A Next-generation Genetically Attenuated *Plasmodium falciparum* Parasite Created by Triple Gene Deletion

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Immunization with live-attenuated *Plasmodium* sporozoites completely protects against malaria infection. Genetic engineering offers a versatile platform to create live-attenuated sporozoite vaccine candidates. We previously generated a genetically attenuated parasite (GAP) by deleting the *P52* and *P36* genes in the NF54 wild-type (WT) strain of *Plasmodium falciparum* (*Pf p52*$^{-}$/*p36*$^{-}$ GAP). Preclinical assessment of *p52*$^{-}$/*p36*$^{-}$ GAP in a humanized mouse model indicated an early and severe liver stage growth defect. However, human exposure to >200 *Pf p52*$^{-}$/*p36*$^{-}$ GAP-infected mosquito bites in a safety trial resulted in peripheral parasitemia in one of six volunteers, revealing that this GAP was incompletely attenuated. We have now created a triple gene deleted GAP by additionally removing the *SAP1* gene (*Pf p52*$^{-}$/*p36*$^{-}$/*sap1*$^{-}$ GAP) and employed flippase (FLP)/flippase recognition target (FRT) recombination for drug selectable marker cassette removal. This next-generation GAP was indistinguishable from WT parasites in blood stage and mosquito stage development. Using an improved humanized mouse model transplanted with human hepatocytes and human red blood cells, we show that despite a high-dose sporozoite challenge, *Pf p52*$^{-}$/*p36*$^{-}$/*sap1*$^{-}$ GAP did not transition to blood stage infection and appeared to be completely attenuated. Thus, clinical testing of *Pf p52*$^{-}$/*p36*$^{-}$/*sap1*$^{-}$ GAP assessing safety, immunogenicity, and efficacy against sporozoite challenge is warranted.

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INTRODUCTION

Plasmodium parasites that cause malaria present a formidable threat to human health, primarily in resource-poor regions of the world.[1] The tremendous morbidity and mortality inflicted by malaria infection could be dramatically diminished by a completely protective malaria vaccine.[2] Fortunately, immunizations with live-attenuated sporozoites in animal models and humans have consistently demonstrated that a malaria vaccine conferring complete, protracted protection is possible.[3–9] Recent work, involving immunizations with infectious *Plasmodium falciparum* sporozoites delivered by relatively small numbers of mosquito bites under concurrent chloroquine prophylaxis (chemoprophylaxis with sporozoites), showed robust, long-lasting protection against infectious sporozoite challenge.[10] Furthermore, recent clinical studies with irradiation-attenuated *P. falciparum* sporozoites administered to humans intravenously showed complete protective efficacy against infectious sporozoite challenge at the highest-dose immunization regimen.[11] This work built on previous studies showing that irradiation-attenuated *P. falciparum* sporozoites administered to humans by the bite of >1,000 infected mosquitoes conferred robust and near-complete protection against infectious sporozoite challenge.[12] However, both chemoprophylaxis with sporozoites and irradiation-attenuated sporozoites do not allow for any intrinsic control over the design of the whole parasite immunogen. In contrast, engineered attenuation of parasites (genetically attenuated parasite (GAP)) offers a platform for controlled and consistent design of a whole parasite immunogen. Previous work with the rodent malaria parasites *P. berghei and P. yoelii* demonstrated the utility of genetic attenuation by deletion of the pre-erythrocytic stage-expressed genes called upregulated in infectious sporozoites (*UIS*)*3* and *UIS4,* both of which resulted in early liver stage developmental arrest.[13–15] Deletion of pre-erythrocytic stage-expressed genes *P52* and *P36* similarly resulted in an early liver stage developmental arrest.[16,17] Immunization of mice with *uis3*$^{-}$, *uis4*$^{-}$, or *p52*$^{-}$ parasites induced complete long-lasting protection against infectious sporozoite challenge, demonstrating that rodent malaria GAPs are highly effective immunogens.[13–15,18] Subsequently, a double gene deletion *p52*$^{-}$/*p36*$^{-}$ GAP was generated in *P. yoelii*.[19] Infection with *Py p52*$^{-}$/*p36*$^{-}$ sporozoites did not cause blood stage infection in Balb/cJ mice, and immunization with *Py p52*$^{-}$/*p36*$^{-}$ sporozoites conferred complete protection against intravenous infectious sporozoite challenge or infectious mosquito bite challenge.[19] Deletion of *P52* and *P36* prevents the parasite from establishing or maintaining a parasitophorous vacuole

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Table 1 Infections of *Plasmodium yoelii p52⁻/p36⁻* and *P. yoelii sap1⁻* in the Balb/cByJ mouse model

Parasite genotype	Number of injected sporozoites	Number of mice injected/number with blood stage patency	Day of blood stage patency[a]
P. yoelii wild type	7.5×10^4	5/5	3
P. yoelii p52⁻/p36⁻	7.5×10^4	30/4	4.7
P. yoelii sap1⁻	7.5×10^4	30/0	–

[a]Mice injected with p52⁻/p36⁻ and sap1⁻ sporozoites were followed for 14 days postinjection. Sporozoites were isolated from mosquito salivary glands at day 14 or day 15 postinfectious blood meal and were intravenously injected into the tail vein of Balb/cByJ mice. The time to blood stage patency (defined as >1 infected red bllod cell (RBC)/10,000 RBCs) was monitored microscopically by Giemsa-stained thin blood smears starting on day 3 postinjection.

membrane, which causes the parasite's attenuated phenotype. To assess orthologous genetic attenuation in *P. falciparum*, we previously generated a double gene deletion GAP lacking *P52* and *P36* (*Pf p52⁻/p36⁻*) in the NF54 strain.[20] Analysis of the knockout (2 KO) parasites *in vitro* and in a humanized mouse model showed that the *Pf p52⁻/p36⁻* parasites suffered a liver stage growth defect early following hepatocyte infection. Subsequent clinical assessment showed that *Pf p52⁻/p36⁻* was not completely attenuated in human infection, and resulted in peripheral blood stage parasitemia in one of six volunteers exposed to >200 *Pf p52⁻/p36⁻*-infected mosquito bites.[21] Therefore, the next step was to introduce a third gene deletion in these parasites that, when combined with the *Pf p52⁻/p36⁻* background, would potentially yield complete attenuation. In an earlier study, we identified the sporozoite asparagine-rich protein 1 (*SAP1*) to be essential for liver stage development of *P. yoelii*.[22] The encoded protein localized to the cytoplasm of salivary gland sporozoites and deletion of *SAP1* led to reduction in abundance of numerous *UIS* transcripts caused by increased RNA degradation.[22,23] *P. yoelii sap1⁻* sporozoites exhibited a complete arrest in early liver stage development and the absence of blood stage parasitemia in mice injected with up to 2×10^6 *sap1⁻* sporozoites.[22] Similar to *P. yoelii*, *sap1⁻ P. berghei* also showed complete attenuation.[24] Thus, we targeted *P. falciparum SAP1* for deletion to generate a triple gene KO (3KO) GAP. Here, we report the successful generation of a *Pf p52⁻/p36⁻/sap1⁻* 3KO GAP and its characterization in preclinical assays.

RESULTS

Complete attenuation of *P. yoelii sap1⁻* in the Balb/cByJ mouse strain

Our initial studies of the *P. yoelii p52⁻/p36⁻* GAP indicated complete attenuation because injection of 1×10^5 sporozoites in Balb/cJ mice did not lead to blood stage parasitemia (breakthrough).[19] Similarly, no blood stage breakthrough infection was observed with the orthologous KO parasite in *P. berghei (Pb p52⁻/p36⁻)* in Balb/cJ mice.[25] However, occasional breakthrough infection did occur in C57BL/6 mice injected with *Pb p52⁻/p36⁻* sporozoites, which are more susceptible to *P. berghei* infection.[25] Thus, we tested if *Py p52⁻/p36⁻* could show breakthrough blood stage infection in a more susceptible mouse strain. We recently observed that Balb/cByJ mice, a congenic strain of Balb/cJ mice, carried significantly higher liver stage burden when compared to Balb/cJ mice (unpublished data). In light of the higher susceptibility of Balb/cByJ mice to *P. yoelii*, we infected Balb/cByJ mice with a high-dose (7.5×10^4) of *P. yoelli p52⁻/p36⁻* sporozoites and indeed observed breakthrough blood stage parasitemia in a fraction of these mice (**Table 1**). To evaluate the breakthrough capacity of *Py sap1⁻* parasites, we then tested the same dose of *P. yoelii sap1⁻* sporozoites in Balb/cByJ mice and observed no breakthrough blood stage parasitemia in any of the challenged mice (**Table 1**). This demonstrated that *Py sap1⁻* parasites are completely attenuated. Thus, we decided to pursue a *SAP1* (PF3D7_1147000) deletion to achieve complete *P. falciparum* liver stage attenuation, reasoning that combining a deletion causing full arrest with two deletions causing severe but incomplete arrest in murine models would generate a parasite highly unlikely to cause breakthrough growth in humans.

Generation of the *P. falciparum* p52⁻/p36⁻/sap1⁻ triple gene KO parasite

Our recent phase 1 clinical study with *Pf p52⁻/p36⁻* GAP showed that this 2KO parasite was severely but incompletely attenuated as one of six volunteers exposed to >200 *Pf p52⁻/p36⁻*-infected mosquito bites developed peripheral blood parasitemia.[21] To further enhance the attenuated phenotype, we additionally targeted *SAP1* in the *Pf p52⁻/p36⁻* genetic background. A clone of *Pf p52⁻/p36⁻* in which the positive selectable marker HsDHFR had been removed[26] allowed for the introduction of a sequential gene deletion. *SAP1* was deleted in this clone using the positive-negative selection strategy that was previously used to delete *P52* and *P36* (**Figure 1**).[20] Following cloning of recombinant parasites by limiting dilution, Southern blotting of genomic DNA (Southern) and genotyping by polymerase chain reaction (PCR) were performed to confirm the successful deletion of *SAP1* in this *Pf p52⁻/p36⁻/sap1⁻* 3KO as well as the absence of any residual *Pf p52⁻/p36⁻* parasites (**Figure 1a,b**).

Pf p52⁻/p36⁻/sap1⁻ parasites show normal blood stage growth and development in mosquitoes

Analysis of a the clonal population of the 3KO showed no observable defect during asexual blood stage replication (**Figure 1c**), in its ability to produce gametocytes (**Figure 1d**) or male gamete exflagellation (**Figure 1e**) when compared to wild type (WT). Evaluation of midgut infection in *Anopheles stephensi* mosquitoes fed with gametocyte cultures showed no significant differences between WT and 3KO parasites in oocyst prevalence (**Figure 2a**) and oocyst numbers per infected mosquito midgut (**Figure 2b**). Importantly, the invasion of mosquito salivary glands appeared unchanged in 3KO, as similar numbers of salivary gland sporozoites were isolated when compared to WT (**Figure 2c**). Additionally, 3KO sporozoites showed robust gliding motility on a solid substrate (**Figure 2d**).

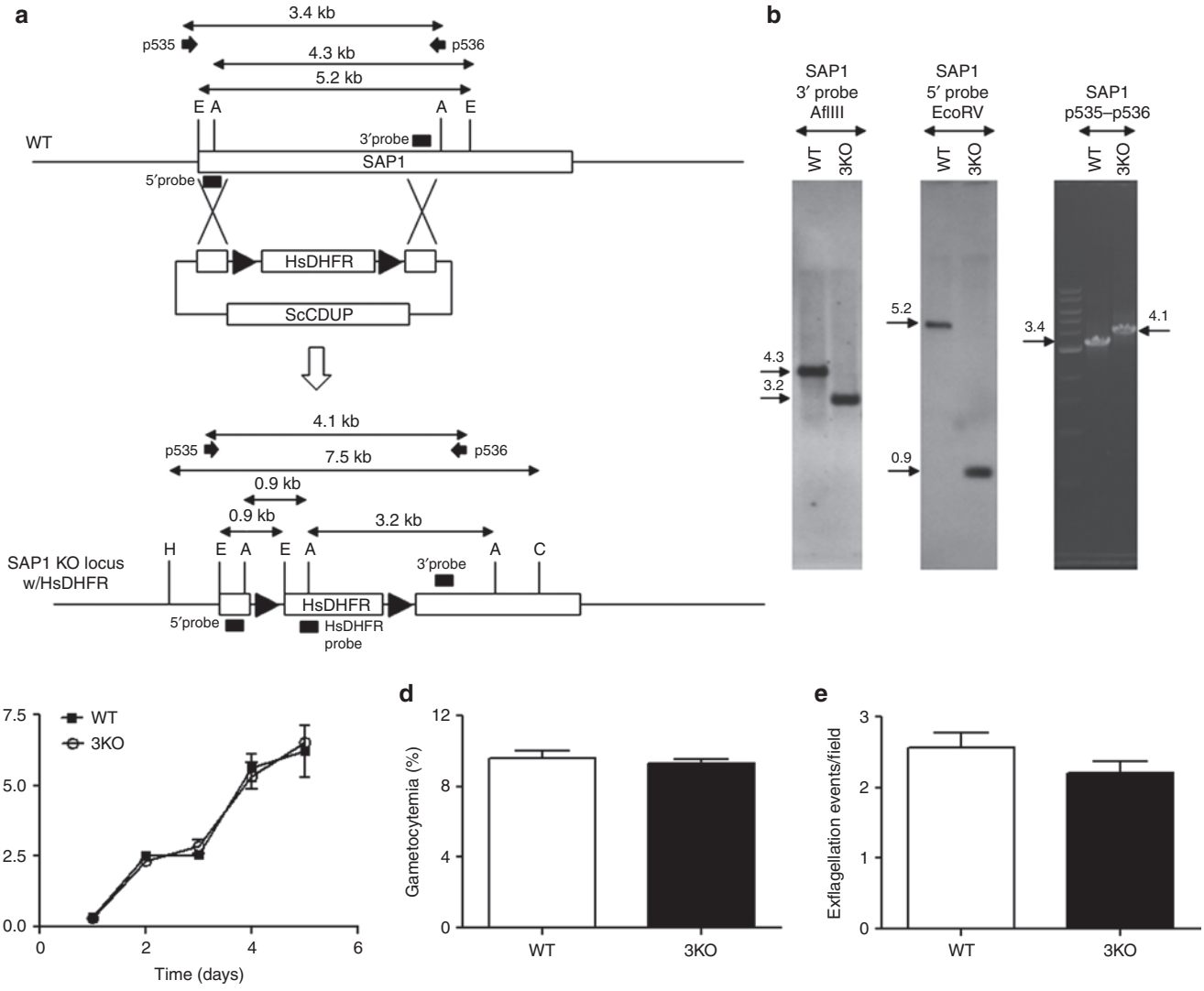

Figure 1 Strategy for targeted gene deletion of *Pf SAP1*, characterization, and phenotypic analysis of the *Pf p52⁻/p36⁻/sap1⁻* 3KO. (**a**) Schematic of strategy for deleting the *SAP1* gene in a *Pf p52⁻/p36⁻* KO clone lacking the HsDHFR marker.[26] Enzymes and probes for Southern blotting and primers for polymerase chain reaction (PCR) are shown. Sizes of genomic DNA fragments and PCR products are indicated in kilobases. (**b**) Southern blotting (left and middle panels) and PCR (right panel) to show deletion of *SAP1* in a *Pf p52⁻/p36⁻* KO clone (*Pf p52⁻/p36⁻/sap1⁻* 3KO). AflIII-digested genomic DNA was hybridized with a 3′ probe yielding a 4.3 and 3.2 kb band for wild type (WT) and 3KO DNA, respectively. Hybridization of EcoRV-digested genomic DNA with a 5′ probe detected a 5.2 and 0.9 kb band for WT and 3KO DNA, respectively. PCR on genomic DNA using primers p535 and p536 yielded a 3.4 and 4.1 kb band for WT and 3KO DNA, respectively. (**c**) Comparison of asexual blood stage growth rates, as measured by increase in parasitemia over time, between WT and 3KO. Cultures were initiated at 0.5% parasitemia and analyzed daily by Giemsa-stained thin blood smears until day 5. Growth assays were performed in triplicate for each line and parasitemia plotted as mean ± SEM. Mann–Whitney *U*-test was used for statistical analysis. (**d**) Comparison of % gametocytemia between WT and 3KO cultures at the time of feeding *in vitro* gametocyte cultures to mosquitoes (14–16 days postinitiation of *in vitro* gametocyte cultures). Gametocytemia was determined three independent times in duplicate cultures for each line and plotted as mean ± SEM. Mann–Whitney *U*-test was used for statistical analysis. (**e**) Comparison of number of male gamete exflagellation events between WT and 3KO gametocyte cultures at the time of feeding to mosquitoes. Exflagellation (plotted as mean ± SEM) was analyzed in several microscopic fields four independent times in duplicate and triplicate cultures for WT and 3KO, respectively. Mann–Whitney *U*-test was used for statistical analysis.

Pf p52⁻/p36⁻/sap1⁻ sporozoites show normal cell traversal and host cell infection

We next investigated if the gene deletions in the 3KO clone would affect the ability of the parasite to traverse or invade hepatocytes, the two steps important for *Plasmodium* parasites to initiate liver infection. No significant differences were observed between WT and 3KO sporozoites in their ability to traverse hepatocytes *in vitro* (**Figure 2e**). Sporozoites of 3KO were also able to infect hepatocytes *in vitro* at levels comparable to WT (**Figure 2f**).

Pf p52⁻/p36⁻/sap1⁻ parasites fail to complete liver stage development in FRG-HuHep mice

To evaluate whether 3KO parasites can complete liver stage development *in vivo*, FRG-HuHep (FRG) mice were injected intravenously with one million WT or 3KO sporozoites. Seven days after sporozoite infection, mice were injected with human red blood cells and subsequently sacrificed to collect the livers and peripheral blood. The blood was used for *in vitro* culture to detect the occurrence of blood stage infection.[27] Blood collected from mice injected with WT

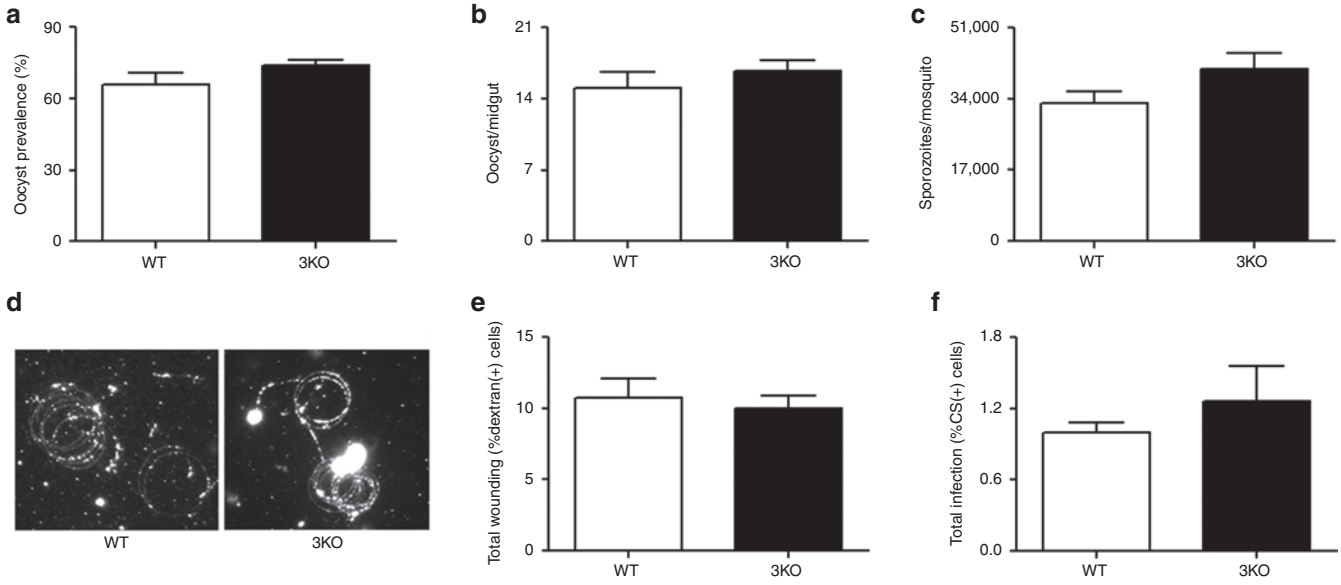

Figure 2 Mosquito stage development, and *in vitro* sporozoite host cell traversal and invasion assays. (**a**) Prevalence of oocysts in mosquito midguts infected with WT and 3KO parasites. Oocyst prevalence (plotted as mean ± SEM) was calculated by dividing the number of dissected midguts containing oocycts by the total number of midguts on day 7 postfeeding of mosquitoes with *in vitro* gametocyte cultures. Prevalence was determined in several mosquitoes four independent times in duplicate and quadruplicate for WT and 3KO, respectively. Mann–Whitney *U*-test was used for statistical analysis. (**b**) Comparison of average number of oocysts per mosquito midgut (plotted as mean ± SEM) between WT and 3KO on day 7 postfeeding of mosquitoes. Oocyts numbers were determined in several mosquitoes four independent times in duplicate and quadruplicate for WT and 3KO, respectively. Mann–Whitney *U*-test was used for statistical analysis. (**c**) Comparison of average number of sporozoites per mosquito (plotted as mean ± SEM) between WT and 3KO on 14–16 days postfeeding of mosquitoes. Sporozoite numbers were determined three independent times in at least duplicate for each line. Mann–Whitney *U*-test was used for statistical analysis. (**d**) Staining of CSP trails using Alexa 488-conjugated anti-*Pf*CS 2A10 antibody in motility assays of salivary gland sporozoites from WT and 3KO. Sporozoites were collected 14–16 days postfeeding of mosquitoes. (**e**) Average total of HC-04 cells in traversal assays with WT and 3KO salivary gland sporozoites (plotted as mean ± SEM) as measured by the fraction of total HC-04 cells in the sample that had taken up FITC-dextran. Total dextran positive cells were determined four independent times in duplicate for each line. Mann–Whitney *U*-test was used for statistical analysis. (**f**) Average total infection of HC-04 cells by WT and 3KO salivary gland sporozoites (plotted as mean ± SEM) as measured by the fraction of total HC-04 cells in the sample that were positive for intracellular parasites as measured by CS staining. Total CS-positive cells were determined four independent times in duplicate for each line. Mann–Whitney *U*-test was used for statistical analysis CS, circumsporozoite; FITC, fluorescein isothiocyanate.

sporozoites consistently produced patent parasitemia in culture. In contrast, none of the *in vitro* cultures of blood collected from the 3KO sporozoite-injected mice produced a positive blood stage parasite culture as evaluated by microscopic examination of blood smears for a period of 3 weeks postcollection (Table 2). Livers from these mice were also removed on day 7 and analyzed by immunofluorescence assay for presence of liver stage parasites. As expected, mature late stage parasites were detected in livers from WT sporozoite-injected mice (**Supplementary Figure S2**) at an average density of one parasite per 74 mm^2 area of liver section (**Supplementary Table S1**). Conversely, no liver stages of any size were detected on day 7 in mice injected with 3KO sporozoites (**Supplementary Table S1**).

Table 2 Analysis of blood stage patency in FRG mice injected with wild type or *Pf p52⁻/p36⁻/sap1⁻* 3KO salivary gland sporozoites

Parasite line	Number of mice infected[a]/ number with blood stage patency[b]
Wild type	3/3
3KO	3/0
WT	3/3
3KO (-H) c2	3/0

[a]Mice were injected with 1×10^6 sporozoites. [b]Patency was checked 3–30 days after initiation of *in vitro* blood stage culture.

A next-generation GAP vaccine candidate for clinical studies

A GAP that could enter clinical development would need to be devoid of any extraneous DNA and not exhibit any drug resistance. Thus, we removed the HsDHFR selectable marker from the *sap1⁻* locus using the FLP/FRT system[26] in 3KO parasites. Two clonal lines that were positive for *Pf p52⁻/p36⁻/sap1⁻* triple gene deletion were also devoid of any drug selectable marker cassettes (clones 3KO (-H) c1 and c2) by Southern and PCR analyses (**Supplementary Figure S1** and **Supplementary Table S2**). These clones were analyzed for retention of vital characteristics needed to produce sporozoites for experimental vaccination. As with the parental 3KO parasite, no defects were observed in blood stage growth (Figure 3a), gametocyte production (data not shown), sporozoite production (Figure 3b), and gliding on solid substrates (Figure 3c). Additionally, 3KO (-H) c1 and c2 were susceptible to the drugs WR99210 and blasticidin, further demonstrating that the cassettes containing selectable markers conferring resistance to these drugs were no longer retained (**Supplementary Table S3**). Three FRG mice were injected with sporozoites of one of the 3KO (-H) clones to determine if the parasites showed the same degree of liver stage attenuation as observed for the marker-containing 3KO. As expected, no liver stage parasites were detected by immunofluorescence assays at

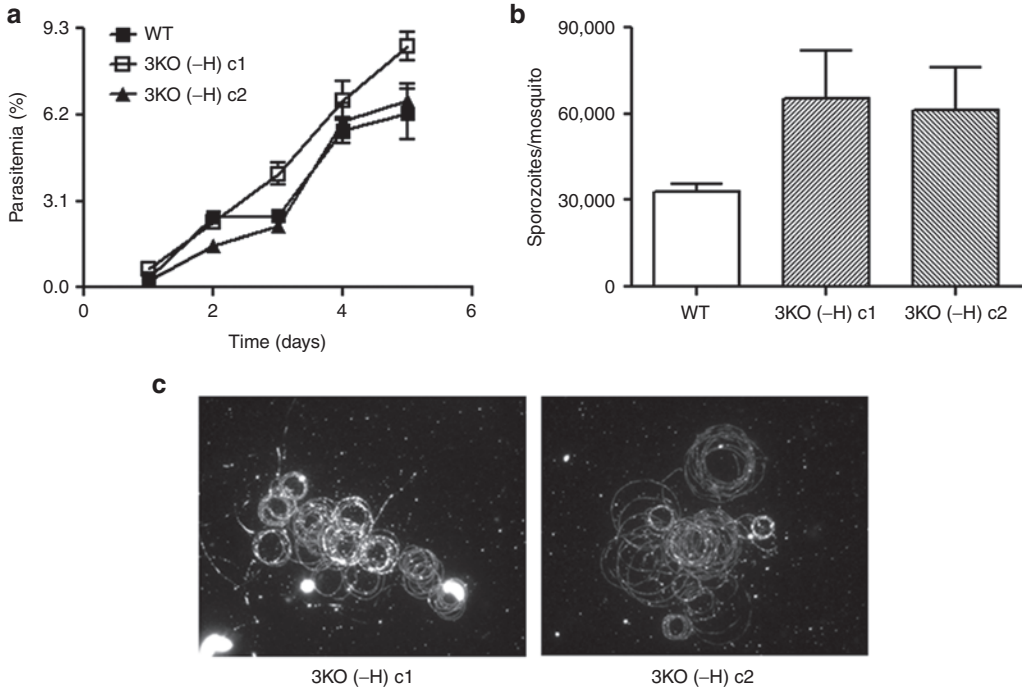

Figure 3 Phenotypic analysis of *Pf p52⁻/p36⁻/sap1⁻* 3KO (-H) clones. (**a**) Comparison of asexual blood stage growth rates between wild type (WT) and 3KO (-H) clones. Cultures were initiated at 0.5% parasitemia and analyzed daily until day 5. Growth assays were performed in triplicate for each line and parasitemia plotted as mean ± SEM. Mann–Whitney *U*-test was used for statistical analysis. (**b**) Comparison of average number of sporozoites (plotted as mean ± SEM) per mosquito between WT and 3KO (-H) clones 14–16 days postfeeding of mosquitoes. Sporozoite numbers were determined three independent times in at least duplicate for each line. Mann–Whitney *U*-test was used for statistical analysis. (**c**) Staining of CS trails using Alexa Fluor 488-conjugated anti-*Pf*CSP 2A10 antibody in motility assays of salivary gland sporozoites of WT and 3KO (-H) clones.

day 7 post-3KO(-H) sporozoite injection, and *in vitro* culturing of blood collected from the 3KO(-H)-infected FRG mice did not show occurrence of blood stage infection during 3 weeks of culture (**Table 2**). Control mice injected with WT sporozoites had detectable liver stage infection and *in vitro* blood cultures were consistently parasitemic shortly after blood transfer from infected FRG mice (**Table 2**). Finally, we used reverse transcriptase polymerase chain reaction (RT-PCR) analysis to test for the presence of *P. falciparum* 18S A-type transcripts in day 7 liver samples from FRG mice infected with either WT or 3KO (-H) salivary gland sporozoites. While we detected a strong signal for 18S in FRG mice infected with WT sporozoites at day 7, no signal for 18S was detected in liver samples from three independent mice that had been injected with 3KO (-H) sporozoites (**Supplementary Figure S3**).

DISCUSSION

Despite the enormous importance of malaria, the goal of developing a *P. falciparum* vaccine that demonstrates high efficacy and confers long-lasting protection remains unrealized.[28,29] Recent data however renewed optimism that a highly protective vaccine might be attainable when based on live-attenuated sporozoites. Seder *et al.*[11] showed that intravenous immunization with repeated high doses of irradiated, cryopreserved *P. falciparum* sporozoites protected six out of six volunteers against infectious *P. falciparum* challenge. This is in agreement with historical data showing that intravenous immunizations with irradiation-attenuated sporozoites in animal models and by mosquito bite delivery in human volunteers induce sterile protection against subsequent sporozoite challenges. The efficacy of these whole live-attenuated sporozoite immunizations have so far been unmatched by any subunit malaria vaccines in development, including the most advanced CSP-based RTS,S vaccine candidate currently being tested in phase 3 clinical studies, which showed 30–50% protective efficacy and a relatively short duration of protection.[30]

Targeted gene deletion that allows for producing intrinsically and uniformly attenuated *Plasmodium* sporozoites with a potential of increased vaccination potency, is a design-based alternative strategy to irradiation-based parasite attenuation.[31] In our previous study, deletion of two pre-erythrocytic stage-expressed genes (*P52* and *P36*) in *P. falciparum* negatively affected the ability of sporozoites to create functional hepatocyte infection and initiate liver stage development, resulting in an attenuated phenotype.[20] Phenotypic analysis of the *P52* and *P36* gene deletions in rodent malaria parasites implicated the lack of a parasitophorous vacuole membrane surrounding the parasites early in hepatocyte infection as the cause for the profound developmental defect in the *p52⁻/p36⁻* parasites.[19,20] Despite this defect, however, the double gene deletion did not completely attenuate *Pf p52⁻/p36⁻* infections. This came to light in a first-in-human proof-of-concept safety study in which one of six volunteers developed peripheral blood parasitemia after exposure to >200 *Pf p52⁻/p36⁻* GAP-infected mosquito bites.[21] Interestingly, it was recently shown that *P. berghei* rodent malaria *p52⁻/p36⁻* parasites could, in rare instances, develop within the hepatocyte without parasitophorous vacuole membrane formation,[32] providing a potential explanation for the occurrence of breakthrough blood stage infections. We have here substantiated this potential for breakthrough in the *P. yoelii*

model by demonstrating the infrequent occurrence of blood stage parasitemia in highly susceptible Balb/cByj mice challenged with *Py p52⁻/p36⁻* sporozoites. Thus, this model will be an important experimental addition to the toolbox that evaluates GAP phenotypes.

To optimize attenuation and prevent blood stage breakthrough of those rare parasites that could develop without P52 and P36 expression, we introduced an additional gene deletion into *Pf p52⁻/p36⁻* parasites. The deletion was selected to complete attenuation based on data from rodent malaria parasite models, which showed that *Py* and *Pb sap1⁻* parasites suffered complete attenuation of early liver stage growth and did not show breakthrough blood stage parasitemia.[22,24] We corroborated this here with the highly sensitive *P. yoelii*-Balb/cByJ model. *SAP1* was also selected for deletion as the putative function of the encoded protein significantly differs from the functions of P52 and P36. While SAP1 is a cytoplasmic protein involved in regulating RNA stability and as such impacts sporozoite gene expression,[22] P52 and P36 are secreted proteins both involved in the formation of the parasitophorous vacuole membrane.[19] Deletions of genes that are involved in independent biological processes should improve the robustness of complete attenuation and further reduce the possibility of compensatory changes in the parasite that might lead to loss of attenuation. We demonstrated here that the *Pf p52⁻/p36⁻/sap1⁻* triple gene deletion had no significant effect on gametocytogenesis, mosquito infectivity, or sporozoite production. Also, despite undergoing extensive sequential genetic manipulation and drug selection *in vitro*, *Pf p52⁻/p36⁻/sap1⁻* 3KO sporozoites showed neither reduced viability nor altered characteristics of initial infection, as measured by the ability of the sporozoites to glide on a solid substrate, traverse and infect hepatocytes.

To directly assess attenuation of the *Pf p52⁻/p36⁻/sap1⁻* 3KO parasite, we employed the robust humanized FRG mouse model harboring human hepatocytes and human red blood cells that allow for complete development of *P. falciparum* liver stages and supports liver stage-to-blood stage transition.[27] Unlike WT parasites, 3KO parasites were undetectable in the livers of infected FRG mice at day 7 after sporozoite infection. Strikingly, no liver-to-blood transition of infection was observed for the 3KO, while WT infections reliably showed this transition. Long-term *in vitro* culture of the isolated blood also did not result in detectable parasitemia for 3KO. This further suggests that the 3KO parasites are fully attenuated and cannot undergo liver stage development. The removal of the drug resistance marker was achieved using the flippase (FLP)/flippase recognition target (FRT) system, which allowed for complete excision of all exogenous DNA from the 3KO genome. As a result, the 3KO (-H) clones are devoid of any drug resistance markers, which is currently a requirement for the use of genetically engineered agents in advanced clinical testing. It is also important to note that several rounds of genetic manipulation and prolonged *in vitro* culturing did not affect the fitness and viability of the 3KO (-H) clones. This further emphasizes that the development of *P. falciparum* genetically attenuated sporozoites for vaccination is feasible.

With the pursuit of a triple gene deletion *Pf* GAP, we have focused on achieving robust sporozoite production, viability, and complete attenuation, which is a prerequisite for the use of attenuated sporozoites in human vaccination. A recent clinical study with *Pf p52⁻/p36⁻* GAP showed that they induce substantial immune responses including functional antibody responses that can effectively block sporozoite infection *in vitro*.[21,33] These data, together with extensive evidence that *sap1⁻* and *p52⁻/p36⁻* rodent GAPs engender sterile protection against sporozoite challenge in mice, give reasons to predict that the *Pf p52⁻/p36⁻/sap1⁻* triple gene deletion GAP will induce protective immune responses in humans.[19,22,24] However, early liver stage arresting GAPs might not yet constitute the optimal live-attenuated immunogen. It was previously shown that late liver stage–arresting *P. yoelii* GAPs, created by gene deletions in the fatty acid biosynthesis pathway (FASII), produce superior immune responses and protection in mice.[34,35] However, recent analysis of FASII gene deletions in *P. falciparum* showed an unexpected detrimental effect on sporozoite formation in oocysts, thus currently precluding the production of FASII KO GAPs for human immunization.[36]

In conclusion, we have developed a next-generation triple gene deletion GAP strain of *P. falciparum*. We used state-of-the-art preclinical tools to evaluate its degree of attenuation and found that 3KO produced viable, infectious sporozoites that arrested early, could not complete liver stage development and could not transition to blood stage infection. A proof-of concept clinical study with the 3KO GAP assessing safety, induction of cellular and humoral immune responses as well as preliminary efficacy against infectious sporozoite challenge is thus warranted.

MATERIALS AND METHODS

Ethics statement. All animal studies were approved by the Institutional Review Board at Seattle BioMed.

In vitro *culturing of parasite lines.* The WT *P. falciparum* NF54 strain and KO lines were propagated *in vitro* in O^+ human blood (Interstate Blood Bank, Memphis, TN) in custom-made Roswell Park Memorial Institute medium containing hypoxanthine, sodium bicarbonate, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Invitrogen, Life Technologies, Grand Island, NY) supplemented with 0.4% Albumax (Invitrogen) and gentamycin (Invitrogen) according to standard procedures for *in vitro P. falciparum* culturing.

Blood stage growth assay. *In vitro* cultures of parasites were set up in triplicate in 12-well plates at 0.5% starting parasitemia and 4% hematocrit (HCT). Media was replaced and parasitemia was determined daily for 5 days by Giemsa (Sigma-Aldrich, St Louis, MO)-stained thin smears to determine rate of growth.

Design of gene-targeting constructs and generation of KO parasite lines. Oligonucleotide primers used to generate 5′ and 3′ homologous recombination regions (5′ and 3′ flanks, respectively) for simultaneous deletion of *P52* and *P36* genes were as follows: *P52* 5′flank: 5′ CCGCGGGGATC TCTATAAATGCATGAGG (sense) and 5′; ACTAGTGAAGTTCCTATAC TTTCTAGAGAATAGGAACTTCCTGGGTGAGTTTTTGCCGTA GTACTAAAAGCATCATTC (antisense); *P36* 3′ flank: 5′ CCTAGGGAA-GTTCCTATTCTCTAGAAAGTATAGGAACTTCGGGAATTTACAT-GCCATTCTATG (sense) and 5′ GGCGCCCCTATACCCTTCCCTT-GTG (antisense). For *P52/P36* targeting plasmid, the 5′ and 3′ flanks were cloned into SacII-SpeI and AvrII-SfoI, respectively in the pCC1 plasmid.[37] Oligonucleotide primers used to generate 5′ and 3′ flanks for deleting *SAP1* gene were as follows: 5′ flank: 5′ CCGCGG*TGA*AGAAAAGGG AAACCAAGACATGTG (*SAP1* start codon mutated; italicized; sense) and 5′ ACTAGTATAACTTCGTATAGCATACATTATACGAAGTT ATGGTGTATTATAACTTTGTGGTGTATTATAAC (antisense); 3′ flank: 5′ GAATTCATAACTTCGTATAATGTATGCTATACGAAGTTATCAGAA

TCAAAATTATATAACCAACC (sense) and 5′ CCTAGGCGTTGTTAAGA TGTGGGTCTATATACG (antisense). 5′ and 3′ flanks for *SAP1* targeting plasmid were cloned into SacII-SpeI and EcoRI-AvrII, respectively in pCC1. WT parasites were transfected (see below) to generate the *p52*$^{-}$/*p36*$^{-}$ double KO parasites.[20,26] Knockout parasites were cloned by limiting dilution. HsDHFR marker was removed by transfecting a clone of *p52*$^{-}$/*p36*$^{-}$ with p-Tet-BSD-FLP plasmid.[26] *SAP1* was deleted in a clone of *p52*$^{-}$/*p36*$^{-}$ lacking the HsDHFR marker yielding the *p52*$^{-}$/*p36*$^{-}$/*sap1*$^{-}$ triple KO. This line was further cloned by limiting dilution. One clone (3KO) was selected and transfected with the pTet-BSD-FLP plasmid generating a 3KO population lacking the HsDHFR marker in the *SAP1* locus. This population was cloned again by limiting dilution and two clones (3KO (-H) c1 and c2) were picked.

Transfection of* P. falciparum *with KO plasmid constructs. Plasmid DNA was extracted by maxi prep (Qiagen, Valencia, CA). *P. falciparum* NF54 (WT) parasites were sorbitol synchronized[38] and transfected with 30–40 µg plasmid by electroporation at 0.31 kV and 950 µF using a BioRad Gene Pulser (BioRad, Hercules, CA).[39] To select for transfectants, cultures were placed under either WR99210 (WR; Jacobus Pharmaceuticals, Princeton, NJ) or Blasticidin (Invivogen, San Diego, CA) 48 hours after transfection depending on whether HsDHFR or AtBSD used as selectable marker, respectively.[26] WR and Blasticidin were used at 2.5 nmol/l and 2.5 µg/ml, respectively.

Negative selection for KO parasites. Knockout parasites were generated by positive-negative drug selection.[37] Briefly, transfectants positively selected using WR were propagated without WR to enrich for parasites that had lost the episomal KO plasmid. Thereafter, WR pressure was reapplied to select for parasites with the KO plasmid integrated in the genome. These parasites were placed under weekly cycles with or without 5-fluorocytosine (770 nmol/l) (Sigma-Aldrich) to select for KO parasites that had undergone target gene deletion by double crossover homologous recombination. 5-fluorocytosine-resistant parasites were genotyped by Southern and PCR.

Parasite cloning by limiting dilution. Knockout parasites were cloned by limiting dilution in 96-well flat bottom plates. Parasitemia (using Giemsa-stained thin smears) and HCT of cultures were accurately determined. Cultures were diluted and plated at a density of 0.5 parasite per well in a 200-µl volume at 2% HCT. Cultures were fed once a week with media containing fresh blood at 0.5% HCT. Parasitemia in wells was checked starting 14 days postinitiation of cloning.

Southern blotting and PCR. Southern blotting for the *P52*/*P36* locus was performed by hybridizing HindIII-ClaI-digested genomic DNA from WT and KO lines with a 3′ probe. AflII or EcoRV-digested DNA was hybridized with 3′ or 5′ probe, respectively to characterize WT and *SAP1* KO locus. Digested DNA was run on a 0.7% Tris-acetate-EDTA agarose gel at 55 V and transferred to Hybond-N membrane (Amersham, GE Healthcare Life Sciences, Pittsburgh, PA) in 20× SSC overnight at room temperature (RT). DNA was UV crosslinked to the membrane and hybridized with digoxygenin-labeled probes prepared using the DIG kit (Roche Diagnostics, Indianapolis, IN). A *P52*/*P36* locus 3′ probe was generated using oligonucleotide primers 5′ TATGTACATGTGAAAGTAGCAAAGAC (sense) and 5′ TTCCCTTGTGGGAAATTACAATGAC (antisense). 3′ probe for *SAP1* locus was generated with oligonucleotide primers 5′ ATTATGAA CATGACAATACAACTAACG (sense) and 5′ CATATTTATGCTACTGT CAGGGATAG (antisense). 5′ probe for *SAP1* locus was generated using oligonucleotide primers 5′ CTAAAATACATAATATACGAAAA AAGTATG (sense) and 5′ TCATATGGCATATAAGATTGTATATCC (antisense). HsDHFR probe was generated with primers 5′ CCTGGCC ACCGCTCAGGAACG (sense) and 5′ TCCTTGTCACAAATAGTTT AAGATGG (antisense). Primers 5′ CTCAAGAAGAATCCACCCTCATTG (sense) and 5′ CCACACATAACCAGAGGGCAGC (antisense) were used to make a BSD probe. Primers 5′ CAACCTGCAAAATCTAAATTGGT (sm002; sense) and 5′ GTAAATATATAAAACACTACAAATAGTAC (mo041; antisense) were used for PCR genotyping the *P52*/*P36* locus, and 5′ TCCAAAAATTGACATTCAGAGTTATAG (p353; sense) and 5′ ACACTTATATGTATAGAAATAGTGTTAC (p536; antisense) for the *SAP1* locus.

Mosquito infections. Gametocyte cultures of WT and KO lines were propagated in O^{+} human blood in custom-made Roswell Park Memorial Institute medium containing supplemented pooled human A^{+} serum (Interstate Blood Bank). Culture media was changed daily and culture volume was maintained around 35 ml. Gametocytogenesis was checked by Giemsa-stained thick smears. Exflagellation was checked by phase contrast microscopy at ×40 magnification beginning 12 days postinitiation of gametocyte cultures. The cultures were fed to mosquitoes when majority of the gametocytes were morphologically mature and vigorously exflagellating. Female *A. stephensi* mosquitoes aged 4–7 days were starved for 1–2 hours and fed for at least 30 minutes at 37 °C on a Baudruche membrane feeder apparatus (Joseph Long, Belleville, NJ). Each cage with 250–300 mosquitoes was fed with concentrated erythrocytes from the 35 ml gametocyte culture mixed with an equal volume of fresh red blood cells and 2 volumes of A^{+} serum. Oocysts prevalence was determined by microdissecting whole midguts and examining them at ×10 magnification using a phase contrast microscope. Midgut and salivary gland sporozoite numbers were determined by microdissecting and grinding whole midguts and salivary glands from mosquitoes on day 7 and days 14–16 postfeeding with *in vitro* gametocyte cultures, respectively, and counting using a hemocytometer.

Traversal and invasion. Traversal and invasion assays were performed as previously described.[40] Briefly, HC-04 cells were plated at 300K cells/well in a 24-well plate the day before in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), penicillin (200 IU/ml)/streptomycin (200 µg/ml) (Corning, Corning, NY), and 5 ml of amphotericin B (Fungizone) (Corning). Salivary gland sporozoites of WT and KO lines were activated in Roswell Park Memorial Institute medium and 20% fetal bovine serum at RT for 15 minutes. Sporozoites were transferred to HC-04-coated chamber slides at 100K/well (3:1 ratio of cells:sporozoites). Fluorescein isothiocyanate-dextran (Invitrogen) was added to the appropriate wells to assess total wounding by sporozoites. The slides were centrifuged at 1,500 rpm for 3 minutes at RT and incubated at 37 °C for 1.5–2 hours. Media was removed and cells were fixed and permeabilized in Cytofix/Cytoperm (Becton Dickinson, Franklin Lakes, NJ). Cells were stained with anti-*Pf*CS mouse monoclonal antibody (2A10) and analyzed by flowcytometry for total wounding (dextran^{+} cells) and total infection (CS^{+} cells).

Motility. Glass coverslips (VWR International, Radnor, PA) were precoated with 10 ng/ml 2A10 antibody in phosphate-buffered saline overnight at RT. Salivary gland sporozoites of WT and KO parasites were activated in Roswell Park Memorial Institute medium containing 20% fetal bovine serum and allowed to glide on antibody-coated coverslips at 37 °C for 2 hours. Coverslips were fixed in 10% neutral buffered formalin (Sigma-Aldrich), blocked in 2% bovine serum albumin–phosphate-buffered saline, stained with Alexa 488-conjugated 2A10 antibody, and mounted in ProLong Gold Antifade Reagent (Life Technologies). Motility was assessed by detecting CS protein shed in gliding trails on the coverslips.

Patency. FRG-HuHep mice (Yecuris Corporation, Tualatin, OR) were injected intravenously with 1×10^{6} each of WT and KO salivary gland sporozoites. On days 6 and 7, these mice were injected with 400 µl of washed O^{+} human blood at 50% HCT. On day 7, 3–4 hours following injection of human blood, the mice were sacrificed, and peripheral blood was collected by cardiac puncture, washed three times, and *in vitro* cultures were set up to determine progression of infection from liver stage to blood stage. Blood stage patency was assessed starting day 2 postblood collection by Giemsa-stained thin smears.

Indirect immunofluorescence assay. After collecting peripheral blood for *in vitro* culturing, the livers of infected mice were perfused with phosphate-buffered saline, dissected out, washed with phosphate-buffered saline, and fixed in 10% neutral buffered formalin. Fifty micrometer sections were cut using a Vibratome apparatus (Ted Pella, Redding, CA). Immunofluorescence assays were performed as previously described.[41] Primary antibodies used were anti-BiP (monoclonal) and anti-ACP (polyclonal).

RT-PCR assay. Liver samples were collected in TRIzol (Life Technologies) from FRG mice day 7 postinfection with 1×10^6 intravenously injected either WT or 3KO (-H) salivary gland sporozoites. Total RNA was extracted using the Direct-zol MiniPrep Kit (Zymo Research, Irvine, CA). cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit (Qiagen). PCR cycling conditions used for amplification of cDNA were 92 °C for 30 seconds for DNA denaturation, 54 °C for 30 seconds for primer annealing, and 62 °C for 1 minute for extension (35 cycles). *P. falciparum* 18S A-type rRNA was amplified using primers 5′ CCAGTAGTCATATGCTTGTCTC and 5′ GAAGCGTATTAAAGCGAAAAGC (~700 bp product). Human ApoA1 was amplified using primers 5′ AGCGTGACCTCCACCTTCAG and 5′ CCTTCACCTCCTCCAGATCCTT (~150 bp product).

SUPPLEMENTARY MATERIAL

Figure S1. Strategy for removal of HsDHFR marker from the *SAP1* KO locus in 3KO.

Figure S2. Immunofluorescence of *in vivo* liver stage development.

Figure S3. RT-PCR of RNA collected on day 7 of liver stage development.

Table S1. Density of liver stage infection in mice injected with WT or 3KO salivary gland sporozoites.

Table S2. Sizes of fragments detected by Southern and PCR products for WT, 3KO and 3KO (-H) clones.

Table S3. Assay for retention of drug resistance markers in 3KO and 3KO(-H) clones.

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