Genetic evidence for erythrocyte receptor glycophorin B expression levels defining a dominant *Plasmodium falciparum* invasion pathway into human erythrocytes

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Abstract

*Plasmodium falciparum*, the parasite that causes the deadliest form of malaria, has evolved multiple proteins known as invasion ligands that bind to specific erythrocyte receptors to facilitate invasion of human erythrocytes. The EBA-175/glycophorin A (GPA) and Rh5/basigin ligand-receptor interactions, referred to as invasion pathways, have been the subject of intense study. In this study, we focused on the lesser characterized sialic acid-containing receptors glycophorin B (GPB) and glycophorin C (GPC). Through bioinformatic analysis, we identified extensive variation in glycophorin B transcript (GYPB) levels in individuals from Benin, suggesting selection from malaria pressure. To elucidate the importance of the GPB and GPC receptors relative to the well-described EBA-175/GPA invasion pathway, we used an *ex vivo* erythrocyte culture system to decrease expression of GPA, GPB or GPC via lentiviral short hairpin RNA transduction of erythroid progenitor cells, with global surface proteomic profiling. We assessed parasite invasion efficiency into knock down cells using a panel of wild type *P. falciparum* laboratory strains and invasion ligand knockout lines, as well as *P. falciparum* Senegalese clinical isolates and a short-term culture-adapted strain. For this, we optimized an invasion assay suitable for use with small numbers of erythrocytes. We found that all laboratory strains and the majority of field strains tested were dependent on GPB expression level for invasion. Collective data suggest that the GPA and GPB receptors are of greater importance than the GPC receptor, supporting a hierarchy of erythrocyte receptor usage in *P. falciparum*. 
Introduction

Malaria is a disease of major global health importance that is caused by parasites of the genus *Plasmodium*, of which *Plasmodium falciparum* is the most virulent (1). The asexual erythrocytic stage of the parasite life cycle is responsible for the symptoms associated with malaria (1). A key step during this stage is parasite invasion of erythrocytes mediated by interactions between parasite invasion ligands and their cognate erythrocyte receptors, which define invasion pathways. Across *Plasmodium* species, invasion ligands are grouped into two families – the Duffy binding-like or Erythrocyte binding-like (DBL/EBL) family, and the Reticulocyte binding-like (RBL) family (2–4). *P. falciparum* has four EBL ligands – EBA-175, EBL-1 (PEBL), EBA-140 (BAEBL) and EBA-181 (JESEBL) and five RBL ligands – Rh1, Rh2a, Rh2b, Rh4 and Rh5. Of these, the cognate receptors of five invasion ligands are known – glycophorin A, B and C, which bind EBA-175 (5), EBL-1 (6, 7) and EBA-140 (8, 9) respectively, and complement receptor 1 (CR1) and basigin (BSG), receptors for Rh4 (10, 11) and Rh5 (12) respectively.

Invasion pathways can be classified according to their dependence on the presence of sialic acid on receptors; pathways involving the EBL invasion ligands and Rh1 are reliant on sialic acid (sialic acid-dependent invasion pathways), while those involving the remaining RBL ligands are not (sialic acid-independent invasion pathways). Although *P. falciparum* has an extensive array of invasion pathways, not all are utilized at the same time. The set of dominant invasion pathways used during invasion is strain-dependent and has led to a broad classification of *P. falciparum* strains as sialic acid-dependent or -independent. However, laboratory-adapted strains have the
ability to switch invasion pathway usage when specific receptors or determinants of interaction are absent from the erythrocyte surface (13, 14). Furthermore, field isolates commonly utilize different sets of invasion pathways (15–20). The virulence of \textit{P. falciparum} has been partly attributed to its extensive set of invasion pathways, which enable it to efficiently invade diverse host erythrocytes harboring different receptor polymorphisms.

Most recently, the Rh5/BSG sialic acid-independent invasion pathway has received the greatest attention owing to the essentiality of the Rh5/BSG invasion pathway (12, 20). Other studies have also shown that the EBA-175/GPA sialic acid-dependent invasion pathway plays a significant role in both sialic acid-dependent and sialic acid-independent strains (21–23). Importantly, naturally occurring anti-EBA-175 and anti-Rh5 invasion-inhibitory antibodies have been identified in malaria-exposed individuals (24–27).

Lesser characterized are the EBA-140/GPC sialic acid-dependent invasion pathway (8, 9, 28, 29), and the sialic acid-dependent parasite invasion ligand EBA-181 for which no receptor has been identified (30–32). The EBL-1/GPB invasion pathway has also been poorly characterized and there are contradictory reports regarding the importance of GPB. One study reported a complete block in invasion of the sialic acid-independent strain, 7G8, into GPB null (S-s-U-) erythrocytes (33), although a prior study showed little inhibition of this strain (34). A subsequent study showed a 40-87% range in invasion efficiency into S-s-U- erythrocytes from five donors (35). Donor to donor blood group differences and differences in receptors may contribute to the variable invasion
phenotypes of GPB null cells and underscore a potential weakness of comparing non-isogenic mutant and wild type erythrocytes.

In a search for novel signatures of *P. falciparum* infection, we performed computational analysis of a published dataset of transcriptomic profiles from malaria-infected and healthy Beninese children (36), which led to the discovery that there is wide variation in GYPB transcripts in healthy individuals. This finding provided the impetus for a detailed study of the use of the GPB receptor in *P. falciparum* invasion. In this study, we used an erythrocyte reverse genetics system (37) to specifically knock down levels of expression of the sialic acid-dependent receptors GPA, GPB and GPC. We report that GPB is a key determinant of *P. falciparum* invasion.

**Materials and Methods**

**Bioinformatic analyses.** A set of 314 host genes with known or potential roles in parasite invasion was defined by combining erythroblast-specific genes from HaemAtlas (38) with blood group genes from the International Society of Blood Transfusion (http://www.isbtweb.org/). Whole-blood raw transcriptomic data from 61 healthy Beninese children (36) were obtained from the Gene Expression Omnibus (GSE34404). Two hundred and thirty-eight genes from the erythroblast-specific/blood group set defined above are expressed in the Benin dataset. This subset was background-corrected using the detection *p*-value and quantile normalized using *limma* (39). The 95th to 5th quantile ratios were calculated to identify the most variable transcripts.

**Ex vivo culture of erythrocytes.** 3-10x10^5 bone marrow-derived CD34+ hematopoietic stem cells (HSCs; Lonza) or CD34+ cells derived from peripheral blood of GCSF-
stimulated donors (obtained from the HSCI-Boston Children’s Hospital FACS Core) were cultured in Iscove’s modified Dulbecco’s medium (IMDM, Biochrom) supplemented with glutamine (Sigma-Aldrich), holo-human transferrin (Scipac), recombinant human insulin (Sigma-Aldrich), heparin Choay (USBioAnalyzed) and 5% solvent/detergent virus-inactivated plasma (Octaplas, Octapharma) as described (40) with the following modifications. On day 6 or 7, cells were transduced with lentivirus harboring short hairpin RNA (shRNA) against GYPA (Clone ID: TRCN0000116453), GYPB (Clone ID: TRCN0000084081) or GYPC (Clone ID: TRCN0000083398) or the empty vector (pLKO). Lentiviral particles were either prepared as previously described (37) or obtained from the RNAi Platform (Broad Institute). Transduction and subsequent selection on 2 µg/ml puromycin dihydrochloride (Sigma-Aldrich) were performed as formerly described (41). From day 12 or 13 to day 18, cells were co-cultured on a murine MS-5 stromal cell layer at a cell density of 3-6x10^5 cells/ml as described (42, 43). On day 18, cells were re-plated on a fresh MS-5 stromal cell layer. Cells were harvested on either day 17 or 18 or day 20 and passed through a 5 µm Supor filter (Pall) to remove residual nucleated cells. Filtered, enucleated cells were stored at 4°C in incomplete RPMI [RPMI-1640 (Sigma-Aldrich) supplemented with 25 mM HEPES and 50 mg L^-1 hypoxanthine] until use in subsequent experiments.

**Flow cytometry-based measurement of erythrocyte receptor expression.** Erythrocytes were washed three times in PBS-3% BSA blocking buffer, then pelleted in a 96-well plate at 5x10^5 erythrocytes per well (cultured erythrocytes) or 1x10^6 (peripheral erythrocytes) and finally resuspended in 50 µl (cultured erythrocytes) or 100 µl (peripheral erythrocytes) of PBS-3% BSA or the appropriate antibody solution. The
following antibodies were used at the indicated dilutions: phycoerythrin (PE)-conjugated anti-DARC (1:10, Miltenyi Biotec), anti-CD71-PE (1:10, Miltenyi Biotec), fluorescein isothiocyanate (FITC)-conjugated anti-GPA (1:50, Clone 2B7, STEMCELL Technologies), anti-GPC-FITC (1:500, BRIC 10, Santa Cruz), anti-band 3-FITC [1:100, BRIC 6-FITC, International Blood Group Reference Laboratory (IBGRL)], anti-BSG [1:1,000, Clone MEM-M6/6, Axxora (Exbio)], anti-CRI (1:200, Santa Cruz), anti-DAF (1:3,000, BRIC 216, IBGRL), anti-RhD (1:20, BRAD3-FITC, IBGRL), and anti-GPA/B (1:8,000, Clone E3, Sigma-Aldrich). To measure GPB expression, erythrocytes were treated with 1 mg/ml trypsin (Sigma-Aldrich) to remove GPA before incubation in the GPA/B antibody. For measurement of GPA and GPC expression in Senegal, the following probes were used in addition to the aforementioned antibodies: anti-GPA – BRIC 256 IgG (IBGRL, 1:100,000) and BRIC 256-FITC fab fragment (1:20); anti-GPC – BRIC 10 IgG (IBGRL, 1:500,000), BRIC 10-FITC fab fragment (1:100) and BRIC 4 IgG (IBGRL, 1:8,000). Fab fragments were produced from whole IgG by papain digestion (Pierce Fab Preparation kit; Thermo Scientific) and FITC-labelled (ProtOn Fluorescein Labelling kit, Vector Laboratories), followed by extensive dialysis in PBS.

Cells were incubated for 1 h at room temperature and washed three times in blocking buffer. Unstained cells and cells stained with directly conjugated antibodies were resuspended in 100 μl PBS for analysis on the MACSQuant flow cytometer [in Boston (Miltenyi Biotec)] or the BD FACScalibur [in Senegal (BD Biosciences)]. Erythrocytes incubated with all other antibodies were then incubated in anti-mouse IgG-Alexa Fluor 488 (1:1,000, Life Technologies) for 30 min at room temperature. Control samples with no prior antibody incubation were incubated in either anti-mouse IgG2a-PE
(1:10, Miltenyi Biotec) or anti-mouse IgG-Alexa Fluor 488. Cells were washed twice and subjected to flow cytometric analyses. The data were analyzed using FlowJo 4 v. 10.0.7 for flow cytometry done in Boston or FlowJo v. 8.8.6 for flow cytometry done in Senegal.

**Quantitative cell surface proteomics.** pLKO control, GPA knockdown, GPB knockdown and GPC knockdown cultured erythrocytes (cRBCs) were prepared as described earlier in the text. The following sets of cells were labeled by aminoxy-biotin as described previously (44, 45) – pLKO cRBCs: 10.2 x 10^6 cells (in duplicate); GPA KD: 16.2 x 10^6 cells; GPB KD: 3.5 x 10^6 cells; GPC KD: 11.2 x 10^6 cells; peripheral RBCs: 10.0 x 10^6 cells (in duplicate).

Briefly, surface sialic acid residues were oxidized with sodium meta-periodate (Thermo) then biotinylated with aminoxy-biotin (Biotium). The reaction was quenched, and the biotinylated cells incubated in a 1% Triton X-100 lysis buffer. Biotinylated glycoproteins were enriched with high affinity streptavidin agarose beads (Pierce) and washed extensively. Captured protein was denatured with dithiotreitol (Sigma-Aldrich), alkylated with iodoacetamide (IAA, Sigma) and digested on-bead with trypsin (Promega) in 200 mM HEPES pH 8.5 for 3 hours. Tryptic peptides were collected and labeled using TMT reagents (Thermo Scientific). The reaction was quenched with hydroxylamine, and TMT-labeled samples combined in a 1:1:1:1:1:1:1 ratio. Labeled peptides were enriched and desalted and then 6% of the total sample was subjected to mass spectrometry.

Mass spectrometry data was acquired using an Orbitrap Fusion coupled with an UltiMate 3000 Nano LC (Thermo). Peptides were separated on a 75 cm PepMap C18 column (Thermo). Peptides were separated using a 180 min gradient of 3 to 33%
acetonitrile in 0.1% formic acid at a flow rate of 200 nL/min. Each analysis used a
MultiNotch MS3-based TMT method (45, 46). The scan sequence began with an MS1
spectrum (Orbitrap analysis, resolution 120,000, 400-1400 Th, AGC target 2 x 105,
maximum injection time 50 ms). MS2 analysis consisted of CID (quadrupole ion trap
analysis, AGC 15,000, NCE 35, maximum injection time 120 ms). The top ten precursors
were selected for MS3 analysis, in which precursors were fragmented by HCD prior to
Orbitrap analysis (NCE 55, max AGC 2 x 105, maximum injection time 150 ms, isolation
specificity 0.5 Th, resolution 60,000).

Sample collection for ex vivo invasion assays and erythrocyte receptor expression.
Collection of clinical isolates and their experimental use were approved by the Ethics
Committee of the Ministry of Health in Senegal and by the Institutional Review Board of
the Harvard T.H. Chan School of Public Health. Sample collection was done in
November 2013, towards the end of the transmission season in Senegal. After informed
consent from patients presenting with uncomplicated malaria in Thies, Senegal, ~4 ml of
whole blood was collected from each patient in Sodium Citrate Vacutainers and
transported to Dakar, Senegal. Samples arrived in the laboratory within 6 hours of draw
and after washes in incomplete RPMI and removal of the buffy coat, a fraction of
parasitized cells was enzyme-treated with *Vibrio cholerae* neuraminidase (Sigma-
Aldrich), chymotrypsin (Worthington) and trypsin and placed in culture conditions until
invasion assay set up.
For measurement of erythrocyte receptor expression in healthy Senegalese, 2-3 ml of
whole blood was collected from healthy donors from the Senegalese National Blood
Transfusion Center in Dakar, Senegal. Flow cytometry was performed on the day of blood collection.

Parasite cultures. *P. falciparum* cultures were maintained in human O+ erythrocytes. Parasites were grown at 2% hematocrit in complete RPMI medium (incomplete RPMI supplemented with 2.57 mM sodium bicarbonate (Sigma-Aldrich), 0.25% AlbuMAX II (Life Technologies) and 0.25% AB+ serum (for *ex vivo* cultures) or 0.5% AlbuMAX II for laboratory strains and the short-term culture-adapted strain. Cultures were kept at 37°C in a modulator incubator chamber, gassed with 1% O₂, 5% CO₂ and 94% N₂.

Enzyme treatments. Ring stage parasite cultures were washed three times in incomplete RPMI and treated with 1 mg/ml trypsin, 1 mg/ml chymotrypsin and 66.7 mU/ml of *Vibrio cholerae* neuraminidase for 1 h at 37°C, with gentle mixing. Enzyme-treated cells were then washed twice in incomplete RPMI and once in complete RPMI, then resuspended at 2% hematocrit (HCT) in complete RPMI and placed in culture.

Invasion Assays. Invasion assays were performed in half-area 96-well plates in duplicate or triplicate. At the late trophozoite or schizont stage, the parasite culture was resuspended and adjusted to 0.5% HCT based on hemocytometer cell counts, for *ex vivo* invasion assays in Senegal, or based on volume, for invasion assays conducted in Boston. GPA-, GPB- and GPC-knockdown cultured erythrocytes (cultured red blood cells; cRBCs) and pLKO cRBCs were adjusted to 0.5% HCT based on hemocytometer cell counts. Parasitized cells were added to cRBCs at an 80:20 ratio (see Fig. 4B). For *ex vivo* invasion assays in Senegal, 10 μl of the total sample volume of 40 μl was used for smears to determine the starting parasitemia by light microscopy. Boston-based invasion assays were performed with a total sample volume of 30 μl. Assays were harvested 30-
40 hours post-set up by making smears or cytospin preparations, which were stained with May-Grünwald and Giemsa to determine final parasitemia by light microscopy. 500-2000 erythrocytes were counted, depending on the experiment. The invasion efficiency based on final parasitemia was determined for each strain after normalization to pLKO control cells.

Statistical Analyses. Univariate analyses were performed using GraphPad Prism v. 5.0 for Mac OS X. Significant differences between each knockdown group and control group were determined using a one-way ANOVA and Dunnett’s multiple comparison test.

Data analysis for quantitative surface proteomics. Mass spectra were processed using a Sequest-based in-house software pipeline as previously described (47). Data were searched using the human Uniprot database (April 2014) concatenated with common contaminants, and filtered to a final protein-level false discovery rate of 1%. Proteins were quantified by summing TMT reporter ion counts across all peptide-spectral matches using in-house software as previously described (47), excluding peptide-spectral matches with poor quality MS3 spectra (a combined signal:noise ratio of less than 250 across all TMT reporter ions). For protein quantitation, reverse and contaminant proteins were removed.

A subset of 78 membrane proteins were identified based on the presence of the following criteria extracted from the UniProt (48) database (type I/II/III/IV transmembrane domain, multipass transmembrane domain, GPI-anchored, lipid anchored) or predictions of transmembrane helices based on the TMHMM 2.0 program (49, 50). Each reporter ion channel was summed across all 78 proteins and normalized assuming equal protein loading across all samples. To compare the relative abundance of
the membrane proteins between the pLKO cRBCs and KD cRBCs, proteins with a coefficient of variation $> 0.25$ in the pLKO technical replicates were excluded from further analysis.

Fold change for each protein was calculated according to signal:noise (GYP KD)/average signal:noise (pLKO controls). $P$-values (Significance A) were calculated and adjusted with the Benjamini-Hochberg method using Perseus v. 1.5.1.6 (51). To compare the abundance of plasma membrane proteins between pLKO cRBCs and peripheral RBCs, for each protein the normalized signal for each reporter channel was renormalized to a total of 1 and the data were clustered using the k-means algorithm in Cluster v. 3.0 with a Euclidean distance metric (52) and subsequently displayed using TreeView v. 1.1.6r4 (53).

**Results**

**Glycophorin B transcript levels vary widely amongst healthy individuals in a malaria-endemic region**

Erythrocyte receptors involved in *P. falciparum* invasion and their regulatory regions harbor polymorphisms, some of which are overrepresented in malaria-endemic regions and are suggested to have arisen as a consequence of the selective force of malaria on the human genome. To determine if we could identify additional polymorphisms that might affect *P. falciparum* infection, we performed computational analysis of transcriptional profiles generated from whole blood of children in Benin, published by Idaghdour *et. al* (36). We first defined a subset of erythroid-specific genes from the transcriptome based on the HaemAtlas published by the Bloodomics
Consortium (38) and from known blood groups. Of 238 erythroid-specific/blood group transcripts from 61 healthy children included in the analysis, we identified four genes with wide expression range (95 quantile to 5 quantile ratio larger than 10, z score > 3): carbonic anhydrase 1 (CA1), hemoglobin zeta chain (HBZ), RAP1 GTPase activating protein (RAP1GAP) and unexpectedly, GYPB (Fig. 1A). Importantly, many transcripts with little variation served as internal controls (Fig. 1A).

To determine if this GYPB transcript variation translated to receptor expression variation, we measured the levels of GPB, as well as GPA, GPC and other blood group receptors in healthy donors in the malaria endemic country of Senegal over two consecutive years (Fig. 1B). The variation in surface expression of GPB in GPB-positive individuals, estimated by the 95/5 quantile ratio, was ~1.5 fold above background, much smaller than the transcriptional variation found in the Beninese children. We also observed modest variation in surface expression of other receptors we measured, with a 95/5 quantile ratio ranging from ~1.2 to 3 (Fig. 1B).

Knockdown of GPA, GPB or GPC in ex vivo cultured erythrocytes

To understand expression level modulation of GPB in *P. falciparum* invasion and to determine the importance of this receptor relative to the other major sialic acid-dependent receptors, GPA and GPC, we knocked down expression of GPB in *ex vivo* cRBCs via shRNA-mediated gene silencing (37). In 6-7 independent experiments, we achieved approximately $53.5 \pm 8.9\%$ standard deviation (SD) knockdown (KD) of GPA ($P \leq 0.001$), $72.3 \pm 14.2\%$ SD KD of GPB ($P \leq 0.001$) and $82.9 \pm 6.5\%$ SD KD of GPC ($P \leq 0.001$) (Fig. 2A and 2B), as determined by flow cytometry. We also measured the
expression level of all of the glycophorins (between three and seven times), and following KD of each receptor, found little change in the expression levels of the others by flow cytometry (Fig. 2B). We further measured surface expression by flow cytometry of other known *Plasmodium* receptors (BSG, band 3, CR1 and DARC) (Fig. S1). We observed a four-fold increase in band 3 levels following GPA KD as previously reported (54) a two-fold increase in BSG levels on GPA KD cells, and a ~25% decrease in BSG levels on GPB KD cells. Further, we found no association between GPB and BSG levels by flow cytometry in Senegalese individuals (Fig. 1B). In addition, KD and pLKO control cRBCs had normal erythrocyte morphology (Fig. 2C).

Global proteomic profiling to determine the level and specificity of GPA, GPB and GPC receptor knockdowns

To establish the similarity between cRBCs and physiologically derived red blood cells (pRBCs), we measured expression of 78 cell surface receptors by plasma membrane profiling (PMP) on cRBCs and pRBCs (Table S1). Firstly, this analysis demonstrated the reticulocyte-like nature of the cRBCs by showing that several proteins that decrease in abundance during reticulocyte maturation, including CD71 (TFRC) (55, 56), CD36 (57, 58), ITGA4 (58), and SLC3A2 (59), were decreased in pRBCs relative to pLKO cRBCs (Cluster 1; Fig. 3A). The level of expression of proteins in Cluster 2 remained unchanged between pRBCs and pLKO cRBCs, while Cluster 3 proteins were enriched on pRBCs, and include GPA, and GPC. (By this method we are unable to distinguish between GPA and GPB peptides, thus due to the ~four-fold greater abundance of GPA receptors, we assume that the signal emanates from a ratio of 4:1 for GPA:GPB.)
We have calculated the numbers of molecules per unit surface area of known *P. falciparum* host receptors (46, 60–66) (Table 1), using published values of the surface area of reticulocytes (142.4 ± 2.0 µm$^2$) and pRBCs (133.6 ± 3.0 µm$^2$) (46). We find that the surface density of receptors between pLKO cells and pRBCs for all known receptors, are within three-fold of each other, except for CR1 which is ~five-fold lower on pLKO cRBCs. GPA/GPB and GPC are found at lower levels on pLKO cells, while BSG is the only major receptor that is found at a higher level. The lower density of GPA/ GPB and GPC on pLKO cells suggests that further limitation of receptor densities by knockdown will elicit receptor densities significantly lower than those observed on pRBCs (Table 1).

To ensure that the specificity of invasion phenotypes would result specifically from the receptors that were depleted, we made a comparison of pLKO and KD cRBCs. As expected, GPA and GPC were reduced in the GPA and GPC KD cRBCs respectively (Fig. 3B, 3D). As mentioned earlier, GPA and GPB peptides are indistinguishable; therefore GPB depletion in the GPB KD cells was not observable by this analysis. Of all the known receptors considered, only BSG levels were reduced in any of the KDs (albeit at borderline significance, $P = 0.047$) (Table S1, Fig. 3C), in GPB KD cells. Calculated surface densities of BSG on GPB KD cells are comparable to those found on pRBCs (Table 1), suggesting that BSG is not limited to levels that would contribute to an invasion phenotype. This analysis also revealed other proteins that have significantly altered levels in the pLKO cRBCs relative to KD cRBCs (Fig. 3B-D, Table S2). To date, no role in invasion has been described for these proteins.

Interestingly, band 3 (SLC4A1) levels were not significantly elevated by proteomic analysis on GPA KD cells (Fig. 3B), in contrast to the significant increase in
band 3 levels determined by flow cytometry (Fig. S1) (67, 68). One potential explanation for the discrepancy is that knockdown of GPA improves accessibility of the band 3 antibody to its epitope during flow cytometry measurements.

**A scaled-down assay for measuring erythrocyte invasion efficiency**

To enable us assess invasion profiles of as many *P. falciparum* strains as possible, we developed a scaled-down invasion assay that makes use of small numbers of cells of interest (Fig. 4A). In our assay design, a *P. falciparum* culture treated with neuraminidase (N), trypsin (T) and chymotrypsin (C), constituting donor cells, is added to cells of interest (acceptor cells) in an 80:20 ratio and parasitemia is determined after a single round of invasion. We used the 80:20 ratio of donor to acceptor cells over other ratios because the 80:20 ratio allowed us to use a limited number of cultured erythrocytes, yet gave us a reasonable, measurable final parasitemia of 3% when assessing invasion of 3D7 into pLKO control cells with a starting parasitemia of ~2% (Fig. 4B). The success of this scaled-down assay depends on effective NTC treatment to prevent re-invasion into donor cells, careful counting of acceptor cells (achieved with a hemocytometer) and a reasonable starting parasitemia in the donor culture (ideally 2.5%), since only 20% of the cells in each sample have intact receptors for invasion.

**Glycophorin B is a major receptor used by *P. falciparum* laboratory strains**

To assess the relative importance of GPB in invasion, we tested invasion of KD cRBCs by a panel of wild type *P. falciparum* laboratory-adapted strains and invasion ligand deletion strains. Using our scaled-down invasion assay, we observed decreased
invasion of the sialic acid-dependent strain, Dd2, into GPB KD cRBCs (56.4 ± 24.2% SD) as well as GPA KD cRBCs (72.9 ± 18.3%, SD) as previously reported (37), but no change in invasion of GPC KD cells (91.8 ± 8.2% SD) relative to pLKO control cells (Table 2).

It has been shown that strain 3D7 uses the sialic acid-dependent EBA-175/GPA invasion pathway to some extent (23). We did not observe a decrease in invasion of 3D7 into GPA KD cRBCs consistent with a previous report (37), however, the parent 3D7 strain and the derived invasion ligand deletion lines, 3D7ΔEBA-175 and 3D7ΔRh2b, had decreased invasion into GPB KD cRBCs (Table 2). This decrease reached statistical significance for 3D7 (66.6 ± 23.3% SD and 3D7ΔRh2b (60.5% ± 26.6% SD), indicating that in 3D7, the GPB receptor is more important than the GPA receptor. None of the 3D7 strains showed decreased invasion into GPC KD cRBCs (Table 2), suggesting a lesser role in invasion for this receptor.

Like 3D7, the sialic acid-independent strains 7G8 and HB3 showed decreased invasion of GPB KD cells, though this decrease was not statistically significant (Table 2). We did not observe decreased invasion of 7G8 or HB3 into GPA or GPC KD cRBCs, relative to pLKO cRBCs. Altogether, our data suggest that GPB is important for invasion by the tested sialic acid-dependent and sialic acid-independent laboratory strains of P. falciparum.

Many P. falciparum field isolates use glycophorin B for invasion.

To determine if GPB is important in invasion by field isolates, we assessed invasion of a culture-adapted Senegalese isolate and fresh clinical isolates from Senegal
into KD cRBCs (Tables 2 and 3). Sen51, a short-term culture-adapted strain, which was sensitive to neuraminidase treatment [defined as invasion efficiency of less than 60% into neuraminidase (Nm)-treated pRBCs that are devoid of sialic acid (Table S3)], showed decreased invasion into GPB KD cRBCs (58.8 ± 16.8% SD) compared to GPA KD cRBCs (109.7 ± 28.3% SD) and GPC KD cRBCs (113.0 ± 3.6% SD) (Table 2).

For invasion of fresh clinical isolates, five out of eight strains had less than 60% invasion into GPB KD cRBCs as well as GPA KD cRBCs, while none of the five had less than 60% invasion into GPC KD cRBCs relative to pLKO cRBCs (Table 3). Of these five isolates, three were sensitive to Nm-treated pRBCs (Th303, Th305 and Th306), while two were resistant (Th304 and Th312). Two other isolates, which had decreased invasion only into GPC KD cRBCs were sensitive to Nm-treated pRBCs (Th266 and Th268), bringing the proportion of Nm-sensitive strains to 75% (six out of eight strains).

Invasion into GPB KD cells ranged from 36.7 ± 5.8% SD to 118.0 ± 66.4% SD, while the range for GPA KD cells was 28.5 ± 3.5% SD to 110.4 ± 60.7% SD and for GPC KD cells, 52.5 ± 16.4% SD to 93.5 ± 42.2% SD (Table 3). Overall, our results indicate that for field isolates, GPB is of comparable importance in invasion as GPA, and more utilized than GPC for most strains tested, though there is more heterogeneity in receptor usage for field isolates than lab lines. Furthermore, there is not a simple relationship between sialic acid-dependence and the invasion phenotype into KD cRBCs.

Discussion

In this study we have used erythrocyte reverse genetics in an isogenic background to specifically and comparatively assess the use of three major sialylated erythrocyte receptors – GPA, GPB and GPC – in invasion of erythrocytes by P. falciparum.
laboratory-adapted and field strains. We used genetically altered cRBCs to evaluate the
contribution of specific invasion ligands in isogenic cells in *ex vivo* parasite invasion
assays. This contrasts with previous studies, which depend on enzyme-treated cells and
non-isogenic naturally occurring mutant erythrocytes to reveal invasion pathways (54).
The cRBCs used in this study express the full complement of *P. falciparum* receptors and
are of a relatively homogeneous age, though their reticulocyte-like nature results in some
differences in surface receptor abundance, relative to pRBCs. Nevertheless, we achieved
limiting numbers of receptors on KD cells, enabling us to determine the relative
importance of specific receptors. In comparing invasion into GPA, GPB, and GPC KD
cells, we observed a dependence on GPB by both sialic acid-dependent and sialic acid-
independent laboratory strains; this reliance on GPB was greater than GPA, which is used
by many laboratory strains regardless of sialic acid-dependence (21, 23). Our data also
suggest that the GPC receptor is less important for invasion in most strains tested.

Bioinformatic analysis of transcriptional profiles of Beninese children revealed a
wide variation in GYPB transcript levels and one factor that might contribute to this is
*GYPB* genetic polymorphisms in Africans. A high prevalence of the GPB null genotype
exists in malaria-endemic regions and amongst individuals of African descent – ranging
from 2-8% in West Africa to as high as 59% amongst the Efe pygmies in the Democratic
Republic of Congo (69, 70) – suggesting that malaria pressure selected for this
polymorphism. The Dantu variant of GPB, representing a hybrid GPA-GPB molecule
with a GPB N-terminal region and a GPA C-terminal region, has been shown to confer
protection against invasion and growth of *P. falciparum in vitro* (71). A recent study has
found evidence for a strong protective effect of the Dantu NE genotype against cerebral
malaria and severe malaria anemia in East Africa (70). The Dantu NE genotype, which results from an intricate structural modification of the \textit{GYPE-GYPB-GYP}A locus including deletion of the 3’ end of \textit{GYPB}, was found to have arisen recently in Kenya. In addition to the Dantu variant, the authors identified multiple examples of deletions and duplications at the \textit{GYPE-GYPB-GYP}A locus. In our study, we did not observe variation in GPB surface expression in healthy Senegalese to the same extent as that seen for transcription in Beninese individuals, suggesting that transcriptional differences in GYPB do not directly reflect surface receptor expression. Alternatively, it is possible that there are country-specific differences in GYPB polymorphisms, such that Beninese, but not Senegalese individuals exhibit GYPB transcriptional variation with concomitant variation in surface expression levels. Further investigation is required to understand the origin and significance of GYPB transcriptional variation.

The observation from this work that GPB is an important receptor for \textit{P. falciparum} invasion is at odds with work showing that some laboratory-adapted strains (72, 73) and field isolates from Kenya (74), Columbia and Peru (75) have either an \textit{ebl}-1 gene deletion, a thymidine insertion or a premature stop codon that results in a truncated EBL-1 product. Prior to these findings, some of the studies that had reported use of the EBL-1/GPB invasion pathway had used \textit{P. falciparum} strains that have a mutated or deleted \textit{ebl}-1 gene, for example 7G8, 3D7 or HB3 (33, 35), suggesting that perhaps there is an additional parasite ligand that binds to GPB. In this study, we observe usage of GPB by laboratory strains that are reported to lack a functional EBL-1 ligand: 7G8, 3D7 (73) and HB3 (72), further suggesting that there is an additional parasite ligand for GPB. Such a ligand may have features in common with the other \textit{P. falciparum} invasion
ligands that bind glycophorins (EBA-175, EBL-1 and EBA-140), such as a DBL-like domain.

Importantly, our work demonstrates that many field strains use GPB for invasion. Given this dependence on GPB in a region with GPB null prevalence, it would be relevant to genotype field isolates to determine if there are any mutations in \textit{ebl-1}, as has been noted in some field isolates in Kenya (74), Columbia and Peru (75), and in the absence of inactivating mutations, to determine if invasion-inhibitory EBL-1 antibodies exist in the Senegalese population. Identifying an alternate invasion ligand that binds to GPB would warrant assessing usage by field isolates and determining the ability of naturally acquired antibodies against this ligand to block invasion, which may lead to consideration of this ligand for inclusion in an invasion-blocking subunit vaccine.

Eight of the nine field strains in this study showed decreased invasion into either GPA, GPB, and/or GPC KD cells, emphasizing the sialic acid-dependent nature of many Senegalese strains. We also found that several field isolates had decreased invasion into Nm-treated pRBCs, consistent with previous field studies reporting decreased invasion of Nm-treated pRBCs (15, 18, 26, 76). However, we did not find a simple concordance between decreased invasion into GPA, GPB and GPC KD cRBCs and sialic acid-dependence (as determined by invasion into Nm-treated pRBCs) (Table S3), underscoring the importance of separately assessing specific receptors. In this study, we have investigated the use of three major sialylated receptors, and in so doing, highlighted the lesser role of GPC compared with GPA or GPB. The minor role of the EBA-140/GPC invasion pathway, especially in laboratory strains, is in concordance with other studies (21, 23). Knockout of the EBA-140 invasion ligand is facile in all laboratory strains (9),
and in contrast to knockouts of EBA-175, does not lead to a change in the use of ligand-receptor interactions (23). Jiang et al., show that chymotrypsin treatment of GPA-null cells (En(a-)) causes an almost complete block in invasion of ten *P. falciparum* laboratory strains (21). Both the EBA-175/GPA and the EBA-140/GPC invasion pathways are chymotrypsin-resistant, indicating minimal use of the EBA-140/GPC invasion pathway in the absence of GPA (21). In addition, the limited requirement of GPC for erythrocyte invasion can be viewed in light of the interaction between the subtelomeric variant open reading frame protein (STEVOR) and GPC (77) for the rosetting of parasite-infected erythrocytes, suggesting an additional role for this erythrocyte receptor.

Recent studies have provided more evidence to demonstrate the importance of deformability in the invasion process (78, 79). We did not measure deformability of KD cRBCs, however, it is unlikely that changes in deformability of cRBCs as a result of receptor KDs account for the invasion phenotypes of *P. falciparum* strains into KD cRBCs. This is because GPA null cells and GPB null cells have similar deformability and membrane mechanical stability as WT pRBCs (80), whereas GPC null cells reportedly have decreased membrane stability and deformability (80). But the majority of *P. falciparum* strains in this study had normal invasion into GPC KD cRBCs.

Altogether, the genetic evidence presented in this study reveals that the GPB invasion pathway is important for invasion of strains tested. Our study provides the impetus for a more detailed investigation into the use of GPB by alternative invasion ligands, and the use of EBL-1 in invasion by field isolates, both of which could contribute to the dominance of GPB as a receptor in the hierarchy of the RBL and EBL invasion ligands.
Acknowledgements.

We are grateful to blood donors in Dakar, Senegal and patients in Thies, Senegal for consenting to participate in this study. MTD and SD conceived experiments. SD performed most erythrocyte genetic and parasitological experiments, and data analyses. MC, UK, and AKB carried out some erythrocyte genetic and parasitological experiments and data analyses. MM and CG assisted with erythrocyte genetic experiments. UK, LVN and MPW carried out surface proteomic experiments and data analyses. RHYJ and JMG performed the bioinformatic analyses. ADA, DN, TND and SM supervised sample collection and processing. MTD and MPW supervised molecular experiments. SD, MC, and MTD wrote the manuscript with input from co-authors. We thank Aziz Kosber for technical assistance.

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Figure Legends

Figure 1. Glycophorin B transcriptional variation and erythrocyte receptor expression amongst individuals in malaria-endemic regions. A. Graph highlighting transcripts, including GYPB, whose abundance varied widely amongst erythroid-specific transcripts from 61 healthy individuals in Benin. Bioinformatic analysis was based on the whole blood transcriptomics study of malaria-infected and healthy children in Benin (36).
B. Expression of GPB and other erythrocyte receptors from blood of healthy Senegalese donors in 2011 (left graph) and 2012 (right graph). The graphs show the 95/5 quantile ratio normalized to the quantile ratio for a control secondary antibody. The quantile ratio was based on mean fluorescence intensity values determined by flow cytometry. In 2011, measurements were made on RBCs from 29 donors (GPB) or 32 donors (CD55, CR1) or 41 donors (RhD), and in 2012, on 11 donors (GPB) or 29 donors (all other receptors). In 2011, one GPB null individual and three RhD null individuals were observed but were excluded from measurement of the 95/5 quantile ratio. In 2012, three different probes were used to measure expression of GPA and GPC (see Materials and Methods).

Figure 2. Knockdown of glycophorin A, B and C in cultured erythrocytes. A. Expression of glycophorin A (GPA), glycophorin B (GPB) and glycophorin C (GPC) on the cell surface of GPA knockdown (KD) (green), GPB KD (blue) and GPC KD (pink) cultured erythrocytes (cRBCs) as determined by flow cytometry. Representative flow cytometry plots are shown. Grey traces: receptor expression on pLKO cRBCs; dashed traces: pLKO cells stained with a control antibody. B. Mean expression ± standard error from three to seven independent experiments, normalized to expression on pLKO cells. For measurement of GPB expression, cRBCs were treated with trypsin to remove GPA and cells stained with a GPA/B antibody. Statistical significance was determined using a one-way analysis of variance (ANOVA) with a Dunnett’s multiple comparison test. Significant differences are indicated by ***P ≤ 0.001. C. May-Grünwald, Giemsa-stained cytospins showing normal morphology of KD and pLKO control cRBCs. cRBCs were
passed through a 5 µm filter to remove nucleated cells prior to flow cytometry and
cytospin preparation.

**Figure 3. Quantitative cell surface proteomics of peripheral erythrocytes and
cultured erythrocytes.** A. Quantitative cell surface proteomic comparison between
pLKO cRBCs and peripheral RBCs (pRBCs). K-means clustering analysis of the 78
surface membrane proteins identified three clusters: cluster 1 – proteins that decrease
between pLKO cRBCs and pRBCs; cluster 2 – proteins that remain at the same level
between pLKO cRBCs and pRBC; and cluster 3 – proteins that increase between pLKO
cRBCs and pRBCs. Scale bar represents normalized relative abundance of each protein.
Note that the peptides detected by this technique did not allow a distinction to be made
between GPA and GPB. B – D. Comparison of the relative abundance of membrane
proteins between the pLKO control cRBCs and either the GPA KD cRBCs (B), GPB KD
cRBCs (C) or GPC KD cRBCs (D). Fold change was calculated as: signal:noise (GP
KD)/average signal:noise (pLKO control). Y-axes show the average signal:noise (S:N)
across all samples. *P*-values were estimated using Benjamini-Hochberg corrected
Significance A, calculated in Perseus v 1.5.1.6.

**Figure 4. Schematic of invasion assay design.** A. A ring stage parasite culture is treated
with neuraminidase (N), trypsin (T) and chymotrypsin (C), returned to culture conditions
and allowed to mature to late trophozoite (troph) or schizont stage. The NTC-treated
culture (donor cells) is mixed in an 80:20 ratio with either knockdown (KD) or control
pLKO cRBCs (acceptor cells). Initial parasitemia and/or final parasitemia after one round
of invasion are determined either by microscopy from counts of 500-2000 erythrocytes or
by flow cytometry of SYBR green I-stained cells. Invasion assays are set up at 0.5%
hematocrit. All acceptor cells are counted by hemocytometer prior to assay setup. B.
Invasion of NTC-treated *P. falciparum* 3D7 into pLKO control cRBCs with varying ratio
of enzyme-treated donor cells to pLKO acceptor cells. 100:0 ratio indicates NTC-treated
3D7 donor cells and no pLKO cRBCs. Initial parasitemia was ~2%. Final parasitemia
was determined by flow cytometry of SYBR green I-stained cells. The assay was
performed once in duplicate. Bars represent the mean ± the range. The 80:20 ratio was
selected for subsequent invasion assays. E: Expected parasitemia based on invasion into
50:50 mixture. O: Observed parasitemia.
Table 1. Comparison of copies per unit surface area of known *P. falciparum* host receptors on peripheral erythrocytes and cultured erythrocytes.

<table>
<thead>
<tr>
<th>Protein</th>
<th>pRBC</th>
<th>pLKO</th>
<th>GPA KD</th>
<th>GPB KD</th>
<th>GPC KD</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPA&lt;sup&gt;(62-64, 66)&lt;/sup&gt;</td>
<td>7.49 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4.42 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.25 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.62 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2.38 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>GPB&lt;sup&gt;(62-64, 66)&lt;/sup&gt;</td>
<td>1.87 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.11 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>3.11 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4.05 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>5.95 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>GPC&lt;sup&gt;(62-64, 65)&lt;/sup&gt;</td>
<td>7.49 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2.57 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.52 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.62 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>9.51 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>BSG&lt;sup&gt;(61, 64)&lt;/sup&gt;</td>
<td>2.25 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>6.26 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>8.72 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>2.21 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>6.52 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>CR1&lt;sup&gt;(64)&lt;/sup&gt;</td>
<td>1.50 - 8.98 x 10&lt;sup&gt;0&lt;/sup&gt;</td>
<td>0.31 - 1.84 x 10&lt;sup&gt;0&lt;/sup&gt;</td>
<td>0.41 - 2.43 x 10&lt;sup&gt;0&lt;/sup&gt;</td>
<td>0.38 - 2.31 x 10&lt;sup&gt;0&lt;/sup&gt;</td>
<td>0.52 - 3.13 x 10&lt;sup&gt;0&lt;/sup&gt;</td>
</tr>
<tr>
<td>Band 3&lt;sup&gt;(61)&lt;/sup&gt;</td>
<td>7.49 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>3.53 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4.68 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.97 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>3.26 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kx&lt;sup&gt;(61)&lt;/sup&gt;</td>
<td>7.49 x 10&lt;sup&gt;0&lt;/sup&gt;</td>
<td>8.81 x 10&lt;sup&gt;0&lt;/sup&gt;</td>
<td>1.05 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>7.04 x 10&lt;sup&gt;0&lt;/sup&gt;</td>
<td>8.36 x 10&lt;sup&gt;0&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD55&lt;sup&gt;(61)&lt;/sup&gt;</td>
<td>1.50 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.59 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.49 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>9.47 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1.42 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD44&lt;sup&gt;(60, 61)&lt;/sup&gt;</td>
<td>2.62 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>4.47 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>6.66 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>3.05 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>3.64 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The reported number of copies of known *P. falciparum* host receptors on peripheral erythrocytes (pRBCs) were used to estimate the number of copies of host receptors on pLKO cRBCs based on the normalized signal:noise ratios from quantitative surface proteomics and the following reported surface areas for mature RBCs: 133.6 ± 3.0 µm<sup>2</sup> and for reticulocytes: 142.4 ± 2.0 µm<sup>2</sup> (46), which we assume to be representative of pLKO cRBCs. Since GPA and GPB peptides are indistinguishable by surface proteomics, estimation was based on the reported relative abundance of GPA and GPB on pRBCs. The densities of host receptors per unit surface area for GPA, GPB, and GPC are within three-fold lower on pLKO cRBCs compared to pRBCs.
Table 2. Invasion efficiency of *P. falciparum* laboratory-adapted strains and a Senegalese culture-adapted isolate into knockdown cultured erythrocytes.

<table>
<thead>
<tr>
<th>Strain</th>
<th>GPA KD</th>
<th>GPB KD</th>
<th>GPC KD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dd2</td>
<td>72.9 ± 18.3*</td>
<td>56.4 ± 24.2**</td>
<td>91.8 ± 8.2</td>
</tr>
<tr>
<td>3D7</td>
<td>90.2 ± 11.4</td>
<td>66.6 ± 23.3***</td>
<td>89.6 ± 9.8</td>
</tr>
<tr>
<td>3D7ΔEBA175</td>
<td>100.4 ± 31.8</td>
<td>53.4 ± 30.0</td>
<td>94.1 ± 16.5</td>
</tr>
<tr>
<td>3D7ΔRh2b</td>
<td>101.2 ± 14.8</td>
<td>60.5 ± 26.6*</td>
<td>100.2 ± 4.8</td>
</tr>
<tr>
<td>7G8</td>
<td>85.0 ± 31.5</td>
<td>56.1 ± 26.8</td>
<td>98.3 ± 21.9</td>
</tr>
<tr>
<td>HB3</td>
<td>101.3 ± 43.7</td>
<td>53.6 ± 18.1</td>
<td>93.0 ± 15.5</td>
</tr>
<tr>
<td>Sen51</td>
<td>109.7 ± 28.3</td>
<td>58.8 ± 16.8*</td>
<td>113.0 ± 3.6</td>
</tr>
</tbody>
</table>

*a* Invasion efficiency into KD cells relative to pLKO control cells; based on final parasitemia. Parasitemia was determined by microscopy from counts of 500-2000 erythrocytes, depending on the experiment. Invasion assays were performed in triplicate, four to six times for 3D7, Dd2, 7G8, and HB3 and three times for 3D7ΔEBA-175, 3D7ΔRh2b, and Sen51. Shown are the mean and standard deviation. Statistical significance was determined using a one-way ANOVA with a Dunnett’s multiple comparison test. Significant differences are indicated by *$P < 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$.**
Table 3. Invasion efficiency of *P. falciparum ex vivo* field isolates into knockdown cultured erythrocytes.

<table>
<thead>
<tr>
<th>Strain</th>
<th>GPA KD</th>
<th>GPB KD</th>
<th>GPC KD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th266</td>
<td>88.7 ± 12.1</td>
<td>66.2 ± 14.2</td>
<td>52.5 ± 16.4</td>
</tr>
<tr>
<td>Th268</td>
<td>100.9 ± 28.1</td>
<td>79.1 ± 17.8</td>
<td>54.8 ± 9.4</td>
</tr>
<tr>
<td>Th275</td>
<td>110.4 ± 60.7</td>
<td>118.0 ± 66.4</td>
<td>70.9 ± 10.0</td>
</tr>
<tr>
<td>Th303</td>
<td>39.0 ± 4.1</td>
<td>55.6 ± 4.6</td>
<td>69.1 ± 2.5</td>
</tr>
<tr>
<td>Th304</td>
<td>40.7 ± 1.6</td>
<td>56.0 ± 4.7</td>
<td>92.3 ± 24.9</td>
</tr>
<tr>
<td>Th305</td>
<td>30.6 ± 0</td>
<td>36.7 ± 5.8</td>
<td>86.7 ± 10.1</td>
</tr>
<tr>
<td>Th306</td>
<td>44.9 ± 0.1</td>
<td>47.6 ± 16.5</td>
<td>93.5 ± 42.2</td>
</tr>
<tr>
<td>Th312</td>
<td>28.5 ± 3.5</td>
<td>51.7 ± 10.1</td>
<td>91.8 ± 2.0</td>
</tr>
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</table>

*Invasion efficiency into KD cells relative to pLKO control cells; based on final parasitemia. Parasitemia was determined by microscopy from counts of 800-1000 erythrocytes. Invasion assays were performed once, in triplicate (Th266, Th268, Th275) or duplicate (Th303, Th304, Th305, Th306, Th312). Errors indicate the standard deviation (Th266, Th268, Th275) or the range (Th303, Th304, Th305, Th306, Th312).*
Figure 1

A

Benin

95/5 Quantile Ratio

Erythroblast Genes

CA1

HBZ

GYPB

GYPE

GYPC

GYPA

RAP1GAP

B

Senegal, 2011

95/5 Quantile Ratio

Normalized to Control Ab

GPB  RhD  CD55  CR1

Senegal, 2012

95/5 Quantile Ratio

Normalized to Control Ab

Clone 2B7  fab
BRIC 256  IgG
BRIC 10  IgG
BRIC 4
GPB  BSG  CR1

GPA  GPC
Figure 4

A

Ring culture → Enzyme-treated Ring culture → Enzyme-treated trophs/schizonts → cRBCs

36-42hrs ➔ 80:20 ➔ 30-40hrs

Donor Cells + cRBCs

Measurement of Re-invasion: Microscopy or Flow cytometry

B

3D7 invasion

<table>
<thead>
<tr>
<th>E: 2.9</th>
<th>2.2</th>
<th>1.5</th>
<th>0.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>O: 3.0</td>
<td>2.1</td>
<td>0.7</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Final Parasitemia (%)

3D7 NTC : pLKO cRBCs

50:50  80:20  85:15  90:10  100:0
**FIGURE S1. Characterization of glycophorin A-, B- and C-depleted cultured erythrocytes.**

A. Expression of *P. falciparum* invasion receptors (CR1 and BSG), band 3 and *P. vivax* receptor DARC on the surface of GPA KD, GPB KD and GPC KD cRBCs as determined by flow cytometry. Representative plots are shown. B. Mean expression ± standard deviation of *P. falciparum* invasion receptors (CR1 and BSG), DARC and band 3 receptors on the surface of GPA KD, GPB KD and GPC KD cRBCs as determined by flow cytometry from two to four experiments. cRBCs were passed through a 5 µm filter to remove nucleated cells prior to flow cytometry.
<table>
<thead>
<tr>
<th>cRBCs</th>
<th>Protein</th>
<th>Change</th>
<th>P-value</th>
<th>Description</th>
<th>Function/ Role</th>
</tr>
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<tbody>
<tr>
<td>GPA KD</td>
<td>PARK7</td>
<td>Decreased</td>
<td>0.0141</td>
<td>Protein DJ-1</td>
<td>Positive regulator of androgen receptor-dependent transcription.</td>
</tr>
<tr>
<td>GPB KD</td>
<td>BCAM</td>
<td>Decreased</td>
<td>0.0312</td>
<td>Basal Cell Adhesion Molecule, Lutheran Blood Group</td>
<td>Member of the immunoglobulin superfamily. Receptor for the laminin extracellular matrix protein.</td>
</tr>
<tr>
<td>GPB KD</td>
<td>RHAG</td>
<td>Decreased</td>
<td>0.0418</td>
<td>Ammonium transporter Rh type A</td>
<td>Transport of ammonium and carbon dioxide across the erythrocyte membrane.</td>
</tr>
<tr>
<td>GPB KD</td>
<td>BSG</td>
<td>Decreased</td>
<td>0.0474</td>
<td>Basigin, Ok Blood Group</td>
<td>Essential role in <em>P. falciparum</em> invasion of erythrocytes.</td>
</tr>
<tr>
<td>GPB KD</td>
<td>SLC7A5</td>
<td>Increased</td>
<td>0.0381</td>
<td>Large neutral amino acids transporter small subunit 1 Isoform 2 of Potassium-transporting ATPase alpha chain 2</td>
<td>Transport of large neutral amino acids.</td>
</tr>
<tr>
<td>GPB KD</td>
<td>ATP12A</td>
<td>Increased</td>
<td>0.0336</td>
<td></td>
<td>ATPase, H+/K+ transporter.</td>
</tr>
<tr>
<td>GPC KD</td>
<td>SLC30A1</td>
<td>Increased</td>
<td>0.0464</td>
<td>Zinc transporter 1</td>
<td>Transport of sugars, bile salts and organic acids, metal ions and amine compounds.</td>
</tr>
<tr>
<td>GPC KD</td>
<td>CD46</td>
<td>Increased</td>
<td>1.8441x10^7</td>
<td>Complement Membrane Cofactor Protein</td>
<td>Complement regulatory protein.</td>
</tr>
</tbody>
</table>
**TABLE S3.** Table of neuraminidase sensitivity of *P. falciparum* strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>IE into Nm pRBCs&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Resistant/Sensitive&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dd2</td>
<td>N/A</td>
<td>S</td>
<td>(1)</td>
</tr>
<tr>
<td>3D7</td>
<td>N/A</td>
<td>R</td>
<td>(2)</td>
</tr>
<tr>
<td>3D7ΔEBA175</td>
<td>N/A</td>
<td>R</td>
<td>(2)</td>
</tr>
<tr>
<td>3D7ΔRh2b</td>
<td>N/A</td>
<td>R</td>
<td>(3)</td>
</tr>
<tr>
<td>7G8</td>
<td>N/A</td>
<td>R</td>
<td>(4)</td>
</tr>
<tr>
<td>HB3</td>
<td>N/A</td>
<td>R</td>
<td>(1)</td>
</tr>
<tr>
<td>Sen51</td>
<td>50.8 ± 8.8</td>
<td>S</td>
<td>This Study</td>
</tr>
<tr>
<td>Th266</td>
<td>17.3 ± 7.5</td>
<td>S</td>
<td>This Study</td>
</tr>
<tr>
<td>Th268</td>
<td>51.1 ± 6.3</td>
<td>S</td>
<td>This Study</td>
</tr>
<tr>
<td>Th303</td>
<td>45.3 ± 11.4</td>
<td>S</td>
<td>This Study</td>
</tr>
<tr>
<td>Th304</td>
<td>105.6 ± 4.6</td>
<td>R</td>
<td>This Study</td>
</tr>
<tr>
<td>Th305</td>
<td>48.9 ± 7.6</td>
<td>S</td>
<td>This Study</td>
</tr>
<tr>
<td>Th306</td>
<td>14.1 ± 3.1</td>
<td>S</td>
<td>This Study</td>
</tr>
<tr>
<td>Th312</td>
<td>61.4 ± 22</td>
<td>R</td>
<td>This Study</td>
</tr>
</tbody>
</table>

<sup>a</sup>Invasion efficiency into neuraminidase-treated (Nm) pRBCs relative to untreated pRBCs; based on final parasitemia. Parasitemia was determined by SYBR green flow cytometry. Mean ± standard deviation (Sen51, Th266, Th268) or range (Th303, Th304, Th305, Th306, Th312) for one biological replicate.

<sup>b</sup>Sensitivity to neuraminidase treatment for field isolates in this study was based on a cutoff of 60%.

**References**