Supplementary Materials for

Malaria parasites target the hepatocyte receptor EphA2 for successful host infection

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Materials and Methods

Mosquito Rearing and Sporozoite Production: For *P. yoelii* sporozoite production, female 6–8-week-old Swiss Webster (SW) mice (Harlan, Indianapolis, IN) were injected with blood stage P. yoelii (17XNL) parasites to begin the growth cycle. Animal handling was conducted according to Institutional Animal Care and Use Committee-approved protocols. Female *A. stephensi* mosquitoes were allowed to feed on infected mice after gametocyte exflagellation was observed. Salivary gland sporozoites were isolated according to standard procedures at days 14 or 15 post blood meal. For each experiment, salivary glands were isolated in parallel in order to ensure sporozoites were extracted from salivary glands under the same conditions.

Cell Lines and Culture: Hepa1-6 Cells were purchased from ATCC. HC04 cells were a kind gift from Jetsumon Sattabongkot Prachumsri. Cells were maintained in DMEM complete media (Dulbecco's Modified Eagle Medium (Cellgro, Manassas, VA), supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO), 100 IU/mL penicillin (Cellgro), 100 µg/mL streptomycin (Cellgro), 2.5 µg/mL fungizone (HyClone/ Thermo Fisher, Waltham, MA) and split 1-2 times weekly.

Primary human hepatocyte experiments: Cryopreserved Human Hepatocytes from single donor (Triangle Research, Research Triangle Park, NC) were plated on collagen-coated 12-well plates at a density of 800k live cells per well. Cells were maintained in InvitroGro HI Hepatocyte Media supplemented with Torpedo antibiotic mix (BioreclamationIVT, Baltimore, MD) for 24 hours at 37 C with 5% CO2. 100k P. falciparum sporozoites per well were added and incubated as above for 90 minutes. Cells were then detached from plate using trypsin, fixed in Cytoperm/Cytofix (BD Biosciences, Franklin Lake, NJ) on ice for 15 minutes, and stained for parasite CSP as described above [antibody 2A10, conjugated to Alexa-488]. After staining, cells were incubated with DNA staining buffer [1 µM Sytox Blue (Thermo Fisher Scientific, Waltham, MA), 0.4 mg/mL RNAse A (ThermoFisher), and 5 mM EDTA in PBS] at room temperature for 30 minutes before flow cytometry on an LSRII (BD Biosciences), with analysis on FlowJo software (Tree Star, Ashland, OR).

Antibody Array: Livers were collected from 7 BALB/cJ and 7 BALB/cByJ mice and flash frozen in liquid nitrogen. Frozen livers were then ground using Retsch100 Planetary Ball Mill. Cyroground liver powder was resuspended at 30 ug/mL in 1X Cell Lysis buffer supplemented with 1mM PMSF. Lysates were centrifuged for 10 minutes at 4^{o}C and supernatant was used in the assay. PathScan Antibody arrays (Cell Signaling Technology) were used to assess levels of active RTKs according to manufacturer instructions. Signal was captured using GenePix 2000 microarray scanner. Spots were aligned and signal analyzed using Mapix software (Innopsys, Chicago, IL).

Immunofluorescence Assay: $1.5x10^5$ Hepa1-6 cells were seeded in DMEM complete medium in each well of an eight-well Permanox slide. Cells were infected with $5x10^4$ *P. yoelii* sporozoites. Slides were centrifuged for 3 min at 515 x g in a hanging-bucket centrifuge to aid in sporozoite invasion. After 90 min, we removed media that contained

sporozoites that had not infected the cells and added fresh media only. We allowed LSs to develop for 24 hours or 48 hours, at which time cells were fixed with 4% paraformaldehyde, blocked, and permeabilized for 1 hour in PBS with the addition of 0.1% Triton X-100 and 2% BSA. Staining steps were performed in PBS supplemented with 2% BSA. We stained cells using an anti-EphA2 antibody (Cell Signaling Technology, clone D4A2) at a 1:175 dilution, and a monoclonal antibody to UIS4 (made against recombinant UIS4 by Promab, clone 8E11B3), used at a final concentration of 16.7μg/ml. Cells were incubated with primary antibodies at 4^0C overnight and then washed several times, and antibodies were visualized with the use of AlexaFluor-488 goat anti-mouse and AlexaFluor-594 goat anti-rabbit secondary antibody (Life Technologies, Grand Island, NY, USA). We used DAPI (1 μg/mL) stain to visualize both hepatocyte and parasite nuclei.

Cloning, protein expression and purification of PyP52 and PyP36: Sequences for PyP52 (UniProt:Q7K5V2) and PyP36 (UniProt:Q7RPW4) were optimized for human codon bias and synthesized commercially (Integrated DNA Technologies, San Jose, CA, USA). For PyP52, the endogenous leader sequence (residues 1-24) was replaced by the tissue plasminogen activator (tPA -UniProt:P00750) signal peptide (residues 1–23) to promote protein secretion. The transmembrane domain was removed (residues 458-480) and replaced by a GS linker and poly-Histidine tag (8X HIS). The final expression construct, containing PyP52 amino acid residues 25-457, was placed under CMV promotion for expression in mammalian cell culture. For the PyP36 expression construct, the two amino-terminus transmembrane domains (residues 25-44 and 51-73) were replaced by the tPA signal, and a GS linker 8XHIS tag was added to the carboxyl-terminus. The final PyP36 expression containing amino acids 74-356 was placed under the CMV promoter for expression in mammalian cells.

The expression constructs were used to transfect suspension HEK293 cells maintained in antibiotic free, serum free FreeStyle 293 Expression medium (Life Technologies) using 293-Free transfection reagent (EMD Millipore, Billerica, MA, USA), according to manufacturer's instructions. Five to six days post-transfection, culture supernatants were harvested and clarified by centrifugation/filtration. The supernatants were then supplemented with 350 mM NaCl (final concentration) and 0.2% sodium azide (final concentration) prior to binding to Ni-NTA agarose. The protein-bound resin was treated with the wash buffer (25 mM Tris pH 8, 300 mM NaCl, 20 mM imidazole), and the protein was eluted with the elution buffer (25 mM Tris pH 7.4, 300 mM NaCl, 200 mM imidazole). Purified protein was further separated by size-exclusion chromatography using a HiLoad 16/600 Superdex-200 pg column (GE Healthcare) in HBS-E (10 mM Hepes pH 7, 150 mM NaCl, 2 mM EDTA) to remove contaminants and protein aggregates. Final purity was assessed by analytical size exclusion chromatography. Quality control analysis by Coomassie gel and size-exclusion trace is shown in Fig. S7.

Cell Treatments, Lysis and Western blots: Hepa1-6 cells were plated at 6 x 10^5 cells per well of a 12-well plate in DMEM complete media. Where indicated, cells were treated with complete media only, 10 μg/mL recombinant P52 (described above), 10 μg/mL recombinant P36 (described above), or a combination of both for 30 min. EphrinA1-Fc

(R&D Systems, Minneapolis, MN, USA) was supplemented at 1 μg/mL 10 min before cell lysis, alone or in combination with recombinant P52 or P36. Dasatinib (Cell Signaling Technology, Danvers, MA) was used at 200 nM. Cells were lysed in SDS lysis buffer (2% SDS, 50mM Tris-HCl, 5% glycerol, 5 mM EDTA, 1 mM NaF, 10mM β-glycerophosphate, 1 mM PMSF, 1 mM activated Na_3VO_4, 1 mM DTT, 1% phosphatase inhibitor cocktail 2; Sigma-Aldrich, St. Loius, MO, USA), 1% PhosSTOP Phosphatase Inhibitor Cocktail Tablet (Roche, Indianapolis, IN, USA), filtered for 30 min at 4000 rpm through AcroPrep Advance Filter Plates (Pall Corporation, Port Washington, NY, USA) and stored at -80^{0}C. Western blots were performed according to manufacturer instruction with the iBlot Dry Transfer System (Life Technologies, Carlsbad, CA, USA). Membranes were probed with antibodies against p-EphA2(clone D4A2) (pY772) (Cell Signaling Technology, Danvers, MA) at a dilution of 1:1000 and βActin (clone 8H10D10) (Cell Signaling Technology, Danvers, MA) at a dilution of 1:2000 in a solution of 5% BSA and 0.1% TritonX100. Signals from immunoblots were detected using either an Alexa 680-conjugated anti-rabbit antibody or an Alexa 800-conjugated anti-mouse antibody used at 1:7500 in the same solution as above (LI-COR Biosciences). Membranes were visualized using an Odyssey infrared imaging system (LI-COR Biosciences).

In vivo experiments in EphA2$^{(-/-)}$ mice: EphA2$^{(-/-)}$ mice were purchased from Jackson labs. WT controls were crossed with EphA2$^{(-/-)}$ mice, then F2 animals were generated by crossing heterozygotes and littermates were used as controls. Mice were infected i.v. with 10^5 *P. yoelii* sporozoites via tail-vein injection. Animals were sacrificed at 42 h post-infection and liver tissue was harvested in TRIzol (Life Technologies, Carlsbad, CA, USA). Animal handling was conducted according to Institutional Animal Care and Use Committee-approved protocols.

Quantification of liver burden by real-time RT-PCR: Total RNA was extracted using TRIzol reagent. cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit according to the manufacturer's instructions (Qiagen, Germantown, MD, USA). All PCR amplification cycles were performed at 95°C for 30 s for DNA denaturation, and 60°C for 4 min for primer annealing and DNA strands extension. Parasite 18S was amplified using primers with sequences: 5'GGGGATTGGTTTTGACGTTTTTGCG3' and 5'AAGCATTAAATAAAGCGAATACATCCTTAT3'. Mouse GAPDH was amplified using sequences 5'CCTCAACTACATGGTTTACAT3' and 5'GCTCCTGGAAGATGGTGATG3'. For quantitative PCR (qPCR), a standard curve was generated using 1:4 dilutions of a reference cDNA sample for PCR amplification of all target PCR products. Experimental samples were compared to this standard curve to give a relative abundance of transcript.

Quantification of liver stages, EphA2 level and PVM formation by FACS: For Cells were cultured as described above. *Plasmodium yoelii* infections, Hepa1-6 cells were seeded at 1x10^5 cells/cm^2, and infected with *P. yoelii* sporozoites at an MOI of 0.3, 16-24 hrs following plating. For *Plasmodium falciparum* infections, HC04 cells were seeded 1x10^5 cells/cm2, and infected with *P. falciparum* sporozoites at an MOI of 0.3, 16-24 hrs

following plating. At the desired time point, cells were detached using a Cell Dissociation Buffer, enzyme-free (Life Technologies). Unless specifically indicated, total, not surface EphA2 was monitored. Surface EphA2 was measured using an antibody against EphA2 (R&D Systems, Minneapolis, MN, Clone #233720) conjugated to PE in 2% BSA prior to permeabilization, after fixation with 4% PFA. Total mouse EphA2 was measured using an antibody against EphA2 conjugated to APC (R&D Systems, Minneapolis, MN, Clone #233720) following permeabilization with Perm/Fix solution (BD Biosciences) with 2% BSA in PBS. Permeabilized cells were blocked in Perm/Wash buffer (BD Biosciences Franklin Lakes, NJ, USA) supplemented with 2% BSA. Additional staining steps were performed in the same buffer. Cells were stained using the monoclonal antibody (clone 2F6, for *P. yoelii* and 2A10 for *P. falciparum*) to circumsporozoite protein (CSP) conjugated to Alexa Fluor® 647 at a final concentration of 2 μg/mL. Human EphA2 was only monitored after permiabilization according to manufacturers' specifications. For PVM identification, *P. yoelii* parasites which express a UIS4-Myc fusion were detected using a human c-Myc antibody (clone 9E10) (R&D Systems, Minneapolis, MN, USA) according to manufacturer's specifications. Permeability dye from Invitrogen (LIVE/DEAD® Fixable Yellow Dead Cell Stain Kit, for 405 nm excitation) was used to manufacturer's specifications. All populations were identified by FACS. Analysis was performed on BD LSRII. Flow cytometric analysis was performed using FlowJo software (TreeStar). All experimental conditions were tested in biological triplicate. All data is representative of three independent experiments.

Statistical Methods: Unless otherwise indicated, p-values were determined using a two-tailed end t-test for samples with unequal variance.

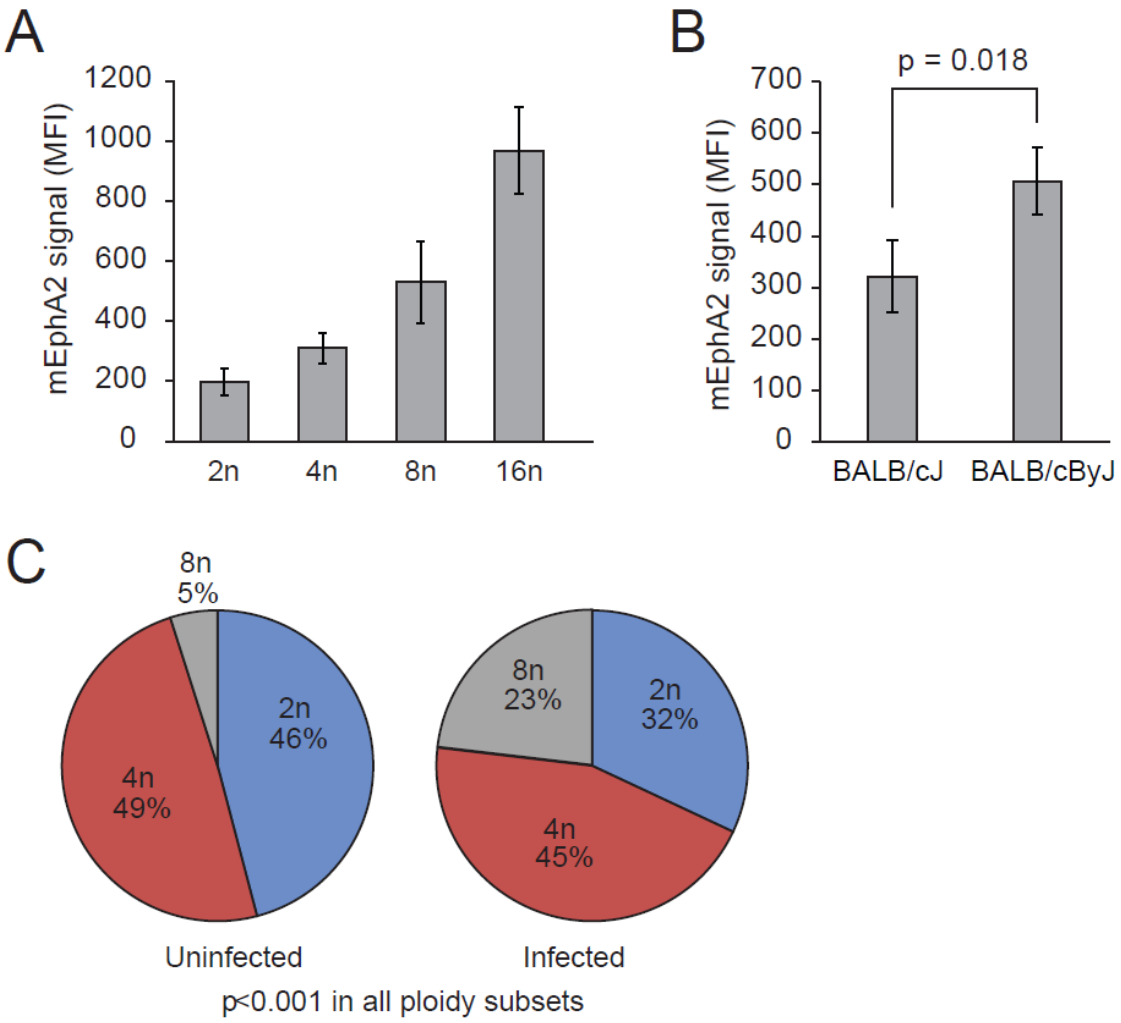

Fig. S1.

EphA2 levels are increased in more susceptible hepatocytes. (**A**) Hepa1-6 cells were stained for EphA2 and DNA and monitored by flow cytometry. EphA2 levels are higher in cells with higher DNA content. (**B**) Hepatocytes were isolated by collagenase-mediated perfusion and percoll gradient from BALB/cJ or BALB/cByJ mice and then stained with EphA2 and assessed by flow cytometry. BALB/cByJ mice have higher levels of EphA2 than BALB/cJ mice. (C) Plated human hepatocytes infected with P. falciparum show a higher percentage of cells with 8n DNA content, and lower percentage of 2n cells, than uninfected cells from the same culture. Results are from single hepatocyte donor; results are representative of multiple donors.

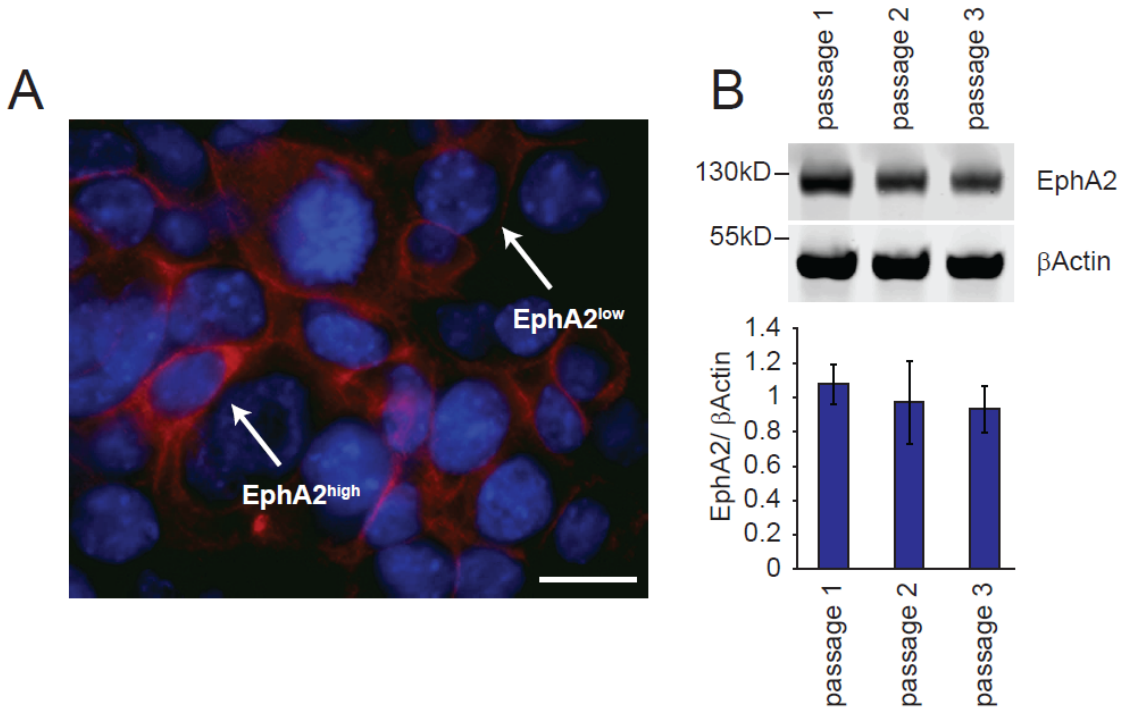

Fig. S2

EphA2 levels vary more within a single culture than between cultures. (**A**) Image showing variability of EphA2 levels between hepatoma cells in a Hepa1-6 culture. EphA2 staining is depicted in red, DNA is visualized by DAPI in blue. Scale bar is 10µm. (**B**) Hepa1-6 cells were passaged for one month and lysed every 1-3 passages. Levels of EphA2 were assessed by western blot and βActin was used as a loading control.

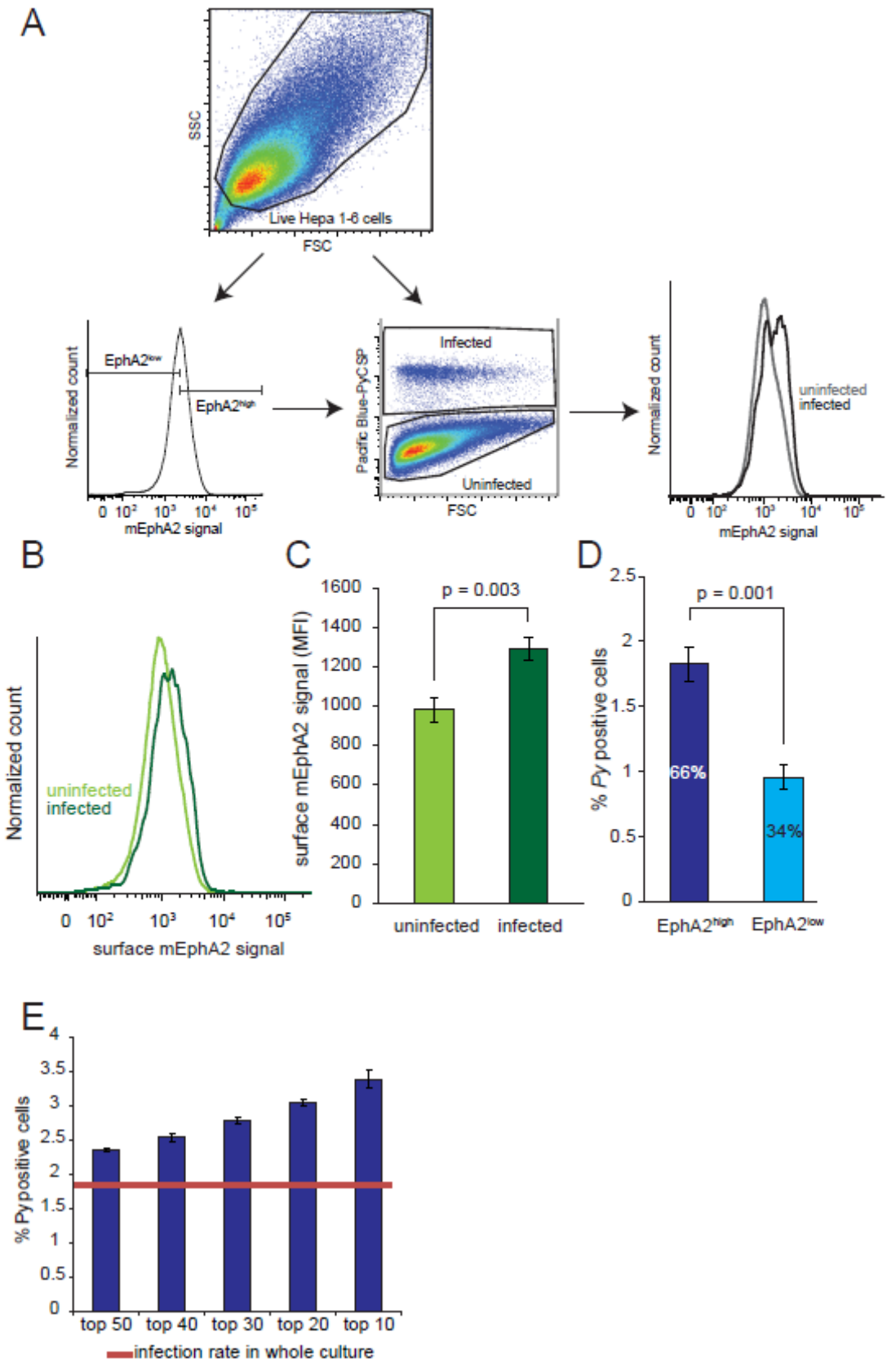
A
SSC
Live Hepa 1-6 cells
FSC
Normalized count
EphA2low
EphA2high
mEphA2 signal
Pacific Blue-PyC SP
Infected
Uninfected
FSC
Normalized count
uninfected
infected
mEphA2 signal
B
Normalized count
uninfected
infected
surface mEphA2 signal
C
surface mEphA2 signal (MFI)
p = 0.003
uninfected
infected
D
% Py positive cells
p = 0.001
66%
34%
EphA2high
EphA2low
E
% Py positive cells
top 50
top 40
top 30
top 20
top 10
infection rate in whole culture

Fig. S3

(**A**) Gating strategy for identifying CSP-positive cells, EphA2^{high} and EphA2^{low} cells. (**B**) *Plasmodium* sporozoites preferentially invade hepatocytes with high EphA2 expression *in vitro* and *in vivo*. Histograms depict the distribution of surface levels of EphA2 in uninfected (light green) and infected (dark green) Hepa1-6 cells. (**C**) Surface EphA2 levels in infected Hepa1-6 cells are higher than uninfected Hepa1-6 cells within a single culture. EphA2 levels are monitored by EphA2-PE and expressed as median fluorescence intensity (MFI). (**D**) Infection rates within EphA2^{high} and EphA2^{low} populations are illustrated. The percentages within each bar graph represent the proportion of infected cells within each subset of EphA2 expression. (**E**) Hepa1-6 cells were infected with *P. yoelii* parasites at a MOI of 0.3. The highest 10, 20, 30, 40 or 50% of EphA2-expressing hepatoma cells were gated, then the infection rate within that subset is shown. The infection rate in the total culture is depicted with a red line.

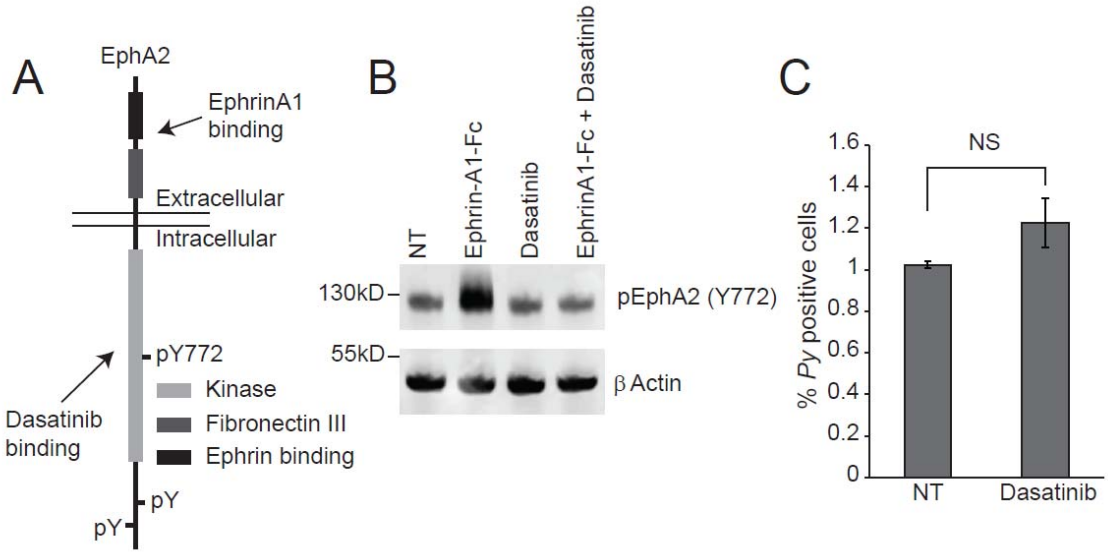

Fig. S4

Dasatinib treatment does not impact initial parasite infection. (A) Schematic describing EphA2 structure. (B) Hepa1-6 cells were stimulated with 1 µg/mL EphrinA1-Fc for 10 min with or without 2 h pretreatment with Dasatinib. Cells were lysed and levels of activated EphA2 (pY772) was assessed by western blot. (C) Untreated or Dasatinib treated Hepa 1-6 cells were infected with *P. yoelii* sporozoites. Infection rate was assessed by staining with an antibody against CSP and flow cytometry. Dasatinib treatment does not impact infection.

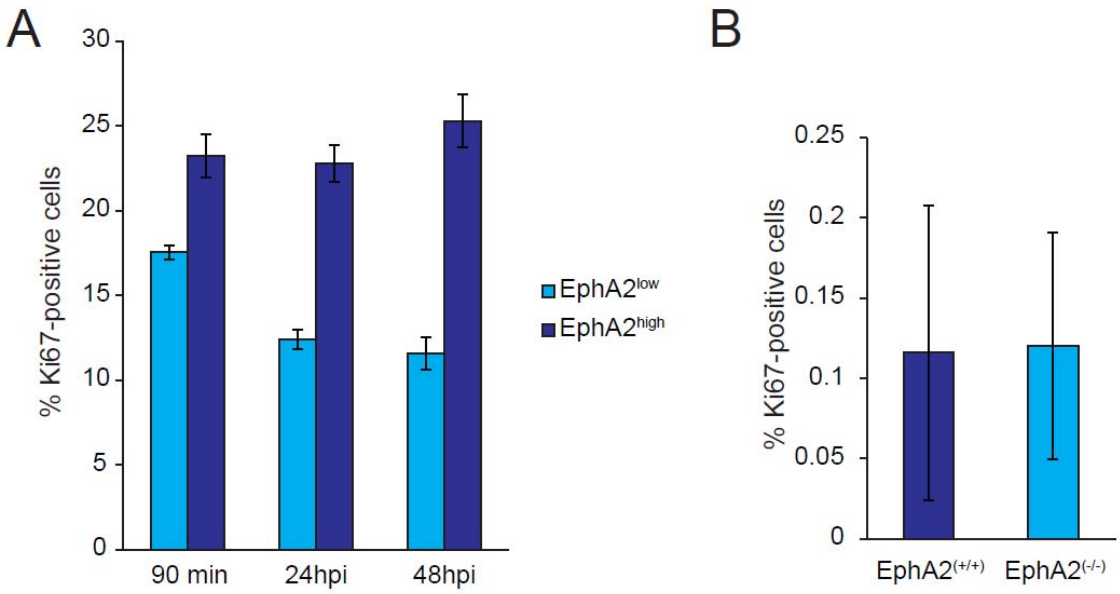

Fig. S5

(**A**) Cell division is increased in EphA2^{high} cells. Hepa1-6 cells were stained for EphA2 and the cell cycle progression marker Ki-67 and assessed by flow cytometry. EphA2^{high} cells have a greater proportion of cycling cells than EphA2^{low} cells. (**B**) Hepatocytes of EphA2$^{(-/-)}$ mice have similar cell division rates when compared to hepatocytes of WT mice. Livers from three WT BALB/cByJ mice and three EphA2$^{(-/-)}$ mice were dissociated by collagenase-mediated perfusion. Hepatocytes were stained for the cell cycle marker Ki-67.

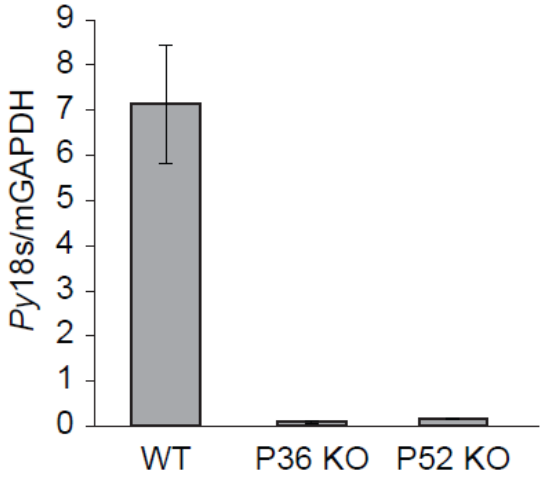

Fig. S6

p52-/p36- parasites are rapidly cleared from liver *in vivo*. BALB/cJ mice were infected with 10^5 *p52-*, *p36-* or WT *P. yoelii* sporozoites. Mice were sacrificed 3 h after infection and assessed for liver stage burden by qRT-PCR.

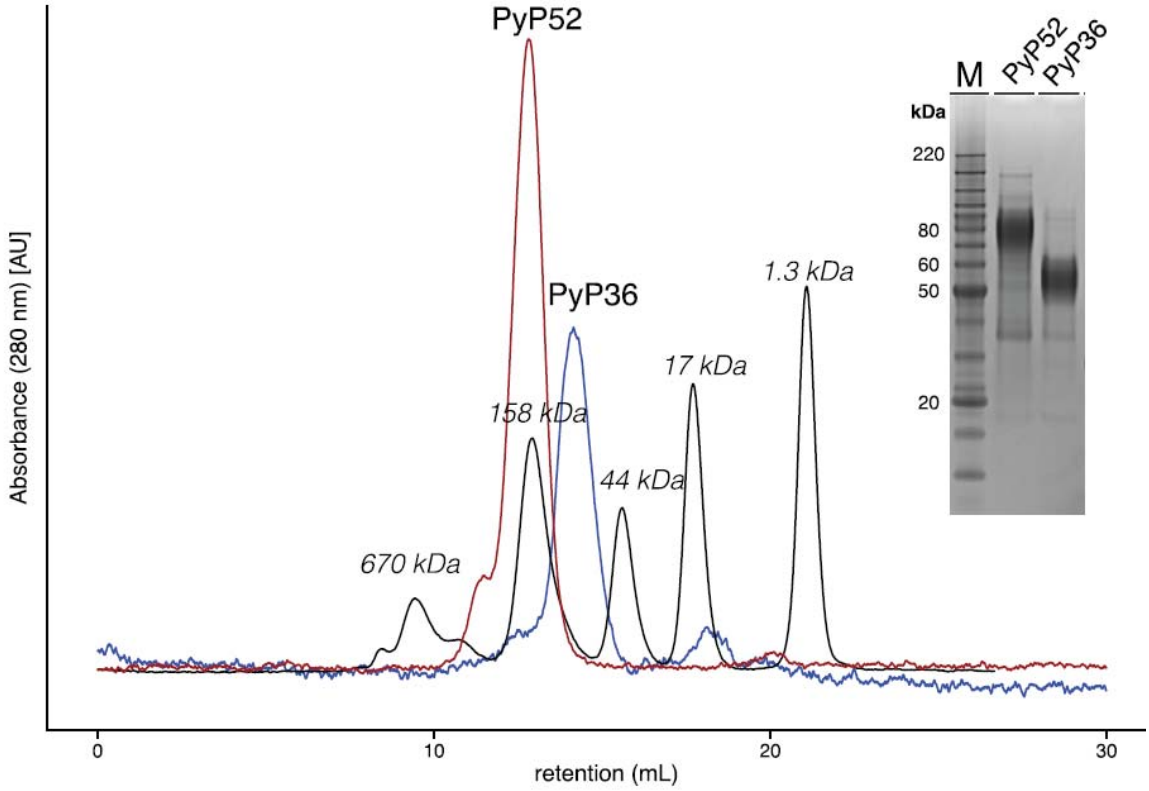

Fig. S7
Recombinant protein production in HEK293 cells. Purified PyP52 (~160-kDa peak; red) and PyP36 (~100-kDa peak; blue) were separated by size-exclusion chromatography on a calibrated Superdex 200 column (molecular weight standards are overlayed in black). Migration patterns of PyP52 and PyP36 under denaturing conditions (SDS-PAGE; inset) suggest that the recombinant proteins are glycosylated and dimerize under native conditions.

Table S1.
Assessment of the activity of 28 receptors in BALB/cJ and BALB/cByJ mouse livers

Receptor*	BALB/cByJ signal	BALB/cJ signal**	p-value
M-CSFR-CSF-1R	**1.314**	**1.000**	**0.001**
c-Kit-SCFR	**1.317**	**1.000**	**0.003**
Met-HGFR	**1.294**	**1.000**	**0.004**
PDGFR	**1.226**	**1.000**	**0.004**
IGF-IR	**1.318**	**1.000**	**0.005**
EGFR-ErbB1	**1.292**	**1.000**	**0.005**
EphA2	**1.218**	**1.000**	**0.006**
Tie2-TEK	**1.289**	**1.000**	**0.006**
EphB1	**1.250**	**1.000**	**0.008**
HER3-ErbB3	1.299	1.000	0.011
EphB3	1.292	1.000	0.012
InsR	1.269	1.000	0.013
EphA1	1.187	1.000	0.014
TrkB-NTRK2	1.137	1.000	0.015
FGFR4	1.159	1.000	0.015
Ron-MST1R	1.280	1.000	0.018
FGFR1	1.157	1.000	0.020
VEGFR2-KDR	1.211	1.000	0.022
EphB4	1.260	1.000	0.028
EphA3	1.242	1.000	0.038
Tyro-3-Dtk	1.156	1.000	0.047
HER2-ErbB2	1.207	1.000	0.048
Axl	1.164	1.000	0.057
TrkA-NTRK1	1.193	1.000	0.074
ALK	1.323	1.000	0.088
FGFR3	1.168	1.000	0.090
FLT3-Flk2	1.223	1.000	0.103
Ret	0.953	1.000	0.599

**Receptors are depicted in bold if they have >20% increase in signal in BALB/cByJ mice with a p-value of <0.01
** All receptor levels were normalized to the average level in BALB/cJ mice