A perforin-like protein mediates disruption of the erythrocyte membrane during egress of *Plasmodium berghei* male gametocytes

**Elena Deligianni,**1† **Rhiannon N. Morgan,**1,2† **Lucia Bertuccini,**3 **Christine C. Wirth,**4,5 **Natalie C. Silmon de Monerri,**6‡ **Lefteris Spanos,**1 **Michael J. Blackman,**6 **Christos Louis,**1,2 **Gabriele Pradel**4,5 and **Inga Siden-Kiamos**1*

1 Institute of Molecular Biology and Biotechnology, FORTH, Heraklion, Greece.
2 Department of Biology, University of Crete, Heraklion, Greece.
3 Dipartimento di Tecnologie e Salute, Istituto Superiore di Sanità, Rome, Italy.
4 Institute for Molecular Biotechnology, RWTH Aachen University, Aachen, Germany.
5 Research Center for Infectious Diseases, University of Würzburg, Würzburg, Germany.
6 Division of Parasitology, MRC National Institute for Medical Research, Mill Hill, London, UK.

**Summary**

Successful gametogenesis of the malaria parasite depends on egress of the gametocytes from the erythrocytes within which they developed. Egress entails rupture of both the parasitophorous vacuole membrane and the erythrocyte plasma membrane, and precedes the formation of the motile flagellated male gametes in a process called exflagellation. We show here that egress of the male gametocyte depends on the function of a perforin-like protein, PPLP2. A mutant of *Plasmodium berghei* lacking PPLP2 displayed abnormal exflagellation; instead of each male gametocyte forming eight flagellated gametes, it produced gametocytes with only one, shared thicker flagellum. Using immunofluorescence and transmission electron microscopy analysis, and phenotype rescue with saponin or a pore-forming toxin, we conclude that rupture of the erythrocyte membrane is blocked in the mutant. The parasitophorous vacuole membrane, on the other hand, is ruptured normally. Some mutant parasites are still able to develop in the mosquito, possibly because the vigorous motility of the flagellated gametes eventually leads to escape from the persisting erythrocyte membrane. This is the first example of a perforin-like protein in *Plasmodium* parasites having a role in egress from the host cell and the first parasite protein shown to be specifically required for erythrocyte membrane disruption during egress.

**Introduction**

Male and female gametocytes of the malaria parasite are formed in the blood of the vertebrate host. They develop from merozoites, which after invasion of red blood cells follow a differentiated developmental pathway leading to the production of mature gametocytes. Similarly to asexual blood-stage parasites, gametocytes develop inside a parasitophorous vacuole (PV) in the cytoplasm of the host red blood cell. After uptake by the mosquito vector in a blood meal, the mature gametocytes are activated and within 10–20 min gametes are formed. These fuse to form the zygote, which later develops into the motile ookinete.

An important step in gametogenesis is egress of the male and female gametocytes from the red blood cell. This results in the formation of a mature female macrogamete, which is competent for fertilization. The development of mature male gametes is considerably more complicated. Simultaneously with egress from the host cell, the DNA is replicated three times and axonemes are assembled. Finally, the axonemes are activated resulting in the release of eight flagellated microgametes from each residual male gametocyte. In rapid swimming movements the microgametes within minutes attach to females and erythrocytes in the blood meal forming so-called exflagellation centres. Fertilization ensues after fusion of the microgamete with the female gamete.

Egress comprises the rupture of two membranes, the PV and erythrocyte membranes surrounding the developing gametes. A recent study of *Plasmodium falciparum* gametocytes (Sologub et al., 2011) concluded that the

---

*Received 26 November, 2012; revised 31 January, 2013; accepted 25 February, 2013. *For correspondence. E-mail inga@imbb.forth.gr; Tel. (+30) 2810 391118; Fax (+30) 2810 391104.
†These two authors contributed equally to this work. ‡Present address: Albert Einstein College of Medicine, Bronx, NY, USA.

© 2013 John Wiley & Sons Ltd
The erythrocyte membrane (EM), on the other hand, persists for several minutes. The parasitophorous vacuole membrane (PVM) is ruptured very rapidly (within 1–2 min) after gametocyte activation. The erythrocyte membrane (EM), on the other hand, persists for several minutes.

Perforin-like proteins are characterized by the presence of a membrane attack complex/perforin (MAC/PF) domain. This domain is a feature of proteins able to form pores in lipid bilayers and was first identified in complement factors of the mammalian immune system. These proteins bind to the surface of target cells upon which they oligomerize and insert into the target membrane forming transmembrane pores. In Apicomplexa a number of perforin-like proteins have been identified (for reviews see Roiko and Carruthers, 2009; Kafsack and Carruthers, 2010). In the related apicomplexan parasite Toxoplasma gondii, a perforin-like protein was shown to be involved in egress of tachyzoites from host cells (Kafsack et al., 2009). In Plasmodium five genes encoding proteins with a MAC/PF domain have been identified; all have a putative secretory signal sequence suggesting that they are secreted proteins (Kaiser et al., 2004). For three of these a function related to cell passage has been established by mutational analysis. MAOP (Membrane Attack Ookinete Protein also named PPLP3, Plasmodium perforin-like protein 3) (Kadota et al., 2004) and PPLP5 (Ecker et al., 2007) have functions in traversal of the mosquito midgut epithelium cells by the ookinete. The third protein, called SPECT2 (Sporozoite Microneme Protein Essential for Cell Traversal or PPLP1) (Ishino et al., 2005), is important for sporozoite cell traversal. Two additional PPL-proteins are found in Plasmodium parasites, but their function has not previously been studied. PPLP4 is expressed in the ookinete stage (Hall et al., 2005; Raibaud et al., 2006); however, attempts to knock out the gene failed (Ecker et al., 2008). Here, we show that PPLP2 is expressed in the gametocytes of two Plasmodium species, and that in Plasmodium berghei the protein is necessary for normal egress of the male gametocyte from the host erythrocyte.

**Results**

**pplp2 is expressed in gametocytes and ookinetes**

Both of the **pplp2** genes of *P. berghei* (PBANKA_143240) and of *P. falciparum* (PF3D7_1216700) comprise a single exon encoding in each case with a centrally located MAC/PF domain, characteristic of perforin-like proteins (Fig. S1). The presence of a predicted signal peptide suggests that the protein is secreted. The C-terminal region of the predicted sequence contains three ApiPLP C-terminal β-pleated sheet (APC-β) domains (Kafsack and Carruthers, 2010). These apicomplexan-specific ~55 amino acid domains have been suggested to play a role in initial recognition of the target membranes (Kafsack and Carruthers, 2010), but their function is unknown. Previous proteomic analyses have detected PPLP2 in merozoites (one single peptide) and in mature stage V gametocytes (nine peptides) in *P. falciparum* (Florens et al., 2002; Silvestrini et al., 2010) and in male gametocytes in *P. berghei* (Khan et al., 2005). To our knowledge, PPLP2 proteins have not been detected in any of the other stages of the life cycle, although transcriptomic data deposited in PlasmoDB suggest that the *P. falciparum* **pplp2** gene is expressed in blood stages and in ookinetes.

To determine the mRNA expression pattern of the **pplp2** gene in *P. berghei* we performed reverse transcriptase PCR (RT-PCR) analysis on total RNA from wild-type (WT) mixed blood stages, which contain asexual stages and gametocytes, mixed asexual blood stages from the HPE strain which does not produce gametocytes, purified oocysts and mosquito midguts containing oocysts. The results (Fig. 1A) show that **pplp2** mRNA is exclusively found in gametocytes and ookinetes. As shown below, we did not detect the protein in oocytocytes, which may indicate that the signal detected in the RT-PCR analysis may originate from gametocytes remaining in our oocinete preparation. Expression of **pplp2** could not be detected in either mixed asexual parasites of the HPE line, which is deficient in the production of gametocytes, or in oocysts.

We used an antiserum developed against a polypeptide within the MAC/PF domain of the *P. falciparum* PPLP2 (Fig. S1A, amino acids 576–661) in immunofluorescence assays (IFA) of *P. berghei* to determine the subcellular location and temporal expression pattern of the protein. The specificity of the antiserum was validated by testing on samples of the knockout mutant described below. In this case no signal was detected (Fig. S1B). Asexual blood stages were not stained by the antibody in concordance with the RT-PCR data (data not shown), and no signal was detected in female gametocytes (Fig. S1C). However, in male gametocytes a clear IFA signal was evident in proximity to the EM (Fig. 1B, a–d) and PVM (Fig. 1B, e–h). In the absence of a specific marker we could not investigate whether the protein is localized to the plasma membrane (PM). To attempt to better localize the PPLP2 signal we used an antiserum against SEP1, a protein residing in the PVM of gametocytes and asexual blood stages, as a marker of the PVM (Birago et al., 2003). In addition we employed a commercial antibody recognizing TER-119, a glycophorin A-associated protein present on the mouse erythrocyte surface, which labels the EM of red blood cells. Finally, an antibody against the nuclear protein SET was used to differentiate between the nuclei of male and female gametocytes (Pace et al., 2006). The IFA signals of the SEP1 and TER-119 antibodies labelling the two membranes were not well resolved and we can therefore only conclude that PPLP2 is...
localized close to both of these two membranes. In samples of activated gametocytes the labelling of PPLP2 was lost at an early time point after activation (Fig. 1B, i–k). No IFA signal for PPLP2 was detected in ookinetes.

**PPLP2 is expressed in gametocytes of P. falciparum**

To extend the above data, the subcellular location of the *P. falciparum* PPLP2 orthologue was also investigated. For this we used a mouse antiserum directed against *P. falciparum* PPLP2 (amino acid residues 501–840 comprising the complete MAC/PF domain; for validation of the antiserum see Fig. S1D). IFA showed PPLP2 expression in gametocytes, which were highlighted by labelling of the sexual stage-specific marker proteins Pfs230 (Fig. 2A) or Pfs25 (Fig. 2B). Pfs230 is a secreted 6-cys protein expressed in gametocytes and gametes of both genders, where it associates with the plasma membrane (reviewed by Williamson, 2003; Pradel, 2007). Pfs25 on the other hand is a female-specific GPI-anchored protein composed of four epidermal growth factor domains which resides in intracellular vesicular structures of mature non-activated macrogametocytes (Scholz et al., 2008). Within a few minutes following activation, Pfs25 then relocates to the surface of the forming macrogamete and is subsequently abundantly expressed on the surface of zygotes and ookinetes (Scholz et al., 2008; Sologub et al., 2011; reviewed by Pradel, 2007). The colocalization studies reveal that in the mature non-activated *P. falciparum* gametocytes, PPLP2 is intracellularly detected, where it is present within vesicular structures (Fig. 2A and B; top panels).

We then investigated PPLP2 labelling in gametocyte following *in vitro* activation with xanthurenic acid. In *P. falciparum* the PVM ruptures within less than 2 min after activation followed approximately 10 min later, by disruption of the EM to enable the release of the newly formed gametes (Sologub et al., 2011; reviewed in Wirth and Pradel, 2012). The IFA showed that during the first 7 min post activation PPLP2 relocates to the periphery of the
Fig. 2. Location of PPLP2 in *P. falciparum* gametocytes during activation. Cultures of gametocytes before activation, and at 7 and 15 min post activation, were labelled with antiserum B directed against PPLP2 (green) and counterstained with (A) anti-Pfs230 antisera (red) or (B) anti-Pfs25. The corresponding differential interference contrast (DIC) images are shown. Note that mature *P. falciparum* gametocytes have a banana-like shape but round up during activation. Bar, 5 μm (A) and 2 μm (B).
gametocytes, which were in the process of rounding up (Fig. 2A and B; centre panels). At 15 min post activation (i.e. after completion of rupture of both PVM and EM), PPLP2 labelling was no longer detected in the parasites. No PPLP2-specific signal was detected in either the exflagellating microgametocytes (Fig. 2A; bottom panel) or the newly formed macrogametes (Fig. 2B; bottom panel). Interestingly, we noticed that in some cases, a punctate PPLP2-specific labelling was detectable associated with the shed EM and PVM membrane fragments which are often found adjacent to the egressed parasites (Rupp et al., 2011) (Fig. S2).

Collectively these results suggest that in both Plasmodium species PPLP2 is present in gametocytes, where it is associated with the cell periphery during egress. The limitations of confocal analysis did not allow us to further pinpoint the exact location of the protein. However, PPLP2 disappears during egress of the gametocytes and then localizes with the shed membranes, indicating that the protein is associated with the enveloping membranes, i.e. PVM or EM, of the activated gametocytes. Whereas, in P. falciparum the protein is found in both sexes in P. berghei the protein is restricted to the males.

To determine the function of PPLP2 we used standard protocols to generate a knockout mutant of P. berghei (Janse et al., 2006) using double-cross-over homologous recombination to remove most of the pplp2 coding region (Fig. S3A). Mutant parasites were readily recovered and grew at normal rates in mice, indicating that the gene has no important function in asexual blood stages. Correct genomic integration of the replacement construct was verified by PCR analysis and Southern blot (Fig. S3B–D). No pplp2 mRNA could be detected in the mutant parasites (Fig. 1A, lane 7) confirming that the gene had indeed been disrupted. Two knockout clones were obtained from two independent transfections, and identical results were obtained for both in the following experiments.

The mutant P. berghei clones, called pplp2(−), formed both female and male gametocytes in similar numbers to the WT [WT: males 3.2%, females 3.5%; pplp2(−): males 2%, females 2.2%; average of three experiments] and exflagellating cells at numbers similar to WT were also detected. However, exflagellation was clearly aberrant (Fig. 3A–C, Movies S1–S4). Ten minutes after activation...
of WT parasites (Movie S1) eight individual flagella radially break out from the residual cell and almost immediately attach to erythrocytes present in the sample, forming exflagellation centres. Such typical events were never detected in any of the two mutant clones (Fig. 3B and C) but, instead, we detected abnormal forms of exflagellation. The most common form was cells where the flagella were strongly attached to each other, visible as a thick ‘super-flagellum’ (Fig. 3A, Movie S2). The super-flagella remained attached to the residual body, although they were beating seemingly normally. However, they did not attach to erythrocytes and therefore these gametes did not form exflagellation centres (Movie S3). The super-flagellum continued to move for extended periods of time, at least for the duration of 1 h. This is in contrast to the WT parasites, where motile gametes are only detected for about 15 min after the first beating flagellum has been detected. In the mutant, sometimes individual flagella could be discerned in the super-flagellum, moving independently (Movie S4), although still in close contact. In other cells we detected two flagella of intermediate size on the exflagellating cell (data not shown).

To further study the super-flagella we stained the axonemes of activated gametocytes with a tubulin-specific antibody (Billker et al., 2002) (Fig. 3C). We measured the diameter of the axonemes in the mutants and compared it to single axonemes of WT flagella (for representative pictures and details of measurements see Fig. S4). The results indicated that the super-flagella contained several axonemes; of the 11 instances investigated eight super-flagella had a diameter corresponding to three axonemes, while two contained two axonemes each, and one flagellum one axoneme only.

The erythrocyte membrane persists during exflagellation of pplp2(−) parasites, while the PVM is disrupted normally

One plausible explanation for the super-flagellum phenotype described above is that rupture of the surrounding membranes did not take place normally. To investigate this further we used IFA with the SEP1 and TER-119-specific antibodies described above as markers of the PVM and EM respectively. In addition, the antiserum against SET allowed specific recognition of the male nucleus. In the first set of experiments labelling was performed with the three antibodies simultaneously. The antibodies against SEP1 and SET were both raised in rabbits and we could therefore not label each protein specifically. However, the labelling of each is restricted to a specific, distinct compartment (PVM versus nucleus), thus allowing differential recognition of the staining pattern. Samples of WT and pplp2(−) gametocytes were fixed at different time points following activation (non-activated, 1 and 8 min), followed by immunolabelling. In the non-activated samples (Fig. 4A, a–f, 0 min) we could clearly discern the PVM as peripheral SEP1 labelling, in proximity to the EM labelled for TER-119. In both WT and mutant parasites reactivity of the SEP1 antibody had disappeared 1 min after activation of gametogenesis, reflecting the fact that the PVM is rapidly ruptured, as previously described for P. falciparum (Sologub et al., 2011) (Fig. 4A, g–l). Thus the PVM rupture was not affected in the pplp2(−) mutant. The EM was present at this early time point in both strains. At 8 min after activation the EM had been ruptured in the WT (Fig. 4A, m–o). However, in the mutant cells the EM was still present at this time point (Fig. 4A, p–r). In conclusion these results indicate that the pplp2(−) mutant is specifically blocked in the rupture of the EM.

We next wanted to determine whether the EM in the pplp2(−) mutant persisted even at later time points. Activated samples were therefore fixed and labelled 16 min after activation. We did not include samples of the WT at this late time point, as egress is complete at 8 min (see above) and fully mature flagellated male gametes have already been released from the residual cell at 10 min (Movie S1). To discern the male gametocytes we used double labelling with either anti-SET (Fig. 4B, a–c) or the anti-tubulin antibody described above, which labels the axonemes (Fig. 4B, d–f). The results revealed that even at this late time point the EM still enclosed the residual cell as well as the super-flagella even though normal motility of the axonemes was detected by live imaging at this time (Movies S2–S4). Taken together, these results indicate that rupture of the EM was severely impaired in the pplp2(−) parasites, and that the motile flagella in bundles were ‘pushing’ on the surrounding membrane, creating the visible super-flagellum.

For a more detailed analysis we used transmission electron microscopy to examine activated pplp2(−) gametocytes (Fig. 5). Gametocytes were fixed 19 min after activation to make sure that most of them had reached optimal exflagellation. This was confirmed by live imaging of an aliquot of the same samples. Although there were differences between individual cells some common characteristics were detected. We did not detect an intact PVM in any case, while an intact EM was clearly seen in all the samples studied. The axonemes had partly exited the residual cell of the gametocyte, an activity known to be strictly dependent on flagellar motility (Sinden et al., 1976) (Fig. 5A and B). However, unlike the WT situation where the flagella exit the residual gametocyte radially, the pplp2(−) mutant flagella were consistently ‘bundled’ together and coated with the EM (Fig. 5B, arrows). In cross-sections two or more axonemes were found to be grouped together within a common membrane, which we interpret to be the
flagellar membrane (Fig. 5C). This corresponds to the super-flagellum, which we saw by live imaging and immunolabelling. An interesting observation was that in most cells the cytoplasm of the red blood cell was clearly present, as well as whorls of membrane, which we propose to be remnants of the PVM. These results confirmed the results of the immunofluorescence assays described above: in the absence of PPLP2 the PVM is disrupted normally while the EM remains intact, suggesting a specific role for PPLP2 in EM disruption.

Fig. 4. Rupture of the erythrocyte membrane is blocked in the pplp2(-) male gametocytes.
A. Comparison of WT male gametocytes (left part of figure) with pplp2(-) mutant gametocytes (right part of figure) at different time points after activation of the gametocytes. Samples were fixed either before activation [0 min (a–f)] or 1 (g–l) or 8 min after activation (m–r). The SET antibody, a marker for the male gametocyte nucleus, and the antibody against SEP1, a protein residing in the PVM, were both recognized with the Alexa-555-conjugated anti-rabbit secondary antibody (a,g,m,d,j,p, red). The erythrocyte membrane was labelled with the TER-119 antibody (b,h,e,k,n,q, green). The PVM is disrupted in both WT and pplp2(-) gametocytes within 1 min after activation, as evidenced by the loss of the red peripheral labelling. The erythrocyte membrane had disappeared at 8 min in the WT, while it persisted in the mutant (see also B). The nuclear SET labelling increased in intensity and size during the time-course, as the genome undergoes three rounds of DNA replication.
B. Exflagellating pplp2(-) mutant male gametocytes stained 16 min after activation with an antibody recognizing the TER-119 erythrocyte surface protein (a,c,d,f, green). The SET protein (b,c, red) is a marker for the nucleus of the male gametocytes. The TAT antibody stains the tubulin of the axoneme (e,f, red). The TER-119 labelling envelopes the whole axoneme (f).
pplp2 is not essential during later mosquito stages

We next examined the formation of ookinetes from the two pplp2(−) mutant clones in parallel to the WT. The results revealed that seemingly normal ookinetes were formed from the pplp2(−) mutant gametocytes. However, the percentage of ookinetes formed was significantly lower compared with the WT parasites (Fig. 6A). This result
suggests that although egress of the gametocyte was impaired male gametes were sometimes able to finally break free of the EM and fertilize female gametocytes. Similarly, oocysts were detected in midguts dissected from mosquitoes fed with the mutant strain, although again the number was significantly reduced compared with the WT (Fig. 6B). The reduction in the number of oocysts formed is roughly proportional to the reduction of ookinetes from mosquitoes fed with the mutant strain, although we cannot rule out a minor role. Finally, an infection could be established in naïve mice fed with the mosquitoes containing oocysts (data not shown). Taken together, these results suggest that PPLP2 functions primarily during male gametocyte egress.

**Saponin and equinatoxin treatment rescue the pplp2(-) phenotype**

The non-ionic glycoside detergent saponin lyses the PVM and EM but does not affect the parasite plasmalemma (Ansorge et al., 1996). In view of the apparent role of PPLP2 in EM disruption, we asked whether treatment of mutant gametocytes could rescue the block in egress. In a first set of experiments we treated gametocytes with saponin immediately after taking a blood sample from the infected mouse, then 5 min later ookinete medium containing xanthurenic acid was added. This experimental design allows saponin-mediated rupture of the PVM and EM before the formation of mature gametes. Treatment in this manner of WT gametocytes revealed that exflagellation still takes place, although the flagellated gametes remained attached to the residual cells and smaller exflagellation centres were formed; these effects are probably due to the fact that most of the surrounding non-parasitized erythrocytes are also lysed by saponin. Remarkably, similar saponin treatment of the pplp2(-) gametocytes resulted in the formation of exflagellating gametocytes with similar morphology to the WT parasites; individual flagella of normal thickness were now clearly detectable and exflagellation centres were formed (Fig. 7A, Movie S5). To quantify the effect of saponin on WT and pplp2(-) parasites we counted the number of oocystes formed from saponin treated blood samples. This revealed a slightly dose-dependent toxic effect of saponin treatment of the WT samples, although we could not detect any specific defect on exflagellation *per se* (Movie S6). Strikingly, the number of oocystes formed from treated pplp2(-) parasites was increased more than twofold after saponin treatment compared with nontreated mutant samples (Fig. 7B). These results showed that an EM-disrupting detergent is capable of partially rescuing the pplp2(-) phenotype. We also used equinatoxin II, a toxin which has been reported to selectively permeabilize the EM in erythrocytes infected with *P. falciparum* asexual blood-stage parasites, without affecting the integrity of the PVM (Jackson et al., 2007). Treatment of pplp2(-) gametocytes with equinatoxin II resulted in normal exflagellation with independent, morphologically normal flagella released from the residual cell (Fig. 7C, Movie S7). Equinatoxin II treatment also resulted in increased ookinete production compared with non-treated mutant samples. WT parasites treated with equinatoxin II produced fewer oocystes than untreated control samples, similar to what was seen when saponin was used (Fig. 7D), probably because of the lytic effect on uninfected erythrocytes. These results are fully consistent with the IFA and electron microscopy analysis, and support a model in which PPLP2 has a direct or indirect role in rupturing the EM during gametocyte egress.

**Saponin treatment does not rescue pplp2(-) after flagellated microgametes have formed**

In the experiments described above we treated the gametocytes with saponin or equinatoxin II before the time point...
Fig. 7. The pplp2(−) phenotype can be rescued by treatment with saponin and equinatoxin.
A. Saponin treatment of pplp2(−) gametocytes 5 min prior to activation rescues the mutant phenotype. A normal exflagellating cell with five normal flagella is seen (asterisks). See Movie S5.
B. Ookinete conversion presented as percentage of non-treated control samples. Samples of WT and pplp2(−) gametocytes treated with different concentrations of saponin. Saponin was added 5 min prior to activation of the gametocytes. The difference between the two strains was found to be statistically significant, \( P < 0.01 \) using two-way ANOVA analysis.
C. Equinatoxin II treatment rescues the mutant phenotype. Equinatoxin II was added to the pplp2(−) gametocytes 2 min before activation, resulting in normal exflagellation. One representative gametocyte with well separated flagellated gametes (asterisks) is shown. See also Movie S7.
D. Ookinete conversion (percentage of non-treated control samples) of WT and pplp2(−) gametocytes treated with different concentrations of equinatoxin II 5 min prior to activation of the gametocytes. The difference between the two strains was found to be statistically highly significant, \( P < 0.0001 \) using two-way ANOVA analysis.
E. Incomplete rescue of the super-flagellum phenotype when saponin is added after initiation of exflagellation. A male gametocyte treated with saponin displaying the super-flagellum. Saponin was added 14 min after activation. See also Movie S8.
F. Saponin treatment after activation removes the erythrocyte membrane of WT and pplp2(−) male gametocytes. Saponin was added to WT and to pplp2(−) gametocytes at 14 min after activation. After 1 min samples were fixed and stained with the TER-119 antibody recognizing the erythrocyte membrane (a,c,d,f, green) and the TAT antibody recognizing tubulin (b,c,e,f, red). a–c. WT with eight separate flagellated gametes still connected to the residual cell. d–f. pplp2(−) mutant, with one super-flagellum. The intensity of the green channel was enhanced in ImageJ to visualize remaining TER-119 labelling on non-infected erythrocytes. Scale bar 5 \( \mu \text{m} \).
when the EM is normally ruptured. We next wanted to investigate whether saponin treatment could rescue the mutant if added after the formation of motile flagella. Exflagellation, as scored by the presence of motile flagella, could first be detected in control samples at 10 min post activation, both in WT and in mutant parasites. We therefore added saponin to the gametocytes 14 min after activation. WT parasites continued normal exflagellation as expected. The *pplp2*(−) mutant gametocytes displayed two different phenotypes. Normally exflagellating cells could be detected, with the radial arrangement of the flagella, but many of the gametocytes retained the superflagella (Fig. 7E, Movie S8). In parallel these samples were stained with the anti-TER-119 and TAT antibodies to verify that the EM was destroyed by the treatment (Fig. 7F). As expected, and in contrast to what we saw in non-treated samples (Fig. 4B), no TER-119 labelling on the exflagellating *pplp2*(−) males was detected. Weak labelling could be detected on some of the surrounding erythrocytes (note that the green signal has been enhanced in Fig. 7F). Taken together, these results show that disruption of the EM of mutant cells at a time point when microgametes have already begun exflagellation cannot rescue all super-flagella. This supports the finding from the electron microscopy study where several flagella were found enclosed within the same flagellar membrane.

*pplp2*(−) female gametocytes are competent to form ookinetes

To rule out any adverse effects of PPLP2 loss on female fertility *per se* we determined ookinete conversion after crossing the *pplp2*(−) mutant to a strain that form only fertile males (*Δ47*). The results (Fig. 8A) clearly show a rescue in this cross, suggesting that the *pplp2*(−) mutant females are competent in forming ookinetes. We also detected normally egressed female gametocytes in the transmission electron microscopy analysis (Fig. 8B). This is consistent with the fact that PPLP2 was not detected in the proteome of female *P. berghei* gametocytes (Khan et al., 2005) and suggests that the function of the protein is restricted to male gametocytes.

Discussion

We show here, for the first time in *Plasmodium*, that a perforin-like protein is necessary for egress of the parasite from the host cell. Specifically, PPLP2 has an important function in egress of male gametocytes from the red blood cell. We provide direct evidence that the role of PPLP2 is in the rupture of the EM; to our knowledge this is the first report of a parasite protein that functions in this process. In addition, our results demonstrate that this protein is only required during male gametogenesis as female gametogenesis proceeded normally in a *P. berghei* mutant lacking PPLP2.

We used antisera developed against PPLP2 to compare the location and timing of expression of the protein in *P. berghei* and *P. falciparum* parasites. In both species the protein is detected in gametocytes and the signal disappears as egress proceeds during gametogenesis. This is consistent with the role of PPLP2 in gamocyte egress that we determined here for *P. berghei*. However, there were also differences between the two parasites. The most important difference is that in *P. berghei* we only detected the protein in the male gametocytes, while in the human parasite the protein is also found in the females. Importantly, other proteins which
have previously been described to be male-specific in *P. berghei*, are not sex-specific in the human parasite. One prominent example is P230, which is male-specific in *P. berghei* (Khan *et al.*, 2005) but is expressed in both males and females in *P. falciparum* (Williamson *et al.*, 1996). Another example is Actin II which is male-specific in the rodent parasite (Deligianni *et al.*, 2011), while in the human parasite Actin II is also detected in females (Rupp *et al.*, 2011). From the phenotypic analysis of the *P. berghei* knockout mutant we concluded that the function is restricted to the male, consistent with the expression of the protein exclusively in these cells. Attempts to genetically modify the *pplp2* locus in *P. falciparum*, have been unsuccessful, even with constructs designed to simply reconstitute a functional gene, suggesting technical issues with the approaches used (N.C.S. and M.B., unpublished). Without a gene disruptant mutant in the human parasite the functional significance of the difference in the sex-specificity of the expression of the gene in the two species cannot be determined.

Mutant *P. berghei* parasites lacking PPLP2 developed normally in the blood of the vertebrate host and formed morphologically normal gametocytes. However, although viable and fertile male gametes developed in this strain, the males remained trapped within the red blood cell. Our analysis revealed that the function of PPLP2 is restricted to the rupture of the EM, but has no function in the destruction of the internal membrane surrounding the developing gametocyte, the PVM. We base this conclusion on several lines of evidence. First, immunofluorescence assays of activated gametocytes from WT and the *pplp2* mutant showed that staining of SEP1, a PVM protein, was rapidly lost in the mutant parasites. On the other hand, the EM persisted in our mutant even when flagellated gametes were readily detected. Second, transmission electron microscopy of activated mutant gametocytes revealed undamaged EM while an intact PVM could not be detected. Occasionally remnants of what we suggest to be the PVM were detected as fragments attached to the gametocyte. Erythrocyte cytoplasm was also detected outside the gametocyte but inside the EM. Third, the use of the pore-forming toxin equinatoxin II, which specifically produces pores in the EM (Jackson *et al.*, 2007), rescued the phenotype and enabled normal exflagellation. One interpretation of our findings is that PPLP2 acts similarly to equinatoxin II, i.e. permeabilizing and presumably destabilizing the mechanical integrity of the EM to enable its eventual rupture. The apparent loss of PPLP2 from the gametocyte as egress proceeds could correspond to its translocation from a previously non-membrane location into a membrane (presumably the EM in this case), as occurs with most pore-forming proteins. While we have not here directly demonstrated that PPLP2 has pore-forming activity, the predicted MAC/PF domain of PPLP2 possesses all the important structural features known to be required for oligomerization and pore formation (Kafszack and Carruthers, 2010). The finding that the erythrocyte cytoplasm also persists in the mutant is completely in accord with our model, since its loss in WT parasites may be a direct result of EM permeabilization by PPLP2.

Previous proteomic data suggest that PPLP2 may be expressed in asexual blood stages of *P. falciparum* as well as gametocytes (Florens *et al.*, 2002). However, it should be born in mind that even highly enriched gametocyte preparations are likely contaminated with asexual blood stages, making it difficult to draw unambiguous conclusions from these data regarding stage-specificity of expression. A more recent analysis (Silvestrini *et al.*, 2010) of mature stage V *P. falciparum* gametocytes obtained results, consistent with increased expression of PPLP2 in gametocytes in accord with our findings here. We obviously cannot rule out expression of PPLP2 in asexual stages, but certainly, the absence of any phenotype in asexual blood stages of the *P. berghei* *pplp2(−) mutant argues against any important role for PPLP2 in asexual blood-stage egress.

Wild-type male gametes consist of one axoneme and one nucleus surrounded by a flagellar membrane (Sinden *et al.*, 1976). During exflagellation the eight axonemes exit the residual, naked cell in a radial pattern. We readily detected motile flagella in the *pplp2(−)* mutant by live imaging. However, the flagella were abnormally thick, and we therefore named them super-flagella. Immunofluorescence analysis and electron microscopy revealed that super-flagella consist of axonemes ‘bundled’ together, in many cases within one flagellar membrane, and bounded with the EM. We could rescue this phenotype by treatment with either saponin or equinatoxin II, but this was only possible if these agents were added before the time point when rupture of the EM normally takes place. When saponin was added after exflagellation had begun, many super-flagella were still visible, confirming the microscopic evidence of flagella with several axonemes within one flagellar membrane. We cannot readily explain this finding, mainly due to the fact that so little is known about this process. One possibility could be that when the EM does not rupture and the erythrocyte cytoplasm persists, as we see here, exit of the axoneme from the cytoplasm of the residual gametocyte is not correctly co-ordinated with the unfolding of the flagellar membrane. The lack of space outside the gametocyte may simply force the axonemes to exit at the same position thus residing inside the same flagellar membrane. Once this has occurred, it presumably cannot be reversed by artificially induced rupture of the EM.

The super-flagellum phenotype shows similarities to the phenotype resulting from treatment of *P. falciparum*...
microgametes with 1,10-phenanthroline, an inhibitor of Zn-dependent metalloproteases (Sologub et al., 2011). In those experiments the EM was found to persist, while the PVM was ruptured and in activated microgametocytes, the axonemes formed bundles that remained attached to the residual body (for an example see Fig. S5). We performed similar experiments and detected gametocytes with motile super-flagella when treated with 1 mM 1,10-phenanthroline. Individual flagella broke out after 30–60 s of vigorous beating, suggesting that the EM was more fragile in the treated samples than in the pplp2(−) mutant (data not shown). It is therefore possible that the activity of both PPLP2 and a putative metalloprotease are both required to rupture the EM. However, the effect of 1,10-phenanthroline, a metal chelating agent, may be nonspecific as a very high concentration of the drug is needed for an effect to be detected, and an effect of the inhibitor on DNA replication has also been reported (Raabe et al., 2009). Therefore, these data must be regarded with great caution until more specific inhibitors are available.

A T. gondii mutant deficient in the perforin-like protein TgPLP1 showed a delay in tachyzoite egress from the host cell (Kafsack et al., 2009). In this case, both the PVM and the host cell membrane initially remained intact, suggesting that in this parasite the perforin-like protein has a role in rupture of the PVM and, possibly, the host cell membrane. It is interesting to note that egress did eventually take place in the Toxoplasma mutant, apparently due to mechanical rupture of the PVM as a result of the vigorous motility of the parasites within the vacuole. Similarly, in our case the pplp2(−) mutation did not result in a complete block of male gametogenesis, as some normal ookinetes were formed and these in turn could traverse the midgut epithelium and develop into sporogonic oocysts. We believe that the rapid movements of the flagella allow some mutant gametes to eventually eliminate the host membrane, and thus become able to fuse with the female gametes.

Two P. berghei knockout mutants with a gametocyte egress phenotype have previously been described, lacking the proteins MDV-1/PEG3 (Ponzi et al., 2009) and PbGEST (Talman et al., 2011) respectively. These two proteins show some interesting similarities, as they are both localized in osmiophilic bodies. Both mutants affected in male as well as female egress due to a block in the disruption of the two surrounding membranes, the PVM and the EM. This is clearly different from what we see in the case of the pplp2(−) mutant, although the super-flagellum phenotype was also noted in the mutant lacking PbGEST. In the mdv-1/peg3 mutant, on the other hand, individual flagella could be seen beating trapped inside the surrounding membranes. In all three mutants motile axonemes are formed and fertility is reduced, but not abolished. With the present data it therefore seems reasonable to suggest that MDV-1/PEG3 and PbGEST have similar functions, while PPLP2 has a distinct role, although further work is clearly needed to fully understand this aspect.

In conclusion, we have identified a fourth protein with a stage-specific role in egress of the P. berghei gametocyte from its surrounding host cell. The previously identified proteins, MDV-1/PEG3, PbGEST and Actin II act specifically on the rupture of the PVM, while PPLP2 has a function restricted to EM rupture. Previous work has shown that egress of asexual blood-stage forms in P. falciparum is dependent on the activity of least one calcium-dependent kinase, CDPK5 (Dvorin et al., 2010), and proteases also play a role in both gametocyte and asexual blood-stage egress in both P. falciparum and P. berghei (see Blackman, 2008; Roiko and Carruthers, 2009; Wirth and Pradel, 2012 for recent reviews). How the functions of these various molecular players are related to each other raises many interesting questions for further investigation, including fundamental questions about how the two bounding membranes are selectively ruptured in a timely manner. The elucidation of these molecular events may also suggest new targets for intervention strategies against the malaria parasite.

**Experimental procedures**

**Parasite strains**

The strains of *P. berghei* used were ANKA 2.34, a gametocyte-producing strain referred to as WT and HPE, a non-gametocyte producing line (Janse et al., 1992). The mutants Δ47 (Khan et al., 2005) and Δ45/48 (van Dijk et al., 2001) have been described. Parasites were maintained in Theiler’s Original mice.

All animal work was carried out in full conformity with Greek regulations: Presidential Decree (160/91) and law (2015/92) which implement the directive 86/609/EEC from the European Union and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes and the protocol was approved by the Ethics Committee of FORTH.

**Parasitology methods**

Activation of gametocytes was achieved by incubating gametocytes at 19°C in exflagellation medium (complete oocyst culture medium supplemented with 50 μM xanthurenic acid). Ookinetes were cultured *in vitro* according to Rodríguez et al. (2002). For cross-fertilization experiments oocyst culture were seeded with equal volumes of blood from mice infected with ANKA 2.34, Δ47, Δ48/45 or pplp2(−). Ookinet conversion rate (the percentage of female gametes developing into ookinetes), was determined by incubating samples from *in vitro* cultures with the mAb 13.1, recognizing a surface antigen of female gametes, zygoetes and ookinetes (Winger et al., 1988). To determine oocyst formation female *Anopheles gambiae* strain G3 mosquitoes were fed on infected mice. Dissections for oocyst counts were done 12 days after feeding.
Mature gametocytes of the *P. falciparum* strain NF54 were cultivated *in vitro* as described (Ilediba and Vanderberg, 1981). Gametocytes were activated by incubating mature gametocyte cultures in 100 μM xanthurenic acid/PBS pH 8.5 at RT.

**Generation of plasmid for gene disruption of the pplp2 locus**

The primers used for the constructs are summarized in the Table S1. The plasmid and DNA sequence of pL0001 are available from MR4 (http://www.mr4.org).

The plasmid p.Dpplp2 (Fig. S2A) for the gene disruption was constructed in the standard vector pL0001. 579 bp from the 5’ end of the ORF of *pplp2* and a 591 bp fragment of the 3’ end were amplified from *P. berghei* genomic DNA. The two fragments were cloned into the KpnI and HindIII sites and the EcoRI and BamHI sites respectively. The plasmid was digested with KpnI and BamHI before transfection. After the double cross-over 1562 bp in the middle of the target gene were replaced by the TgDHFR/TS cassette.

**Transfection, cloning and genotyping**

Parasites were transfected and cloned as described (Janse *et al.*, 2006). Correct integration was verified by PCR with the primers indicated (Table S1); primers L635, L665, L739 and L740 have been described previously (Franke-Fayard *et al.*, 2004). Southern blot analysis was performed according to Siden-Kiamos (1997). Genomic DNA of the WT and mutant was digested with either HindIII or EcoRI and the fragments separated on an agarose gel. The DNA was transferred to a nitrocellulose membrane filter and hybridized at 60°C with a mixed probe corresponding to the 5’ and 3’ targeting regions.

**RT-PCR**

Each sample of RNA from mixed blood stages was obtained from 100 μl of infected blood. Ookinet RNA was isolated from ookinetes which had been enriched using the 13.1 antibody coated magnetic bead technique (Siden-Kiamos *et al.*, 2000); this sample also contains female gametes and zygotes. Finally, the oocyst samples were obtained by dissection of midguts from infected mosquitoes. Total RNA was isolated using the TRI reagent from Sigma. The cells were pelleted and then resuspended in 500 μl of TRI; alternatively midguts were homogenized in 500 μl of TRI and the fragments separated on an agarose gel. The DNA was transferred to a nitrocellulose membrane filter and hybridized at 60°C with a mixed probe corresponding to the 5’ and 3’ targeting regions. After activation in exflagellation medium at 19°C. Fixation was done in 4% paraformaldehyde in PBS for 15 min, after which the gametocytes were collected on poly-L-lysine-coated coverslips (13 mm) by centrifugation at 500 g for 15 min. The fixative was removed and the cells were permeabilized with 0.2% saponin in PBS for 30 s. Permeabilization with 0.5% Triton X-100 was also tested, but no labelling was detected after this treatment. The following steps were done as described below. In all other experiments gametocytes, activated or non-activated, were incubated in 4% paraformaldehyde in microtubule stabilizing buffer (MTSB, 10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, 5 mM MgCl2, pH 6.9) for 1 h. The parasites were collected on coverslips as described above. After removal of the fixative samples were permeabilized with 0.5% Triton X-100 in PBS for 10 min. After rinsing twice in PBS the primary antibodies were added diluted in PBS with 5% normal goat serum. Incubation was either for 1 h at room temperature or overnight at 4°C. Cells were washed twice with PBS for 5 min and incubated with secondary antibodies, washed twice with PBS for 5 min before mounting in Vectashield (Vector laboratories). All steps were carried out at room temperature, unless indicated otherwise. The samples were analysed using a Zeiss LSM 510 confocal laser scanning microscope attached to a Zeiss Axioskop 2 plus microscope. Images were analysed with ImageJ software (http://rsbweb.nih.gov/ij/).

**Antibodies**

The anti-SET and anti-SEP1 antibodies have been described (Birago *et al.*, 2003; Pace *et al.*, 2006). Both antibodies were produced in rabbits. The mAb TAT recognizes *P. berghei* tubulin (Billker *et al.*, 2002). Conjugated anti-mouse TER-119 was purchased from Biozol. Secondary antibodies were anti-mouse (Alexa-488, Alexa-555) and anti-rabbit (Alexa-488, Alexa-546) conjugated to Alexa Fluor (Invitrogen) or anti-rabbit conjugated to Cy-3 (Jackson Research).

**Expression of recombinant protein and derivation of mouse antisera A against P. falciparum PPLP2**

Amino acids 576–661 of PPLP2 (Plasmodb gene-ID: PF3D7_1216700) were expressed as an N-terminal GST and C-terminal 6xHis fusion protein in *Escherichia coli*. The *pplp2* fragment was amplified by PCR, using primers 3F (5’-GCCGAATTTAAGAATGCTGGATCCAAATTAAAAGTAC-3’) and 35R (5’-CTTAAATAATATTCAATTATGCTGCAGGTTGCTGTGTTCCC-3’) with *P. falciparum* 3D7 genomic DNA as a template. The resulting fragment was cloned into pGEX6.1 (GE Healthcare) producing the plasmid pgeX6.1-L2-6xHis. SHuffle *E. coli* (Eurogentec) were transformed with pgeX6.1-L2-6xHis. Four hundred millilitres of culture was induced for 4 h at 37°C with 1 mM Isopropyl β-D-1-thiogalactopyranoside. Cells were pelleted by centrifugation and lysed using Bugbuster (Novagen). Inclusion bodies were resuspended in 30 ml of sodium chloride Tris-EDTA (STE) buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA) and DTT was added to a final concentration of 5 mM. Lysis was achieved by the addition of 1.5% N-laurylsarcosine and sonication for 1 s pulses for 1 min with a Vibracell sonicking microprobe (Sonics & Materials). The lysate was clarified by centrifugation at 10 000 g for 5 min at 4°C and adjusted to 4% Triton X-100 preceding incubation at 4°C on a rotator for 15 min.

© 2013 John Wiley & Sons Ltd, *Cellular Microbiology*, 15, 1438–1455
The supernatant was incubated with S-linked glutathione agarose (GSHA) (Sigma) for 15 min at 4°C on a rotating wheel. The GSHA was washed five times with ice-cold PBS by repeated centrifugation at low speed. Protein was eluted with 1 M reduced glutathione in PBS, and used for immunization of mice at 1 mg ml⁻¹.

Expression of recombinant protein and derivation of mouse antisera B against P. falciparum PPLP2

Recombinant protein for PPLP2 (Plasmodb gene-ID: PF3D7_1216700) was expressed as a maltose-binding protein-tagged fusion protein of 79 kDa, which comprises the MACPF domain (amino acids 501–840). Cloning was mediated by EcoRI restriction sites (underlined), using primers 5′-ATGAATTCCGTGAATAACGTCCAAGGAAA-3′ and 5′-TAGAATTCCTAGGAATACTTATTATCGGTG-3′, and the amplified PCR product was inserted into vector pEH902 (kindly provided by K. Williamson, Chicago; Williamson et al., 1995). Recombinant proteins were expressed in E. coli BL21 (DE3) RIL cells according to the manufacturer’s protocol (Stratagene) and purified via amylose resin (New England BioLabs) as described (Williamson et al., 1995) with the following modifications: Bacterial pellet was directly resuspended in lysis buffer. After incubation on ice for 20 min cells were disrupted by pressure (3 ¥ 1000 psi) and 4 min by sonication (50 cycles/50% intensity). DNase treatment was omitted. Elution of the amylose-bound fusion protein was achieved using the batch purification method according to the manufacturer’s instructions (New England BioLabs). Specific immune sera were generated by immunization of mice. Housing and handling of the animals followed the guidelines of the animal welfare committee of the government of Lower Franconia.

Immunofluorescence analysis (IFA) of P. falciparum gametocytes

Mature non-activated and activated NF54 gametocyte cultures were air dried on Teflon slides and fixed for 10 min in methanol at −80°C. For membrane permeabilization and blocking of non-specific binding, fixed cells were incubated for 30 min in 0.01% saponin/0.5% BSA/PBS and 1% neutral goat serum (Sigma-Aldrich) in PBS. Specimens were then incubated for 1.5 h at 37°C with mouse anti-PPLP2 antiserum diluted in 0.01% saponin/0.5% BSA/PBS. After washing in 0.01% saponin/0.5% BSA/PBS, the specimens were incubated with rabbit anti-Pls230 antisera (raised against a recombinant protein of immunogenic region C as described in Williamson et al., 1995) or rabbit anti-Pls25 antibody (ATCC) to highlight the gametocytes. Mouse antisera against the MBP tag were used as negative control (Fig. S1D). Binding of primary antibody was visualized using AlexaFluor488-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Alexa Fluor488 or Alexa Fluor 596; Molecular Probes) diluted in 0.01% saponin/0.5% BSA/PBS. Nuclei were highlighted by incubating the specimens with Hoechst 33342 (Invitrogen) for 1 min. Specimens were microscopically examined using a Leica TCS SP2 confocal laser scanning microscope. Digital images were processed using AdobePhotoshop CS software.

Transmission electron microscopy

Infected mice were treated for 36 h with 15 mg ml⁻¹ sulfadiazine in the drinking water (Beetsma et al., 1998). The following stages were carried out at room temperature. The infected blood was immediately diluted 20-fold in gametocyte medium (GM) (RPMI 1640 containing 25 mM Hepes, 2 mM L-glutamine, supplemented with freshly made 0.2% NaHCO₃ and 1 mg ml⁻¹ BSA, pH 7.3). The gametocytes were layered on a cushion of 15% Nycodenz diluted in GM and centrifuged at 500 g for 20 min. The interphase was recovered and the gametocytes were pelleted at 500 g for 5 min and resuspended in GM. Gametogenesis was induced by diluting the gametocytes 1:5 in exflagellation medium and incubated at 19°C. After 19 min the gametocytes were fixed in 2% glutaraldehyde, 2% paraformaldehyde, 2 mM CaCl₂ in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4°C, and processed 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 RT, treated with 1% tannic acid in 0.05 M cacodylate buffer for 30 min and rinsed in 1% sodium sulfate in 0.05 M cacodylate buffer for 10 min. Post-fixed specimens were washed, dehydrated through a graded series of ethanol solutions (30–100% ethanol) and embedded in Agar 100 (Agar Scientific, UK). Ultrathin sections, obtained by a UC6 ultramicrotome (Leica), were stained with uranyl acetate and lead citrate and examined by an EM208 Philips electron microscope.

Acknowledgements

We thank A. Pikoulas and A. Karagouni-Dalakoura for assistance in this project. The equinatoxin II was kindly provided by Prof. Gregor Anderluh. We thank Oliver Billker, Robert E. Sinden and Marta Ponzi for gifts of antibodies. R.M. was funded by InterMal training, a Marie Curie Initial Training Network (Grant No. PITN-GA-2008-215281 to I.S.-K.) and N.S.M. was in receipt of a UK Medical Research Council PhD studentship. C.L. and M.J.B. are members of the EVIMalaR network (Grant Agreement # 242095), which partially funded this research. Funding was also received from Transmalaria bloc (HEALTH-F3-2008-223736) and the Priority Programme SPP1580 of the Deutsche Forschungsgemeinschaft.

References

Ansorge, I., Benting, J., Bhakdi, S., and Lingelbach, K. (1996) Protein sorting in Plasmodium falciparum-infected red blood cells permeabilized with the pore-forming protein streptolysin O. Biochem J 315 (Part 1): 307–314.
Beetsma, A.L., van de Wiel, T.J., Sauerwein, R.W., and Eling, W.M. (1998) Plasmodium berghei ANKA: purification of large numbers of infectious gametocytes. Exp Parasitol 88: 69–72.
Billker, O., Shaw, M.K., Jones, I.W., Ley, S.V., Mordue, A.J., and Sinden, R.E. (2002) Azadirachtin disrupts formations of organised microtubule arrays during microgametogenesis of Plasmodium berghei. J Eukaryot Microbiol 49: 489–497.
Birago, C., Albanesi, V., Silvestrini, F., Picci, L., Pizzi, E., Alano, P., et al. (2003) A gene-family encoding small exported proteins is conserved across Plasmodium genus. Mol Biochem Parasitol 126: 209–218.
Blackman, M.J. (2008) Malarial proteases and host cell egress: an ‘emerging’ cascade. Cell Microbiol 10: 1925–1934.

© 2013 John Wiley & Sons Ltd, Cellular Microbiology, 15, 1438–1455
Deligianni, E., Morgan, R.N., Bertuccini, L., Kooij, T.W., Laforge, A., Nahar, C., et al. (2011) Critical role for a stage-specific actin in male exflagellation of the malaria parasite. *Cell Microbiol* 13: 1714–1730.

van Dijk, M.R., Janse, C.J., Thompson, J., Waters, A.P., Braks, J.A., Dodemont, H.J., et al. (2001) A central role for P48/45 in malaria parasite male gamete fertility. *Cell* 104: 153–164.

Dvorin, J.D., Martyn, D.C., Patel, S.D., Grimley, J.S., Collins, C.R., Hopp, C.S., et al. (2010) A plant-like kinase in *Plasmodium falciparum* regulates parasite egress from erythrocytes. *Science* 328: 910–912.

Ecker, A., Pinto, S.B., Baker, K.W., Kafatos, F.C., and Sinden, R.E. (2007) *Plasmodium berghei*: plasmodium perforin-like protein 5 is required for mosquito midgut invasion in *Anopheles stephensi*. *Exp Parasitol* 116: 504–508.

Ecker, A., Bushell, E.S., Tewari, R., and Sinden, R.E. (2008) Reverse genetics screen identifies six proteins important for malaria development in the mosquito. *Mol Microbiol* 70: 209–220.

Florens, L., Washburn, M.P., Raine, J.D., Anthony, R.M., Grainger, M., Haynes, J.D., et al. (2002) A proteomic view of the *Plasmodium falciparum* life cycle. *Nature* 419: 520–526.

Franke-Fayard, B., Trueman, H., Ramesar, J., Mendoza, J., van der Keur, M., van der Linden, R., et al. (2004) A *Plasmodium berghei* reference line that constitutively expresses GFP at a high level throughout the complete life cycle. *Mol Biochem Parasitol* 137: 23–33.

Hall, N., Karras, M., Raine, J.D., Carlton, J.M., Kooij, T.W., Berriman, M., et al. (2005) A comprehensive survey of the *Plasmodium* life cycle by genomic, transcriptomic, and proteomic analyses. *Science* 307: 82–86.

Ifediba, T., and Vanderberg, J.P. (1981) *Complete in vitro maturation of Plasmodium falciparum* gametocytes. *Nature* 294: 364–366.

Ishino, T., Chinzei, Y., and Yuda, M. (2005) A *Plasmodium* sporozoite protein with a membrane attack complex domain is required for breaching the liver sinusoidal cell layer prior to hepatocyte infection. *Cell Microbiol* 7: 199–208.

Jackson, K.E., Spielmann, T., Hanssen, E., Adisa, A., Separovic, F., Dixon, M.W., et al. (2007) Selective permeabilization of the host cell membrane of *Plasmodium falciparum*-infected red blood cells with streptolysin O and equinatoxin II. *Biochem J* 403: 167–175.

Janse, C.J., Ramesar, J., van den Berg, F.M., and Mons, B. (1992) *Plasmodium berghei*: in vivo generation and selection of karyotype mutants and non-gametocyte producer mutants. *Exp Parasitol* 74: 1–10.

Janse, C.J., Franke-Fayard, B., Mair, G.R., Ramesar, J., Thiel, C., Engelmann, S., et al. (2006) High efficiency transfection of *Plasmodium berghei* facilitates novel selection procedures. *Mol Biochem Parasitol* 145: 60–70.

Kadota, K., Ishino, T., Matsuyama, T., Chinzei, Y., and Yuda, M. (2004) Essential role of membrane-attack protein in malarial transmission to mosquito host. *Proc Natl Acad Sci USA* 101: 16310–16315.

Kafasack, B.F., and Carruthers, V.B. (2010) Apicomplexan perforin-like proteins. *Commun Integr Biol* 3: 18–23.

Kafasack, B.F., Pena, J.D., Coppens, I., Ravindran, S., Boothroyd, J.C., and Carruthers, V.B. (2009) Rapid membrane disruption by a perforin-like protein facilitates parasite exit from host cells. *Science* 323: 530–533.

Kaiser, K., Camargo, N., Coppens, I., Morrissey, J.M., Vaidya, A.B., and Kappe, S.H. (2004) A member of a conserved *Plasmodium* protein family with membrane-attack complex/perforin (MACPF)-like domains localizes to the micronemes of sporozoites. *Mol Biochem Parasitol* 133: 15–24.

Khan, S.M., Franke-Fayard, B., Mair, G.R., Lasonder, E., Janse, C.J., Mann, M., and Waters, A.P. (2005) Proteome analysis of separated male and female gametocytes reveals novel sex-specific Plasmodium biology. *Cell* 121: 675–687.

Pace, T., Olivier, A., Sanchez, M., Albanesi, V., Picci, L., Siden-Kiamo, I., et al. (2006) Set regulation in asexual and sexual *Plasmodium* parasites reveals a novel mechanism of stage-specific expression. *Mol Microbiol* 60: 870–882.

Perry, M.M., and Gilbert, A.B. (1979) Yolk transport in the ovarian follicle of the hen (*Gallus domesticus*): lipoprotein-like particles at the periphery of the oocyte in the rapid growth phase. *J Cell Sci* 39: 257–272.

Ponzi, M., Siden-Kiamo, I., Bertuccini, L., Curra, C., Kroeze, H., Camarda, G., et al. (2009) Egress of *Plasmodium berghei* gametes from their host erythrocyte is mediated by the MDV-1/PEG3 protein. *Cell Biochem* 11: 1272–1288.

Pradel, G. (2007) Proteins of the malaria parasite sexual stages: expression, function and potential for transmission blocking strategies. *Parasitol* 134: 1911–1929.

Raabe, A.C., Bilker, O., Vial, H.J., and Wengelnik, K. (2009) Quantitative assessment of DNA replication to monitor microgametogenesis in *Plasmodium berghei*. *Mol Biochem Parasitol* 168: 172–176.

Raibaud, A., Brahim, K., Roth, C.W., Brey, P.T., and Faust, D.M. (2006) Differential gene expression in the ookinete stage of the malaria parasite *Plasmodium berghei*. *Mol Biochem Parasitol* 150: 107–113.

Rodriguez, M.C., Margos, G., Compton, H., Ku, M., Lanz, H., Rodriguez, M.H., and Sinden, R.E. (2002) *Plasmodium berghei*: routine production of pure gametocytes, extracellular gametes, zygotes, and ookinetes. *Exp Parasitol* 101: 73–76.

Roiko, M.S., and Carruthers, V.B. (2009) New roles for perforins and proteases in apicomplexan egress. *Cell Microbiol* 11: 1444–1452.

Rupp, I., Sologub, L., Williamson, K.C., Scheuermayer, M., Reininger, L., Doerig, C., et al. (2011) Malaria parasites form filamentous cell-to-cell connections during reproduction in the mosquito midgut. *Cell Res* 21: 683–696.

Scholz, S.M., Simon, N., Lavazec, C., Dude, M.A., Templeton, T.J., and Pradel, G. (2008) PfCCp proteins of *Plasmodium falciparum*: gametocyte-specific expression and role in complement-mediated inhibition of egress. *Int J Parasitol* 38: 327–340.

Siden-Kiamo, I. (1997) Southern/northern blotting and hybridization techniques. In The Molecular Biology of *Insect Disease Vectors: A Methods Manual*. Crampton, J.M., Beard, C.B., and Louis, C. (eds). London: Chapman and Hall, pp. 230–243.
Siden-Kiamos, I., Viachou, D., Margos, G., Beetsma, A., Waters, A.P., Sinden, R.E., and Louis, C. (2000) Distinct roles for pbs21 and pbs25 in the in vitro ookinete to oocyst transformation of Plasmodium berghei. J Cell Sci 113 (Part 19): 3419–3426.

Silvestrini, F., Lasonder, E., Olivieri, A., Camarda, G., van Schaikj, B., Sanchez, M., et al. (2010) Protein export marks the early phase of gametocytogenesis of the human malaria parasite Plasmodium falciparum. Mol Cell Proteomics 9: 1437–1448.

Sinden, R.E., Canning, E.U., and Spain, B. (1976) Gametogenesis and fertilization in Plasmodium yoelii nigeriensis: a transmission electron microscope study. Proc Natl Acad Sci USA 193: 55–76.

Sologub, L., Kuehn, A., Kern, S., Przyborski, J., Schillig, R., and Pradel, G. (2011) Malaria proteases mediate inside-out egress of gametocytes from red blood cells following parasite transmission to the mosquito. Cell Microbiol 13: 897–912.

Talman, A.M., Lacroix, C., Marques, S.R., Blagborough, A.M., Carzaniga, R., Menard, R., and Sinden, R.E. (2011) PbGHOST mediates malaria transmission to both mosquito and vertebrate host. Mol Microbiol 82: 462–474.

Williamson, K.C. (2003) Pfis230: from malaria transmission-blocking vaccine candidate toward function. Parasite Immunol 25: 351–359.

Williamson, K.C., Keister, D.B., Muravtova, O., and Kaslow, D.C. (1995) Recombinant Pfis230, a Plasmodium falciparum gametocyte protein, induces antisera that reduce the infectivity of Plasmodium falciparum to mosquitoes. Mol Biochem Parasitol 75: 33–42.

Williamson, K.C., Fujioka, H., Akaiwa, M., and Kaslow, D.C. (1996) Stage-specific processing of Pfs230, a Plasmodium falciparum transmission-blocking vaccine candidate. Mol Biochem Parasitol 78: 161–169.

Winger, L.A., Tirawanchai, N., Nicholas, J., Carter, H.E., Smith, J.E., and Sinden, R.E. (1988) Ookinetes antigens of Plasmodium berghei. Appearance on the zygote surface of an Mr 21 kD determinant identified by transmission-blocking monoclonal antibodies. Parasite Immunol 10: 193–207.

Wirth, C.G., and Pradel, G. (2012) Molecular mechanisms of host cell egress by malaria parasites. Int J Med Microbiol 302: 172–178.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** A. CLUSTALW alignment of PPLP2 encoded by *P. berghei* gene PBANKA_143240 (top) and *P. falciparum* gene PF3D7_1216700 (bottom). The predicted signal peptide is indicated by a yellow line and the membrane attack complex/perforin (MACPF)-like domain by a green dashed line (amino acids 488–712 in *P. berghei, 562–786 in P. falciparum*). The apicomplexan perforin-like (ApiPLP) motif ([W-x(2)-[FL]-[FL]-x(2)-[FY]-G-T-H-x(7)-G-G] is marked with a solid blue line (all below the sequences). The regions chosen for production of the two PPLP2 antisera are indicated by a light red line (antisera A) and by a dark red line (antisera B); both lines above the sequences.

B. Specificity of PPLP2 antiserum A. No PPLP2-specific signal was detected in *P. berghei* male gametocytes lacking *pplp2*. The SET protein (green) was used as a marker of the nucleus of male gametocytes.

C. No PPLP2 signal was detected in *P. berghei* female gametocytes. The characteristic small female nucleus was stained with the SET antibody and the PVM was stained for the SEP1 protein (both green). Both of these antisera were produced in rabbits. The PPLP2 antiserum A is of mouse origin and the secondary antibody conjugated to Alexa Fluor 555 (red). These experiments were carried out in parallel with labelling of WT male gametocytes, which were positive for PPLP2.

D. Specificity of PPLP2 antiserum B. No labelling was detected on gametocytes immunolabelled with a mouse antisera against the male-specific binding protein (MBP) tag. An antibody against the Pfs230 antigen (red) identified the gametocyte.

**Fig. S2.** Immunofluorescence assays of *P. falciparum* gametocytes at 15 min post activation showed PPLP2 labelling (green) in association with shed membranes (indicated by arrows). The exflagellating microgametocyte is highlighted by labelling of Pfs230 (red). Arrowheads indicate microgametes. The corresponding differential interference contrast (DIC) images are shown. Bar, 5 μm.

**Fig. S3.** Experimental genetic strategy for disruption of *pplp2* in *Plasmodium berghei*. A. Maps of the WT *pplp2* locus, the disruption vector p*Δpplp2*, and the *pplp2* locus after integration. The WT locus comprises a single exon encoding the PPLP2 protein. A double-cross-over strategy was employed to replace the middle of the coding region with the DHFR/TS cassette. The two targeting fragments are depicted as double arrows in blue. Primers used for diagnostic PCR (black arrows), the expected fragments (purple double arrows) and their size are indicated. Note that the DHFR/TS cassette is not shown to scale.

B. Diagnostic PCR reactions on gDNA from WT (lanes 1, 4 and 7) and the clone *pplp2(−) cl1* (lanes 2, 5 and 8). A non-cloned population from an independent transfection was also included (lanes 3, 6 and 9). The absence of contaminating WT parasites in the transfected parasites is evidenced by the primer pair peraF/peraR. The primer pairs are indicated on the top and molecular weight markers to the left.

C and D. Southern blot analysis of *pplp2(−) cl1*. A. The *pplp2* locus of WT (top) and mutant parasite (bottom) gDNA. The DNA was digested with EcoRI and HindIII and the sizes of the fragments generated are shown. B. Southern blot analysis of gDNA from WT and the *pplp2(−) cl1* digested by EcoRI and HindIII. The blot was hybridized with a mixed probe containing the two fragments used for the targeting construct (red lines in C).

**Fig. S4.** Measurements of the diameter of the super-flagella of the *pplp2(−)* mutant. A. Exflagellating male gametocytes were stained with the TAT mAb recognizing the tubulin of the axonemes. (a–k) Axonemes of the *pplp2(−)* mutant. (l) A representative example of WT single *gameto- cyes*. The characteristic small female nucleus was stained with the SET antibody and the PVM was stained for the SEP1 protein. Scale bar 5 μm.

B. Measurement of axonemes of the *pplp2(−)* mutant and comparison with WT single axonemes. The diameter of the axonemes was measured using ImageJ. Forty WT axonemes were measured and the average size determined was 300 nm ± 62 nm (standard deviation). The average diameters of three measurements per mutant axonemes and the calculated
number of axonemes per flagella are shown in the table for each panel a–k.

**Fig. S5.** Examples of super-flagella of 1,10-phenanthroline-treated *P. falciparum* gametocytes. Gametocytes were activated in the presence of 1,10-phenanthroline as described (Sologub *et al.*, 2011). At 20 min post activation, the PVM had ruptured, while the EM was still detectable. (A and B) Axonemes had clustered to bundles, forming a super-flagellum. The axonemes reside inside the same flagellar membrane. AX, axoneme; EM, erythrocyte membrane; PDM, parasite-derived membrane; SF, super-flagellum. Bar, 2 μm (A), 0.5 μm (B).

**Table S1.** Sequence of the oligonucleotide primers used in this work.

**Movie S1.** Exflagellation of WT male gametocyte. Separate flagella are visible. In the final frames an exflagellation centre is formed. Images captured at one frame per second for 100 s. (avi)

**Movie S2.** Exflagellation of *pplp2*(*−*) mutant. A single beating super-flagellum is visible. Images captured at one frame per second for 54 s. (avi)

**Movie S3.** Four exflagellating *pplp2*(*−*) mutant males, each having one super-flagellum. No exflagellation centres are formed. Images captured at one frame per second for 90 s. (avi)

**Movie S4.** One exflagellating *pplp2*(*−*) cell. In this case two flagella are seen in close proximity to each other. Images captured at one frame per second for 58 s. (avi)

**Movie S5.** *pplp2*(*−*) sample treated with saponin before activation. Eight separate flagella are attached to the residual cell. Images captured at one frame per second for 84 s. (avi)

**Movie S6.** WT sample treated with saponin before activation. Eight separate flagella are attached to the residual cell. Images captured at one frame per second for 120 s. (avi)

**Movie S7.** *pplp2*(*−*) sample treated with equinatoxin II before activation. Eight separate flagella are attached to the residual cell. Images captured at two frames per second for 88.5 s. (avi)

**Movie S8.** *pplp2*(*−*) sample treated with saponin 14 min after activation. A male with a persisting super-flagellum is visible. Images captured at one frame per second for 82 s. (avi)