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Vital and dispensable roles of *Plasmodium* multidrug resistance transporters during blood- and mosquito-stage development

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Summary

Multidrug resistance (MDR) proteins belong to the B subfamily of the ATP Binding Cassette (ABC) transporters, which export a wide range of compounds including pharmaceuticals. In this study, we used reverse genetics to study the role of all seven *Plasmodium* MDR proteins during the life cycle of malaria parasites. Four *P. berghei* genes (encoding MDR1, 4, 6 and 7) were refractory to deletion, indicating a vital role during blood stage multiplication and validating them as potential targets for antimalarial drugs. Mutants lacking expression of MDR2, MDR3 and MDR5 were generated in both *P. berghei* and *P. falciparum*, indicating a dispensable role for blood stage development. Whereas *P. berghei* mutants lacking MDR3 and MDR5 had a reduced blood stage multiplication *in vivo*, blood stage growth of *P. falciparum* mutants *in vitro* was not significantly different. Oocyst maturation and sporozoite formation in *Plasmodium* mutants lacking MDR2 or MDR5 was reduced. Sporozoites of these *P. berghei* mutants were capable of infecting mice and life cycle completion, indicating the absence of vital roles during liver stage development. Our results demonstrate vital and dispensable roles of MDR proteins during blood stages and an important function in sporogony for MDR2 and MDR5 in both *Plasmodium* species.

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Introduction

ATP Binding Cassette (ABC) transporters are membrane proteins that translocate diverse compounds at the expense of ATP and have been well conserved in many organisms. They are organized into either two domains (half-transporter), consisting of a transmembrane domain (TMD) and a nucleotide-binding domain (NBD) required for ATP-hydrolysis or into four domains (full-transporter), consisting of two TMDs alternated with two NBDs (Fig. 1A). Half-transporters require homo- or hetero-dimerization to become functional (Jones *et al.*, 2009).

In humans, ABC-transport proteins are well known for their involvement in multidrug resistance against antiviral, antimicrobial or anti-cancer drugs (Dean *et al.*,

2001). Especially transporters of the ABCB, ABCC and ABCG subfamilies play important roles in resistance, of which the most studied multidrug resistance (MDR) protein is MDR1. This protein belongs to the ABCB subfamily, which contains both half- and full-transporters (Dean *et al*., 2001). Amplification of the MDR1 encoding gene has been shown to confer resistance of cancer cells to chemotherapeutic drugs through increased excretion (Roninson *et al*., 1986). However, these transporters also play important roles in physiological cellular processes such as membrane biogenesis, autocrine pathways and homeostasis, as their substrates may vary from lipids and sterols to heavy metals and amphipathic or conjugated xenobiotic compounds (Borst and Elferink, 2002).

In the human malaria parasite *P. falciparum*, 16 ABC genes have been identified (Koenderink *et al*., 2009). Based on phylogenetic analysis of the conserved nucleotide binding domains, seven are recognized as members of the B family of ABC transporters. PfMDR1 (PF3D7_0523000) has been most intensively studied for its involvement in antimalarial drug resistance. Amplifications and polymorphisms of PfMDR1 have been associated with decreased sensitivity towards multiple antimalarial drugs, including chloroquine and artemisinin (Foote *et al*., 1989; Reed *et al*., 2000; Veiga *et al*., 2011). Its primary localization on the membrane of the digestive vacuole may indicate involvement in drug accumulation, where several antimalarial compounds interfere with polymerization of reactive free heme released upon hemoglobin digestion (Sullivan *et al*., 1996). Hence, Pf*mdr1* polymorphisms have been shown to reduce drug accumulation resulting in antimalarial resistance (Reed *et al*., 2000). Furthermore, a single nucleotide polymorphism (SNP) in Pf*mdr2* (PF3D7_1447900), a heavy metal transporter (Rosenberg *et al*., 2006), is associated with *in vitro* resistance to pyrimethamine (Briolant *et al*., 2012). Recently, another SNP in Pf*mdr2* showed a statistically significant association ($p = 2 \times 10^{-10}$) with artemisinin resistance (Miotto *et al*., 2015). Moreover, varying indels in the Pf*mdr6* gene (PF3D7_1352100) have been associated with altered artesunate and piperaquine sensitivity (Mu *et al*., 2003; Anderson *et al*., 2005; Wang *et al*., 2012; Okombo *et al*., 2013), although transfection-based confirmation of that association is lacking. Apart from a role in drug resistance, the physiological function as well as substrate specificity of the *Plasmodium* MDR proteins remains largely unknown, also for PfMDR5 (PF3D7_1339900), which is localized at the plasma membrane of intra-erythrocytic parasites (Kavishe *et al*., 2009).

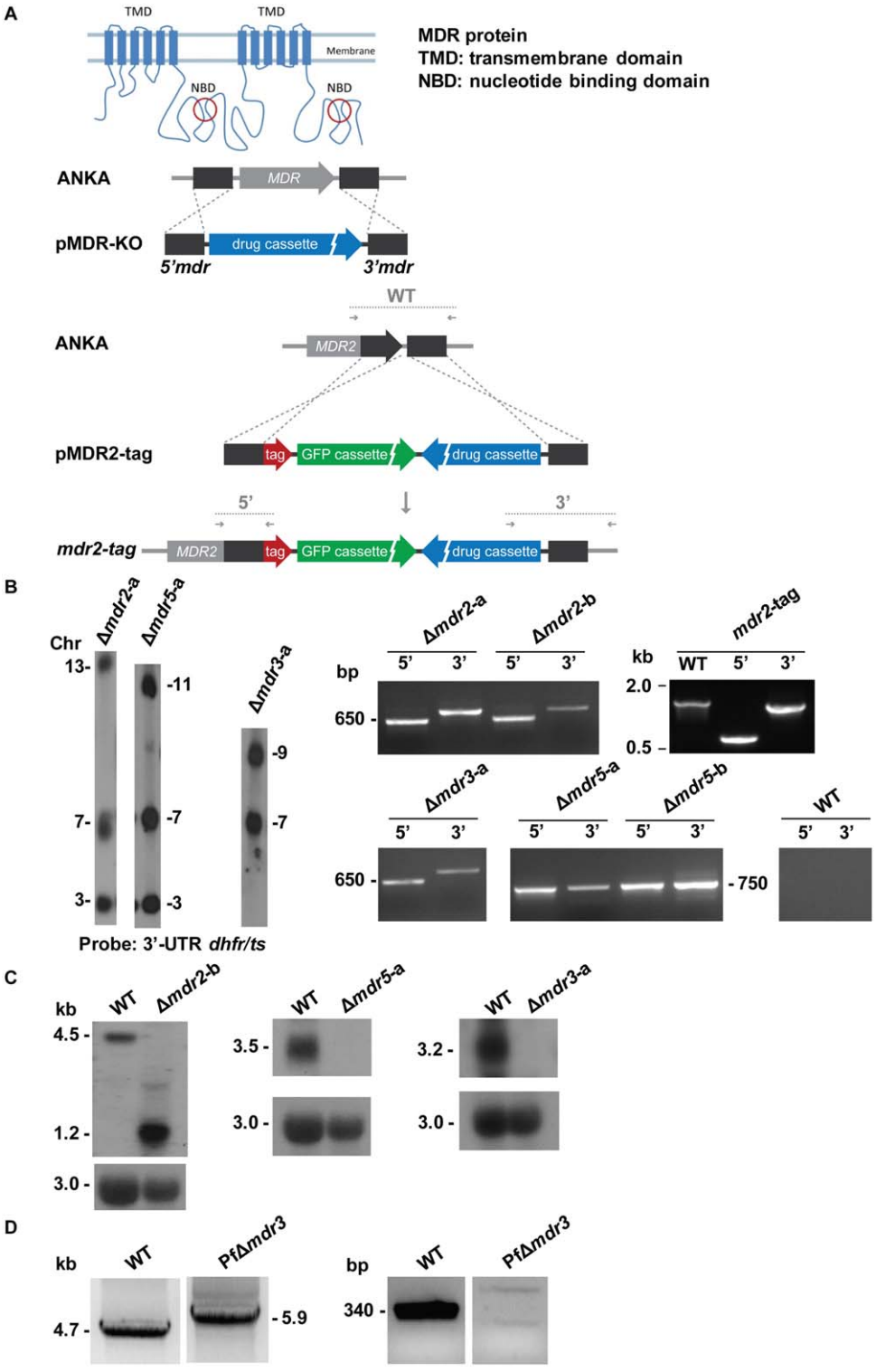
To gain insight into the function of these proteins and their role in the *Plasmodium* life cycle, we first systematically targeted the seven MDR proteins by experimental genetics in the murine malaria model parasite *Plasmodium berghei*. We found that four out of seven ABCB encoding genes (*mdr1*, *mdr4*, *mdr6* and *mdr7*) are refractory to gene deletion. We were able to select *P. berghei* mutants lacking *mdr2*, *mdr3* and *mdr5*, which is indicative of a dispensable role for the encoded proteins during asexual blood stage growth *in vivo*. We subsequently targeted *mdr2*, *mdr3* and *mdr5* for deletion in *P. falciparum* and were able to select mutants *in vitro*, confirming a non-essential role for blood stage development. Both *P. berghei* and *P. falciparum* mutants lacking expression of MDR2 and MDR5 showed reduced oocyst formation. Additionally, the mutants lacking PbMDR2, PbMDR5 and PfMDR2 showed reduced sporozoite production. Furthermore, PbMDR2::mCherry expression was observed during blood- and mosquito stage development. Combined, our results provide evidence that *Plasmodium* express several MDR proteins that have specific functions during sporogony.

Results

Dispensable roles of P. berghei MDR2, MDR3 and MDR5 for blood stage development

We systematically targeted all seven *mdr* genes by experimental genetics in the rodent malaria parasite *Plasmodium berghei*. Multiple attempts to disrupt Pb*mdr1*, *4*, *6* and *7* by DNA constructs that integrate through homologous double cross-over integration (Fig. 1A) using standard methods for genetic modification of *P. berghei* (Janse *et al*., 2006b) were unsuccessful (Supporting Information Table S1). Mutant parasites lacking these MDR encoding genes could not be isolated, indicating an essential role of these proteins for blood stage multiplication. Evidence for expression of these genes in blood stages has previously been reported (Supporting Information Table S2). We successfully targeted the Pb*mdr4* gene with a DNA-construct for C-terminal tagging with an HA-tag (Supporting Information Fig. S2A and B), indicating that the failure to delete the gene from blood stages is not due to inaccessibility of the locus for genetic modification. However, we were unable to detect expression of the HA-tagged MDR4 by immunofluorescence using anti-HA antibodies, possibly due to low expression levels in blood stages (Supporting Information Fig. S2C).

We were successful in obtaining mutant parasites lacking Pb*mdr2*, Pb*mdr3* and Pb*mdr5*, in which correct deletion was demonstrated by Southern analysis of separated chromosomes and diagnostic PCR for 5′ and 3′ TR integration (Fig. 1B). We also generated a mutant



expressing PbMDR2 tagged at the C-terminus with an mCherry-3xMyc tag (Pb*mdr2*::*mCherry*; Fig. 1A). Correct tagging was shown by diagnostic PCR for 5′ and 3′ TR integration of the tagging construct (Fig. 1B) and Southern analysis of separated chromosomes (Supporting Information Fig. S4A).

Northern analysis of transcription in blood stages indicates that all three genes are transcribed in blood

Fig. 1. MDR transporters targeted for deletion in *P. berghei* and *P. falciparum*.
A. Schematic overview of ABC transporter structure (top), double crossover deletion mechanism (middle) and mdr tagging strategy (bottom).
B. Southern analysis of separated chromosomes and diagnostic PCR confirms correct disruption of the mdr genes in Δ*mdr2*, Δ*mdr3* and Δ*mdr5* (left panel). Separated chromosomes were hybridized using a 3′UTR *pbdhfr* probe that recognizes the DNA-construct integrated into the *mdr2, mdr3* and *mdr5* loci on chromosome 13, 9 and 11, respectively, and the endogenous *dhfr/ts* on chromosome 7. In addition in Δ*mdr2* and Δ*mdr5* it recognizes the GFP-luciferase reporter cassette in the *230p* locus on chromosome 3 of the parent line. Diagnostic PCR analysis showing correct integration of the gene targeting construct using primers (see Supporting Information Table S4 for the primer sequences) that amplify both the 5′ and 3′ side of the integration regions after targeting Pb*mdr2* both for deletion and *mCherry-3xMyc* tagging as well as Pb*mdr3* and Pb*mdr5* for deletion(right panel).
C. Northern analysis showing the presence of transcripts of all three *mdr* genes in blood stages of WT parasites. In blood stages of both the Δ*mdr3* and Δ*mdr5* transcripts are absent. In Δ*mdr2* a small transcript of 1.2kb is observed, which encodes for the first 85 amino acids of MDR2, while the full size transcript of 4.5kb is present in WT.
D. Diagnostic PCR using either Long Range (left panel) or intra-ORF (right panel) PCR amplification of genomic DNA from both wild type (NF54) and PfΔ*mdr3* lines confirming deletion of Pf*mdr3* using specific primers (Table S4), respectively. The Long Range PCR shows an increased product size in the PfΔ*mdr3* line resulting from integration of the selectable marker cassette. The intra-ORF PCR amplifies the expected fragment of 340 bp in WT, whereas this fragment is absent in the mutant PCR as a result of Pf*mdr3* gene excision.

stages (Fig. 1C). Also RNAseq analyses of synchronized *P. berghei* blood stages showed expression in asexual trophozoites/schizonts (Supporting Information Table S2). No transcript could be detected in PbΔ*mdr3* and PbΔ*mdr5* blood stage parasites (Fig. 1C). We did observe a small transcript of 1.2kb in PbΔ*mdr2* parasites, which encodes for the first 85 amino acids of MDR2, while the full size transcript of 4.5kb was present in WT parasites, which encodes for the protein of 948 amino acids (Fig. 1C).

For PbΔ*mdr5* parasites, asexual growth was significantly decreased resulting in a multiplication rate of 6.8 per 24 h (SD 0.8; $n = 5$), which is 10 per 24 h in WT (SD 0, $n = 10$) (Janse *et al.*, 2003). For PbΔ*mdr2* the multiplication rate of was comparable to WT (Table 1). For PbΔ*mdr3* we have only been able to select mutants in a single transfection experiment. Both clones from this experiment showed a strongly reduced growth rate of asexual blood stages (mean multiplication rate of 4.2x per 24 h (SD 0.6; $n = 2$). In all other transfection experiments ($n=9$) targeting Pb*mdr3* we were unable to select for parasites with a disrupted *mdr3* locus, probably due to the strong reduction in growth rate. Because of the failure to select an independent secondary mutant we decided to discontinue further analysis of the PbΔ*mdr3* phenotype.

Combined, these results show that PbMDR2, 3 and 5 are dispensable for blood stage development of *P. berghei*, although the lack of both MDR3 and MDR5 appears to affect the growth rate of blood stage parasites.

Dispensable roles P. falciparum MDR2, MDR3 and MDR5 for blood stage development

We have previously generated *P. falciparum* mutants lacking expression of MDR2 and MDR5 (van der Velden *et al.*, 2015). Generation of these mutants indicate that both MDR2 and MDR5 are dispensable for blood stages and confirms the observations in *P. berghei*. In this study we additionally targeted *mdr3* and *mdr6* for deletion with the pHHT-FRT-(GFP)-Pf*mdr3* and pHHT-FRT-(GFP)-Pf*mdr6* deletion constructs (Supporting Information Fig. S1) (Duraisingh *et al.*, 2002; Maier *et al.*, 2006). In agreement with our findings in *P. berghei*, we were unable to isolate *P. falciparum* parasite mutants lacking *mdr6* in three transfection experiments (data not shown), which indicates an essential role of the MDR6 protein for blood stage development. In contrast, we were able to readily select mutants lacking expression of MDR3. Correct deletion of Pf*mdr3* was validated using a diagnostic LR-PCR in which the expected larger amplification product (including selectable marker cassette) was obtained for parasites lacking this gene compared to WT (Fig. 1D). In addition, an intra-exonic PCR confirmed absence of the Pf*mdr3* gene in the mutant line (Fig. 1D).

Our ability to generate *P. falciparum mdr2*, *mdr3* and *mdr5* mutants indicates dispensable roles during blood stage development. We next analyzed the *in vitro* growth rate of *P. falciparum* blood stages over a period of 7 days using a modified pLDH method (Gamo *et al.*, 2010). In this assay, mutants lacking MDR2, MDR3 and MDR5 showed a normal growth rate, whereas the maximal parasitemia that was reached in cultures of PfΔ*mdr2* parasites was significantly lower at day 7 compared to WT parasites of the NF54 strain (Fig. 2). However, when we subsequently compared exponential growth of PfΔ*mdr2* to WT for an extended period the blood stage multiplication rate was not significantly different form wild type (Supporting Information Fig. S3).

MDR2 and MDR5 of P. berghei play a role during mosquito stage sporogony

We explored the phenotypes of PbΔ*mdr2* and PbΔ*mdr5* during sexual, mosquito and liver stages. The gametocyte and ookinete conversion rate resembled that of WT parasites (Table 1). Whereas oocysts were readily detected in mosquito midguts that were fed on mice infected with *P. berghei* parasites lacking expression of MDR2 or MDR5, the subsequent sporogonic

Table 1. Phenotypes of *P. berghei* mutants lacking expression of MDR proteins.

Lines	Asexual multiplication rate[a] (SD)	Gametocyte production[b] % (SD)	Ookinete production[c] % (SD)	Oocyst production day 12–13[d] (SD)	Oocyst production day 16–17[d] (SD)	Sporozoite production[e] $\times 10^3$ (SD)	Oocyst size day 12[f] µM (SD)	Oocyst size day 16[f] µM (SD)
WT								
1037m1f1m0c1 (a)	10 (0) *n*=10	15–25[f]	50–90[f]	375 (228)	266 (117)	35 (12.4)		
676m1cl1 (b)	10 (0) *n*=10	15–25[f]	50 90[f]	224 (134)	151 (242)	58.8 (7.8)	33 (3)	33 (6)
Mutants								
Δ*mdr2-a*	10 (0) *n*= 4	ND	ND	152 (89.0)	17.8 (20.9)	1.2 (0.5)		
Δ*mdr2-b*	10 (0) *n*= 2	16.9 (1.0)	80.7 (9.1)	145 (151)	20.6 (10.9)	3.5 (1.0)	19 (5)	27 (6)
Δ*mdr5-a*	6.8 (0.8) *n*=2	18.2 (1.4)	71.7 (6.5)	147 (102)	50.2 (29.1)	4.5 (1.3)		
Δ*mdr5-b*	6.7 (0.7) *n*=3	ND	ND	168 (169)	94.5 (26.0)	9.8 (2.9)	21 (2)	33 (3)

a. The multiplication rate per 24 hour of blood stage parasites in mice infected with a single parasite.
b. The percentage of blood stage parasites developing into gametocytes *in vivo*.
c. The percentage of female gametes developing into mature ookinetes *in vitro*.
d. The mean number of oocysts per mosquito.
e. The mean number of salivary gland sporozoites per mosquito (day 19–22).
f. The developmental data for wild type parasites are shown as the range of mean values of > 10 experiments.
ND, not determined.

development was significantly affected. The mean number of Pb*Δmdr2-b* and Pb*Δmdr5-b* GFP-positive oocysts was 48 ± 13 and 63 ± 16, respectively, between day 9 and 23 after mosquito feeding (Fig. 3A) and was significantly reduced compared to WT (128 ± 24; $p < 0.05$). Moreover, this effect was increased in time as at later time points (day 16–17) fewer oocysts could be detected for both lines when compared to earlier time points (day 12–13) (Table 1). Many of the MDR2 and MDR5 deficient oocysts appeared immature (Fig. 3E) and their average size was significantly smaller at day 12 compared to WT (Table 1). However, at day 16, mutant oocysts measured similar sizes as WT oocysts, suggesting that the lack of the MDR2 or MDR5 protein resulted in a delayed growth (Table 1). Maturation of oocysts could be specifically monitored in the 1037m1f1m0cl1 parent line. This reference line expresses the reporter fusion protein GFP-Luciferase under the control of the *ama-1* promoter. In mosquito stages, this promoter is only active in mature oocysts when sporozoites are formed. We therefore counted GFP-positive oocysts in mutant and WT-infected mosquitoes at day 17 after infection. Developmental retardation was evident and significant for the Pb*Δmdr2-a* and Pb*Δmdr5-a* gene deleted parasites as only 1.0% (SD 1.6%) and 23% (SD 11%) of the oocysts in the respective deletion mutants reached full maturation as shown by GFP expression, compared to 82% (SD 9.7%) in WT (Fig. 3F). This also resulted in strongly reduced numbers of sporozoites. Only 3.4–6.0% and 13–17% of WT sporozoite numbers could be isolated from salivary glands of Pb*Δmdr2* and Pb*Δmdr5* infected mosquitoes, respectively (Table 1; Fig. 3B).

These results indicate that both MDR2 and MDR5 play a role during sporogony. No published data is available on expression of these proteins in *P. berghei* oocysts or sporozoites, although evidence has been reported for transcription of MDR5 in sporozoites (Supporting Information Table S1). Moreover, *P. falciparum* MDR5 has been found in proteomes of sporozoites. MDR2 has only been detected in proteomes of *P. falciparum* asexual blood stages and gametocytes. We analysed expression of PbMDR2 in blood stages, oocysts and sporozoites of the mutant Pb*mdr2*::*mCherry* by fluorescence microscopy. Analysis of oocyst and sporozoite production of Pb*mdr2*::*mCherry* parasites show that both oocyst and sporozoite numbers of the Pb*mdr2*::*mCherry* parasites are not different from wild type parasites and in addition the infectivity of sporozoites is similar as wild type parasites as shown by similar prepatent periods after inoculation of isolated sporozoites (Supporting Information Fig. S4). These results indicate that the mCherry tag does not affect the function of MDR2 in these stages.

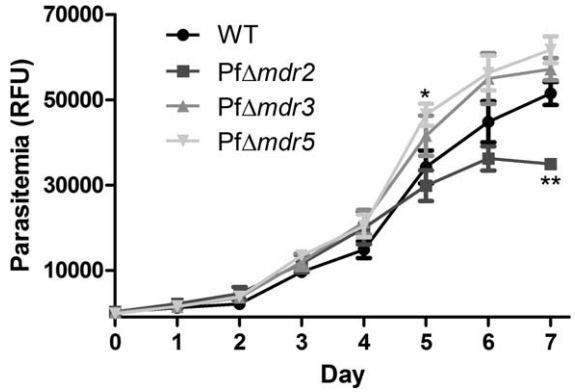


Fig. 2. Reduced maximal parasitemia in PfΔ*mdr2* parasites. Growth of asexual blood stages of WT and two clonal lines for PfΔ*mdr2*, PfΔ*mdr3* and PfΔ*mdr5* (data of two clones per mutant was pooled) was monitored over a 7-day period in the *in vitro* multiplication assay. *In vitro* parasite cultures were started at 0.1% parasitemia and growth was determined by measuring relative fluorescence units (RFU) in three independent experiments (triplicate samples per time point) using a modified pLDH method (Gamo *et al*., 2010). Asexual multiplication was comparable for PfΔ*mdr3* and PfΔ*mdr5*, however, maximal parasitemia of PfΔ*mdr2* was significantly reduced at day 7.

In both asexual blood stages and gametocytes of Pb*mdr2*::*mCherry* fluorescence signals were detected, which is in agreement with MDR2 detection in the *P. falciparum* proteomes of these stages. The fluorescence signals are mainly associated with hemozoin granules (in both asexual stages and gametocyte; Fig. 4A, Supporting Information Fig. S4C), which may suggest that this protein is located on the food vacuole membrane as *P. berghei* trophozoites and gametocytes have many small food vacuoles. In mature schizonts these food vacuoles merge into one or two large vesicles containing hemozoin, and it is in these vesicles that we also observe fluorescent signal; Fig. 4A). In addition, we found fluorescence signals associated with the plasma lemma of blood stages, which is especially clear in merozoites of mature schizonts.

We also observed fluorescence signals during oocyst development (Fig. 4B). From day 10 onwards, before sporozoite formation, we observe a highly structured fluorescence pattern within the oocyst possibly associated with early sporoblast formation (Meszoely *et al*., 1989). In more mature oocysts, when sporozoite formation is observed, the fluorescence signal becomes more diffuse but it is only associated with the areas within the oocyst where sporozoites are present and are budding from the sporoblasts.

Infectivity of PbΔ*mdr2* or PbΔ*mdr5* salivary gland sporozoites was tested by intravenous injection of 10,000 parasites into mice. All mice (n=3) developed blood stage infection after a prepatent period of 5–6 days which is comparable to the prepatent period of WT sporozoites (Annoura *et al*., 2012).

In conclusion, PbMDR2 and PbMDR5 play an important role in both oocyst formation and maturation as well as sporozoite development. In the mutants lacking these proteins, lower total numbers of oocysts were observed, and in addition to a lower percentage of fully mature oocysts, this resulted in strongly reduced sporozoite formation. No evidence was found that sporozoites that were produced in parasites lacking MDR2 or MDR5 expression had a lower infectivity to mice.

P. falciparum MDR2 and MDR5 play a role during mosquito stage sporogony

Oocyst and sporozoite formation was also analyzed in the *P. falciparum* mutants PfΔ*mdr2*, PfΔ*mdr3* and PfΔ*mdr5*. Gametocytes of these parasites were fed to *Anopheles stephensi* mosquitoes using a standard membrane feeding assay (Ponnudurai *et al*., 1989). First, oocysts in mosquito midguts were quantified 7 days post infection in 1 to 7 independent feeding experiments. Oocyst production in PfΔ*mdr3*-infected mosquitoes (50 ± 6 oocysts; n=1, 20 mosquitoes) was not significantly different ($p > 0.05$) from wild type infected mosquitoes (75 ± 7 oocysts; n=7, 120 mosquitoes). This is in contrast with PbΔ*mdr2* and PbΔ*mdr5* infected mosquitoes, where the number of oocysts was significantly reduced (both $p < 0.0001$) to only 18 ± 2 oocysts (n=6, 120 mosquitoes) and 11 ± 2 oocysts (n=4, 60 mosquitoes), respectively (Fig. 3C). Sporozoite formation was analyzed by determining the mean in 10 mosquitoes per experiment by salivary gland dissection at 14–16 or 18 days post infection. In infected mosquitoes, the number of salivary gland sporozoites was significantly reduced to only 17.2% ($p < 0.05$) for PfΔ*mdr2* (n=3) and borderline significant ($p = 0.05$) to 30.3% for PfΔ*mdr5* (n=3) compared to NF54 WT (n=6) (Fig. 3D). In contrast, mosquitoes fed on PfΔ*mdr3* (n=3) had wild type levels of salivary gland sporozoites (Fig. 3D).

Discussion

In this study, we analyzed the role of seven MDR proteins during the life cycle of *P. berghei* and *P. falciparum* malaria parasites using reverse genetic methods. As a first screen, all seven genes were targeted for deletion in the *P. berghei* rodent model since for this parasite highly efficient and standardized methods exist for deletion of genes by double cross-over homologous constructs using linear DNA constructs. Despite multiple attempts to delete the *mdr1*, *mdr4*, *mdr6* and *mdr7* genes from the *P. berghei* genome, we could not select

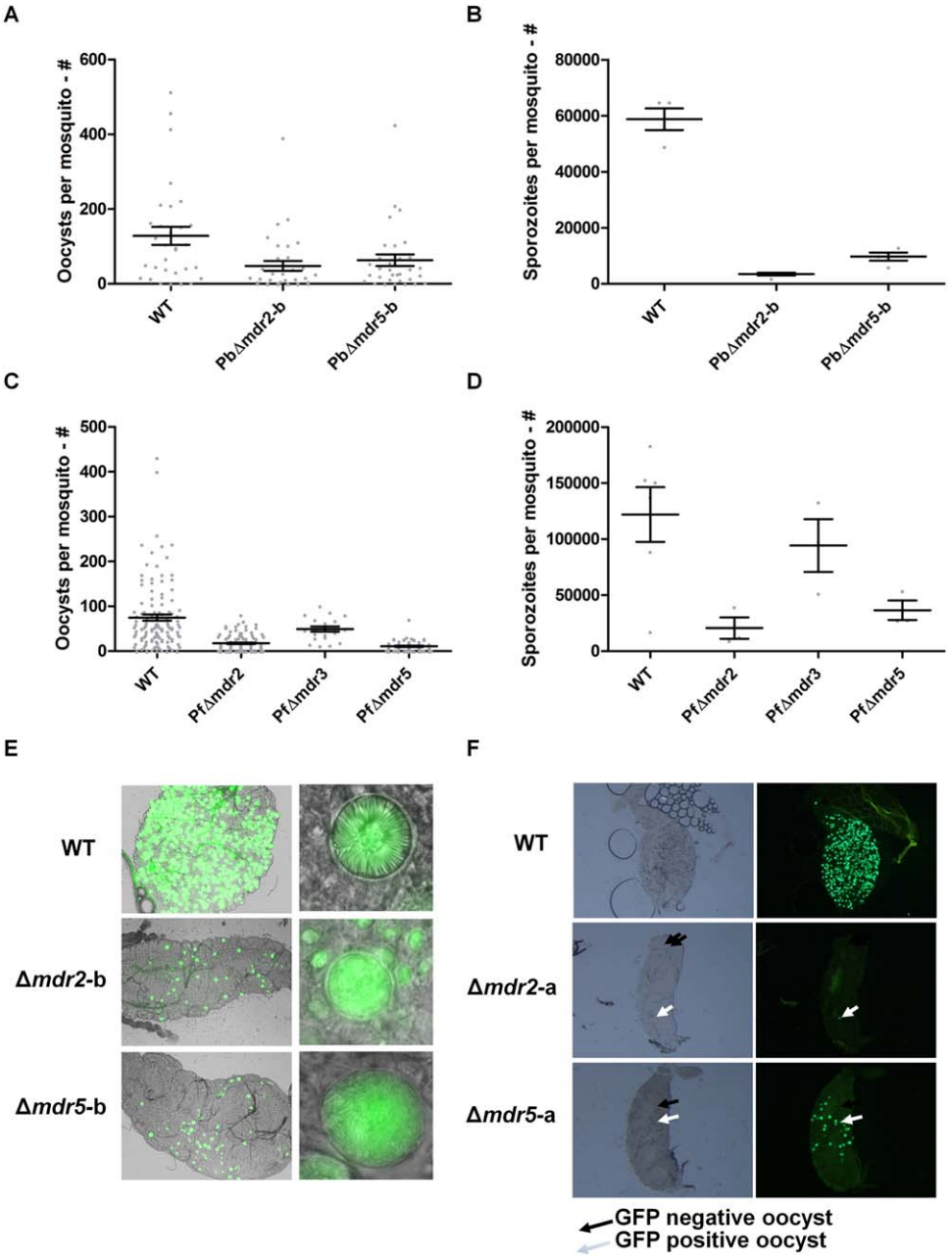


Fig. 3. Altered mosquito stage development of mutants lacking expression of MDR2 and MDR5.
A. Oocyst production in *A. stephensi* mosquitoes of *P. berghei* parasites lacking MDR2 and MDR5 is significantly reduced ($p < 0.05$).
B. Sporozoite production in *A. stephensi* mosquitoes of *P. berghei* parasites lacking MDR2 and MDR5 is significantly reduced ($p < 0.0001$).
C. Following *P. falciparum* standard membrane feeding assays using female *A. stephensi* mosquitoes, oocysts were counted at day 7 post infection in wild type NF54, PfΔ*mdr2*, PfΔ*mdr3* and PfΔ*mdr5* parasites. Oocyst production of parasites lacking MDR2 and MDR5 expression was significantly reduced ($p < 0.0001$), however, oocyst production of PfΔ*mdr3* was unaffected ($p > 0.05$).
D. The number of sporozoites that could be isolated from the salivary glands of the infected mosquitoes was decreased for PfΔ*mdr2* ($p < 0.05$) and PfΔ*mdr5* ($p = 0.05$), but not for PfΔmdr3 ($p > 0.05$) parasites isolated at day 14–16 or 18 post infection. Each data point represents the average number of sporozoites from 10 dissected mosquitoes in an experiment.
E. Oocyst number and maturity was substantially decreased at day 16 in *P. berghei* gene deletion lines compared to WT, as shown by GFP positivity in whole midguts and sporogony per oocyst in phase-contrast microscopy.
F. Strongly decreased formation of mature oocysts in PbΔ*mdr2* and PbΔ*mdr5* as visualized by mature GFP-expressing oocyst. Both WT and mutant parasites express GFP under control of the *ama1* promotor that is only active in mature oocysts that undergo sporogony.

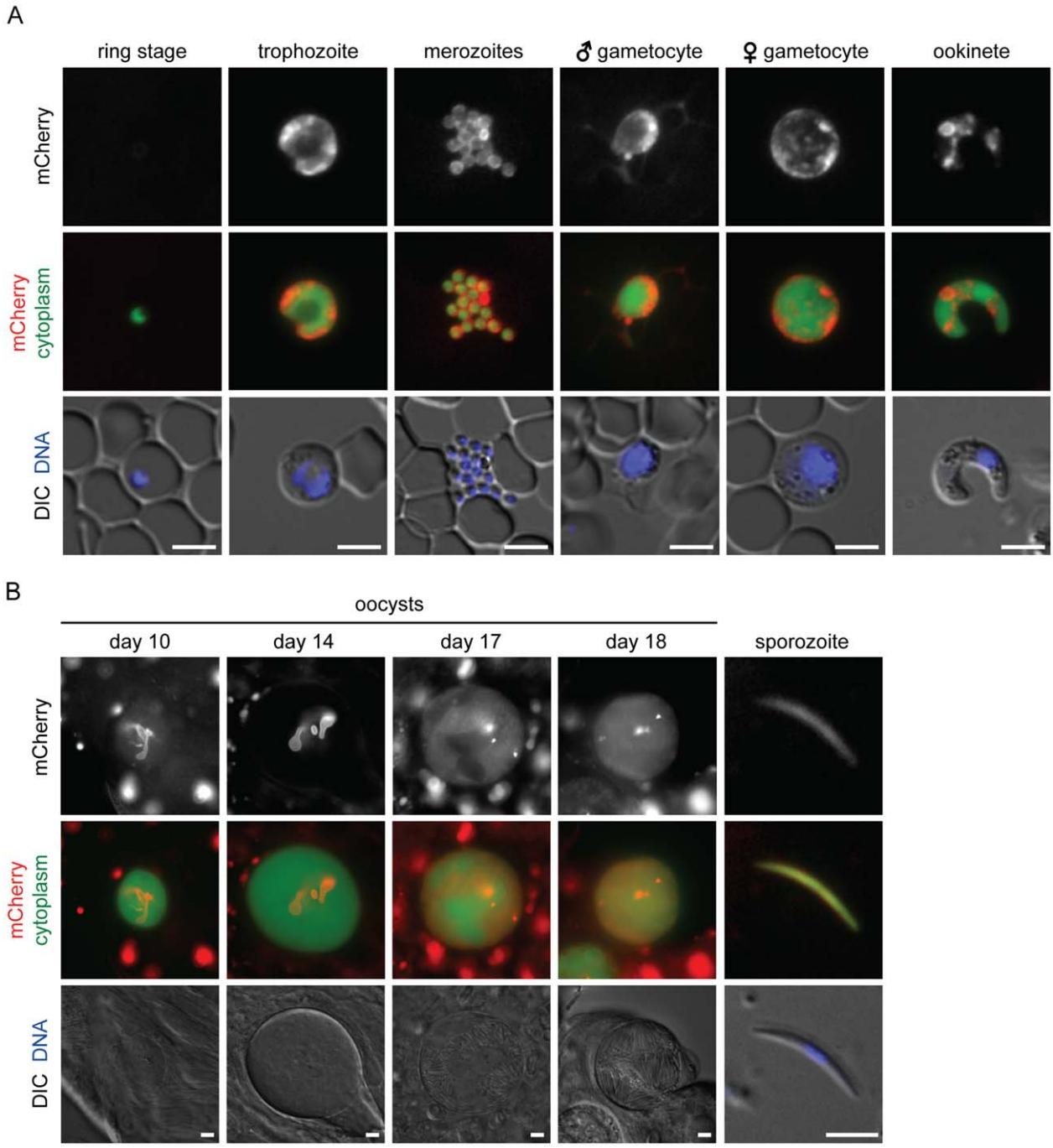


Fig. 4. Localization of PbMDR2::mCherry
Expression of MDR2::mCherry during *P. berghei* blood (A) and mosquito (B) stage development. Upper row represents mCherry expression, middle row is combined expression of cytoplasmic GFP (green) and MDR2::mCherry (red), lower row shows phase-contrast and nuclear Hoechst33342 (blue) staining.
A. Shown are parasites during ring, trophozoite, merozoite and male as well as female gametocyte stages and ookinetes.
B. Shown are oocysts 10, 14, 17 and 18 days after the mosquito bloodmeal and a salivary gland-associated sporozoite.

gene deletion mutant parasites. These results suggest that these genes are essential for blood stage multiplication. In addition, the unsuccessful attempts to delete the genes encoding for MDR1 (Sanchez *et al.*, 2010) and MDR6 (this study) in *P. falciparum* supports our findings in *P. berghei* that these proteins are essential for blood stage development of malaria parasites. We therefore focused on the function of the *mdr2*, *mdr3* and *mdr5*

genes that could be deleted from the *P. berghei* genome and generated equivalent mutants in *P. falciparum*.

Mdr2, *mdr3* and *mdr5* were successfully deleted in *P. berghei* and *P. falciparum*. In *P. berghei*, data from Northern analysis (this article) and RNAseq analysis (Otto *et al*., 2014) indicated that these three genes are transcribed in blood stages, suggesting that they play a role during blood stage development. Also by analyzing a mutant expressing a mCherry-tagged version of MDR2 we found expression in asexual blood stages and in gametocytes. The fluorescence signals were associated both with hemozoin crystals and with the surface of parasites suggesting a location in both the membrane of food vacuoles surrounding the hemozoin crystals and in the plasma lemma membrane. Although the ability to delete these genes may indicate that other proteins compensate for the loss MDR transporter function, the reduced growth we observed for *P. berghei* blood stages lacking expression of MDR3 and MDR5 does suggest a role of these proteins in these stages. The lack of a growth phenotype in blood stages of the *P. berghei* mutant lacking MDR2 suggests either the absence of a function in blood stages or complete compensation of its role by other proteins. Also for the *P. falciparum* genes evidence is present for blood stage expression, both at the transcript and protein level (Supporting Information Table S2). However, and in contrast to the *P. berghei* results, we did not find evidence for a decreased growth rate for the Pf*mdr3* and Pf*mdr5* gene deletion mutants. Nevertheless, the maximal parasitemia that was reached in cultures of PfΔ*mdr2* parasites was significantly lower at day 7 compared to WT parasites, although monitoring exponential growth over a 12-day period did not show any defect in the multiplication of blood stages. Combined, this may indicate that the function of the orthologous MDR proteins differ between blood stages of *P. berghei* and *P. falciparum* or that in *P. falciparum* blood stages the loss of the MDR3 and MDR5 proteins can be completely compensated by other proteins, whereas in *P. berghei* blood stages only the function of MDR2 can be fully compensated by (an)other protein(s). However, these discrepancies may also be due to different assays used for analysis of blood stage growth. For *P. berghei*, blood stage growth was determined *in vivo* whereas *P. falciparum* growth was analyzed under *in vitro* conditions. Although here we describe Pf*mdr2* as a dispensable gene *in vitro*, the T484I polymorphism is identified as a genetic background marker for *kelch13* mutations (Miotto *et al*., 2015), highlighting the possible importance of this gene for parasite survival within artemisinin-treated hosts.

Further analysis of the mutants lacking expression of MDR2 and MDR5 demonstrated that these proteins play a role in the formation of oocysts and sporozoites in both *P. berghei* and *P. falciparum*. Whereas *P. falciparum* MDR5 expression has been shown previously by analysing proteomes of sporozoites (Supporting Information Table S2), MDR2 expression had not been reported in mosquito stages. MDR2 had only been detected in proteomes of *P. falciparum* asexual blood stages and gametocytes. By analyzing the Pb*mdr2*::*mCherry* mutant, we show here for the first time expression of MDR2 in oocysts and in sporozoites. Expression in these stages is in agreement with our observations of reduced sporogony in mutants lacking expression of MDR2. Absence of these proteins results in strongly reduced oocyst and sporozoite (except for PfΔ*mdr5*) formation compared to WT parasites. Whereas in *P. berghei* we found a more pronounced reduction of PbΔ*mdr2* compared to PbΔ*mdr5* oocyst numbers, in *P. falciparum* the effect was more severe in mutants lacking MDR5 expression. The effect of MDR2 or MDR5 absence on *P. berghei* maturation was most clearly visualized in the parasite mutants where only fully mature oocysts express GFP, as a strong and significant reduction in GFP-positive oocysts was observed compared to the parent line. In both *P. berghei* and *P. falciparum*, the absence of MDR2 and MDR5 did not result in a complete block of sporozoite formation. We demonstrated that *P. berghei* sporozoites lacking either MDR2 or MDR5 were infectious to mice, resulting in blood stage infections with prepatent periods that were comparable to WT sporozoites. These observations suggest that these proteins have no function during sporozoite invasion of hepatocytes or during subsequent liver stage development.

ABC transport proteins play an important role in maintaining homeostasis in many organisms, and could be of special importance for parasites interacting with their host cell environment from which they require essential nutrients and where they dispose toxic waste products. Identification of the vital role of these transporters in different stages of the parasite life cycle may reveal novel drug targets for inhibition of parasite development and transmission prevention. In this study, we present evidence that *P. berghei mdr1*, *4*, *6*, *7* and *P. falciparum mdr6* are likely to be essential for asexual multiplication, highlighting the potential of these transporters or their substrates as drug targets for treating blood stage malaria. Furthermore, *mdr2* and *5* play a significant role in mosquito-stage development in both species. Further unraveling their involvement in physiological pathways could lead to novel strategies to target parasite transmission, and the determination of substrate specificity of these transporters is therefore an essential next step.

Experimental procedures

Experimental animals and P. berghei ANKA reference lines

Animal experiments were conducted in female C57BL/6, BALB/c and Swiss OF1 mice (6–8 weeks; Charles River) and approved by the Animal Experiments Committee of the Leiden University Medical Center (DEC; 12042; 12111), or in C57BL/6 mice as approved by the ethics committee of the Berlin state authority (Landesamt für Gesundheit und Soziales Berlin, permit number G0469/09. Both the Dutch Experiments on Animal Act and the German 'Tierschutzgesetz in der Fassung vom 22. Juli 2009' are established under European guidelines (EU directive no. 86/609/EEC regarding the Protection of Animals used for Experimental and Other Scientific Purposes).

Two different *P. berghei* ANKA parasite lines were used to generate gene-deletion mutants: 676m1cl1 (PbGFP-LUC_{con}; mutant RMgm-29; www.pberghei.eu) and 1037cl1 (PbGFP-Luc_{schiz}; mutant RMgm-32; www.pberghei.eu), originating from the cl15cy1 wild type (WT) (Janse *et al.*, 2006b) which was used to generate the Pb*mdr2*::*mCherry* tagged parasites. In both reporter lines, a *gfp-luc* expression cassette is stably integrated into the *Pb230p* locus in absence of a drug-selectable marker (Janse *et al.*, 2006a, Spaccapelo *et al.*, 2010). These lines thus express the GFP-Luciferase fusion protein under the control of either the constitutively active *eef1α* promoter (676m1cl1) or the schizont-specific *ama1* promoter (1037cl1).

Generation of P. berghei ABC transporter gene deletion and tagged mutants

Genes encoding ABC transporters were deleted using a double cross-over strategy based on homologous recombination of targeting constructs into the genome of the parasite. Targeting sequences were PCR amplified from *P. berghei* ANKA (cl15cy1) genomic DNA using primers specific for the 5′UTR or 3′UTR regions of the different ABC transporter genes (Supporting Information Table S3). These targeting regions (TR) were cloned into the pL0001 plasmid (www.mr4.com), flanking the pyrimethamine resistant *Toxoplasma gondii* (*Tg*) dihydrofolate reductase-thymidylate synthase (*dhfr/ts*) as a selectable marker (SM) under control of the *P. berghei dhfr/ts* promoter. Tagging of *mdr4* was performed using a similar double crossover recombination strategy as described above. A DNA construct containing an 3xHA tag was used that target *mdr4*. This construct was obtained from the Sanger Institute (PbGEM-084058; http://plasmogem.sanger.ac.uk/designs/final_vector/84058; named in this study plasmid pL1995) (Schwach *et al.*, 2015). This construct aims at integration at the 3′end of *mdr4*, replacing the stop codon with an HA tag and contains the pyrimethamine resistant human dihydrofolate reductase-thymidylate synthase (*hdhfr*). Transfection and pyrimethamine selection of mutant parasites with was performed as described for generation of the gene deletion mutants.

For the Pb*mdr2*::*mCherry* tagging construct, fragments at the 3′ region and the carboxy-terminal end of the coding sequence were amplified using specific primers (Supporting Information Table S3). These fragments were cloned into the pBAT-SIL6 vector (Kooij *et al.*, 2012) using the indicated restriction enzymes (Supporting Information Table S3). The carboxy-terminus was cloned in frame with the *mCherry-3xMyc* tag. Gene-deletion and tagging constructs were verified by Sanger sequencing and linearized with the appropriate restriction enzymes (Supporting Information Table S3). Transfection and selection of transformed parasites with pyrimethamine was performed using the high efficiency transfection and selection technology for genetic modification of *P. berghei* (Janse *et al.*, 2006b). Clonal lines of all gene-deletion mutants were generated through limiting dilution of the parasites in mice. Deletion of the targeted genes or *mdr2* tagging by correct integration of the DNA constructs was verified by diagnostic PCR analysis covering the 5′TR and 3′TR integration using gene-specific primers (Supporting Information Table S4) and Southern analysis of pulsed-field gel electrophoresis separated chromosomes that were hybridized with a 3′*pbdhfr/ts* probe (FIGE; Supporting Information Table S4).

Transcription of the *mdr* genes was determined by Northern analysis of RNA obtained from WT and gene-deletion mutant blood stages of asynchronous *in vivo* infections or from synchronous blood infections of WT parasites (Janse and Waters, 1995). Northern blots were hybridized with a 5′-UTR fragment of the *mdr* genes, PCR amplified from genomic WT DNA using the appropriate primer pairs (Supporting Information Table S3) and with the *a/b-large subunit rRNA* probe as control (primer 644) (Mu *et al.*, 2003).

In vivo multiplication rate of asexual P. berghei blood stage parasites

During the cloning procedure of the gene-deletion mutants, the multiplication rate of asexual blood stage parasites in mice was determined as described before (Spaccapelo *et al.*, 2010). Parasitemias in percentages in Swiss OF1 mice injected with a single parasite are determined at day 8 to 11 in Giemsa-stained blood films. Per mouse, an estimated number of 1.2×10^{10} erythrocytes is used to calculate the 24-h multiplication rate. The percentage of infected erythrocytes in mice infected with reference lines of the *P. berghei* ANKA strain consistently ranges between 0.5% and 2% at day 8 post infection, resulting in a mean multiplication rate of 10 per 24 h (Janse *et al.*, 2003).

In vivo P. berghei gametocyte production and in vitro ookinete production

The gametocyte conversion rate was determined as the percentage of ring forms that develop into mature gametocytes in synchronized infections in mice that are pre-treated with phenylhydrazine-HCl (Janse and Waters, 1995). Ookinete production was analyzed by standard *in vitro* fertilization and ookinete maturation assays (van Dijk *et al.*, 2001). Gametocytes for these assays were obtained from infected mice that had been pre-treated with phenylhydrazine-HCl to increase gametocyte numbers. The ookinete conversion rate is defined by the percentage of female gametes that

develop into mature ookinetes under standardized *in vitro* culture conditions for activation of gametocytes, fertilization and ookinete maturation. The percentage of females that developed into ookinetes was determined by counting female gametes and mature ookinetes in Giemsa-stained blood smears, made at 16–18 h post activation.

P. berghei oocyst and sporozoite production in Anopheles stephensi mosquitoes

For mosquito transmission experiments, female *A. stephensi* mosquitoes were fed on mice infected with WT parasites or gene-deletion mutants. Oocyst development, oocyst production and sporozoite production was monitored in infected mosquitoes as described (Billker *et al*., 1997). Oocyst numbers were counted and measured in midguts of infected mosquitoes at 9, 12, 14, 16, 19, 21 and 23 days post infection, while sporozoite numbers were counted at day 21–22. Salivary gland sporozoites were isolated and counted as described (Annoura *et al*., 2012). Mean differential oocyst (± SEM) and sporozoite numbers between WT and mutant lines were determined using a one-way ANOVA with Dunnett's post-test, or two-tailed students' *t*-test for mCherry tagged PbMDR2 parasites.

Localization of HA-tagged PbMDR4 and mCherry-tagged PbMDR2

Detection of the HA-tagged PbMDR4 protein was performed after fixing asexual parasites with 4% paraformaldehyde (PFA), quenching with 0.1 M glycine, blocking with 10% FCS and permeabilization with 1% Triton-X-100. Localization of was performed using specific antibodies against HA raised in rat (Roche, 1:1000), visualized with anti-rat ALEXA488 (green). Nuclei were stained with DAPI (red).

Live protein localization of the PbMDR2::mCherry tagged protein was performed only minutes after sample collection and Hoechst 33342 DNA staining, using either conventional slides and coverslips or concanavalin A-coated ibidi μ-Dishes (35 mm, low; Grid500) with pre-warmed RPMI 1640 medium containing 20% fetal calf serum. Images for live protein localization were recorded on a Zeiss AxioObserver Z1 epifluorescence microscope, equipped with a Zeiss AxioCam MRm camera and processed minimally with FIJI (Schindelin *et al*., 2012).

P. berghei sporozoite infectivity and liver stage development

P. berghei sporozoites were collected at day 21–22 post infection by hand-dissection of the salivary glands. Salivary glands were collected in DMEM (Dulbecco's Modified Eagle Medium from GIBCO) and homogenized in a home-made glass grinder. The number of sporozoites was determined by counting these in 10 salivary glands in duplicate in a Bürker-Türk counting chamber using phase-contrast microscopy.

To determine *in vivo* infectivity of sporozoites, Swiss OF1 mice were infected with 10^4 salivary gland sporozoites by intravenous injection, as previously described (Billker *et al*., 1997). Blood stage infections were monitored by analysis of Giemsa-stained thin smears of tail blood collected on day 4–8 after inoculation of sporozoites. The prepatent phase (measured in days post sporozoite infection) ends at the day that blood stage infection with 0.5–2% parasitemia is observed.

P. falciparum reference lines and culture conditions

Plasmodium falciparum wild type (NF54) and mutant parasites were maintained in a semi-automated culture system and gametocyte formation was induced as reported previously (Ifediba and Vanderberg, 1981; Ponnudurai *et al*., 1982; Ponnudurai *et al*., 1989). Briefly, *in vitro* parasites were grown in RPMI medium supplemented with human serum (complete medium) and 5% hematocrit. Medium was changed twice daily and fresh human red blood cells were obtained weekly from the Dutch national blood bank (Sanquin).

Generation and genotyping of P. falciparum mdr2, mdr3 and mdr5 gene deletion mutants

For phenotype analysis, we used the previously generated *P. falciparum mdr2* and *mdr5* gene deletion lines lacking the h*dhfr::gfp* selectable marker (van der Velden *et al*., 2015). In addition, for the parasite development within mosquitoes, we used two independently generated additional Pf*Δmdr2* and Pf*Δmdr5* clones in which the selectable marker was not removed. For deletion of the *P. falciparum mdr3* and *mdr6* genes, we followed a homologous double crossover strategy as described (Duraisingh *et al*., 2002; Maier *et al*., 2006). The deletion construct was made by replacing the Pf*52* homologous regions in the pHHT-FRT-(GFP)-Pf52 construct (kindly provided by Ben van Schaijk) with *mdr3* and *mdr6* target regions (TR) (Supporting Information Fig. S1) (van Schaijk et al., 2010). These regions were amplified from *P. falciparum* NF54 genomic DNA (gDNA) using *PfuUltra* II Fusion HS DNA Polymerase (Agilent Technologies) with primer pairs for the 5′ and 3′ target regions of *mdr3*, respectively (Supporting Information Table S3), and a similar approach was applied to amplify the *mdr6* target regions. The 5′ and 3′ target regions were cloned into the pHHT-FRT-(GFP)-Pf52 construct using *BssHII* plus *BsiWI* and *XmaI* plus *NheI* restriction enzymes after TOPO TA subcloning (Invitrogen) and sequence validation. This resulted in the deletion constructs pHHT-FRT-(GFP)-Pf*mdr3* and pHHT-FRT-(GFP)-Pf*mdr6*. Transfection and selection procedures we performed as described previously (Duraisingh *et al*., 2002; Maier *et al*., 2006), and clonal lines were obtained by limiting dilution.

Genotyping of the Pf*Δmdr3* mutant was performed by diagnostic PCR using Expand Long Range dNTPack (Roche) PCR (LR-PCR). Mixed asexual blood stage gDNA from NF54 wild type (WT) and Pf*Δmdr3* was isolated using the QIAamp DNA Blood Mini Kit (Qiagen). Primers flanking the 5′ and 3′ TR of *mdr3* were used to amplify WT and

mutant DNA using LR-PCR to validate correct double homologous crossover integration (Supporting Information Table S4). The LR-PCR was performed as described previously (van der Velden *et al.*, 2015), with an annealing temperature of 43.5°C. An additional diagnostic PCR was performed as reported (van der Velden *et al.*, 2015) on gDNA using primers designed within the open reading frame (ORF) of *mdr3* (Supporting Information Table S4).

P. falciparum in vitro *multiplication rate of asexual blood stages*

Asexual growth of *P. falciparum* WT and two independent clones from each of the mutant lines PfΔ*mdr2*, PfΔ*mdr3* and PfΔ*mdr5* was monitored in three consecutive experiments during 7 days. Briefly, parasites were inoculated in triplicate at 0.1% parasitemia in complete medium (at 2.5% hematocrit) and were incubated in 96-wells plates at 37°C under candle jar culture conditions (Jensen and Trager, 1977). After sedimentation of RBCs, complete medium was refreshed daily. Exponential growth of WT and a representative PfΔ*mdr2* clone was also monitored in three consecutive experiments during 12 days. Briefly, parasites were inoculated in triplicate at 0.5% parasitemia in complete medium (at 4% hematocrit) and were incubated in 96-wells plates at 37°C under candle jar culture conditions. On odd days, complete medium was refreshed after sedimentation of RBCs, while on even days, parasite cultures were diluted with fresh red blood cells (1:5). Each day, (a subset of) iRBC samples were resuspended and transferred to black-side clear-bottom 96-wells cell culture plates (Greiner Bio-One) and frozen at −20°C until readout using a modified *Plasmodium* lactate dehydrogenase (pLDH) method (McNamara *et al.*, 2013). In this assay, pLDH activity was determined by adding 70 μL fresh reaction mixture (286 mM 3-acetyl pyridine adenine dinucleotide (Sigma-Aldrich), 5.66 U/mL diaphorase (Worthington), 357.5 μM resazurin (Sigma-Aldrich), 286 mM sodium L-lactate (Sigma-Aldrich), 20 mM Tris-HCl pH 8.0) to all plates of three independent experiments. After incubation in the dark for 30–60 min following excitation at 530 nm, absorbance was measured at 590 nm using a Synergy 2 Multi-Mode Microplate Reader (Bio-Tek). Uninfected red blood cells served as background and growth rate was measured in relative fluorescent units (RFU) and plotted using GraphPad Prism version 5.03 (GraphPad Software). Difference in growth rate of all mutant lines (two independently transfected clones per mutant line were pooled, except in the case of exponential growth comparison where a single PfΔ*mdr2* clone was used) was compared to WT values at all time points using 2-way ANOVA with Bonferroni's post-test.

P. falciparum oocyst and sporozoite production in Anopheles stephensi *mosquitoes*

To study parasite mosquito development of the PfΔ*mdr2*, PfΔ*mdr3* and PfΔ*mdr5* mutant lines, standard membrane feeding assays using female *A. stephensi* mosquitoes were performed as described (Ponnudurai *et al.*, 1987; Ponnudurai *et al.*, 1989). Midgut oocysts were counted at day 7 post infection in wild type NF54, PfΔ*mdr2*, PfΔ*mdr3* and PfΔ*mdr5* parasites from 7, 6, 1 and 4 independent experiments containing a total of 120, 120, 20 and 60 mosquitoes, respectively. Salivary gland sporozoites were determined at day 14–16 or 18 post infection in 10 mosquitoes per experiment (of which the means are represented in the graph) in 6, 3, 3 and 3 independent assays for NF54, PfΔ*mdr2*, PfΔ*mdr3* and PfΔ*mdr5*, respectively. For PfΔ*mdr2* and PfΔ*mdr5*, two independent clones were used and the resulting oocyst and sporozoite data was plotted using GraphPad Prism version 5.03 (GraphPad Software). We only included experiments in which at least 70% of the mosquitoes were infected. Mean differential oocyst and sporozoite numbers (± SEM) between WT and mutant lines were determined using a one-way ANOVA with Dunnett's post-test.

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Supporting information

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