Supplemental Information for

Dual RNAseq shows the human mucosal immunity protein, MUC13, is a hallmark of *Plasmodium* exoerythrocytic infection

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Materials and Methods

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Parasites

P. berghei-GFP (Pb-GFP) (Franke-Fayard et al., 2004) and *P. berghei* luciferase (Swann et al., 2016) sporozoites were obtained by dissection of infected *An. stephensi* mosquito salivary glands. Dissected salivary glands were homogenized in a glass homogenizer, then filtered twice through nylon cell strainers (25 μm pore size) and counted using a Neubauer hemocytometer and kept on ice until infection. Pb-GFP infected *An. stephensi* mosquitoes were obtained from the Insectary Core Facility at New York University.

Cell Culture

Huh7.5.1, HC04, and HepG2-CD81 cells were cultured at 37 °C and 5% CO_2 in DMEM High glucose media (Invitrogen), supplemented with 10% FCS (Corning), 200 U/ml penicillin, and 200 μg/ml streptomycin (Invitrogen). During infection, cell media was supplemented with 12 μM 5-fluorocytosine (Cayman), 50 μg/ml gentamicin sulfate (Gemini Bio-Products), and 100 μg/ml neomycin trisulfate salt hydrate (SIGMA).

Infection of Hepatocytes for RNASeq and Western Blot analysis

24 hours prior to infection, hepatocytes were either seeded in 24 well (RNA extraction - 120,000 per well) or 96 well plates (Luciferase growth assays - 30,000 per well). Hepatocytes were infected *in vitro* with *P. berghei* sporozoites freshly dissected from infected *An. stephensi* mosquitoes at a ratio of 0.3 sporozoites per seeded cell. Plates were centrifuged at 330 x g for 4 minutes to bring sporozoites closer to cells, and plates were then incubated at 37°C 5% CO_2 for 2 hours to promote sporozoite invasion (Prudencio et al., 2006). After 2 hours, the cells were washed, fresh media containing 12 μM 5-fluorocytosine (Cayman), 50 μg/ml gentamicin sulfate (Gemini Bio-Products), and 100 μg/ml neomycin trisulfate salt hydrate (SIGMA) was added and the cells were returned to the incubator.

Separation of Infected and Uninfected Hepatocytes and RNA Isolation

Cells were dissociated from plates at time zero (uninfected hepatocytes and sporozoites before infection), 24 hpi (when the sporozoites have transformed into trophozoites), and 48 hpi (when the trophozoites have transformed into liver stage schizonts), by the addition of 500 μL TrypLE (ThermoFisher), washed and resuspended in fresh media, and passed through a 40 μm cell strainer (Falcon). Uninfected cells were isolated from infected cells by FACS sorting with a BD Influx cell sorter, with gating based upon GFP mean fluorescence intensity (MFI). Cells were sorted directly into 600 μl Qiazol reagent (Qiagen) and total RNA was isolated using a Qiagen miRNEasy kit (Qiagen).

Dual RNA-sequencing

Total RNA was assessed for quantity and quality using an Agilent Tapestation. RNA libraries were generated using Illumina's TruSeq Stranded Total RNA Sample Prep Kit using at least 100 ng of RNA. RNA libraries were multiplexed and sequenced with 100 base pair (bp) paired single end reads (SR100) to a depth of approximately 25 million reads per sample on an Illumina HiSeq2500.

Dual RNA-sequencing analysis

FastQC (Andrews, 2010) was used to perform quality control. Raw reads were first aligned to the human genome (GRCh38, release 25) using STAR2.5.2b (Dobin et al., 2013) and sorted using samtools1.2 (Li et al., 2009). Gene expression was quantified using HTSeq (Anders et al., 2015). Unmapped reads were extracted using Picard tools1.141 (http://broadinstitute.github.io/picard), and aligned to *P. berghei* (release 32 in PlasmoDb). Parasite gene expression was obtained using the same workflow as the human transcripts. Differential expression analysis was performed using DESeq2 (Love et al., 2014). A multifactorial design was used for the paired differential expression analysis for both infected and non-infected samples at 48 hours.

Sequencing Data Deposition

All RNA sequencing files were deposited in the short read sequence archive (http://www.ncbi.nlm.nih.gov/sra) under BioProject ID PRJNA390648. All statistics for these sequencing runs are available in Table S6.

Gene Validation (qPCR and Western)

For real-time qPCR, RNA was extracted as indicated above, then converted to cDNA using superscript II (Invitrogen) and random hexamers, according to the suggested protocol. qPCR was performed on a Bio-Rad CFX96, using the Perfecta SYBR green master mix (Quanta) and primers indicated in Table S7. For western blots, Huh7.5.1 and HC04 cells were plated at a concentration of 120,000 cells per well in a 24-well plate. After infection and sorting as described above, 1,000 infected or 100,000 uninfected cells were washed twice with cold 1X PBS. Cells were then lysed within the plate via the addition of 200 µl RIPA buffer plus 1:100 protease inhibitor (Halt – Thermo Fisher Scientific). Equal numbers of cells, due to the low numbers of infected cells, were loaded for protein analyses, and proteins were loaded onto BioRad anyKD gels. Proteins were transferred to membranes and were probed with primary anti *HsMUC13* (Rabbit polyclonal - Lifespan Biosciences #LS-C345092) and α- Vinculin (Rabbit monoclonal – Abcam #ab129002) antibodies at 1:1,000 dilution overnight at 4°C, probed with goat anti-rabbit HRP secondary (Life Technologies #G21234) and detected using SuperSignal West Pico and Femto (4:1 ratio Pico:Femto) Chemiluminescent Substrate (Thermo Fisher). Densitometry was calculated using ImageJ (http://rsbweb.nih.gov/ij/) after image inversion, and is shown relative to loading control.

***Plasmodium* ExoErythrocytic Form (EEF) culture for cellular localization**

For *P. berghei* imaging, 96-well glass bottom plates (MatTek Corporation) were coated with Poly-L-Lysine 0.01% (v/v) (SIGMA) and subsequently seeded with Huh7.5.1 or HC04 cells (110,000 cells per well) 24 hours before infection. *P. berghei* sporozoites were freshly isolated from infected *An. stephensi* mosquitoes as above and resuspended in DMEM media, but additionally supplemented with 0.5 µM posaconazole (Cayman), 12 µM 5-fluorocytosine (Cayman), 50 µg/ml gentamicin sulfate (Gemini Bio-Products), and

100 µg/ml neomycin trisulfate salt hydrate (SIGMA). Pre-seeded well plates were infected with *P. berghei* sporozoites using a 1:3 infection ratio (sporozoite to cell) and incubated for 2 hours at 37°C in 5% CO_2. After 2 hours, media was replaced and plates were incubated for 48 hours.

For *P. vivax* imaging*, P. vivax* sporozoites were freshly isolated from infected *An. darlingi* mosquitoes from a laboratory-established colony in the Peruvian Amazon region (Moreno et al., 2014). *P. vivax* EEF culture was performed as follows, 24 hours prior to mosquito dissection and infection, 8-well Nunc Lab-Tek chamber slides (Thermo Scientific) were coated with Poly-L-Lysine 0.01% (v/v) (SIGMA) and seeded with HC04 cells (45,000 cells per well). Accudenz purified *P. vivax* sporozoites were diluted in antibiotics and antifungals supplemented DMEM as above. Slides were infected using a 1:2 infection ratio (sporozoite to HC04 cell) and incubated for 4 hours at 37°C under 5% CO_2 atmosphere. After this initial incubation period, infection media was replaced and slides were incubated for 7 days, with cell culture media replaced every 48 hours.

Plasmodium EEF and *MUC13* immunofluorescence microscopy and high content imaging

Slide chambers and 96 well plates, generated as described above, were fixed with 4% paraformaldehyde-PBS (Affymetrix) for 20 min, permeabilized with 0.1% Triton X-100 (SIGMA) for 5 min and stained overnight at 4°C using one of three antibodies. The first antibody was a HSP70 (*Plasmodium* heat shock protein 70) mouse polyclonal antibody (dilution 1:500, 1 mg/ml stock), developed by GenScript using a codon-optimized sequence of *P. berghei* HSP70 (PBANKA_0711900.1). The identity of this amino acid sequence between *P. berghei* and *P. vivax* is 95% and the antibody recognizes HSP70 from both *Plasmodium* species.   The following amino acid sequence was used to generate the antibody:

MVGGSTRIPK IQTLIKEFFN GKEACRSINP DEAVAYGAAV QAAILSGDQS NAVQDLLLLD
VCSLSLGLET AGGVMTKLIE RNTTIPAKKS QIFTTYADNQ PGVLIQVYEG ERALTKDNNL
LGKFHLDGIP PAPRKVPQIE VTFDIDANGI LNVTAVEKST GKQNHITITN DKGRLSPEEI
DRMVNDAEKY KAEDEENKKR IEARNSLENY CYGVKSSLED QKIKEKLQPN EVETCMKSVT
SILEWLEKNQ LAKDEYEAK QKEAEAVCSP IMSKIYQDAG AAAGGMPGGM PGGMPGGMPG
GMPGGMNFPG GMPGGMGAPA GAPAGSGPTV EEVD.

The conserved HSP70 fragment, which was codon optimized for *E. coli*, was expressed in *E. coli*, double purified, and used for mice immunization according to GenScript protocols. The second antibody was a commercially obtained *Pb*UIS4 (*P. berghei* up-regulated in infective sporozoites gene 4) goat polyclonal antibody, used at a 1:200 dilution from 1 mg/ml (Biorbyt #orb11636). The third antibody was *Hs*MUC13 (human Mucin 13, c-terminus region) rabbit polyclonal antibody, used at a 1:500 dilution from a 1 mg/ml stock (MUC13 Antibody #1 - LifeSpan BioSciences #LS-C345092). A second antibody specific for the extracellular domain of *Hs*MUC13 (MUC13 Antibody #2 - LifeSpan BioSciences #LS-A8191) was also used for confirmation, using the same experimental conditions, in Figure S6. All primary antibodies concentrations were determined by performing 15 serial dilutions (1 mg/ml stock concentration) ranging between 1:10 to $1:1\times10^6$ and a fixed concentration of secondary antibodies (1.5 mg/ml stock concentration, dilution 1:500).

Once the primary antibody concentrations were optimized, four different secondary dilutions, starting from a 1.5 mg/ml stock concentration were tested: 1:500, 1:600, 1:1000, and 1:1500. The following secondary antibodies and dilutions were ultimately used: 1) Alexa Fluor 488-conjugated AffiniPure Goat Anti-Rabbit IgG, Fc Fragment (Jackson ImmunoResearch Lab Inc #111-545-008) (dilution 1:600) and 2) Alexa Fluor 647-conjugated AffiniPure Goat Anti-Mouse IgG, Fc Fragment (Jackson ImmunoResearch Lab Inc #111-605-008) (dilution 1:500) and 3) Alexa Fluor 647-conjugated AffiniPure Fab Fragment Bovine Anti-Goat IgG, Fc fragment specific (Jackson ImmunoResearch Lab Inc # 805-607-008) (dilution 1:500). Nuclei and hepatocyte plasma membrane were detected using Hoechst 33342 (Thermo Fisher) at 500 µM and CellMask deep red (Thermo Fisher Scientific) at 1X. After immunofluorescence staining, chambers were removed from *P. vivax* infected Lab-Tek systems, slides were mounted with Vectashield (Vector Labs) and #1.5 glass coverslips were affixed using nail polish. *P. berghei* infected well plates were resuspended in PBS and covered with aluminum foiled seals. Images were acquired using a PerkinElmer UltraView Vox Spinning Disk Confocal (100X or 60X oil objective); UltraVIEW laser power was set to 50% for 405 nm, 488 nm, 561 nm, and 640 nm. Exposure values of 474 and 611 milliseconds (ms) were used on

the green and red channel, respectively. Some 96 well plates were also scanned using the Operetta high content imaging system (PerkinElmer) (Table S8).

Liver-stage *P. berghei* drug *in vitro* assay

In vitro drug assays were performed in coated 96-well glass bottom plates (MatTek Corporation) seeded with HC04 cells and infected with *P. berghei* sporozoites as previously described. Infected wells were treated with 4-point serial 10-fold dilutions (1 μM highest concentration) of atovaquone (Santa Cruz biotechnology # sc-217675) or puromycin dihydrochloride (Santa Cruz biotechnology # sc-108071B). Independent drug treatments were performed 2, 12, and 24 hpi. Positive controls of growth with DMSO 0.1% (v/v) and uninfected wells were also included. Plates were incubated for 48 hours, fixed with PBS-paraformaldehyde and stained as previously described. *P. berghei* EEF quantification was performed by indirect immunofluorescence using the Operetta High Content Screening system (PerkinElmer). Images were collected using a 40X objective and the acquisition parameters described in Table S8. Parasites EEFs, hepatocytes, and nuclei were labeled using a P.sppHSP70 mouse polyclonal antibody (dilution 1:500, 1 mg/ml stock), *HsMUC13* , c-terminus region rabbit polyclonal antibody (dilution 1:500, 1 mg/ml stock) (LifeSpan BioSciences #LS-C345092), and Hoechst 33342, respectively. Primary antibody detection was performed with goat anti-mouse (Alexa Fluor 647, red) and anti-rabbit (Alexa Fluor 488, green) secondary antibodies. Images were analyzed using the Harmony Software (PerkinElmer). Objects likely to be parasites, hepatocytes, or nuclei were identified based on fluorescence intensity, and morphology. Cell area ($μm^2$) was calculated using the "measure ellipse" feature from Harmony.

Knockdown of *MUC13*

MUC13 was knocked down using an shRNA pool, containing 3 shRNA hairpins, targeting *MUC13* packaged in a lentiviral vector (Santa Cruz Biotechnology). HC04 and Huh7.5.1 cells were plated at 120,000 cells per well in a 24 well plate, then 24 hours after plating media was replaced with complete media (as above) plus 5 μg/ml polybrene. Virus was added at an multiplicity of infection of 0.5, cells were spun at 800 x g for 30 min, then incubated overnight at 37°C. The following day media was changed to

complete media and cells were again incubated overnight at 37°C. Cells were then moved to a 6-well plate and selected using complete media plus 2.5 µg/ml puromycin. After 7 days of selection, splitting as required, and knockdown efficiency was confirmed via western blot.

Ethics Statements

Human subjects protocols were approved by the Human Research Protection Program of the University of California San Diego (approval number 120652) and Universidad Peruana Cayetano Heredia (R-157-13-14). Written informed consent was obtained from all study participants.
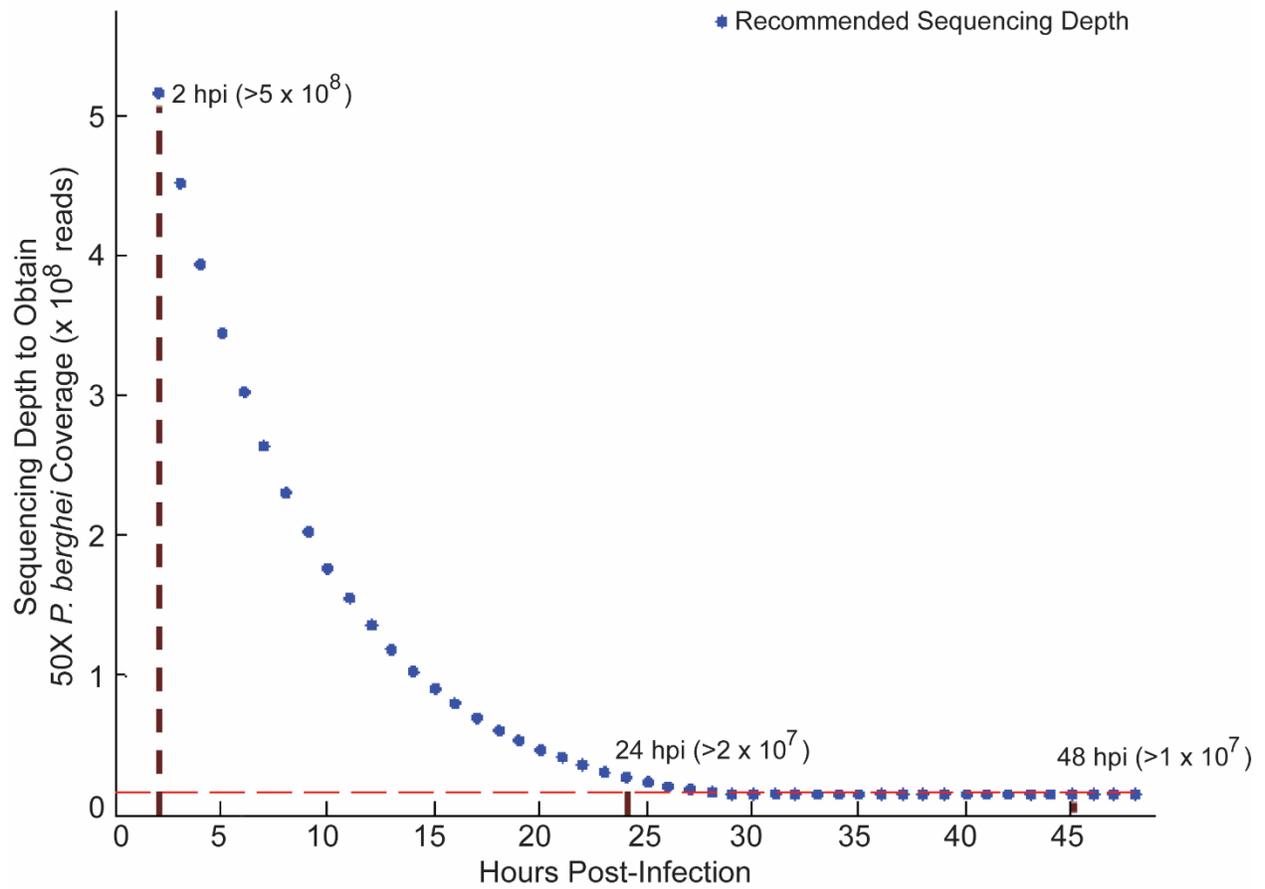
Supplementary Figures

Figure S1: Analysis of required sequencing depth needed to ensure adequate *P. berghei* sequencing coverage. Dashed lines indicate position of 2 hpi (dark red) and point of lowest coverage requirement (light red).
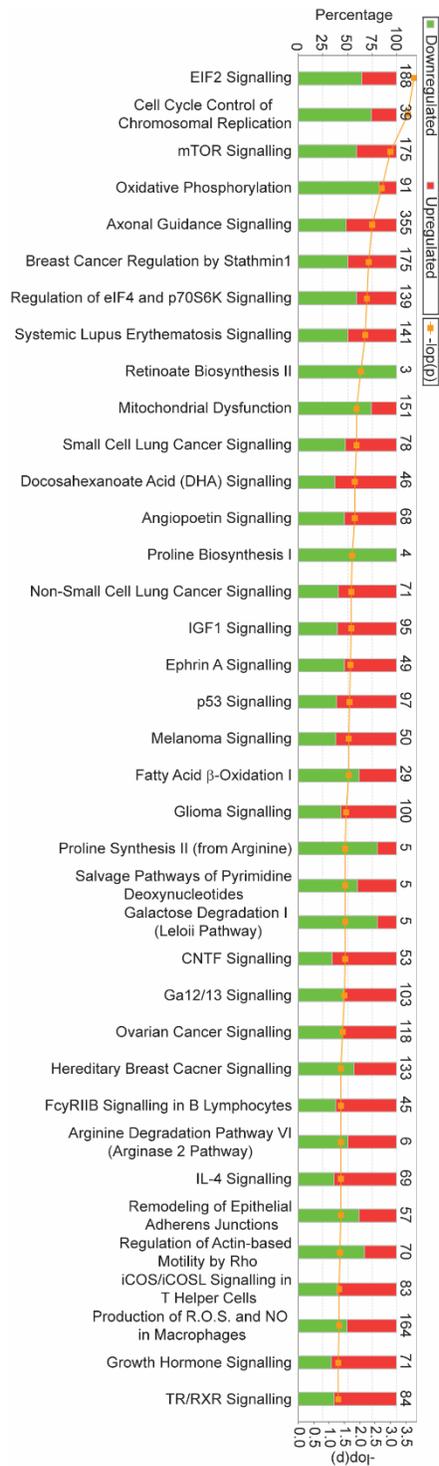
Figure S2: Identification of Host transcriptional pathways whose expression was altered using Ingenuity pathway analysis. Comparison was made between all 7 48 hpi infected datasets indicated in Figure 1A (4 Huh7.5.1, 2 HC04 and 1 Huh7.5.1) versus 7 matched uninfected datasets. Pathways are indicated by percentage of dysregulated genes versus total genes in the pathway and the -logP-value.
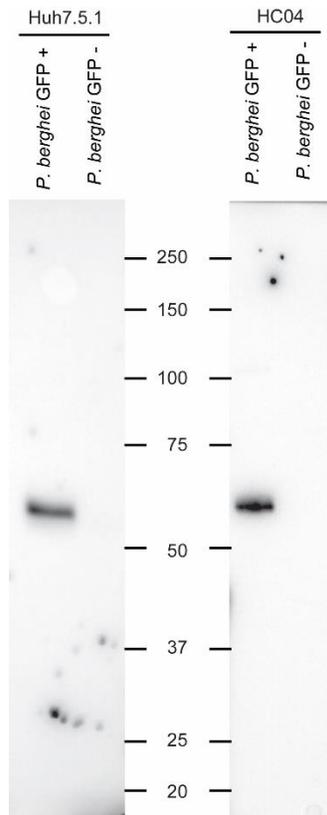
Figure S3: Full-length western blots of *MUC13* for the westerns shown in figure 1D. Molecular weights, in kDa, are indicated. A 1:500 dilution of rabbit polyclonal antibody to the MUC13 intracellular domain (LifeSpan BioSciences #C345092) was used to label the protein, and the presence of MUC13 was identified using a 1:5,000 dilution of goat anti-rabbit secondary conjugated with HRP.
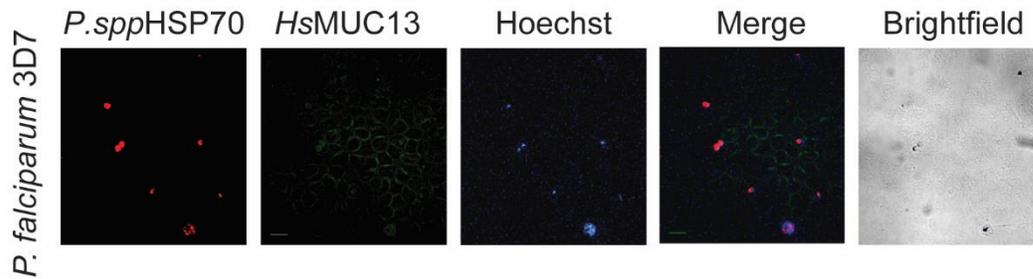
Figure S4: Staining of *MUC13* in *P. falciparum asexual blood stages.*
Thin smears of asynchronous *P. falciparum* 3D7 culture were fixed in cold methanol for 5 minutes and washed twice in cold PBS. Slides were stained overnight with a 1:700 dilution (1 mg/ml stock) of mouse polyclonal antibody to *P.spp*HSP70 and a 1:500 dilution of rabbit polyclonal antibody to *Hs*MUC13 extracellular domain (MUC13 Antibody #1 - LifeSpan BioSciences #LS-C345092). Primary antibody localization was visualized with goat anti-mouse (Alexa Fluor 647, red) and goat anti-rabbit (Alexa Fluor 488, green) secondary antibodies, respectively. Nuclei were stained with Hoechst 33342 (blue). Merged images between *Hs*MUC13, *P.spp*HSP70, and Hoechst and unstained Brightfield merged with Hoechst 33342 are shown. Scale bars 10 µm; 60X oil objective.
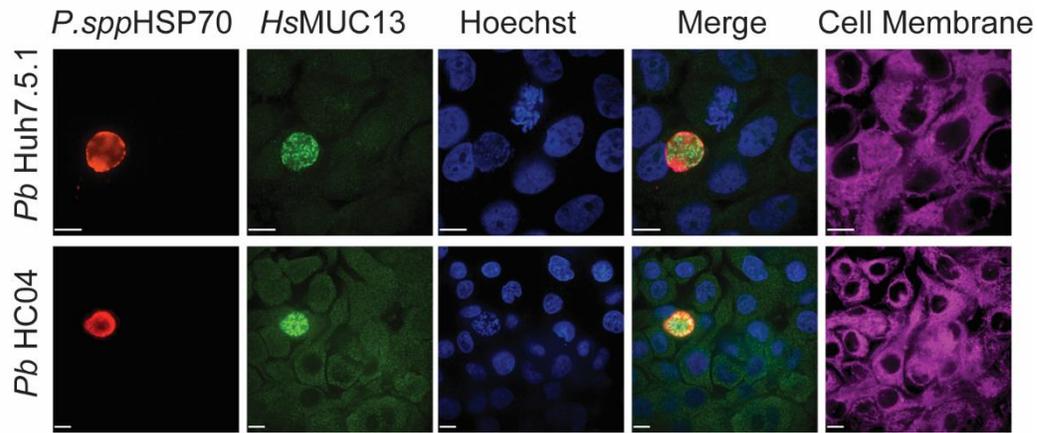
Figure S5: Detection of *MUC13* in *P. berghei*-infected HC04 cells at 48 hpi using immunofluorescence microscopy and an alternative *HsMUC13* antibody. Cells were fixed and stained with a 1:500 dilution (1 mg/ml stock) of mouse polyclonal antibody to *P.spp*HSP70 (see methods) and a 1:500 dilution of rabbit polyclonal antibody to MUC13 extracellular domain (MUC13 Antibody #2 - LifeSpan BioSciences #LS-A8191). Primary antibody localization was visualized with goat anti-mouse (Alexa Fluor 647, red) and goat anti-rabbit (Alexa Fluor 488, green) secondary antibodies, respectively. Nuclei were stained with Hoechst 33342 (blue) and cell membranes with CellMask deep red (purple). Scale bars 10 μm; 60X oil objective.
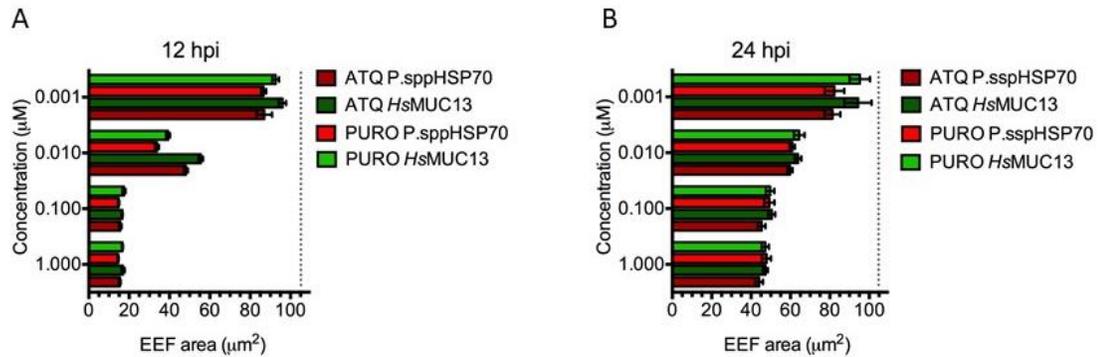
Figure S6: MUC13 as a biomarker for *Plasmodium* EEF detection. Effect of atovaquone (ATQ) and puromycin (PURO) on growth (cell area) of *P. berghei* EEF when treated at two different time points: 12 hpi (A) and 24 hpi (B). The assay was incubated for 48 hpi total. Dashed line represents DMSO control. Four technical replicates were performed; error bars show SD. *P. berghei* was labeled with a *P.spp*HSP70 mouse polyclonal antibody (dilution 1:500, 1 mg/ml stock) and visualized with goat anti-mouse antibody (Alexa Fluor 647, red). Cells were labeled with a rabbit polyclonal antibody recognizing the intracellular domain of MUC13 (MUC13 antibody #1-LifeSpan BioSciences #C345092) (1:500 dilution, 1 mg/ml stock) and detected with a goat anti-rabbit antibody (Alexa Fluor 488, green). Hoechst 33342 was used for cell and parasite nuclei identification.

Supplemental Tables

Table S7: RT-PCR primers used in this study.

Gene Name	Forward Primer Sequence (5'->3')	Reverse Primer Sequence (5'->3')
MUC13	ATGGCTGTAACCAGACTGCG	CTTGAGACTGGAAGCAACGC
NR4A3	TCCGCTCCTCCTACACTCTC	GACGCAGGGCATATCTGGAG
NEAT1	AAACGCTGGGAGGGTACAAG	ATGCCCAAACTAGACCTGCC
SLC22A8	CTTTGTGCCCTTGGACTTGC	GGAAGAGGCAGCTGAAGGAG
RASSF9	GGAAATCCAGACACCCCCAC	TTGGAGATCTGTTTTTATGCCGAG
B2M	AAGATGAGTATGCCTGCCGT	TGCTTACATGTCTCGATCCCAC

Table S8: Acquisition parameters used in Harmony software 3.5 – Operetta.

Filter	Excitation (nm)	Emission (nm)	Acquisition time (ms)	Height (μm)
Alexa Fluor 488	460-490	500-550	250	-1.5
Alexa Fluor 647	620-640	650-760	200	-1.5
Hoechst 3342	360-400	410-480	30	-1.5

Additional Data

Table S1: Gene expression (RNAseq) for the combined 48 hpi *P. berghei* datasets generated in this study with fold changed determined for infected versus uninfected. The columns in Table S1 are as follows: GeneID: designation of each gene; baseMean: The base mean is the mean of normalized counts of all samples, normalizing for sequencing depth. log2 Fold Change: Average change in RNA expression levels, converted to log2 values; log2 Fold Change SE: Standard error of the log2 FC, based upon read depth for a given gene; DESeq2 stat: The statistic used to determine significance, measured as Log2 FC divided by log2 fold change SE; pvalue: The measure of significance of a log2 fold change based upon the coverage, as determined by the DE2Seq stat; padj: The p-value adjusted for the false discovery rate cutoff.

Table S2: Gene expression (RNAseq) for Huh7.5.1 datasets, for both Human and *P. berghei*, with fold changed determined for infected versus uninfected, generated at 24 and 48 hpi in this study. The columns in Table S2 are as follows: GeneID: designation of each gene; baseMean: The base mean is the mean of normalized counts of all samples, normalizing for sequencing depth. log2 Fold Change: Average change in RNA expression levels, converted to log2 values; log2 Fold Change SE: Standard error of the log2 FC, based upon read depth for a given gene; DESeq2 stat: The statistic used to determine significance, measured as Log2 FC divided by log2 fold change SE; pvalue: The measure of significance of a log2 fold change based upon the coverage, as determined by the DE2Seq stat; padj: The p-value adjusted for the false discovery rate cutoff.

Table S3: The gene matrix, indicating the total number of reads for each gene within all RNA samples obtained in this study, used to generate heat map presented in Figure 1A. Cell Line identity and time point for each RNA sample is as indicated in the top row, genes are identified by both name and ensemble ID, and total reads are indicated for each.

Table S4: Human Pathways shown to be downregulated during parasite infection, as determined by Metascape. Pathways were identified as enriched using the 618 downregulated genes which were selected from 21941 differentially expressed genes based upon a criteria of p< 0.01 and FC> 2. The "Annotation" sheet contains all gene identifiers, gene annotations and information (including known function, localization, etc), and presence in the specified gene ontology group (presented in binary present or absent (1.0 or 0.0) form). The "Enrichment" sheet contains all enriched terms for the gene list indicated in the annotation set and the functional identifier for that gene cluster. The most significant term within a group is chosen as the group summary. Groups are presented

with significance measured via <u>LogP</u> and <u>Log(q-value)</u> (P value adjusted for FDR), and <u>InTerm_InList</u> (how many genes are enriched – InTerm, versus how many total genes are in a given functional category – InList).

Table S5: Human Pathways shown to be upregulated during parasite infection, as determined by Metascape. Pathways were identified as enriched using the 840 upregulated genes which were selected from 21941 differentially expressed genes based upon the criteria of p< 0.01 and FC> 2. The "Annotation" sheet contains all gene identifiers, gene annotations and information (including known function, localization, etc), and presence in the specified gene ontology group (presented in binary present or absent (1.0 or 0.0) form). The "Enrichment" sheet contains all enriched terms for the gene list indicated in the annotation set and the functional identifier for that gene cluster. The most significant term within a group is chosen as the group summary. Groups are presented with significance measured via <u>LogP</u> and <u>Log(q-value)</u> (P value adjusted for FDR), and <u>InTerm_InList</u> (how many genes are enriched – InTerm, versus how many total genes are in a given functional category – InList).

Table S6: Sequencing Statistics for all sequencing samples conducted in this study. Columns are labeled as follows: <u>Sample Name</u>: The RNA sample for which these statistics apply; <u>Total Reads</u>: The total number of 100bp paired end reads obtained within each sample; <u>Map to Human</u>: The total number of reads which map to the human genome; <u>Human mapping rate</u>: The proportion of total reads, presented as a ratio of Human Reads: Total Reads, which map to the Human genome; <u>Map to Malaria</u>: The total number of reads which map to the *Plasmodium berghei* genome; <u>Malaria mapping rate</u>: The proportion of total reads, presented as a ratio of *P. berghei* Reads: Total Reads, which map to the *P. berghei* genome; <u>Total Mapping Rate</u>: The proportion of total reads, presented as a ratio of (*P. berghei* Reads+Human Reads):Total Reads, which map to either the *P. berghei* or Human genome

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