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CHAPTER 6

Generation of Growth- and Virulence-Attenuated Blood-stage Malaria Parasites

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Abstract

Immunization with killed or attenuated *Plasmodium* blood-stage parasites, or with live parasites under curative chemotherapy, can induce protective immunity against a malaria infection. Such infection-based immunization is being pursued not only to characterize potential live-attenuated blood-stage vaccines, but also to identify the critical host and pathogen components involved in development of protective immunity and pathology. We targeted 41 *Plasmodium berghei* genes for disruption in order to generate genetically modified blood stage parasites (GAP_{BS}) that are growth- and virulence- attenuated and that may serve as immunogens and as tools to study protective immunity. Using mutants generated in this and in previous studies, we examined their infection and virulence characteristics by assessing experimental cerebral malaria (ECM) in C57BL/6 mice and the development of hyper-parasitemia in BALB/c mice. Blood stage infections of 9 mutants showed significant reduction in *in vivo* growth rates. Seven of these 9 growth-attenuated mutants did not induce ECM in C57BL/6 mice. Two single-gene deletion mutants, lacking expression of either aminopeptidase P or leucyl aminopeptidase and a double gene-deletion mutant that lacks expression of both plasmepsin-4 and berghepain-2, did not induce hyper-parasitemia in the majority of BALB/c mice. These mice resolved the infection and the convalescent mice were protected against infections with wild type parasites.

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Introduction

Licensed human vaccines available today principally belong to three categories – live attenuated microbes (e.g. measles, mumps), killed/inactivated microbes (e.g. Polio, rabies) or protein subunit/conjugate (e.g. Hepatitis B, HPV) (http://www.cdc.gov/vaccines/). A large number of subunit-vaccine candidates against malaria parasites, *Plasmodium*, have been tested in animal models and humans, mainly as a protein (antigen) formulation, or expressed by a (DNA or viral) vector system in order to generate protective immunity [1]. Most malaria antigens that have been selected as subunit-vaccine candidates have been characterized as targets of natural immunity, most often associated with strong antibody responses [2]. However, the most advanced leading subunit pre-erythrocytic vaccine candidate RTS,S showed only limited efficacy as in Phase 3 testing with clinical malaria episodes in children being reduced by only 30–50% [3,4]. Clinical trials of erythrocytic (blood stage) subunit-vaccines have also shown modest protection; the testing of more than 10 candidate subunit vaccines targeting *Plasmodium* blood stages have not progressed to or further than Phase 2 trials, with only three candidates having reached Phase 2b trials [5]. The limited success with subunit-vaccine development has renewed interest in developing vaccines consisting of whole, killed or attenuated parasites [6]. While sustained and sterile immunity has been achieved using live *Plasmodium* liver stage parasites attenuated by radiation or genetic modification or administered under curative doses of chemoprophylaxis [7–9], full protective immunity to malaria with either killed sporozoites or killed blood stage parasites have so far been unsuccessful [7,10].

Whole *Plasmodium* blood-stage formulations used in immunization studies usually consist of infected red blood cells (iRBC). These formulations have included killed parasites in adjuvant, radiation-attenuated iRBC, or infection with wild-type iRBC administered under curative doses of chemotherapy and these have been used to immunize both rodents and primate models of malaria [6,10–12]. The results of these immunizations, while varied in their protective efficacies for the different combinations, have demonstrated protective immunity including complete protection against a challenge with wild type parasites (for a review see [10]). Furthermore, in a small immunization study of humans, evidence was found for the generation of complete protective immunity against *P. falciparum* that was achieved through repeated inoculations of very low numbers of iRBC (~30) resulting in sub-patent infections that were controlled using curative dose of chemotherapy [13]. These studies were remarkable in that they showed that not only immunization with whole blood stages can induce complete protective immunity in humans, but also that protective immunity could be achieved using only limited amounts of parasite material and in the absence of a major antibody response [6].

Currently practical limitations exist for immunization strategies that require humans be infected with parasites inside red blood cells, either killed or attenuated, for example it is unclear if regulatory authorities would approve, as part of a mass vaccination program, the intravenous administration of infected red blood cells to humans [2,6]. Nonetheless, such immunization studies can provide important insights into how protective immune responses can be induced and maintained against *Plasmodium* blood stages [14,15]. Similar to immunization studies using genetically attenuated parasites that arrest in the liver (GAP_{LS}) [8], studies into blood-stage immunization would clearly benefit from creating genetically attenuated blood stage parasites (GAP_{BS}) in animal models that induce limited, self-resolving infections that are virulence-attenuated and that can provoke strong and long-lasting immunity without the induction of malarial symptoms or additional pathologies. Such parasites can be instrumental tools to uncover important correlates of protection, to both better understand how iRBC are detected and eliminated by the host immune response, and also to identify correlates of disease.

A number of gene-deletion mutants generated in both rodent and human parasites have been reported to exhibit moderate to severe reduction in their blood-stage multiplication rates. However, the first growth- and virulence-attenuated GAP_{BS} was only recently reported for the rodent model malaria parasite *P. yoelii* YM (a lethal strain) [16]. This GAP_{BS}, which lacks the gene encoding purine nucleoside phosphorylase (PNP), is virulence-attenuated and produces a self-resolving infection in mice. Importantly, after a single infection with this parasite, all convalescent mice were protected against subsequent wild-type parasite challenge for prolonged periods (>5 months) [16]. Since then, other rodent malaria GAP_{BS} have been also reported, which show growth- and virulence-attenuation and induce self-resolving infections after which mice are protected against wild type challenge. This includes the GAP_{BS} that lacks genes encoding for nucleotide transporter 1 (NT1) [17] which was based on a study performed in *P. falciparum*, where an equivalent gene-deletion created parasites that grow only when purines are provided at supra-physiological concentrations to the culture medium and has been proposed as a potential *P. falciparum* GAP_{BS} candidate [18]. Others GAP_{BS} characterized in the rodent system include GAP_{BS} lacking expression of rhomboid 1 [19], plasmepsin-4 (PM4) [20], and a GAP_{BS} that lacks both PM4 and MSP7, a merozoite-specific protein [21]. The GAP_{BS} that have been created in *P. berghei* ANKA do not cause experimental cerebral malaria (ECM) in ECM-susceptible mice as wild type parasite do. These studies show that not only is it possible to generate growth- and virulence-attenuated blood stages parasites by targeting specific genes in the parasite genome, but also that strong and long-lasting protective immune responses can be induced in mice that have resolved their infections. However, despite growth- and virulence-attenuation, most of the reported GAP_{BS} still

produce infections with relatively high parasitemias (parasite loads). An ideal GAP_{BS} should result in infections with low level parasitemias that spontaneously resolve shortly after the parasites are introduced into the blood. An infection with low (sub-patent) parasitemias was only achieved by the Δ*nt1* mutant generated in non-lethal *P. yoelii* XNL when infected with low dose of parasites [17]. These sub-patent, self-resolving, infections generated strong cellular and humoral immune responses that provided complete protection in BALB/c, C57BL/6 and Swiss mice [17]. However, this GAP_{BS} was created in a virulent rodent parasite line (i.e. *P. yoelii* YM or *P. berghei* ANKA), where the kinetics and virulence phenomena of a gene-deletion mutant might be substantially different. We have targeted 41 genes for targeted disruption in the virulent rodent parasite *P. berghei* ANKA in order to generate GAP_{BS} that are both growth- and virulence-attenuated and can serve as protective immunogens. Specifically we aimed to create virulence-attenuated GAP_{BS}, which induce short-term blood infections with low parasitemias that are resolved by the host and induce protective immunity. The genes selected for targeted disruption were based on published roles of their encoding proteins in blood stages, or based on a reported delay in growth phenotype in *P. falciparum* mutants [22]. From the 41 genes selected 19 were refractory to targeted disruption. We generated 7 single gene-deletion mutants and 2 double gene-deletion mutants that showed significant reduction in blood stage asexual multiplication rates. From these mutants we identified seven GAP_{BS} that were both growth- and virulence-attenuated and 3 of these mutants did not generate hyper-parasitemia in BALB/c mice. These mice were able to resolve their infection and were protected against an infection with wild type parasites.

Results

Selection of genes for analysis by targeted gene deletion

For the generation of mutant blood stage parasites that are growth- and/or virulence-attenuated we selected a total of 41 genes for analysis by targeted deletion (Table 1). The first group consists of 8 genes encoding all *Plasmodium* rhomboid proteases (‘rhomboid genes’). We chose the genes coding for these proteins, because of the critical roles identified for several rhomboid proteases in host cell invasion and pathogenesis of apicomplexan parasites [23,24]. In addition, it has been shown that *P. berghei* and *P. yoelii* mutants lacking rhomboid 1 show a reduction in their blood stage growth rates [19,25]. Gene targeting experiments for the 8 rhomboids, generation and characterization of mutants lacking expression of rhomboid proteases has been described in Chapter 4. In Table 1 we show an overview of all the gene deletion experiments performed and in Table 2 we show growth- and virulence-characteristics of the mutants that we were able

to generate.

The second group (consisting of 12 genes) constitutes genes encoding 8 putative hemoglobinases and 4 other enzymes possibly involved in the *Plasmodium* hemoglobin (Hb) digestion ('hemoglobin digestion genes'). We chose genes coding for these proteins, because of the important role Hb digestion has in parasite growth [26]. In addition, gene disruption studies of hemoglobinases in *P. falciparum* demonstrate that this system is redundant and the enzymes have overlapping activities in Hb degradation [27–32]. Mutants lacking expression of certain hemoglobinases, while viable, show reduced growth rates and the equivalent mutants in *P. berghei* are both growth- and virulence-attenuated [20,30]. Gene targeting experiments for 12 genes and the generation and characterization of mutants lacking expression of hemoglobinases has been described in Chapter 5. Here, we analyse the growth rates of all mutants (Table 2) and provide data on the virulence-characteristics of these mutants (see below and Table 2).

The third group (of 8 genes) was selected based on *P. falciparum* mutants that exhibited a growth-delay phenotype ('*P. falciparum* growth-related genes'). These mutants were generated in a forward genetic screen based on random *piggyBac* mutagenesis ([22] and J.H. Adams unpublished observations).

The last group (consisting of 13 genes) is a heterogeneous group, which encode a variety of proteins expressed in asexual blood stages ('other genes'). These have been selected based on a proven, or putative effect on growth of *Plasmodium* blood stages. It includes 3 members of Rab GTPase family. Rab GTPases are key regulators of vesicular traffic in eukaryotic cells and in *Plasmodium* 11 *rab* genes have been identified of which 10 are transcribed in the iRBC and they possibly have overlapping functions [33]. Two genes were selected that encode enzymes involved in carbon metabolism: phosphoenolpyruvate carboxylase (PEPC) and carbonic anhydrase (CA). Carbon dioxide (CO_2) is thought to be essential for the growth of intraerythrocytic malaria parasites in order to synthesize pyrimidine through CO_2 fixation and to regulate the intracellular pH of the parasite [34]. PEPC is thought to catalyse CO_2 fixation with phosphoeno/pyruvate in the absence of pyruvate carboxylase in *Plasmodium* and thereby supplying the cytosol with oxaloacetate (OAA) [34]. *P. falciparum* mutants lacking expression of PEPC showed a strong reduction in growth of trophozoites and mutants could only be selected by supplying additional malate to cultures of the blood stages [34]. Carbonic anhydrase (CA) facilitates CO_2 transport across the plasma membrane and inhibitors of *Plasmodium* CA affect the growth of *P. falciparum* blood stages [35,36]. Two genes were selected that encode putative transporters, putative amino acid transporter (AAT) and nucleoside transporter 1 (NT1). NT1 is a plasmamembrane permease which is involved in uptake of

purines [37,38] and asexual blood stages of *P. yoelli* and *P. berghei* NT1-deficient mutants show a very reduced growth in mice [17,39]. Three genes were selected that play a role in the Kennedy phospholipid biosynthesis pathway, choline kinase (*ct*), choline/ethanolaminephosphotransferase (cept) and a putative ethanolamine kinase (*ek*). It was unclear at the initiation of these studies if the generation of *Plasmodium* phospholipids were only derived by *de novo* synthesis, or could be also derived from an alternative scavenging pathway. If the parasite would make use of both systems, deletion of one of these genes may not have a deleterious effect, but may affect growth rate of the parasites [40]. We also targeted the gene encoding a putative hemolysin, as it is implicated to play a role in parasite egress from the RBC [41,41]. As multiple genes are important for parasitophorous vacuole (PV) formation and since *P. berghei* liver stages can survive and replicate inside a hepatocyte with a compromised/absent PV [42], we attempted to delete the PV resident protein hepatocyte erythrocyte protein 17 kDa (*hep17*; also known as exported protein 1) [43]. Finally, we attempted to disrupt a gene encoding a putative DNA (cytosine-5)-methyltransferase (*dnmt2*). DNA methylation plays an important role in gene silencing/activation, deletion of the equivalent gene (pmt1) in yeast resulted in decreased rates of vegetative growth [44].

Genes which were refractory to targeting deletion

A total of 19 out of the 41 genes were refractory to targeting deletion in multiple transfection experiments (Table 1). The multiple unsuccessful attempts to disrupt these genes indicate that these have a critical function for asexual blood stage growth, although a failure to disrupt a gene is not an unequivocal proof that the encoded protein is essential for blood stage multiplication.

These genes include 4 'rhomboid genes' (*rom4*, *6*, *7* and *8;* see Chapter 4) and 3 'hemoglobin digestion genes' (*bln*, *aap*, *hdp;* see Chapter 5). Four out of 8 '*P. falciparum* growth-related genes' were refractory to gene deletion in *P. berghei* (*i.e. caf1*, *pp2c*, *ApiAP2* and PBANKA_020890). The unsuccessful attempts to disrupt one of these genes, *caf1* encoding CCR4-associated factor 1 (PBANKA_142620), has been published [45]. Of the 'other genes', the 3 *rab* genes were refractory to deletion and attempts to delete *ck*, *cept* and *ek*, were also unsuccessful, and this was supported by a recent study that also showed that genes of the Kennedy phospholipid biosynthesis pathway were refractory to genetic disruption in *P. berghei* [40]. The genes encoding hemolysin, and HEP17 were also refractory to disruption. See Table 1 and Table S1 for details of these unsuccessful gene-deletion attempts and primers used to amplify the targeting sequences, generate the gene-deletion constructs and for genotyping. Information on failed attempts to disrupt

genes including DNA constructs and primers have been submitted to the RMgmDB database of genetically modified rodent malaria parasites (www.pberghei.eu).

Table 1. Selected genes for analysis by targeted gene deletion

Gene name	*P. berghei* Gene ID	*P. falciparum* Gene ID	Product name in PlasmoDB	Successful targeting deletion?	DNA construct name	Experiment No. Mutant name[1]	RMgmDB ID[2]
Genes that encode rhomboid proteases **(8 genes)**							
rom1	PBANKA _093350	PF3D7_ 1114100	rhomboid protease ROM1	yes	Mg031	538cl2	RMgm-177
					pL1533	1496cl4	RMgm-761
rom3	PBANKA _070270	PF3D7_ 0828000	rhomboid protease, putative	yes	pL1097	430cl1, 687cl1	RMgm-178
rom4	PBANKA _110650	PF3D7_ 0506900	rhomboid protease, putative (ROM4)	no	pL1078	653, 684, 695	RMgm-187
rom6	PBANKA _135810	PF3D7_ 1345200	rhomboid protease ROM6, putative	no	PCR1916	2118, 2119, 2140	RMgm-758
rom7	PBANKA _113460	PF3D7_ 1358300	rhomboid protease ROM7, putative	no	PCR1917	2120, 2121, 2141	RMgm-759
rom8	PBANKA _103130	PF3D7_ 1411200	rhomboid protease, putative	no	PCR1918	2122, 2123, 2142	RMgm-760
rom9	PBANKA _111470	PF3D7_ 0515100	rhomboid protease, putative	yes	PCR1919	2124cl1, 2125cl1	RMgm-762
rom10	PBANKA _111780	PF3D7_ 0618600	rhomboid protease ROM10, putative	yes	Mg011	468cl2	RMgm-179
Genes that encode enzymes involved in hemoglobin digestion pathway **(12 genes)**							
pm4	PBANKA _103440	PF3D7_ 1407800	plasmepsin 4	yes	PCR1597	1688cl1	RMgm-808
bp2	PBANKA _093240	PF3D7_ 1115700	berghepain-2	yes	pLTgPain2	Pain2cl8	
					pL1602	1619cl1	RMgm-809
bln	PBANKA _113700	PF3D7_ 1360800	bergheilysin	no	PCR1541	1502	RMgm-804
					pL1557	1543	
					pLTgLysin	lysinko 1-2-3	
dpap1	PBANKA _093130	PF3D7_ 1116700	dipeptidyl amino-peptidase 1	yes	pLDPA	DPAkocl5	
					PCR1833	1962cl1	RMgm-810
app	PBANKA _131810	PF3D7_ 1454400	aminopeptidase P	yes	PCR1924	2129cl2, 2248cl1	RMgm-813
aap	PBANKA _141030	PF3D7_ 1311800	M1- family alanyl aminopeptidase	no	PCR1877	2058, 2087, 2111	RMgm-806
					pLTgAPN	aapko 1-2-3	
lap	PBANKA _130990	PF3D7_ 1446200	M17-family leucyl aminopeptidase	yes	PCR1878	2112cl3	RMgm-814
dap	PBANKA _083310	PF3D7_ 0932300	M18-family aspartyl aminopeptidase	yes	PCR1879	2060cl1	RMgm-815
hdp	PBANKA _131060	PF3D7_ 1446800	heme detoxification protein	no	PCR1690	1748, 1778, 2212	RMgm-807
					PCR1762	2208, 2213	
					pPhHDP	hdpko 1-2-3	

bp1	PBANKA _132170	PF3D7_ 1458000	berghepain 1	yes	pL1976	2250cl1	RMgm-816
dpap2	PBANKA _146070	PF3D7_ 1247800	dipeptidyl aminopeptidase 2	yes	PCR1875	2056cl1	RMgm-811
dpap3	PBANKA _100240	PF3D7_ 0404700	dipeptidyl aminopeptidase 3	yes	PCR1876	2057cl1, 2110cl1	RMgm-812
***Genes selected based on P. falciparum piggyBac insertion mutants with a growth phenotype* (8 genes)**							
caf1	PBANKA _142620	PF3D7_ 0811300	CCR4-associated factor 1	no	PCR1518 PCR1585	1463, 1489 1591, 1615	RMgm-639
cdc25	PBANKA _140400	PF3D7 _1305500	conserved *Plasmodium* protein, unknown function	yes	PCR1524	1492cl1	RMgm-829
pp2c	PBANKA _123070	PF3D7 _0615900	conserved *Plasmodium* protein, unknown function	no	PCR1699 PCR1827	1782 1957	RMgm-827
ApiAP2	PBANKA _135600	PF3D7_ 1342900	transcription factor with AP2 domain(s), putative (ApiAP2)	no	PCR1831	2007, 2330, 2337	RMgm-913
	PBANKA _020890	PF3D7_ 0104200	conserved *Plasmodium* protein, unknown function	no	PCR1691 PCR1774	1799 1893	RMgm-828
	PBANKA _112890	PF3D7_ 0630100	conserved *Plasmodium* protein, unknown function	yes	PCR1830	2329cl1	RMgm-860
	PBANKA _030100	PF3D7_ 0203000	conserved *Plasmodium* protein, unknown function	yes	PCR1883	2331cl1	RMgm-861
Rpus	PBANKA _111100	PF3D7_ 0511500	RNA pseudouridylate synthase, putative, fragment	yes	PCR1775	1894cl1	RMgm-830
***Other genes* (13 genes)**							
rab5a	PBANKA _030800	PF3D7_ 0211200	Rab5a, GTPase, putative	no	PCR1548	1526, 1587, 1608, 1647, 1648, 1680, 1681	RMgm-821
rab5b	PBANKA _140910	PF3D7_ 1310600	Rab5b, GTPase, putative	no	PCR1709	1785, 1786	RMgm-822
rab11b	PBANKA _135410	PF3D7_ 1340700	Rab GTPase 11b	no	PCR1710	1787, 1788	RMgm-823
ck	PBANKA _104010	PF3D7_ 1401800	choline kinase	no	PCR1549	1527, 1609, 1649, 1682	RMgm-818
cept	PBANKA _112700	PF3D7_ 0628300	choline/ethanolamine-phophotransferase	no	PCR1550	1528, 1610, 1650, 1683	RMgm-819
ek	PBANKA _092370	PF3D7_ 1124600	ethanolamine kinase, putative	no	PCR1643	1673, 1695	RMgm-820
nt1	PBANKA _136010	PF3D7_ 1347200	nucleoside transporter 1	yes	PCR1693 PCR1776	1781cl1 1912	RMgm-831
aat	PBANKA _112830	PF3D7_ 0629500	amino acid transporter, putative	yes	PCR1925	2130cl1	RMgm-832
pepc	PBANKA _101790	PF3D7_ 1462700	phophoenolpyruvate carboxylase	yes	PCR1777	1895cl1	RMgm-833
ca	PBANKA _090900	PF3D7_ 1140000	carbonic anhydrase, putative	yes	PCR1881	2114cl1	RMgm-834

hemolysin	PBANKA _131910	PF3D7_ 1455400	hemolysin, putative	no	PCR1591	1594, 1618	RMgm-824
hep17	PBANKA _092670	PF3D7_ 1121600	circumsporozoite-related antigen	no	PCR1555	1542, 1611	RMgm-825
dnmt2	PBANKA _021140	PF3D7_ 0727300	DNA (cytosine-5)-methyltransferase, putative (DNMT2)	yes	pL1789	1935cl1, 1965cl1	RMgm-835

[1] Experiment number for independent transfection experiments: the unsuccessful attempts (X3) and the experiment number/clone of the gene deletion mutants

[2] The ID number of the mutants (or of the unsuccessful attempts for gene deletion) in the RMgmDB database (www.pberghei.eu) of genetically modified rodent malaria parasites

Successfully generated gene deletion mutants and analysis of growth- and virulence- attenuation

Successful gene deletion mutants were generated for 22 out of the 41 genes (Table 1). Successful deletion of these genes demonstrates that they are not essential for asexual blood stage growth under the conditions used for selection of the gene-deletion mutants. These genes include 4 'rhomboid genes' (*rom1*, *3*, *9*, 10; see Chapter 4) and 9 'hemoglobin digestion genes' (*pm4*, *bp2*, *dpap1*, *app*, *dap*, *lap*; *bp1*, *dpap2*, *dpap3*; see Chapter 5). Four out of 8 '*P. falciparum* growth-related genes', were successfully deleted (*cdc25*, PBANKA_030100, PBANKA_112890 and *Rpus*) (Figure S1). In addition, for five out of 13 'other genes' (*nt1*, *aat*, *pepc*, *ca*, *dnmt2*), it was possible to generate deletion mutants (Figure S2). In addition to these single gene-deletion mutants, we also generated two 'double' gene-deletion mutants. In one mutant (Δ*pm4*Δ*bp2*) both plasmepsin-4 (*pm4*) and berghepain-2 (*bp2*) were sequentially deleted. The proteins encoded by these genes are responsible for initial cleavage of native hemoglobin (Chapter 5). In the second double gene-deletion mutant (Δ*pm4*Δ*smac*), both plasmepsin-4 (*pm4*) and the gene *smac* were deleted (Figure S2). The *smac* gene encodes SMAC (schizont membrane-associated cytoadherence protein, PBANKA_010060), which is involved in *P. berghei* ANKA schizont sequestration [46]. See Table 1 and Table S1 for details of the successful gene-deletion mutant generation and primers used to amplify the targeting sequences, generate the gene-deletion constructs and for genotyping. All information on the gene deletion mutants, including DNA constructs and primers, has been submitted to the RMgmDB database of genetically modified rodent malaria parasites (www.pberghei.eu).

For all gene-deletion mutants, we confirmed the correct integration of the constructs and the successful disruption of the gene by diagnostic PCR and/or Southern analyses of separated chromosomes (Figures S1-2). For all mutants we determined the *in vivo* asexual multiplication rates (i.e. growth rate), which is calculated during the initial phase of infection after mice are infected with a single parasite and is defined as the daily-fold increase in parasite numbers [20] (Table 2). When mutants were observed to have

a significantly reduced growth rate we also confirmed the lack of transcription of the disrupted genes by Northern analyses of blood stage mRNA from the mutant parasites (Figures S2). In addition, for mutants with a significant growth defect (see below; 9 out of 22 mutants), we determined their virulence characteristics in C57BL/6 and BALB/c mice. In C57BL/6 mice we determined whether the mutant parasites induce experimental cerebral malaria (ECM). *P. berghei* ANKA is a frequently used model to study ECM in C57BL/6 mice. When these mice are infected with 10^4 to 10^6 wild type (wt) *P. berghei* ANKA parasites, more than 90% of mice develop features of ECM between day 6 and 9 after infection [47]. In BALB/c mice we determined the development of hyper-parasitemia. BALB/c mice infected with wt *P. berghei* ANKA do not develop ECM, but succumb to hyper-parasitemia (>50%), anemia and general organ failure in the second or third week after infection [48].

Gene-deletion mutants that exhibit normal growth rates

Wild-type *P. berghei* ANKA parasites have a consistent 10-fold increase in parasitemia per 24 hour [20], and we observed that 15 out of 22 single gene-deletion mutants we generated had no significant reduction in their asexual multiplication rates (Table 2). The '*P. falciparum* growth-related gene mutants' Δ*cdc25*, Δ*PBANKA_030100*, Δ*PBANKA_112890* and Δ*Rpus* have wt multiplication rates (Table 2), which are different from the reduced growth rates of their corresponding *P. falciparum piggyBac* insertion mutants. The cell cycle of mutant *Pf*Δ*cdc25* is prolonged by 10% (J. H. Adams, unpublished data); mutants Δ*PF3D7_0630100* (ortholog of PBANKA_030100), Δ*PF3D7_0203000* (ortholog of PBANKA_112890) and *Pf*Δ*Rpus* exhibit 45–65% reduction in RBC invasion compared to wt *P. falciparum* NF54 [22]. Other mutants with wt growth rates were 4 'rhomboid gene mutants' (Δ*rom1*, Δ*rom3*, Δ*rom9*, Δ*rom10;* Chapter 4, Table 2), 4 'hemoglobin digestion gene mutants' (Δ*bp2*, Δ*dap*, Δ*dpap2*, Δ*dpap3;* Chapter 5, Table 2) and 3 'other gene mutants' (Δ*aat*, Δ*ca* and Δ*dnmt2*). The wt-like growth of Δ*rom1* and Δ*ca* mutants is unexpected. In other studies it has been found that *P. berghei* and *P. yoelii* mutants lacking rhomboid 1 show a reduction in their blood stage growth rates [19,25]. The normal growth rate of Δ*ca* is also unexpected, given CA has been considered a potential drug target and the antimalarial activity of CA inhibitors against both *P. falciparum* and *P. berghei* has been reported [35,36].

Gene-deletion mutants that exhibit significant reduced growth rates

In infections of mice, 7 out of 22 single gene-deletion mutants show a reduction in their growth rates and have a significantly lower than the 10-fold increase in parasitemia per 24 hour observed in wt *P. berghei* ANKA infections. Specifically, 5 'hemoglobin digestion

gene mutants' (Δ*pm4*, Δ*dpap1*, Δ*app*, Δ*lap* and Δ*bp1;* Chapter 5, Table 2) and 2 'other gene mutants' (Δ*nt1* and Δ*pepc*) (Table 2). Recent analyses of the growth of *P. yoelli* and *P. berghei* mutants lacking expression of NT1 show a reduction in asexual blood stages growth rate [17,39], which is similar to that we report in this study (Table 2). The reduced growth rate of the *P. berghei* Δ*pepc* corresponds to the reduced growth of *P. falciparum* blood stages lacking expression of PEPC [34]. In addition to the 7 single gene-deletion mutants, the two double gene-deletion mutants, Δ*pm4*Δ*smac* and Δ*pm4*Δ*bp2*, showed a strong reduction in their multiplication/growth rates (Table 2).

Table 2. Growth and virulence characteristics of blood stages of gene deletion mutants.

Mutant	Day to 0.5-2% parasitemia[1]	Multiplication rate[2]	ECM in C57BL/6[3]	Self-resolving in C57BL/6[4]	Hyper-parasitemia in BALB/c[5]	Self-resolving in BALB/c[4]
wt [7]	8 (0.2), n=40	10.0 (0.7)	6/6	0/6	6/6	0/6
***Mutants without significant reduction in asexual multiplication rates* (15 mutants)**						
Δ*rom1*-p	8 (0), n=2	10.0 (0.0)	6/6	0/6	6/6	0/6
Δ*rom1*-c	8 (0), n=3	10.0 (0.0)	6/6	0/6	6/6	0/6
Δ*rom3*	8 (0), n=3	10.0 (0.0)	n.d	n.d	n.d	n.d
Δ*rom9*	8 (0), n=4	10.0 (0.0)	n.d	n.d	n.d	n.d
Δ*rom10*	8 (0), n=4	10.0 (0.0)	n.d	n.d	n.d	n.d
Δ*bp2*-a	8 (0), n= 5	10.0 (0.0)	6/6	0/6	6/6	0/6
Δ*bp2*-b	8 (0), n=6	10.0 (0.0)	6/6	0/6	n.d	n.d
Δ*dap*	8 (0), n=3	10.0 (0.0)	6/6	0/6	n.d	n.d
Δ*dpap2*	8.3 (0.4), n=4	9.4 (1.0)	n.d	n.d	n.d	n.d
Δ*dpap3*-a	8.3 (0.6), n=3	9.2 (1.3)	n.d	n.d	n.d	n.d
Δ*dpap3*-b	8 (0), n=5	10.0 (0.0)	n.d	n.d	n.d	n.d
Δ*cdc25*	8 (0), n=4	10.0 (0.0)	n.d	n.d	n.d	n.d
Δ*PBANKA_030100*	8.2 (0.4), n=5	9.5 (1.0)	n.d	n.d	n.d	n.d
Δ*PBANKA_112890*	8 (0), n=4	10.0 (0.0)	n.d	n.d	n.d	n.d
Δ*Rpus*	8 (0), n=7	10.0 (0.0)	n.d	n.d	n.d	n.d
Δ*aat*	8 (0), n=5	10.0 (0.0)	n.d	n.d	n.d	n.d
Δ*ca*	8 (0), n=4	10.0 (0.0)	n.d	n.d	n.d	n.d
Δ*dnmt2*-a	8.4 (0.5), n=5	9.1 (1.2)	5/5	0/5	n.d	n.d
Δ*dnmt2*-b	8 (0), n=3	10.0 (0.0)	n.d	n.d	n.d	n.d
***Mutants with significant reduction in asexual multiplication rates* (7 mutants)**						
Δ*pm4*	9 (0), n=2	7.7 (0.0) ***	0/6	0/6	6/6	0/6
Δ*dpap1*-a	9.5 (0.7), n=2	7.0 (1.0) ***	yes[6]	none[7]	6/6	n.d
Δ*dpap1*-b	9 (0), n=4	7.7 (0.0) ***	6/6	0/6	n.d	n.d
Δ*app*-a	12 (0), n=1	4.6 (0.0) ***	0/6	3/6	0/6	6/6
Δ*app*-b	12 (0), n=4	4.6 (0.0) ***	0/6	6/6	n.d	n.d
Δ*lap*	15.5 (0.7), n=2	3.3 (0.2) ***	0/6	6/6	0/6	6/6
Δ*bp1*	9.7 (0.6), n=3	6.8 (0.8) ***	0/6	0/6	n.d	n.d

Δ*nt1*	9.8 (0.5), n=4	6.7 (0.7) ***	0/5	0/5	6/6	n.d
Δ*pepc*	13.7 (0.6), n=3	3.9 (0.2) ***	6/6	0/6	6/6	n.d
Double gene-deletion mutants **(2 mutants)**						
Δ*pm4*Δ*smac*	13.5 (0.7), n=2	3.9 (0.3) ***	0/6	5/6	5/5	n.d
Δ*pm4*Δ*bp2*-a	12, 16, 20, n=3	3.4 (1.1) ***	0/6	6/6	2/12	10/10
Δ*pm4*Δ*bp2*-b	21, 24, n=2	2.3 (0.1) ***	0/6	6/6	3/6	3/3

n.d, not determined

[1] The day on which the parasitemia reach 0.5–2% in mice infected with a single parasite during cloning assays. The mean of one cloning experiment and standard deviation are shown. n, the number of mice tested. For the Δ*pm4*Δ*bp2* mutants, due to large variation, the days of the individual clone are shown.

[2] The multiplication rate of asexual blood stages per 24 hours as determined in the cloning assays. Mean values and standard deviations of each line were shown, student T-test, *, P<0.01; ***, P<0.0001.

[3] Development of symptoms of experimental cerebral malaria (ECM)

[4] Mice with parasitemias <50% that resolving infections in C57BL/6 or BALB/c.

[5] Hyper-parasitemia infections in BALB/c mice is defined as a parasitemia > 50%.

[6] Spaccapelo R, *et al*, 2011 (ref[21])

[7] Spaccapelo R, unpublished data

Gene-deletion mutants that exhibit normal growth and virulence characteristics

We tested 3 out of 15 mutants with normal (wt) growth rates for their ablity of inducing ECM in C57BL/6 mice (i.e. Δ*rom1*, Δ*bp2* and Δ*dap*). It has been reported that *P. berghei* and *P. yoelii* mutants lacking rhomboid 1 exhibit a slightly reduced growth rate and are less virulent in mice than wt parasites [19,25]. In particular, the *P. berghei* Δ*rom1* mutant as reported by Srinivasan *et al*. did not cause ECM in Swiss mice [19]. We therefore determined the virulence of two independent Δ*rom1* mutants (Chapter 4) in C57BL/6 mice. We found that both mutants induced ECM at day 5–6 as wt *P. berghei* ANKA parasites (Chapter 4, Table 2).

Gene-deletion mutants that exhibit reduced growth rates but still cause ECM

Infections with 2 of the 9 growth-attenuated mutants, Δ*dpap1* and Δ*pepc*, still induced ECM in C57BL/6 mice. The Δ*dpap1*-b mutant caused ECM on day 7–9 post infection in mice infected with 10^5 parasites; in comparison, a wt infection initiated with the same number of parasites produced a higher parasitemia and mice succumbed to ECM 1 or 2 days earlier (i.e. day 6–7; Figure 1A). Most mice infected with Δ*dpap1*-a developed ECM (R. Spaccapelo, unpublished data). Interestingly, while we observed Δ*pepc* infections have a strong reduction in growth when infections are initiated with a single parasite, the growth rates in mice infected with 10^5 parasites (intraperitoneally) was, unexpectedly, not strongly reduced compared to a wt infection in 2 independent experiments and all

mice developed ECM (Figure 1B). Unfortunately, we have been unable to select a second independent Δ*pepc* mutant yet, despite 6 seperate transfection experiments targetting *pepc*. Confirmation of the effects on parasite growth rates in mice when infected with different parasite numbers awaits either the generation/characterization of a second independent mutant or restoration of the wt phenotype when *pepc* gene is re-introduced into the Δ*pepc* genome (i.e. genetic complementation).

Gene-deletion mutants that that exhibit reduced growth rates and reduced virulence

Infections with 7 out of 9 growth-attenuated mutants also showed virulence-attenuation, with respect to ECM. Specifically, Δ*pm4*, Δ*app*, Δ*lap*, Δ*bp1*, Δ*nt1*, Δ*pm4*Δ*bp2* and Δ*pm4*Δ*smac* did not induce ECM in C57BL/6 (Table 2). Furthermore, C57BL/6 mice infected with four of these mutants were able to spontaneously resolve infections to different degrees (Table 2): 3 out of 6 mice survived a Δ*app*-a infections, while all (6/6) mice resolved infections with either with Δ*app*-b or Δ*lap* (Table 2). Whereas Δ*pm4*-infected C57BL/6 were not able to resolve infections [20], 12 out of 12 mice resolved infections with the double gene-deletion mutants Δ*pm4*Δ*bp2*-a or Δ*pm4*Δ*bp2*-b and 4 of these resolved infections without developing hyper-parasitemia (Figure 1C). For the double gene-deletion mutant Δ*pm4*Δ*smac*, 5/6 mice resolved the infection and these mice cleared parasites in 3 weeks before parasitemia reaching 20%. One of the six mice did not control the infection and developed hyper-parasitemia (>50%) (Figure 1C).

In addition to virulence characeristics in C57BL/6 mice we analysed the growth (parasitemia and self-resolving infections) of 6 growth-attenuated mutants in BALB/c mice (Δ*pm4*, Δ*dpap1*-a, Δ*app*, Δ*lap*, Δ*pm4*Δ*smac* and Δ*pm4*Δ*bp2*; Table 2). BALB/c mice infected with wt *P. berghei* ANKA do not succumb to ECM, but still are unable to resolve the infection and mice die of sustained hyper-parasitemia (>50%) and anemia. Mutants Δ*pm4* and Δ*dpap1*-a induced parasitemias in excess of 50% in BALB/c mice, as did the double gene-deletion mutant Δ*pm4*Δ*smac* that had shown reduced parasitemias in C57BL/6 mice which could resolve the infections. As mice were sacrificed at parasitemias between 50 and 70%, we did not determine whether these mice were able to resolve the infections as had previously shown with Δ*pm4* infections [20]. All BALB/c mice (n=6) infected with 10^5 Δ*app* or 10^5 Δ*lap* parasites, and 13 out of 18 BALB/c mice infected with the 10^5 or even 10^6 Δ*pm4*Δ*bp2* mutants did not develop hyper-parasitemias and resolved their infections (Figure 2).

All mice that had resolved their infections (both C57BL/6 and BALB/c) were challenged with 10^5 wt parasites by intraperitoneal (i.p) injection, at least 1 month after clearance of

the parasites. All mice are protected against wild type *P. berghei* ANKA challenge (data not shown).

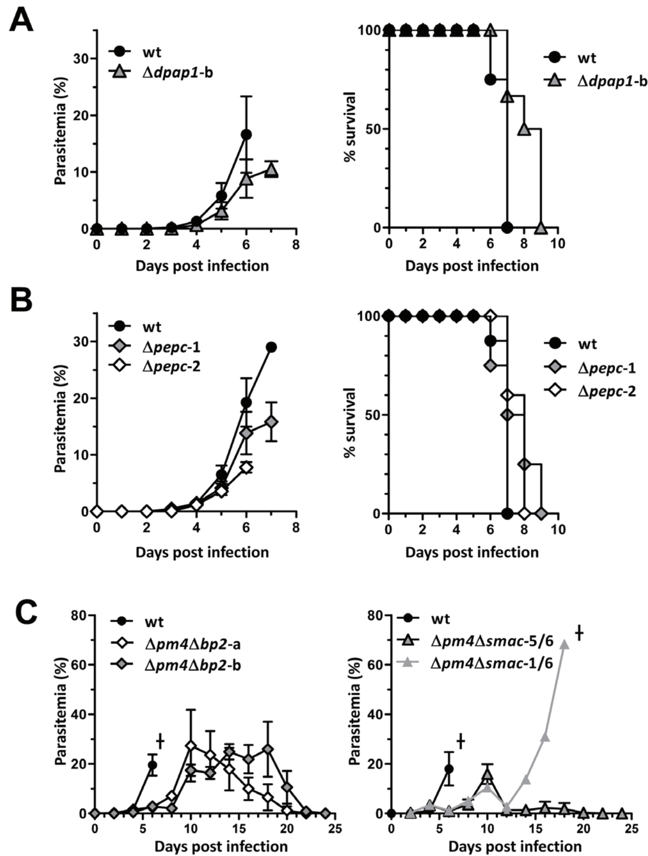

Figure 1. The course of infection of wild type and mutant *P. berghei* parasites in C57BL/6 mice.

A. The course of infection (left panel) and survival curve (right panel) in C57BL/6 mice (n=6) i.p infected with 10^5 wild-type (wt, cl15cy1) or Δ*dpap1*-b parasites. Δ*dpap1*-b infection produced a lower parasitemia and mice succumbed to ECM 1 or 2 days later compare to wt parasites.

B. The course of infection (left panel) and survival curve (right panel) in C57BL/6 mice (n=6) i.p infected with 10^5 wt (cl15cy1) or Δ*pepc* parasites in 2 independent experiments. Δ*pepc* infection produced a lower parasitemia compared wt infection, but still caused ECM on day 6-9 after infection.

C. Course of parasitemia in C57BL/6 mice. Mice (n=6) were i.p infected with 10^5 wt (cl15cy1), 10^5 Δ*pm4*Δ*bp2*-a, 10^6 Δ*pm4*Δ*bp2*-b (left panel) or 10^5 Δ*pm4*Δ*smac* parasites (right panel). All wt-infected mice developed cerebral complications at day 6 after infection, whereas none of the mice infected with Δ*pm4*Δ*bp2* or Δ*pm4*Δ*smac* parasites developed ECM. All mice infected with Δ*pm4*Δ*bp2* parasites resolved infections resulting in undetectable parasitemia by microscopic analysis between day 22 and 24 post infection. Five out of 6 mice infected Δ*pm4*Δ*smac* parasites resolved infections in 3 weeks with peak parasitemia less than 25%. One mouse developed hyper-parasitemia (>50%).

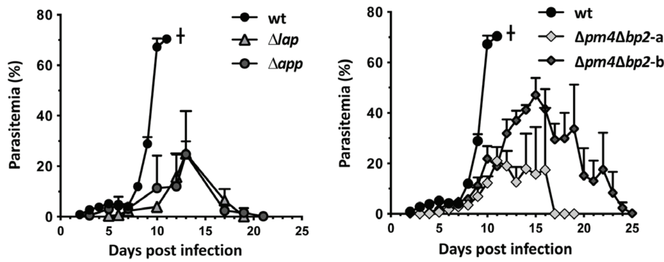

Figure 2. The course of infection of wild type and mutant *P. berghei* parasites in BALB/c mice.

Mice (n=6) were i.p infected with 10^5 wt (cl15cy1), 10^5 Δ*lap*, 10^5 Δ*app* (left panel); 10^5 Δ*pm4*Δ*bp2*-a (n=12) or 10^6 Δ*pm4*Δ*bp2*-b (n=6) (right panel). All wt-infected mice developed hyper-parasitemia on day 10-11 after infection, whereas none of the mice infected with Δ*lap* or Δ*app* parasites developed hyperparasitemia and resolved infections resulting in undetectable parasitemia by microscopic analysis between day 20 and 22 post infection (p.i). Ten out of 12 mice infected with 10^5 Δ*pm4*Δ*bp2*-a parasites and 3 out of 6 mice infected with 10^6 Δ*pm4*Δ*bp2*-b resolved infections without developing hyperparasitemia.

Discussion

In this study we examined *P. berghei* gene-deletion mutants in order to identify genetically attenuated blood stage parasites (GAP_{BS}), specifically, mutants that are both growth- and virulence-attenuated and that may serve as immunizing agents and as tools to study correlates of disease and protection. Using mutants generated in this and previous studies, we first examined their growth characteristics and established the multiplication rates for blood stages of each mutant in cloning assays. For those mutants with a significant reduction in growth, we examined their virulence by assessing experimental cerebral malaria (ECM) in C57BL/6 mice and for a number of these mutants we also examined if the infection in BALB/c mice resulted in hyper-parasitemia (i.e. >50%). We analysed the course of parasitemia in both C57BL/6 and BALB/c mice as we aimed to identify GAP_{BS} that induce only low-parasitemia, self-resolving infections that are cleared soon after parasites are introduced into the blood. Until now, most of the reported virulence-attenuated GAP_{BS} that do not induce ECM in C57BL/6 mice, still produce infections in BALB/c with relatively high parasitemias [20,21]. We identified 9 mutants that had a strongly reduced asexual multiplication rate (>20% reduction compared to wt). Seven of these mutants did not induce ECM, suggesting that the growth rate of blood stages is an important factor for inducing ECM. The absence of ECM in mice infected with the double gene-deletion mutants Δ*pm4*Δ*bp2* and Δ*pm4*Δ*smac* was expected, since mice infected with Δ*pm4* parasites also do not develop ECM [20]. Interestingly, while it has been reported that C57BL/6 mice infected with Δ*pm4* cannot resolve their infections

and die from hyper-parasitemia [20], we found that all C57BL/6 mice infected with Δ*pm4*Δ*bp2*, and most mice (5/6) infected with Δ*pm4*Δ*smac* can resolve their infections. These results demonstrate that it is possible to generate further virulence-attenuated parasites through the deletion of multiple genes, as was also reported by Spaccapelo *et al.* [21] with mutants that lack expression of both PM4 and MSP7. The Δ*pm4*Δ*bp2* mutant lacks PM4 and BP2, the two key enzymes involved in hemoglobin digestion, as described in Chapter 5. This mutant has a further reduced multiplication rate compared to Δ*pm4*, which may contribute to the capacity of C57BL/6 mice to resolve infections with this mutant. The Δ*pm4*Δ*smac* mutant lacks in addition to plasmepsin-4, expression of SMAC, a parasite protein involved in the adherance of *P. berghei* schizonts (in a CD36-dependent manner) to host endothelium [46]. It has been shown that the reduced growth of parasites lacking SMAC is in large part due to the clearance of unsequestered SMAC-deficient schizonts by the spleen. We found that the growth rate of Δ*pm4*Δ*smac* is strongly reduced compared to either Δ*pm4* or Δ*smac*, which may explain why C57BL/6 mice are able to resolve infections.

While 7 of the 9 'slow-growing' mutants were virulence-attenuated, two of these mutants (Δ*dpap1* and Δ*pepc*) still induced ECM in mice, suggesting that factors other than a delay in growth contribute to induction of ECM. We found that all mutants (i.e. Δ*pm4*, Δ*app*, Δ*pm4*Δ*bp2* and Δ*pm4*Δ*smac*) that had reduced hemozoin (Hz) production (data shown in Chapter 5) do not induce ECM. Hz is released into the circulation at schizont rupture and it is rapidly removed by phagocytosis mainly in the liver and spleen. Upon phagocytosis Hz cannot be further degraded and persists for prolonged periods in host tissue and has long been considered as a virulence factor. It has been shown that the number of Hz-containing leukocytes in the peripheral blood correlates with disease severity in *P. falciparum*-infected patients [49,50]. Several inflammatory and immune-modulatory effects of Hz have been reported (reviewed in [51,52]). Therefore, the amount of Hz that is released by the parasite may play a critical role in both inducing inflammatory responses and severe pathology in the host. Since induction of ECM correlates with pro-inflammatory status of the host [53], Hz may be a critical factor involved in inducing ECM. However, the amount of Hz, like growth, may not be the only factor responsible for inducing ECM, since blood stages of several growth-attenuated mutants (Δ*nt1*, Δ*lap* and Δ*bp1*) have normal Hz production and do not induce ECM (Chapter 5, Table 2; unpublished results). However, the absence of ECM in these mutants could still be related to reduced amounts of Hz released in the circulation early in an infection. Mice infected with slow-growing parasites can be expected to release less Hz compared to wt parasites and therefore the Hz levels may be below the threshold that is required to induce inflammatory responses during the acute phase of the infection necessary to

produce ECM. Clearly, further research is required to unravel the relative contributions of the critical parasite (and host) factors that result in severe disease and protection. So far we have been unable to select mutants that do not induce ECM, but that have both a normal growth rate and Hz production. The selection of such mutants would indicate that other factors in addition to growth rate and Hz levels contribute to ECM. Despite reduced growth rates and lack of ECM in C57BL/6 mice, the Δ*pm4*, Δ*nt1* and Δ*pm4*Δ*smac* mutants produced hyper-parasitemia infections in BALB/c mice. However, we found that BALB/c infected with three mutants, Δ*lap*, Δ*app* and Δ*pm4*Δ*bp2*, are able to resolve without developing hyper-parasitemias.

Combined, our results show that it is possible to generate mutants with strongly reduced growth rates that do not induce ECM and that through the deletion of one or multiple genes it is possible to create mutants that produce self-resolving infections in mice without producing hyper-parasitemia. However, mice infected with these mutants still develop parasitemias ranging between 10–50%. Up to now we have not yet been able to generate mutants that produce 'low-level' infections that resolve shortly after parasite inoculation into the blood and without developing high parasitemias. Even with parasites that have strongly reduced growth rates, both C57BL/6 and BALB/c mice are unable to rapidly mount an effective immune response that can control an acute infection. It is, however, important to note that in all our experiments, the mice were infected with relatively high numbers of parasites (10^5–10^6). It is possible that starting infection with lower numbers of parasites would allow the mice to control infections before developing high parasitemias, or would lead to infections with very low or even sub-patent parasitemias [17]. For *P. falciparum*, it has been postulated that infection with low numbers of infected RBC (under curative chemotherapy) generates protective immune responses that are defined by the absence, or low levels of antibodies and strong cell-mediated responses, including upregulation of nitric oxide synthase, CD4+/CD8+ proliferative T-cell and INF-γ responses [13,54,55]. Protective immunity with *P. berghei* infections in mice have been mainly reported from immunization requiring repeated, prolonged infections cleared by drug treatment, or after a self-resolving and sustained infection with an avirulent parasite line [10,16,20,56–59]. The protective immune responses in these mice are largely antibody-dependent, where the iRBC of wt challenge are opsonized and then removed in the spleen by phagocytosis [20,60,61]. These studies and those of experimental *P. falciparum* infections in humans, where protective cellular immune responses are induced with low numbers of iRBC, would suggest that the induction of protective immunity might require different parasite loads depending on the nature of the immune (cellular or humoral) responses required. Clearly, further research is required to determine both the parasite and host factors that can induce protective immune responses against blood stages.

The use of attenuated blood stage parasites can be extremely useful tools to better understand induced rather than acquired immunity against *Plasmodium* and may help to create an effective and the boardest anti-parasite vaccine.

Material and Methods

Animals and parasites

Female C57BL/6, BALB/c and Swiss OF1 mice (6–8 weeks old; Charles River/Janvier) were used. All animal experiments of this study were approved by the Animal Experiments Committee of the Leiden University Medical Center (DEC 10099; 12042; 12120). The Dutch Experiments on Animal Act is established under European guidelines (EU directive no. 86/609/EEC regarding the Protection of Animals used for Experimental and Other Scientific Purposes).

Three reference *P. berghei* ANKA parasite lines were used for generation of the gene-deletion mutants and the transgenic parasites: the 'wild type' (wt) line cl15cy1 [62] and two reporter lines, i.e. *Pb*GFP-LUC$_{con}$ (line 676m1cl1; mutant RMgm-29; www.pberghei.eu) and *Pb*GFP-Luc$_{schiz}$ (line 1037cl1; mutant RMgm-32; www.pberghei.eu). Both reporter lines were generated in the cl15cy1 parent line and express the fusion protein GFP-Luciferase either under the control of the constitutive *eef1α* promoter or the schizont-specific *ama1* promoter, respectively. The *gfp-luc* expression cassette is stably integrated into the *pb230p* locus without introduction of a drug-selectable marker [20,63].

6

Generation of gene-deletion mutants

To generate targeted gene deletion mutants, the replacement constructs (Table S1) were generated using conventional cloning method or a modified two step PCR method [64]. Plasmid construct pL1789 targeting *dnmt2* was constructed in plasmid pL0035 (www.mr4.com), which contains the h*dhfr*::y*fcu* selectable marker (SM) under the control of the *eef1α* promoter [65]. The h*dhfr*::y*fcu* marker is a fusion gene of the positive selection marker human *dihydrofolate reductase* and the negative selection marker, which is a fusion gene of yeast *cytosine deaminase* and *uridyl phosphoribosyl transferase* [65]. The 5'- and 3'- targeting regions (TR) of *dnmt2* were amplified from wild type *P. berghei* ANKA (cl15cy1) genomic DNA (primers used were shown in Table S1) and cloned into restriction sites of *Hind*III/ *Sac*II and *Xho*I/*Eco*RV of plasmid pL0035. Prior to transfection the DNA-construct was linearized with *Hind*III and *Eco*RV. Constructs targeting *rab5a*, *rab5b* and *rab11b* were kindly provided by Dr. Gordon Langsley (Faculte de Medecine, Universite

Paris Descartes) as a collaborative project. Other replacement constructs were generated by the modified two step PCR method (Figure S1A). Briefly, in the first PCR reaction two fragments of 5'- and 3'-TR were amplified from wild type genomic DNA with the primer sets P1/P2 and P3/P4 respectively (primers sequences shown in Table S1). The reverse primers of 5'TR (P2) and the forward primers of 3'TR (P3) have 5' extensions homologous to the h*dhfr* SM from pL0040 or to h*dhfr*::y*fcu* SM from pL0048. In the second PCR reaction, the 5'- and 3'-TR were annealed to either side of the selectable marker cassette, and the joint fragment was amplified by the external anchor-tag primers 4661/4662, resulting in the PCR-based targeting constructs. Before transfection, the PCR-based constructs were digested with appropriate restriction sites (as indicated in primer sequences in Table S1) to remove the 'anchor-tag' and with *Dpn*I that digests any residual uncut plasmids (Figure S1A).

Transfection and selection of transformed parasites with pyrimethamine was performed using standard technology for the genetic modification of *P. berghei* [62]. All information on the generation of gene-deletion mutants (as well as unsuccessful disruption attempts), such as DNA constructs and primers, has been submitted to the RMgmDB database of genetically modified rodent malaria parasites (www.pberghei.eu).

Clonal parasite lines were obtained from all gene-deletion mutants by the method of limiting dilution. Correct integration of DNA constructs and disruption of the genes was verified by diagnostic PCR analyses (see Table S2 for primers used) and/or Southern analyses of chromosomes separated by pulsed-field gel electrophoresis hybridized with probes specific for the selectable maker [62]. See Table S2 for primers used.

Northern analysis of blood stage mRNA were performed to confirm absence of transcripts. Total RNA was isolated from mixed blood-stages of wild type *P. berghei* ANKA (cl15cy1) and the different gene-deletion mutant lines. Northern blots were hybridized with probes specific for the open reading frame (ORF) of each gene after PCR amplification from wt *P. berghei* ANKA genomic DNA (primers shown in Table S2). As a loading control, Northern blots were hybridized with the oligonucleotide probe L644R that recognizes the large subunit ribosomal RNA (rRNA) [66].

The double gene-deletion mutant Δ*pm4*Δ*smac* which lacks expression of both PM4 and SMAC (schizont membrane-associated cytoadherence protein, PBANKA_010060) was generated by targeting *pm4* using construct PCR1597 in mutant Δ*smac3*$^{-sm}$, which lacks expression of SMAC and is free of SM (the generation of Δ*smac3*$^{-sm}$ is described in [46]).

The generation of the double gene-deletion mutant Δ*pm4*Δ*bp2* (lacking genes coding PM4 and BP2) is described in Chapter 5 using the same method as described for Δ*pm4*Δ*smac*.

Analysis of growth-attenuation

To determine growth-attenuation of the mutants, we determined their growth rate (multiplication rate) of asexual blood stages in mice. The multiplication rate of asexual blood stages in mice is determined during the cloning procedure [20] and is calculated as follows: the percentage of infected erythrocytes in Swiss OF1 mice injected with a single parasite is quantified at day 8 to 11 on Giemsa-stained blood films. The mean asexual multiplication rate per 24 hour is then calculated assuming a total of 1.2×10^{10} erythrocytes per mouse (2mL of blood). The percentage of infected erythrocytes in mice infected with reference lines of the *P. berghei* ANKA strain consistently ranges between 0.5–2% at day 8 after infection, resulting in a mean multiplication rate of 10 per 24 hour [20,67].

Analysis of Virulence-attenuation

The capacity of mutants to induce ECM was analysed in C57BL/6 mice. Groups of 6 mice were intraperitoneally (i.p) infected with 10^5–10^6 wild type *P. berghei* ANKA, or different mutant parasites. Onset of ECM in *P. berghei* infection was determined by measurement of a drop in body temperature below 34°C [20]. The body temperature of infected mice was measured twice a day from day 5 to day 8 after infection using a laboratory thermometer (model BAT-12, Physitemp Instruments Inc., Clifton, NJ) with a rectal probe (RET-2) for mice. When infected mice showed a drop in temperature (below 34°C), the mice were sacrificed. In addition to ECM in C57BL/6 mice we determined the course of parasitemia in BALB/c mice. Groups of 5–6 BALB/c mice were i.p infected with 10^4–10^6 mutants or wild type parasites. The course of parasitemia was determined by Giemsa-staining of blood smears once in every two days or every day during acute and peak infection. When mice developed high parasitemias (50–70%), the mice were sacrificed.

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Supplementary Material

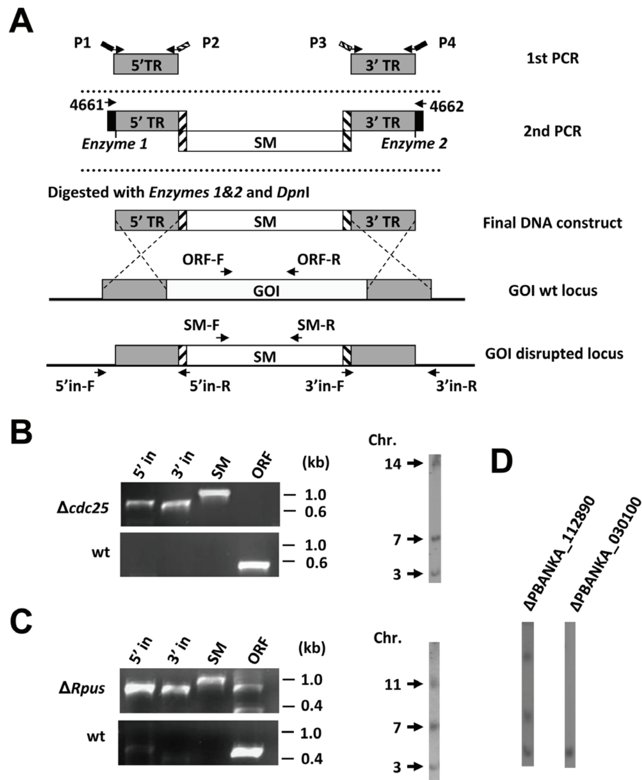

Figure S1. Generation of the *P. berghei* mutants Δ*cdc25*, Δ*Rpus*, ΔPBANKA_112890 and ΔPBANKA_030100.

A. Schematic representation of the double cross-over gene-deletion constructs generated using a modified two-step PCR method and the wild type (wt) loci of the gene of interest (GOI) before and after disruption. In the first PCR reaction, 5'- and 3'- targeting region (TR, grey boxes) of the gene of interest (GOI) were amplified from *P. berghei* ANKA genomic DNA with the primer sets P1/P2 and P3/P4. Primers P2 and P3 have 5'- extensions homologues to the selectable marker cassette (SM) (hatched boxes). This SM cassette was excised from plasmid pL0040 (h*dhfr*) or pL0048 (h*dhfr*::y*fcu*) digested with *Xho*I and *Not*I. Primers P1 and P4 have 5'-terminal extensions (black boxes) for the second PCR reaction. In the second PCR reaction, the 5'- and 3'- targeting sequences annealed to either side of the SM, and the joint fragment was amplified by the external anchor-tag primers 4661/4662. Before transfection, the PCR construct was digested with 1 (or 2) restriction enzymes that were introduced in primers P1 and P4 to remove the anchor-tag and with *Dpn*I to digest any residual plasmid. See Table S1 for primer sequences used to amplify the target regions. Primer positions (arrows) for diagnostic PCRs are shown (see Table S2 for primer sequences and expected product sizes).

B. Diagnostic PCRs (left) and Southern analysis of pulsed field gel-separated chromosomes (right) confirm correct disruption of *cdc25* in mutant Δ*cdc25*. The following primers were used for diagnostic PCRs: 5' integration (5' in): 5033/4770; 3' integration (3' in): 4771/5100; SM (h*dfhr*): 307C/3187; *cdc25* ORF: 5034/5035. Separated chromosomes were hybridized using a 3'UTR *pbdhfr* probe that recognizes the DNA-construct integrated into the *cdc25* locus on chromosome 14, the endogenous *dhfr/ts* on chromosome 7 and the GFP-luciferase cassette in the *230p* locus on chromosome 3.

C. Diagnostic PCRs (left) and Southern analysis of pulsed field gel-separated chromosomes (right) confirm

correct disruption of *Rpus* in mutant Δ*Rpus*. The following primers were used for diagnostic PCRs: 5' in: 5880/4770; 3' in: 4771/5881; SM (h*dfhr*::y*fcu*): 4698/4699; *Rpus* ORF: 5882/5883. Separated chromosomes were hybridized using a 3'UTR *pbdhfr* probe that recognizes the DNA-construct integrated into the *Rpus* locus on chromosome 11, the endogenous *dhfr*/*ts* on chromosome 7 and the GFP-luciferase reporter cassette in the *230p* locus on chromosome 3.

D. Southern analyses of pulsed field gel-separated chromosomes confirm correct disruption of PBANKA_112890 and PBANKA_030100 in ΔPBANKA_112890 and ΔPBANKA_030100, respectively. Separated chromosomes of ΔPBANKA_112890 were hybridized using a 3'UTR *pbdhfr* probe that recognizes the DNA-construct integrated into the PBANKA_112890 locus on chromosome 11, the endogenous *dhfr*/*ts* on chromosome 7 and the GFP-luciferase reporter cassette in the *230p* locus on chromosome 3. Separated chromosomes of ΔPBANKA_030100 were hybridized using an h*dhfr* probe that recognizes the DNA-constructs integrated into the PBANKA_030100 locus on chromosome 3.

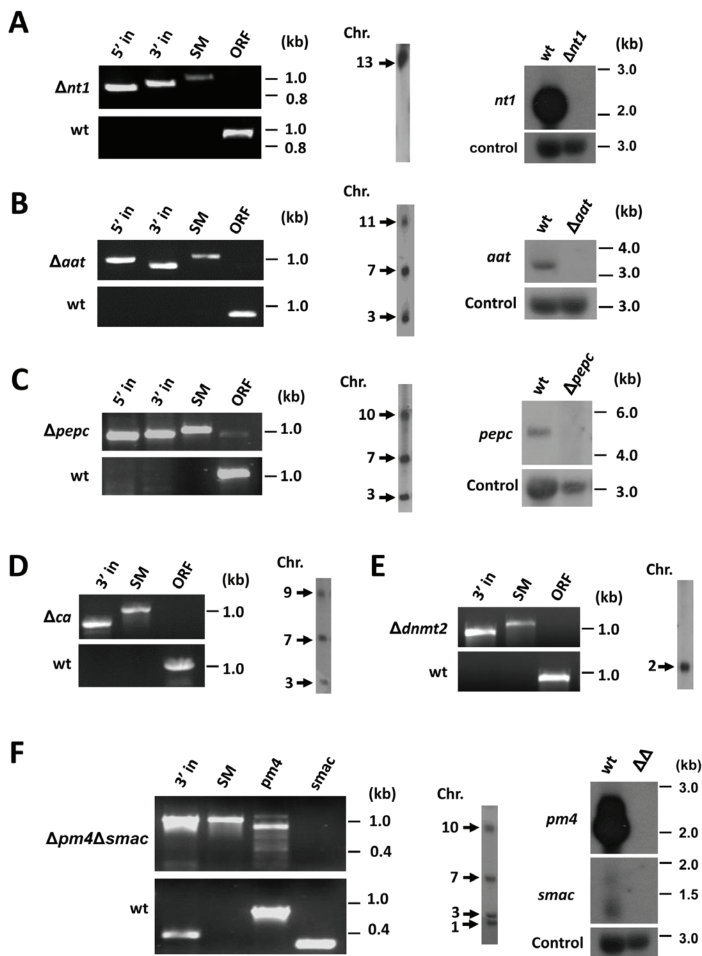

Figure S2. Genotype analysis of the *P. berghei* mutants Δ*nt1*, Δ*aat*, Δ*pepc*, Δ*ca*, Δ*dnmt2* and Δ*pm4*Δ*smac*

A. Diagnostic PCRs (left) and Southern analysis of pulsed field gel-separated chromosomes (center) confirm

correct disruption of *nt1* in mutant Δ*nt1*. Northern analysis of blood-stage mRNA (right) confirms the absence of *nt1* transcripts in Δ*nt1*. The following primers were used for diagnostic PCRs: 5' integration (5' in): 5855/4770; 3' integration: (3' in) 4771/5856; SM (h*dfhr*): 307C/3187; *nt1* ORF: 5857/5858. Separated chromosomes were hybridized using an h*dhfr* probe that recognizes the DNA-construct integrated into the *nt1* locus on chromosome 13. Northern blot was hybridized using a PCR probe recognizing the *nt1* ORF (primers 5857/5858) and with an oligonucleotide probe L644R that recognizes the large subunit ribosomal RNA (as loading control).

B. Diagnostic PCR (left) and Southern analysis of pulsed field gel-separated chromosomes (center) confirms correct disruption of *aat* in mutant Δ*aat*. Northern analysis of blood-stage mRNA (right) confirms the absence of *aat* transcripts in the Δ*aat*. The following primers were used for diagnostic PCRs: 5' in: 7115/4770; 3' in: 4771/7116; SM (h*dfhr*::y*fcu*): 4698/4699; *aat* ORF: 7117/7118. Separated chromosomes were hybridized using a 3'UTR *pbdhfr* probe that recognizes the construct integrated into the *aat* locus on chromosome 11, the endogenous *dhfr*/*ts* on chromosome 7 and the GFP-luciferase reporter cassette in the *230p* locus on chromosome 3. Northern blot was hybridized using a PCR probe recognizing the *aat* ORF (primers 7117/7118) and with an oligonucleotide probe L644R recognizing the large subunit rRNA (as loading control).

C. Diagnostic PCR (left) and Southern analysis of pulsed field gel-separated chromosomes (center) confirms correct disruption of *pepc* in mutant Δ*pepc*. Northern analysis of blood-stage mRNA (right) confirms the absence of *pepc* transcripts in the Δ*pepc*. The following primers were used for diagnostic PCRs: 5' in: 5977/4770; 3' in: 4771/5978; SM (h*dfhr*::y*fcu*): 4698/4699; *pepc* ORF: 5979/5980. Separated chromosomes were hybridized using a 3'UTR *pbdhfr* probe that recognizes the construct integrated into the *pepc* locus on chromosome 10, the endogenous *dhfr*/*ts* on chromosome 7 and the GFP-luciferase reporter cassette in the *230p* locus on chromosome 3. Northern blot was hybridized using a PCR probe recognizing the *pepc* ORF (primers 5979/5980) and with an oligonucleotide probe L644R recognizing the large subunit rRNA (as loading control).

D. Diagnostic PCRs (left) and Southern analysis of pulsed field gel-separated chromosomes (right) confirm correct disruption of *ca* in mutant Δ*ca*. The following primers were used for diagnostic PCRs: 3' in: 4771/6984; SM (h*dfhr*::y*fcu*): 4698/4699; *ca* ORF: 6985/6986. Separated chromosomes were hybridized using a 3'UTR *pbdhfr* probe that recognizes the DNA-construct integrated into the *ca* locus on chromosome 9, the endogenous *dhfr*/*ts* on chromosome 7 and the GFP-luciferase reporter cassette in the *230p* locus on chromosome 3.

E. Diagnostic PCRs (left) and Southern analysis of pulsed field gel-separated chromosomes (right) confirm correct disruption of *dnmt2* in mutant Δ*dnmt2*. The following primers were used for diagnostic PCRs: 3' in: 4239/5990; SM (h*dfhr*::y*fcu*): 4698/4699; *dnmt2* ORF: 5373/5374. Separated chromosomes were hybridized using an h*dhfr* probe that recognizes the DNA-construct integrated into the *dnmt2* locus on chromosome 2.

F. Diagnostic PCRs (left) and Southern analysis of pulsed field gel-separated chromosomes (middle) confirm correct disruption of *pm4* in the Δ*smac3*$^{-sm}$ mutant background. Northern analysis of blood-stage mRNA (right) confirms the absence of *pm4* and *smac* transcripts in the mutant Δ*pm4*Δ*smac.* The following primers were used for diagnostic PCRs: 3' in: 1662/5517; SM (h*dfhr*::y*fcu*): 4698/4699; *pm4* ORF: 5518/5519; *smac* ORF: 4204/4205. Separated chromosomes were hybridized using a 3'UTR *pbdhfr* probe that recognizes the DNA-construct integrated into the *pm4* locus on chromosome 10, the endogenous *dhfr*/*ts* on chromosome 7, the GFP-luciferase reporter cassette in the *230p* locus on chromosome 3 and the 3'*pbdhfr* sequence in in the disrupted *smac* locus on chromosome 1. Northern blot was hybridized using a PCR probe recognizing the *pm4* ORF (primers 5518/5519) or the *smarc* ORF (4204/4205) and with an oligonucleotide probe L644R recognizing the large subunit rRNA (as loading control). See Table S2 for primers used for generation of the probes.

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Table S1. Targeting constructs and primers

Gene	Construct	Basic construct	Descrip-tion	No.	Sequences	Restriction sites	Description
caf1	PCR1518	pL0040	P1	4674	GAACTCGTACTCCTTGGTGACGGGTACCATTGGGGAACTTGTTCAC	*Asp718*I	5'- *caf1* targeting region F
			P2	4762	CCTTCAATTTCGGATCCACTAGTTTCAGACTTCGACAACCAAG		5'- *caf1* targeting region R
	PCR1585	pL0040	P1	5342	GAACTCGTACTCCTTGGTGACGGGTACCTCCTTATGTAGCCATTGAC	*Asp718*I	5'- *caf1* targeting region F
			P2	5343	CATCTACAAGCATCGTCGACCTCCCAAGCCATATATAATACCTG		5'- *caf1* targeting region R
			P3	4727	CCTTCAATTTCGGATCCACTAGTGTGGCTTGTGTTAAAGAC		3'- *caf1* targeting region F
			P4	4675	AGGTTGGTCATTGACACTCAGCAGTACTGCCTCTTCCCATTATTCTG	*Sca*I	3'- *caf1* targeting region R
pp2c	PCR1699	pL0040	P1	5844	GAACTCGTACTCCTTGGTGACGGGTACCATATATCATTCTACGCATATCC	*Asp718*I	5'- *pp2c* targeting region F
	PCR1827	pL0048	P2	5845	CATCTACAAGCATCGTCGACCTCTTAGCATGTATTAGTTTGGAG		5'- *pp2c* targeting region R
			P3	4725	CCTTCAATTTCGGATCCACTAGCAAGAGAAATGAAGGAACAG		3'- *pp2c* targeting region F
			P4	4673	AGGTTGGTCATTGACACTCAGCAGTACTTCCGATGCGATAATTAGC	*Sca*I	3'- *pp2c* targeting region R
ApiAP2	PCR1831	pL0048	P1	5999	GAACTCGTACTCCTTGGTGACGGGTACCTATATACATCAACGATGCATTC	*Asp718*I	5'- *ApiAP2* targeting region F
			P2	6000	CATCTACAAGCATCGTCGACCTCTTTTTCCCCTCTTCTTGTC		5'- *ApiAP2* targeting region R
			P3	6001	CCTTCAATTTCGGATCCACTAGCCAGAACCTCCTATTTTCC		3'- *ApiAP2* targeting region F
			P4	6002	AGGTTGGTCATTGACACTCAGCAGTACTGTTCACCATTGGGATCAG	*Sca*I	3'- *ApiAP2* targeting region R
PBANKA_020890	PCR1691	pL0040	P1	5868	GAACTCGTACTCCTTGGTGACGGGTACCAATTGGTGCCATAACCAG	*Asp718*I	5'-PBANKA_020890 targeting region F
	PCR1774	pL0048	P2	5869	CATCTACAAGCATCGTCGACCTCTCGATCAACCGATTTAGC		5'-PBANKA_020890 targeting region R
			P3	5870	CCTTCAATTTCGGATCCACTAGCACTTGCATGCTATTGTC		3'-PBANKA_020890 targeting region F
			P4	5871	AGGTTGGTCATTGACACTCAGCGGTACCCATGATGCCTTCATATGTG	*Asp718*I	3'-PBANKA_020890 targeting region R
PBANKA_112890	PCR1830	pL0048	P1	6672	GAACTCGTACTCCTTGGTGACGAAGCTTAGAAACCCGATATCCCAGTG	*Hind*III	5'- PBANKA_112890 targeting region F
			P2	6673	CATCTACAAGCATCGTCGACCTCCGCGGCATGACAAAATACATATACAC	*Sac*II	5'- PBANKA_112890 targeting region R
			P3	6674	CCTTCAATTTCGGATCCACTAGCTCGAGAAGCACATGGAGAGAAAGG	*Xho*I	3'- PBANKA_112890 targeting region F
			P4	6675	AGGTTGGTCATTGACACTCAGCGATATCTTAGCTCTTGAAATATGATTTGC	*Eco*RI	3'- PBANKA_112890 targeting region R
PBANKA_030100	PCR1883	pL0048	P1	6731	GAACTCGTACTCCTTGGTGACGAAGCTTAAAAAGCTCCGTTTAAATTGG	*Hind*III	5'- PBANKA_030100 targeting region F
			P2	6732	CATCTACAAGCATCGTCGACCTCCGCGGTTTCCTCCATACTAGTTTGTC	*Sac*II	5'- PBANKA_030100 targeting region R
			P3	6733	CCTTCAATTTCGGATCCACTAGGTACCAAATGGGAATTTAATTATG	*Kpn*I	3'- PBANKA_030100 targeting region F
			P4	6734	AGGTTGGTCATTGACACTCAGCGATATCACCTCCATGTGTCATTTTTAC	*Eco*RV	3'- PBANKA_030100 targeting region R
cdc25	PCR1524	pL0040	P1	4676	GAACTCGTACTCCTTGGTGACGGGTACCCCAACTAATACATCATCACTC	*Asp718*I	5'-*cdc25* targeting region F

			P2	4728	CATCTACAAGCATCGTCGACCTCATATATTTTGGGATGCTTCTG		5'-*cdc25* targeting region R
			P3	4729	CCTTCAATTTCGGATCCACTAGCATTTTGAAGTTCCATAATAGTC		3'-*cdc25* targeting region F
			P4	4677	AGGTTGGTCATTGACACTCAGCAGTACTGTTATCGAGCCATATTTGC	*Sca*I	3'-*cdc25* targeting region R
Rpus	PCR1775	pL0048	P1	5876	GAACTCGTACTCCTTGGTGACGGGTACCTTCATGATTTGTACCTAATCTC	*Asp718*I	5'-*Rpus* targeting region F
			P2	5877	CATCTACAAGCATCGTCGACCTCCTTGTTTTCCTCCTAATACG		5'-*Rpus* targeting region R
			P3	5878	CCTTCAATTTCGGATCCACTAGTGATAACGAATCCTCATGTG		3'-*Rpus* targeting region F
			P4	5879	AGGTTGGTCATTGACACTCAGCGGTACCCCTAATGTGTTCATAGTATTCC	*Asp718*I	3'-*Rpus* targeting region R
ck	PCR1549	pL0040	P1	5193	GAACTCGTACTCCTTGGTGACGGGTACCAATATTTAGATCTTGTACAATTATAATTC	*Asp718*I	5'- *ck* targeting region F
			P2	5194	CATCTACAAGCATCGTCGACCTCACTTGAGATTTTTTTATTTTGATATG		5'- *ck* targeting region R
			P3	5195	CCTTCAATTTCGGATCCACTAGTCTATTGATTATTCTACAGACAC		3'- *ck* targeting region F
			P4	5196	AGGTTGGTCATTGACACTCAGCAGTACTATATAATTCAAAATGTTTGAAAGTG	*Sca*I	3'- *ck* targeting region R
cept	PCR1550	pL0040	P1	5205	GAACTCGTACTCCTTGGTGACGGGTACCCATTTTCATAAATGCATAACTG	*Asp718*I	5'- *cept* targeting region F
			P2	5206	CATCTACAAGCATCGTCGACCTCTCTTCATAACTTGCATTTCTC		5'- *cept* targeting region R
			P3	5207	CCTTCAATTTCGGATCCACTAGGACGGGTAAAATATACATCG		3'- *cept* targeting region F
			P4	5208	AGGTTGGTCATTGACACTCAGCAGTACTGATCATTAGCATTATGGTGTG	*Sca*I	3'- *cept* targeting region R
ek	PCR1643	pL0040	P1	5794	GAACTCGTACTCCTTGGTGACGGGTACCGCATCATTTCCCCTTATCG	*Asp718*I	5'- *ek* targeting region F
			P2	5795	CATCTACAAGCATCGTCGACCTCAACGGTAATGCAATTTCAG		5'- *ek* targeting region R
			P3	5796	CCTTCAATTTCGGATCCACTAGTCCCAACGTTATAATTTACTG		3'- *ek*targeting region F
			P4	5797	AGGTTGGTCATTGACACTCAGCAGTACTCGCCAAATGAACTAGCTC	*Sca*I	3'- *ek* targeting region R
nt1	PCR1693	pL0040	P1	5851	GAACTCGTACTCCTTGGTGACGGGTACCTTGTCCATCGTTATATTTATCC	*Asp718*I	5'- *nt1* targeting region F
	PCR1776	pL0048	P2	5852	CATCTACAAGCATCGTCGACCTCTTTATGAAAAATGGAGAATTCG		5'- *nt1* targeting region R
			P3	5853	CCTTCAATTTCGGATCCACTAGAATAAATCAATGTGTGCCTC		3'- *nt1* targeting region F
			P4	5854	AGGTTGGTCATTGACACTCAGCAGTACTATCTCGAATGGCTATTGC	*Sca*I	3'- *nt1* targeting region R
amino acid transporter	PCR1925	pL0048	P1	7111	GAACTCGTACTCCTTGGTGACGTCGCGATTGCTGCTGTATTTTTATTCTGG	*Nru*I	5'-*aat* targeting region F
			P2	7112	CATCTACAAGCATCGTCGACCTCAATAGATGCAATCATTCATACC		5'-*aat* targeting region R
			P3	7113	CCTTCAATTTCGGATCCACTAGGAAGTGTGCTTTTTACTTTATACC		3'-*aat* targeting region F
			P4	7114	AGGTTGGTCATTGACACTCAGCTCGCGATGCAGTTTATAAGCCGAGCTTG	*Nru*I	3'-*aat* targeting region R
pepc	PCR1777	pL0048	P1	5973	GAACTCGTACTCCTTGGTGACGGGTACCGATAATGTCCTACTTTTTCTTTG	*Asp718*I	5'-*pepc* targeting region F
			P2	5974	CATCTACAAGCATCGTCGACCTCTATATAGCTGCTTGAGACAC		5'-*pepc* targeting region R
			P3	5975	CCTTCAATTTCGGATCCACTAGGCAAAATACCGGATAACTC		3'-*pepc* targeting region F
			P4	5976	AGGTTGGTCATTGACACTCAGCGGTACCTTTAGGAAACCAATCAAAGAG	*Sca*I	3'-*pepc* targeting region R

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ca	PCR1881	pL0048	P1	6979	GAACTCGTACTCCTTGGTGACGTCGCGACCTTTGATATTACAAACATATTATAC	*Nru*I	5'-*ca* targeting region F
			P2	6980	CATCTACAAGCATCGTCGACCTCGATTTACAAAACTGGATAATCAC		5'-*ca* targeting region R
			P3	6981	CCTTCAATTTCGGATCCACTAGTTTTTTTTGCTGAATGATTAGG		3'-*ca* targeting region F
			P4	6982	AGGTTGGTCATTGACACTCAGCTCGCGACCTGGACATATTTTCAATATTAC	*Nru*I	3'-*ca* targeting region R
hemolysin	PCR1591	pL0040	P1	5386	GAACTCGTACTCCTTGGTGACGGGATCCTTCTAAAATCCCCATATACAC	*BamHI*	5'- *hemolysin* targeting region F
			P2	5387	CATCTACAAGCATCGTCGACCTCCTTTGGGGTTTTATGTGAG		5'- *hemolysin* targeting region R
			P3	5388	CCTTCAATTTCGGATCCACTAGATATGTCCCAATCAAATACAC		3'- *hemolysin* targeting region F
			P4	5389	AGGTTGGTCATTGACACTCAGCAGTACTATTACTTGAAACATACGCAC	*Sca*I	3'- *hemolysin* targeting region R
hep17	pL1415	pL0037	P1	3953	ATGCTCGTCGACATATTGTACATAAGCCATTTTGCC	*Sal*I	5'- *hep17* targeting region F
			P2	3955	ATGCTCAAGCTTAGGCCATGAAAAGGAGGAGC	*Hind*III	5'- *hep17* targeting region R
			P3	3596	ATGCTCGAATTCGTTAGCCTTACTAAGGTCATGCG	*Eco*RI	3'- *hep17* targeting region F
			P4	3597	ATGCTCCCCGGGTGTATCCTGCTTATATCGATTTGTGC	*Xma*I	3'- *hep17* targeting region R
	PCR1555	pL0040	P1	5213	GAACTCGTACTCCTTGGTGACGGGTACCTATTTTTATGTAGCTCCTCC	*Asp718*I	5'- *hep17* targeting region F
			P2	5214	CATCTACAAGCATCGTCGACCTCAGAAAATATAGTGCTATATGTG		5'- *hep17* targeting region R
			P3	5215	CCTTCAATTTCGGATCCACTAGTATCATAAAAAGTTTCGACTC		3'- *hep17* targeting region F
			P4	5216	AGGTTGGTCATTGACACTCAGCAGTACTTTAATGTCCCCAATTATGG	*Sca*I	3'- *hep17* targeting region R
dnmt2	pL1789	pL0035	P1	6468	GCCCAAGCTTATAAAGCCGTGGAAAGGTG	*Hind*III	5'-*dcm* targeting region F
			P2	6469	TTCCCCGCGGCCCCATAATATACACAAGTGC	*Sac*II	5'-*dcm* targeting region R
			P3	6373	CCGCTCGAGAGCTTTAAAACACAGTTAAGAAAATTG	*Xho*I	3'-*dcm* targeting region F
			P4	6374	GCGCGGATATCGTTAAATACTAGCATGTAAATTGG	*Eco*RV	3'-*dcm* targeting region R
pm4	pL1873	pL0048	P1	L6861	GAACTCGTACTCCTTGGTGACGTCGCGACCTTGTCGGGGTACTCAG	*Nru*I	*pm4* 5'-targeting sequence, F
			P2	L6862	CATCTACAAGCATCGTCGACCTCAAGCTTCCCAATCTCTTTAATAAGG		*pm4* 5'-targeting sequence, R
			P3	L6863	CCTTCAATTTCGGATCCACTAGACACGTACCATAACATGC		*pm4* 3'-targeting sequence, F
			P4	L6864	AGGTTGGTCATTGACACTCAGCTCGCGATTCCTACAAATCAAATATCACG	*Nru*I	*pm4* 3'-targeting sequence, R
		anchor-tag primers					
				4661	GAACTCGTACTCCTTGGTGACG		anchor-tag primer, F
				4662	AGGTTGGTCATTGACACTCAGC		anchor-tag primer, R

Red: restriction sites

Blue: 5'- extensions homologues to the h*dhfr*::y*fcu* selectable marker cassette from pL0048

Green: 5'- extensions homologues to the anchor tag primers 4661/4662

Table S2. Primers for genotyping

Genes	No.	Primer sequences	Description	Integration PCR Pair	Expected product size (bp)
Primers for PCR analyses					
caf1	5029	CATGTATGGATACAATTTAATCG	*caf1* 5' in-F for pL1518	4770	801
	2849	aaacaattgAAAATCGTAGATGTATGG	*caf1* 5' in-F for pL1585	4770	788
	5030	GTTTACATCACTTCCATAGTC	*caf1* 3' in-R	4771	837
	5031	GTTGTTAGTATTGGCACAC	*caf1* ORF-F		578
	5032	TTCATAGCACAATTGTTACTC	*caf1* ORF-R		
pp2c	5846	AGATTGGTGTATATAAAAGACTG	*pp2c* 5'in-F	4770	932
	4978	CCGATTAATGATATGCGTG	*pp2c 3'*in-R	4771	853
	5847	CGGCATTTTAGAATGTATGAC	*pp2c* ORF-F		1022
	4980	GGAACTCCGGTATTTGAG	*pp2c* ORF-R		
ApiAP2	6003	GCGAATGGTTATTTATACATGC	*ApiAP2* 5'in-F	4770	859
	6004	TGTAACTATTTGTTCGTTTCC	*ApiAP2 3'*in-R	4771	877
	6005	GTGATAAATTTCCATGAATTGC	*ApiAP2* ORF-F		850
	6006	AGAGGTTAGATGATTGATGTG	*ApiAP2* ORF-R		
PBANKA_020890	5872	TCGAAAATTAGCATATGAAGG	PBANKA_020890 5'in-F	4770	870
	5873	CCAATTACACCAAAATTTCAC	PBANKA_020890 3'in-R	4771	610
	5874	ATATTAGAAGAAGCACTTATGG	PBANKA_020890 ORF-F		618
	5875	TTCATAAGGAGCATCATGAC	PBANKA_020890 ORF-R		
cdc25	5033	TCTACTATTTCTCATTTCTTCAC	*cdc25* 5' in-F	4770	893
	5100	TAATGTGAAGCCACATCC	*cdc25* 3' in-R	4771	835
	5034	GGAAAATAACAGCGTCAG	*cdc25* ORF-F		567
	5035	CCTACATAGACGTTGTCAC	*cdc25* ORF-R		
Rpus	5880	ACGTGTAATGTGATTATATACC	*Rpus* 5' in-F	4770	856
	5881	TTAATTGAAATCGAACATTTGG	*Rpus* 3' in-R	4771	831
	5882	CCCCAAAGATTCTCACAC	*Rpus* ORF-F		597
	5883	CCAGCATTTTCGTTAACTC	*Rpus* ORF-R		
Rab5a	5348	CCAGCAAATATCATATGGAG	*rab5a* 5'in-R	3189	1193
	5349	CATGAATCCAAAGTATTTATGTG	*rab5a* 3'in-F	4239	1015
	5350	AATAATAATAACGGTGATAATCG	*rab5a*ORF-R		506
	5351	TTTGTTTTTTGTTGTTTTTCAC	*rab5a*ORF-F		
Rab5b	6909	TTAAAATTGTTAGTTGCTTTGTG	*rab5b* 5'in-F	3189	1221
	6910	TATGCCAAATTTAATAGAAAATTCAG	*rab5b* 3'in-R	4239	1015
	6911	GCAGCTTTTTTGCACCATAC	*rab5b*ORF-F		555
	6912	TTACCTCTGAATTTATTTTTTGTG	*rab5b*ORF-R		
Rab11b	6297	CTTTACCAATTTTGCTAAATAAGG	*rab11b* 5'in-F	3189	853
	6298	TCTATTTCAAAGGTGCAAGAG	*rab11b* 3'in-R	4239	896
	6299	CCAGGTAAAACACATTTATTGTC	*rab11b*ORF-F		992
	6300	GCACTTTCATATGTTTCATGAC	*rab11b*ORF-R		
ck	5840	GCATTTGTTTATATATCACAGAG	*ck* 5' in-F	4770	640
	5197	GTAGCATGGAAAATGTTCTC	*ck* 3' in-R	4771	786
	5198	TGAAGTATATGAAACGATGAG	*ck* ORF-F		474
	5199	GTAGCTATGAAATTATATCCAG	*ck* ORF-R		
cept	5972	TTATCATAATATAAGGCATCTACC	*cept* 5' in-F	4770	942
	5210	TGATGATCTCGAATATACAG	*cept* 3' in-R	4771	726

	5211	TTATGCGAACCGTATTGG	*cept* ORF-F		546
	5212	AAACGTACTGAAGTAATTGC	*cept* ORF-R		
ek	5836	TTGTTTATTTTAAGCACTTTCC	*ek* 5' in-F	4770	917
	5837	GATGCACAAAATGATGCAG	*ek* 3' in-R	4771	759
	5838	ATACAGAAATTCCAGAAAAACG	*ek* ORF-F		1042
	5839	CGGGTTGGTATTAAATTCC	*ek* ORF-R		
nt1	5855	CGTCAACTTAAAAATTGTATGC	*nt1* 5' in-F	4770	791
	5856	TGTTTTACGGATTAAAGATCAC	*nt1* 3' in-R	4771	883
	5857	CTGTTTTAGCCCTTTTCG	*nt1* ORF-F		995
	5858	GTATAAGCATGTGGTTAGC	*nt1* ORF-R		
aat	7115	AAAATGAAATTAATCCAAAACAATAC	*aat* 5' in-F	4770	1034
	7116	ATTATACCCATAGCAAGAATTGTG	*aat* 3' in-R	4771	885
	7117	TGATGTGGTTCAAAATATAGTG	*aat* ORF-F		883
	7118	TAATGGGAGCACTAATAAGC	*aat* ORF-R		
pepc	5977	GGGCTTTATACTATTTTTTTGTC	*pepc* 5' in-F	4770	954
	5978	TATCGTGGTAGAGTAAAACTG	*pepc* 3' in-R	4771	997
	5979	CATGATTTATCCGAAAAATATAGTG	*pepc* ORF-F		1003
	5980	GTGCTTTATATACATATACAACAC	*pepc* ORF-R		
ca	6983	ACCCCAACTTATTTAAAGATAG	*ca* 5' in-F	4770	798
	6984	CAAAGATTCGATTATTCAAAGAG	*ca* 3' in-R	4771	836
	6985	AGAGCGAATATTATTTGAATTGC	*ca* ORF-F		1013
	6986	CATAATCATAGATCTCATTAGTACTG	*ca* ORF-R		
hemolysin	5390	ACTGTATATGGATGCATGG	*hemolysin* 5' in-F	4770	810
	5391	AATTTCTTTTGGGTTTGACG	*hemolysin* 3' in-R	4771	734
	5392	ATGAAAAAACGCTGCTGAG	*hemolysin* ORF-F		561
	5393	TGAGGAAATAAGACATACCAG	*hemolysin* ORF-R		
hep17	4355	ttgcatactcgagCAAACCCGAGAATAAAATTAAATTATTC	*hep17* 5' in-F	4770	1121
	4356	aataatactcgagCAAATGGTGATCCAAATATAAAGGCC	*hep17* 3' in-R	4771	899
	3942	CGATTCAAAAAATATAATAATGTAGAG	*hep17* ORF-F		476
	3911	GGCTAACATTTTCTAAAAGTAGAG	*hep17* ORF-R		
dnmt2	5990	ATTACTATTTACAACGGATGC	*dcm* 3' in-R	4239	953
	5373	TGATTCGGAGGAAAATTCAC	*dcm* ORF-F		936
	5374	TGCTTGAAATTATTTACCACC	*dcm* ORF-R		
pm4	5517	CATGCGAATAAATGCTCAG	*pm4* 3' in-R	1662	1122
	5518	TCCGAATATTTAACAATTCGTG	*pm4* ORF-F		869
	5519	ATGAAAGGTACTGGAATACTC	*pm4* ORF-R		
smac	4204	CACCATGGATAAATACGATAACAATGGAAAATCATTGG	*smac* ORF-F		328
	4205	AATGATCTTAGAATTATGTCTTAGCCTTTCC	*smac* ORF-R		
Universal primers					
	4770	CATCTACAAGCATCGTCGACCTC	5'*pbeef1a* R, 5'in-R		
	4771	CCTTCAATTTCGGATCCACTAG	3'*pbdhfr/ts* F, 3'in-F		
	3189	CTGGTGCTTTGAGGGGTG	5' *eef1a* R, 5'in-R		
	4239	GATTTTTAAAATGTTTATAATATGATTAGC	3'*pbdhfr/ts* F, 3'in-F		
	1662	GATTCATAAATAGTTGGACTTG	3'*pbdhfr/ts* F		
	307C	GCTTAATTCTTTTCGAGCTC	h*dhfr* F, SM-F		1009
	3187	GTGTAGTCTGTGTGCATGTC	3'*pbdhfr/ts* R, SM-R		
	4698	GTTCGCTAAACTGCATCGTC	h*dhfr* F, SM-F		1108
	4699	GTTTGAGGTAGCAAGTAGACG	y*fcu* R, SM-R		

Other Primers for generation of probes				
	692	CTTATATATTTATACCAATTG	3'*pbdhfr/ts* F	404
	693	GTTTTTTTTTAATTTTTCAAC	3'*pbdhfr/ts* R	
	886	GGAAGATCTATGGTTGGTTCGCTAAACTGCATCG	h*dhfr* F	582
	887	GGAAGATCTTTAATCATTCTTCTCATATACTTC	h*dhfr* R	
	L644R	GGAAACAGTCCATCTATAATTG	*lsu rrna* (A-type)	

pb = *P. berghei*, h = human, y = yeast

5' in=5' integration PCR; 3' in=3' integration PCR