Egress of *Plasmodium berghei* gametes from their host erythrocyte is mediated by the MDV-1/PEG3 protein

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Summary

Malaria parasites invade erythrocytes of their host both for asexual multiplication and for differentiation to male and female gametocytes – the precursor cells of *Plasmodium* gametes. For further development the parasite is dependent on efficient release of the asexual daughter cells and of the gametes from the host erythrocyte. How malarial parasites exit their host cells remains largely unknown. We here report the characterization of a *Plasmodium berghei* protein that is involved in egress of both male and female gametes from the host erythrocyte. Protein MDV-1/PEG3, like its *Plasmodium falciparum* orthologue, is present in gametocytes of both sexes, but more abundant in the female, where it is associated with dense organelles, the osmiophilic bodies. ∆mdv-1/peg3 parasites in which MDV-1/PEG3 production was abolished by gene disruption had a strongly reduced capacity to form zygotes resulting from a reduced capability of both the male and female gametes to disrupt the surrounding parasitophorous vacuole and to egress from the host erythrocyte. These data demonstrate that emergence from the host cell of male and female gametes relies on a common, MDV-1/PEG3-dependent mechanism that is distinct from mechanisms used by asexual parasites.

Introduction

The *Plasmodium* life cycle is a sequence of alternating invasive and replicating stages within the cells of the vertebrate and invertebrate host. In contrast to the increasing knowledge of invasion of host cells (Baum et al., 2008), the molecular mechanisms underlying egress from the host cell are largely unknown. Only recently, have the first proteins been characterized that are involved in egress from malaria parasites from the host red blood cell (Blackman, 2008) while those involved in the release of parasites from the infected liver cell are yet to be identified. Malaria parasites invade red blood cells both for asexual multiplication and for the formation of gametocytes, the precursor cells of gametes. All bloodstream forms of *Plasmodium* are dependent on efficient egress from the host erythrocyte for continued development. Egress of the daughter merozoites is mediated by a subpopulation of dense granules in the schizont, the exosomes (Yeh et al., 2007). Rupture of these organelles releases in the parasitophorous vacuole parasite protease Subtilisin1, which cleaves at least two putative proteases of the SERA family, and is essential to merozoite release. Interestingly, the product of a member of the SERA family, ECP-1, also plays a role in sporozoites release from the oocyst (Aly and Matuschewski, 2005), and multiple papain-like proteases are present in liver stages (Sturm et al., 2006; Sturm and Heussler, 2007; Schmidt-Christensen et al., 2008), suggesting that a similar protease activity also plays a role in the egress of the liver merozoites.
In sexual differentiation, both the proteins and mechanisms of the egress of the female and male gametes from the erythrocyte are unknown. As with merozoite, gamete egress involves rupture of two membranes, the parasitophorous vacuole membrane (PVM) and the infected erythrocyte membrane. However, unlike merozoites, a possible role of cysteine and papain-like proteases in the process of gamete emergence has not yet been identified, and analyses of the proteomes of gametocytes have not detected such proteases (Hall et al., 2005; Khan et al., 2005). Inhibition of male gamete exflagellation by protease inhibitors has been reported in P. berghei (Torres et al., 2005) and P. falciparum (Rupp et al., 2008), although a specific role of these compounds on emergence remains to be demonstrated. It is also thought that the mechanical shear forces generated by male gametes during exflagellation may also contribute to the rupture of surrounding membranes. Recently, a protein has been characterized associated with the emergence of female gametes and conserved in Plasmodium, Pfg377 (PFL2405c; de Koning-Ward et al., 2008). Female gametes of P. falciparum deficient in Pfg377 expression emerged from their host erythrocyte significantly less efficiently. Interestingly, this protein is associated with electron-dense organelles, the osmiophilic bodies (OBs), suggesting that these organelles might be functionally equivalent to the electron-dense exosomes of asexual schizonts. OBs are membrane-bound vesicles, found predominantly in female gametocytes, and the release of their contents into the parasitophorous vacuole has been postulated to aid in the escape of gametocytes from the erythrocyte (Alano et al., 1995). The association between OBs and gamete egress is strengthened by the observation that the Pfg377 gametocytes have reduced numbers of OBs (de Koning-Ward et al., 2008). To date, only one other protein is known to be associated with OBs, Pfg377 (protein of early gametocyte 3) or Plmg3 (male development-1) (PFL0795c; Silvestrini et al., 2005; Furuya et al., 2005). However, this protein was shown to be associated not only with the OBs but also with the parasite membrane and all membranous compartments derived from the parasitophorous vacuole (Furuya et al., 2005; Lanfrancoti et al., 2007). P. falciparum parasites deficient in expressing Plmg3 were affected in their capacity to produce functional gametocytes, which were unable to be transmitted through mosquitoes. A recent analysis of the Plmg3 orthologue of the rodent parasite P. berghei suggested a role of this protein in the production of fertile female gametes, and disruption of the gene encoding this protein resulted in a significantly reduced formation of zygotes, ookinetes and oocysts (Lal et al., 2009).

In order to define the possible role of MDV-1/PEG3 in the emergence and formation of fertile gametes, we have analysed the localization and function of this protein in more detail in P. berghei. Our results also show that in parasites lacking MDV-1/PEG3 the formation of fertile gametes and subsequent fertilization were strongly inhibited. However, closer examination revealed that the ΔMDV-1/PEG3 phenotype resulted chiefly from a defect in emergence of both female and male gametes from the host cell, thus indicating that a second Plasmodium protein is associated with OBs and is involved in egress of gametocytes from the host cell.

Results

Characterization of the gene encoding MDV-1/PEG3 of P. berghei

Homology searches with the PfMDV-1/PEG3 protein sequence in Plasmodium genome databases identified a single P. berghei MDV-1/PEG3 orthologue, as well as MDV-1/PEG3 single copy orthologues in other rodent, human and simian malarias. The identified mdv-1/peg3 orthologous genes (PB000248.02.0 in P. berghei, PC000315.05.0 in P. chabaudi, PY00179 in P. yoelii, PKH_143370 in P. knowlesi and Pv123505 in P. vivax), encoded highly similar proteins ranging from 214 to 289 amino acids in length, characterized by a signal peptide, a predicted largely alpha-helical structure, and regions of significant local homology possibly representing functionally conserved domains (Fig. S1). No obvious homologies to eukaryotic conserved functional or structural domains were otherwise detected which could drive hypotheses on the function of the MDV-1/PEG3 proteins. Southern blot analysis with a pbmdv-1/peg3-specific probe on separated P. berghei chromosomes produced a single hybridization signal on chromosome 14 (data not shown). As the P. falciparum orthologous gene is located 660 kb from the left telomere of chromosome 12 (Furuya et al., 2005), location of the pbmdv-1/peg3 gene on rodent chromosome 14 is consistent with the reported synteny between the largest portion of P. falciparum chromosome 12 and that of P. berghei chromosome 14 (Kooij et al., 2005).

P. berghei MDV-1/PEG3 is predominantly expressed in gametocytes

Antibodies raised against a recombinant portion of the PbMDV-1/PEG3 protein were used to analyse expression of the protein during blood stage development by both immunofluorescence and Western blot analysis. Control mouse pre-immune serum did not react with the different blood stages (data not shown), whereas the antibodies against PbMDV-1/PEG3 specifically stained gametocytes but did not react with asexual blood stages (Fig. 1A). Western blot analysis of proteins obtained from purified,
mature gametocytes and from asexual stages confirmed the gametocyte-specific expression of MDV-1/PEG3. The antibodies recognized a major band at approximately 25 kDa, in reasonable agreement with the molecular weight of 21 kDa predicted from the protein sequence without the signal peptide (Fig. 1B, left panel). The presence of MDV-1/PEG3 during subsequent zygote and ookinete formation was investigated by Western blot analysis on protein samples collected from a culture in which 60–70% of the female gametocytes were fertilized and had transformed into zygotes (4 h after induction of gamete formation) and ookinetes (18 h). This experiment revealed that in unfertilized gametes/zygotes or ookinetes either no or a very weak signal specific for MDV-1/PEG3 was detectable in contrast to the strong signal obtained in gametocyte preparations (Fig. 1B, central panel). In order to test whether MDV-1/PEG3 was possibly released at gametogenesis, a preparation of mature gametocytes was obtained, divided in two aliquots, and one was induced to undergo gametogenesis. After 30 min, supernatants from both aliquots were analysed by Western blot with anti-MDV-1/PEG3 antibodies. This experiment clearly showed that MDV-1/PEG3 is readily detectable in the supernatant of induced gametocytes, and absent from that of non-induced gametocytes (Fig. 1B, right panel). These results in conclusion indicate that the MDV-1/PEG3 protein is predominantly produced in gametocytes, and it is mostly released extracellularly during gamete formation, being minimally present in unfertilized gametes, zygotes and during further development of the ookinete.

Sex-specific expression of MDV-1/PEG3

To investigate the sex-specific expression of MDV-1/PEG3, we analysed the staining pattern of the above antibodies on male and female gametocytes of a transgenic reporter *P. berghei* line that expresses GFP exclusively in gametocytes, and in a sex-specific manner. This line, pG-SET/GFP, expresses a GFP-tagged version of the nuclear protein SET (PB001284.02.0) under control of a gametocyte-specific promoter, producing a more intense, nucleus-associated fluorescence in male compared with female gametocytes (Pace et al., 2006). Analysis of immature gametocytes (20–24 h post invasion; hpi) and mature gametocytes (28 hpi) of this line revealed a significantly stronger staining of the cytoplasm of female gametocytes with the MDV-1/PEG3 antibodies compared with the male gametocytes, and a clear increase in staining intensity during maturation of the gametocytes (Fig. 2A). In addition, this analysis showed a clear difference in the staining pattern of the MDV-1/PEG3 antibodies in the two sexes, being more localized throughout the whole cytoplasm in female gametocytes, while confined in one or a few spots in males (Fig. 2A).

To further investigate the expression regulation of MDV-1/PEG3, we analysed the activity of the promoter of the *pbmdv-1/peg3* gene by producing a transgenic reporter parasite (line 707cl1) where a GFP reporter gene was placed under the control of 1.5 kb of *pbmdv-1/peg3* genomic upstream region. This construct was stably integrated into the c-type *ssu-rrna* locus on chromosome 5 by single cross-over integration, as confirmed by Southern blot analysis of separated chromosomes (Fig. S2A and B) and by diagnostic PCR analysis (data not shown). GFP expression of these parasites was analysed by fluorescence microscopy of the blood stages during synchronized development in vivo and in vitro. In the first 16 hpi, corresponding to development of ring forms into mature trophozoites, no GFP expression could be detected. The first GFP-positive cells were detected at 17 h (Fig. 2B) and their number rapidly increased between 17 and 24 hpi, although the percentage of GFP-positive parasites never exceeded 15%. This, and the observation that no schizonts were ever found to be GFP-positive, strongly suggested that the GFP fluorescent cells were gametocytes.
purified mature gametocytes from this line by fluorescence microscopy and FACS identified two populations with clearly different fluorescence intensities (Fig. 2B). FACS sorting and Giemsa staining of the cells from the two populations showed that cells with the lower GFP fluorescence were male and those being more strongly fluorescent were female gametocytes (Fig. 2B). In conclusion, the observations that the promoter of pbmdv-1/peg3 is switched on only in gametocytes from around 17 hpi, and that it is more active in female than in male gametocytes are in full agreement with the results of the immunofluorescence analyses, which detected higher levels of the MDV-1/PEG3 protein in female gametocytes.

Subcellular localization of MDV-1/PEG3 in gametocytes shows association with OBs in female gametocytes

In order to identify the subcellular localization of MDV-1/PEG3, the above specific antibodies were used in immunoelectron microscopy analysis of purified gametocytes. As fixation protocols designed to optimally preserve subcellular structures failed to reveal specific signals, a comparatively harsher protocol was used in order to improve antibody accessibility to the protein in the fixed cells. In this experiment, anti-MDV-1/PEG3-specific gold particles were clearly observed in association with OBs, the electron-dense organelles preferentially present in the cytoplasm of female gametocytes (Fig. 3A), while the
pre-immune serum did not give specific signals (Fig. 3B).

Production of gametocytes is not affected in parasites deficient in expression of MDV-1/PEG3 ($\Delta$mdv-1/peg3 parasites)

To analyse the function of MDV-1/PEG3, different parasite lines deficient in expression of the protein were generated by disruption of the $pbmdv-1$/$peg3$ gene. The construct used for replacement of the $pbmdv-1$/$peg3$ coding region contained the $tdhfr$ selection cassette flanked by 5′- and 3′-UTR $pbmdv-1$/$peg3$ regions necessary to target the construct by double cross-over integration into the chromosomal locus by homologous recombination (Fig. S3A). In two independent experiments the construct was transfected in the reference ANKA strain of $P. berghei$ (line cl15cy1) and two transformed parasite clones were obtained, $\Delta$mdv-1/peg3a (clone 678cl1) and $\Delta$mdv-1/peg3b (clone 721cl1). Correct integration of the construct was confirmed by diagnostic PCR and Southern blot analysis (Fig. S3B and C). A third line deficient in expression of MDV-1/PEG3 was generated using a parental line ($wt$-fluo; line 820cl1m1cl1) which contains, stably integrated into the genome, the $gfp$ and the red fluorescent protein (rfp) reporter genes under the control of a male and of a female-specific promoter respectively (Fig. S4A and B). The purpose of this line was to perform the quantitative analysis of fertilization and meiosis described below. As this line no longer contains drug selectable markers, the above DNA construct was used to disrupt the $pbmdv-1$/$peg3$ gene, yielding the line $\Delta$mdv-1/peg3-fluo (928cl1). Correct integration of the DNA construct and disruption of $mdv-1$/$peg3$ were confirmed by diagnostic PCR and Southern analysis of separated chromosomes (Fig. S4D).

The preliminary morphological analysis on the $\Delta$mdv-1/peg3 parasites revealed that the capacity to produce male and female gametocytes was not affected in the absence of MDV-1/PEG3. Gametocyte counts in Giemsa stained blood films obtained from mice with synchronized infections indicated that, under standard conditions, 18% of trophozoites of $\Delta$mdv-1/peg3a (range 16–23%; three experiments) and 20% of the $\Delta$mdv-1/peg3b (range 18–22%; four experiments) developed into morphologically mature gametocytes, compared with the 15–25% of the reference wild-type line cl15cy1 (van Dijk et al., 2001; Janse and Waters, 2005). In synchronized infections of
mice pretreated with phenylhydrazine the average female/male ratios in the defective lines were 1.2 and 1.4 (range 1.2–1.5) compared with ratios ranging between 1 and 1.8 in wild-type parasites (van Dijk et al., 2001; Janse and Waters, 2005). Northern analysis of mRNAs collected from purified gametocytes showed absence of the pbmdv-1/peg3 transcript in both Δmdv-1/peg3a and Δmdv-1/peg3b (Fig. S5A). Both Western blot and immunofluorescence analyses with MDV-1/PEG3 antibodies failed to detect the protein in Δmdv-1/peg3 purified gametocytes (Fig. S5B and C and data not shown). Finally, immunoelectron microscopy analysis with the same antibodies indicated that OBs are present in the Δmdv-1/peg3 gametocytes but could not be decorated with MDV-1/PEG3-specific gold particles (Fig. S5D).

Δmdv-1/peg3 male and female gametocytes are strongly affected in their capacity to produce viable zygotes and ookinetes

The fertility of the Δmdv-1/peg3 gametocytes was analysed in standard in vitro fertilization and ookinete maturation assays. Gametocytes of all three Δmdv-1/peg3 parasite lines were strongly impaired in their capacity to produce mature ookinetes (Fig. 4A). Although small numbers of ookinetes were nevertheless formed, they represented at most 6% (range 0–6%, 12 exp.) of the total ookinete population. This reduction in ookinete formation was due to a very low zygote conversion rate (Fig. 4A). In wild-type parasites, the zygote conversion rate was determined to be 79% (mean ± SD, 79 ± 1%). This rate was strongly reduced in Δmdv-1/peg3 parasites (Fig. 4A). The frequency of zygotes per ookinete in Δmdv-1/peg3 parasites varied between 2% and 8% (mean ± SD, 6 ± 2%). This reduced zygote conversion rate in Δmdv-1/peg3 parasites indicates a strong impairment at the step of zygote formation.

Fig. 4. Analysis of fertilization and formation of zygotes and ookinetes of Δmdv-1/peg3 parasites.
A. The ookinete conversion rate, defined as the percentage of female gametes developing into ookinetes, is strongly reduced in Δmdv-1/peg3 and in crossings of the Δmdv-1/peg3 parasites with fertile males (♂♂♂♂) or fertile females (♀♀♀♀) of line Δp47. Purple colour in each bar indicates standard deviation interval.
B. FACS analysis of the DNA content (ploidy) of female gametocytes, gametes and zygotes at 4 h after induction of gametogenesis shows the strongly reduced formation of tetraploid zygotes in Δmdv-1/peg3. Female cells and zygotes were selected on RFP expression. Hoechst 33258 fluorescence intensity measurement of the DNA content (ploidy) shows the presence of two distinct populations in wild-type parasites (wt/fluo) after fertilization: unfertilized female gametes, escaped from the erythrocyte (Gate P1), and zygotes with a tetraploid DNA content (Gate P3). In the Δmdv-1/peg3-fluo mutants most females have intermediate fluorescence intensity (Gate P2), representing female gametocytes/gametes inside the erythrocytes (see Results and Fig. S7).
C. Strongly reduced rates of fertilization and zygote formation in Δmdv-1/peg3 parasites as shown by the percentages (mean and SD) of free, unfertilized female gametes (Gate P1), zygotes (Gate P3) and female gametocytes/gametes inside erythrocytes (Gate P2) determined as described in B. In wild-type (wt-fluo) parasites most females (>50%) are fertilized (Gate P3), whereas more than 60% of the females of Δmdv-1/peg3-fluo are still inside their host cell (Gate P2), also after crossing with fertile males of Δp47. As a control, a mutant is shown (Δp48/45-fluo) that produces fertile free female gametes but no fertile males. Crossing with fertile males of Δp47 shows wild-type fertilization rates (>50%).
ookinete production observed in wild-type parasites (Fig. 4A). To further analyse the fertility of male and female gametocytes separately, standard in vitro cross-fertilization assays were performed, in which the Δmdv-1/peg3 gametes were cross-fertilized with gametes of two mutant lines producing either only fertile males (line 270cl1: Δp47; Khan et al., 2005) or only fertile females (line 137cl8: Δp48/45; van Dijk et al., 2001). While the control cross-fertilization between Δp47 and Δp48/45 gametocytes produced wild-type numbers of ookinetes, all combinations of crosses involving Δmdv-1/peg3 gametocytes produced instead significantly lower numbers of ookinetes (Fig. 4A). These results demonstrate that both the male and female gametocyte/gametes of the Δmdv-1/peg3 lines are strongly affected in their capability of producing ookinete.

As the few ookinetes analysed 18 h after gametogenesis showed a normal morphology in Giemsa-stained smears, comparable to that of wild-type ookinetes, this suggested that Δmdv-1/peg3 parasites were defective either in gamete formation or in fertilization. The rate of fertilization and meiosis was therefore determined by FACS analysis at 4 h after induction of gametogenesis (Fig. 4B). This experiment was conducted with the Δmdv-1/peg3-fluo line, in which the female gametocytes and gametes/zygotes express RFP allowing for FACS selection of the RFP-positive females to be analysed. Before FACS analysis cells were stained with the DNA-specific dye Hoechst 33258 to measure DNA content and determine the ploidy of female gametocytes and gametes/zygotes (Khan et al., 2005; Janse and Vianen, 1994). In the wild-type parasites of parent line wt-fluo, more than 60% of the female gametes were fertilized and underwent meiosis at 4 h, producing zygotes with a tetraploid DNA content (gate P3 in Fig. 4B and C). A comparable rate of zygote formation was observed when Δp47 fertile males were crossed with Δp48/45 fertile females (Fig. 4C). In contrast, the percentage of Δmdv-1/peg3-fluo female cells reaching the tetraploid DNA content was reduced to less than 25%. In addition, cross-fertilization of the Δmdv-1/peg3 female gametocytes/gametes with Δp47 fertile males did not result in a significant increase of zygotes (Fig. 4B and C). This analysis therefore strongly suggested that Δmdv-1/peg3 female gametes are unable to be fertilized even in the presence of fertile male gametes.

An intriguing observation in the FACS analysis was that most of the activated Δmdv-1/peg3 female cells showed both a slight increase of Hoechst33258 fluorescence intensity and an increase in size (forward size scatter) compared with wild-type activated unfertilized gametes (Fig. 4B and C; gate P2). As previous work showed that Hoechst 33258-stained parasites within the host erythrocyte exhibit a higher level of fluorescence compared with free parasites, due to host erythrocyte background fluorescence (Janse et al., 1987), we hypothesized that the increased fluorescence intensity of the Δmdv-1/peg3-Fluo-activated female gametes was due to the fact that they were still contained within the erythrocyte.

ΔMDV-1/PEG3 gametocytes are strongly affected in their capacity to form gametes able to emerge from the host erythrocyte

In order to test this hypothesis the process of gamete formation was analysed in more detail in both female and male gametes of the Δmdv-1/peg3 lines. Light microscopy analysis of live gametes at 15–30 min after induction confirmed that most of the round-shaped female gametes were still surrounded by the red blood cell membrane. While light microscopy analysis was not straightforward for the female gametes, it was more reliable for the male gametes. Trapped Δmdv-1/peg3 exflagellating male gametes were readily detectable by the actively moving gametes within the red blood cells, resulting in characteristic protuberances of the erythrocyte membrane. These male gametes were unable to break out of their host cell during the observation period of 30 min (see Movie S1). The mean percentage of Δmdv-1/peg3 male gametes that did not emerge after 30 min was 60.4% (range 45–72%; SD 9.6; three experiments), compared with 11.6% (range 8–15%; SD 3.3; three experiments) observed in wild-type male gametes. The male gametes, collected between 30 min and 1 h after induction of gametogenesis, were also analysed by immunofluorescence with an anti-mouse-spectrin antibody, specific for the erythrocyte membrane, and an anti-tubulin-I antibody reacting with the male gamete flagella. This analysis showed that in the Δmdv-1/peg3 parasites staining of anti-tubulin antibodies was in most cases confined within round-shaped cells stained also with the anti-spectrin antibodies, which represented the trapped exflagellating male gametes in contrast to the free, fully elongated flagella readily observed in similar preparations of wild-type parasites (Fig. 5). A further intriguing feature of the Δmdv-1/peg3 male gametogenesis was that the few defective male gametes which were able to emerge failed to attach to other uninfected and infected erythrocytes to produce the classically described exflagellation centres.

The emergence of female gametes was also analysed using the RFP-positive female gametes from the Δmdv-1/peg3-fluo line, which allowed for a better discrimination of free and entrapped gametes in live preparations observed by fluorescence microscopy. This experiment showed that at 30 min after activation, the mean percentage of non-emerged female gametes was 88% (range 79–95%; SD 5.5; three experiments) compared with 17.3% (range
14–26; SD 5.6; three experiments) of the wild-type female gametes. To further characterize the intracellular location of activated \( \Delta mdv-1/peg3 \) male and female gametes, electron microscopy analysis was conducted on activated gametes at 30 min after induction of gametogenesis. In the analysis of \( \Delta mdv-1/peg3 \) male gametes, the ultrastructural sections mainly showed non-emerged gametes showing apparently fully assembled flagella trapped within the parasitophorous vacuole and the erythrocyte membranes (Fig. 6A). Analysis of \( \Delta mdv-1/peg3 \) female gametes clearly revealed in most cases the presence of the erythrocyte membrane and host cell cytoplasm around the round shaped non-emerged gamete (Fig. 6B). A. Wild type. B and C. \( \Delta mdv-1/peg3 \). Male gametes from wild-type (A) and \( \Delta mdv-1/peg3 \) parasites (B and C) were collected 30 min after induction of gametogenesis, fixed and analysed by staining with an anti-tubulin-I antibody detecting the male gamete flagella and an anti-spectrin antibody, specific for the erythrocyte surface. In B and C male flagella entrapped within the erythrocyte are clearly visible. Scale bar indicates 5 \( \mu m \).

In the above experiments formation of low numbers of \( \Delta mdv-1/peg3 \) ookinetes were observed, which showed a similar morphology compared with wild-type ookinetes. To analyse the viability/infectivity of the \( \Delta mdv-1/peg3 \) ookinetes, \( Anopheles gambiae \) mosquitoes were allowed to feed on mice infected with \( \Delta mdv-1/peg3a \) in three independent experiments. In all three experiments oocysts could be detected at day 10 after infection (Table S2), although their number was significantly lower than in infections with wild-type parasites (P-values between < 0.001 and < 0.01), in agreement with the strongly reduced oocyste production observed \textit{in vitro}.
Fig. 6. Electron microscopy analysis of gamete emergence in \(\Delta\text{mdv-1/peg3}\) parasites.

A. An activated male gametocyte with completely organized flagella (arrowheads) confined in the parasitophorous vacuole membrane (PVM) and erythrocyte membrane (EM). PM is the parasite plasmamembrane.

B. An activated female gamete still trapped in the PVM and EM. PM, PVM and EM are clearly distinguishable in the enlargement shown in the panel. Osmiophilic bodies are absent.

C. Activated male gamete showing spindle microtubules (SMT) associated with a centriolar plaque and reaching the cytoplasm through the nuclear pore (p), in an area of nuclear budding.

D. An example of a budded nucleus in an activated male gametocyte.

E. Transverse section across a male gamete showing an axoneme (Ax) and a nucleus (Nu*) with non-condensed chromatin as evident from the comparison with the condensation of the residual larger male gametocyte nucleus (Nu).

F. Transverse section across a \(\Delta\text{mdv-1/peg3}\) male gamete showing condensed chromatin in the nucleus comparable to chromatin condensation of nuclei of wild-type microgametes. Mc, mitochondrion; Hz, haemozoin; ER, endoplasmic reticulum.
Sporulating oocysts could be observed containing fully formed sporozoites, and motile sporozoites were found in salivary glands at 18 days after feeding (Fig. S6). In order to analyse the infectivity of these sporozoites to the mammalian host, Δmdv-1/peg3a parasites were fed to *Anopheles stephensi* mosquitoes, and the resulting infected mosquitoes were used to infect two mice. Both mice developed a patent blood stage infection at day 6 after mosquito bite (parasitaemia between 0.5% and 1%) and genotype analysis by Southern blot analysis of separated chromosomes confirmed the mutant genotype of the transmitted Δmdv-1/peg3a parasites (data not shown). These results demonstrate that a small proportion of Δmdv-1/peg3 parasites are able to progress through complete development in the mosquito and are able to re-infect the rodent host, indicating that the function of the MDV-1/PEG3 protein is restricted to the process of gametogenesis.

**Discussion**

*MDV-1/PEG3 of P. berghei is a sexual stage-specific protein expressed from early stage of gametocytogenesis*

The gene *pbmdv-1/peg3* is not expressed in asexual stages, and starts to be transcribed in gametocytes from 17 h after erythrocyte invasion, ranking *pbmdv-1/peg3* mRNA production as one of the earliest events detected during the development of *P. berghei* gametocytes (Janse *et al.*, 2003). We show that the stage-specific expression of the gene is governed by DNA sequences contained in 1.5 kb of the *pbmdv-1/peg3* genomic upstream region, and that protein production, as determined by GFP fluorescence intensity of individual cells, is greater in female than in male gametocytes, in agreement with PbMDV-1/PEG3 abundance observed in wild-type gametocytes. In addition, the immunofluorescence studies show distinct PbMDV-1/PEG3-specific staining patterns in the two sexes. This indicates a sex-specific localization of the protein, which was confirmed to be a component of female gametocyte OBs by immunoelectron microscopy, while the precise location of PbMDV-1/PEG3 in male gametocytes remains to be identified. In terms of the timing of appearance of PbMDV-1/PEG3 in gametocytogenesis, and of its abundance and distribution in the two sexes, the above data are consistent with the classically reported appearance of multiple OBs scattered in the cytoplasm of female gametocytes from 18 hpi, and their scarce presence in male gametocytes (Mons, 1986; Janse *et al.*, 2003).

The function of MDV-1/PEG3 is dispensable for the production and maturation of *P. berghei* gametocytes

Gametocyte production, sex ratio and maturation of gametocytes of Δmdv-1/peg3 parasites were indistinguishable from those of wild-type parasites, indicating that the PbMDV-1/PEG3 protein is dispensable for the development of mature gametocytes. Also in a recent analysis it has been shown that the lack of MDV-1/PEG3 in *P. berghei* had no significant effect on gametocyte development and sex ratio (Lal *et al.*, 2009). Phenotypic characterization of the function of MDV-1/PEG3 in *P. berghei* demonstrated a lack of conservation with *P. falciparum*. In the latter species mutants that lack expression of PfMDV-1/PEG3 showed both a reduced gametocyte production and earlier developmental defects defined by accumulation of abnormal membrane structures in gametocytes, mainly in male gametocytes. As the *P. falciparum* disrupted parasites all derived from a single transfection experiment, it is impossible to rule out that the above defects could be ascribed to an independent mutation arisen in the selection process. However, differences also exist in the subcellular localization of the protein in the two species, as the PfMDV-1/PEG3 protein shows an expanded range of cellular localization compared with the *P. berghei* protein. PfMDV-1/PEG3 is not only present in the OBs of gametocytes, but is also associated with the gametocyte PVM and to virtually all membrane compartments derived from it in gametocytes of both sexes (Furuya *et al.*, 2005; Lanfrancotti *et al.*, 2007). Despite the orthologous relationship and conserved nature of MDV-1/PEG3 in different *Plasmodium* species, these results in conclusion might suggest a different spectrum of roles for this protein in gametocytes of *P. berghei* and *P. falciparum*. It would therefore be of interest to analyse the function of MDV-1/PEG3 in other *Plasmodium* species which might provide insight whether the importance of this protein for gametocyte development is specific for *P. falciparum*.

PbMDV-1/PEG3 is released at gametogenesis and is involved in emergence from the erythrocyte of both male and female gametes

PbMDV-1/PEG3 rapidly disappears after gamete formation and is released extracellularly shortly after or during gamete formation. This indicates that the protein is quickly mobilized from the respective compartments in both sexes during the process of gamete formation, suggesting that PbMDV-1/PEG3 mobilization may be functionally relevant to this process. While our data show that most of the PbMDV-1/PEG3 protein is released, a recent analysis also showed a rapid re-localization of PbMDV-1/PEG3 to the gametocyte/gamete surface after induction of gametogenesis and an association with the periphery of the gametocytes/gametes (Lal *et al.*, 2009). This study also showed that, interestingly, a small remaining patch containing MDV-1/PEG3 was identified at the apical tip of developing zygotes/ookinetes, which possibly explains
the residual signal detected in our Western blot analysis of proteins from zygotes and ookinetes. We identified a striking defect in the process of gametogenesis of the $\Delta$mdv-1/peg3 parasites: the majority of both the flagellated male gametes and the spherical female gametes had failed to emerge from the red blood cells after 30 min after induction of gametogenesis, while emergence in the wild-type parasites was largely completed within approximately 10–15 min, showing the important role of PbMDV-1/PEG3 in emergence of gametes of both sexes. This is the first $P.$ berghei protein shown to be involved in gamete emergence. The only other protein proposed to be functionally involved in this process is the $P.$ falciparum protein Pfg377, which is generally conserved in Plasmodium. Interestingly, this female-specific gene product is also localized in the OBs of the gametocytes. Mutants lacking expression of Pfg377 showed a reduced efficiency in emergence of female gametes but not of male gametes (de Koning-Ward et al., 2008). The absence of Pfg377 was associated with the absence or much reduced numbers of OBs, which may suggest that this protein is involved in the formation of such organelles. This suggests a functional difference with the role of PbMDV-1/PEG3, whose absence has no apparent effect on the presence of OBs and their disappearance at gametogenesis in the $\Delta$mdv-1/peg3 parasites.

Our analysis of the defective emergence of the $\Delta$mdv-1/peg3 gametes showed both common and sex-specific features in the two sexes. During male gametogenesis flagella are formed but most male gametes remain trapped within the red blood cell due to the failure of the parasite to disrupt the PVM and the erythrocyte membrane. In live observations, confined flagella are nevertheless highly motile and actively deform the red blood cell membrane. These observations indicate a severe defect in emergence of the male gametes, as time-course analysis of gametogenesis of wild-type $P.$ berghei demonstrates that emergence rapidly follows the formation of the flagella (Billker et al., 2002). These observations also show that mechanical forces produced by the flagellar movements of the male gametes are not sufficient per se to free the male gametes from the host cell, and perhaps counter-intuitively indicate that such forces do not play a major role in male gamete egress. A surprising observation was that the few $\Delta$mdv-1/peg3 male gametes that were nevertheless able to emerge do not appear to attach to uninfected red blood cells, resulting in the lack of the erythrocyte rosettes usually described as exflagellation centres. This may suggest that absence of PbMDV-1/PEG3 is also associated to yet unidentified molecular defect(s) on the surface of male gametes. A comparable lack of attachment to uninfected erythrocytes has been so far described only in one $P.$ falciparum mutant which lacked expression of protein Pfs230 on the surface of male gametes (Eksì et al., 2006).

During female gametogenesis, $\Delta$mdv-1/peg3 gametes are able to round up within seconds from induction, but most of them remain surrounded by both the PVM and the erythrocyte membrane. Interestingly, our analysis shows that the $\Delta$mdv-1/peg3 non-emerged female gametes are devoid of OBs, similarly to wild-type gametes that have emerged from the host erythrocyte. This observation shows that the lack of MDV-1/PEG3 does not affect the activation and discharge of the content of OBs shortly after induction of gametogenesis. On the other hand our observation that MDV-1/PEG3 is released upon gametogenesis, and the fact that discharge of the content of these bodies in the absence of MDV-1/PEG3 is not sufficient for the female gametes to disrupt the PVM and erythrocyte membrane, strongly suggest that the MDV-1/PEG3 protein plays a major role in disruption of the PVM and erythrocyte membrane.

In the present work we observed both in vitro and in vivo that the few $\Delta$mdv-1/peg3 parasites escaping from the host erythrocyte can complete their development in the mosquito, producing sporozoites able to establish a blood infection in mice. This further indicates that the function of the PbMDV-1/PEG3 protein is restricted to the process of gamete emergence.

Towards a molecular dissection of the mechanisms of gamete emergence

PbMDV-1/PEG3 is the first molecule to be identified in malaria parasites which plays a role in the emergence of gametes of both sexes. Specific mechanisms governing MDV-1/PEG3 production, storage, trafficking and delivery are likely to exist in each gender because both the amount and the localization of the protein differ in female and in male gametocytes. At emergence, sex-specific processes, such as the discharge of other proteins present in the OBs in female gametes or the production of flagellae mechanical forces in male gametes, appear to have a minor effect on the disruption of the PVM in the absence of MDV-1/PEG3. The present work suggests that PbMDV-1/PEG3 is directly involved in PVM destabilization, and that lysis of the red blood cell membrane does not proceed with a still intact PVM in both male and female gametogenesis. Whether PbMDV-1/PEG3 itself or additional mechanisms play a role in disrupting the erythrocyte membrane after PVM lysis to complete gamete release remains to be elucidated. Recent analyses of the egress mechanisms used by liver and blood stage merozoites to escape hepatocytes (Sturm et al., 2006) and erythrocytes (Yeoh et al., 2007; Blackman, 2008), respectively, strongly suggested that also in these stages the PVM is disrupted prior to the lysis of the host cell membrane in a...
protease-sensitive fashion. The present work on PbMDV-1/PEG3 indicates that egress of *Plasmodium* gametes from the erythrocyte appears to occur via a stage-specific mechanism, and suggests that a more detailed knowledge of this pathway may be exploited to generate novel approaches to block parasite transmission.

**Experimental procedures**

**DNA constructs, parasite lines, transfection and selection of transgenic parasites**

A gene targeting construct was made to disrupt mdv-1/peg3 (PB000248.02.0) by modifying a standard plasmid (pL0001) designed for targeted gene disruption by double-crossover homologous recombination. The protein-coding sequence of the *mdv-1/peg3* gene was replaced with the selection cassette containing the pyrimethamine-resistant dhfr-ts of *Toxoplasma gondii* by cloning the 5'- and 3' flanking regions of the gene upstream and downstream of the selection cassette of pL0001. A 960 bp 5'-targeting region and a 778 bp 3' targeting region were amplified from *P. berghei* genomic DNA using primer pairs L2156/L2157 and L2158/L2159 respectively (Table S3). A methylated CiaI site in primer2157 made us deviate from the original strategy for introducing the target regions into pL0001. PCR products were purified and subcloned in the pCR2.1-TOPo vector (Invitrogen). The 3' targeting region was digested from its respective TOPo vector and introduced into EcoRV/BamHI double-digested pL0001. The resulting plasmid was subsequently digested with Asp718 and the 5'-targeting region, digested from its respective TOPo vector using the Asp718 site of the TOPo vector, was introduced yielding the pl1057 mdv-1/peg3 disruption vector. The correct orientation of the 5'-targeting region in the TOPo vector and in pL1057 was determined by Asp718 and HindIII/EcoRI restriction digestion analysis respectively. The vector was linearized by MluNI/XbaI double digestion for integration into the genome by double-crossover homologous recombination into the *mdv-1/peg3* locus on chromosome 14 (Fig. S3A). The Δmdv-1/peg3 mutant lines 678c1 (Δmdv-1/peg3a) and 721c1 (Δmdv-1/peg3b) were generated by transfecting the ANKA strain reference line clone15cy1 (Janse et al., 2006) with construct pL1057 and mutant line 928c1 (Δmdv-1/peg3-fluo) was generated by transfecting transgenic line 820c1m1c1 (wt-fluo) with a construct to disrupt the *p48/45* gene by double cross-over integration as described in van Dijk et al. (2001). See below for a description of the generation of the transgenic line 820c1m1c1.

Transmission of parasites and selection and cloning of the transgenic parasites was performed as previously described (Janse et al., 2006). Correct integration of DNA constructs in the cloned lines 678c1, 721c1, 707c1, 928c1 and 1197c1 was determined by PCR and Southern analysis of digested genomic DNA and chromosomes separated by field inversion gel electrophoresis as previously described (Janse et al., 2006). The plasmids pL0001 and pL0017 are available from the Malaria Reference and Research Reagent Resource Center (MR4; http://www.malaria.mr4.org).

**Generation of a reporter line *P. berghei* line (820c1m1c1, wt-fluo) that expresses RFP in female gametocytes, gametes and zygotes**

To analyse fertilization and meiosis by FACS we generated a reporter line that expresses RFP in females and GFP in males. To generate this line a construct was made that contains (i) a male GFP expression cassette; (ii) a female RFP expression cassette; and (iii) a drug selection cassette containing a fusion of the positive selectable marker hdhfr and the negative selectable marker yfcu (Braks et al., 2006).

**Male GFP expression cassette.** The gfp mutant 3 gene (BamHI/XbaI) of pL0017 was exchanged for the e-gfp gene of plasmid pEGFP-NI (Clontech, subcloned SacII/NotI in pBluescript-SK). Then the 3'UTR pdhfr/tts (3' dhfr) of pL0017gFP was replaced by the 3'UTR of the *P. berghei calmodulin* gene (PB000866.00.0; 3'cam, Asp718/XbaI; 2566: 3'-cggGTACCGACCATATAAGAATTACCC and 2567: 3'-tcgTCTAGAGTTATATATATGTTGTTTACGCCGG primers). Next, the eef1aα promoter was replaced by the male-specific promoter of PB000791.03.0 from plasmid pL0012 (1531 bp EcoRV/BamHI fragment; Khan et al., 2005).

**Female RFP expression cassette.** The 3'dhfr of pL1102 was replaced by the 3'utr of the p48/45 gene (PB001525.02.0; 3' 48/45; Asp718/XbaI; 1882: 3'-cggGTACCGCCGATATCGAATATATGATGTTGGATGTTTACGCCGG primers). Subsequently, the eef1aα promoter was exchanged with the female-specific promoter of ccp2 (PB000504.02.0) of plasmid pL1118 (EcoRV/BamHI fragment; Khan et al., 2005). Finally the RFP expression cassette (5'ccp2-rfp-3' 48/45) was cloned into...
Drug-selection cassette. The eef1aa-gfp-3 dhfr cassette of pl0023 was replaced by the amal-gfp-3 dhfr cassette of pBSKamal-gfp-3-UTR (Franke-Fayard et al., 2008) to obtain pl1141. Subsequently, the 230p integration cassette of pl1141 (Asp788/NotI blunt) was introduced in pl0035 that contains the fusion gene of dhfr and yfcu (Braks et al., 2006; Asp718/EcoRV) to create plasmid FCU230p. We used the dhfr-yfcu selection cassette from pl0035 to be able to remove the complete drug selectable marker by negative selections as described in Braks et al. (2006). Finally the Asp718 fragment of the male/female expression cassette of pBSFeRFP-MaGFP was cloned into plasmid FCU230p to make pl1186 (Fig. S4A for a schematic representation of pl1186). Plasmids and sequences of pL0017, pL0023, pL0035 and pL1102 are available from MR4 (http://www.malaria.mr4.org). Parasites of the reference line of the ANKA strain c15cy1 (Janse et al., 2006) were transfected with pl1186 and pyrimethamine-resistant parasites were selected by positive selection with pyrimethamine as described in Janse et al. (2006) resulting in parasite line 820. Before applying negative selection, parasites were cloned by limiting dilution and we selected 820cl1 for further analysis. Correct integration of construct pl1186 in 820cl1 was shown by diagnostic PCR (data not shown) and Southern analysis of digested genomic DNA and separated chromosomes (Fig. S4B). Parasites of 820cl1 were then subjected to negative selection with 5-fluorocytosine (5-FC), essentially as described (Braks et al., 2006); four mice infected with 820cl1 were treated with 5-FC starting at a parasitaemia of 0.1–0.5% with a daily single dose of 0.5 ml of a solution of 20 mg ml\(^{-1}\) a day for a period of 4 days. Resistant parasites were collected between days 5 and 7 after start of the 5-FC treatment and the genotype analysed by diagnostic Southern analysis for removal of the drug-selectable marker dhfr-yfcu, by a recombination event between the two 3' dhfr sequences (Fig. S4B). Parasites from one of the four mice (mouse 1) that had been treated with 5-FC were cloned by limiting dilution, resulting in line 820cl1m1cl1 (wt-fluo). Correct expression of the reporter proteins GFP en RFP in male and female gametocytes and absence in asexual stages was analysed by fluorescence microscopy of live blood stages obtained from tail blood of infected mice (data not shown) and of purified gametocytes (Fig. S4C).

Production and purification of recombinant

PbMDV-1/PEG3 protein and production of mouse antiserum

Bacterially expressed recombinant protein was produced using the pGEX-6P-1 vector into which a 366 bp PCR fragment, corresponding to a region within the coding region of mdv-1/peg3 gene, was inserted. This fragment was amplified using the primers: sn: GACCGGATCCAAAGACTTAGATGATTTAAAG ATG and dx: GACCGGCGGCCTCTATTTATCAGTTAAAT CATCTGCG. This recombinant protein was used to produce specific immune serum in BALB/c mice. Fifty micrograms of the recombinant protein and complete Freund’s adjuvant were mixed to form a stable emulsion and injected intraperitoneally in the stomach area. The immunization procedure was repeated using 25 μg of antigen in incomplete Freund’s adjuvant 28, 42, 56, 70 days after the first injection. At day 84 the immunized mice were bled to obtain immune serum. Before the immunization cycle, blood (100 μl) was collected from the submandibular vein of each mouse to obtain pre-immune serum.

Immunofluorescence analysis

For immunolocalization experiments, infected red blood cells were fixed in 4% EM grade pararormaldehyde and 0.0075% EM grade glutaraldehyde in phosphate buffered saline (PBS) as described by Tonkin et al. (2004). Immune sera were diluted in PBS containing 1% BSA. Images were taken using a Leica DMRB microscope and a 100X/PL FLUOTAR objective, and pictures recorded using a SPOT RT digital camera and Unisot software. Images were processed using Adobe Photoshop.

Preparation of parasite protein extracts and

Western blot analysis

Infected blood from 10 mice (10 ml) was pooled and a 2 ml aliquot was immediately diluted in PBS (pH 7.3) for collection of gametocytes, which were then enriched by Nycodenz density centrifugation (Janse and Waters, 1995). The remaining blood (8 ml) was diluted with RPMI-1640 ookinete culture medium (pH 8.0) and incubated at 21°C to allow for fertilization and zygote/ookinete formation. Purified zygote and ookinete preparations were obtained as described in Rodriguez et al. (2002). Briefly: after 3 h a sample containing zygotes (corresponding to 2 ml blood) was collected, which were subsequently purified by Nycodenz gradient centrifugation. At 18 h after incubation the remaining culture material, containing the mature ookinetes, was collected and the red blood cells were lysed with 0.17 M ice cold ammonium chloride and the parasites pelleted after washing with PBS. From all samples Giemsa stained smears were made for the determination of the percentage of gametocytes, zygotes and ookinetes. The final preparation of ookinetes contained 6 × 10⁷ ookinetes from 60 ml of culture. Aliquots of the pelletted enriched parasites were resuspended in 100 μl 0.1 M sodium phosphate buffer, to which 1 mM PMSF (phenylmethanesulfonyl fluoride) had been added, and subjected to sonication (four bursts of 15 s with cooling on ice). The protein extracts, each sample corresponding to 1/80th of the original blood sample, were loaded on a 15% SDS-PAGE Laemmli gels. After separation the proteins were transferred to nitrocellulose filters, which were processed for standard Western blot analysis, using antibodies against MDV-1/PEG described above. As a loading control, a monoclonal antibody against parasite actin (Siden-Kiamos et al., 2006) was used. Secondary antibodies were conjugated to horseradish peroxidase and the Super Signal kit from Pierce was used for detection by chemoluminescence.

Immunoelectron microscopy

Nycodenz-purified gametocytes (Janse and Waters, 2005) of wild-type c15cy1 and mutant 678c1 were processed for immunoelectron microscopy with slight modifications to the protocol described in Scala et al. (1992). Briefly, samples were fixed
overnight at 4°C with 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M sodium cacodylate buffer (TAAB). They were subsequently included in 2% agar noble in H2O, dehydrated in ethanol serial dilutions and embedded with Unicryl resin (British BioCell International). Ultrathin sections, collected on gold grids, were sunk in 100% ethanol for 3 min, immersed in 0.05 M Tris buffer (pH 10.0) in PCR tubes, and then kept at 99°C for 1 h using a constant temperature box (Saito et al., 2003). Immunostaining was performed as follows: the grids were floated on a mixture of normal goat serum 5% in PBS-BSA 1% (pH 7.2) for 30 min, rinsed and incubated overnight at 4°C with mouse polyclonal anti-PbMDV-1/PEG3 serum (1:50 in PBS-BSA 0.1%). After washing, grids were incubated for 1 h with 10 nm gold-conjugated goat anti-mouse IgG (SIGMA) diluted 1:20, rinsed in buffer and in distilled water, and air-dried. As controls, the sections were stained without any heating, without the primary antibody and with the diluted anti-mouse IgG-gold serum or with the diluted mouse pre-immune serum in place of the first antibody. Finally, sections were stained with 2% uranyl acetate in H2O and observed in a 208 Philips transmission electron microscope.

Transmission electron microscopy

Nycodenz purified gametocytes (Janse and Waters, 1995) of wild-type and Δmdv-1/peg3 lines were activated for gamete formation and fixed after 30 min with 2.5% glutaraldehyde, 2% paraformaldehyde and 2 mM CaCl2 in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4°C, according to (Perry and Gilbert, 1979). Parasites were washed in cacodylate buffer and post-fixed with 1% OsO4 in 0.1 M sodium cacodylate buffer for 1 h at room temperature, treated with 1% tannic acid in 0.05 M cacodylate buffer for 30 min and rinsed in 1% sodium sulfate in 0.05 cacodylate for 10 min. Fixed specimens were washed, dehydrated through a graded series of ethanol solutions (30–100% ethanol) and embedded in Agar 100 (Agar Scientific Ltd, UK). Ultrathin sections obtained by a MT-2B Ultramicrotome (LKB – Pharmacia) were stained with uranyl acetate and lead citrate and examined by an EM 208 Philips electron microscope.

In vitro (cross) fertilization/ookinete maturation assays

The fertility of the gametes of the mutant lines was analysed by standard in vitro fertilization and ookinete maturation assays (Khan et al., 2005; van Dijk et al., 2001). Gametocytes for these assays were either obtained from infected blood from mice that have been pretreated with phenylhydrazine to increase gamete numbers or from infected blood of mice that has been treated with the antimalarial drug sulfadiazine to obtain highly pure populations of gametocytes (Beetsma et al., 1998). In these experiments mice were fed on mice infected with wild-type parasites and Δmdv-1/peg3 parasites and uninfected mosquitoes were removed the next day. At day 10 and day 14 after feeding oocysts were counted in dissected midguts using a phase contrast microscope. At day 21 after feeding, dissected salivary glands were analysed for the presence of sporozoites. Infected A. stephensi mosquitoes were allowed to feed on naïve mice on day 21 after the infectious blood meal.

Fertilization and meiosis by FACS

Fertilization and meiosis in the mutant lines was analysed by determination of the DNA content (or ploidy) by FACS measurement of the fluorescence intensity of female gametes and zygotes stained with the DNA-specific dye Hoechst-33258 (Janse et al., 1987; Janse and Vianen, 1994). For these experiments we used the mutant line Δmdv-1/peg3-fluo (928cl1) and Δp48/45-fluo (1197cl1) which are generated in the parent line wt-fluo (820c1m1cl) that expresses RFP in the female gametocyte/gamete and during further development into the zygote/ookinete. The stage-specific RFP expression allows selection of female gametes and zygotes stages in the process of FACS analysis of the DNA content of cells (Fig. S4C and D).

Activation of gametocytes was performed in in vitro (cross) fertilization/ookinete maturation assays as described above. At 4 hpa cells were stained for 1 h at room temperature with Hoechst-33258 (10 μM) and subsequently analysed by FACS using a LSR-II flow cytometer (Becton Dickinston). Cells were analysed at room temperatures with the following filters (parameters/thresholds): UB 440/40 (Hoechst) (400/5000); BE 575/26 (RFP) (500/5000); BF 530/30 (GFP) (500/5000); FSC (250/2000); SSC (200/5000).

The cells for analysis were selected on size by gating on FSC and SSC. A total of 10 000–500 000 cells were analysed per sample and all measurements were performed on triplicate cultures. Subsequently the female gametes and zygotes were selected based on their RFP expression (Fig. S4E and F). To determine the Hoechst-fluorescence intensity from the populations of unfertilized female gametes and zygotes, gates were set as shown in the figures. Data processing and analysis was performed using the program FlowJo (http://www.flowjo.com).

Mosquito transmission experiments

For mosquito transmission experiments females of Anopheles gambiae (strain G3) or A. stephensi were fed on mice infected with wild-type parasites and Δmdv-1/peg3 parasites and unfed mosquitoes were removed the next day. At day 10 and day 14 after feeding oocysts were counted in dissected midguts using a phase contrast microscope. At day 21 after feeding, dissected salivary glands were analysed for the presence of sporozoites. Infected A. stephensi mosquitoes were allowed to feed on naïve mice on day 21 after the infectious blood meal.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Multiple sequence alignment of the Pmdv-1/peg3 orthologous proteins. Sequence alignment of the PMDV-1/PEG3 orthologous proteins of P. berghei (Pb), P. falciparum (Pf), P. yoelii (Py), P. chabaudi (Pc), P. vivax (Pv) and P. knowlesi (Pk).

Fig. S2. Generation of transgenic line 707cl1 for the analysis of the activity of the promoter of pbmdv-1/peg3.
A. Schematic representation of construct pL1108 for introduction of gfp under the control of the mdv-1/peg3 promoter into the genome of P. berghei. The constructs integrate into the c/d-ssurma locus on chromosome 5/6 by single cross-over integration.
B. Southern blot analysis of separated chromosomes of wild-type parasites and of parasites of line 707cl1, showing integration of construct pL1108 in chromosome 5/6 of clone 707cl1. Chromosomes were hybridized to a probe specific for the 3′-UTR of the dhfr gene which hybridizes to the integrated pL1108 construct in the ssurma locus on chromosome 5/6 and to the endogenous dhfr gene on chromosome 7.
C. Purified gametocytes of line 707cl1 expressing GFP.

Fig. S3. Disruption of the pbmdv-1/peg3 locus.
A. The mdv-1/peg3 genes was disrupted using the replacement vectors pL1057. The mdv-1/peg3 locus is shown before and after integration of construct pL1057. Primers used for diagnostic PCR (B and D) and the restriction fragments used for Southern analysis of the genotype (C) are shown.
B. Diagnostic PCR using genomic DNA from wild-type parasites and Δmdv-1/peg3 lines 678cl1 and 721cl1.
C. Southern blot analysis of genomic DNA from wild-type parasites and Δmdv-1/peg3 lines 678cl1 and 721cl1 with the pL1057-specific probe.
D. Diagnostic PCR analysis of genomic DNA and Southern blot analysis of separated chromosomes of mutant 928cl1 (Δmdv-1/peg3-fluo), showing correct disruption of the locus. Chromosomes were hybridized to a probe specific for the 3′-UTR of the dhfr gene which hybridizes to the integrated pL1057 construct in the mdv-1/peg3 locus on chromosome 14, in addition to the endogenous dhfr gene on chromosome 7 and to the construct with the reporter genes rfp and gfp integrated into the 230p locus on chromosome 3.

Fig. S4. Generation and analysis of parasite reference line 820cl1m1cl1 (wt-fluo) that stably expresses GFP in male gametocytes and RFP in female gametocytes.
A. Schematic representations of (i) the vector used to introduce the gfp/rfp male/female expression cassette into the 230p locus; (ii) the 230p genomic locus; (iii) the resulting integration in the genome of parasites after positive selection with pyrimethamine and (iv) the genomic locus after negative selection with 5-FC. Vector pL1186 is linearized at the KspI sites. Integration of pL1186 into the genome occurs by double cross-over homologous recombination results in a 1 kb deletion of the nonessential 230p gene of the parasites that are selected with pyrimethamine. After negative selection with 5-FC, parasites are selected in which the positive/negative selectable marker cassette (hdfr-yfcu) has been excised from the integrated construct by a recombination event between the two 3′dhfr sequences (shown in red).
Arrows indicate the position and size of expected restriction site fragments in Southern analysis.

B. Genotype analysis of parasites after positive selection (line 820) and after negative selection from four mice (m1–m4). Southern analysis of separated chromosomes and restricted DNA shows the correct integration of pL1186 in the 230p locus on chromosome 3. Southern analysis of PstI/Ncol-digested DNA of parasites after 5-FC treatment (m1–m4) show the presence of the GFP-positive DNA fragment with a reduced size (1.6 kb instead of 3.3 kb in line 820) after recombination has resulted in the excision of the selectable marker cassette. Parasites of 820clm1cl1 were cloned by limiting dilution yielding line 820clm1cl1 (wt-fluo).

C. GFP and RFP expression in purified males and females respectively, of wt-fluo parasites.

D. Detection of RFP-positive female gametocytes and GFP-positive males by FACS analysis in blood infected with wt-fluo parasites. Gametocyte populations are clearly separated from the (large) population of uninfected red blood cells and red blood cells infected with the asexual blood stages.

E. RFP and Hoechst-33258 fluorescence of a female gametocyte, zygote and ookinete of wt-fluo. Zygotes and ookinetes were collected at 4 h after activation (hpa) and 18 hpa respectively, and stained for 1 h at room temperature with Hoechst 33258. Note the increased Hoechst fluorescence intensity of the nuclei of zygotes/ookinetes as result of fertilization and meiotic DNA replication, resulting in tetraploid nuclei.

F. FACS analysis of Hoechst-33258 fluorescence intensity of female gametocytes and zygotes of wt-fluo, selected on RFP fluorescence. In the upper panel haploid female gametes are shown before fertilization and the lower panel shows unfertilized females and zygotes collected at 4 hpa.

**Fig. S5.** Analysis of Δmdv-1/peg3 gametocytes.

A. Northern analysis of mRNA from purified gametocytes using a pbmdv-1/peg3-specific probe showing the absence of transcripts in Δmdv-1/peg3 (lines 678cl1 and 721cl1) and presence in wild type (WT). Transcripts are also absent in mRNA from blood stages of the non-gametocyte producer clone 2.33 of *P. berghei*. As a control, a crossing between these males and fertile females of line 1097cl1 is shown, resulting in wild-type fertilization rates (> 50%). This latter line lacks expression of the male fertility factor P48/45 and is generated in the RFP-expressing parent line 820m1clm1.

**Table S1.** The number (and percentage) of macrogametes 30 min after induction of gametogenesis that are surrounded by the PVM and erythrocyte membrane (Non-emerged), or without PVM and erythrocyte membrane (Emerged) as determined by electron microscopy. Only independent macrogametes were counted in a single ultrathin section, and only cells were selected which showed most of the osmiophilic bodies discharged and an eccentric, small nucleus and extended endoplasmic reticulum.

**Table S2.** Oocyst production by MDV-1/PEG3 mutant parasites (line 678cl1) in *A. gambiae* mosquitoes.

**Table S3.** Primers used in this study.

**Movie S1.** PbMDV-1/PEG3-defective male gametes entrapped within the host red blood cell.

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