amino acids. Since the first peptide insert occupying dodecameric channel renders it more compact than GS I of other organisms, the stability of ΔI1r*Pf*GS was examined. While the activity of ΔI1r*Pf*GS was comparable with r*Pf*GS when exposed to higher concentrations of urea or NaCl, ΔI1r*Pf*GS was thermally less stable. Exposure of ΔI1r*Pf*GS to increased temperatures in the febrile range of 37 °C to 42 °C could lead to visible precipitation and almost 80% reduction in activity. Size-exclusion chromatography showed the dissociation of oligomers. Interestingly, deletion of the second peptide insert (ΔI2r*Pf*GS) led to a complete loss of activity. To gain additional insights, molecular dynamics (MD) simulations were performed for the two adjacent subunits constituting the active site from upper and lower hexamers. A comparison of intra-chain and inter-chain hydrogen bonds suggested 25 and 12 interactions that were unique for *Pf*GS and ΔI2*Pf*GS, respectively. This also included some of the residues that are directly associated with the enzyme activity. Interestingly, of the 25 unique interactions in *Pf*GS, only two of them were from the second peptide insert. There were changes in the overall protein conformation with deviations in Cα backbone, residue flexibility, hydrogen bond formation, and solvent accessible surface area. r*Pf*GS lacking both the inserts (ΔI1I2r*Pf*GS) was also found to be inactive (Figure 2). These results suggest that the evolution of the second peptide insert has occurred with compensatory changes that affect *Pf*GS activity when the second peptide insert was deleted.

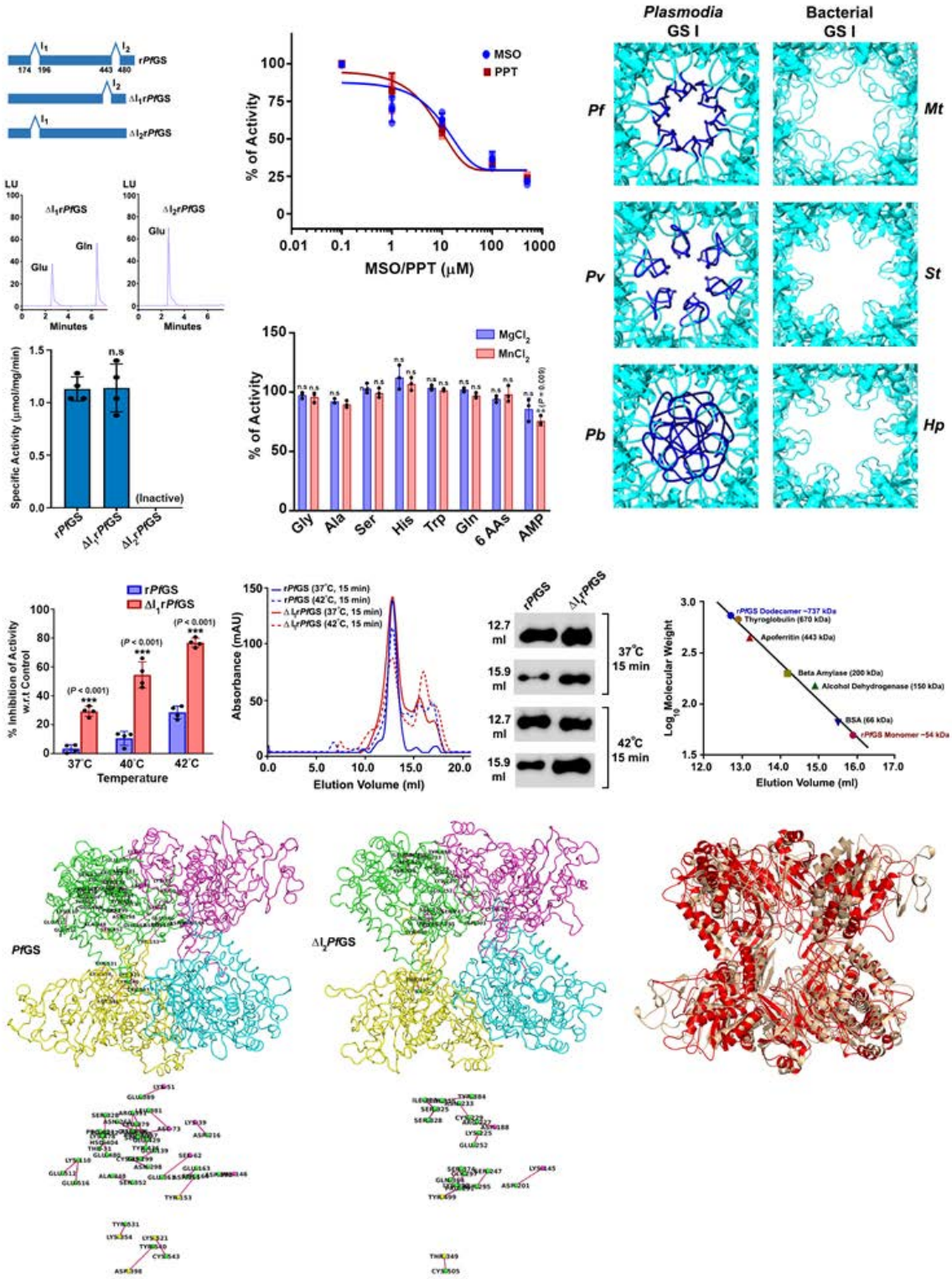

Figure 2: Characterization of r*Pf*GS lacking the first and second peptide inserts.

Plasmodium GS is cytosolic and expressed in the entire life cycle

Immunofluorescence and Western analyses suggested that parasite GS is an abundant cytosolic enzyme expressed in all the asexual stages. GS activity was around 0.21 and 0.29 nmol mg^{-1}

total protein min^{-1} for *Pf* and *Pb* lysates, respectively. GS expression was also detected in *Pf* and *Pb* gametocytes, and ookinete, oocyst, sporozoite and exo-erythrocytic stages of *Pb* (Figure 3). These evidences suggest that native parasite GS is cytosolic and active, and abundantly expressed in all the stages.

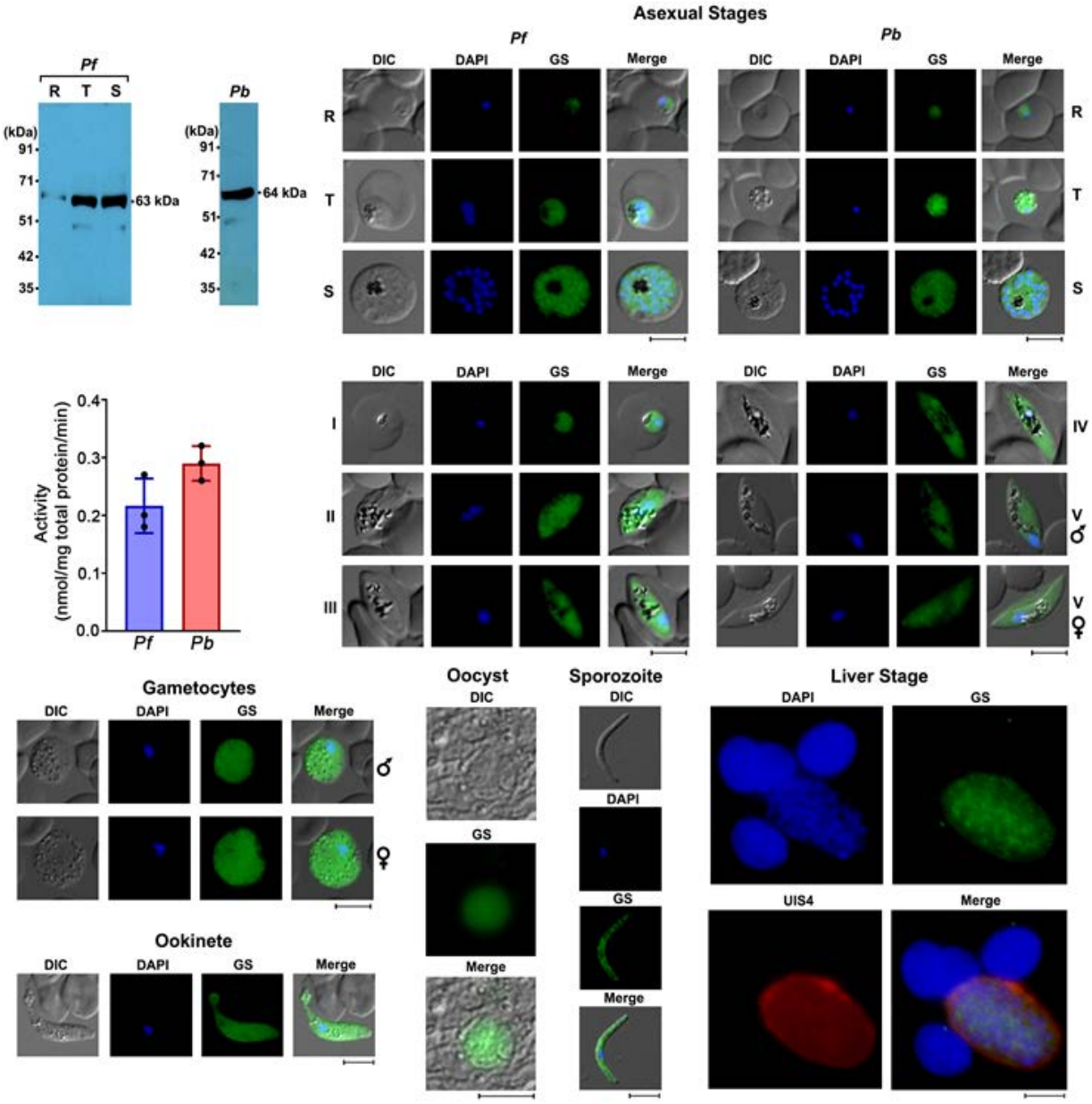

Figure 3: Expression of GS in the life cycle of *Plasmodia*.

GS is essential for *P. falciparum*, but non-essential for *P. berghei*

Our repeated attempts to generate GS knockout (KO) in *Pf* turned out to be unsuccessful. Therefore, a conditional, mislocalizing, knock sideways (cKS) strain (*Pf*GS^{cKS}) was generated through selection-linked integration approach wherein, GS was fused in-frame with FKBP and

GFP through linkers, followed by a neomycin selectable marker separated by skip peptide. The FKBP-GFP fusion led to almost 70% reduction in the specific activity of *Pf*GS activity, and *Pf*GS^{cKS} parasites displayed ~60% reduction of growth. While *Pf*3D7 cultures could be maintained continuously in a glutamine-free medium, *Pf*GS^{cKS} parasites failed to grow and no viable parasites could be detected after 4 days of glutamine removal. The results suggest that intervention of endogenous GS activity affects the growth of *Pf* asexual stages. Rapamycin-induced GS mislocalization was then performed by transfecting *Pf*GS^{cKS} strain with a plasmid that expressed plasma membrane-targeting Lyn peptide fused with FRB and mcherry. GS was knocked sideways from cytosol to the plasma membrane by the addition of rapamycin. Since the active sites of GS are formed by adjacent monomers, membrane anchoring driven by rapamycin-induced dimerization of GS-FKBP-GFP with Lyn-FRB-mCherry disrupted the oligomerization of GS and abrogated GS activity leading to almost 90% decrease in the parasite growth.

Western analysis and enzyme assays carried out for the cytosol and membrane fraction of rapamycin-induced *Pf*GS$^{cKS+Lyn}$ parasites confirmed the mislocalization of GS and the loss in GS activity. The formation of mature, stage V gametocytes was also affected (Figure 4). These findings demonstrate the requirement of endogenous GS for *Pf* asexual stages and gametocytes, signifying that Hb-derived and extracellular glutamine are inadequate. Interestingly, in contrast to *Pf*, GS could be deleted in *Pb*. The growth of GSKO parasites was similar to that of wildtype (WT) parasites with no significant difference in the mortality of GSKO-infected mice. *Pb*GSKO parasites could undergo sexual stage development in mosquitoes with ~40% reduction in the sporozoite formation. GSKO sporozoites could complete exo-erythrocytic stage development and there was no significant difference in the growth of sporozoite-derived GSKO asexual stages (Figure 5). These results suggest the dispensable nature of GS in the entire life cycle of *Pb*.

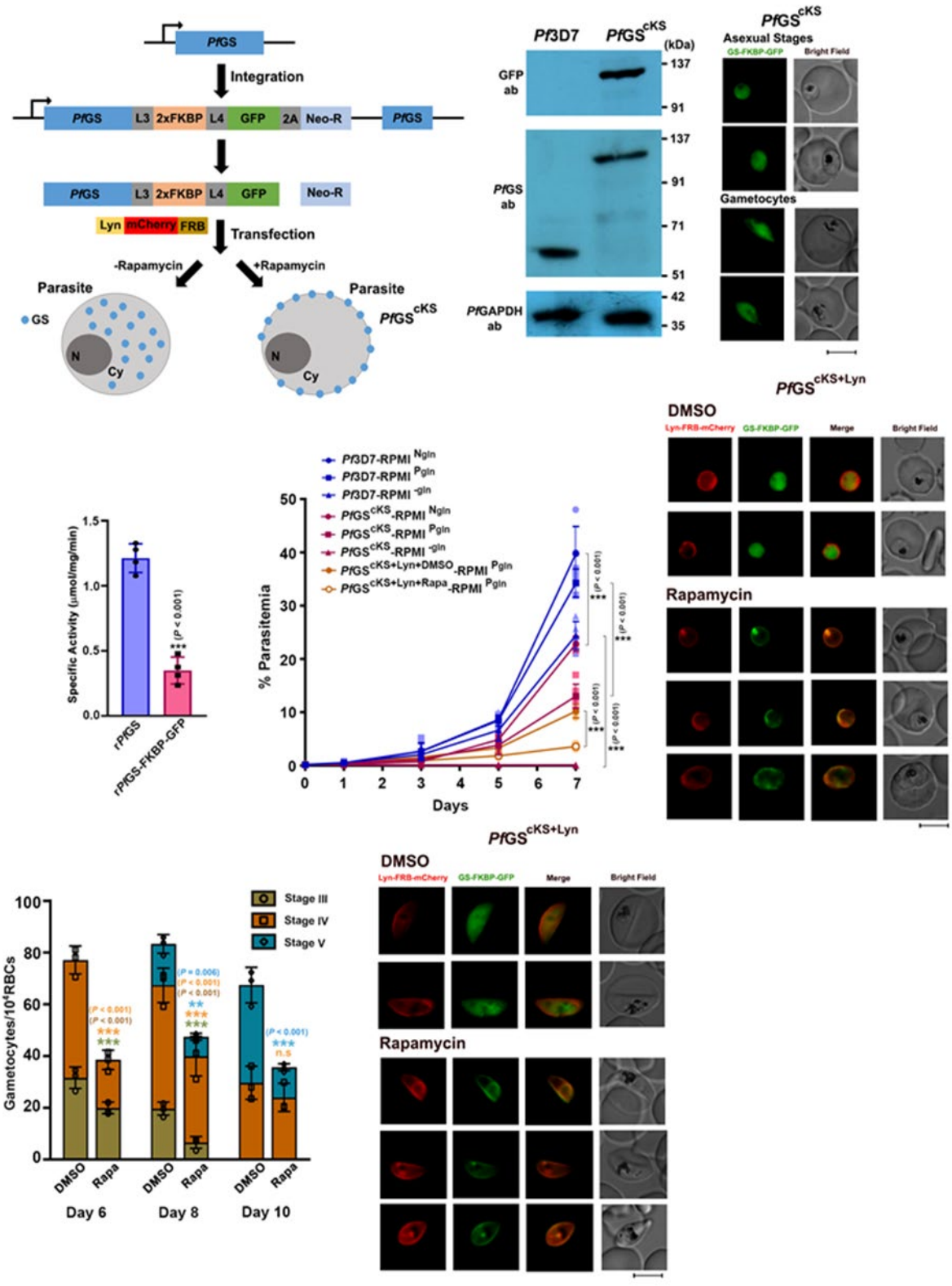

Figure 4: GS is essential for the development of *P. falciparum* asexual stages and gametocytes.

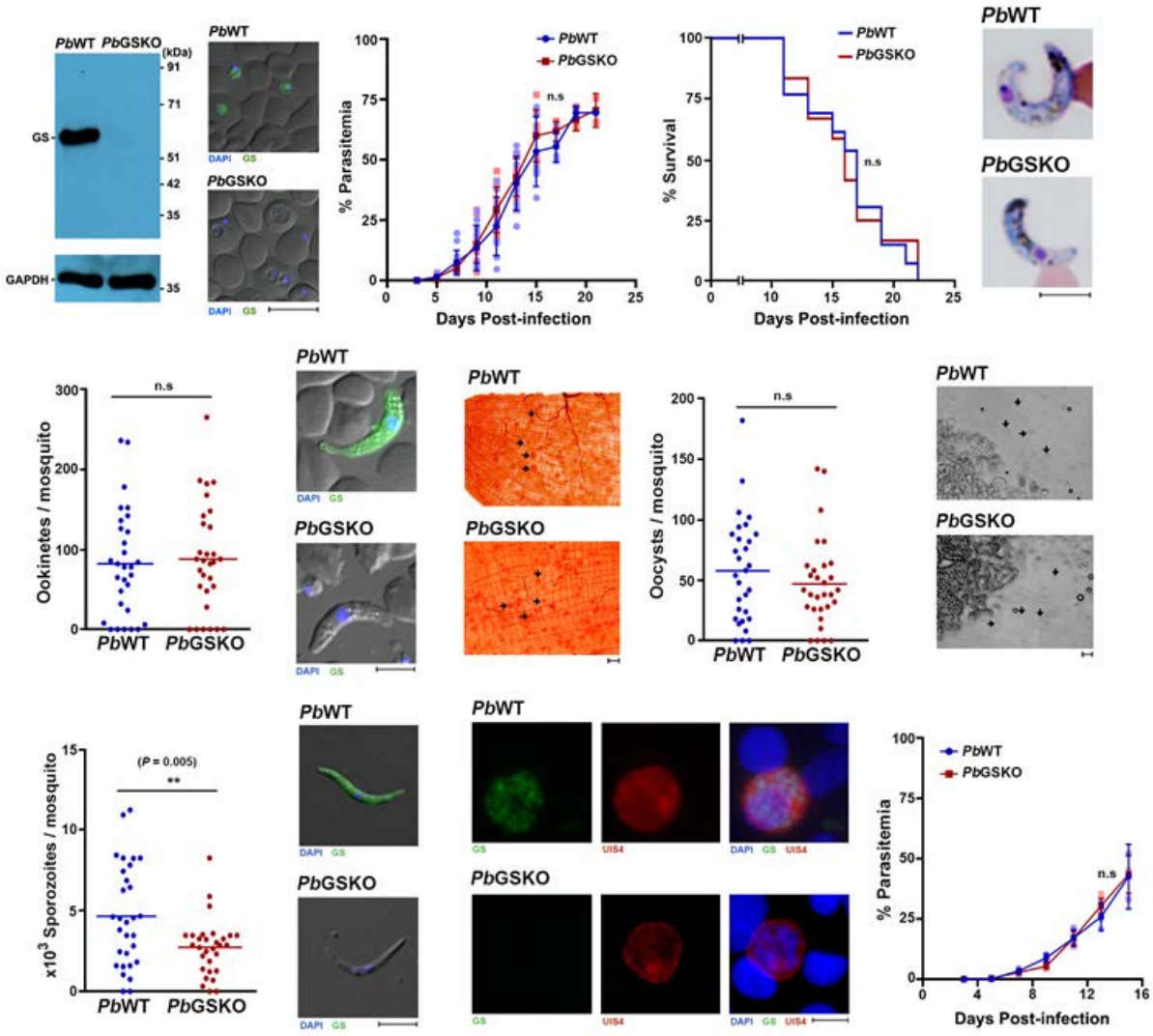

Figure 5: GS is dispensable for the entire life cycle of *Pb*.

MSO and PPT inhibit the growth of *P. falciparum*, but not of *P. berghei*

In agreement, MSO and PPT could inhibit the *in vitro* growth of *Pf*, but not the *in vitro* and *in vivo* growth of *Pb*. They could inhibit the growth of *Pf* asexual stages in the presence and absence of glutamine, and Giemsa-stained smears showed the presence of stressed, arrested, pyknotic and dead parasites. On the contrary, MSO and PPT did not inhibit the growth of *Pb* in *in vitro* cultures. Similar effect was observed *in vivo* when *Pb*WT-infected mice were treated with MSO and PPT. The growth of *Pb*WT in the treated mice was similar to that of untreated control with no significant changes in the mortality of mice (Figure 6). Besides confirming the requirement of GS in *Pf*, the data obtained with the chemical inhibition studies indicate the effect of targeting GS for *Pf* infections.

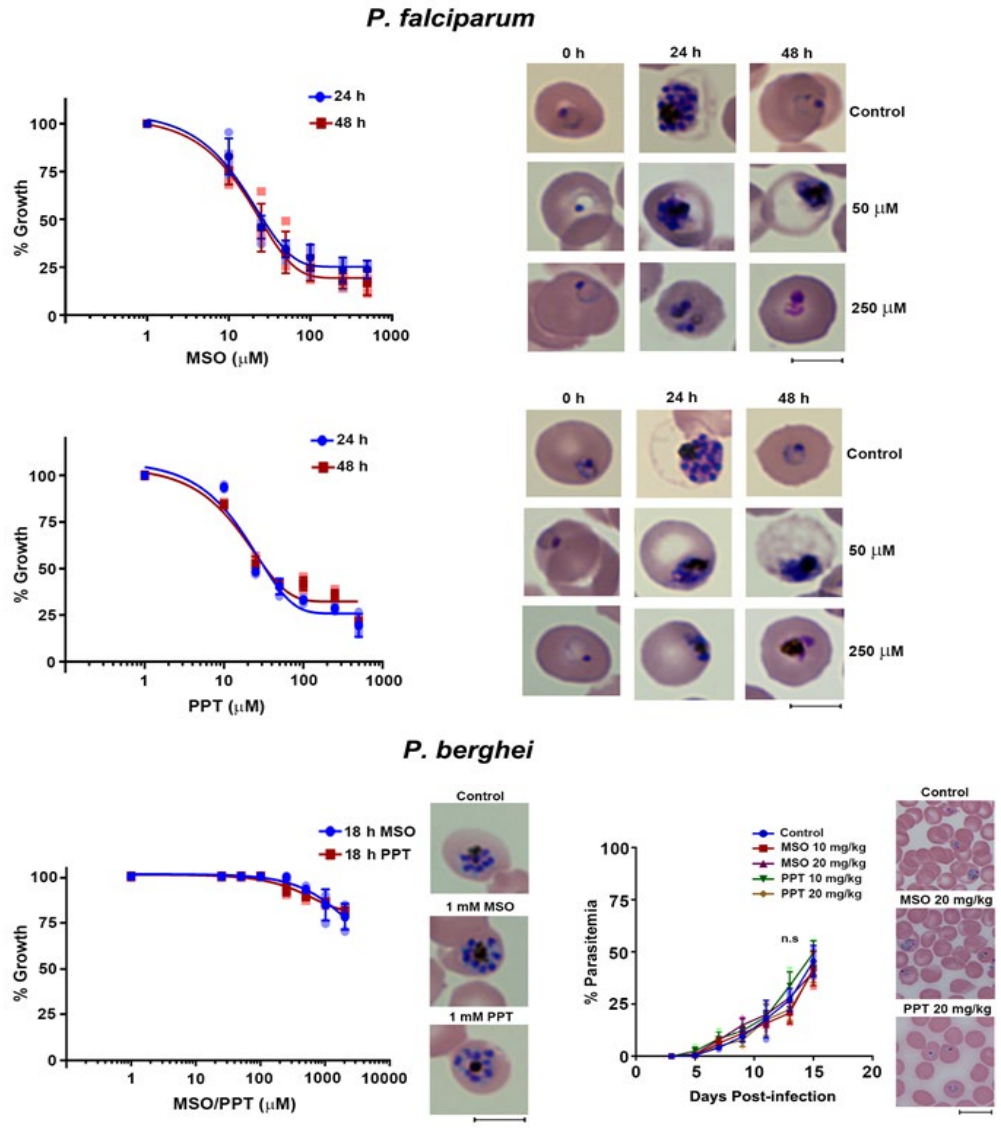

Figure 6: MSO and PPT inhibit the growth of *P. falciparum*, but not of *P. berghei*

Targeting GS affects the protein synthesis in *P. falciparum*

Metabolic labeling of proteins was carried out using [^{35}S]-Methionine and -Cysteine in *in vitro* cultures of *Pf* and *Pb*. Interestingly, MSO and PPT could inhibit protein synthesis in *Pf*, but not in *Pb*. Examination of free glutamine levels in *Pf* parasites treated with MSO and *Pb*GSKO parasites showed a similar decrease in the glutamine levels. However, the free asparagine levels were reduced to the extent of 1.5-2.0 fold in *Pf* parasites, and almost remained unaltered in *Pb*GSKO parasites. Since *Pf* proteins are rich in asparagine, it was examined whether GS inhibition by MSO in *Pf* can lead to eIF2α phosphorylation - a molecular signature leading to the inhibition of protein synthesis. Interestingly, a short-term exposure of *in vitro* cultures to MSO for 6 h could lead to prominent phosphorylation of eIF2α in *Pf*, and no such

phosphorylation could be detected in *Pb*. Proteomic studies suggested that eIF2α phosphorylation was reflected on *Pf* proteome with an overall decrease in protein synthesis that included important asparagine-rich proteins (Figure 7). These findings suggest that *Pf*-specific GS essentiality is due to a unique functional requirement of glutamine for asparagine synthesis that is crucial for asparagine-rich proteins in *Pf*.

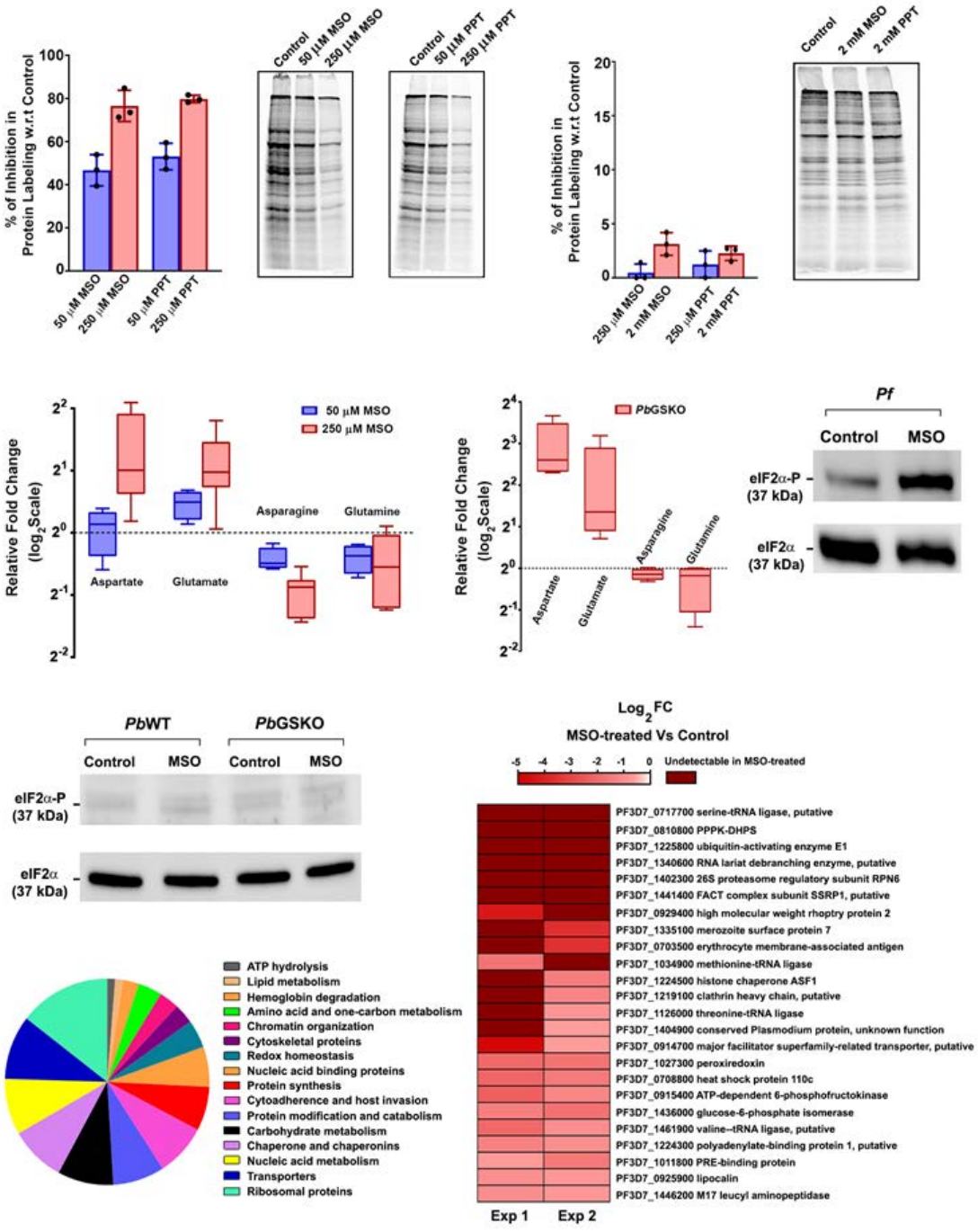

Figure 7: Inhibition of GS affects protein synthesis in *P. falciparum*.

Effect of GS inhibitors on *P. vivax* (*Pv*) and ART-resistant *Pf*Cam3.I^{R539T} parasites

To investigate whether the non-essentiality of GS in *Pb* could be corroborated with human parasite *Pv* whose proteins are not asparagine-rich, *Pf* and *Pv* clinical isolates were treated with MSO and PPT. MSO and PPT could inhibit the growth of *Pf* clinical samples, but not of *Pv*. This in turn confirmed that GS requirement is restricted to *Pf* mainly due to the asparagine-rich nature of its proteins containing asparagine repeats. GS levels are upregulated in ART-resistant *Pf* parasites during ART exposure favoring them to synthesize glutamine for nitrogen storage and prepare for starvation[28]. Interestingly, the exposure of ART or DHA in combination with MSO for 6 h led to ~80% reduction in viable parasites with respect to ART or DHA whereas, the exposure of *Pf*Cam3.I^{R539T} rings to MSO alone could only lead to 10–20% inhibition with respect to the untreated control. Similar results were obtained for PPT although PPT was slightly less effective than MSO (Figure 8). These data suggest the potential of inhibiting GS to combat artemisinin resistance.

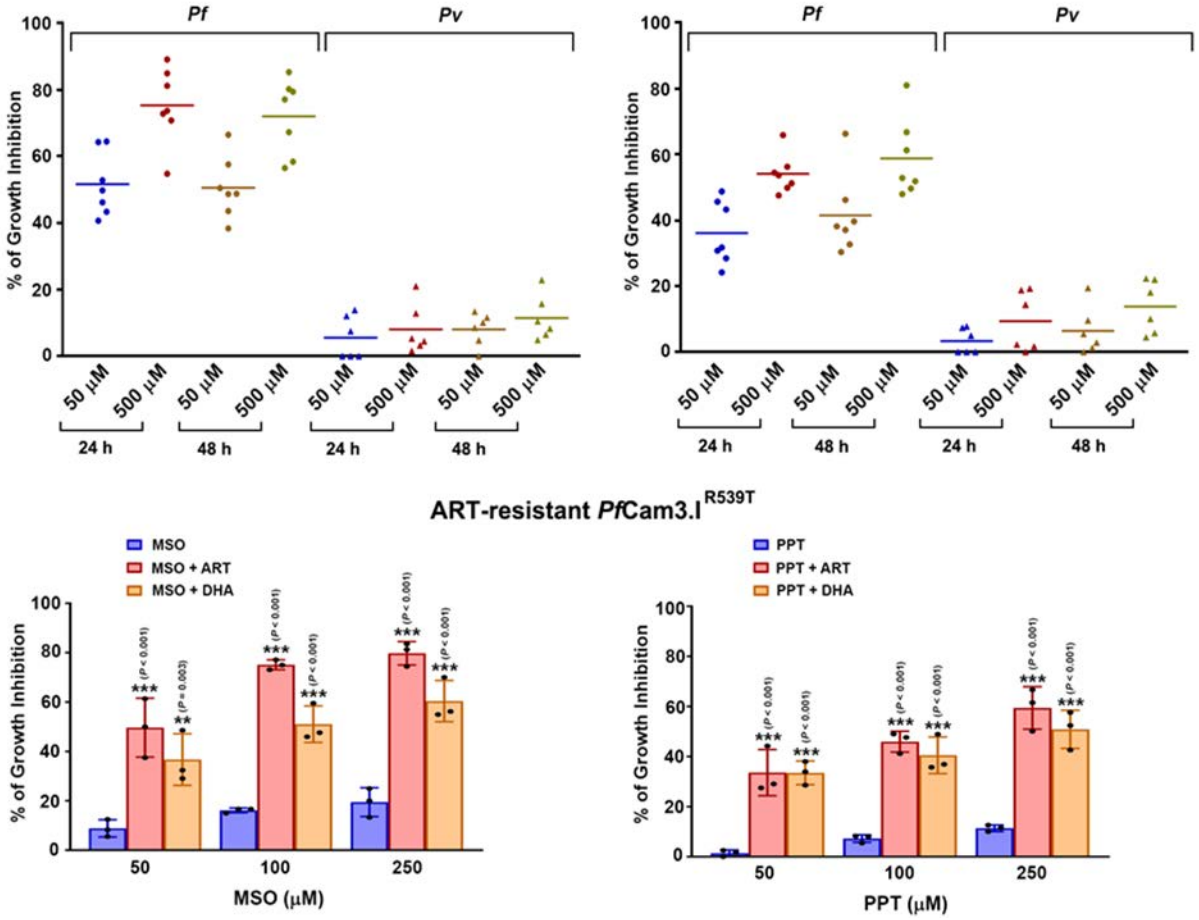

Figure 8: Effect of MSO and PPT on *Pf* and *Pv* clinical isolates and ART-resistant *Pf*Cam3.IR^{539T} strain.

Summary:

The findings suggest that parasite GS has evolved to adapt to the asexual stage metabolism (Figure 9). The asexual stages acquire ~75% of host Hb and degrade it in the FV, releasing amino acids in millimolar concentrations. The parasite utilizes approximately one-fifth of them and effluxes the surplus. Therefore, the absence of feedback inhibition by amino acids could render parasite GS withstanding its activity in the cytosol where there is a continuous release of amino acids from FV. Likewise, the lack of regulation by adenylylation could be a metabolic adaptation. α-ketoglutarate, a TCA cycle intermediate, is a key allosteric regulator of GS and low intracellular concentrations of α-ketoglutarate inactivate GS by bringing out adenylylation[27]. It is known that asexual stages support their rapid growth and proliferation by continuously deriving ATP through glycolysis and the TCA cycle is minimally functional[29]. Most of the TCA cycle enzymes could be deleted without any significant effect on asexual stages. However, TCA cycle is essential for mosquito stage development[30]. The lack of regulation by adenylylation could provide the flexibility for parasite GS to function in all the life cycle stages.

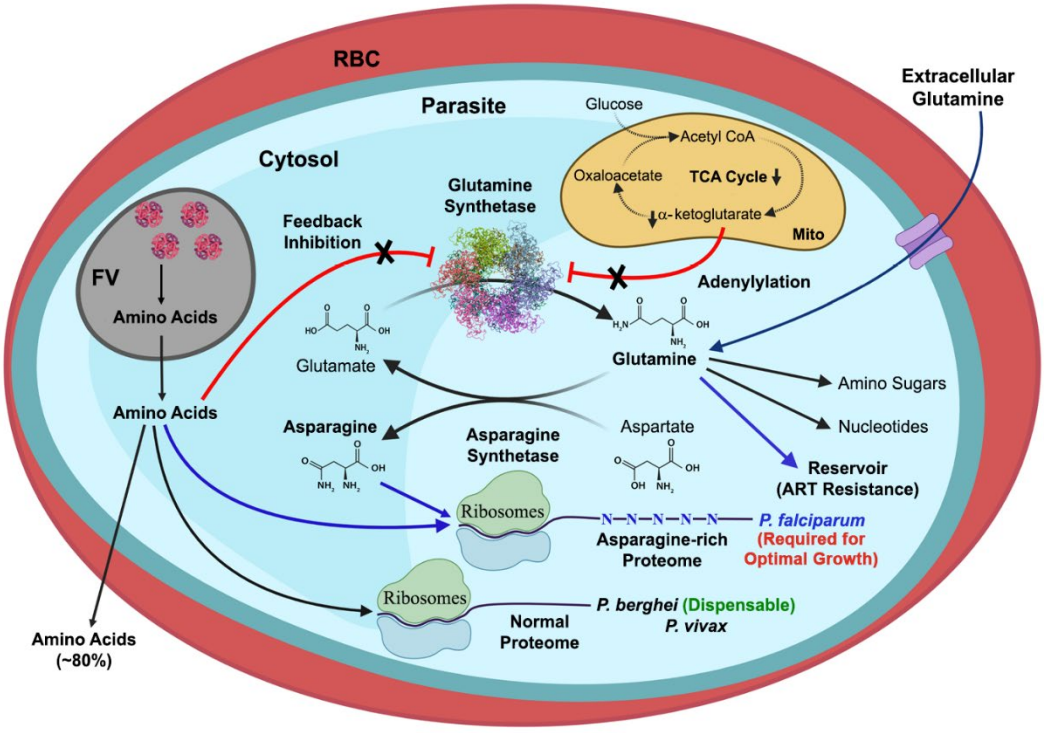

Figure 9: Model depicting the distinct evolution of *Plasmodium* GS and its significance in *P. falciparum.*

Another interesting feature is the presence of two characteristic peptide inserts. The first insert occupying dodecamer channel contributes to the stability of *Pf*GS at febrile temperatures. Parasites are known for extensive adaptations to febrile temperatures, with *Pf* hsp110 stabilizing the asparagine repeat-rich proteins and *Pf*AP2-HS transcription factor protecting the parasites[31,32]. However, deletion of second insert renders parasite GS completely inactive. It would be of interest to examine the compensatory structural rearrangements that have occurred in *Plasmodium* GS to accommodate this insert and their impact on GS activity. It is also possible that this insert is responsible for the lack of feedback inhibition by amino acids. Altogether, the findings shed insights on unique biochemical and structural features of parasite GS that complement the metabolic signature and febrile niche of asexual stage parasites.

Another important finding is the recognition of species-specific differences in the requirement of *Plasmodium* GS. By combining reverse genetics with chemical inhibition studies and by correlating the results with clinical samples, it is shown that GS is required for *Pf* growth, but non-essential for *Pb* and *Pv*. The findings clearly suggest that Hb-derived and extracellular glutamine are inadequate to support optimal *Pf* growth in asexual and sexual stages when the endogenous GS activity is compromised. *Pf* encodes more than 5000 proteins and the unique feature of *Pf* proteins is their asparagine-rich nature that spans across 30% of the proteome[31]. The asparagine-rich proteome imposes a selective burden in *Pf* for glutamine besides its requirement for other metabolic pathways. ART-resistant parasites depend on glutamine as a nitrogen reservoir[28] and exhibit fitness loss in amino acid and nutrient limitation conditions[33,34]. Targeting GS inhibits the emergence of viable ART-resistant parasites upon ART exposure. In summary, GS can serve as a unique target for *Pf* responsible for more than 90% of malaria deaths and ART resistance. The unique features of unusual type I parasite GS can serve as a platform for developing *Plasmodium*-specific GS inhibitors.

References:

1) Nagaraj, V. A. et al. Malaria parasite-synthesized heme is essential in the mosquito and liver stages and complements host heme in the blood stages of infection. PLoS Pathog. 9, e1003522 (2013).

2) Nagaraj, V. A. & Padmanaban, G. Insights on heme synthesis in the malaria parasite. Trends Parasitol. 33, 583–586 (2017).

3) Gratraud, P. et al. Oleic acid biosynthesis in Plasmodium falciparum: characterization of the stearoyl-CoA desaturase and investigation as a potential therapeutic target. PLoS One 4, e6889 (2009).
4) Kloehn, J., Harding, C. R. & Soldati-Favre, D. Supply and demand-heme synthesis, salvage and utilization by Apicomplexa. FEBS J. 288, 382–404 (2020).
5) Hayashi, M. et al. Vacuolar H(+)-ATPase localized in plasma membranes of malaria parasite cells, Plasmodium falciparum, is involved in regional acidification of parasitized erythrocytes. J. Biol. Chem. 275, 34353–34358 (2000).
6) Lin, J. et al. Replication of Plasmodium in reticulocytes can occur without hemozoin formation, resulting in chloroquine resistance. J. Exp. Med 212, 893–903 (2015).
7) Hopp, C. S. et al. Deletion of the rodent malaria ortholog for falcipain-1 highlights differences between hepatic and blood stage merozoites. PLoS Pathog. 13, e1006586 (2017).
8) World Health Organization. WHO Model Prescribing Information: Drugs Used in Skin Diseases
9) Petersen, A. B., Ronnest, M. H., Larsen, T. O. & Clausen, M. H. The chemistry of griseofulvin. Chem. Rev. 114, 12088–12107 (2014).
10) Sahoo, A. K. & Mahajan, R. Management of Tinea corporis, Tinea cruris, and Tinea pedis: a comprehensive review. Indian Dermatol Online J. 7, 77–86 (2016).
11) Liu, K. et al. A metabolomic perspective of griseofulvin-induced liver injury in mice. Biochem. Pharmacol. 98, 493–501 (2015).
12) Oh, Y. J. et al. Cytochrome b5 reductase 1 triggers serial reactions that lead to iron uptake in plants. Mol. Plant 9, 501–513 (2016).
13) Antonny, B., Vanni, S., Shindou, H. & Ferreira, T. From zero to six double bonds: phospholipid unsaturation and organelle function. Trends Cell Biol. 25, 427–436 (2015).
14) Riggle, B. A., Miller, L. H. & Pierce, S. K. Desperately seeking therapies for cerebral malaria. J. Immunol. 204, 327–334 (2020).
15) Varo, R. et al. Adjunctive therapy for severe malaria: a review and critical appraisal. Malar. J. 17, 47 (2018).
16) Francis, S. E., Sullivan, D. J. Jr. & Goldberg, D. E. Hemoglobin metabolism in the malaria parasite Plasmodium falciparum. Annu. Rev. Microbiol. 51, 97–123 (1997).
17) Babbitt, S. E. et al. Plasmodium falciparum responds to amino acid starvation by entering into a hibernatory state. Proc. Natl Acad. Sci. USA 109, E3278–E3287 (2012).
18) Bannister, L. H. & Sherman, I. W. Plasmodium. In: Encyclopedia of Life Sciences (ELS) (John Wiley & Sons, Ltd: Chichester, 2009).

19) Graewe, S., Stanway, R. R., Rennenberg, A. & Heussler, V. T. Chronicle of a death foretold: Plasmodium liver stage parasites decide on the fate of the host cell. FEMS Microbiol. Rev. 36, 111–130 (2012).

20) Cruzat, V., Macedo Rogero, M., Noel Keane, K., Curi, R. & Newsholme, P. Glutamine: metabolism and immune function, supplementation and clinical translation. Nutrients 10, 1564 (2018).

21) Uchida, K., Ohmori, D., Yamakura, F., & Suzuki, K. Changes in free amino acid concentration in the hemolymph of the female *Culex pipiens pallens* (Diptera: Culicidae), after a blood meal. *Journal of medical entomology*, *27*(3), 302–308 (1990).

22) Gill, H. S. & Eisenberg, D. The crystal structure of phosphinothricin in the active site of glutamine synthetase illuminates the mechanism of enzymatic inhibition. Biochemistry 40, 1903–1912 (2001).

23) Janssen, P. J., Jones, W. A., Jones, D. T. & Woods, D. R. Molecular analysis and regulation of the glnA gene of the gram-positive anaerobe Clostridium acetobutylicum. J. Bacteriol. 170, 400–408 (1988).

24) Liaw, S. H., Pan, C. & Eisenberg, D. Feedback inhibition of fully unadenylylated glutamine synthetase from Salmonella typhimurium by glycine, alanine, and serine. Proc. Natl Acad. Sci. USA 90, 4996–5000 (1993).

25) Brown, J. R., Masuchi, Y., Robb, F. T. & Doolittle, W. F. Evolutionary relationships of bacterial and archaeal glutamine synthetase genes. J. Mol. Evol. 38, 566–576 (1994).

26) Okano, H., Hwa, T., Peter Lenz, P. & Yan, D. Reversible adenylylation of glutamine synthetase is dynamically counterbalanced during steady-state growth of Escherichia coli. J. Mol. Biol. 404, 522–536 (2010).

27) Theron, A. et al. Differential inhibition of adenylylated and deadenylylated forms of M. tuberculosis glutamine synthetase as a drug discovery platform. PLoS One 12, e0185068 (2017).

28) Mok, S. et al. Artemisinin-resistant K13 mutations rewire Plasmodium falciparum's intra-erythrocytic metabolic program to enhance survival. Nat. Commun. 12, 530 (2021).

29) MacRae, J. I. et al. Mitochondrial metabolism of sexual and asexual blood stages of the malaria parasite Plasmodium falciparum. BMC Biol. 11, 67 (2013).

30) Ke, H. et al. Genetic investigation of tricarboxylic acid metabolism during the Plasmodium falciparum life cycle. Cell Rep. 11, 164–174 (2015).

31) Muralidharan, V. & Goldberg, D. E. Asparagine repeats in Plasmodium falciparum proteins: Good for Nothing? PLoS Pathog. 9, e1003488 (2013).

32) Tintó-Font, E. et al. A heat-shock response regulated by the PfAP2-HS transcription factor protects human malaria parasites from febrile temperatures. Nat. Microbiol. 9, 1163–1174 (2021).
33) Bunditvorapoom, D. et al. Fitness loss under amino acid starvation in artemisinin-resistant Plasmodium falciparum isolates from Cambodia. Sci. Rep. 8, 12622 (2018).
34) Nair, S. et al. Nutrient limitation magnifies fitness costs of antimalarial drug resistance mutations. Antimicrob. Agents Chemother. 66, e01529–21 (2022).

Copy of one or two specific publications/research papers of the applicant, relevant to the research work on which the award is claimed.

The following two publications relevant to the research work on which the award is claimed are enclosed. The actual size of the pdf versions of these manuscripts are around 7-9 Mb and in addition, there are multiple supplementary files. Because of the allowed size limit of 2.5 Mb for the files to be uploaded, I could only upload the medium-resolution pdf files where the image qualities may be slightly compromised. High-resolution figures and supplementary files shall be accessed through the journal page and the online links are provided below.

1) Chandana et al. Malaria parasite heme biosynthesis promotes and griseofulvin protects against cerebral malaria in mice (2022). Nature Communications, 13, 4028. https://doi.org/10.1038/s41467-022-31431-z
2) Ghosh et al. Distinct evolution of type I glutamine synthetase in *Plasmodium* and its species-specific requirement (2023). Nature Communications, 14, 4216. https://doi.org/10.1038/s41467-023-39670-4

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