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RESEARCH ARTICLE

# Heme Detoxification Protein (*Pf*HDP) is essential for the hemoglobin uptake and metabolism in *Plasmodium falciparum*

**Priya Gupta**[1] | **Rajan Pandey**[2] | **Vandana Thakur**[3] | **Sadaf Parveen**[1] | **Inderjeet Kaur**[1] | **Ashutosh Panda**[1] | **Rashmita Bishi**[1] | **Sonali Mehrotra**[1] | **Asif Akhtar**[1] | **Dinesh Gupta**[2] | **Asif Mohmmed**[3] | **Pawan Malhotra**[1]

[1]Malaria Biology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India

[2]Translational Bioinformatics Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India

[3]Parasite Cell Biology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India

**Correspondence**
Pawan Malhotra, Malaria Biology Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi-110067, India.
Email: pawanm@icgeb.res.in; pawanmal@gmail.com

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## Abstract

Hemoglobin degradation is crucial for the growth and survival of *Plasmodium falciparum* in human erythrocytes. Although the process of Hb degradation has been studied in detail, the mechanisms of Hb uptake remain ambiguous to date. Here, we characterized Heme Detoxification Protein (*Pf*HDP); a protein localized in the parasitophorus vacuole, parasite food vacuole, and infected erythrocyte cytosol for its role in Hb uptake. Immunoprecipitation of *Pf*HDP-GFP fusion protein from a transgenic line using GFP trap beads showed the association of *Pf*HDP with Hb as well as with the members of PTEX translocon complex. Association of *Pf*HDP with Hb or *Pf*exp-2, a component of translocon complex was confirmed by protein–protein interaction and immunolocalization tools. Based on these associations, we studied the role of *Pf*HDP in Hb uptake using the *Pf*HDP-HA-GlmS transgenic parasites line. *Pf*HDP knockdown significantly reduced the Hb uptake in these transgenic parasites in comparison to the wild-type parasites. Morphological analysis of *Pf*HDP-HA-GlmS transgenic parasites in the presence of GlcN showed food vacuole abnormalities and parasite stress, thereby causing a growth defect in the development of these parasites. Transient knockdown of a member of translocon complex, *Pf*HSP101 in HSP101-DDDHA parasites also showed a decreased uptake of Hb inside the parasite. Together, these results advocate an interaction between *Pf*HDP and the translocon complex at the parasitophorus vacuole membrane and also suggest a role for *Pf*HDP in the uptake of Hb and parasite development. The study thus reveals new insights into the function of *Pf*HDP, making it an extremely important target for developing new antimalarials.

**Abbreviations:** Exp-2, exportin2; Hb, hemoglobin; HDP, Heme Detoxification Protein.

## 1 | INTRODUCTION

*Plasmodium falciparum* is a leading cause of malaria with 241 million malaria cases and 627,000 (12% more than in 2019) deaths reported worldwide in the year 2020 (World malaria report 2021). Although drug therapies and vector control mechanisms for the containment of the disease have been developed, eradication of malaria has not been achieved yet.[2] The spread of resistance against the available antimalarials and unavailability of a highly efficacious vaccine poses a greater challenge to eradicate this dreaded disease. WHO recommended the use of RTS,S malaria vaccine to prevent malaria in children living in malaria endemic regions (World malaria report 2021).[1] Early resistance against the artemisinin combination therapies, a present-day front-line therapy, had been reported in western Cambodia, later spreading across an expanding area of the Greater Mekong subregion.[3] Hence, there is an urgent need to identify new drug targets and molecules that can be targeted to develop effective vaccines to prevent the disease spread.

During the asexual stage of life cycle inside the human erythrocyte, the parasite digests Hb in a specialized organelle, the food vacuole. Hb is digested by the sequential action of a complex of proteases including plasmepsins, falcipains, and falcilysin inside the food vacuole.[4–7] These proteases cleave Hb to short peptides and finally to amino acids.[8] The free toxic by-product, heme generated during the process is subsequently converted into an inert insoluble polymer; hemozoin.[9–11] *Pf*HDP (Heme Detoxification Protein) has been shown to be extremely potent in converting heme to hemozoin.[12] *Pf*HDP, a food vacuole-associated protein, possesses two heme-binding sites and a Hb-binding site[13] and is a part of a ~200 kDa complex with other proteins including falcipain 2/2, Plasmepsin II, Plasmepsin IV, and histo-aspartic protease inside the food vacuole.[14] The interaction of *Pf*HDP and Hb paved the way for a hypothesis suggesting that *Pf*HDP might be playing some role in the uptake of Hb from the erythrocyte cytoplasm.[13]

Although the mechanism of Hb degradation has been extensively studied; the mechanisms of uptake of the Hb from infected erythrocyte cytosol to the parasite remain poorly understood. Four distinct pathways have been proposed to assist in the uptake of Hb.[15,16] The uptake begins with the folding of parasites around erythrocyte cytoplasm followed by the development of vesicles and cytostome that continue uptake of Hb inside the parasite. After this step, phagosomes appear, which assist in the trafficking of Hb. Finally, cytostomal invaginations elongate to form tubes that connect to the digestive vacuole at one end and to the parasite surface at the other end, opening to the erythrocyte cytosol.[15,16] Despite all the microscopic evidence available, the molecules and adaptors that participate in delivering Hb to the food vacuole hold ambiguity. Based on immuno-electron microscopy, *Pf*HDP was shown to be present in the vesicles that traffic Hb from the erythrocyte to the food vacuole of the parasite.[12] The trafficking (outward-inward) of *Pf*HDP does not follow the classical secretory pathway and is not affected by brefeldin A treatment.[12] *Pf*HDP lacks a classical N-terminal signal sequence or PEXEL motif, which usually assists in the sorting and transporting of any protein to a destined site using the translocon complex. The translocon complex consisting of PTEX150, Exportin 2, PTEX88, HSP101, and Trx2, is used by the parasite to export proteins from the parasite to the erythrocyte cytoplasm.[17] However, there is no evidence available for the outbound trafficking mechanisms of *Pf*HDP.

In this study, we unravel one of the pathways involved in the uptake of Hb from the erythrocyte cytosol. Downregulation of *Pf*HDP in the *Pf*HDP-HAGlmS transgenic parasites led the parasites to take up less Hb from the erythrocyte and induced parasite stress. Immunoprecipitation of parasite cell lysates using GFP trap beads showed an association of *Pf*HDP with Hb and with the components of the translocon complex, such as exportin-2, PTEX150 and HSP101. In silico and in vitro protein–protein interaction studies confirmed the association of *Pf*HDP with *Pf*exp-2. Hence, we propose that *Pf*HDP participates in the uptake of Hb from erythrocyte. Functional insights into the role of *Pf*HDP can help us design better inhibitors targeting both the heme and Hb-binding *Pf*HDP domains.

## 2 | METHODS

### 2.1 | Maintenance of *P. falciparum* cultures and transfection

The *P. falciparum* parasite line 3D7 was maintained as described previously.[18] To generate a GFP overexpressing transfection vector construct, the entire open reading frame of *Pf*HDP was amplified using HDPGFP-FP 5′GCAGATCTTTTTTCATCAGTATGAAAAAT AGATTTTATTAT 3′ and HDPGFP-RP 5′GC CCTAGGAAAAATGATGGGCTTATCTACTATAT3′ primer set and cloned into the pSSPF2 vector to create a C terminal *Pf*HDP-GFP fusion protein under the control of *hsp86* promoter. *P. falciparum* 3D7 ring-stage parasites were transfected with 100 µg of plasmid DNA by electroporation (310 V, 950 µF) and the transfected parasites were selected using 2.5 nM blasticidin.[19] The expression of the *Pf*HDP-GFP fusion protein in transgenic *P. falciparum* blood-stage parasites was examined by western blotting

and immunofluorescence. Protein bands were visualized using an ECL kit (Thermo Scientific).

For transfection of knockdown constructs, C-terminal region of *Pf*HDP was amplified using gene-specific primers, HDP GlmSHA FP 5′GCAGATCTTTGAACATA AGCCTGTAAAAAGGA C 3′ and HDP GlmSHA RP 5′ GCCTGCAGAAA AATGATGGGCTTATCTACTAT3′ and cloned into the transfection vector pHA-glmS using *Pst*I and *Bgl*II restriction sites to create a fusion of desired gene of interest (GOI) with HA-glmS at the 3′ UTR under the control of native promoter. The ring-stage parasites were transfected as mentioned and transgenic parasites were selected on alternate WR22910 drug ON and OFF cycles to ensure genomic integration of *Pf*GOI-HA-glmS constructs. The transgenic parasites were then subjected to clonal selection by serial dilution to obtain parasite line from a single genome integrated clone. The integration was checked by PCR amplification of genomic fragments using different sets of primers: (1) (a/b) HDP GlmSHA FP/ HDP GlmSHA RP (2) (c/d) HDPint check 5′ GTAGAATGTATTTTTCATCAGT3′/ HAintcheck 5′TACGGATACGCATAATCGG3′.

## 2.2 | Cloning, recombinant expression, and purification of exportin 2 protein

The forward and reverse primers used for the cloning of C-terminal *Plasmodium falciparum* exportin 2 (*Pf*exp-2) were 5′ CCCCATGGGATCCATGAACAATTAAAGATATTTA 3′ and 5′ GCGCGGCCGCTTCTTTATTTTCATCTTTTTT 3′, respectively. The PCR product of these primers was cloned into a pJET vector and subsequently subcloned into a pET28b vector. The gene cloned in the pET-28b vector was expressed in codon+ *Escherichia coli* cells and protein localized to inclusion bodies, which were isolated as described. Briefly, the inclusion bodies were solubilized in an 8 M urea buffer (500 mM Tris, 150 mM NaCl). The suspension was incubated for 1 h at room temperature (RT) and then centrifuged at 12,000 × *g* for 30 min at RT. The supernatant containing solubilized protein was kept for binding with Ni-NTA$^{+}$ resin overnight at RT with constant shaking. After binding, the suspension was packed in a purification column, and flow-through was collected. The resin was washed with an 8 M urea buffer containing 10 mM imidazole. Bound protein(s) was eluted in an 8 M urea buffer containing different concentrations of imidazole. Eluted protein fractions were analyzed by 12% SDS-PAGE. The eluted fractions containing the purified protein, were pooled and concentrated. The refolding method was adopted from a standard universal protocol.[20] The protein was refolded gradually by decreasing the urea concentration (6, 4, 2, 1, 0 M) in the refolding buffer (0.05 M Tris, pH 8, 1 mM EDTA, 0.5 M arginine, 0.4 mM Triton X-100, 1 mM reduced glutathione, 0.5 mM oxidized glutathione). The refolded proteins were concentrated and dialyzed against 0.05 M Tris, pH 8, and 0.15 M NaCl and stored at −80°C. The purified protein was analyzed on a 15% SDS polyacrylamide gel followed by western blotting with anti-His HRP antibody.

## 2.3 | Generation of antibodies against recombinant *Pf*HDP and *Pf*exp-2

Antibodies against recombinant *Pf*HDP and *Pf*exp-2 were raised in mice and rabbit. For this, 5- to 6-week-old female BALB/c mice were immunized with 25 μg of recombinant HDP protein emulsified in Freund's complete adjuvant on day 0 followed by three boosts of proteins emulsified with Freund's incomplete adjuvant on days 14, 28, and 42. The animals were bled for serum collection on day 49. In case of rabbits, New Zealand white female rabbits were immunized with 200 μg of recombinant *Pf*HDP and *Pf*exp-2, respectively, emulsified in Freund's complete adjuvant on day 0 followed by three boosts emulsified with Freund's incomplete adjuvant on days 21, 42, and 63. The animals were bled for serum collection on day 70. The antibody titer in serum samples was quantified by enzyme-linked immunosorbent assay (ELISA).

### 2.3.1 | In vitro protein–protein interaction analysis

ELISA-based protein–protein interaction analysis was performed as described previously.[21] Briefly, a 96-well microtiter plate was coated overnight at COC with 50 ng recombinant *Pf*HDP protein. Another unrelated *Plasmodium falciparum* recombinant protein, Ddi was coated as a negative control. After blocking the wells with 5% milk in PBS for 2 h, recombinant *Pf*exp-2 was added in different amounts ranging from 0 to 100 ng, and the plate was incubated for 3 h at 37°C. The interaction was detected using antibodies against exportin 2 (1:500). Incubation with HRP-conjugated anti-rabbit antibodies (1:3000) was done for 1 h and quantified after adding the substrate OPD by measuring the resulting absorbance at 490 nm.

Far western assays were performed according to the protocol described earlier.[22] Briefly, 1–5 μg of recombinant *Pf*HDP and an unrelated *Plasmodium* protein *Pf*MLH/ MBP were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The proteins were first denatured and then renatured on the membrane itself. The membranes were blocked with 5% skimmed milk and incubated with 2 μg/ml of purified interacting bait proteins,

that is, Hb and exp 2 in protein-binding buffer (100 mM NaCl, 20 mM Tris [pH 7.6], 0.5 mM EDTA, 10% glycerol, and 1 mM DTT) for 2 h at room temp. After washing the non-specific proteins, membranes were incubated with rabbit anti- Hb/ anti-exp-2 followed by incubation with HRP-conjugated anti-rabbit antibodies (1:100000) for 1 h at RT. Finally, membranes were imaged with a Biorad ECL chemidoc.

For in vitro co-immunoprecipitation, 1 µg of each protein *Pf*HDP and Hb was incubated together at room temperature for 2 h in a reaction volume of 100 µl containing 1× binding buffer (50 mM phosphate buffer at pH 7.0, 75 mM NaCl, 2.5 mM EDTA at pH 8.0, and 5 mM $MgCl_2$), 0.1% Nonidet P 40, and 10 mM DTT. The reaction mix was incubated for 2 h at 4°C with 20 µl of pre-equilibrated Protein A/G-conjugated antibody beads. The beads were centrifuged at 1000× g for 5 min, washed with 200 µl of binding buffer containing 400 mM NaCl, boiled for 5 min in SDS/PAGE reducing loading buffer, electrophoresed, immunoblotted, and probed. Protein A/G beads conjugated to preimmune sera were used as a negative control.

The Surface Plasmon Resonance analysis was carried out on the Biacore T200 instrument (GE Healthcare). Over 9500 Response Units of the *recombinant* HDP protein were immobilized on S-Series CM5 sensor chip (GE Healthcare) using 10 mM sodium acetate pH 4.0 solution (GE Healthcare). The surface of the sensor chip was blocked with 1 M ethanolamine-HCl pH 8.5 (GE Healthcare). Recombinant *Pf*exp-2/Hb at increasing concentrations was injected over the immobilized HDP and on the reference flow cell at a flow rate of 20 µl $min^{-1}$. The kinetic parameters of the interaction and binding responses in the steady-state region of the sensogram were analyzed using Biacore evaluation software, version 4.1.1 (GE Healthcare).

## 2.4 | Indirect immunofluorescence assay

Briefly, thin smears of parasite cultures were made on a glass slide and fixed with chilled methanol at −80°C. Slides were blocked in blocking buffer (PBS, 3% BSA) for 2 h at 37 °C. Immunostaining was performed using primary antibodies (anti-*Pf*HDP antibody 1:100, anti-Hb antibody 1:100, anti-*Pf*exp-2 antibody 1:50, anti-*Pf*PTEX150 antibody 1:50) and appropriate secondary antibody Alexa flour 594 goat anti-mice (1:500) and Alexa flour 488 goat anti-rabbit (1:500). For liquid staining, the parasite samples were fixed in 4% paraformaldehyde/glutaraldehyde. The fixed samples were permeabilized using 0.1% triton X100. The cells were blocked in 10% FBS for 2 h at RT. Immunostaining was then performed using primary antibodies overnight at 4°C. Appropriate secondary antibody, Alexa flour 594 goat anti-mice (1:500) and Alexa flour 488 goat anti-rabbit (1:500) was then added to stain the parasites for 1 h at RT. The nucleus of the parasites was stained using DAPI. For imaging, a drop of the suspension was taken on a slide and viewed under the microscope.

The transgenic parasite suspension was incubated with DAPI (2 ng/ml) in PBS at RT for 10 min and parasites were observed under a microscope to visualize the GFP expression. The images were captured using a Nikon A1 Confocal Microscope and exported as 8-bit RGB files. Images were analyzed using Nikon NIS Elements v 4.0software. Imaris image was created using the software IMARIS v 4.

## 2.5 | Immunoprecipitation reaction

Immunoprecipitation experiments were performed using the Pierce Crosslink Immunoprecipitation Kit (Product #26147). Briefly, synchronized *Plasmodium falciparum* mid-late trophozoites were enriched from the uninfected population using density-based percoll treatment. The parasite pellet obtained was treated with Streptolysin O to lyse the erythrocyte membrane. The pellet containing the parasite surrounded by the parasitophorous vacuolar membrane was washed with PBS until lysis of RBC stopped by centrifugation at 15,000 × g for 1 min and the pellet was then resuspended in PBS. The parasite pellet was then lysed using the RIPA buffer (250 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol: pH 7.4) containing protease and phosphatase inhibitor cocktails (Roche) for 30 min at 4°C with intermittent mixing. The lysate was clarified by centrifugation at 15,000 × g for 30 min. The supernatant protein concentration was determined by the BCA Protein estimation assay kit (Pierce) using BSA standards as reference. The SLO-treated trophozoite stage lysate of *Pf*HDP-GFP transgenic parasites was immunoprecipitated using GFP-Trap®_A Kit (Chromotek)following the manufacturer's instructions. GFP-Trap®_A beads were allowed to bind to parasite lysate by tumbling the tube end-over-end. Proteins were eluted in a 50 µl elution buffer. Proteins in the immunoprecipitated samples were digested by in-solution trypsin digestion. Samples were brought to a final volume of 100 µl in 50 mM Ammonium Bicarbonate (Sigma) buffer to adjust the pH to 7.8, reduced with 10 mM DTT for 1 h at RT followed by alkylation with 40 mM Iodoacetamide (Sigma) for 1 h at RT under dark conditions. Proteins were digested by the addition of Promega sequencing grade-modified trypsin (V511A) at a ratio 1:50 (w/w) of trypsin: protein. For complete digestion, samples were placed in a water-bath at 37°C for 16 h. After digestion, extracted peptides were acidified with 0.1% formic acid and analyzed by mass spectrometry. 3D7

parasites treated using the same protocol were used as a negative control.

## 2.6 | Conditional knockdown assay

The functional role of *Pf*HDP was determined by knocking down the HDP mRNA with glucosamine. The effect of knockdown on parasite invasion was evaluated with 3D7 strain of *P. falciparum* as the control. The parasite lines (*Pf*HDP-HA-GlmS transgenic and 3D7) were synchronized using 5% sorbitol and the growth assay was set at the mid-ring stage with a hematocrit and parasitemia of synchronized ring stage culture adjusted to 2% and 1%, respectively. Glucosamine was added to the parasite culture at varying concentrations (0, 1.25, 2.5, and 5 mM). Parasite growth was monitored microscopically by Giemsa-stained smears. The parasitemia was estimated after an incubation of 40 h in the next cycle and also in the second cycle using flow cytometry. Briefly, cells from samples were pelleted and washed with PBS followed by staining with ethidium bromide (10 μg/ml) for 20 min at 37°C in the dark. The cells were subsequently washed twice with PBS and analyzed on FACS calibur (Becton Dickinson) using the Cell Quest software. Fluorescence signal (FL2) was detected with the 590 nm band pass filter using an excitation laser of 488 nm collecting 100,000 cells per sample. Uninfected RBCs stained in a similar manner were used as control. Following acquisition, data were analyzed for percentage parasitemia of each sample by determining the proportion of FL2-positive cells using Cell Quest.

## 2.7 | Protein–protein docking analysis

PlasmoDB release (release 48) was used to retrieve sequences of *Pf*exp-2 and *Pf*HDP proteins.[23] The predicted 3D model of *Pf*HDP was used as reported previously.[13] The cryo-EM structure of *Pf*exp-2 has been resolved,[24] hence its corresponding PDB structure (6E10.pdb) was retrieved from the RCSB PDB. The protein–protein docking was performed using PatchDock based on shape complementarity principles.[25] Energy refinement was performed for top 100 docked conformations using FireDock.[26] Different conformations showing global energy ≤−5.0 kcal/mol for *Pf*exp-2-*Pf*HDP complexes were further analyzed. PyMol was used to visualize protein complexes and generate images (https://pymol.org/2/). DIMPLOT was used to retrieve and visualize residues within 4 Å of interacting docked *Pf*exp- 2-*Pf*HDP complexes.[27] Protein interaction calculator (PIC) was used to identify *Pf*exp-2-*Pf*HDP interactions which recognize various kinds of interactions, such as disulfide bonds, hydrophobic interactions, ionic interactions, hydrogen bonds, aromatic-aromatic interactions, aromatic-sulfur interactions, and cation–π interactions within a protein or between proteins in a complex.[28]

# 3 | RESULTS

## 3.1 | *Pf*HDP interacts with Hb as well with the members of *Plasmodium* translocon complex

To identify the protein(s) associated with *Pf*HDP, we generated a parasite line expressing C- terminal GFP-tagged HDP in *Plasmodium falciparum*. *Pf*HDP-GFP was expressed in an episomal construct; pSSPF2 vector (Figure S1A–D) with GFP tag at 3′ end under the control of HSP86 promoter. Western blot analysis using anti-GFP antibodies recognized two bands in *Pf*HDP-GFP in transgenic parasite lysates, one corresponding to *Pf*HDP-GFP ~50 kDa and the other at ~25 kDa. The lower band could be a result of processing of HDP-GFP in the lysate samples (Figure S1E). Indirect immunofluorescence assay of transgenic *Pf*HDP-GFP asexual blood stages showed that *Pf*HDP is localized in the punctate structures trafficked to the food vacuole (Figure S1A). Co-staining of these transgenic parasites with BODIPY ceramide stain followed by live-cell imaging revealed that the punctate structures corresponding to *Pf*HDP-GFP are present at the parasitophorus vacuole membrane as well as near the food vacuole indicating that *Pf*HDP is trafficked to the food vacuole as well as to the erythrocyte cytoplasm (Figure 1B). To ascertain whether *Pf*HDP-GFP was trafficked to the erythrocyte cytosol, we fractionated the transgenic parasites using streptolysin O to separate the parasite and erythrocyte cytoplasm fractions. Western blot analysis of both fractions using anti-GFP antiserum recognized bands at ~50 kDa and ~25 kDa in parasite cytoplasm and a band at ~25 kDa in erythrocyte cytoplasm (Figure S1F). Together, these results demonstrated that *Pf*HDP-GFP was trafficked to the erythrocyte cytosol besides being transported to the food vacuole for the hemozoin formation. Western blot analysis of Streptolysin O treated fractions of infected erythrocytes and immunofluorescence assays using anti-*Pf*HDP antibodies further validated that *Pf*HDP is present both inside the parasite as well as in the erythrocyte cytoplasm (Figure S2A,B).

We next examined the *Pf*HDP-GFP interactome in the *Pf*HDP-GFP parasite line using a GFP pull-down assay. Briefly, *Pf*HDP-GFP protein was pulled down from cell lysates together with interacting partners, if any, using GFP-Trap beads. Bound and eluted proteins were digested with trypsin and the released peptides were analyzed by mass spectrometry to identify the interacting partners. In addition

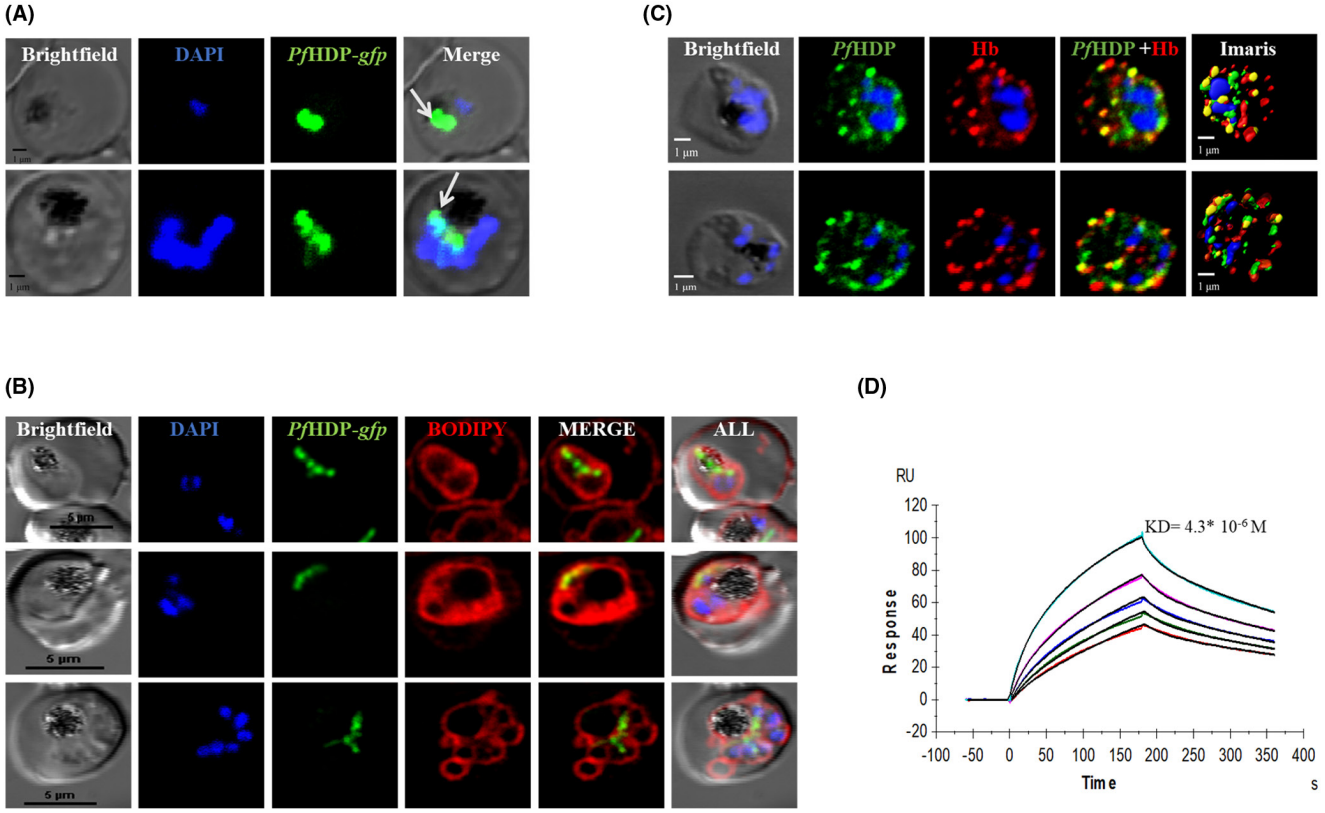


**FIGURE 1** (A) Subcellular localization of *Pf*HDP in *Pf*HDP-GFP transgenic lines. The image shows *Pf*HDP is trafficked in punctate vesicle-like structures in the parasite (B) The BODIPY-TR ceramide stain, which stains the lipid membranes shows *Pf*HDP-GFP are trafficked to the parasite plasma membrane (C) *Pf*HDP colocalizes with Hb, both inside the parasite as well as in the cytoplasm of erythrocyte (Pearson coefficient co-relation - 0.8) (D) *Pf*HDP interacts with Hb in an SPR experiment. The interaction is a two-state reaction, and the observed dissociation constant is 4.3* $10^{-6}$ M for the reaction.

**TABLE 1** List of proteins pulled down by GFP- Trap beads from lysates of *Pf*HDP-GFP parasites from *P. falciparum*

| Gene ID | Protein | MW (kDa) | Peptides |
|---|---|---|---|
| PF3D7_1446800 | Heme Detoxification Protein (HDP) | 24.3 | 3 |
| PF3D7_1471100 | Exported protein 2 (EXP-2) | 33.4 | 8 |
| PF3D7_1345100 | Thioredoxin 2 (TRX2) | 18.6 | 1 |
| PF3D7_1105600 | Translocon component PTEX88 (PTEX88) | 90.7 | 1 |
| PF3D7_1116800 | Heat shock protein 101 (HSP101) | 102.8 | 4 |

to the food vacuole proteases like Plasmepsin and falcipain 2, which are already shown to be a part of hemozoin formation complex,[14] we found several proteins in the pull-down results including Hb as an interacting protein of *Pf*HDP. Additionally, members of the translocon complex including *Pf*exp-2, PTEX150, and PTEX88 were identified in the immunoprecipitate (Table 1, Figure S3). None of these proteins were pulled down from the lysates of *P. falciparum* 3D7, which served as a negative control. Together, these results suggested an association of *Pf*HDP with Hb as well as with the components of the translocon complex.

To provide further evidence(s) for the association between Hb and *Pf*HDP, co-localization and in vitro protein–protein interaction studies such as co-immunoprecipitation and far-western analysis were performed. For the far-western analysis, recombinant HDP protein (5 µg) that served as a bait was resolved on the SDS-PAGE gel and Hb (2 µg/ml) was allowed to interact with bait protein on the nitrocellulose membrane. The bound protein was probed with anti-Hb antibody. A high-affinity interaction between *Pf*HDP and Hb was observed (Figure S4A). *Pf*MLH, an unrelated nuclear protein (Tarique et al, 2012), was

used as a negative control, which did not show any binding with Hb. Second, co-immunoprecipitation analysis between *Pf*HDP and Hb was carried out using anti-rabbit HDP antiserum. As seen in Figure S4B, anti-HDP antisera could pull down Hb from a mixture of the two proteins as analyzed by western blot analysis using anti-human Hb antibody. Recombinant ClpQ protein (Jain et al. 2013), a mitochondrial protein, was used as a negative control. Immunolocalization studies were next performed to localize *Pf*HDP and human Hb protein together in trophozoite stage parasites using anti-*Pf*HDP and anti-Hb antibodies. *Pf*HDP co-localized considerably with human Hb with a Pearson coefficient of 0.81, both inside the parasite as well as in the erythrocyte cytosol (Figure 1C). We further analyzed the kinetics of the binding of *Pf*HDP to Hb using Surface Plasmon Resonance. The recombinant HDP protein was immobilized on a CM5 SPR chip and Hb was allowed to interact with the immobilized protein at different concentrations ranging from 31.125 to 500 µg/ml. The sensogram showed a dose-dependent increase in binding of the Hb with time to the immobilized *Pf*HDP protein with an equilibrium dissociation constant of 4.3* $10^{-6}$ M (Figure 1D). The binding followed a two-state reaction suggesting more than one binding site for binding of *Pf*HDP to Hb.

To validate the interaction of components of translocon complex with *Pf*HDP, co-localization studies were performed by immunostaining trophozoite stage parasites with the respective antibodies. Immunofluorescence assay showed that *Pf*HDP co-localized with the two components of the translocon complex: *Pf*exp-2 and *Pf*PTEX150, at the parasitophorus vacuolar membrane (Figure 2A). The Pearson coefficient of the correlation was found to be above ~0.6. Together, these results point towards a likely transient interaction of the *Pf*HDP with the translocon complex at the parasitophorus vacuole membrane.

## 3.2 | *Pf*HDP interacts with exportin 2 at the parasitophorus vacuolar membrane

We next sought to analyze the interaction of *Pf*HDP with one of the members of the translocon complex; *Pf*exp-2 that has been suggested to be a pore-forming protein on the parasitophorus vacuolar membrane.[29,30] To study the interaction between *Pf*HDP and *Pf*exp-2, we cloned and expressed a C-terminal fragment of *Pf*exp-2 encompassing amino acids 139–285 in *E. coli* (Figure S5A). The full-length protein could not be expressed in *E.coli*. The protein was purified to near homogeneity using the Ni-NTA$^{+}$ column. The purified protein migrated at a molecular size of ~20 kDa as seen in Coomassie-stained SDS-PAGE and western blot experiment performed using anti-His-HRP antibody (Figure S5B,C). Specific antibodies were generated against recombinant *Pf*exp-2 protein in rabbit. The antisera recognized a band of ~33 kDa in the 3D7 parasite cell lysate, which corresponds to the monomeric form of native *Pf*exp-2 (Figure S5D).

We further employed in vitro protein–protein interaction tools such as in vitro ELISA-based protein-binding assay and far-western-binding analysis to assess *Pf*HDP interaction with *Pf*exp-2. Recombinant *Pf*HDP protein interacted with *Pf*exp-2 in a concentration-dependent manner in an ELISA experiment (Figure S5E). A nonspecific recombinant *Pf*Ddi protein (unpublished) was used as a negative control, which showed no significant interaction with *Pf*exp-2. For the far-western-binding analysis, recombinant *Pf*HDP protein was used as a bait and *Pf*exp-2 was allowed to interact with bait protein on the nitrocellulose membrane and an interaction if any was probed with anti-exp-2 antibody. A nonspecific, purified MBP protein was used as a negative control. As evident in Figure 3B, *Pf*HDP interacted specifically with *Pf*exp-2 (Figure 2B). To know whether *Pf*HDP interacts with *Pf*exp-2 in the cell, parasite lysate from *Pf*HDP-GFP transgenic line was immunoprecipitated using GFP-TRAP column and elutes from the GFP pull-down assay were analyzed by western blot analysis using anti-*Pf*exp-2 antibody. The parasite lysate from 3D7 parasite line was used as a negative control. As seen in Figure S4F, *Pf*HDP-GFP fusion protein interacted with *Pf*exp-2, which was detected in the western blot analysis, whereas 3D7 lysate did not show any band corresponding to *Pf*exp-2.

To study the kinetics of the binding of *Pf*HDP to *Pf*exp-2, the Surface Plasmon Resonance analysis was performed by immobilizing the recombinant HDP protein on a CM5 chip using EDC-NHS coupling. Recombinant *Pf*exp-2 was allowed to interact with the immobilized protein at different concentrations ranging from 0.625 µM to 10 µM. The sensogram showed a dose-dependent increase in binding of the *Pf*exp-2 with time to the immobilized *Pf*HDP, with an equilibrium dissociation constant of 1.1* $10^{-6}$ M (Figure 2C). Together, these binding studies unequivocally suggested an interaction between *Pf*HDP and *Pf*exp-2.

We further carried out in silico interaction analysis using the already known structure of the malaria translocon complex, 6E10.pdb and HDP structure model to identify the potential interaction sites for the *Pf*exp-2 and *Pf*HDP. In silico docking analysis of *Pf*exp-2 monomer with *Pf*HDP using PatchDock and energy refinement on top 100 conformations were carried out using default parameters. Next, the docked conformations, with global-binding energy better than −5 kcal/mol were analyzed. Interestingly, the docking analysis showed that *Pf*HDP binds to the multiple sites of *Pf*exp-2, which includes

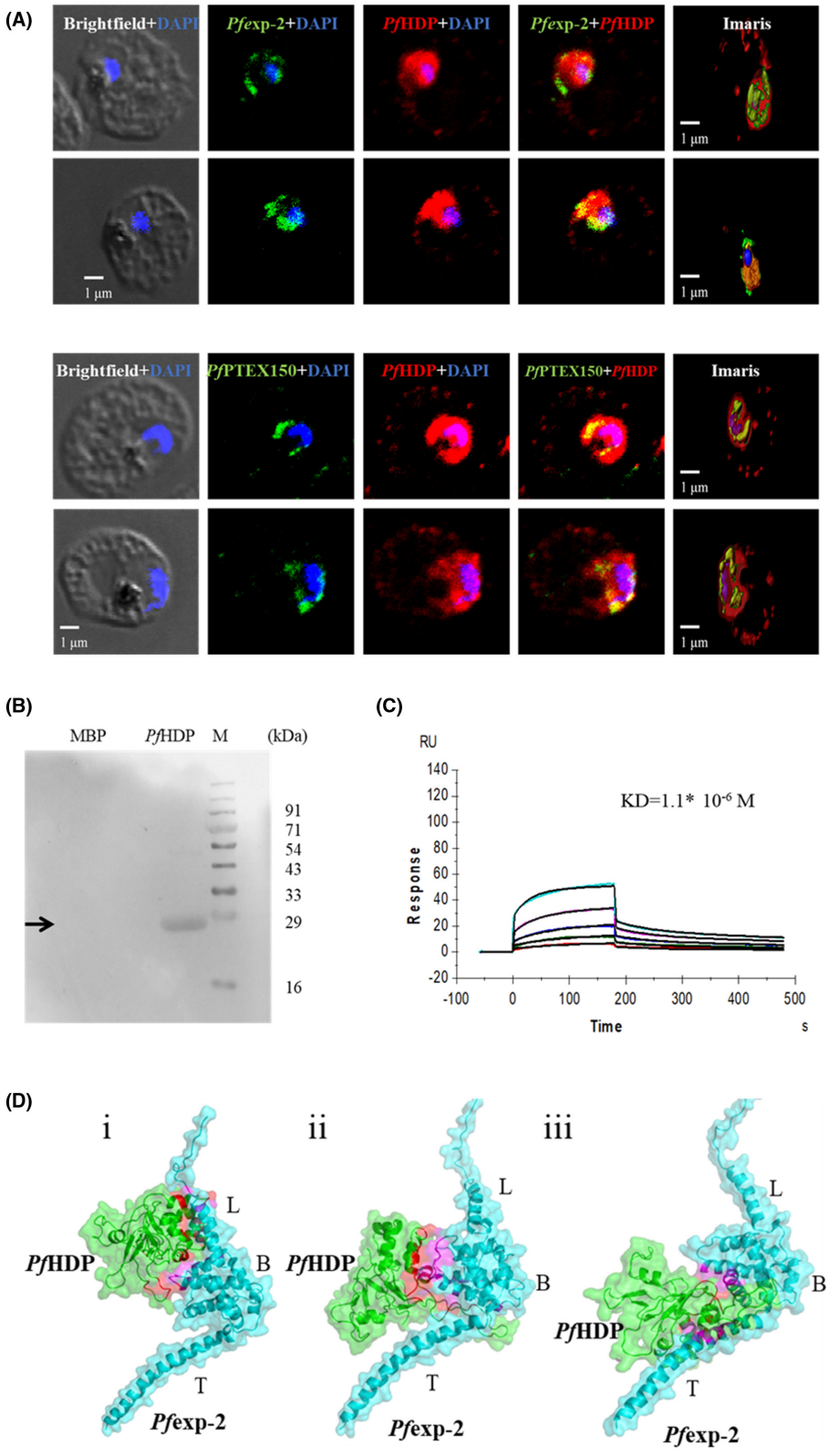


**FIGURE 2** (A) *Pf*HDP colocalizes with the members of translocon components; *Pf*exp-2 and *Pf*PTEX150 at the parasitophorus vacuolar membrane with a Pearson coefficient of 0.74 and 0.60, respectively. (B) *Pf*exp-2 interacts with *Pf*HDP in a far western experiment. An unrelated protein, MBP was used as a negative control. (C) *Pf*HDP interacts with recombinant C-terminal *Pf*exp-2 in an SPR experiment. The interaction is a two-state reaction, and the dissociation constant is 1.1* 10–6 M. (D) Conformational docking patterns (i–iii) were observed for The *Pf*exp-2 (monomer)-*Pf*HDP complex. Green-*Pf*HDP, Cyan-*Pf*exp-2, Red—*Pf*exp-2 interacting region within 4 Å of *Pf*HDP, Magenta - *Pf*HDP interacting region within 4 Å of *Pf*exp-2.

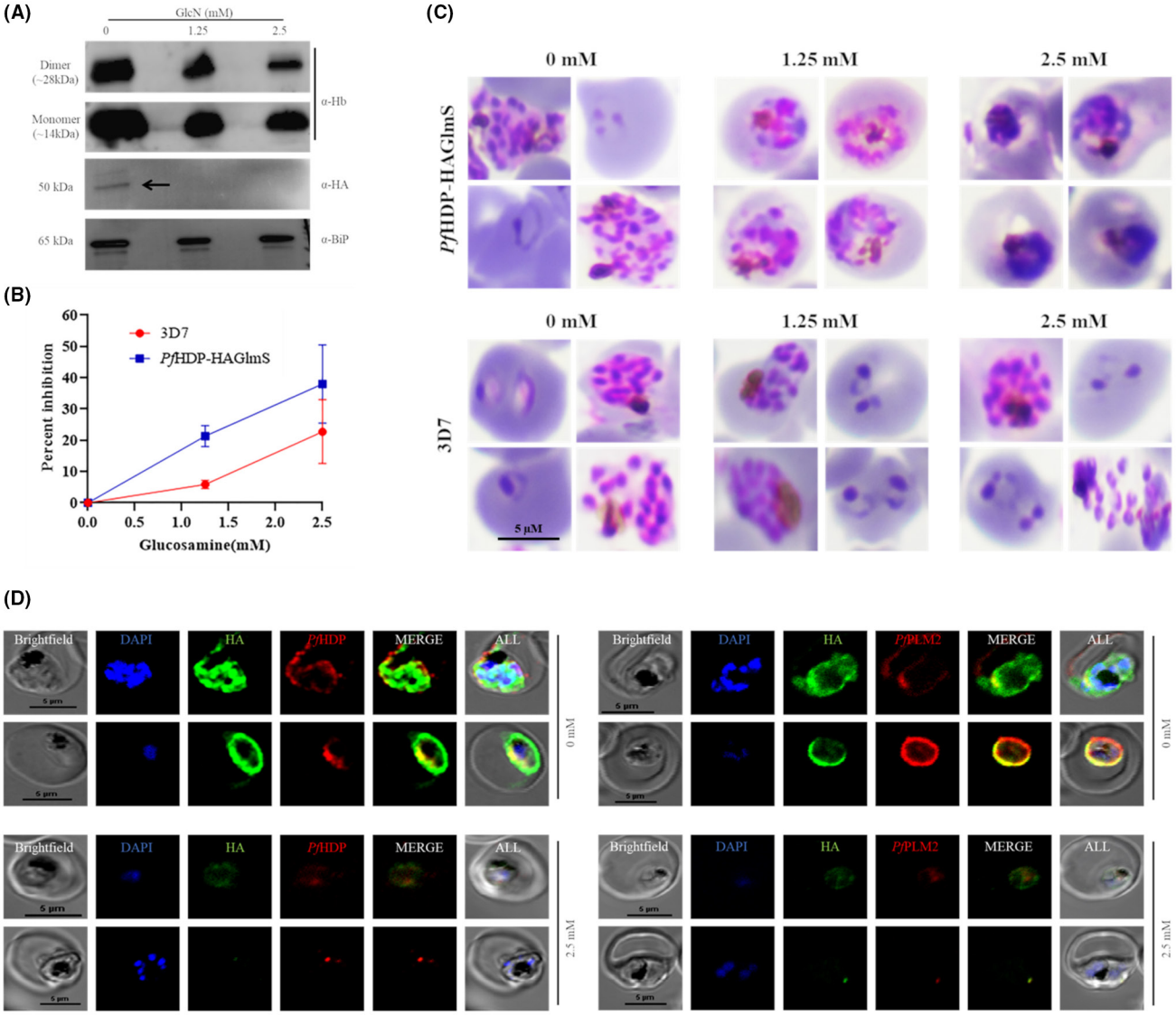


FIGURE 3 (A) Western blot analysis of lysate from *Pf*HDP-HA-GlmS line with α-HA rat serum and α-Hb antibody. Hb uptake is reduced in the knockdown parasites. Anti BiP was used as the loading control. (B) Conditional knockdown of *Pf*HDP in parasites showing up to 45% invasion inhibition at 2.5 mM glucosamine concentration. Data represent mean ± SD; $n = 3$ experiments. (C) Representative Giemsa-stained smears highlight the parasite stress in the trophozoite stage following *Pf*HDP knockdown. (D) Immunofluorescence assays show the low levels of *Pf*HDP and plasmepsin-2 in *Pf*HDP-HA-GlmS knockdown lines.

Linker helix (L), globular domain body (B) and transmembrane domain (T) of *Pf*exp-2 (Figure 2D) with the highest binding energy observed for *Pf*HDP-*Pf*exp-2 in the Linker region (−39.57 kcal/mol, pose 1). Similarly, the best binding score observed for *Pf*HDP binding to the globular domain body (B) and transmembrane domain (T) of *Pf*exp-2 was −17.08 (pose 2) and −6.35 kcal/mol (pose 3), respectively. The PPI analysis showed gradual changes in the *Pf*HDP residues, which interact with different pockets of *Pf*exp-2 (Table S1) however *Pf*HDP TYR56 and ASN59 residues binding remain consistent and were present within 4 Å of *Pf*exp-2 in almost all conformations analyzed for *Pf*exp-2-*Pf*HDP complexes (Figure S6). Furthermore, our docking analysis with heptameric *Pf*exp-2 indicates that it could be a potential transporter for *Pf*HDP (Figure S6; Table S2). Thus, our bioinformatics analysis further supported an interaction between *Pf*exp-2 and *Pf*HDP.

## 3.3 | Knockdown of *Pf*HDP results in parasite stress and low levels of Hb inside the parasite

To illustrate the functional importance of *Pf*HDP protein in parasite biology in particularly for Hb metabolism at asexual blood stages, a transgenic line with the

genomic locus of *Pf*HDP fused to GlmS ribozyme system was generated. The GlmS ribozyme is transcribed along with the gene. This inducible knockdown system uses glucosamine as an inducer. In the presence of glucosamine, the inducer binds to ribozyme inducing the cleavage of the chimeric mRNA and thereby knocking down the respective targets (Figure S8A). The transgenic line was generated using pHA-GlmS vector-based constructs (Figure S8B,C). The integrants were selected using WR22910 selection, followed by clonal selection by limiting dilution to obtain a pure conditional knockdown parasite clone. The integration was confirmed by PCR amplification using different combinations of primer sets (Figure S8D). Expression of the HA-tagged fusion protein was confirmed by western blot analysis of the transgenic parasites using anti-HA antibody, which detected a single band at ~50 kDa, corresponding to the size of dimeric native protein in the parasite (Figure S8E). No such band was detected in 3D7 parasite lysate that served as a negative control.

To study the effect of the knockdown on the expression of *Pf*HDP protein, the late trophozoite stages of transgenic parasites were treated with different concentrations of GlcN (0, 1.25, and 2.5, respectively). The parasites were harvested in the next cycle at 42–44 hpi and saponin-treated parasites were lysed in RIPA buffer. The expression of the fusion protein was analyzed by western blot analysis of the lysates from transgenic parasites using anti-HA antibody. A considerable reduction in the expression of *Pf*HDP protein was seen in the presence of different concentrations of GlcN (Figure 3A). *Pf*BiP, a constitutively expressed endoplasmic reticulum chaperone protein, was used as a loading control. Densitometry analysis was performed for the western blot (Figure S9) We further studied the effect of the knockdown of *Pf*HDP on the growth of the parasites. GlcN was added at the ring stage parasites 16–20 hpi at varying concentrations (0, 1.25, 2.5 mM) and the growth was monitored till the formation of new rings up to two invasion cycles. In the first cycle, a slight decrease in the parasitemia was observed at 2.5 mM GlcN concentration. However, when the parasites were allowed to progress to the second cycle, growth inhibition of ~40% was observed in *Pf*HDP-HAGlmS knockdown parasites (Figure 3B). The inability of GlcN to inhibit more than 50% parasite growth could be attributed to the incomplete knockdown of the protein in the first cycle, as the synthesis of *Pf*HDP begins as early as the ring stages of the parasite. Giemsa smears in the second cycle of growth demonstrated an induction of parasite stress and food vacuole abnormalities (Figure 3C). The 3D7 parasites treated similarly with varied GlcN concentrations were used as a negative control. We next analyzed the Hb levels in the knockdown parasites by western blotting using anti-Hb antibody. A considerable decrease in the amounts of Hb was seen inside the GlcN-treated parasites (Figure 3A). *Pf*BiP was used as a loading control and it did not show any change in expression. We extended the study further and studied the expression/localization of *Pf*HDP and Plasmepsin 2 (which is a member in the Hemozoin formation complex) in the *Pf*HDP knockdown parasites. Interestingly, we observed an inappropriate expression for Plasmepsin 2, a food vacuole protease in these stressed parasites (Figure 3D). Overall, these results demonstrated a role of *Pf*HDP in Hb uptake and its impact on the growth of parasites.

### 3.4 | Parasites expressing low levels of *Pf*HSP101 protein display low levels of Hb inside the parasite

To understand the role of the translocon complex in Hb metabolism if any, we next studied the uptake of Hb in *Pf*HSP101-DDDHA[31] knockdown line. For the same, the *Pf*HSP101-DDD-HA parasites were grown in the presence of trimethoprim (TMP) (Figure 4A); removal of TMP causes functional interference of *Pf*HSP101 protein, thereby leading to its knockdown.[31] Briefly, the late trophozoite stages of parasites were treated with different concentrations of TMP (0, 2.5, 5 μM, respectively) and these untreated versus treated parasites were harvested in the next cycle at 42–44 hpi. The saponin lysed parasites were subjected to lysis in RIPA buffer and Hb levels in the control versus knockdown parasites were analyzed by western blot analysis using anti-Hb antibody. A remarkable decrease in levels of Hb was observed in the TMP-untreated parasites where *Pf*HSP101 protein had been reduced considerably in comparison to the TMP-treated parasites (Figure 4B). *Pf*BiP was used as a loading control. Reduction in *Pf*HSP101 levels was confirmed in TMP-untreated parasite lysates using anti-HA antibody.

In summary, these results advocate a role for *Pf*HDP and translocon complex in Hb metabolism in the parasite. Based on the subcellular localization of *Pf*HDP, its interaction with the Hb and *Pf*exp-2, and its effect on the uptake of Hb in knockdown parasites, we propose a model suggesting that *Pf*HDP plays a crucial role in the uptake and metabolism of Hb in infected erythrocyte. The interaction of *Pf*HDP with *Pf*exp-2 and other members of the translocon complex suggests that it might be transported to the erythrocyte using the complex. The *Pf*HDP-Hb complex is subsequently taken up by the parasite and gets translocated to the food vacuole by vesicular trafficking.

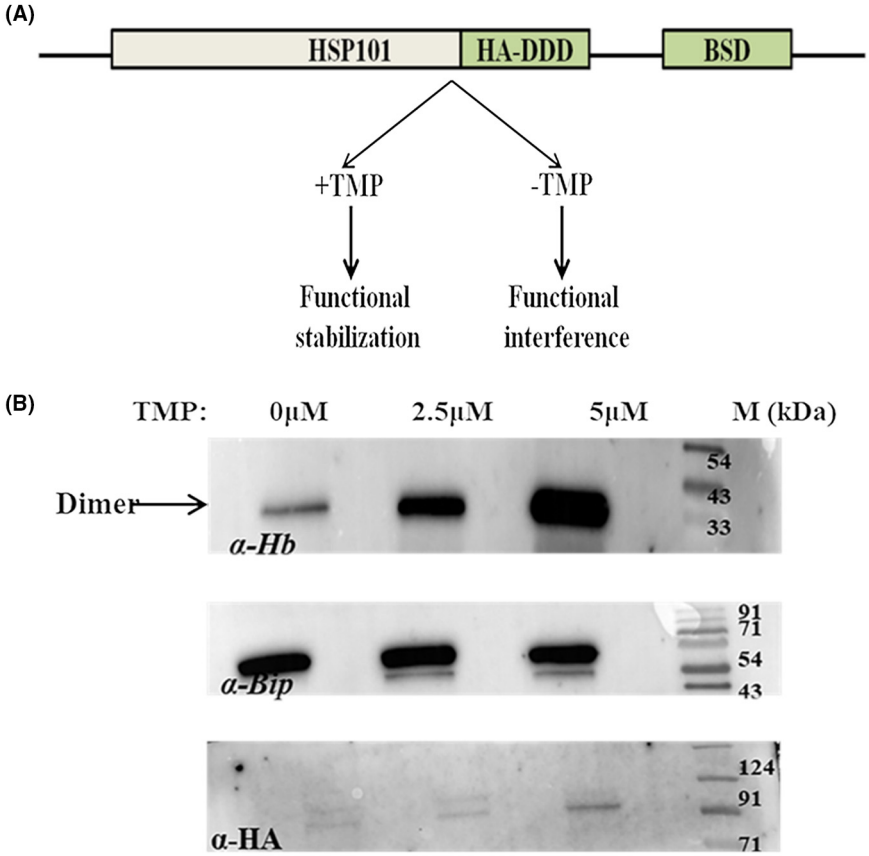


**FIGURE 4** HSP101 DDDHA knockdown parasites take up less Hb from the host erythrocyte cytoplasm. (A) Illustration of the HSP101 DDDHA transgenic construct (B) western blot to detect the Hb levels inside the parasite in HSP101 knockdown parasites. BiP is used as a loading control.

## 4 | DISCUSSION

Hb uptake and its degradation are highly crucial processes for the growth of *P. falciparum*. The degradation of Hb results in the generation of amino acids that are utilized by the parasite for its protein synthesis. Heme generated as a by-product during the process of Hb catabolism is highly toxic for the survival of parasites. Heme Detoxification Protein has earlier been shown to be involved in the conversion of heme to an inert polymer hemozoin.[12] It has been reported earlier that *Pf*HDP is exported into the infected erythrocyte cytosol and takes a circuitous trafficking route to reach the food vacuole and catalyzes Hz formation in the food vacuole.[12] The authors further showed using anti-*Pf*HDP antibody that the trafficking of *Pf*HDP to the cytosol of RBCs does not occur via the classical secretory pathway and the inbound *Pf*HDP protein is trafficked via cytostomal uptake in vesicles containing Hb.[12] However, the question that remains unanswered is how *Pf*HDP is exported into the erythrocyte cytosol. Furthermore, it is also important to understand the function of *Pf*HDP exported to the erythrocyte cytoplasm. In the present study, we aimed to understand the Hb uptake/trafficking inside the infected parasites and the possible role of *Pf*HDP in Hb metabolism. To gain insights into the mode of trafficking of *Pf*HDP into the infected parasites from RBC cytosol, a C-terminal GFP fusion HDP overexpressing parasite line under the control of Hsp86 promoter was generated. Confocal microscopy studies showed that the *Pf*HDP-GFP protein is trafficked via punctate, vesicle-like structures. Colocalization of the GFP signal in these parasites with lipid-binding dye, BodiPY ceramide, showed that it is present at the parasite membrane suggesting that it is trafficked to the erythrocyte cytoplasm. Streptolysin O treatment of these transgenic parasites showed the expression of *Pf*HDP-GFP fusion protein in the erythrocyte compartment suggesting that *Pf*HDP-GFP protein is exported to PVM and to the erythrocyte cytoplasm. In order to identify the trafficking mechanism of *Pf*HDP inside the erythrocyte cytoplasm, immunoprecipitation analysis of *Pf*HDP-GFP transgenic line lysates with GFP trap was performed. The immunoprecipitate showed the presence of Hb along with the members of the translocon complex[17] such as *Pf*PTEX150, *Pf*Exp-2, and *Pf*HSP101. Previous studies have indicated that *Pf*HDP is present in vesicles containing Hb endocytosed from the erythrocyte cytoplasm (Jani et al 2008). However, there is no evidence on the mechanisms of export of this protein. *Pf*HDP lacks any classical signal sequence that could lead to its trafficking to the erythrocyte. Our immunoprecipitation results point to a likely role of the translocon complex in the outward transport of *Pf*HDP.

To validate the proposed hypothesis that the translocon complex is involved in the transport of *Pf*HDP, we expressed a C-terminal fragment of *Pf*exp-2 and raised

specific antibodies against *Pf*exp-2. *Pf*HDP colocalized well with either *Pf*exp-2 or *Pf*PTEX150 at the parasitophorus vacuolar membrane with a Pearson's coefficient of >0.5. To support further the role of translocon complex in the export of *Pf*HDP protein in the erythrocyte cytoplasm, interaction studies between *Pf*HDP and *Pf*exp-2 were performed bioinformatically and in vitro protein interaction strategies. A recent near-atomic resolution cryoEM structure of endogenous translocon complex revealed that *Pf*exp-2 and *Pf*PTEX150 intermingle to form a static, funnel-shaped pseudo-sevenfold symmetric protein conducting channel spanning the vacuole membrane (Chi-Min Ho et al., 2018). The in silico docking analysis of *Pf*exp-2 from the available structure with *Pf*HDP further supports the interaction between *Pf*HDP and *Pf*exp-2. Furthermore, in vitro interaction study showed that recombinant HDP interacted with both Hb as well as *Pf*exp-2 with considerable affinities, thereby suggesting that *Pf*HDP-Hb complex formed in infected erythrocyte cytoplasm. Surface Plasmon resonance results further showed a two-state binding reaction between *Pf*HDP and *Pf*exp-2, suggesting the presence of more than one binding site on HDP for binding to *Pf*exp-2. Additionally, downregulation of a translocon component *Pf*HSP101 in a HSP101-DDDHA expressing line showed decreased Hb levels inside the parasite. This observation could be an indirect result of a decreased export of *Pf*HDP in the cytoplasm of erythrocyte, leading to decreased uptake of Hb inside the parasites.

We next studied the relevance of the *Pf*HDP exported to the erythrocyte cytoplasm. To study the functional relevance of *Pf*HDP in the parasite in Hb metabolism, a *Pf*HDP-HAGlmS inducible knockdown transgenic line was generated, although our attempts to knockout *Pf*HDP gene failed. Knockdown of HDP in the parasite line using the inducer glucosamine induced food vacuole abnormalities and parasite stress. An approximate 40% growth inhibition was observed in glucosamine-treated parasites. A reduced level of *Pf*HDP inside the transgenic lines also led to the reduction in the uptake of Hb from the parasite cytosol. The food vacuole was not properly developed in the *Pf*HDP knocked down parasites and hence the parasites exhibited gross morphological deformities. The induction of food vacuole stress leads to inhibition of parasite growth. These parasites also showed mislocalized or poorly expressed Plasmepsin 2 protein, a part of the hemozoin formation complex inside the food vacuole.

In summary, here we characterized *Pf*HDP for its role in Hb uptake in addition to its previously characterized function in heme degradation. To study the *Pf*HDP transport inside the parasite, here we generated a *Pf*HDP-GFP transgenic line. Immunoprecipitation of highly synchronized culture of trophozoite from *Pf*HDP-GFP line using GFP-TRAP beads followed by LC–MS/MS analysis showed the association of Hb and *Pf*HDP with each other and with the members of translocon complex such as Exp-2, PTEX150, PTEX88, HSP101, and Trx2, a complex known to export parasite proteins. Hence, we also propose a role of the complex in transport of *Pf*HDP into the host cell. *In*silico analysis and in vitro protein–protein interaction techniques confirmed the interaction of *Pf*HDP with *Pf*exp-2. We further showed that *Pf*HDP is highly crucial in maintaining food vacuole and parasite health as attempts to knockdown the protein-induced parasite stress. Hb uptake is severely affected in these transgenic parasites. The study thus emphasizes on the dual roles of Heme Detoxification Protein in Hb uptake as well as in conversion of heme to hemozoin. Looking at the multiple roles of *Pf*HDP in the parasite life cycle, *Pf*HDP thus appears to be an important target for new antimalarial discovery.

## AUTHOR CONTRIBUTIONS

PM conceived the idea. PM and PG designed the experiments. PG performed literature analysis. PG, RP, VT, SP, IK, AP, RB, AA and SM performed experiments. RP and DG performed bioinformatics analysis. AM, DG, and PM supervised the study. PG and PM wrote the manuscript, and all authors read and approved the manuscript.

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## ETHICS STATEMENT

The animal work performed in this study was approved by the Institutional Animals Ethics Committee of ICGEB (IAEC-ICGEB). Rotary blood bank provided human red blood cells.

## ORCID

*Pawan Malhotra* https://orcid.org/0000-0002-7384-6280

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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