

Loss-of-function analyses defines vital and redundant functions of the *Plasmodium* rhomboid protease family

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Summary

Rhomboid-like proteases cleave membrane-anchored proteins within their transmembrane domains. In apicomplexan parasites substrates include molecules that function in parasite motility and host cell invasion. While two *Plasmodium* rhomboids, ROM1 and ROM4, have been examined, the roles of the remaining six rhomboids during the malaria parasite's life cycle are unknown. We present systematic gene deletion analyses of all eight *Plasmodium* rhomboid-like proteins as a means to discover stage-specific phenotypes and potential functions in the rodent malaria model, *P. berghei*. Four rhomboids (ROM4, 6, 7 and 8) are refractory to gene deletion, suggesting an essential role during asexual blood stage development. In con-

trast ROM1, 3, 9 and 10 were dispensable for blood stage development and exhibited no, subtle or severe defects in mosquito or liver development. Parasites lacking ROM9 and ROM10 showed no major phenotypic defects. Parasites lacking ROM1 presented a delay in blood stage patency following liver infection, but in contrast to a previous study blood stage parasites had similar growth and virulence characteristics as wild type parasites. Parasites lacking ROM3 in mosquitoes readily established oocysts but failed to produce sporozoites. ROM3 is the first apicomplexan rhomboid identified to play a vital role in sporogony.

Introduction

Rhomboid-like proteins are intramembrane serine proteases that hydrolyse a substrate within its transmembrane (TM) spanning domain (Freeman, 2004). The first rhomboid protease described was *Drosophila melanogaster* Rhomboid-1 that initiates cell signalling by cleaving the membrane-resident Spitz, an epidermal growth factor (EGF)-like ligand precursor, releasing it as an activated molecule from the membrane and promoting subsequent EGF-receptor signalling (Urban *et al.*, 2001). With the exception of viruses, rhomboid-like proteins are found in all kingdoms of life and share a conserved core structure of six TM domains that contains the catalytic serine-histidine diad (Koonin *et al.*, 2003; Wang *et al.*, 2006; Wu *et al.*, 2006; Ben-Shem *et al.*, 2007; Lemberg and Freeman, 2007). Interestingly, as a family of proteins, they share only around 5% sequence identity in the core region (Urban *et al.*, 2002b) and have highly variable amino termini. Some members have additional TM domains at either N- or C-termini of the core six, but their precise functions are unknown. Rhomboids function in cell–cell signalling in metazoans (Urban *et al.*, 2002a; Sundaram, 2004), quorum sensing between bacteria (Stevenson *et al.*, 2007), facilitate mitochondrial membrane fusion in yeast (McQuibban *et al.*, 2003) and regulate apoptosis (Cipolat *et al.*, 2006).

Many eukaryotic organisms contain large rhomboid gene families. Multiple rhomboid-like enzymes are also found in the genomes of apicomplexan parasites such as *Toxoplasma gondii*, *Plasmodium* spp., *Eimeria tenella*, *Cryptosporidium* spp., *Theileria* spp. and *Babesia bovis*.

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Based on a phylogenetic clustering of rhomboid-like proteins, a nomenclature has been defined according to the initial assignment of *T. gondii rhomboids* (Dowse and Soldati, 2005) which expresses six rhomboid-like proteins TgROM1–6. Only four TgROMs (i.e. ROM1, 3, 4 and 6) have direct *Plasmodium* homologues (Dowse and Soldati, 2005); there are no direct homologues of TgROM2 and TgROM5 in *Plasmodium* and ROM7–10 are only present in *Plasmodium* but not in *T. gondii*. Although *Plasmodium* species do not have a direct TgROM5 homologue, *P. falciparum* ROM1 and ROM4 share substrate specificities with TgROM5 (Baker *et al.*, 2006). Apicomplexan ROM6 is most likely an evolutionarily ancient mitochondrial PARL-like rhomboid, whereas all the others seem to be unique to apicomplexan parasites (Dowse and Soldati, 2005). Interestingly, *Plasmodium* ROM9 also has a putative mitochondrial-targeting sequence and clusters with PARL-like rhomboids (Santos *et al.*, 2011b).

Although *in vitro* certain rhomboids of apicomplexan parasites can cleave adhesins that are known to mediate recognition of, and attachment to host cells, their *in vivo* substrate specificities are largely unknown (Freeman, 2009; Santos *et al.*, 2011b). Understanding the biological functions of apicomplexan rhomboids is an active area of research because of the critical roles identified for several of these molecules in host cell invasion and pathogenesis (Freeman, 2009; Buguliskis *et al.*, 2010; Santos *et al.*, 2011b). In *T. gondii*, TgROM4 has been shown to process surface adhesins including MIC 2, 3 (microneme proteins) and apical membrane antigen 1 [AMA1 (Buguliskis *et al.*, 2010)]; this TgROM4-mediated AMA1 cleavage critically regulates the parasite's switch from an invasive to a replicative mode (Santos *et al.*, 2011a). TgROM1 is localized in micronemes (Brossier *et al.*, 2005) and although it is critical for parasite growth, TgROM1 is not essential for parasite invasion (Brossier *et al.*, 2008). From the eight *Plasmodium* rhomboids, only ROM1 and ROM4 have been analysed in more detail. *In vitro* substrates of the *P. falciparum* proteins include the merozoite-specific proteins AMA1 and proteins of the EBL (erythrocyte binding ligands) and RBL (reticulocyte binding ligands) families as well as several proteins of the invasive ookinete- and sporozoite-stages, such as TRAP (thrombospondin-related anonymous protein), CTRP (circumsporozoite- and TRAP-related protein) and MAEBL (merozoite adhesive erythrocytic binding protein) (Baker *et al.*, 2006). Structural analysis of the RBL protein EBA175 (Erythrocyte-binding antigen 175) and TRAP provided evidence for shedding of these proteins by a rhomboid-like protease (O'Donnell *et al.*, 2006) and indeed both proteins are cleaved *in vitro* by ROM4 (O'Donnell *et al.*, 2006; Ejigiri *et al.*, 2012). In *P. falciparum* only a small fraction of AMA1 is shed by ROM1 and the intramembrane cleavage can be reduced to undetectable levels by mutagenesis

without discernible phenotypic consequences (Olivieri *et al.*, 2011). The successful generation of *P. berghei* and *P. yoelii* mutants that lack expression of ROM1 also demonstrated that ROM1 is not essential for blood stage multiplication (Srinivasan *et al.*, 2009; Vera *et al.*, 2011). Blood stages of these mutants suffer only from a minor reduction in growth, again indicating that intramembrane cleavage of AMA1 by ROM1 is not essential for invasion of erythrocytes. ROM1 mutants in *P. yoelii* also showed a slight growth defect during liver stage development which has been attributed to reduced cleavage of the parasitophorous vacuole protein UIS4 (upregulated in sporozoites 4) by ROM1 (Vera *et al.*, 2011). Whether additional proteins are processed by *Plasmodium* ROM4 or ROM1 *in vivo* is still unknown. The non-essential nature of ROM1 during the complete life cycle indicates that other rhomboids or proteases from other families can cleave substrates of ROM1. ROM4 on the other hand has been reported to be essential for blood stage growth as attempts to mutate the *rom4* gene of *P. falciparum* failed (O'Donnell *et al.*, 2006). However, differences in cellular localization and *in vitro* substrate specificities of ROM1 and ROM4 (Baker *et al.*, 2006; O'Donnell *et al.*, 2006) raise questions as to whether these rhomboids cleave the same substrates *in vivo*. In blood stage *P. falciparum* ROM1 is located in micronemes of merozoites and ROM4 is embedded into the merozoite plasma membrane (O'Donnell *et al.*, 2006).

Due to the absence of data defining *Plasmodium* rhomboid expression, cellular localization, or importance during the parasite's life cycle, it is largely unknown which, if any rhomboids are biologically essential, redundant or perhaps share overlapping functions. We therefore undertook a genetic screen of all eight rhomboids by targeting each gene for deletion by homologous recombination using the genetically most tractable malaria parasite, *P. berghei*. Multiple attempts to disrupt genes encoding ROM4, ROM6, ROM7 and ROM8 failed, suggesting that these proteins have essential, non-redundant functions during blood stage development. We show that in addition to ROM1, three other rhomboid-like proteases – ROM3, ROM9 and ROM10 – are redundant for asexual blood stage development in mice. Parasites lacking ROM9 and ROM10 showed no major phenotypic defects during the entire life cycle; however, parasites lacking ROM1 were slightly compromised with respect to their ability to develop in the liver. Although already transcribed strongly in the female gametocyte, ROM3 is crucial during oocyst development inside the mosquito. Parasites lacking ROM3 produce normal numbers of ookinetes that readily establish oocysts in the mosquito. Although normal in size and number, oocysts fail to sporulate, suggesting that ROM3 plays a key role in the regulation of cytokinesis and production of individual sporozoites.

Table 1. Expression profile of *Plasmodium rhomboids*.

Protein	Gene ID	mRNA				Protein			
<i>P. berghei</i>									
		As^{a,b}	Gct^{a,b}	Ook^a	Sp^{a,b}	As^c	Gct^d		Sp^e
ROM1	PBANKA_093350	+ (-)	+ (-)	++	+ (++)	- (+ ^e)	±		- (+ ^e)
ROM3	PBANKA_070270	- (++)	+ (-)	-	- (-)	-	-		-
ROM4	PBANKA_110650	+ (++)	+ (-)	++	+ (++)	- (+ ^f)	-		- (+ ^f)
ROM6	PBANKA_135810	+ (-)	+ (-)	-	- (+)	-	-		-
ROM7	PBANKA_113460	+ (-)	- (-)	-	- (+)	-	-		-
ROM8	PBANKA_103130	+ (-)	- (-)	-	- (-)	-	-		-
ROM9	PBANKA_111470	± (-)	± (-)	-	+ (-)	-	-		-
ROM10	PBANKA_111780	+ (++)	± (-)	+	- (-)	-	-		-
<i>P. falciparum</i>									
		As^g	Gct^g		Sp^h	As^c	Gct^e	Ooc^c	Sp^c
ROM1	PF3D7_1114100	+	±		+	+	-	-	-
ROM3	PF3D7_0828000	±	++		±	±	-	-	-
ROM4	PF3D7_0506900	+	++		+	++	-	-	+
ROM6	PF3D7_1345200	±	±		±	-	±	-	-
ROM7	PF3D7_1358300	±	-		-	-	-	-	-
ROM8	PF3D7_1411200	+	+		+	-	-	-	-
ROM9	PF3D7_0515100	±	±		±	-	±	-	-
ROM10	PF3D7_0618600	+	±		±	±	+	-	-

a. RT-PCR results in this study (see Fig. S1).

b. PlasmoDB EST data in parentheses (-, no data; +, 1 EST; ++, 2–7 ESTs).

c. PlasmoDB proteome data (-, no data; ±, 1–2; +, 3–10; ++, > 10).

d. PbANKA Male vs female gametocyte proteome (Khan *et al.*, 2005).

e. IFA evidence using anti-PbROM1 serum (Srinivasan *et al.*, 2009).

f. Western and IFA evidence using anti-PbROM4 serum (Ejigiri *et al.*, 2012).

g. PlasmoDB RNAseq data (-, no data; ±, 0–6; +, 6–10) RPKM (log2).

h. PlasmoDB oligo array (-, no data; ±, 1–100; +, 100–1000; ++, > 1000) RMA value.

AS, asexual stages; Gct, gametocytes; Ook, ookinetes; Ooc, oocysts; Sp, sporozoites.

Results

The gene expression profiles of rhomboids across the parasite life cycle

The genome of the rodent malaria parasite *P. berghei* contains eight genes encoding rhomboid proteases (Dowse and Soldati, 2005) (Gene IDs are shown in Table 1). Each member shows a high level of sequence identity (70–85%) with its rhomboid orthologue from the human malaria parasite *P. falciparum* and shares a syntenic location in the genome (<http://www.plasmodb.org>) with an identical exon/intron gene structure.

We compared expression data of all rhomboid genes in *P. berghei* and *P. falciparum* in publicly available literature as well as transcriptome and proteome datasets across the life cycle (Table 1) (<http://www.plasmodb.org> and Khan *et al.*, 2005). Immunofluorescence assays (IFA) on HA-tagged ROM1 and ROM4 show expression of these proteins in dividing schizonts and merozoites in *P. falciparum* (O'Donnell *et al.*, 2006). A similar pattern of expression of *P. berghei* and *P. yoelii* ROM1 has been shown in schizonts and merozoites by IFA (Srinivasan *et al.*, 2009; Vera *et al.*, 2011). In both *P. berghei* and *P. falciparum* ROM1 is located in the micronemes. *P. berghei* ROM1 and ROM4 of *P. falciparum* and *P. berghei* have been detected in the sporozoite stage (Srinivasan *et al.*, 2009; Ejigiri *et al.*, 2012) (Table 1). Our

RT-PCR analyses (Fig. S1) demonstrate transcription of *P. berghei rom1* and *rom4* in blood stages and sporozoites. These different observations indicate that both ROM1 and ROM4 of human and rodent parasites have comparable patterns of expression and cellular location. For other rhomboids the information on stage-specific expression is much more limited. Proteome analyses indicate that ROM6, ROM9 and ROM10 are expressed in *P. falciparum* gametocytes (Table 1); however, proteome evidence for expression of these proteins in *P. berghei* gametocytes or other blood stages is absent. ROM7 and ROM8 are absent in all *P. falciparum* and *P. berghei* proteomes and ROM3 is only detected in *P. falciparum* blood stages. EST, RNAseq (Table 1) and RT-PCR analyses (Fig. S1), however, indicate that ROM3, ROM7 and ROM8 are expressed in blood stages and therefore the absence in proteomes may suggest that rhomboids are relatively low abundant proteins, although we have to take into account the difficulties of proteomic sequencing multi-pass TM proteins. *P. falciparum* RNAseq analyses indicate that transcription of *rom3* and *rom4* is upregulated in gametocytes compared with asexual stages (Table 1). RNAseq analyses of different *P. berghei* blood stages also showed strongly increased transcripts levels of *rom3* and *rom4* in gametocytes (W.A.M. Hoeijmakers, A. Religa, C.J. Janse, A.P. Waters and H.G. Stunnenberg, unpubl. data). We con-

firmed transcription of these rhomboids in *P. berghei* gametocytes by RT-PCR (Fig. S1).

The similarities between the eight rhomboid genes of *P. falciparum* and *P. berghei* and similarities in expression patterns and cellular locations of several rhomboids point towards a conserved function between rhomboid orthologues across the parasites' life cycle in both species. Since most rhomboids appear to be expressed in multiple life cycle stages, the expression pattern is, however, not a good indicator for a putative role at a distinct life cycle stage.

Evidence for an essential role of ROM4, 6, 7 and 8 in asexual blood stage parasites – ROM1, 3, 9 and 10 are dispensable

As a first step towards understanding the roles of rhomboid proteases during the *Plasmodium* life cycle, we undertook a systematic, individual gene deletion approach for each of the eight *P. berghei* rhomboid genes and provide here a detailed analysis of the growth characteristics of gene deletion mutants for four rhomboids. Using well-established standard assays, the following phenotypes of the gene deletion mutants were characterized throughout the complete life cycle including blood, mosquito and liver stages: *in vivo* asexual blood stage multiplication rate, *in vivo* gametocyte production, *in vitro* ookinete formation, oocyst and sporozoite development in *Anopheles stephensi* mosquitoes and the prepatent period in mice after injection of sporozoites. The prepatent period is defined as the time taken to achieve a 0.5–2% blood stage parasitemia in mice after intravenous inoculation of 10^4 sporozoites.

Standard genetic modification technologies used to replace entire *rhomboid* genes by double cross-over integration with a drug resistant marker resulted in gene deletion mutants for *rom1*, *3*, *9* and *10*, while multiple attempts to disrupt *rom4*, *6*, *7* and *8* were unsuccessful (see Tables S1, S2 and S3 for details of these unsuccessful gene-deletion attempts including primers used to amplify the targeting sequences, generate the gene-deletion constructs and genotype). We demonstrated by RT-PCR that *rom4*, *6*, *7* and *8* are transcribed in blood stages (Fig. S1) and the multiple unsuccessful attempts to disrupt *rom4*, *6*, *7* and *8* may indicate that these genes have a critical function for asexual blood stage growth and multiplication. While a failure to disrupt a gene is not an unequivocal proof that the encoded protein is essential for blood stage growth/multiplication for *rom4* we show that the failure of disruption is not due to refractoriness of the genetic locus to genetic modification by creating a transgenic mutant (*rom4::mCherry*) that expresses a C-terminally mCherry-tagged ROM4 (Fig. S2). Genotype analysis of the *rom4::mCherry* parasites and analysis of ROM4::mCherry

expression (Fig. S2) demonstrates that correct genetic modification of the *rom4* locus is possible. Immunofluorescence analysis of ROM4::mCherry revealed its expression in gametocytes (Fig. S2); however, the very weak fluorescence signals in asexual blood stages prevented confirmation of merozoite surface location observed in *P. falciparum* (Fig. S2). The normal asexual multiplication rate (Table 2) indicates that the mCherry-tagged ROM4 functions normally during blood stage development. All information on the failed attempts to disrupt *rom4*, *6*, *7* and *8*, including DNA constructs and primers have been submitted to the RMgmDB database of genetically modified rodent malaria parasites (<http://www.pberghei.eu>).

The correct integration of the constructs and successful disruption of the other *rhomboids*, *rom1*, *3*, *9* and *10*, were confirmed by diagnostic PCR and Southern analysis of separated chromosomes (Figs 1 and 2). Northern analyses showed transcription of all four genes in blood stages of wild type *P. berghei* ANKA parasites and confirmed the lack of transcription in the blood stages of the respective gene-deletion mutants (Figs 1 and 2). The clonal lines of the gene-deletion mutants $\Delta rom1$, $\Delta rom3$, $\Delta rom9$ and $\Delta rom10$ showed no change in asexual growth rates *in vivo* (Table 2). Despite the evidence for transcription and protein expression of ROM1, 9 and 10 in asexual blood stages (Figs 1, 2 and S1; Table 1) this indicates a non-essential, redundant function for these proteins during asexual blood stage multiplication.

ROM9 and ROM10 are dispensable for the entire life cycle

Two independent gene-deletion mutants were generated for *rom9*: $\Delta rom9$ -a and $\Delta rom9$ -b (Fig. 2C). Asexual blood stage multiplication, gametocyte production and ookinete production of both $\Delta rom9$ mutants were similar to that of wild type *P. berghei* parasites.

We analysed $\Delta rom9$ -a parasites during development in mosquitoes and in hepatocytes. The $\Delta rom9$ parasites produced wild type levels of oocysts (Table 2). Although we showed that *rom9* is transcribed in salivary gland sporozoites (Fig. S1), we found no significant differences between $\Delta rom9$ parasites and wild type parasites with respect to the production of sporozoites, their gliding motility (data not shown), traversal and invasion of hepatocytes and in the prepatent period in mice after injection of sporozoites (Table 2). Immunofluorescence analyses of liver stage parasites stained with antibodies against markers for parasite development (HSP70), parasitophorous vacuole membrane (PVM) (UIS4 and EXP1) and merozoite formation (MSP1), also revealed no distinct differences in morphology between $\Delta rom9$ and wild type liver stage parasites at 24 h or 48 h after sporozoite invasion (Fig. 3A). However, $\Delta rom9$ parasite loads between 53 and 57 h after

Table 2. Phenotypes of *P. berghei* mutants lacking expression of rhomboids.

Lines	Asexual multiplication rate ^a (s.d.)	Gametocyte production ^b % (s.d.)	Ookinete production ^c % (s.d.)	Oocyst production ^d (s.d.)	Sporozoite production ^e × 10 ³ (s.d.)	Sporozoite traversal ^f % (s.d.)	Sporozoite invasion rate ^g % (s.d.)	Prepatent period compared with wt ^h
Mutants								
<i>Δrom1-p</i>	10 (0) n = 2	16.4 (0.5)	56.6 (1.8)	156.3 (151.8)	ND	ND	ND	ND
<i>Δrom1-c</i>	10 (0) n = 3	17.3 (1.0)	68.9 (9.5)	155.6 (118.9)	35.9 (3.3)	27.3 (7.6)	23.3 (8.5) ⁱ	+1 (n = 3)
				220.0 (70.3)	49.0 (1.6)	ND	35.6 (10.6) ^j	+1 (n = 3)
				ND	ND	ND	58.5 (6.8)	ND
<i>Δrom3-a</i>	10 (0) n = 3	17.9 (1.3)	61.3 (5.9)	148.4 (136.8)	NA	NA	NA	NA
<i>Δrom3-b</i>	10 (0) n = 2	18.9 (3.0)	71.0 (6.1)	259.7 (155.8)	NA	NA	NA	NA
<i>Δrom9-a</i>	10 (0) n = 4	18.9 (4.1)	74.3 (11.0)	145.8 (123.8)	43.2 (6.5)	21.1 (2.1)	51.7 (9.2)	+0 (n = 5)
<i>Δrom9-b</i>	10 (0) n = 4	19.0 (2.7)	74.2 (4.6)	ND	ND	ND	ND	ND
<i>Δrom10</i>	10 (0) n = 4	18.1 (2.6)	63.8 (7.4)	191.8 (147.2)	44.7 (8.0)	ND	53.3 (4.5)	+0 (n = 3)
Tagging mutants								
<i>rom3::gfp</i>	10 (0) n = 3	18.0 (2.7)	66.5 (4.7)	298.2 (110.7)	51.4 (8.9)	ND	ND	ND
<i>rom4::mCherry</i>	10 (0) n = 7	ND	ND	ND	ND	ND	ND	ND
<i>wf</i>	10 (0) n > 10	15–25	50–90	120–290	35–80	19.3–25.7	53.0–61.8	+0

a. The multiplication rate per 24 h 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 ookinets *in vitro*.

d. The mean number of oocysts per mosquito (days 11–14).

e. The mean number of salivary gland sporozoites per mosquito (days 19–22).

f. Percentage of Dextran positive cells in Huh7 cell cultures 2 h after post-infection.

g. The percentage of intracellular sporozoites at 3 h post-infection of Huh7 cell cultures.

h. The prepatent period (measured in days post sporozoite infection) is defined as the day when a blood stage infection with a parasitemia of 0.5–2% is observed (+0 = similar to wild type; +1 = 1 day delay compared with wild type).

i. The developmental data for wild type parasites are shown as the range of mean values of > 10 experiments.

j. *P* < 0.05, determined by Student's *t*-test as compared with wild type control run in parallel.

ND, not done; NA, not applicable.

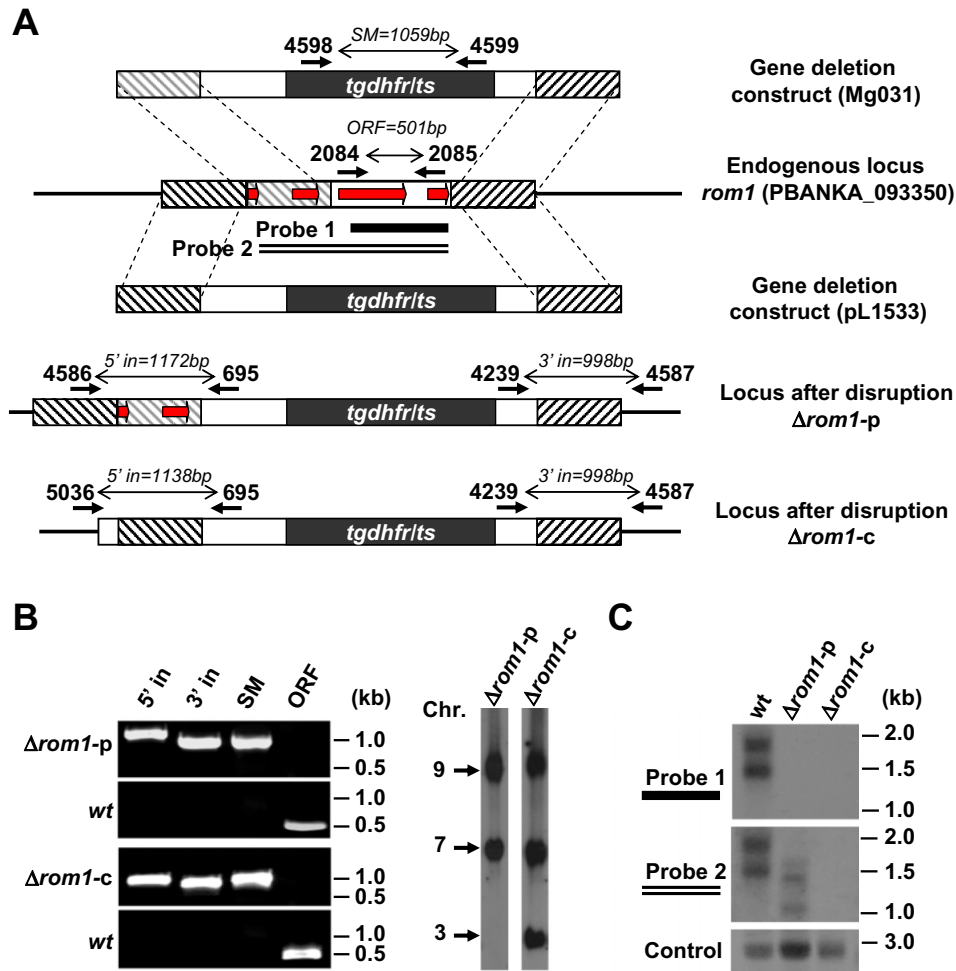


Fig. 1. Generation of mutants lacking expression of *P. berghei* rhomboid 1.

A. Schematic representation of the constructs Mg031 and pL1533 targeting *rhomboid-1* for gene deletion by double cross-over homologous recombination at the target regions (hatched boxes), and the locus before and after disruption. Each construct contains the *tgdhfr/ts* selection cassette (black box). Mg031 replaces the 3rd and 4th exons (red arrows) of the ORF but retains 1st and 2nd exons in mutant $\Delta rom1$ -p. pL1533 replaces the complete ORF in $\Delta rom1$ -c. Primer positions for diagnostic PCRs, amplicon sizes and the location of two PCR probes for the Northern blot analyses are shown (see Table S3 for primer sequences).

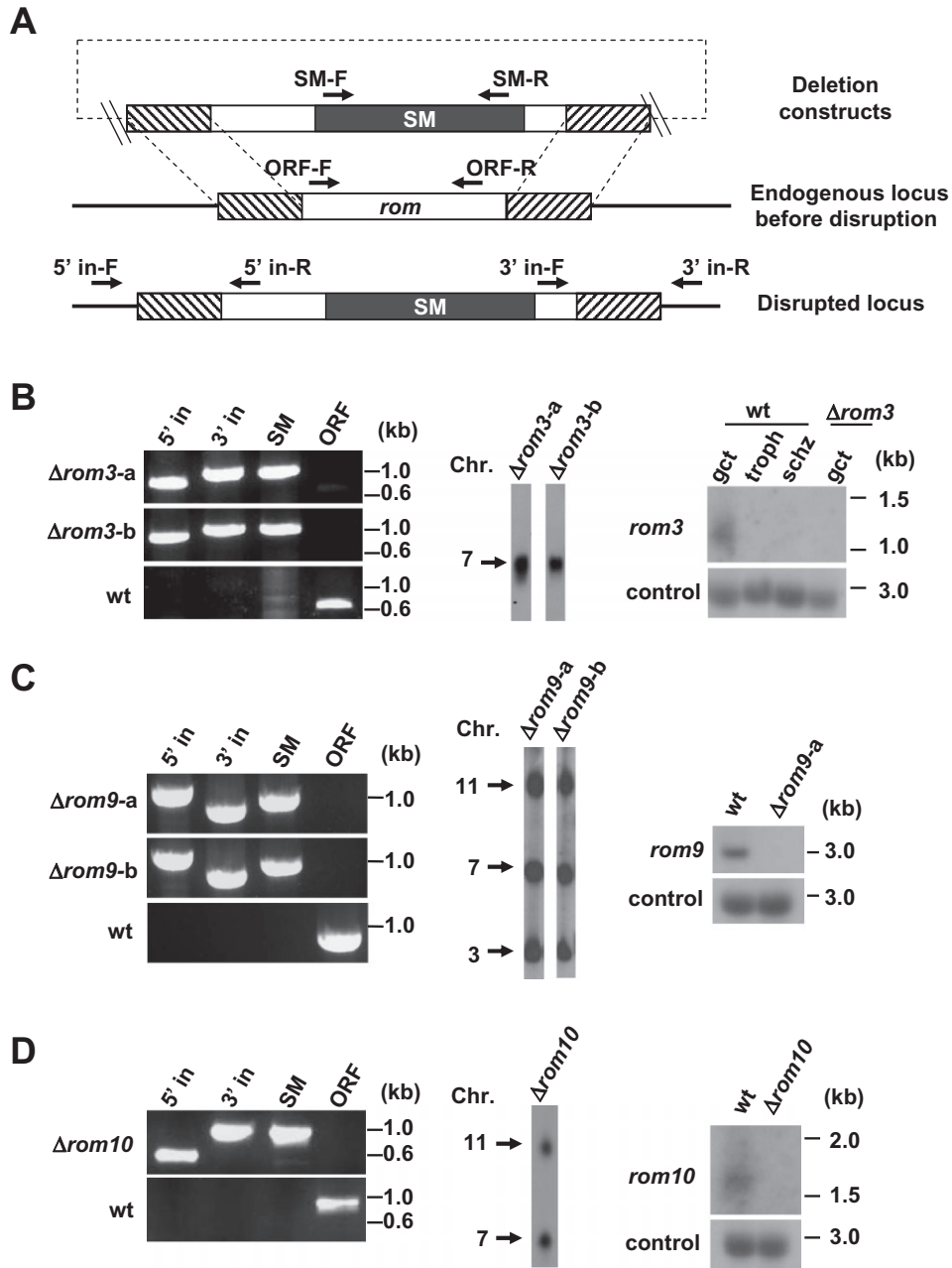
B. Diagnostic PCRs (left) and Southern analyses of separated chromosomes (right) confirm the correct integration of the constructs in $\Delta rom1$ -p and $\Delta rom1$ -c. Primer pairs and amplicon sizes are shown in A: 5'/3' in, integration PCR; SM, amplification of the *tgdhfr/ts* selection cassette; ORF, deleted ORF. For Southern analyses, pulsed field gel-separated chromosomes were hybridized with a 3'UTR *pbdfhr* probe that recognizes the constructs integrated into *rom1* locus on chromosome 9 and the endogenous *dhfr/ts* locus on chromosome 7; in the $\Delta rom1$ -c line, the probe also hybridizes to the GFP-luciferase reporter cassette in the *230p* locus on chromosome 3.

C. Northern blot analyses of mRNA from mixed blood stage parasites confirm the loss of wild type *rom1* transcripts in $\Delta rom1$ -p and $\Delta rom1$ -c. Locations of the PCR probes used for hybridization were shown in A (PCR probes were generated by PCR-amplification from wild type *P. berghei* genomic DNA using primers 2084/2085 for Probe 1, and primers 2082/2085 for Probe 2; see Table S3 for primer sequences). In blood stages of $\Delta rom1$ -p, truncated transcripts were detected with Probe 2, recognizing exons 1 and 2 of *rom1*. In $\Delta rom1$ -c, no transcripts were detected. As a loading control, hybridization was performed with probe L644R that recognizes the large subunit ribosomal RNA. wt, wild-type *P. berghei* ANKA.

infection (measured by RT-PCR, FACS and luciferase assay) were consistently lower both *in vitro* and *in vivo* (Fig. 3B and C) which may indicate that this enzyme plays a (minor) role during liver stage development.

We generated one gene deletion mutant for *rom10* and analysed this mutant throughout the entire life cycle. The phenotype of $\Delta rom10$ parasites was similar to that of wild type parasites in all developmental assays (Table 2), spe-

cifically *in vivo* asexual multiplication rate, *in vivo* gametocyte production, *in vitro* ookinete production, oocyst and sporozoite production and *in vivo* liver stage development. In addition, $\Delta rom10$ sporozoites showed normal rates of gliding (data not shown), *in vitro* hepatocyte invasion rate and prepatent period (Table 2). These results indicate that this protein is redundant and/or that its function can be fulfilled by other (rhomboid) proteases.



Δrom1 parasites have wild type blood stage growth and virulence characteristics, but show delayed liver stage development

In the course of this study we generated two independent *P. berghei* $\Delta rom1$ mutants; the first mutant $\Delta rom1$ -p is a partial gene deletion lacking the 3rd and 4th exons of *rom1* that contain the catalytic diad (Fig. 1A and B); the second mutant $\Delta rom1$ -c lacks the entire open reading frame (ORF) (Fig. 1A and B). Northern analyses of *rom1* transcripts in blood stages using two PCR-amplified probes specific to 3rd and 4th exons, or to the whole ORF,

showed that $\Delta rom1$ -p lacks transcripts containing the 3rd and 4th exons but still produces stable, truncated transcripts consisting of the 1st and 2nd exons. As expected, $\Delta rom1$ -c had no detectable *rom1* transcripts (Fig. 1C). Both $\Delta rom1$ -p and $\Delta rom1$ -c showed *in vivo* multiplication rates that were similar to wild type *P. berghei* parasites when analysed in the cloning-assay (Table 2). To determine possible differences in growth rate and virulence during prolonged infections in mice, we analysed the growth rate of $\Delta rom1$ and wild type parasites in C57BL/6 and in BALB/c mice (Fig. 4A and B). C57BL/6 mice are sensitive to experimental cerebral malaria (ECM) and cer-

Fig. 2. Generation of mutants lacking expression of *P. berghei* rhomboid 3, 9 and 10.

A. Schematic representation of the DNA construct used for targeting rhomboid genes for deletion and the gene locus before and after disruption. The constructs which contain a drug-selectable marker cassette (SM; black box) disrupt the genes by double cross-over homologous recombination at the target regions (hatched boxes). The locations of primers for diagnostic PCRs are shown.

B. Diagnostic PCRs, Southern and Northern analyses confirm correct disruption of *rhomboid 3* (*rom3*) in 2 independent mutants, $\Delta rom3$ -a and $\Delta rom3$ -b. For diagnostic PCRs (left), the following primers were used: 5' integration (5' in): 2389/695; 3' integration: (3' in) 4239/1886; amplification of the *tgdhfr/ts* selection cassette (SM): 4598/4599; deleted ORF (ORF): 1812/1813. For Southern analyses of separated chromosomes (middle), pulsed field gel-separated chromosome were hybridized using a *tgdhfr/ts* probe that recognizes the construct integrated into *rom3* locus on chromosome 7. Northern blot analysis (right) of mRNA of different blood stages shows transcripts only present in wild type gametocytes (gct). No transcripts are detected in trophozoites (troph) or schizonts (schz). The analysis also confirms the loss of *rom3* transcripts in gct of $\Delta rom3$. Hybridization was performed using a PCR probe recognizing *rom3* ORF (primers 1812/1813). As a loading control, hybridization was performed with probe L644R that recognizes the large subunit ribosomal RNA.

C. Analysis of two independent mutants, $\Delta rom9$ -a and $\Delta rom9$ -b, lacking expression of ROM9. For diagnostic PCRs (left), the following primers were used: 5' in: 7083/4906; 3' in: 4239/7084; SM (*hdhfr::yfcu*): 4698/4699; ORF: 7085/7086. For Southern analyses (middle), chromosomes were hybridized using a 3'UTR *pbdhfr* probe that recognizes the construct integrated into *rom9* on chromosome 11, the *dhfr/ts* on chromosome 7 and the GFP-luciferase reporter cassette in the *230p* locus on chromosome 3. Northern blot was hybridized with a PCR probe recognizing *rom9* ORF (primers 7085/7086) (right).

D. Analysis of $\Delta rom10$ lacking expression of ROM10. For diagnostic PCRs (left), the following primers were used: 5' in: 6939/4179; 3' in: 4239/2088; SM (*tgdhfr/ts*): 4598/4599; ORF: 6940/2066. Chromosomes were hybridized using a 3'UTR *pbdhfr* probe that recognizes the construct integrated into *rom10* on chromosome 11 and the *dhfr/ts* on chromosome 7 (middle). Northern blot was hybridized with a PCR probe recognizing *rom10* ORF (primers 6940/2066) (right). See Table S3 for all primer sequences and product sizes.

ebrial complications develop 6–8 days post-infection with *P. berghei* ANKA parasites, whereas BALB/c mice are ECM-resistant and develop fulminating (and lethal) parasitemias peaking 2–3 weeks after infection. In both C57BL/6 and BALB/c mice $\Delta rom1$ parasites showed infection patterns that were highly similar to infections initiated with wild parasites (Fig. 4A and B); all $\Delta rom1$ infected C57BL/6 mice developed ECM symptoms at day 6 or 7 after infection (Fig. 4A).

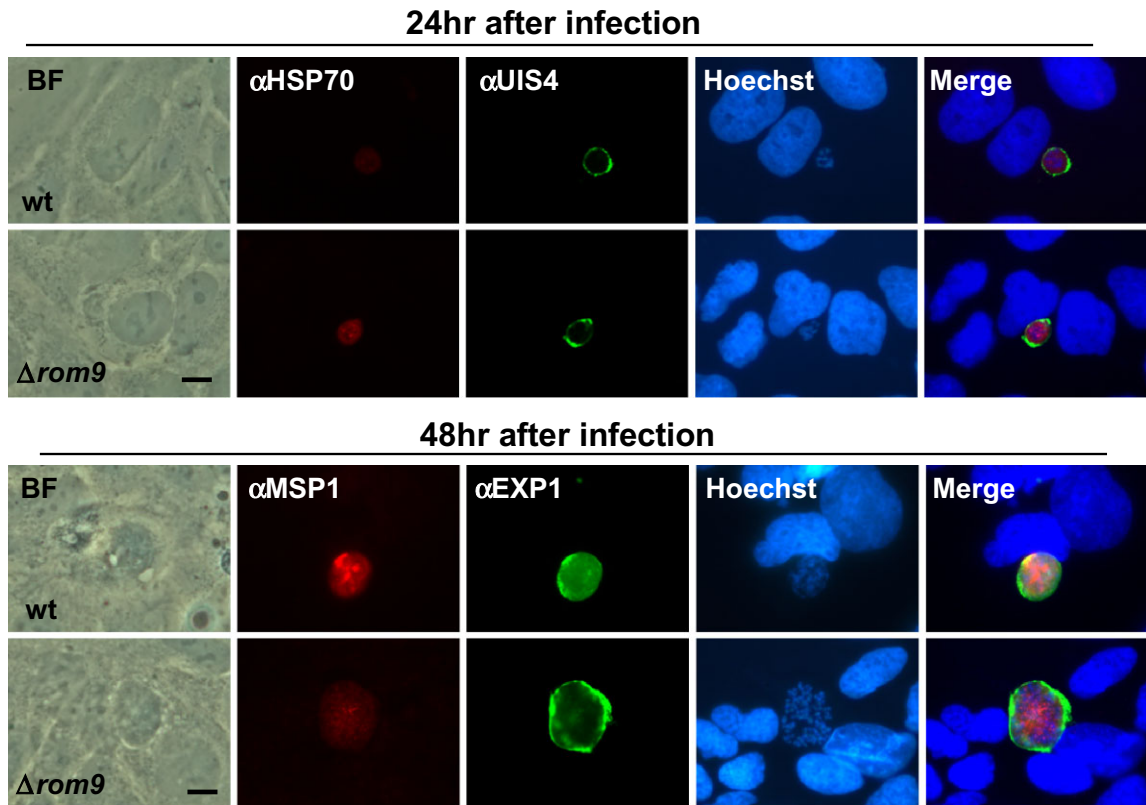
$\Delta rom1$ parasites were next examined during mosquito and liver stage development. These parasites produced numbers of ookinetes, oocysts and salivary gland sporozoites similar to those of wild type parasites (Table 2). However, liver-stage development was reduced as shown by a 1-day extension of the prepatent period in mice following the inoculation with 10^4 purified sporozoites. While gliding motility (data not shown) and the rate of cell traversal of sporozoites were similar to wild type parasites (Table 2), we observed in two out of three experiments a reduction in sporozoite *in vitro* invasion rates (Table 2) that could explain (in part) the delay in the prepatent period. Immunofluorescence analyses of liver stage parasites stained with antibodies against markers for parasite development (HSP70), PVM (UIS4 and EXP1) and merozoite formation (MSP1), revealed no distinct differences in morphology and size between $\Delta rom1$ and wild type liver stages at 24 h or 48 h after sporozoite invasion (Fig. 4C). Although a significant reduction in parasite loads and expression of UIS4 have been reported for liver stages of *P. yoelii* mutants lacking expression of ROM1 (Vera *et al.*, 2011), we only observed a slight, but not significant, reduction in numbers of liver stages as determined by anti-UIS4 antibody staining (Fig. 4D) and we did not observe that these mutants had unusual PVM morphology. Liver stages of $\Delta rom1$ and wild-type parasites were also comparable in size at 48 h post-infection (Fig. 4D).

Δrom3 parasites establish oocysts but fail to produce sporozoites

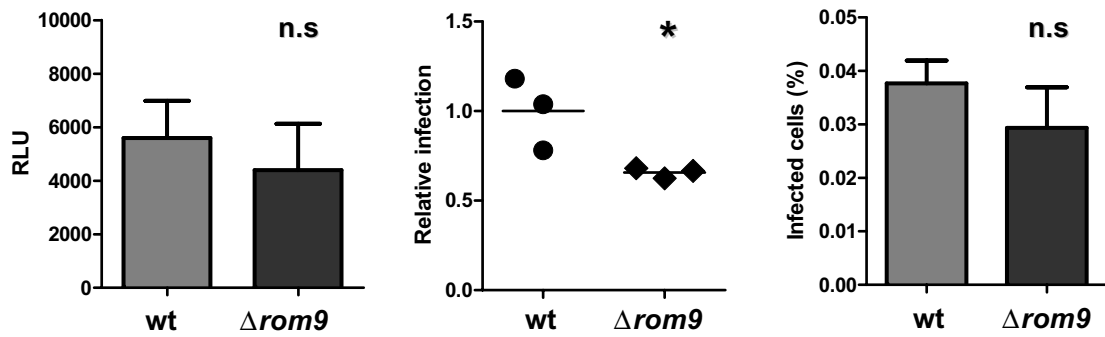
Two independent gene-deletion mutants, $\Delta rom3$ -a and $\Delta rom3$ -b, were generated (Fig. 2B). Asexual blood stage multiplication rates of both mutants determined in the cloning assay were normal and $\Delta rom3$ parasites produced wild type levels of gametocytes, ookinetes and oocysts (Table 2). By light microscopy, $\Delta rom3$ ookinetes have the characteristics of fully mature wild-type ookinetes such as an elongated 'banana' shape, hemozoin clusters and a centrally located, enlarged nucleus and the $\Delta rom3$ and wild type ookinetes show a similar (tetraploid) DNA content (Fig. S3). In addition, by electron microscopy analyses we were unable to detect differences in the ultrastructural morphology of wild-type and $\Delta rom3$ ookinetes, with respect to the nucleus, crystalloid body and the structure of the apical complex (Fig. S5). All membranes of these organelles exhibit a normal structure and the apical complex of $\Delta rom3$ ookinetes had the characteristic features of abundant micronemes, and the presence of microtubules and the inner membrane complex.

However, no sporozoites were detected in salivary glands of mosquitoes infected with $\Delta rom3$ -a or $\Delta rom3$ -b in three independent experiments. Inspection of $\Delta rom3$ oocysts by light-microscopy revealed normal oocyst production but a complete absence of sporozoite formation; mature oocysts had clearly vacuolated cytoplasm and there were no signs of sporulation (Fig. 5A). Western analysis of circumsporozoite protein (CSP) expression using anti-CSP antibody in oocysts-containing midguts at day 10 after infection, showed that CSP is almost absent in the $\Delta rom3$ oocysts (Fig. 5B). An indirect IFA on wild type and $\Delta rom3$ oocysts confirmed the strongly reduced CSP levels in $\Delta rom3$ oocysts (Fig. 5C). There was also

A



B



C

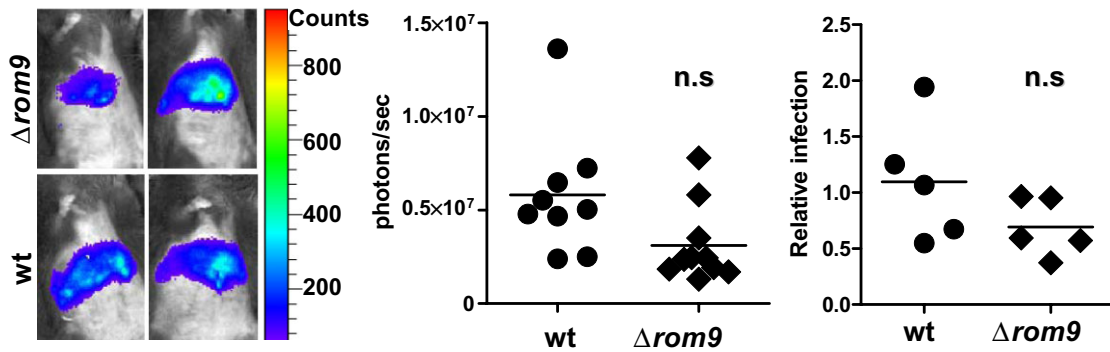


Fig. 3. Liver stage development of $\Delta rom9$ parasites.

A. Immunofluorescence assays of liver stages of $\Delta rom9$ and the wild type (wt) control *PbGFP-Luc_{schz}* at 24 h and 48 h after sporozoite infection of cultured Huh7 cells show a comparable morphology of $\Delta rom9$ and wild type parasites. Anti-HSP70 (red) antibody highlights the parasite's cytoplasm while anti-UIS4 (green) antibody decorates the PVM. Anti-MSP1 (red) antibody recognizes developing merozoites and anti-EXP1 (green) antibody the PVM. Nuclei were stained with Hoechst-33342 (Blue). BF, bright field; scale bars equals 10 μ m.

B. Parasite loads at 57 h in cultured Huh7 cells after infection with 5×10^4 $\Delta rom9$ or wild type control *PbGFP-Luc_{schz}* sporozoites as determined by luciferase assay (left), qRT-PCR (middle) and FACS (right). All assays show a slight reduction of mutant parasite loads, however, only the relative infection value determined by qRT-PCR shows significant reduction (Student's *t*-test: n.s.: not significant; **P* < 0.5).

C. Parasite loads in C57BL/6 mice at 53 h after injection of 1×10^4 $\Delta rom9$ sporozoites or wild type control *PbGFP-Luc_{schz}* sporozoites as determined by *in vivo* imaging (left and middle) and qRT-PCR (right). Representative rainbow images of luminescence in livers of live mice (left) and the corresponding luminescence levels (photons s⁻¹) of livers in whole mice (middle) are shown (Student's *t*-test: n.s.: not significant). The lines indicate mean values and the error bars indicate standard deviations. This figure is available in colour online at wileyonlinelibrary.com.

reduced intensity of Hoechst DNA-staining indicating decreased DNA replication in these oocysts (Fig. 5C). The crucial role of ROM3 in oocyst maturation/sporogony is unexpected given the high transcript levels detected in gametocytes (Table 1, Fig. S1B). When we analysed ROM3::GFP expression in *rom3::gfp* parasites (with the endogenous *rom3* C-terminally tagged with GFP), we observed GFP signal in all female gametocytes (but not in male gametocytes) that persisted into mature ookinetes but was undetectable in oocysts (days 6–17) and sporozoites (Fig. 5D).

The normal development throughout the life cycle (Fig. 5D, Table 2) indicates that the C-terminal GFP-tag does not affect the essential function of ROM3 during sporozoite formation. We attempted to use the *rom3::gfp* parasites to gain a better understanding of the localization of ROM3 in female gametocytes and ookinetes. Specifically, we extracted soluble and insoluble protein fractions of *rom3::gfp* gametocytes and ookinetes and then examined the presence of ROM3 protein using antibodies against the GFP tag. Unfortunately, we were unable to unambiguously identify ROM3::GFP in either fraction, presumably due to the very low expression of ROM3 in these stages (data not shown).

Discussion

The discovery of critical roles for certain rhomboid proteins in motility, host cell invasion and pathogenesis of apicomplexan parasites has attracted considerable attention. However, 10 years after their initial discovery, only two of the eight rhomboids of malaria parasites, ROM1 and ROM4, have been analysed in more detail. Multiple roles have been suggested for ROM1 and ROM4: invasion of red blood cells (RBC) (O'Donnell *et al.*, 2006), sporozoite gliding motility (Ejigiri *et al.*, 2012) and the formation of the PVM in infected hepatocytes (Vera *et al.*, 2011). In this study, we investigated expression patterns of all eight *P. berghei* rhomboids and examined the effects of rhomboid gene deletion across the entire life cycle of the parasite in order to pinpoint additional, new role for this intramembrane protease family. In line with previously published work, our studies support the observations that

ROM1 is dispensable for parasite development throughout the life cycle (Srinivasan *et al.*, 2009; Vera *et al.*, 2011) and that ROM4 is essential for asexual blood stage development (O'Donnell *et al.*, 2006). We identified three more rhomboids that are dispensable for asexual blood stage development: ROM3, ROM9 and ROM10. Mutants lacking expression of ROM9 and ROM10 can complete the entire life cycle without major developmental defects. These observations indicate functional redundancy for several rhomboids (ROM1, 9 and 10) and suggest that processing of essential substrates may be fulfilled by more than one protease. ROM4 and ROM1 are targeted to different cellular locations in the merozoite – surface and microneme (O'Donnell *et al.*, 2006) – making it unlikely that they process identical substrates that would allow ROM4 to compensate for ROM1 in the $\Delta rom1$ mutant, and clearly not vice versa.

The repeated failure to delete ROM4, ROM6, ROM7 and ROM8 genes indicate that these rhomboids play key and most likely independent roles during blood stage development that cannot be met by other proteins. Located on the merozoite surface, *P. falciparum* ROM4 is strongly implicated in the shedding of the erythrocyte binding antigen 175 (EBA175) during merozoite invasion (O'Donnell *et al.*, 2006). Rodent malaria parasites lack EBA175 but express a homologue of EBA140 (BAEBL) which also belongs to the same EBL (erythrocyte-binding-like) TM protein family. Similar to EBA175, *P. falciparum* EBA140 is also an *in vitro* substrate of ROM4 (Baker *et al.*, 2006), and in *P. yoelii* EBA140 (PY04764) is essential for RBC invasion (Otsuki *et al.*, 2009). Interestingly, in *P. berghei* the related protein, PBANKA_133270, also contains a putative cleavage site, similar to the one found in EBA140. Our successful C-terminal tagging of *P. berghei* ROM4 strengthens the notion that the failure to disrupt *rom4* is not due to inaccessibility of this specific locus to genetic modification, but is due to its key role in the enzymatic processing of surface molecules like EBA140. Combined these data suggest that ROM4 shares essential roles in both human and rodent malaria parasites through cleavage of one or more merozoite surface proteins involved in RBC invasion, which cannot be compensated by other rhomboid or non-rhomboid

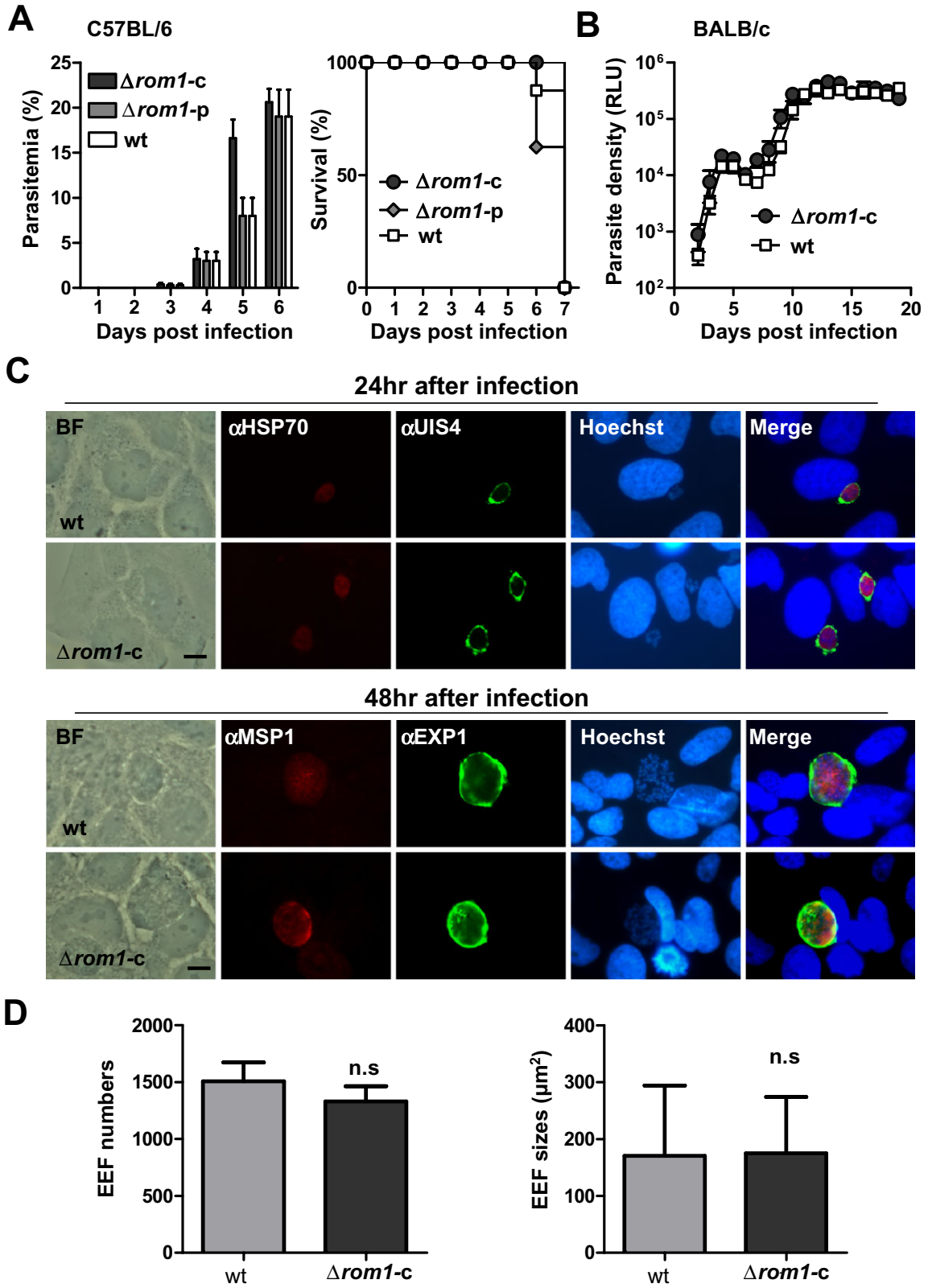


Fig. 4. Blood and liver stage development of $\Delta rom1$ parasites.

A. The course of infection (left panel) and survival curve (right panel) in C57BL/6 mice ($n = 6$) infected with 10^5 wild-type (wt, cl15cy1), $\Delta rom1$ -p or $\Delta rom1$ -c. The parasitemia developed in mice infected with wt, $\Delta rom1$ -p and $\Delta rom1$ -c parasites are highly comparable during the whole course of infection (left). All C57BL/6 mice infected with wild-type (wt, cl15cy1), $\Delta rom1$ -p and $\Delta rom1$ -c parasites developed ECM complications on day 6–7 as indicated by a drop in body temperature below 34°C ; mice were sacrificed at this point (right).

B. The course of infection in BALB/c mice ($n = 6$) infected with 10^4 parasites of wild-type (wt, GFP-luc_{con}) or $\Delta rom1$ -c parasites. Parasite densities were determined by measuring luciferase activity indicated as relative light unit (RLU) in the IVDL-assay. Error bars indicate standard deviations in A and B.

C. Immunofluorescence analyses of $\Delta rom1$ -c and the wild type (wt, GFP-Luc_{con}) exo-erythrocytic forms (EEF) at 24 h and 48 h after infection with 1×10^4 $\Delta rom1$ -c or wild type (GFP-Luc_{con}) parasites compared with wild type control. Anti-HSP70 (red) and anti-UIS4 (green) antibodies recognize the parasite's cytoplasm and the PVM. Anti-MSP1 (red) antibodies recognize developing merozoites and anti-EXP1 (green) antibodies stain the PVM. Nuclei were stained with Hoechst-33342 (Blue). BF, bright field; scale bars equals 10 μm .

D. EEF numbers per well of cultured Huh7 cells at 24 h (left) and the sizes of EEFs at 48 h (right) after sporozoite infection. The numbers of EEFs were determined by counting anti-UIS4 staining positive EEFs in the immunofluorescence assay, and the EEF sizes were determined by measuring the sizes of anti-Hsp70 staining positive parasites. The lines indicate mean values and the error bars indicate standard deviations. This figure is available in colour online at wileyonlinelibrary.com.

proteases. ROM4 from another apicomplexan parasite, *T. gondii*, has been established as a sheddase during gliding motility and invasion (Parussini *et al.*, 2012). In addition to ROM1 and ROM4, *P. falciparum* ROM7 and ROM8 are also expressed in merozoites (Baker *et al.*, 2006). It is, however, unlikely that these proteins process the same TM spanning proteins as ROM1 or ROM4, because they do not show the same *in vitro* substrate specificities as ROM1 or ROM4 (Baker *et al.*, 2006). *Plasmodium* ROM7 only has orthologues in the apicomplexan parasites, *Babesia bovis* and *Theileria annulata*, while ROM8 does not cluster with any other apicomplexan rhomboids (Santos *et al.*, 2011b).

Plasmodium ROM6 is the orthologue of *T. gondii* ROM6, which clusters with the PARL-like mitochondrial rhomboids and localizes to the single mitochondrion (Santos *et al.*, 2011b); however, a mitochondrial location in *Plasmodium* has yet to be confirmed. These ROM6 proteins share common features in their TM domains and catalytic sites, which are characteristic for PARL-type rhomboids (Lemberg and Freeman, 2007), and contain predicted mitochondrial-targeting sequence (Verma *et al.*, 2010). Known conserved substrates of PARL-type rhomboids are dynamin-related proteins (McQuibban *et al.*, 2003; Dowse and Soldati, 2005). *Plasmodium* species express two conserved dynamins: DYN1 (PBANKA_090360) and DYN2 (PBANKA_052040). Interestingly, phylogenetic analyses show that *Plasmodium* DYN2 clusters with dynamins of other species that are mainly implicated in the division of the mitochondrial outer membrane (Charneau *et al.*, 2007). Attempts to disrupt the *dyn2* gene were unsuccessful (Gunnar R. Mair and Chris J. Janse, unpubl. data; <http://www.pberghei.eu/index.php?rmgm=765>), suggesting its essential role in asexual stage parasites. Further research is needed to determine whether DYN2 is the substrate of ROM6 and protein localization studies with tagged proteins or antibodies may reveal whether both proteins are indeed located in the mitochondria in blood stages.

Although ROM1 is not essential for blood stage parasites, it has been reported that *P. berghei* and *P. yoelii* mutants lacking ROM1 exhibit a slight growth delay and appear less virulent in mice than wild type parasites (Srinivasan *et al.*, 2009; Vera *et al.*, 2011). In contrast, we were unable to detect either a growth or virulence-attenuation phenotype in experiments conducted with two independent *P. berghei* $\Delta rom1$ lines. The cause for these discrepancies in blood stage phenotypes between our and the *P. berghei* mutant reported by Srinivasan *et al.* (2009) is unknown. In the study of Srinivasan *et al.* (2009), the mutant clone examined was derived from a single transfection experiment that generated a 3' end truncation of the gene encoding ROM1, preserving a large part of the ROM1 protein. We were able to detect a stable, although truncated, transcript transcribed from the 5' end of the gene in the $\Delta rom1$ -p mutant; whether these are translated into stable, truncated proteins that are inserted into the membrane exerting a dominant-negative effect is unknown. Cloned lines of wild type *P. berghei* ANKA parasites are known to differ in growth and virulence characteristics (Amani *et al.*, 1998) and environmental factors have been shown to influence the course of infections in mice (Levander *et al.*, 1995); such differences between laboratories may influence the outcome of phenotypic analyses of genetically identical mutants. Therefore, the reported growth and virulence phenotype may be unrelated to the disruption of *rom1*.

In contrast to normal blood stage infections of the mutants reported in this study, we found that $\Delta rom1$ parasites have a slight defect in liver stage development with a consistent delay of 1 day in blood stage patency following sporozoite infection. A prolonged prepatent period was also observed by Srinivasan *et al.* (2009). This finding was confirmed by a twofold reduction in liver stage development of *P. yoelii* $\Delta rom1$ parasites (Vera *et al.*, 2011). It has been suggested that this reduction in *P. yoelii* liver development results from reduced cleavage of the PVM protein UIS4 (upregulated in sporozoites 4) (Vera *et al.*,

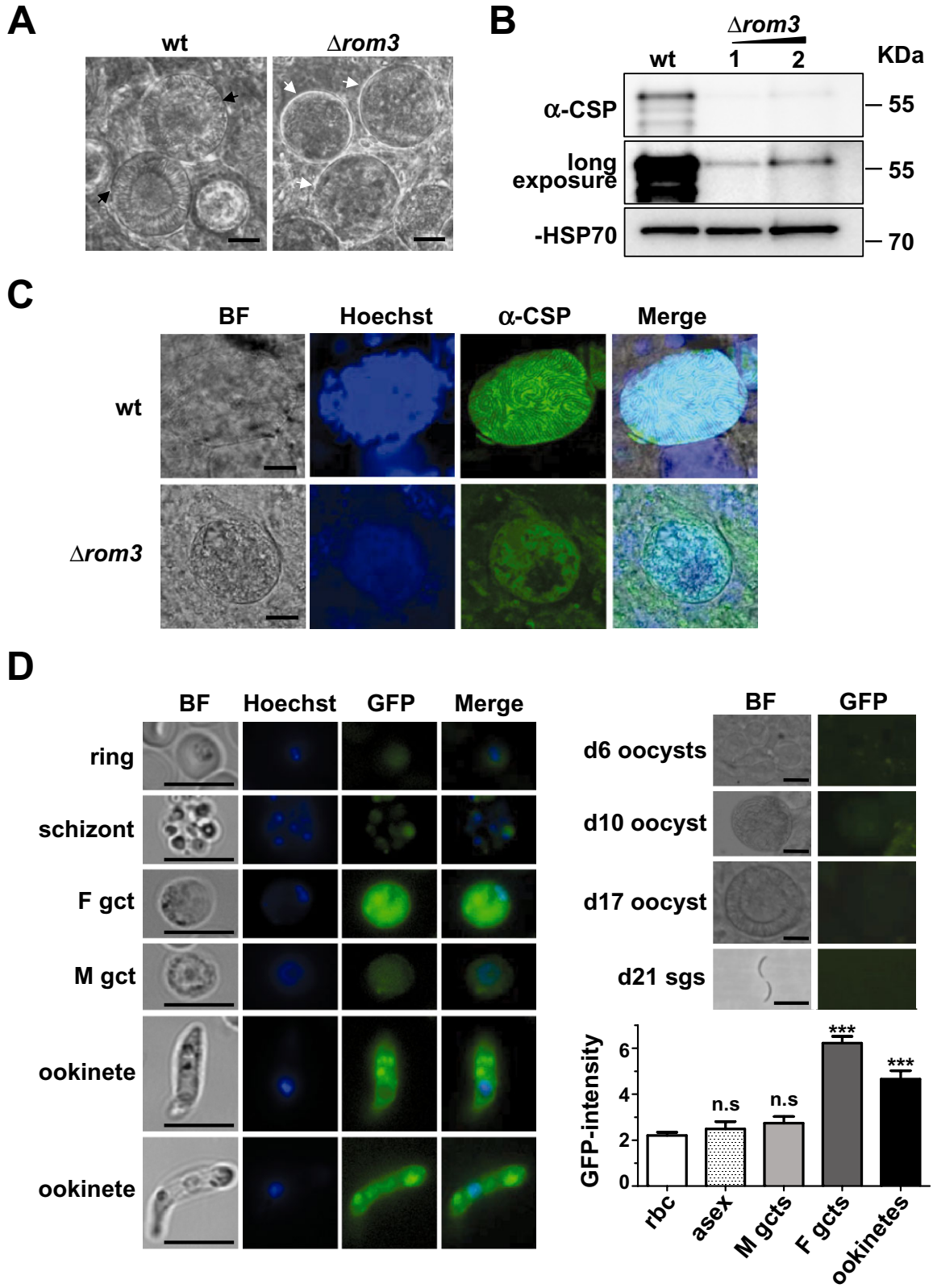


Fig. 5. Rhomboid 3 is essential for sporozoite formation in oocysts.

A. Phase contrast microscopy of wild type and aberrant, vacuolated $\Delta rom3$ oocysts on day 12 after mosquito infection. Mutants lack any signs of sporozoite formation (white arrow) whereas wild type (wt) oocysts collected on the same day show clear sporulation (black arrow). Scale bar 10 μ m.

B. Western blot analysis of midguts from infected mosquitoes. Midguts from mosquitoes infected with $\Delta rom3$ (1 or 2 midguts, day 10) and wild-type (wt, day 10) were separated on SDS-PAGE and stained with anti-CSP and anti-HSP70 antibodies. A longer exposure of the anti-CSP blot is also shown.

C. Developmental defects of $\Delta rom3$ parasites examined by immunofluorescence assays. Compared with wild-type (wt), $\Delta rom3$ oocyst show decreased CSP expression as shown by staining oocysts with anti-CSP (green) antibody and reduced staining with the DNA-specific dye Hoechst-33342 (blue), indicating less DNA replication. BF, bright field. Scale bar 10 μ m.

D. All $rom3::gfp$ female gametocytes (F gct; $n = 50$) and ookinetes ($n > 100$) exhibited a GFP signal that was significantly above background values (** $P < 0.0001$, Student's t -test). No GFP signals above background were detected in either male gametocyte (M gct; $n = 30$) or asexual blood stages. In the mosquito vector no GFP signals were detected in either $rom3::gfp$ oocysts (6–17 days after mosquito infection) or in salivary gland sporozoites (sgs, day 21). Nuclei are stained with Hoechst-33342 (blue). BF, bright field. Scale bar 10 μ m. n.s., not significant, Student's t -test. This figure is available in colour online at wileyonlinelibrary.com.

2011). However, when we analysed liver stage development of *P. berghei* $\Delta rom1$ parasites, we did not find evidence for the aborted liver stage development or parasites with unusual PVM morphology. In addition to the prolonged prepatent period, we did observe a decrease in sporozoite invasion rate in two out of three experiments. However, since there are discrepancies in $\Delta rom1-c$ sporozoite-hepatocyte invasion rates, we cannot conclude that a reduction in invasion is responsible for the delay in the prepatent period. Altogether, the phenotypic observations of different $\Delta rom1$ parasites prove that ROM1 is not essential throughout the complete life cycle in both *P. berghei* and *P. yoelii*. In addition to ROM1, our loss-of-function analyses indicate that there is large degree of functional redundancy of *Plasmodium* rhomboids. We identified two other rhomboids, ROM9 and ROM10, which are dispensable throughout the complete life cycle. Both rhomboids are exclusive to *Plasmodium* (Santos *et al.*, 2011b). Interestingly, ROM9 carries a mitochondrial targeting sequence (MitoProtII <http://ihg.gsf.de/ihg/mitoprot.html>) but its predicted topology is atypical of mitochondrial PARL-like rhomboids (Lemberg and Freeman, 2007). ROM10 lacks key residues predicted to be critical to rhomboid proteolysis (Baker *et al.*, 2006; Lemberg and Freeman, 2007) and is therefore likely an inactive rhomboid. Although the expression of ROM10 was detected in multiple stages in *P. falciparum*, and our RT-PCR and Northern analyses also confirmed its expression in blood stage, the gene-deletion mutant showed no phenotypic defect throughout the entire life cycle.

In contrast to the $\Delta rom1$, $\Delta rom9$ and $\Delta rom10$ parasites, mutants lacking ROM3 expression exhibit a strong and distinct phenotype. The gene encoding ROM3 is highly transcribed in gametocytes and in the $rom3::gfp$ mutant, the GFP signal was clearly observed in female gametocytes through to the ookinete stage but was absent from developing oocysts and sporozoites. *P. berghei* mutants lacking ROM3 are capable of producing normal numbers of oocysts; however, these oocysts show a complete absence of sporozoite formation. This is the first apicom-

plexan rhomboid identified to play such a vital role in sporogony. Mutant oocysts show clear signs of stalled DNA replication and a failure to form individual sporozoites, and remain highly vacuolated. This 'delayed phenotype' in mutants which lack proteins normally expressed in female gametocytes/gametes but only manifest the consequences of the loss-of-function in maturing oocysts is not unique. Examples include several members of the LCCL/lectin adhesive-like protein (CCp/LAP) family with their distinct Lgl1(LCCL)-lectin adhesive domains (Raine *et al.*, 2007). Deletion of these also female gametocyte expressed genes produces a phenotype in maturing oocysts comparable to the one observed in the $\Delta rom3$ parasites; vacuolated oocysts and absence of sporozoite formation (Raine *et al.*, 2007; Ecker *et al.*, 2008; Lavazec *et al.*, 2009). CCp/LAP proteins have no TM domains and therefore are unlikely ROM3 substrates. They are localized to the crystalloid body in ookinetes which has been postulated to constitute a reservoir of proteins synthesized by the gametocyte to be used during oocyst growth and sporozoite development (Garnham *et al.*, 1962; 1969; Saeed *et al.*, 2010). However, ultrastructure analysis of the $\Delta rom3$ and wild-type ookinetes revealed no distinct differences with respect to their crystalloid bodies. In addition, although we observed a punctate location of ROM3::GFP in ookinetes, we did not observe the crystalloid-type location that was shown for LAP2 and LAP3 (Saeed *et al.*, 2010). A failure of oocysts to sporulate also occurs in the absence of three other membrane-bound proteins: glycosyl phosphatidyl inositol (GPI) anchored CSP (PBANKA_040320) (Menard *et al.*, 1997), or TM domain containing plasmepsin VI (PBANKA_040970) (Ecker *et al.*, 2008) and PBANKA_130960 (Lasonder *et al.*, 2008). CSP is localized on the oocyst plasma membrane and on the inner surface of the oocyst capsule; when budding begins, large amounts of CSP cover the surface of sporoblasts and sporozoites (Thathy *et al.*, 2002). Our analyses of CSP expression and processing in $\Delta rom3$ parasites show that although CSP expression is strongly reduced (Figs 5B and S4), process-

ing appears to be normal (Fig. S4). Given that CSP contains a GPI anchor it is unlikely to be processed by ROM3. As most identified natural substrates of rhomboid proteases contain only a single TM domain, it is questionable if PBANKA_130960 is a substrate since it has several putative TM domains (<http://www.plasmodb.org>). In Table S5 we provide a list of putative substrates of ROM3, based on the published proteome data of oocysts and sporozoite proteins (<http://www.plasmoDB.org>), which are predicted to contain a single TM domain and encode a signal peptide. Whether plasmepsin VI is the substrate of ROM3 and contributes to the phenotype observed in ROM3-deficient mutants remains to be investigated. Since we observe expression of ROM3 in gametocytes and ookinetes and not in developing and mature oocysts, it is very much possible that the ROM3 substrate(s) is (are) also present and cleaved in gametocytes/ookinetes.

In this study we have examined all eight rhomboid proteases encoded by *P. berghei* and found four of them (ROM4, 6, 7 and 8) to be critical for asexual development, and one (ROM3) essential for mosquito development. While some member of these rhomboids may govern processes common to a large number of eukaryotic species (for example, the mitochondrial PARL-like rhomboid ROM6), most of them are unique to Apicomplexa and some unique to *Plasmodium*. These specific rhomboids and their substrates offer themselves as targets for anti-parasite interventions. While the exact nature of the function of most rhomboids and their substrate range remain to be elucidated, this study helps provide a clear framework of expression, function in parasite development and the specific and redundant enzymatic activities of this important class of proteases.

Experimental procedures

Experimental animals and *P. berghei* ANKA reference lines

Female C57BL/6, BALB/c and Swiss OF1 mice (6–8 weeks) and male C57BL/6 (6–8 weeks) from Charles River were used. All animal experiments performed at the LUMC were approved by the Animal Experiments Committee of the Leiden University Medical Center (DEC 07171; DEC 10099). 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). Animals used at the Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, were housed in the Specific Pathogen Free facilities of the Institute. All experimental procedures were carried strictly within the rules of the Portuguese official Veterinary Directorate (Direcção Geral de Veterinária), which complies with the European Guideline 86/609/EC and follows the FELASA (Federation of European Laboratory Animal Science Associations) guidelines and recommendations concerning laboratory animal welfare.

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 (Janse *et al.*, 2006b) and two reporter lines, i.e. *PbGFP-LUC_{con}* (line 676m1cl1; mutant RMgm-29; <http://www.pberghei.eu>) and *PbGFP-Luc_{schz}* (line 1037cl1; mutant RMgm-32; <http://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 *eef1 α* 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 (Janse *et al.*, 2006a; Spaccapelo *et al.*, 2010).

RT-PCR analyses

To investigate the transcription pattern of the different rhomboid genes, RT-PCR was performed for each gene using cDNA from (i) asexual blood stages of a non-gametocyte producer *P. berghei* ANKA parasite line (HPE) (Janse *et al.*, 1989), (ii) purified gametocytes of wild type *P. berghei* ANKA (cl15cy1) parasites and (iii) salivary gland sporozoites of wild type *P. berghei* ANKA (cl15cy1). Primers were designed specific to the ORF of each gene and across introns when possible in order to distinguish amplicons from gDNA and cDNA. Details of the primers and expected sizes are shown in Table S4.

Generation of gene deletion mutants

To disrupt the *rhomboid* genes, the following replacement constructs were generated. Plasmid constructs targeting *rom1*, *3*, *4*, and *10* were constructed in the generic plasmid pL0001 (<http://www.mr4.com>) which contains the pyrimethamine resistant *Toxoplasma gondii* (*tg*) dihydrofolate reductase-thymidylate synthase (*dhfr/ts*) as a selectable marker (SM) under the control of *P. berghei* *dhfr/ts* promoter. Targeting regions for homologous recombination were PCR-amplified from *P. berghei* ANKA (cl15cy1) genomic DNA using primers specific for the 5' or 3' ends of each *rhomboid* gene (see Table S2 for the primer sequences) and cloned in upstream and downstream, respectively of the SM; this allows integration of the construct into the targeting regions by double cross-over homologous recombination and complete replacement of the ORF. For transfection the gene-deletion constructs were linearized with the appropriate restriction enzymes (Table S2).

Constructs targeting *rom6*, *8* and *9* were generated using a modified two-step PCR method (Lin *et al.*, 2011). Briefly, in the first PCR reaction two fragments of 5' and 3' targeting regions (TR) were amplified from *P. berghei* ANKA (cl15cy1) genomic DNA with the primer sets shown in Table S2. The reverse primers of 5'TR and the forward primers of 3'TR have 5' extensions homologous to the *dhfr::yfcu* selectable marker cassette from pL0048. In the second PCR reaction, the 5'TR 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 *NruI* (as indicated in primer sequences in Table S2) to remove the 'anchor-tag' and with *DpnI* that digests any residual uncut pL0048 plasmid.

Transfection and selection of transformed parasites with pyrimethamine was performed using standard technology for the genetic modification of *P. berghei* (Janse *et al.*, 2006b). 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 (<http://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 S3 for primers used) and Southern analyses of chromosomes separated by pulsed-field gel electrophoresis hybridized with probes specific for the selectable marker (see Table S3 for primers used) (Janse *et al.*, 2006b).

Generation and analyses of parasites expressing ROM3::GFP and ROM4::mCherry

To tag ROM3 C-terminally with GFP, 1.6 kb upstream of the stop codon of *rom3* containing the entire ORF were PCR-amplified from wild-type *P. berghei* ANKA genomic DNA, TOPO-cloned and sequence analysed (see Table S2 for the primers). The fragment was released from pCR2.1-TOPO (Invitrogen) through digestion with *EcoRV* and *BamHI* and ligated into the generic GFP-tagging plasmid pL1200 (Mair *et al.*, 2010), resulting in construct pL1079 containing the pyrimethamine resistant *tgdhfr/ts* as a selectable marker. To tag ROM4 with mCherry, the complete ORF of *rom4* (except the stop codon) was PCR-amplified from wild type *P. berghei* ANKA genomic DNA (see Table S2 for the primers used). This ORF was digested with *SpeI* and *BglII*, and ligated into *SpeI/BamHI* digested vector pL1646 (containing a C-terminal mCherry tag and the *tgdhfr/ts* selectable marker cassette), resulting in construct pL1920. Before transfection, pL1709 was linearized with *SpeI*, and pL1920 was linearized with *BamHI*. Transfection, selection and cloning of transgenic parasites with pyrimethamine was carried out as described above, generating the following transgenic lines, *rom3::gfp* (line 654cl1) and *rom4::mCherry* (line 2143cl1) that express endogenous C-terminally tagged ROM3 and ROM4 respectively. The live GFP or mCherry signals of parasites of the transgenic mutants were examined by fluorescence microscopy (Leica DM-IRBE Flu) after staining with Hoechst-33342 (2 $\mu\text{mol l}^{-1}$, Sigma, NL) for 15 min at room temperature. The fluorescence intensity was measured using ImageJ software.

Analysis of transcription of rhomboid genes in blood stages of wild type and gene-deletion parasites

Transcript levels were analysed by standard Northern blot analyses. Total RNA was isolated from mixed blood stages or different stages of wild type *P. berghei* ANKA (cl15cy1), non-gametocyte producer line (HPE) and the different gene-deletion mutant lines. Northern blots were hybridized with

probes specific for each rhomboid ORF, which had been PCR-amplified from wild-type *P. berghei* ANKA genomic DNA (primers shown in Table S3). As a loading control, Northern blots were hybridized with the oligonucleotide probe L644R that recognizes the large subunit ribosomal RNA (van Spaendonk *et al.*, 2001).

In vivo multiplication rate of asexual blood stages

The multiplication rate of asexual blood stages in mice is determined during the cloning procedure of each gene-deletion mutant (Spaccapelo *et al.*, 2010) and is calculated as follows: the percentage of infected erythrocytes in Swiss OF1 mice injected with a single parasite is quantified at days 8–11 on Giemsa-stained blood films. The mean asexual multiplication rate per 24 h is then calculated assuming a total of 1.2×10^{10} erythrocytes per mouse (2 ml of blood). 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 after infection, resulting in a mean multiplication rate of 10 per 24 h (Janse *et al.*, 2003; Spaccapelo *et al.*, 2010).

Course of parasitemia, virulence and experimental cerebral malaria in mice infected with $\Delta rom1$ parasites

The course of parasitemia was determined in BALB/c mice. Groups of six mice were intraperitoneally (i.p) infected with 10^4 $\Delta rom1$ -c parasites or equal numbers of the parental reporter line *PbGFP-LUC_{con}*. The course of parasitemia was determined in a luciferase assay (IVDL-assay) (Franke-Fayard *et al.*, 2008); *in vivo* parasite growth in mice is quantified by measuring the luciferase activity of GFP-Luciferase expressing parasites in tail blood. The IVDL-assay generates growth-curves that are identical to those obtained by manual counting of parasites in Giemsa-stained smears. In brief, 10 μl tail blood was collected daily from all mice using heparinized capillaries. Samples were stored at -80°C in Eppendorf tubes till further processing for the luciferase assay. Luciferase activity (luminescence) in the samples was measured as described (Franke-Fayard *et al.*, 2008; Lin *et al.*, 2013).

The capacity of $\Delta rom1$ to induce features of ECM was analysed in C57BL/6 mice. Groups of six mice were infected with 10^5 *P. berghei* ANKA (cl15cy1), $\Delta rom1$ -p or $\Delta rom1$ -c parasites. Onset of ECM in *P. berghei* infection was determined by measurement of a drop in body temperature below 34°C (Spaccapelo *et al.*, 2010). 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, 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.

Gametocyte and ookinete production

Gametocyte production is defined as the percentage of ring forms developing into mature gametocytes during synchronized infections (Janse and Waters, 1995). Ookinete produc-

tion was determined in standard *in vitro* fertilization and ookinete maturation assays and is defined as the percentage of female gametes that develop into mature ookinetes under standardized *in vitro* culture conditions (van Dijk *et al.*, 2001). Female gamete and mature ookinete numbers were determined in Giemsa-stained blood smears made 16–18 h post-activation.

Electron microscopy

Ookinetes were cultured *in vitro* as previously described (van Dijk *et al.*, 2001); briefly, gametocytes for these assays were obtained from infected mice that had been pretreated with phenylhydrazine and treated with the antimalarial drug sulfadiazine (dissolved in the drinking water at a concentration of 30 mg l⁻¹) to obtain highly pure gametocyte populations (Beetsma *et al.*, 1998). Ookinetes from these cultures were purified using a Nycodenz density gradient centrifugation. The purified ookinetes were diluted in RPMI medium (without serum) and fixed by resuspending them in an equal amount of 3% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.2 M sodium cacodylate pH 7.4 (1 h at RT), washed twice with cacodylate buffer (spinning for 2 min at 425 g), post-fixed with 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA) in cacodylate buffer (1 h at RT), and washed again with cacodylate buffer. Subsequently, the samples were stained with an aqueous solution of 1% uranyl acetate for 40 min at RT, washed twice with demineralized water, resuspended in 3% agar (Difco Laboratories, Detroit, Michigan; in demineralized water at 60°C), centrifuged (for 2 min at 425 g) and stored at 4°C until the agar became solid. The samples were dehydrated in series of washes 70% (overnight), 80% (10 min), 90% (10 min) and 100% ethanol (1 h, refreshing two times), infiltrated with a 1:1 mixture of epon LX 112/propylene oxide (1 h) and pure epon (3 h), followed by embedding in epon and polymerization for 48 h at 60°C. The samples were cut using a Leica UC6 ultramicrotome at RT into 100 nm sections with a Diatome ultra 45° diamond knife (Diatome, Switzerland) at a cutting speed of 1 mm s⁻¹. The sections were attached to slot copper grids (Stork Veco BV, Eerbeek, the Netherlands), covered with 1% formvar film and a 7 nm carbon layer; no post-staining was applied prior to data collection. Imaging was performed in a Tecnai 12 Twin transmission electron microscope (FEI Company). Transmission electron microscope was operated at an acceleration voltage of 80 kV, and binned images (2k × 2k) were acquired with a FEI Eagle CCD camera (FEI Company).

Oocyst and sporozoite production in *Anopheles stephensi* mosquitoes

For mosquito transmission experiments female *A. stephensi* mosquitoes were fed on mice infected with wild-type parasites or mutants. Oocyst development, oocyst production and sporozoite production was monitored in infected mosquitoes as described (Sinden, 1997). Oocyst and sporozoites numbers were counted in infected mosquitoes at 11–14 days and 19–22 days after infection respectively. Salivary gland sporozoites were isolated and counted as described (Annoura *et al.*, 2012).

For Western blot analysis of CSP expression in oocysts or sporozoites, we isolated oocysts containing midguts from infected mosquitoes, or 100 000 midgut sporozoites, and proteins were separated on 8% polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. CSP expression was detected by incubation of membranes with monoclonal anti-CSP antibody (Potocnjak *et al.*, 1980) followed by incubation with horseradish-peroxidase-conjugated anti-mouse antibody (Sigma). Immunostained protein complexes were visualized by enhanced chemiluminescence (Amersham). For the IFA, midguts of infected mosquitoes were isolated in RPMI-Medium and immobilized with 2% formaldehyde, 0.2% glutaraldehyde, 2 mM magnesium chloride, 0.02% Triton X-100 in phosphate-buffered saline (PBS). Subsequently midguts were permeabilized, using 2% Saponin in PBS and oocysts stained with monoclonal anti-CSP antibody and monoclonal Alexa-Fluor® 488 goat anti-mouse antibody. DNA was stained with Hoechst-33342.

Sporozoite infectivity and liver stage development

Sporozoites were collected at days 19–25 after infection by hand-dissection of the salivary glands as described (Annoura *et al.*, 2012). Gliding motility of sporozoites was determined in assays that were performed on anti-*P. berghei* CSP antibody (3D11, monoclonal mouse antibody 25 µg ml⁻¹, 100 µl per well) pre-coated 10-well cell-line diagnostic microscope slides (7 mm, Thermo Scientific) to which 1 × 10⁴ sporozoites were added (van Dijk *et al.*, 2005). After 30 min of incubation at 37°C sporozoites were fixed with 4% paraformaldehyde and after washing with PBS, the sporozoites and the trails ('gliding circles') were stained with anti-CSP-antiserum (Yoshida *et al.*, 1980) and anti-rabbit IgG-secondary antibody (Alexa Fluor® 488 Goat Anti-rabbit IgG; Molecular Probes®, Invitrogen). Slides were mounted with Vectorshield (Vector Laboratories) and 'gliding circles' were analysed using a Leica DMR fluorescence microscope at 1000× magnification.

Huh-7 cells, a human hepatoma cell line, were used in *in vitro* analysis of sporozoite infectivity. Huh-7 cells were cultured in 'complete' RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum (FBS), 1% (vol/vol) penicillin-streptomycin, 1% (vol/vol) GultaMAX (Invitrogen), and maintained at 37°C with 5% CO₂. Sporozoite hepatocyte traversal was determined in assays as described previously (Mota *et al.*, 2001). Briefly, Huh-7 cells were suspended in 1 ml of 'complete' medium and were seeded in 24-well plates (10⁵ cells ml⁻¹). After the Huh7 monolayers were > 80% confluent, 10⁵ sporozoites were added with the addition of FITC- or Alexa-647-labelled dextran (Invitrogen, NL) for 2 h. No sporozoites were added to the negative control wells. *In vitro* invasion rates of mutants lacking expression of rhomboids and wild type parasites were determined by the ratio between the number of sporozoites inside the cells and the total number of sporozoites (both inside and outside of cells). Huh-7 cells (5 × 10⁴ cells per well) were seeded into coverslips in 24-well plates and on the following day, cells were infected with 5 × 10⁴ sporozoites. Three hours after infection, cells were fixed with 4% paraformaldehyde in PBS for 20 min. For the double staining, cells were incubated for 30 min in blocking buffer (10% FBS in PBS) followed by 1 h incubation with anti-CSP serum against *P. berghei* CSP protein (Yoshida

et al., 1980) diluted 1:500 in the same buffer. Cells were then washed with PBS and incubated for 45 min with a secondary antibody (anti-rabbit Alexa Fluor® 568) diluted 1:500 in blocking buffer. This procedure only stained the parasites outside the cells. After washing with PBS, cells were fixed with 4% paraformaldehyde in PBS for 30 min, then incubated in for 30 min in permeabilization buffer (1% Triton X-100 in PBS) followed by 1 h incubation with anti-CSP serum diluted 1:500 in the same buffer. Cells were then washed with PBS and incubated for 45 min with a secondary antibody (anti-rabbit Alexa Fluor® 488) diluted 1:500 in blocking buffer. This second staining allows visualization of all the parasites, whether inside or outside the cells. Nuclei were stained with Hoechst-33342. The parasites in both green and red channels were analysed using a DM-IRBE Flu Leica fluorescence microscope.

For analysis of *in vitro* EEF (exo-erythrocytic form) development, 5×10^4 sporozoites were added to a monolayer of Huh7 cells on coverslips in 24-well plates in 'complete' RPMI 1640 (see above). At different time points after infection, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS, blocked with 10% FBS in PBS, and subsequently stained with primary and secondary antibodies for 2 h and 1 h respectively. Primary antibodies used were anti-PbEXP1 [raised in chicken (Sturm *et al.*, 2006)] and anti-UIS4 [raised in rabbit (Mueller *et al.*, 2005)], detecting the PVM-resident proteins; anti-PbHSP70 [raised in mouse (van Dijk *et al.*, 2005)], detecting the cytoplasmic heat shock protein 70 (PBANKA_081890); and anti-MSP1 (mouse; MRA-78 from MR4; <http://www.MR4.org>) detecting MSP1 of *P. yoelii* and *P. berghei*. Anti-mouse, -chicken and -rabbit secondary antibodies, conjugated to Alexa Fluor® 488 and 594, were used for visualization (Invitrogen). Nuclei were stained with Hoechst-33342. Cells were mounted in Vectashield (Vector Laboratories) and examined using a DM-IRBE Flu Leica fluorescence microscope.

In addition, intracellular parasite development was determined by measuring the area of the EEFs inside fixed Huh-7 hepatoma cells. Huh-7 cells (5×10^4 cells per well) were seeded into coverslips in 24-well plates and, the following day, cells were infected with 4×10^4 sporozoites. Forty-eight hours after infection, cells were fixed with 4% paraformaldehyde in PBS for 20 min. Cells were stained by incubation for 30 min in blocking/permeabilization buffer (0.5% Triton X-100, 1% BSA in PBS) followed by 1 h incubation with monoclonal antibody 2E6 against *P. berghei* HSP70 diluted 1:500 in the same buffer. Cells were then washed with 0.5% Triton X-100 in PBS and incubated for 45 min with a secondary antibody (anti-mouse Alexa488) diluted 1:400 in blocking/permeabilization buffer. Nuclei were stained with DAPI. Images were acquired with a Zeiss Axiovert 200 M widefield fluorescence microscope and processed using ImageJ.

Hepatocyte infection was determined by measuring the luminescence intensity in Huh-7 cells infected with either the firefly luciferase-expressing $\Delta rom9$ and the corresponding control line PbGFP-Luc_{schz}. Huh-7 cells infection and culture conditions were as described above. At 57 h after infection, 50 μ l of D-Luciferin (Firefly Luciferase Assay Kit, Biotium) were added to 30 μ l of lysed samples in white 96-well plates. Luminescence intensity of the samples was measured using a microplate reader (Tecan Infinite M200). The viability of

Huh-7 cells was assessed by the AlamarBlue assay (Invitrogen, UK) according to the manufacturer's protocol.

To determine parasite loads by qRT-PCR, sporozoites (5×10^4) were added to a monolayer of Huh7 cells (seeded the day before with 10^5 cells) in 24-well plates in 'complete' DMEM (Dulbecco's modified Eagle's medium) (see above). At different time-points after adding the sporozoites, culture medium was removed, cells washed once with PBS, and cells were resuspended in 200 μ l of RLT buffer (Qiagen's MicroRNeasy kit). RNA from these samples was extracted following the manufacturer's instructions. The transcripting first-strand cDNA synthesis kit (Roche) was used according to the manufacturer's recommendations to make single-stranded cDNA. Real time PCR analysis of *P. berghei* 18S rRNA and human β -actin was performed as described (Ploemen *et al.*, 2009).

Flow cytometry analysis was used to determine the parasite load of $\Delta rom9$ in Huh-7 cell (57 h p.i.) compared the wild type control (PbGFP-Luc_{schz}). The infection and culture condition was as described above. Fifty-seven hours after infection, samples for were washed with PBS, incubated with trypsin for 5 min at 37°C and collected in 400 μ l of 10% v/v FBS in PBS. Cells were then centrifuged at 0.1 g for 5 min, resuspended in 300 μ l of 2% v/v FBS in PBS. All samples were analysed on a LSR Fortessa cytometer with the appropriate settings for the fluorophores used. Data acquisition and analysis were carried out using the BD FACSDiva (BD Biosciences) and FlowJo (v6.4.7, FlowJo) software packages respectively.

To determine *in vivo* infectivity of sporozoites, Swiss OF1 mice were infected with 1×10^4 salivary gland sporozoites by intravenous injection, as previously described (Sinden, 1997). Blood stage infections were monitored by analysis of Giemsa-stained thin smears of tail blood collected on days 4–8 after inoculation of sporozoites. The prepatent period (measured in days post sporozoite infection) is defined as the day when a blood stage infection with a parasitemia of 0.5–2% is observed. In addition we determined parasite loads in livers of infected mice by *in vivo* imaging as described (Ploemen *et al.*, 2009). In addition, a group of 10 male C57BL/6 mice were inoculated by i.v. injection of 1×10^4 freshly isolated sporozoites of $\Delta rom9$ or its wild type control PbGFP-Luc_{schz}. Luciferase activity in the livers of the animals was visualized 44 h after infection using an *in vivo* Imaging System (IVIS Lumina), following i.p. injection of D-luciferin dissolved in PBS (100 mg kg⁻¹). Animals were kept under anesthesia during the measurements, which were performed within 3–5 min after the injection of D-luciferin. Bioluminescence imaging was acquired with a 10 cm field of view, medium binning factor and an exposure time of 180 s. Quantitative analysis of bioluminescence was performed by measuring the luminescence signal intensity using the ROI settings of the Living Image® 3.0 software. ROI measurements are expressed in total flux of photons. qRT-PCR is also used to determine the *in vivo* parasite load in liver. After measuring luminescence, livers from 5 mice were collected, homogenized in 4 ml denaturing solution (4 M guanidine thiocyanate; 25 mM sodium citrate pH 7, 0.5% sarcosyl and 0.7% β -Mercaptoethanol in DEPC-treated water) and total RNA was extracted using the RNeasy Mini kit (Qiagen). cDNA was obtained by reverse transcription (First-strand cDNA synthe-

sis kit, Roche) and qRT-PCR using the SybrGreen method (DyNAmo™ HS SYBR® Green qPCR Kit, Finnzymes) was performed with primers specific for *P. berghei* 18S rRNA for quantification of parasite load in the liver of each mouse. Relative amounts of *P. berghei* mRNA were calculated against the Hypoxanthine Guanine Phosphoribosyl Transferase (HPRT) housekeeping gene (Ploemen *et al.*, 2009).

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