Supporting Information

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SI Text

Recombinant Protein Expression and Antisera Production. The nucleotide sequence for the signal peptide and the GPI anchor were excluded from the expression constructs for genes encoding the P36 and P52 proteins. The P36 expression construct contains coding sequence started from G_{48} through the end of the protein, S_{353}, into pEU-E01-GST plasmid between the XhoI and BamHI sites. P52 expression construct contains S_{44} through K_{458}, as above. Both recombinant proteins were expressed in the wheat germ cell-free protein expression system as GST-fusion proteins (1) and were purified by using the glutathione-Sepharose 4B column (GE Healthcare Bio-Sciences). The affinity-purified proteins were used to obtain immune sera in mice or rabbits. The animals were immunized 3 times in 3-week intervals s.c. using Freund's adjuvant. Preimmune sera and antisera obtained from animals immunized with GST in Freund's adjuvant were used as negative controls.

Immunofluorescent Labeling of P52 Protein in Sporozoites. Salivary gland sporozoites were extracted from mosquitoes by dissection and were deposited on slides at 3,000 per well. After air-drying, the slides were fixed with ice-cold acetone and blocked with 5% nonfat dry milk in PBS for 30 min at 37 °C. Sporozoites were incubated for 1 h at 37 °C with mouse or rabbit anti-P52 sera and anti-TRAP sera, washed with ice-cold PBS, and incubated with anti-rabbit and anti-mouse secondary antibodies conjugated either with AlexaFluor 488 or 546 (Invitrogen), and DAPI (2 μg/mL) for 30 min at 37 °C. After further washing, slides were mounted with Prolong Gold Antifade Reagent (Invitrogen). The images were taken by confocal scanning laser microscopy (LSM5 PASCAL; Carl Zeiss MicroImaging).

Design and Production of Gene-Targeting Constructs. Primers used to produce targeting constructs were as follows: *P52* 5′ flank 5′-GGATCTCTATAAATGCATGAGG-3′ and 5′-CTGGGTGAGTTTTTGCCG-3′, 3′ flank 5′-CAAGGAAAAAAATTAAGGGTTGTG-3′ and 5′-GTTCATTTATATATTTGGAAATATCATC-3′; *P36* 5′ flank 5′-GGAGAGTATAGCAAAATGTTGC-3′ and 5′-GTGCATGTTTCATTAGCATAATCC-3′, 3′ flank 5′-GGGAATTTACATGCCATTCTATG-3′ and 5′-CCTATACCCTTCCCTTGTG-3′. Primers used for simultaneous deletion of P52 and P36 were as follows: P52 5′ flank: 5′-GGATCTCTATAAATGCATGAGG-3′and 5′ CTGGGTGAGTTTTTGCCG-3′; P36 3′ flank 5′-GGGAATTTACATGCCATTCTATG-3′ and 5′-CCTATACCCTTCCCTTGTG-3′.

Design and Generation of *p52*$^{-}$/*p36*$^{-}$ Double-Gene Deletion Parasite Lines. For *P52* and *P36* targeting plasmid, the primers for the P52 5′ flank and the P36 3′ flank were used in combination to target the loci simultaneously. Restriction sites in the MCS were SacII/SpeI for the 5′ flank and AvrII/SfoI for the 3′ flank. Sequencing was performed to confirm inserts. The NF54 *P. falciparum* parasites were transfected to produce *p52*$^{-}$/*p36*$^{-}$ double-gene knockout parasites. To confirm correct integration of the targeting plasmid in the parasite genome and the genetic homogeneity of the knockout population, Southern blotting methodology was used. The enzymes used for the Southern blots of the *p52*$^{-}$/*p36*$^{-}$ KO populations were HindIII and ClaI.

Transfection of *P. falciparum* with Targeting Constructs. Plasmid DNA was extracted by maxi-prep kit (Qiagen). The 3D7 or NF54 *P. falciparum* parasites (Walter Reed Army Institute for Research, Silver Spring, MD) were synchronized at ring stage with sorbitol 2 days before transfection. On the following day, trophozoites were selected for cytoadherence properties by incubation in RPMI plus Gelofusine (Braun). Transfection of *P. falciparum* ring stages with 100 μg of DNA was performed by electroporation at 0.31 kV and 950 μF with a BioRad Gene Pulser (BioRad). Cultures were placed on the positive-selection drug WR99210 (Jacobus Pharmaceuticals) 6 h after transfection and were maintained as described previously (2, 3).

Southern Blotting. Genomic DNA from WT 3D7 and knockout lines was digested for 2–16 h with the following enzymes: P52, 5′ and 3′ HindIII/ClaI; P36, 5′ EcoRI/EcoRV and 3′ EcoRI. Digested DNA was run on a 1% Tris-acetate-EDTA agarose gel at 15 V for 18 h and transferred to Hybond-N membrane (Amersham) overnight at room temperature, UV cross-linked, and prehybridized with herring sperm DNA for 2.5 h. A digoxygenin-labeled probe was prepared by PCR per supplier protocol (Roche) by using the cloning primers. Hybridization was carried out for 18 h at 55 °C. The blot was exposed to film for 10–60 min and developed per standard protocol.

In Vitro Invasion Assay. To assess invasion, 25,000 sporozoites were added per well to an HC-04 cell monolayer on 8-well glass Labtek chamber slides. They were incubated 3 h at 37 °C and 5% CO_2. Slides were washed with PBS and fixed with cold methanol. Slides were incubated 30 min with anti-CSP mAb at room temperature, washed with PBS, and blocked with 0.1% BSA/PBS, followed by a 1:200 HRP goat anti-mouse IgG (Kirkegaard and Perry) as a secondary antibody for 30 min at room temperature. To visualize CSP staining, diaminobenzidine reagent was used according to the manufacturer's instructions (KPL). Slides were mounted by using Permount (Fisher Scientific). Intracellular sporozoites were counted by using microscopy at 200× magnification in triplicate wells (4).

In Vivo Assessment of Infection in a Hepatic Chimera Murine Model. SCID *Alb-uPA* mice 5–14 days old received an inoculation, by intrasplenic injection, with 10^6 human hepatocytes that had been isolated (with informed consent) from surgically resected liver specimens by collagenase digestion and Percoll gradient centrifugation (5). Mice were screened 8 weeks after transplantation for successful hepatocyte engraftment by serum analysis for human α1-antitrypsin by ELISA (Table S7). Chimeric mice received an i.v. tail vein injection of 1×10^6 to 1.5×10^6 *P. falciparum* WT ($n = 3$) or *p52*$^{-}$/*p36*$^{-}$ sporozoites ($n = 3$) and were euthanized by CO_2 overdose at 1 day or 4 days after infection, and their livers were removed for cryosectioning or RNA extraction. Livers were rinsed in PBS, and the lobes were cut into separate pieces. Selected lobes were embedded in Tissue-Tek O.C.T. compound (Miles Scientific) and frozen in an isopentane/liquid N_2 bath, whereas other pieces were flash-frozen in liquid N_2 for RNA extraction. Tissue sections (7 μm) were cut on a Leica CM1900 (Leica Microsystems), fixed in absolute methanol, and stored at −80 °C until used. Four nonserial tissue sections were used for parasite quantification. Diluted antisera (anti-CSP and anti-HSP70 and anti-LSA1) were then applied to the tissue section (in a volume sufficient to cover the tissue), and the slides were then incubated for 30 min at 37 °C in a humidity chamber. Liver-section slides were placed in a staining dish and washed 3 times for 5 min with PBS. A

fluorescein-conjugated IgG (Kirkegaard and Perry) was used as the secondary antibody. The secondary antibody was diluted 1:40 into PBS containing 0.02% Evans blue. The Evans blue was added to act as a counterstain to suppress any autofluorescence in the tissue. The diluted secondary antibody was added and the slides placed in a humidity chamber in the dark and incubated at 37 °C for 30 min. Tissue sections were then washed and the slides mounted by using Vectashield mounting medium (Vector Laboratories). The stained slides were screened with a Nikon Eclipse E600 epifluorescent microscope, and digital images were collected with a SPOT digital camera (Diagnostic Instruments).

RNA for use in RT-PCR analysis was isolated from infected livers as described previously (6). Briefly, first-strand cDNA was generated from total RNA by using the SuperScript First-Strand Synthesis System for RT-PCR kit (Life Technologies). cDNA synthesis was performed by priming RNA isolated from the different parasite samples with random hexamers, and then incubation with reverse transcriptase (RT+). As a control for the presence of genomic DNA, reactions were done omitting the reverse transcriptase (RT−). Amplification of specific *P. falciparum* 18S gene sequences or hGAPDH was accomplished by PCR using a hot-start TaqDNA polymerase from the HotStarTaq PCR kit (Qiagen). One microliter from the cDNA reaction was added to a PCR master mix with 18S primers (forward, 5′-AATCTTGAACGAGGATGCC-3′; reverse, 5′-GGAAACCTTGTTACGACTTCTCC-3′) or GAPDH primers (forward, 5′-GAAGGTGAAGGTCGGAGTC-3′; reverse, 5′-GAAGATGGTGATGGGATTTC-3′). PCR products were electrophoresed on a 1% agarose gel and visualized by staining with ethidium bromide.

1. Tsuboi T, et al. (2008) Wheat germ cell-free system-based production of malaria proteins for discovery of novel vaccine candidates. *Infect Immun* 76:1702–1708.
2. Trager W, Jenson JB (1978) Cultivation of malarial parasites. *Nature* 273:621–622.
3. Crabb BS, et al. (2004) Transfection of the human malaria parasite Plasmodium falciparum. *Methods Mol Biol* 270:263–276.
4. Hollingdale MR, Leland P, Leef JL, Schwartz AL (1983) Entry of Plasmodium berghei sporozoites into cultured cells, and their transformation into trophozoites. *Am J Trop Med Hyg* 32:685–690.
5. Mercer DF, et al. (2001) Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* 7:927–933.
6. Sacci JB, Jr., et al. (2006) Plasmodium falciparum infection and exoerythrocytic development in mice with chimeric human livers. *Int J Parasitol* 36:353–360.

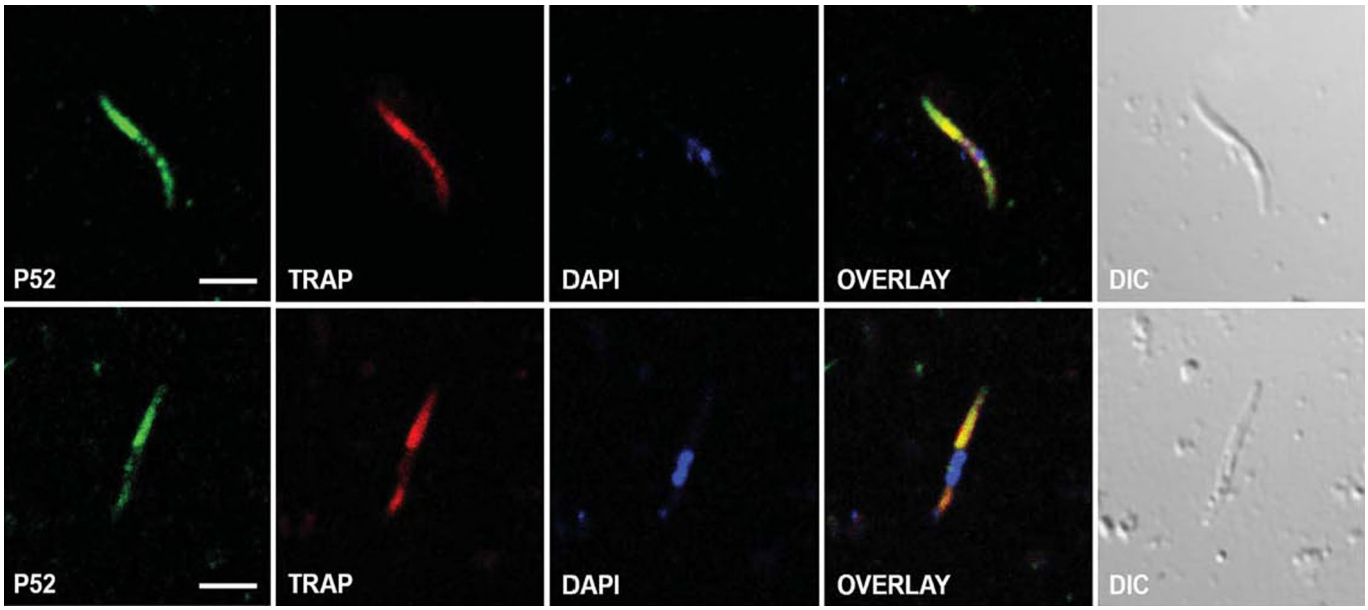

Fig. S1. *P. falciparum* P52 localizes to the invasive organelles of sporozoites. Immunofluorescence assays with *P. falciparum* salivary gland sporozoites were performed by using anti-PfP52 rabbit antisera at ×500 dilution (*Upper*) or mouse antisera at ×200 dilution (*Lower*). Antisera were raised against a wheat germ cell-free expressed PfP52-GST fusion protein. The PfP52 staining is internal to the sporozoite and extensively colocalizes with staining for the micronemal protein TRAP. Preimmune sera and anti-GST antisera did not react with sporozoites. (Scale bar: 5 μm.)

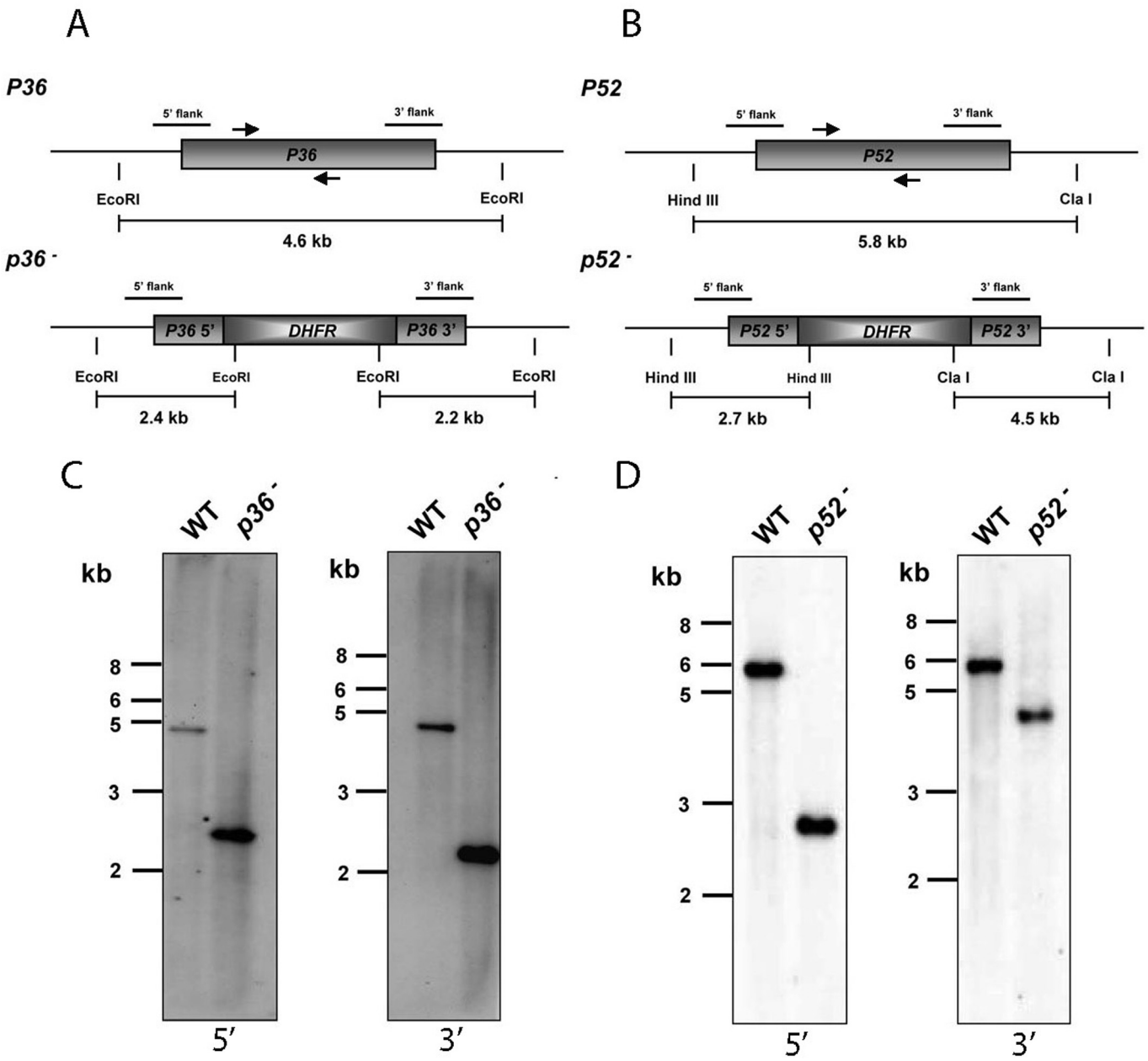

Fig. S2. Targeted deletion of *P. falciparum P36* and *P52*. *P36* (PlasmoDB identifier PFD0210c) and *P52* (PFD0215c) are adjacent loci on chromosome 4. (*A* and *B*) Schematics showing the WT *P36* (*A*) and *P52* (*B*) loci (*Upper*) and the knockout (KO) *p52*$^{-}$ (*B*) and *p36*$^{-}$ (*A*) loci (*Lower*). Arrows indicate the position of the primers used in RT-PCR analysis. The position of probes is indicated by bars above the loci. The expected restriction fragments resulting from enzymatic digestion of the WT loci and KO loci are indicated. (*C* and *D*) Probes specific to the 5′ and 3′ targeting flanks of each locus were hybridized to genomic DNA fragments from KO and WT parasite lines. Analysis of clonal KO parasite lines showed specific bands recognized by the probes at predicted sizes, indicating successful deletion of each locus. For *P36* (*C*), the fragments recognized by the 5′ probe (*Left*) are 4.58 kb for the WT locus and 2.42 kb for the KO locus. The fragments recognized by the 3′ probe (*Right*) are 4.58 kb for the WT locus and 2.17 kb for the KO locus. For *P52* (*D*), fragments recognized by the 5′ probe (*Left*) are 5.8 kb for the WT locus and 2.7 kb for the KO locus. The fragments recognized by the 3′ probe (*Right*) are 5.8 kb in WT locus and 4.5 kb for the KO locus.

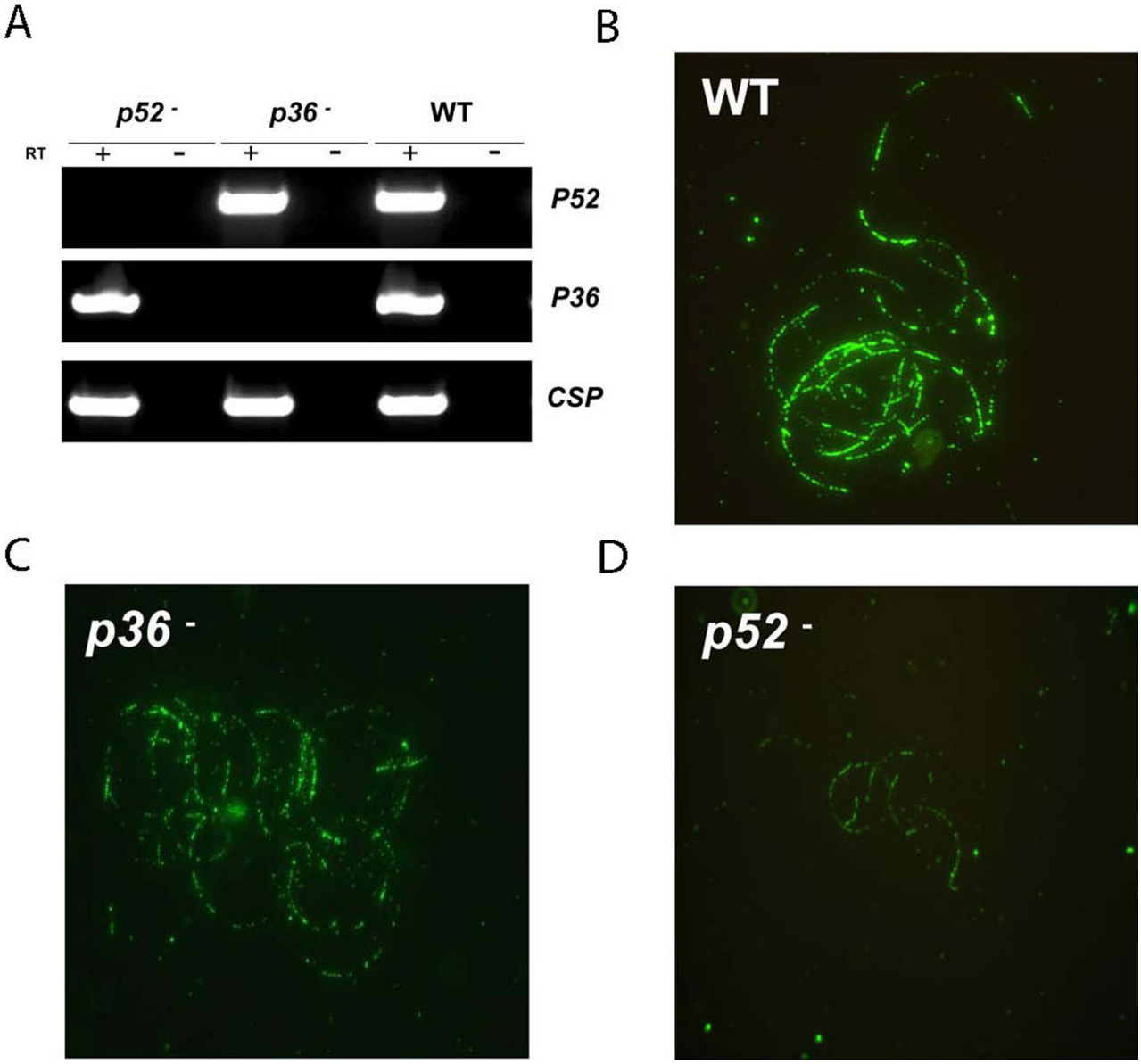

Fig. S3. Knockout lines lack *P52* and *P36* coding transcripts and show normal gliding motility. (*A*) RT-PCR analysis of sporozoites from knockout lines confirmed lack of intact transcripts for *P52* and *P36*. Primers used were specific for the *P. falciparum P52* and *P36* loci; Pf circumsporozoite protein (*CSP*) primers were used as a positive control. Reactions were carried out in the presence (+) or absence (−) of reverse transcriptase for 35 cycles on RNA from salivary gland sporozoites of WT *P. falciparum* and the $p52^{-}$ and $p36^{-}$ parasite lines. Transcripts of *p52* and *p36* were absent from the respective KO parasites, confirming successful deletion of the endogenous loci and absence of contamination by nonrecombinant parasites. (*B–D*) *P52* and *P36*-deficient parasites show normal gliding motility. Salivary gland sporozoites isolated from infected mosquitoes and placed on a glass surface shed CSP as they move. This in vitro assay demonstrates functionality of the motility apparatus that is essential for hepatocyte invasion. Sporozoite trails on glass slides were stained with anti-CSP monoclonal antibody and observed. Sporozoites of the $p52^{-}$ and $p36^{-}$ *P. falciparum* lines produced typical CSP trails. (Magnification: 400×.)

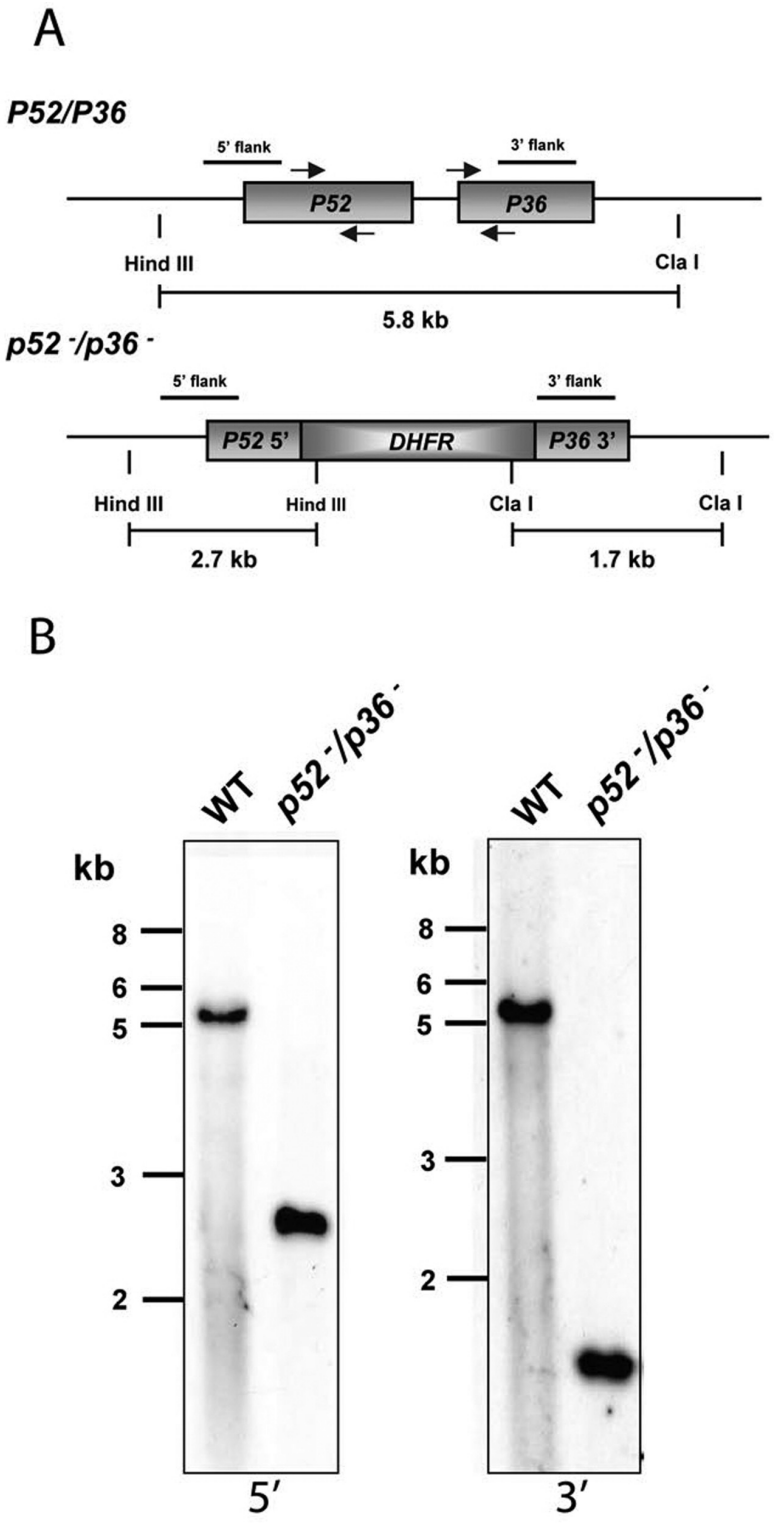

Fig. S4. Simultaneous deletion of *P36* and *P52* in *P. falciparum*. (*A*) *P36* and *P52* are tandem-arranged genes on chromosome 4. Schematics show the WT *P36* and *P52* loci (*Upper*) and the knockout (KO) $p52^{-}/p36^{-}$ loci (*Lower*). Arrows indicate position of primers used in RT-PCR analysis. The position of probes is indicated by bars above the loci. The expected restriction fragments resulting from enzymatic digestion of the WT loci and KO loci are shown. (*B*) Probes specific to the 5′ and 3′ targeting flanks of *P52* and *P36* locus were hybridized to genomic DNA fragments from KO and WT parasite lines. Analysis of KO parasite lines showed specific bands recognized by the probes at predicted sizes, indicating successful deletion of the loci. For *P52*/*P36* locus, the fragment recognized by the 5′ probe (*Left*) is 5.8 kb for the WT locus and 2.7 kb for the KO locus. The fragment recognized by the 3′ probe (*Right*) is 5.8 kb for the WT locus and 1.7 kb for the KO locus.

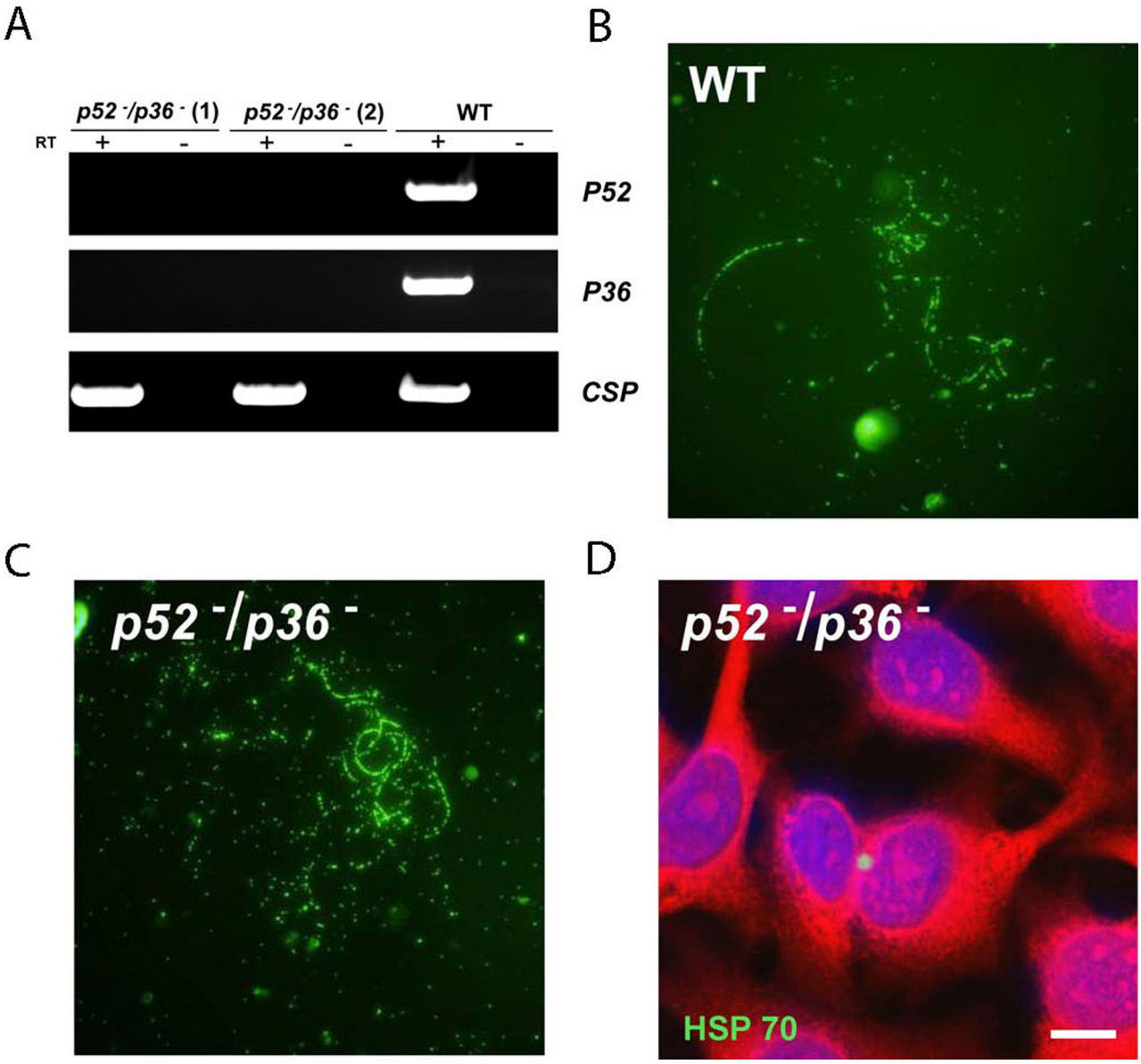

Fig. S5. *p52*$^{-}$/*p36*$^{-}$ knockout lines lack *P52* and *P36* transcription, show normal gliding motility, and are completely attenuated at early liver-stage development. (*A*) RT-PCR analysis of sporozoites from *p52*$^{-}$/*p36*$^{-}$ knockout lines confirmed lack of transcripts for *P52* and *P36*. Primers used were specific for the *P. falciparum P52* and *P36* loci; Pf circumsporozoite protein (*CSP*) primers were used as a positive control. Reactions were carried out in the presence (+) or absence (−) of reverse transcriptase for 35 cycles on RNA from salivary gland sporozoites of WT *P. falciparum* and the *p52*$^{-}$/*p36*$^{-}$ parasite lines. (*B* and *C*) The *p52*$^{-}$/*p36*$^{-}$ parasites show normal gliding motility. Salivary gland sporozoites isolated from infected mosquitoes and placed on a glass surface shed CSP as they move. This in vitro assay demonstrates functionality of the motility apparatus that is essential for hepatocyte invasion. Sporozoite trails on glass slides were stained with anti-CSP monoclonal antibody and observed. (Magnification: 400×.) (*D*) Development of *p52*$^{-}$/*p36*$^{-}$ liver stages was assessed in vitro by using cultured cells of the HC-04 human hepatocyte line. Parasite growth was monitored over 3 days, and liver stages were visualized by immunofluorescence microscopy at 400× magnification with anti-HSP70. *P. falciparum p52*$^{-}$/*p36*$^{-}$ parasites exhibited early-arrested intrahepatocytic development and were rarely detected at day 3 after infection. No intrahepatocytic parasites were detected at day 4 or 6 after infection. (Scale bar: 10 μm.)

Table S1. Sporozoite gliding assay (20,000 sporozoites per well)

Parasite line	Trails per well*			Mean
Pf WT	129	148	122	133
Pf p36^{-}	121	114	126	120
Pf WT	128	74	269	157
Pf p52^{-}	109	64	283	152

*Three replicates.

Table S2. Sporozoite invasion assay (25,000 sporozoites per well)

Parasite line	LS per well*			Mean
Pf WT	312	272	252	279
Pf p36^{-}	350	273	219	281
Pf WT	153	153	130	145
Pf p52^{-}	158	172	127	152

*Determined at 3 h after infection. LS, liver stages (three replicates).

Table S3. Liver-stage development assay (60,000 sporozoites per well)

Parasite line	LS per well*			Mean
Pf WT	108	155	169	144
Pf p36^{-}	84	74	130	96
Pf WT	90	92	88	90
Pf p36^{-}	60	34	53	49
Pf WT	71	61	102	78
Pf p36^{-}	53	72	55	60
Pf WT	75	75	87	79
Pf p52^{-}	89	76	84	83
Pf WT	75	75	87	79
Pf p52^{-}	61	69	71	67
Pf WT	87	92	82	87
Pf p52^{-}	97	95	93	95

*Determined at 72 h after infection. LS, liver stages (three replicates).

Table S4. Sporozoite gliding assay (20,000 sporozoites per well)

Parasite line	Trails per well			Mean
Pf WT	272	217	222	237
Pf p52^{-}/*p36*$^{-}$	135	163	209	169
Pf p52^{-}/*p36*$^{-}$	155	167	158	160
Pf WT	282	241	251	258
Pf p52^{-}/*p36*$^{-}$	162	180	192	178
Pf p52^{-}/*p36*$^{-}$	160	81	275	186
Pf WT	212	191	210	198
Pf p52^{-}/*p36*$^{-}$	169	142	139	150
Pf p52^{-}/*p36*$^{-}$	148	152	156	152

Table S5. Sporozoite invasion assay (60,000 sporozoites per well)

Parasite line	LS per well*			Mean
Pf WT	287	279	301	289
Pf p52^{-}/*p36*$^{-}$	166	152	165	161
Pf p52^{-}/*p36*$^{-}$	273	237	258	256
Pf WT	142	162	143	149
Pf p52^{-}/*p36*$^{-}$	133	126	164	141
Pf p52^{-}/*p36*$^{-}$	136	167	123	142
Pf WT	242	224	269	245
Pf p52^{-}/*p36*$^{-}$	219	244	260	241
Pf p52^{-}/*p36*$^{-}$	217	224	269	245

*Determined at 3 h after infection. LS, liver stages (three replicates).

Table S6. Liver-stage development assay (60,000 sporozoites per well)

Parasite line	LS per well*			Mean
Pf WT	37	44	36	39
Pf p52⁻/p36⁻	0	0	0	0
Pf p52⁻/p36⁻	0	0	0	0
Pf WT	59	55	54	57
Pf p52⁻/p36⁻	0	0	0	0
Pf p52⁻/p36⁻	0	0	0	0
Pf WT	41	40	39	40
Pf p52⁻/p36⁻	0	0	0	0
Pf p52⁻/p36⁻	0	0	0	0

*Determined at 96 h after infection. LS, liver stages (three replicates).

Table S7. Serum values for human alpha 1-antitrypsin in a hepatic chimera murine model

Time after infection	Parasite line	Alpha 1-antitrypsin, μg/mL*		
24 h	*Pf* WT	154	164	217
	Pf p52⁻/p36⁻	117	127	123
4 days	*Pf* WT	112	371	118
	Pf p52⁻/p36⁻	117	98	241

*Determined at 8 weeks after transplantation for individual mice (three replicates).

Table S8. Invasion assay at 3 h (25,000 sporozoites per well)

Parasite line	LS per well*			Mean	Invasion, %
Pf WT (NF54)	192	234	222	216	0.864
Pf WT (3D7)	197	239	242	226	0.904
Pf WT (NF54)	189	191	193	191	0.764
Pf WT (3D7)	196	197	186	193	0.772

*Determined at 3 h after infection. LS, liver stages (three replicates).

Table S9. Liver-stage development assay (60,000 sporozoites per well)

Parasite line	LS per well*			Mean
Pf WT (NF54)	191	206	185	194
Pf WT (3D7)	169	197	192	186

*Determined at 72 h after infection. LS, liver stages (three replicates).