Original Research *Feature article*

Localization and function of a *Plasmodium falciparum* protein (PF3D7_1459400) during erythrocyte invasion

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Impact statement

Plasmodium falciparum malaria is a global health problem. Erythrocyte invasion by *P. falciparum* merozoites appears to be a promising target to curb malaria. We have identified and characterized a novel protein that is involved in erythrocyte invasion. Our data on protein subcellular localization, stage-specific protein expression pattern, and merozoite invasion inhibition by α-peptide antibodies suggest a role for PF3D7_1459400 protein during *P. falciparum* erythrocyte invasion. Even more, the human immunoepidemiology data present PF3D7_1459400 protein as an immunogenic antigen which could be further exploited for the development of new anti-infective therapy against malaria.

Abstract

Nearly 60% of *Plasmodium falciparum* proteins are still uncharacterized and their functions are unknown. In this report, we carried out the functional characterization of a 45 kDa protein (PF3D7_1459400) and showed its potential as a target for blood stage malaria vaccine development. Analysis of protein subcellular localization, native protein expression profile, and erythrocyte invasion inhibition of both clinical and laboratory parasite strains by peptide antibodies suggest a functional role of PF3D7_1459400 protein during erythrocyte invasion. Also, immunoreactivity screens using synthetic peptides of the protein showed that adults resident in malaria endemic regions in Ghana have naturally acquired plasma antibodies against PF3D7_1459400 protein. Altogether, this study presents PF3D7_1459400 protein as a potential target for the development of peptide-based vaccine for blood-stage malaria.

Keywords: *Plasmodium falciparum*, malaria vaccine, erythrocyte invasion, peptide antibodies, naturally acquired antibodies

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Introduction

Malaria has been a major health problem that has impacted on the lives of people residing in the tropics or sub-Saharan Africa.[1,2] Efforts to effectively control malaria face challenges from drug-resistant parasite strains, insecticide-resistant vectors, and limited knowledge of the parasite biology which impedes the development of an effective malaria vaccine.[3]

Plasmodium falciparum erythrocyte invasion is a complex molecular process that involves a cascade of receptor-ligand interactions occurring at the parasite-host cell interface.[4–7] Proteomics/microarray expression analysis,[8,9] saturation mutagenesis strategies,[10] and immuno-epidemiological studies[11–13] have all proven to be valuable strategies for the identification of antigens as promising malaria vaccine targets.

Despite these significant advancements made over the years in profiling *P. falciparum* antigens for malaria vaccine development, there is no effective malaria vaccine with broad operational impact. A considerable number of genes in the genome of the malaria parasite still have no-known function. Therefore, it is important to functionally characterize novel proteins that could be potential targets for the development of an effective malaria vaccine.

Previously, an *in silico* analysis was performed that identified PF3D7_1459400 hypothetical gene amongst a catalogue of conserved hypothetical genes that encode proteins recruited by apicomplexan parasites for cell

invasion.[14] Also, it was reported that knockout of the parasite adhesin, *Plasmodium falciparum* reticulocyte homology protein-2b (PfRh2b) resulted in >2-fold upregulation of PF3D7_1459400 gene.[15] The disruption of *PfRh2b* gene and inhibition of merozoite invasion by PfRh2b specific antibodies shows that the protein plays a key role in the parasite.[16,17]

More importantly, the piggyBac transposon insertional mutagenesis strategy has been used to demonstrate the essentiality of the *PF3D7_1459400 gene* in *P. falciparum*.[10] Even though the above evidences suggest that PF3D7_1459400 gene may play key role during erythrocyte invasion, it is surprising that this gene remained uncharacterized.

Using a combination of protein informatics and molecular methods, we report for the first time, the important role of a 45 kDa *P. falciparum* protein (PF3D7_1459400) in erythrocyte invasion. Our analysis show that the protein is expressed in both asexual and sexual stage parasites. Functional antibodies against the different epitopes of the protein inhibited erythrocyte invasion at varying thresholds. Also, immuno-epidemiological data show that humans have naturally acquired α-PF3D7_1459400 antibodies indicating that the protein is immunogenic. Overall, we have identified the important epitopes within PF3D7_1459400 protein that elicit potent antibodies that inhibit *P. falciparum* merozoite invasion of erythrocytes.

Materials and methods

Antibodies and labeling dyes

Anti-*Plasmodium falciparum* 48/45-kDa Gamete Surface Protein (Pfs48/45) monoclonal antibody was contributed by Louis H. Miller and Alan Saul through the National Institute for Allergy and Infectious Diseases (NIAID) BEI Resources (product number MRA-316A). PKH26 red fluorescent cell linker dye was obtained from Sigma-Aldrich, Co., Saint Louis, MO. Anti-*Plasmodium falciparum* Glideosome Associated Protein (α-PfGAP45) rabbit antibody was generously provided by Dr. Julian C. Rayner. All Alexa fluorophores were obtained from Invitrogen, Thermo Fisher Scientific, Life technologies corporation, Eugene, Oregon.

Molecular informatics

The Eukaryotic Linear Motif (ELM) platform (http://elm.eu.org/) was used to analyze the amino acid sequence of PF3D7_1459400 protein as described previously.[18] Similarly, we employed the use of Iterative Threading ASSEmbly Refinement (I-TASSER)[10,19] to predict the structural characteristics of the protein that may suggest its involvement in parasite invasion.

Gene synthesis and sub-cloning

The codon-optimized genes coding for PF3D7_1459400 protein (Leu74- Lys339 aa) and *Plasmodium falciparum* Acylated Pleckstrin Homology domain-containing protein (PfAPH); (Met1-Lys235 aa) were synthesized and sub-cloned into pET24b vector with Nde1 and Xho1 sites by GenScript, Hong Kong and BioBasic, Canada, respectively. The recombinant plasmids were individually transformed in *E. coli* competent cells for optimal protein expression of the C-terminal, hexa histidine tag (6xHis) proteins.

Recombinant protein expression and purification

The recombinant plasmids were transformed into BL21-Codon Plus (DE3)-RIPL *E. coli* competent cells and the inoculated cultures were induced at an optical density of 0.6–0.8 with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C for 4 h. The recovered bacteria pellet was subjected to lysis and sonication procedures and the solubilized inclusion body pellet (PF3D7_1459400 protein) or soluble protein (PfAPH) were fractionated on immobilized metal affinity column (NI-NTA resin). The protein expression samples along with the eluted fractions obtained from the affinity columns were resolved on SDS-PAGE and analyzed by immunoblotting using α-6xHIS mouse monoclonal antibody or antigen-specific antibodies.

Design of synthetic peptides and generation of anti-peptide antibodies

Two chemically synthesized peptides; **P2**: CEPPQIK YRPVKQTK and **P3**: CKKPKPISVALLNNK corresponding to the conserved domains of the PF3D7_1459400 protein were designed and synthesized by GenScript, Hong Kong using its proprietary optimumAntigen design tool. Similarly, the control immunogenic peptide (PfSRA P3-CSNNKKKKKNDKKKK) and the poorly immunogenic control peptide (R1-LFSKFGSRMHILKC) were synthesized in an earlier study.[20]

All antibodies against PF3D7_1459400 peptides and the control peptides were generated in New Zealand rabbits by GeneScript. Immunizations were performed using a proprietary adjuvant that increases the efficiency of immunizations and decreases the turnaround time for antibody production to 45 days.

Animal ethics

PfAPH rat immunization was performed at the Centre for Plant Medicine Research, Mampong, Akuapim, Ghana in accordance with the institutional animal ethics committee guidelines (CPMR/M.10-PT9/2019).

PfAPH rat immunization

Eight rats were acclimatized for two weeks at the animal facility situated at the Centre for Plant Medicine Research, Mampong, Akuapim, Ghana. Non-immune pre-bleeds were drawn twice within two weeks for use as control sera. The rats were immunized intramuscularly with 50 μg of the purified recombinant PfAPH. The recombinant PfAPH protein was emulsified using complete Freund's adjuvant (CFA) (sigma, St. Louis, MO) during the priming stage of the rats on day 14. This was followed by two booster immunizations with the PfAPH protein formulated in incomplete Freund's adjuvant (IFA) (Sigma, St. Louis

MO) on days 28 and 56. Terminal bleeds were collected on day 70. Anti-PfAPH rat antibodies were tested for specific recognition of the recombinant protein by immunofluorescence assays and immunoblotting. The immunized sera obtained, and pre-bleed sera were stored at −20°C freezer for future use.

Parasite culture

3D7, Dd2, and MISA011 strains of *P. falciparum* were cultured using human O^+ erythrocytes as described previously.[21] The relative synchrony of the parasites was obtained after two rounds of sorbitol treatments followed by Percoll-alanine gradient centrifugation.[22,23] Smears for asexual stage parasites and gametocytes (resulting from high parasitemia) were made from 3D7 parasite cultures for use in subsequent experiments.

Native protein expression analysis

Tightly synchronized cultures of schizont-stage parasites were prepared, and the parasite pellet was obtained by 0.05% Saponin lysis. The recovered parasite pellet was lysed with immunoprecipitation lysis buffer to obtain schizont lysates. The lysate was resolved on 12% SDS-PAGE and subjected to immunoblotting using α-PF3D7_1459400 peptide antibodies to detect the native parasite protein.

Carbonate extraction

Carbonate extraction of 3D7 schizont pellets was performed as described previously.[24] Briefly, schizont stage parasites were hypotonically lysed to remove the cytoplasmic fraction and the resultant membrane pellet was further incubated with 100 mM sodium carbonate, pH 11.5 on ice for 2 h. The sample was centrifuged to obtain both soluble and particulate fractions. The particulate fraction was reconstituted with 4× reducing sample buffer. Samples obtained from both the soluble and particulate fractions were analyzed by immunoblotting.

Immunofluorescence assays

Air-dried blood smears from 3D7 cultures were fixed in chilled methanol for 30 min at −80°C freezer. After fixation, the slides were blocked at room temperature for an hour using 3% BSA formulated in phosphate buffer saline (PBS). The target and subcellular marker antibodies were applied at 1:100 dilution for an hour. Next, the slides were probed with Alexa Fluor 488 and Alexa Fluor 594 secondary antibodies respectively for 45 min. The slides were repeatedly washed and mounted with VECTASHIELD, VECTOR laboratories (Burlingame, CA) containing 4′,6′-diamidino-2-phenylindole (DAPI). At least 50 immunofluorescence assay (IFA) images for the different channels were captured on a fluorescence microscope (Olympus BX-41, Hamamatsu Photonics K.K, Japan) and processed using Fiji-Image J software (National Institutes of Health, USA).

Invasion assays

Laboratory strains and a clinical isolate at ring stage were subjected to two rounds of sorbitol synchronization and invasion assays were performed at schizont stage as described previously.[25] Parasitemia levels were determined by flow cytometry analysis on BD FORTESSA X-20 with FlowJo software (BD, Belgium). Parasite growth inhibitory activity of anti-PF3D7_1459400 specific rabbit antibodies were evaluated by comparison to percent invasion in the presence of protein-G purified rabbit antibodies from the pre-bleed sera.

Ethical statement for human plasma samples

Ethical approval was obtained from the Ethics Committees of the Ghana Health Service and Noguchi Memorial Institute for Medical Research, University of Ghana, Legon.[26,27] The research was performed in accordance with the prescribed guidelines/regulations.

Study design

Plasma sampling for this study was carried out in Obom, GA-West, Accra. Three hundred and nine plasma samples were collected from adults in the community at the beginning of the study during the dry season. The *P. falciparum* infection status of the study participants was determined by polymerase chain reaction (PCR) and microscopy. The plasma samples collected during the dry season were classified as symptomatic, asymptomatic, and uninfected samples (based on undetectable level of parasitemia). A follow-up sampling from the same community was conducted to ascertain the infection status of the study participants during the rainy season. During the raining season (malaria season), we categorized 207 plasma samples into symptomatic ($n = 78$), asymptomatic (73), and uninfected ($n = 56$). An exclusion criterion was included that accounted for 102 plasma samples corresponding to adults that were asymptomatic and uninfected during the dry season, but their infection status changed to symptomatic during the rainy season. We performed a comparative analysis of plasma antibody profiles for all three categories of adults by ELISA to determine if PF3D7_1459400 protein is a target for protective immunity.

Immunoreactivity screens

Immunoreactivity screens were performed by enzyme linked immunosorbent assays (ELISA) as described previously[12] with slight modifications. Ten micrograms of PF3D7_1459400 (P2 and P3), PfSRA-P3 and R1 peptides in phosphate-buffered saline, pH 7.2 were individually coated in 96-well microtiter plates and incubated overnight at 4°C. The plates were washed five times with phosphate-buffered saline (PBS) and PBS containing 0.05% Tween 20 (PBST). The plates were blocked with 3% BSA (Bovine Serum Albumin) in PBS overnight at 4°C. After the washing steps, the plates were incubated with plasma samples (1:100 dilution) from uninfected, symptomatic, and asymptomatic adults for 90 min at room temperature. The washing steps were repeated, and the plates were incubated

with goat anti-Human IgG (H + L) horseradish peroxidase conjugated secondary antibody (ThermoFisher Scientific #31410), (1:5000 dilution) at 37°C for an hour. After the incubation period, the plates were washed five times with PBST and PBS, and 3, 3′, 5, 5′-tetramethylbenzidine (TMB) was added to develop the reaction during a 20-min incubation in the dark. Optical Density was read at 450 nm using a VARIOSKAN LUX multi-mode microplate reader (Thermo Fischer Scientific, USA). While PfSRA-P3 was used as the positive control immunogenic peptide, the poorly immunogenic R1 peptide (R1-LFSKFGSR MHILKC) was used as negative control to normalize the data and exclude background ELISA readouts. Malaria-naïve European donor plasma samples were used as negative experimental control. The cut-off value for ELISA was calculated as mean + 2SD of the ODs for the naïve control samples.

Results

PF3D7_1459400 protein architecture and structural prediction

The domain architecture of PF3D7_1459400 protein shows that it has different characteristic features (Figure 1(a)) including a transmembrane domain at the amino terminus but without the classical signal peptide. Also, multiple sequence alignment analysis (Figure 1(b)) shows that PF3D7_1459400 protein is conserved across apicomplexans with six positionally conserved cysteine residues. Three-dimensional (3D) structural prediction using I-TASSER indicated that PF3D7_1459400 protein shares structural similarities with the cryo-electron microscopy (Cryo-EM) structure of human **A**taxia **T**elangiectasia-mutated and **R**ad3 (ATR)-related protein kinase-ATR **I**nteracting **P**rotein (ATRIP), PDB 5YZ0. Interestingly, the N-terminal part of the ATR monomer harbors **H**untingtin, **E**longation factor 3, the **A** subunit of protein phosphatase 2A (PP2A), and the signaling kinase **T**OR1 (HEAT) repeat regions. We have shown both cartoon and surface representations of the ATR-ATRIP protein complex (Figure 1(c), i–ii). This structural resemblance presents PF3D7_1459400 protein as a candidate antigen for characterization considering that HEAT repeats are potential docking sites for protein–protein interactions.

PF3D7_1459400 and PfAPH recombinant protein expression and purification

The entire PF3D7_1459400 protein is globular but our repeated attempts to express the full-length recombinant protein were unsuccessful. However, when we excluded the transmembrane region, a partial construct (74–340 aa) of the protein was successfully expressed in *E. coli*.

Immunoblotting analysis of the expressed recombinant PF3D7_1459400 protein, solubilized inclusion body protein, and the purified fractions from the metal affinity column is shown in Figure S1(a). Similarly, PfAPH protein was expressed in *E. coli* and the recombinant protein (32 kDa) was detected by immunoblotting using α-6xHis mouse monoclonal antibody (Figure S1(b)). The recombinant

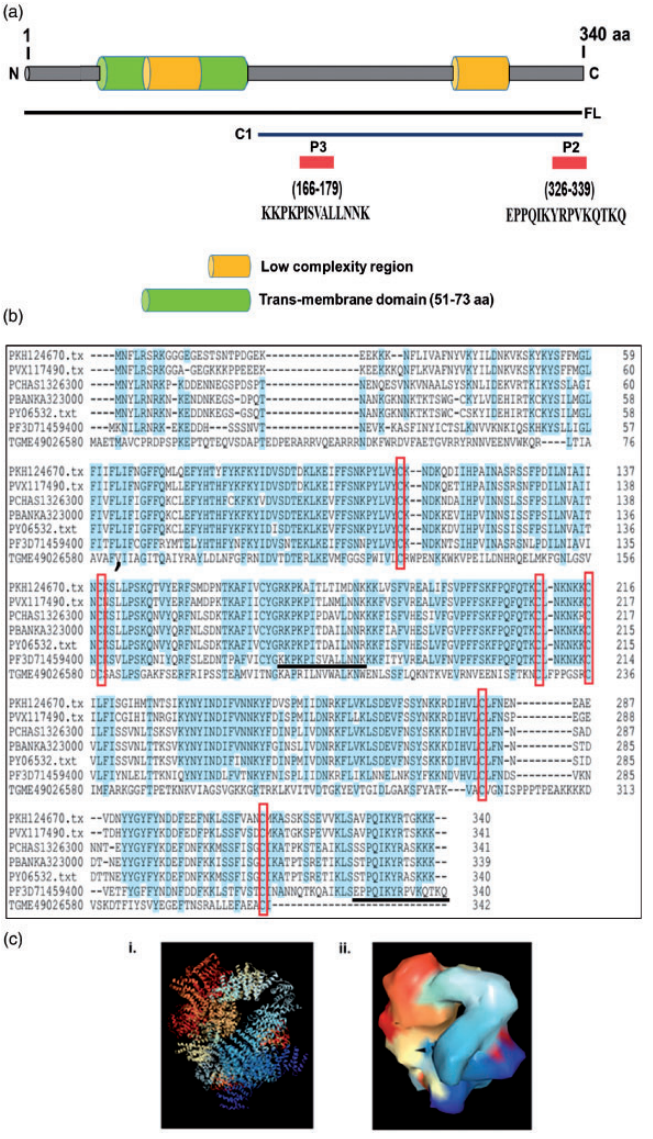

Figure 1. PF3D7_1459400 protein architecture, sequence conservation level, and the predicted protein structure. (a). PF3D7_1459400 protein has a trans-membrane domain at the amino terminus and lacks a signal peptide. B-cell epitope mapping was a basis for the design of two-chemically synthesized peptides (P2 and P3) mapped on the domain architecture of the protein. The black and blue bars represent the full-length protein and the partial construct respectively. (b). Multiple sequence alignment analysis using clone manager shows that PF3D7_1459400 is conserved across apicomplexans with six positionally conserved cysteines. The synthetic peptide sequences have been underlined in the amino acid sequence for the protein. (c). Homology-based modeling for the three-dimensional structural prediction of PF3D7_1459400 protein using I-TASSER showed similarity with the cryo-electron microscopy (Cryo-EM) structure of human Ataxia Telangiectasia-mutated and Rad3 (ATR)-related protein kinase-ATR Interacting Protein (ATRIP). It is the N-terminal part of the ATR monomer that harbors HEAT repeat regions that function as docking sites for protein–protein interactions. (A color version of this figure is available in the online journal.)

PfAPH protein was purified on metal affinity column to apparent homogeneity as shown on SDS-PAGE gel (Figure S1(c)). Anti-PfAPH rat antibody was validated by immunoblotting using 3D7 detergent-treated schizont lysates and the native PfAPH protein was detected at 32 kDa, the theoretical molecular weight (Figure S1(d)).

The partial PF3D7_1459400 recombinant protein (74–340 aa) construct which was expressed in *E. coli* was detected by immunoblotting using α-6xHIS mouse monoclonal antibody (Figure 2(a), i). We performed B-cell epitope mapping which was a basis for the design of two-chemically synthesized peptides marked on the architecture of the protein. Both α-PF3D7_1459400 peptide antibodies (α-P2 and α-P3 rabbit antibodies) detected the recombinant protein during immunoblotting (Figure 2(a), ii–iii). The control antibody (α-R1-peptide rabbit antibody) did not detect the recombinant protein (Figure 2(a), iv) indicating the specificity of α-P2 and -P3 rabbit antibodies.

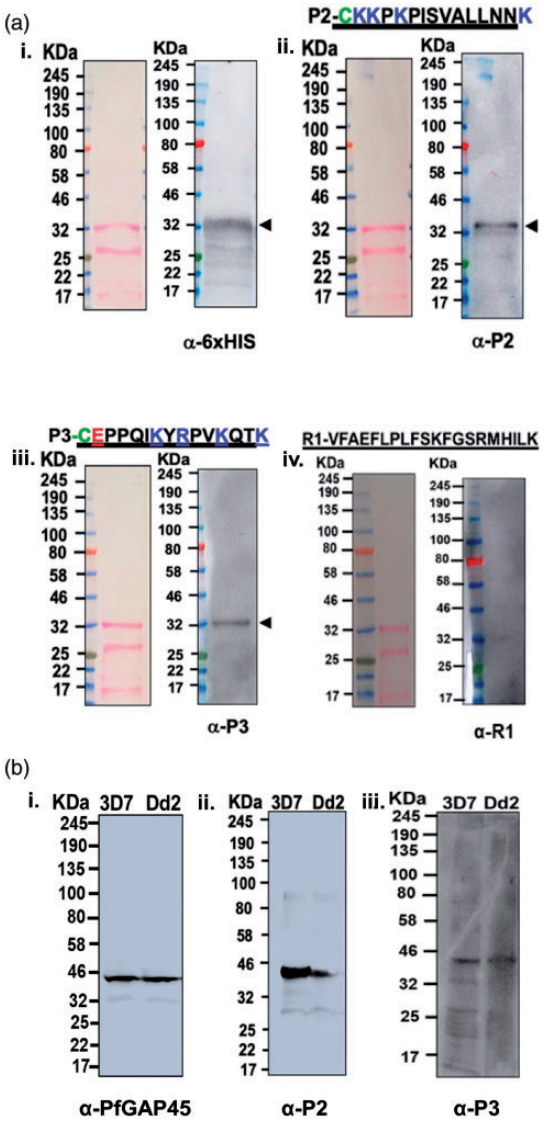

Figure 2. Immunoblotting showing the detection of both recombinant and native PF3D7_1459400 protein. (a, i). The partial construct of PF3D7_1459400 recombinant protein (74–340 aa) was expressed in *E. coli* which migrated as a 32 kDa protein and was detected by α-6xHIS mouse monoclonal antibody. (a, ii–iii). α-PF3D7_1459400 peptide antibodies (α-P2 and -P3 rabbit antibodies) detected the recombinant protein. Ponceau stained membranes are shown by the side of each blot. (a, iv). Anti-R1 peptide rabbit antibody was used as a control to probe the recombinant protein and no signal was detected. (b, i). Anti-PfGAP45 rabbit antibody detected the native parasite protein (45 KDa) during immunoblotting of detergent-treated schizont lysates from 3D7 and Dd2 strains. (b, ii–iii). Anti-P2 and -P3 rabbit antibodies detected the full-length native parasite protein which migrated at 45 kDa. (A color version of this figure is available in the online journal.)

To further validate the specificity of α-P2 and -P3 peptide rabbit antibodies against the native parasite protein, we performed immunoblotting with schizont lysates. The positive control antibody (α-PfGAP45 rabbit antibody) detected the native parasite protein (45 kDa) in 3D7 and Dd2 detergent-treated schizont lysates (Figure 2(b), i). Anti-P2 and -P3 rabbit antibodies detected the native PF3D7_1459400 protein (45 kDa) in both 3D7 and Dd2 schizont lysates (Figure 2(b), ii–iii).

Subcellular localization of PF3D7_1459400 protein during asexual stages

To determine the solubility of PF3D7_1459400 protein, we performed carbonate extraction experiments. The protein was detected in both soluble and membranous fractions indicating that a larger proportion of the protein was embedded in the membrane (Figure 3(a), i). The pre-immune sera did not detect any protein signal indicating the specificity of α-P2 rabbit antibody.

Stage-specific IFA analysis for PF3D7_1459400 native protein indicated that α-P2 rabbit antibody which strongly detected the parasite protein by immunoblotting, also labeled the native protein (Figure 3(b)). PKH26 red fluorescent cell linker dye was used to demarcate erythrocyte membrane and α-P2 rabbit antibody labeled ring, trophozoite, and schizont stages, but no visible fluorescent signal was observed during merozoite attachment to human erythrocytes (Figure 3(b)). Consistent with membrane fractionation experiments, α-P2 rabbit antibody labeled the periphery of schizonts (Figure 3(b)). Stage-specific IFAs revealed that native PF3D7_1459400 protein exhibits dynamic subcellular localization which is supported by a previous report suggesting that the protein is palmitoylated.[28] Therefore, it was imperative to assess the subcellular distribution of the protein using a marker antibody against a known palmitoylated protein, since palmitoylation modulates the subcellular localization of proteins.

Dual immunofluorescence microscopy was performed using the microneme surface marker, α-PfAPH rat antibody and α-P2 rabbit antibody. Both antibodies overlapped in the ring, trophozoite, schizont, and free merozoite stages (Figure 3(c)) suggesting that PF3D7_1459400 protein could be localized on microneme surface in late asexual stages. Both rabbit and rat pre-immune sera were used as IFA controls and no visible fluorescent signals were observed indicating the specificity of protein labeling observed for α-PfAPH rat and α-P2 rabbit antibodies (Figure 3(c)).

PF3D7_1459400 protein is expressed in gametocytes

To determine the expression of native PF3D7_1459400 protein in gametocytes, we performed IFAs as described earlier for asexual stage parasites. Co-labeling of the individual anti-peptide rabbit antibodies (α-P2 and -P3) and the *P. falciparum* gametocyte surface marker antibody (α-Pfs48/45 mouse antibody) indicated that α-P2 rabbit antibodies labeled gametocytes (Figure 4, i). However, α-P3 rabbit

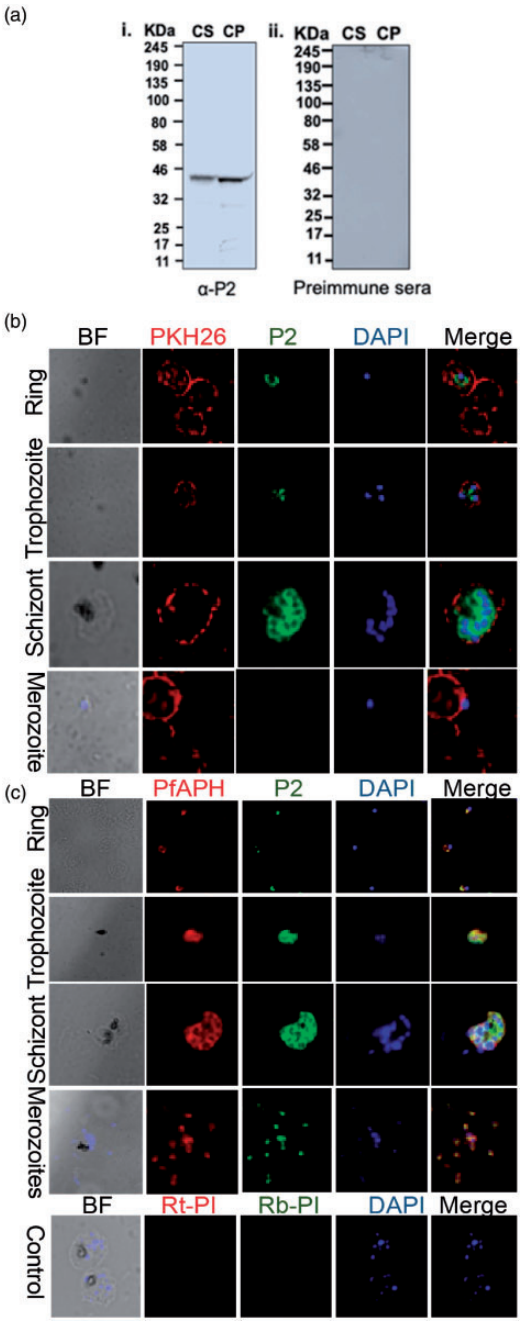

Figure 3. PF3D7_1459400 protein expression profile in asexual stages. (a, i). Carbonate extraction experiments indicated that PF3D7_1459400 is detectable in both soluble and particulate fractions. (a, ii). The pre-immune sera were used to probe the protein and no signal was detected. (b). Stage-specific IFAs performed under permeabilized conditions show that α-PF3D7_1459400 P2 rabbit antibody (green) (1:50) labels rings, trophozoites, and schizonts, but no visible fluorescent signal was observed during merozoite attachment to human erythrocytes. PKH26, the red fluorescent cell linker dye was used to demarcate the erythrocyte membranes. (c). Dual immunofluorescence labeling using α-PF3D7_1459400 P2 rabbit antibody (green), (1:50) and the marker antibody, anti-*Plasmodium falciparum* Acylated Pleckstrin Homology domain-containing protein (α-PfAPH) rat antibody (red), (1:50) revealed that PF3D7_1459400 protein could be localized on microneme surface. Both antibodies labeled ring, trophozoite, schizont, and released merozoites. Rabbit and rat pre-bleed sera were used as negative controls and no detectable fluorescent signals were observed. The secondary antibody used is Alexa 488-conjugated goat α-rabbit IgG, (1:100; Life technologies, Eugene, USA). Alexa 568-conjugated goat α-rat IgG (red), (1:100); Life technologies, Eugene, USA). Nuclei were stained with DAPI (blue). Exposure times were identical for all images of the same channel. BF: bright field; DAPI: 4,6-diamidino-2-phenylindole); Rt-PI: rat pre-immune; Rb-PI: rabbit pre-immune; CS: carbonate supernatant; CP: carbonate pellet. (A color version of this figure is available in the online journal.)

antibody did not label the protein in gametocytes (Figure 4, ii). Currently, the proteolytic processing status of native PF3D7_1459400 protein is unknown. This could be a plausible explanation for the differential α-peptide antibody-labeling patterns observed in gametocytes. The labeling of α-P2 rabbit antibody was predominantly in the cytoplasmic area in gametocytes unlike the α-Pfs48/45 mouse antibody that labeled the surface membrane. Since RNAseq data deposited in PlasmoDB shows that PfAPH is expressed in gametocytes, we co-labeled gametocytes using α-PfAPH rat antibody and α-P2 rabbit antibody which showed that both proteins overlapped in the cytoplasmic compartment (Figure 4, iii).

Anti-PF3D7_1459400 antibodies inhibit *P. falciparum* erythrocyte invasion

Anti-P2 and -P3 rabbit antibodies were tested in invasion assays against 3D7, Dd2, and the clinical isolate (MISA011) at a concentration range of 25–100 μg/mL. Anti-P2 and -P3 rabbit antibodies moderately inhibited parasite invasion in a concentration-dependent manner (30–45% at 100 μg/mL for 3D7 and the clinical isolate) when compared with the pre-immune control (Figure 5). Also, the level of invasion inhibition by α-P2 and -P3 rabbit antibodies in Dd2 strain was about 10–25% at 100 μg/mL when compared with the pre-immune control (Figure 5). The control antibody generated against a shorter construct of the R1 peptide that blocks AMA1-RON4 interaction showed marginal level of invasion inhibition across all the parasite strains tested (Figure 5).

Humans have naturally acquired α-PF3D7_1459400 antibodies

A recent study indicated that the presence of α-PF3D7_1459400 protein specific antibodies in participants in Lira, Northern Uganda was associated with protection against clinical malaria.[29] Therefore, it was of interest to determine how accessible, the inhibitory epitopes (P2 and P3) are to the humoral immune system. We hypothesized that asymptomatic individuals resident in malaria endemic regions may have high levels of α-PF3D7_1459400 protein antibodies. Therefore, we performed plasma immunoreactivity screens by ELISA using uninfected, symptomatic, and asymptomatic plasma samples collected from adults before and during malaria seasons in Obom, GA-West, Accra. The data showed that plasma antibody reactivity to PF3D7_1459400 peptides (P2 and P3) and the control immunogenic peptide (PfSRA-P3) did not discriminate between symptomatic and asymptomatic adults (Figure 6). However, there was a statistical difference between the immunoreactivity patterns for symptomatic and asymptomatic adults when compared with uninfected adults having undetectable levels of parasitemia (Figure 6, i–iii). This observation is consistent with an earlier report on plasma antibody immunoreactivity patterns for other malaria vaccine candidates.[11]

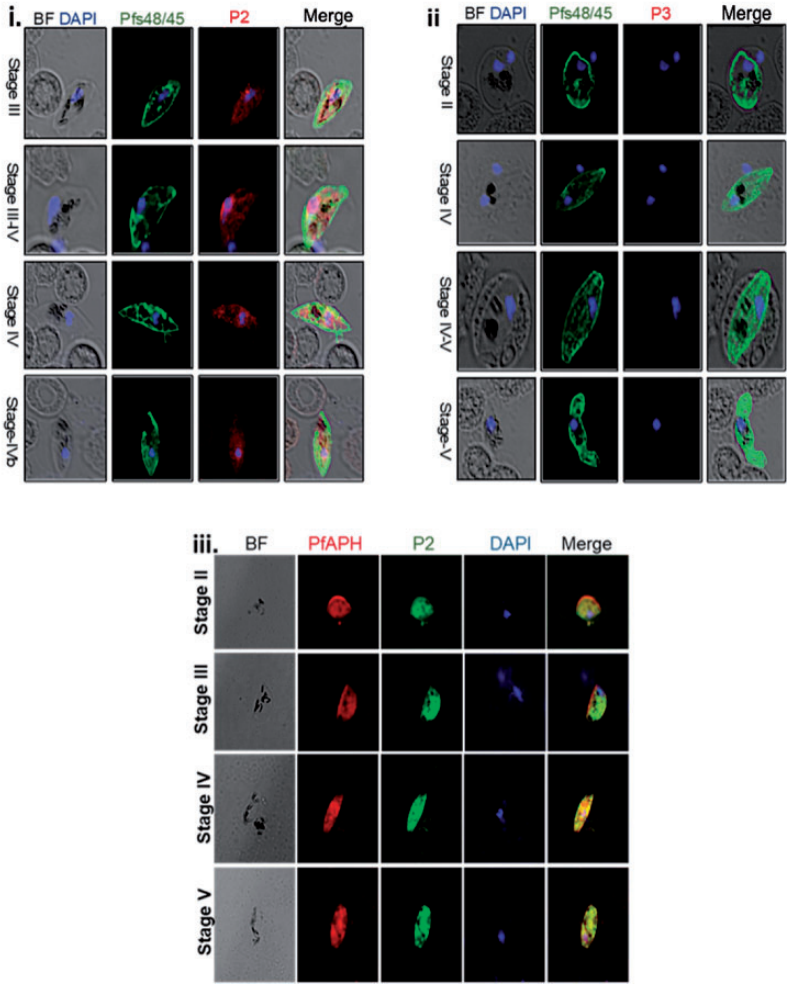

Figure 4. PF3D7_1459400 protein expression in gametocytes. Methanol-fixed *P. falciparum* 3D7 gametocytes were stained with α-PF3D7_1459400 -P2 and -P3 rabbit antibodies (red), (1:50) and α-Pfs48/45 rabbit antibody (green), (1:100). (i–ii): α-PF3D7_1459400 P2 rabbit antibody (red), and α-Pfs48/45 mouse antibody (green) stained different stages of gametocytes but α-PF3D7_1459400 peptide 3 rabbit antibody did not show any discernible staining pattern on gametocytes. (iii). Dual immunofluorescence imaging using α-PfAPH rat antibody (1:50); red and α-P2 rabbit antibody (1:50); green indicated that both PfAPH and PF3D7_1459400 native proteins overlap in the cytosolic compartment in gametocytes. Exposure times were identical for all images of the same channel. Secondary antibodies used are Alexa 568-conjugated goat α-rabbit IgG (red), (1:100), Alexa 568-conjugated goat α-rat IgG (red), (1:100); Life technologies, Eugene, USA). Nuclei were stained with DAPI (blue). BF: Bright field; DAPI: 4,6-diamidino-2-phenylindole. (A color version of this figure is available in the online journal.)

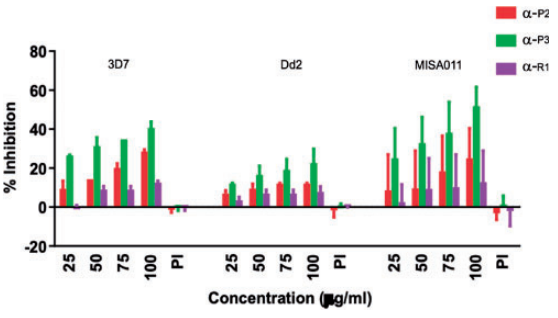

Figure 5. Anti-PF3D7_1459400 peptide antibodies inhibit erythrocyte invasion in both laboratory parasite strains and a clinical isolate. (a). α-PF3D7_1459400 P2 and P3 rabbit antibodies were tested in invasion assays against 3D7, Dd2, and MISA011 strains at a concentration range of 25–100 μg/mL. There was no statistical difference in the observed level of parasite invasion inhibition between α-P2 and -P3 rabbit antibodies ($P = 0.07$ at 100 μg/mL). The pre-immune sera were used as a negative control. The data shown in both assays are two independent assays performed in duplicates. (A color version of this figure is available in the online journal.)

Discussion

Sequencing of *P. falciparum* genome has revealed more than 5000 genes, providing the foundation for systematic approaches to discover candidate malaria vaccine antigens.[20] In an effort to functionally characterize hypothetical genes in the parasite genome with unknown function, we performed a systematic screen for uncharacterized *P. falciparum* invasion-related genes previously, an effort towards the identification of potential blood-stage malaria vaccine candidates.[30] Amongst other hypothetical invasion-related genes that code for merozoite proteins which we identified, PF3D7_1459400 gene was selected for evaluation as a potential blood-stage malaria vaccine candidate.

We expressed the recombinant PF3D7_1459400 protein construct in *E. coli* and the protein was deposited in inclusion bodies which resulted in low recovery of the correctly folded protein in soluble forms. This presented a major hurdle in functional assays that requires protein with

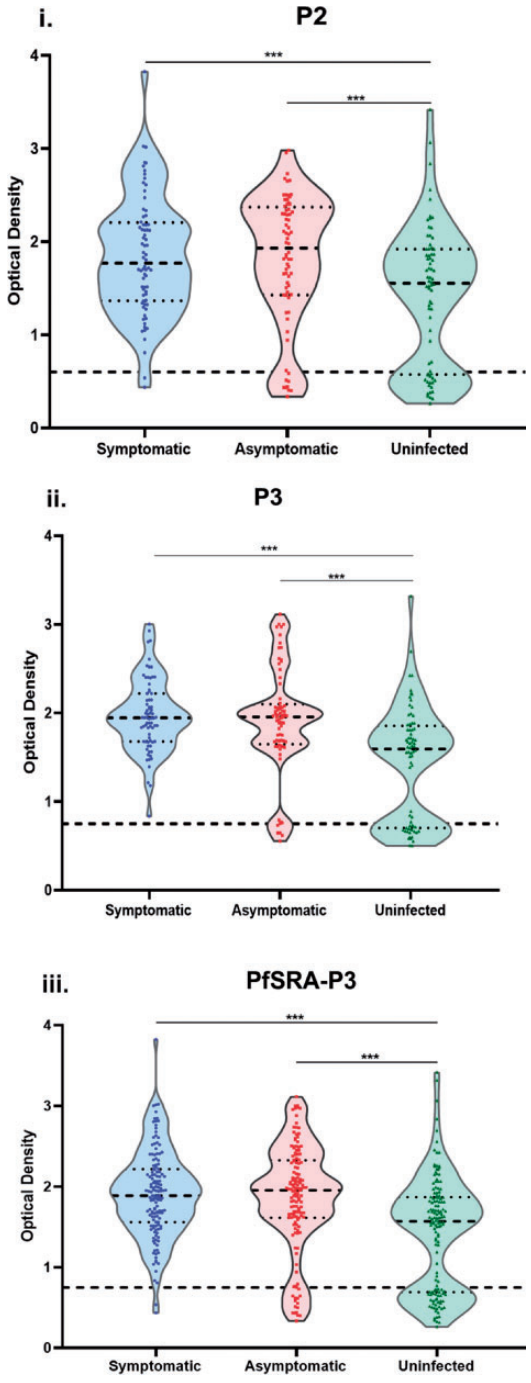

Figure 6. Adults have naturally acquired α-PF3D7_1459400 antibodies. Three hundred and seven adults were recruited as study participants during the dry season and 207 ($n = 207$) plasma samples were categorized into symptomatic ($n = 78$), asymptomatic (73), and uninfected ($n = 56$). We performed a comparative analysis of plasma antibody profiles for all three categories of adult plasma to determine if the protein is a target for protective immunity. The ELISA data showed that the immunoreactivity patterns observed for PF3D7_1459400 peptides (P2 and P3); (6, i–ii) and the immunogenic control peptide (PfSRA P3); (6, iii) did not discriminate between symptomatic and asymptomatic adults. Five different pools of naïve sera were used as controls. The reactivities of IgG levels in the respective plasma samples at 1:100 dilutions were expressed as optical density at 450 nm. Data were analyzed using GraphPad Prism v.8. One-way analysis of variance (ANOVA) and then Turkey's multiple comparison test were conducted to determine the level of statistical significance. The dotted lines in each figure represent the mean + 2SD of the ODs for the control samples. The statistical level of significance is represented below. $^{*}P = 0.03$, $^{**}P = 0.002$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (A color version of this figure is available in the online journal.)

conformational epitopes which may be lacking due to improper protein folding.[31] Due to the insolubility of the recombinant PF3D7_1459400 protein, we considered peptide immunogens since they are an attractive and effective means of inducing host immune responses. Besides, they offer several advantages over the use of recombinant proteins as discussed previously.[32]

PF3D7_1459400 peptide-specific antibodies detected the recombinant protein during immunoblotting at the expected theoretical molecular weight. Also, we observed that the native parasite protein in detergent-treated schizont lysates migrated at a higher molecular weight (45 kDa) than the recombinant protein (32 kDa). This was not surprising because we expressed a partial construct and not the full-length recombinant protein.

Immunofluorescence microscopy data showed that PF3D7_1459400 protein could be localized on microneme surface. Anti-PF3D7_1459400 (P2) rabbit antibody labeled free merozoites, but no detectable signal was observed during merozoite attachment to human erythrocytes. This could imply that the protein may be degraded and might not be relevant during initial merozoite attachment processes.

Also, the protein was localized in the cytoplasmic compartment at different stages of the parasite development, which is substantiated by an existing mass spectrometry data for the native PF3D7_1459400 protein in trophozoites, schizonts, and gametocytes.[33,34]

Protein palmitoylation plays an important role in parasite invasion since *Toxoplasma gondii* apical membrane antigen 1 (TgAMA1) that functions during invasion[35–37] is palmitoylated. PF3D7_1459400 protein is palmitoylated (acylated) based on predictions from clustering and scoring strategy for palmitoylated proteins (CSS-Palm) analysis, Click chemistry/Acyl Biotin Exchange assays performed in a published report on *P. falciparum* palmitome.[28] Therefore, it is not surprising that we observed differential subcellular localization of the protein because palmitoylation has been identified as an important post-translational rheostat for controlling protein function in eukaryotes.[38] More so, palmitoylation-depalmitoylation cycles could be a regulatory mechanism deployed by parasites to alter protein subcellular localization or their membrane binding capacities. Importantly, protein palmitoylation has been shown to play a regulatory role during microneme secretion in *P. falciparum* merozoites.[39]

The knockout of *PfRh2b* gene results in >2-fold upregulation of *PF3D7_1459400* gene[15] suggesting an important role of the gene during erythrocyte invasion by *P. falciparum* merozoites. We have found important epitopes within the PF3D7_1459400 protein that elicit functional anti-peptide antibodies which inhibits erythrocyte invasion of both laboratory and clinical parasite strains.

Interestingly, a previous report indicated that human α-PF3D7_1459400 protein antibodies from study participants in Lira, Northern Uganda are associated with protection from clinical malaria.[29] To this end, we performed immunoreactivity screens using plasma samples from adults who are symptomatic, asymptomatic, and uninfected during the dry and rainy seasons. The data show

that these individuals have high levels of α-PF3D7_1459400 plasma antibodies but the plasma antibody reactivity profiles for PF3D7_1459400 peptides (P2 and P3) and the positive control immunogenic peptide (PfSRA-P3) did not discriminate between symptomatic and asymptomatic adults.

Altogether, our data on protein subcellular localization, stage-specific protein expression pattern, and merozoite invasion inhibition by α-peptide antibodies suggest a role for PF3D7_1459400 protein during *P. falciparum* erythrocyte invasion. Even more, the human immunoepidemiology data present PF3D7_1459400 protein as an immunogenic antigen which could be further exploited for the development of new anti-infective therapy against malaria.

Authors' contributions: EA and GAA conceived the study; EA, PBN, GO, DID, PI, HMB, GA, OA, EAA, and DAB performed the experiments, EA, PBN, and KAK analyzed the data, EA and GAA drafted the manuscript; All authors critically reviewed and edited the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

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SUPPLEMENTAL MATERIAL

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REFERENCES

1. Hotez PJ, Kamath A. Neglected tropical diseases in Sub-Saharan Africa: review of their prevalence, distribution, and disease burden. *PLoS Negl Trop Dis* 2009;**3**:e412
2. Bhatt S, Weiss DJ, Cameron E, Bisanzio D, Mappin B, Dalrymple U, Battle KE, Moyes CL, Henry A, Eckhoff PA, Wenger EA, Briët O, Penny MA, Smith TA, Bennett A, Yukich J, Eisele TP, Griffin JT, Fergus CA, Lynch M, Lindgren F, Cohen JM, Murray CLJ, Smith DL, Hay SI, Cibulskis RE, Gething PW. The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. *Nature* 2015;**526**:207–11
3. WHO. *Responding to antimalarial drug resistance*. Geneva: Author, 2018
4. Reddy KS, Amlabu E, Pandey AK, Mitra P, Chauhan VS, Gaur D. Multiprotein complex between the GPI-anchored CyRPA with PfRH5 and PfRipr is crucial for *Plasmodium falciparum* erythrocyte invasion. *Proc Natl Acad Sci U S A* 2015;**112**:1179–84
5. Volz Jennifer C, Yap A, Sisquella X, Thompson Jenn K, Lim Nicholas TY, Whitehead Lachlan W, Chen L, Lampe M, Tham W-H, Wilson D, Nebl T, Marapana D, Triglia T, Wong W, Rogers KL, Cowman AF. Essential role of the PfRh5/PfRipr/CyRPA complex during *Plasmodium falciparum* invasion of erythrocytes. *Cell Host Microbe* 2016;**20**:60–71
6. Cowman AF, Tonkin CJ, Tham W-H, Duraisingh MT. The molecular basis of erythrocyte invasion by malaria parasites. *Cell Host Microbe* 2017;**22**:232–45
7. Wong W, Huang R, Menant S, Hong C, Sandow JJ, Birkinshaw RW, Healer J, Hodder AN, Kanjee U, Tonkin CJ, Heckmann D, Soroka V, Søgaard TMM, Jørgensen T, Duraisingh MT, Czabotar PE, de Jongh WA, Tham W-H, Webb AI, Yu Z, Cowman AF. Structure of *Plasmodium falciparum* Rh5–CyRPA–ripr invasion complex. *Nature* 2018;**565**:118–21
8. Bozdech Z, Llinás M, Pulliam BL, Wong ED, Zhu J, DeRisi JL. The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*. *PLoS Biol* 2003;**1**:e5
9. Le Roch KG. Discovery of gene function by expression profiling of the malaria parasite life cycle. *Science* 2003;**301**:15030–8
10. Zhang M, Wang C, Otto TD, Oberstaller J, Liao X, Adapa SR, Udenze K, Bronner IF, Casandra D, Mayho M, Brown J, Li S, Swanson J, Rayner JC, Jiang RHY, Adams JH. Uncovering the essential genes of the human malaria parasite *Plasmodium falciparum* by saturation mutagenesis. *Science* 2018;**360**: eaap7847
11. Crompton PD, Kayala MA, Traore B, Kayentao K, Ongoiba A, Weiss GE, Molina DM, Burk CR, Waisberg M, Jasinskas A, Tan X, Doumbo S, Doumtabe D, Kone Y, Narum DL, Liang X, Doumbo OK, Miller LH, Doolan DL, Baldi P, Felgner PL, Pierce SK. A prospective analysis of the Ab response to *Plasmodium falciparum* before and after a malaria season by protein microarray. *Proc Natl Acad Sci U S A* 2010;**107**:6958–63
12. Osier FH, Mackinnon MJ, Crosnier C, Fegan G, Kamuyu G, Wanaguru M, Ogada E, McDade B, Rayner JC, Wright GJ, Marsh K. New antigens for a multicomponent blood-stage malaria vaccine. *Sci Transl Med* 2014;**6**:247ra102
13. Bustamante LY, Powell GT, Lin Y-C, Macklin MD, Cross N, Kemp A, Cawkill P, Sanderson T, Crosnier C, Muller-Sienerth N, Doumbo OK, Traore B, Crompton PD, Cicuta P, Tran TM, Wright GJ, Rayner JC. Synergistic malaria vaccine combinations identified by systematic antigen screening. *Proc Natl Acad Sci U S A* 2017;**114**:12045–50

14. Huynh M-H, Carruthers VB. A *Toxoplasma gondii* ortholog of plasmodium GAMA contributes to parasite attachment and cell invasion. *mSphere* 2016;**1**:00012–6
15. Baum J, Maier AG, Good RT, Simpson KM, Cowman AF. Invasion by *P. falciparum* merozoites suggests a hierarchy of molecular interactions. *PLoS Pathog* 2005;**1**:e37
16. Duraisingh MT, Triglia T, Ralph SA, Rayner JC, Barnwell JW, McFadden GI, Cowman AF. Phenotypic variation of *Plasmodium falciparum* merozoite proteins directs receptor targeting for invasion of human erythrocytes. *EMBO J* 2003;**22**:1047–57
17. DeSimone TM, Bei AK, Jennings CV, Duraisingh MT. Genetic analysis of the cytoplasmic domain of the PfRh2b merozoite invasion protein of *Plasmodium falciparum*. *Int J Parasitol* 2009;**39**:399–405
18. Dinkel H, Van Roey K, Michael S, Kumar M, Uyar B, Altenberg B, Milchevskaya V, Schneider M, Kühn H, Behrendt A, Dahl SL, Damerell V, Diebel S, Kalman S, Klein S, Knudsen AC, Mäder C, Merrill S, Staudt A, Thiel V, Welti L, Davey NE, Diella F, Gibson TJ. ELM 2016 – data update and new functionality of the eukaryotic linear motif resource. *Nucleic Acids Res* 2015;**44**:D294–D300
19. Roy A, Kucukural A, Zhang Y. I-TASSER: a unified platform for automated protein structure and function prediction. *Nat Protoc* 2010;**5**:725–38
20. Amlabu E, Mensah-Brown H, Nyarko PB, Akuh OA, Opoku G, Ilani P, Oyagbenro R, Asiedu K, Aniweh Y, Awandare GA. Functional characterization of *Plasmodium falciparum* surface-related antigen as a potential blood-stage vaccine target. *J Infect Dis* 2018;**218**:778–90
21. Jensen JB, Trager W. *Plasmodium Falciparum* in culture: establishment of additional strains. *Am J Trop Med Hyg* 1978;**27**:7434–6
22. Kanaani J, Ginsburg H. Metabolic interconnection between the human malarial parasite *Plasmodium falciparum* and its host erythrocyte. Regulation of ATP levels by means of an adenylate translocator and adenylate kinase. *J Biol Chem* 1989;**264**:3194–9
23. Awandare GA, Nyarko PB, Aniweh Y, Ayivor-Djanie R, Stoute JA. *Plasmodium falciparum* strains spontaneously switch invasion phenotype in suspension culture. *Sci Rep* 2018;**8**:5782
24. Mitra P, Gupta ED, Sahar T, Pandey AK, Dangi P, Reddy KS, Chauhan VS, Gaur D. Evidence for the nucleo-apical shuttling of a beta-catenin like *Plasmodium falciparum* armadillo repeat containing protein. *PLoS One* 2016;**11**:e0148446
25. Theron J, Eugene CT, De Kwaadsteniet M. Current molecular and emerging nanobiotechnology approaches for the detection of microbial pathogens. *Crit Rev Microbiol* 2010;**36**:318–39
26. Mensah-Brown HE, Amoako N, Abugri J, Stewart LB, Agongo G, Dickson EK, Ofori MF, Stoute JA, Conway DJ, Awandare GA. Analysis of erythrocyte invasion mechanisms of *Plasmodium falciparum* clinical isolates across 3 malaria-endemic areas in Ghana. *J Infect Dis* 2015;**212**:1288–97
27. Mensah-Brown HE, Abugri J, Asante KP, Dwomoh D, Dosoo D, Atuguba F, Conway DJ, Awandare GA. Assessing the impact of differences in malaria transmission intensity on clinical and haematological indices in children with malaria. *Malar J* 2017;**16**:96
28. Jones ML, Collins MO, Goulding D, Joyti SC, Rayner JC. Analysis of protein palmitoylation reveals a pervasive role in plasmodium development and pathogenesis. *Cell Host Microbe* 2012;**12**:246–58
29. Kanoi BN, Takashima E, Morita M, White MT, Palacpac NMQ, Ntege EH, Balikagala B, Yeka A, Egwang TG, Horii T, Tsuboi T. Antibody profiles to wheat germ cell-free system synthesized *Plasmodium falciparum* proteins correlate with protection from symptomatic malaria in Uganda. *Vaccine* 2017;**35**:873–81
30. Doolan DL, Mu Y, Unal B, Sundaresh S, Hirst S, Valdez C, Randall A, Molina D, Liang X, Freilich DA, Oloo JA, Blair PL, Aguiar JC, Baldi P, Davies DH, Felgner PL. Profiling humoral immune responses to *P. falciparum* infection with protein microarrays. *Proteomics* 2008;**8**:4680–94
31. Helb DA, Tetteh KKA, Felgner PL, Skinner J, Hubbard A, Arinaitwe E, Mayanja-Kizza H, Ssewanyana I, Kamya MR, Beeson JG, Tappero J, Smith DL, Crompton PD, Rosenthal PJ, Dorsey G, Drakeley CJ, Greenhouse B. Novel serologic biomarkers provide accurate estimates of recent *Plasmodium falciparum* exposure for individuals and communities. *Proc Natl Acad Sci U S A* 2015;**112**:E4438–E47
32. Schoenherr RM, Saul RG, Whiteaker JR, Yan P, Whiteley GR, Paulovich AG. Anti-peptide monoclonal antibodies generated for immuno-multiple reaction monitoring-mass spectrometry assays have a high probability of supporting Western blot and ELISA. *Mol Cell Proteom* 2014;**14**:382–98
33. Silvestrini F, Lasonder E, Olivieri A, Camarda G, van Schaijk B, Sanchez M, Younis Younis S, Sauerwein R, Alano P. Protein export marks the early phase of gametocytogenesis of the human malaria parasite *Plasmodium falciparum*. *Mol Cell Proteom* 2010;**9**:1437–48
34. Oehring SC, Woodcroft BJ, Moes S, Wetzel J, Dietz O, Pulfer A, Dekiwadia C, Maeser P, Flueck C, Witmer K, Brancucci NMB, Niederwieser I, Jenoe P, Ralph SA, Voss TS. Organellar proteomics reveals hundreds of novel nuclear proteins in the malaria parasite *Plasmodium falciparum*. *Genome Biol* 2012;**13**:11–R108
35. Hehl AB, Lekutis C, Grigg ME, Bradley PJ, Dubremetz JF, Ortega-Barria E, Boothroyd JC. *Toxoplasma gondii* homologue of plasmodium apical membrane antigen 1 is involved in invasion of host cells. *Infect Immun* 2000;**68**:7078–86
36. Lamarque MH, Roques M, Kong-Ha PM, Tonki NML, Rugarabamu G, Marq JB, Penarete-Vargas DM, Boulanger MJ, Soldati-Favre D, Lebrun M. Plasticity and redundancy among AMA-RON pairs ensure host cell entry of toxoplasma parasites. *Nat Commun* 2014;**5**:4098
37. Mital J, Meissner M, Soldati D, Ward GE. Conditional expression of *Toxoplasma gondii* apical membrane antigen-1 (TgAMA1) demonstrates that TgAMA1 plays a critical role in host cell invasion. *Mol Biol Cell* 2005;**16**:4341–9
38. Yadav P, Ayana R, Garg S, Jain R, Sah R, Joshi N, Pati S, Singh S. Plasmodium palmitoylation machinery engineered in E. coli for high-throughput screening of palmitoyl acyl-transferase inhibitors. *FEBS Open Bio* 2019;**9**:248–64
39. Siddiqui MA, Singh S, Malhotra P, Chitnis CE. Protein S-Palmitoylation is responsive to external signals and plays a regulatory role in microneme secretion in *Plasmodium falciparum* merozoites. *ACS Infect Dis* 2020;**6**:379–92

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