Materials and Methods

Experimental animals

Female Swiss Webster mice (6 to 8 weeks old) and female BALB/c mice were purchased from Harlan Company (Indianapolis, IN). Animal handling was conducted according to Institutional Animal Care and Use Committee-approved protocols.

Generation of *Pys1*$^{-}$ parasites

The targeted deletion of *PyS1* by gene replacement was carried out as described in detail by Mikolajczak et al. [1], and the selection of transgenic parasites was carried out as described in Labaied et al. [2]. *PyS1* was replaced with DsRed under the control of the *P. berghei* elongation factor 1α promoter. All parental populations were cloned using limited dilution. The sequences of all oligonucleotide primers used for this study are listed in Table S1. Similarly, *Pbs1*$^{-}$ parasites were created by targeted deletion of *PbS1* by gene replacement.

Reverse transcriptase PCR (RT-PCR)

To evaluate transcript expression, the following samples were processed for RNA extraction: 8 million PyWT 17XNL oocyst sporozoites were isolated from infected *Anopheles stephensi* midguts 10 days after an infections blood meal, 1.5 million PyWT 17XNL and *Pys1*$^{-}$ salivary gland sporozoites were isolated from infected *Anopheles stephensi* salivary glands 14 days after an infectious blood meal, liver stages were extracted from whole liver lysates of mice injected with 1 million sporozoites and blood stages from 500 µl of infected blood. RNA was extracted using TRIzol reagent

(Invitrogen) and treated with DNase (Invitrogen). cDNA synthesis was performed using the Super Script III Platinum 2-step qRT-PCR kit (Invitrogen). *Pys1*$^{-}$ and *P. yoelii* circumsporozoite protein (PyCS) specific primers were used in a standard PCR amplification (35 cycles).

Phenotypic analysis of *Pys1*$^{-}$ parasites in the mosquito

Anopheles stephensi mosquitoes were infected with *Pys1*$^{-}$ parasites or PyWT 17XNL parasites by blood feeding for 6 min on the first day and 10 min on the following day on infected Swiss Webster mice and subsequently maintained under a cycle of 12.5 h light/11.5 h dark and 70% humidity at 24.5°C. Gametocyte exflagellation capacity was evaluated microscopically before mosquito blood meal. Infected mosquitoes were dissected (20 mosquitoes for each dissection) on day 10 after the first blood meal to determine the mean number of midgut oocyst sporozoites. Similarly, dissections on day 14 after the first blood meal were used to determine the mean number of salivary gland sporozoites.

Hepatoma cell line maintenance and cell-traversal assay

All *in vitro* assays were conducted using the human hepatoma cell line HepG2-CD81 [3]. The cell line was cultured in DMEM-F12 medium supplemented with antibiotics and 10% fetal bovine serum (FBS) at 37°C and 5% CO_2.

HepG2-CD81 cells were seeded in chamber slides (Permanox eight-well chamber slide; Nalge Nunc International, Rochester, NY) at a density of 60 x 10^3 cells per well two

days before the assay. A total of 20 x 10^3 sporozoites per well of PyWT 17XNL or *Pys1*$^-$, as well as uninfected mosquito salivary gland extracts (mock), were re-suspended in incomplete DMEM-F12 medium with 3% bovine albumin serum (BSA) and 2 mg ml^{-1} fluorescein isothiocyanate (FITC)-dextran (Invitrogen-Molecular Probes, Eugene, OR). Sporozoites and mock suspensions were added to the cells, centrifuged for two min at 199 rcf and incubated for one h at 37°C. Thereafter, the cells were washed thoroughly with PBS and complete DMEM-F12 medium twice to remove any extracellular dextran, and the cells were incubated for a further three h in complete DMEM-F12 medium. Flow cytometric quantitative analysis of dextran-positive cells was conducted using a flow cytometer BD LSRII (BD Biosciences, San Jose CA) and the flow cytometry analysis program FlowJo version 7.2 (TreeStar, Ashland, OR).

Mouse infections with PyWT 17XNL and *Pys1*$^-$

For sporozoite infection and subsequent liver stage development and onset of blood stage parasitemia, BALB/c mice were injected intravenously (IV) with 100 or 1,000 PyWT 17XNL or *Pys1*$^-$ sporozoites re-suspended in RPMI 1640. Blood stage patency was monitored daily by evaluation of Giemsa-stained thin blood smears from day 1 to day 5 post sporozoite infection. For blood stage growth experiments, BALB/c mice were injected IV with 10^3 or 10^6 PyWT 17XNL or *Pys1*$^-$ erythrocytic asexual stages in RPMI 1640. Blood stage patency was monitored daily by evaluation of Giemsa-stained thin blood smears from day 1 to day 24 post infection.

Transgene expression

Expression of DsRed was determined throughout the parasite life cycle by fluorescence microscopy. The different life cycle stages were visualized as follows: blood stage – wet mount; oocysts – dissected mosquito midguts were placed on a microscope slide under a glass cover slip; salivary gland sporozoites – infected salivary glands were dissected and ground to release sporozoites which were placed on a microscope slide under a glass cover slip. For the liver stage, infected cells were fixed at 24 hrs post infection in 4% formalin for 10 min, then blocked and permeabilized in PBS with 10% bovine serum albumin and 0.2% Triton-X 100 (PBS/BSA/Triton) for IFA. IFA was carried out in PBS/BSA. The staining was performed using a rabbit poly-clonal anti-*P. yoelii* UIS4 primary antibody, a parasite parasitophorous vacuole membrane marker. Fluorescent staining was achieved with Alexa Fluor-conjugated secondary antibodies (Invitrogen Corporation, Carlsbad, CA) specific to rabbit (Alexa Fluor 488, green) IgG. Cells were stained with DAPI to visualize the DNA and mounted with FluoroGuard anti-fade reagent (Bio-Rad, Hercules, CA). Preparations were analyzed using a fluorescence inverted microscope (Eclipse TE2000-E; Nikon), and images were acquired using Olympus 1 x 70 Delta Vision deconvolution microscopy.

References:

[1] Mikolajczak SA, Aly AS, Dumpit RF, et al. An efficient strategy for gene targeting and phenotypic assessment in the Plasmodium yoelii rodent malaria model. Mol Biochem Parasitol 2008; 158:213-216.

[2] Labaied M, Harupa A, Dumpit RF, et al. Plasmodium yoelii sporozoites with simultaneous deletion of P52 and P36 are completely attenuated and confer sterile immunity against infection. Infect Immun 2007; 75:3758-3768.

[3] Silvie O, Rubinstein E, Franetich JF, et al. Hepatocyte CD81 is required for Plasmodium falciparum and Plasmodium yoelii sporozoite infectivity. Nat Med 2003; 9:93-96.

Table S1 PCR oligonucleotide primer sequences used in this study

Primer	Sequence
To test expression throughout the life cycle	
PyS1ORFtest/FWD	GAATACGTCTATTGAAATAAAGC
PyS1ORFtest/RVS	TCCACTGATTCATACATTTT
To test PyS1 gene structure	
PyS1/Exon-1/FWD	CTCTCCAAAAAAAGAGAAAATGT
PyS1/Exon-2/RVS	CGTGAAATAATGCACTTATACA
PyS1/Intron/RVS	GCCAGTCGATAGAAGAGAAA
PyS1/postExon-2/RVS	GGGCGAAAATGACACATAAG
To create PyS1 knockout plasmid	
SP/pyS1-1	CGGATATCAGACACTTATAAAGCTAAAGAAG
ASP/pyS1-2	GAAGAAATATGGGCGCCTACAAATTTCGATGCACTCA
SP/pyS1-3	GAAATTTGTAGGCGCCCATATTTCTTCTCATTTTCC
ASP/pyS1-4	ATGCGGCCGCGATGAGAATAATAATATGTAGATAA
To genotype Pys1- parasites	
S1 5' test FWD	GATCATAAAAATGTGCAACGTC
S1 5' test RVS	GTAATACGACTCACTATAG
S1 3' test FWD	GGCTACGTCCCGCACGGACGAATCCAGATGG
S1 3' test RVS	GTATTTGTTTACATACATATGTGT
S1test FWD	GATCATAAAAATGTGCAACGTC
S1test RVS	CCACAAAGATGATTATTGCAAGGT
To test for presence of PyS1 transcript	
S1/mRNA/FWD	ACTGCAACTATGCCCATGTG
S1/mRNA/RVS	AAATTTCTCAACTATTTTGTTCG
PyCS rt for	AAGAAGTGTACCATTTTAGTTGTAGCGTCAC
PyCS rt rev	CACTACTGGTTGATTCAATTTATTTTGAGCCTC
To genotype Pbs1- parasites	
PbS1 5' test FWD	TACACCCTTAAACAAAAAGCCTAATGGA
PbS1 5' test RVS	CGCATTATATGAGTTCATTTTACACAATCC
PbS1 3' test FWD	GGCTACGTCCCGCACGGACGAATCCAGATGG
PbS1 3' test RVS	CCTATGTATATTATGAACACG
PbS1test FWD	TAATGGTACCGTGTATTATGTAGATGTA
PbS1test RVS	TCCCCGCGGCGAATATTGTTATTCATATCTGTAC

Figure S1

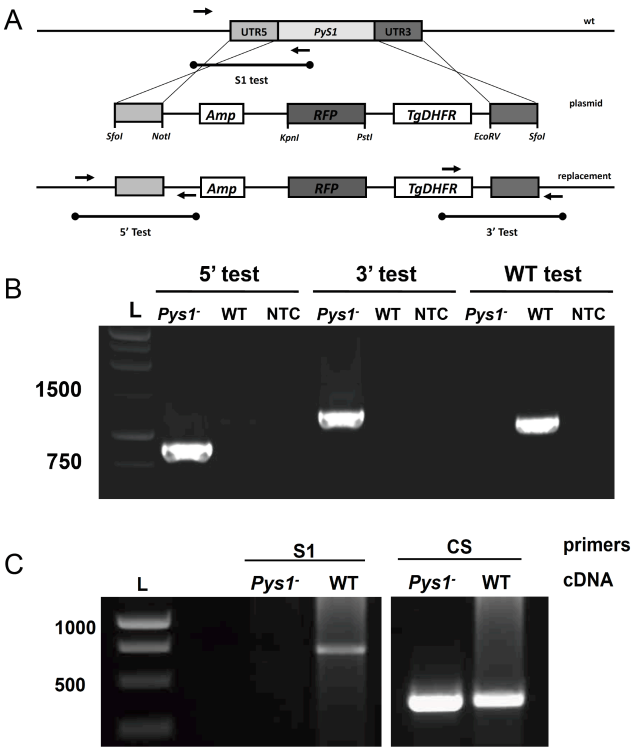

Figure S1: Generation of *P. yoelii* parasites with a deletion in the S1 gene (*Pys1*$^{-}$). *Pys1*$^{-}$ parasites were created by replacing *PyS1* with a plasmid containing the *Toxoplasma gondii* dihydrofolate reductase gene and a red fluorescent protein (DsRed) cassette by double crossover homologous recombination. (A) A graphical representation of the double crossover homologous recombination strategy. (B) Gene replacement analysis of the *Pys1*$^{-}$ clone G4. 5' test evaluates whether the plasmid has integrated 5' of the ORF; 3' test evaluates whether the plasmid has integrated 3' of the ORF, and WT test evaluates whether the ORF is present. The ladder lane is indicated with an "L" with relevant sizes noted to the left. The gDNA templates, *Pys1*$^{-}$, WT, and no template control (NTC), are indicated below each test. (C) No *PyS1* transcript was detected in *Pys1*$^{-}$ parasites. Total RNA was isolated from *Pys1*$^{-}$ and WT salivary gland sporozoites and cDNA was generated. The cDNA from *Pys1*$^{-}$ or WT was used in a PCR reaction with *PyS1* and circumsporozoite (*CS)* specific primers. The ladder lane is indicated with an "L" with relevant sizes noted to the left.

Figure S2

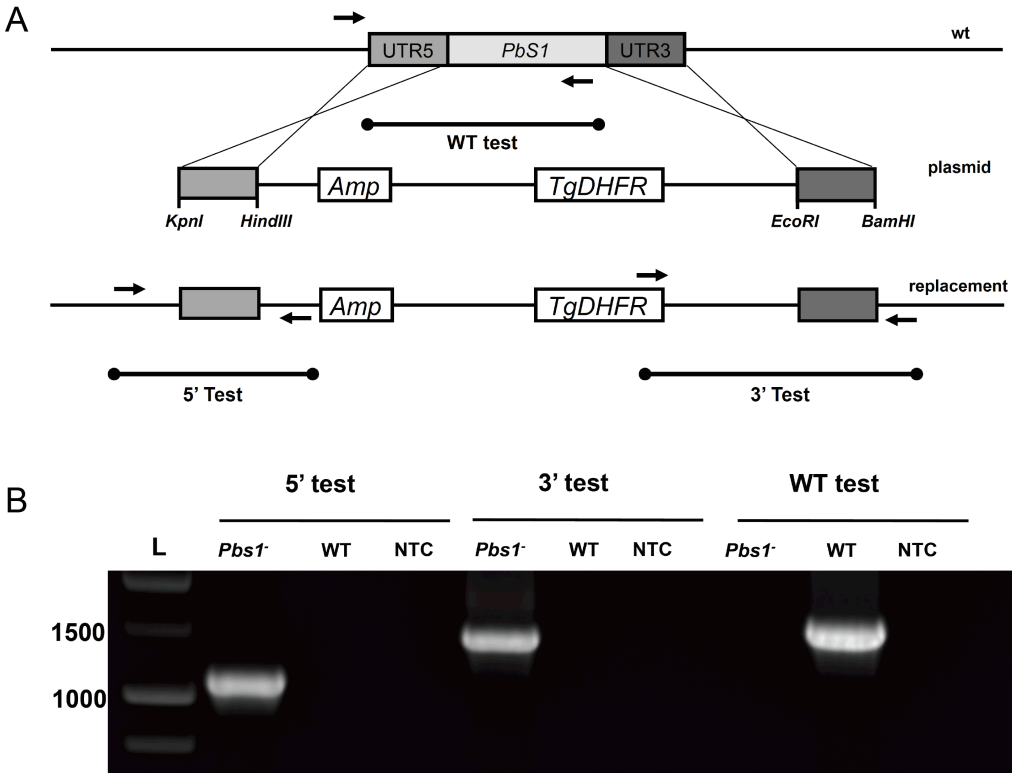

Figure S2: Generation of *P. berghei* parasites with a deletion in the S1 gene (*Pyb1*$^{-}$). *Pbs1*$^{-}$ parasites were created by replacing *PbS1* with a plasmid containing the *Toxoplasma gondii* dihydrofolate reductase gene and a red fluorescent protein (DsRed) cassette by double crossover homologous recombination. (A) A graphical representation of the double crossover homologous recombination strategy. (B) Gene replacement analysis of the *Pbs1*$^{-}$ clone 1. 5' test evaluates whether the plasmid has integrated 5' of the ORF; 3' test evaluates whether the plasmid has integrated 3' of the ORF, and WT test evaluates whether the ORF is present. The ladder lane is indicated with an "L" with relevant sizes noted to the left. The gDNA templates, *Pbs1*$^{-}$, WT, and no template control (NTC), are indicated below each test.

Figure S3

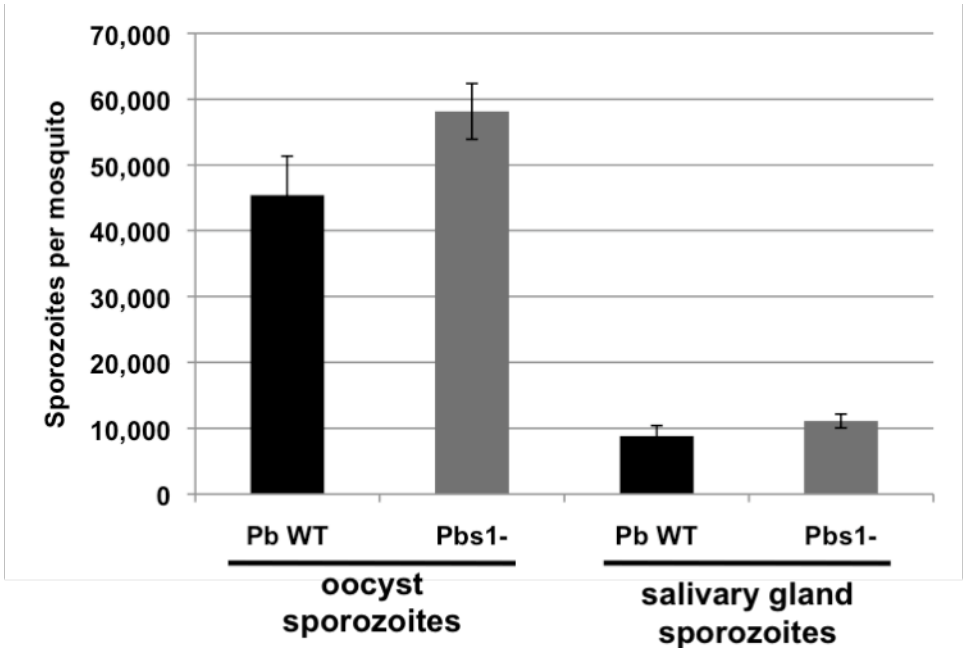

Figure S3: Phenotypic analysis of *Pbs1*$^{-}$ parasites. *Pbs1*$^{-}$ and PbWT infected mosquitoes were evaluated for numbers of midgut and salivary gland sporozoites at day 14 and 21, respectively.

Table S2 Determination of *Pbs1*$^{-}$ pre-patent period

	No. positive/No. injected	pre-patant period (days)
Pbs1^{-}	5/5	3
Pb WT	5/5	3

Table S2: Determination of *PbS1*$^{-}$ pre-patent period. 10,000 *PbS1*$^{-}$ or PbWT sporozoites were injected IV into 5 C57BL/6 mice. Blood stage parasitemia was followed from day three through five to assess pre-patent period.