Malaria proteases mediate inside-out egress of gametocytes from red blood cells following parasite transmission to the mosquito

Ludmilla Sologub,1† Andrea Kuehn,1† Selina Kern,1 Jude Przyborski,2 Rebecca Schillig1 and Gabriele Pradel1*
1Research Center for Infectious Diseases, University of Würzburg, Josef-Schneider-Strasse 2/D15, 97080 Würzburg, Germany.
2Department of Parasitology, Philipps University Marburg, Karl-von-Frisch-Strasse 8, 35043 Marburg, Germany.

Summary
Malaria parasites reside in human erythrocytes within a parasitophorous vacuole. The parasites are transmitted from the human to the mosquito by the uptake of intraerythrocytic gametocytes during a blood meal, which in the midgut become activated by external stimuli and subsequently egress from the enveloping erythrocyte. Gametocyte egress is a crucial step for the parasite to prepare for fertilization, but the molecular mechanisms of egress are not well understood. Via electron microscopy, we show that Plasmodium falciparum gametocytes exit the erythrocyte by an inside-out type of egress. The parasitophorous vacuole membrane (PVM) ruptures at multiple sites within less than a minute following activation, a process that requires a temperature drop and parasite contact with xanthurenic acid. PVM rupture can also be triggered by the ionophore nigericin and is sensitive to the cysteine protease inhibitor E-64d. Following PVM rupture the subpellicular membrane begins to disintegrate. This membrane is specific to malaria gametocytes, and disintegration is impaired by the aspartic protease inhibitor EPNP and the cysteine/serine protease inhibitor TLCK. Approximately 15 min post activation, the erythrocyte membrane ruptures at a single breaking point, which can be inhibited by inhibitors TLCK and TPCK. In all cases inhibitor treatment results in interrupted gametogenesis.

Introduction
Subcellular compartmentalization is a feature of intracellular-living pathogens to avoid the host immune system. These include malaria parasites, which for the most parts of their life cycle reside in a parasitophorous vacuole (PV) within a host cell. During sexual reproduction, however, the parasites have to exit the enveloping erythrocyte in order to form gametes and to prepare for fertilization. Sexual reproduction exclusively takes place following parasite transmission from the human to the mosquito and thus plays a crucial part in the spread of the disease.

The malaria sexual phase begins with the differentiation of sexual precursor cells, the intraerythrocytic gametocytes, which are taken up by the female anopheline mosquito during a blood meal (reviewed in Pradel, 2007; Kuehn and Pradel, 2010). When entering the mosquito midgut together with the meal, the gametocytes become activated by external stimuli, i.e. a drop in temperature and the contact with the mosquito-derived molecule xanthurenic acid (XA). Gametocyte activation leads to rounding up, followed by parasite egress from the enveloping erythrocyte and the formation of gametes. During this period the microgametocyte replicates its genome three times in order to produce eight motile microgametes, a process termed exflagellation. Within approximately 20 min, the fertile gametes have formed. After fertilization, the zygote transforms into an infective ookinete within 1 day, which leaves the midgut lumen and thus marks the end of the malaria sexual phase.

Parasite egress from the host erythrocyte follows a fixed programme and involves the rupture of two membranes, the membrane of the parasitophorous vacuole (PVM) and the erythrocyte membrane (EM). The sequence of membrane rupture remains hitherto unclear. Particularly two models of egress are currently in discussion, the inside-out model, in which the PVM ruptures before the EM, and the outside-in model, in which the EM is degraded first (reviewed in Blackman, 2008).
A number of studies identified proteases that mediate the emergence of malaria parasites from the host cell. In the blood stages of the human malaria parasite *Plasmodium falciparum*, the cysteine protease falcipain-2 and the aspartic protease plasmepsin II were reported to degrade the erythrocytic cytoskeletal proteins 4.1, ankyrin and spectrin, leading to host cell destablity (Le Bonniec et al., 1999; Dua et al., 2001; Hanspal et al., 2002). Furthermore, serine-rich antigen (SERA) proteins, which were originally identified in the PV of blood stage schizonts (e.g. Aoki et al., 2002; Miller et al., 2002), are proposed to mediate PVM rupture. In this context it was shown that *P*SERASE5 is proteolytically activated by the cysteine protease dipeptidyl peptidase *P*DPAP3 and the subtilisin-like serine protease *P*SUB1 (Yeoh et al., 2007; Arastu-Kapur et al., 2008). *P*SUB1-mediated cleavage of *P*SERASE5 releases two polypeptides, one of which binds to the emerging merozoites (Li et al., 2002; Okitsu et al., 2007).

Serine-rich antigen proteins are further involved in the egress of ex erythrocytic stages. In the rodent malaria model *Plasmodium berghei*, *PbSERASE3* was reported to be involved in merozoite egress from hepatocytes and proposed to play a role in host cell death by activating other parasite or host-derived proteases (Schmidt-Christensen et al., 2008). Further, loss-of-function mutants lacking the *P*SERASE8 orthologue *PbSERASE5* (termed *P*bECP1 in this study) are impaired in the egress of midgut sporozoites from the oocyst (Aly and Matuschewski, 2005).

A more recent study showed that besides the involvement of parasite-derived proteases, *Plasmodium* also hijacks a host protease, the erythrocyte-derived calpain-1, for egress (Chandramohanadas et al., 2009). The authors propose that calpain-1 is activated by intracellular calcium and is involved in the cleavage of cytoskeletal proteins. In conclusion, both parasite- as well as host-derived proteases appear to exhibit multiple functions during egress of the malaria parasite from its host cell, ranging from cytoskeleton destabilization to activation of proenzymes and mediation of signalling pathways.

Protease activity during membrane rupture was also investigated by treating blood stage parasites with specific inhibitors, particularly the cysteine protease inhibitor E-64, which resulted in contradictory data (reviewed in Blackman, 2008; Roiko and Carruthers, 2009). The inhibitor was either reported to block degradation of the PVM (Salmon et al., 2001; Wickham et al., 2003; Soni et al., 2005) or the EM (Glushakova et al., 2009). In *P. berghei*, treatment of liver stage parasites with E-64 blocked PVM rupture during ex erythrocytic schizogony and also inhibited translocation of *PbSERASE3* to the hepatocyte cytosol (Schmidt-Christensen et al., 2008).

Two previous studies investigated the influence of different types of protease inhibitors on microgametogenesis. The first study was performed on activated *P. berghei* gametocytes and reported that exflagellation can be blocked by the cysteine/serine protease inhibitors TLCK and TPCK (Torres et al., 2005). The results were subsequently confirmed in *P. falciparum* and showed that treatment of activated gametocytes with TPCK, TLCK or the serine protease inhibitor PMSF reduced the formation of microgametes (Rupp et al., 2008), pointing to the involvement of serine and cysteine proteases in microgametogenesis. Furthermore, the aspartic protease inhibitor EPNP interfered with rounding up of gametocytes (summarized in Table 1).

In this study we investigated on the ultrastructural level the distinct steps of gametocyte egress following activation, and determined which types of protease inhibitors interfere with each of the steps eventually leading to the formation of fertile gametes. Via electron microscopy, we showed that activated gametocytes emerge from the red blood cells by an inside-out mode of egress, in which rupture of the PVM precedes EM rupture. PVM rupture occurs at multiple perforation sites and is particularly sensitive to the cysteine protease inhibitor E-64d, while EM rupture is mediated by TLCK/TPCK-sensitive proteases. These findings form a solid basis for the development of novel antimalarial protease inhibitors with transmission blocking activity.

**Results**

*Gametocytes exit erythrocytes by an inside-out mode of egress*

In an initial set of experiments we studied the timeline of gametocyte egress from the host erythrocyte in untreated parasites. Mature gametocyte cultures were activated and fixed at 0, 2, 6, 12, 15 and 30 min post activation (p.a.). Specimens were embedded for transmission electron microscopy and the progress of gametocyte egress from its host erythrocyte was documented (Fig. 1) and quantified (Table 2).

In ultrathin sections, the typical crescent shape of non-activated mature gametocytes was reflected by a ‘shoeprint’ form (Fig. 1, 0 min, left panel). The gametocyte was lying in a PV within the erythrocyte host cell, which was reduced to a small electron-dense hem. Separating the erythrocyte from the parasite cytoplasm, three membranes were distinguishable, i.e. the PVM, the parasite plasma membrane (PPM) and an electron-dense layer of the so-called subpellicular membrane (SPM) complex (Fig. 1, 0 min, right panel). This complex is a typical feature of gametocytes, which consists of a SPM vacuole subtended by an array of longitudinally oriented microtubules (Sinden, 1982). Interestingly, the SPM was absent in the area of the cytostome, which is used by the parasite...
for the uptake of erythrocyte cytoplasm (Fig. 1, 0 min, right panel). Underneath the SPM, osmiophilic bodies were detected, electron-dense organelles of gametocytes, which were reported to be expressed in gametocytes stages IV and V and which are more abundant in female macrogametocytes than in male microgametocytes (Sinden, 1982; Ponnudurai et al., 1986).

Within 2 min p.a., the PVM had ruptured in 90% of the activated gametocytes (Table 2). PVM rupture occurred at multiple sites (Fig. 1, 2 min, left panel, asterisks) with an average of 5 ± 1.8 ruptures sites per gametocyte section (15 gametocyte sections investigated). In female gametocytes, 78% of visible osmiophilic bodies were associated with regions of PV rupture (15 macrogametocyte sections investigated; Fig. S1A), indicating that they might play a role in membrane lysis. Assemblies of up to five osmiophilic bodies were regularly observed (Fig. 1, 2 min, right panel, arrowheads; Fig. S1A right panel, arrowheads). At the same time, the erythrocyte cytoplasm became electron-light and started disintegrating (Fig. 1, 2 min, left panel).

At 6 min p.a., the PVM had disappeared in all of the activated gametocytes and the erythrocyte cytoplasm was dissolved in 80% of activated gametocytes (Table 2), which were in the process of rounding up (Fig. 1, 6 min, left panel). Furthermore, the SPM started disintegrating (Fig. 1, 6 min, right panel). At this stage, the parasite was solely surrounded by the host EM. In activated microgametocytes, axonemes were detectable in the cytoplasm (Fig. S1B, 6 min). In some occasions, fragments of the PVM were observed adhering to the PPM (Fig. 1, 6 min, left panel). Vesicular structures with an approximate diameter of 200–500 nm were regularly observed, which appeared to originate from parasite-derived membrane vesicles (PDMs) within the erythrocyte (Fig. S1C). At 12 min p.a., the PVM and the erythrocyte cytoplasm had disappeared in all of the activated gametocytes (Table 2). At 15 min p.a., the parasite had fully rounded up and 80% of gametocytes had stripped off the EM (Table 2; Fig. 1, 15 min, left panel). In contrast to the PVM, the EM ruptured at a single site only (50 gametocytes

---

### Table 1. Effect of protease inhibitors on gametogenesis.

| Inhibitor Type of inhibition | IC_{50} (µM) | Mode of action |
|-----------------------------|-------------|---------------|
| Cysteine/serine protease inhibitors | Initially reversible, then irreversible | 12.6 ± 5.40* | no MG formation, EM rupture inhibited, SPM disintegration impaired |
| TLCK | 16.1 ± 2.57* | no MG formation, EM rupture inhibited |
| TPCK | 211.2 ± 68.23* | no MG formation, EM rupture inhibited |
| PMSF | Irreversible | 101.4 ± 8.97 | no MG formation, EM rupture inhibited, PVM rupture impaired |
| Cysteine protease inhibitors E-64d | Irreversible | 101.4 ± 8.97 | no MG formation, EM rupture inhibited, PVM rupture impaired |
| Aspartic protease inhibitors EPNP | Irreversible | 225.8 ± 20.50* | MG formation reduced, rounding up reduced, SPM disintegration impaired |
| Metalloprotease inhibitors 1,10-phenanthroline | Reversible; targeting zinc metalloproteases | 33.2 ± 6.31* | Clustering of MGs, MG motility impaired, EM rupture impaired |

* Previously shown (Rupp et al., 2008).

MG, microgamete.

---

### Table 2. Rupture of membranes during gametocyte egress at different time points p.a.

| Time p.a. | EC Present | EM Absent | PVM Intact | PVM Ruptured | SPM Intact | SPM Disint. |
|-----------|------------|-----------|------------|--------------|------------|-------------|
| 0 min     | 100%       | 0         | 100%       | 0            | 100%       | 0           |
| 2 min     | 100%       | 0         | 100%       | 0            | 10%        | 90%         |
| 6 min     | 20%        | 80%       | 75%        | 25%          | 0          | 100%        |
| 12 min    | 0          | 100%      | 40%        | 60%          | 0          | 100%        |
| 15 min    | 0          | 100%      | 20%        | 80%          | 0          | 100%        |
| 30 min    | 0          | 100%      | 0          | 100%         | 0          | 100%        |

20 sections investigated for each time point.
EC, erythrocyte cytoplasm.
investigated) (Fig. 1, 15 min, left panel; Fig. S1C). Furthermore, microgametes had formed, which originated from the residual body of the activated microgametocyte and which were partly covered by fragments of the EM (Fig. S1B, 15 min). In 75% of activated gametocytes, the SPM had disintegrated at 15 min p.a. (Table 2), thus giving the previously smooth surface of the gametocyte an uneven pattern (Fig. 1, 15 min, right panel). At 30 min p.a., the parasite had fully emerged from the host erythrocyte and was devoid of PVM and EM. The SPM was absent (Table 2).

The PVM ruptures immediately after receiving external stimuli

To investigate the time point of PVM rupture in more detail, we fixed gametocytes at 30, 60, 90 and 120 s following activation. As described above, non-activated gametocytes exhibited a complex of three membranes, i.e. the PVM, the PPM and the SPM, which separates erythrocyte and parasite cytoplasm (Fig. 2A, 0 s). Microtubules were regularly observed underneath the SPM of non-activated gametocytes. At 30 s p.a. the PVM was still
intact in 75% of activated gametocytes (Table 3), but exhibited an undulated shape in all of the activated gametocytes (50 gametocytes investigated; Fig. 2A, 30 s). Subpellicular microtubules were still detectable. From 60 s p.a. on, the microtubules had disappeared, and in 70–90% of activated gametocytes the PVM had ruptured at multiple sites (Table 3; Fig. 2A, 60 s, 90 s, asterisks). Interestingly, the PVM often increased in thickness and then consisted of multiple layers (Fig. 2B, left panel). These membrane layers appeared to originate from PDMs, which attached to the ruptured PVM upon activation (Fig. 2B, right panel; Fig. S2A).

It was previously described that initiation of gametogenesis requires a drop in temperature by approximately 5°C (Sinden, 1983; Sinden et al., 1996), and the presence of XA (Billker et al., 1998; Garcia et al., 1998). An additional signal reported to induce gametocyte activation is an increase in pH from 7.4 to about 8 (Nijhout and Carter, 1978; Sinden, 1983), but such a pH shift was later discussed to be an artificial inductor of exflagellation (Alano and Billker, 2005). We therefore wanted to know, if a combination of all signals is required to initiate PVM rupture, or if the signals are perceived by the parasite sequentially and if a single stimulus is sufficient for PVM lysis.

Gametocytes were incubated for 30 min in RPMI medium under different conditions and PVM rupture was subsequently quantified by electron microscopy in a total number of 20 gametocytes respectively. Non-activated

Table 3. PVM rupture during gametocyte egress at different time points p.a.

| Time p.a. | 0 s | 30 s | 60 s | 90 s | 120 s |
|-----------|-----|------|------|------|-------|
| PVM intact | 100% | 75%  | 30%  | 30%  | 10%   |
| PVM ruptured | 0%  | 25%  | 70%  | 70%  | 90%   |

20 sections investigated for each time point.
gametocytes were often deformed (Fig. S2C). Further investigations revealed that BAPTA-AM-treated activated gametocytes fully egressed (Fig. 2C). Ultrastructural while in the DMSO control lacking BAPTA-AM, the pre-tests was shown to fully inhibit exflagellation resulted BAPTA-AM before activation in a concentration that in wanted to know, if the increase in intracellular calcium is erythrocyte (McRobert 2004). In this context, a recent study reported that treat-while in the presence of XA (Fig. 2C). On the other hand, the PVM was ruptured and the majority of parasites had egressed, when receiving temperature drop and XA at a pH of 7.4. Interestingly, the two signals together were not able to induce PVM rupture in gametocytes at a pH of 6.6 (Fig. 2C). The highest rate of gametocyte egress initiated by temperature drop and XA was observed, when the RPMI medium was supplemented with human serum at a pH of 7.4 (Fig. 2C).

We aimed at investigating the influence of external pH and potassium on gametocyte egress in more detail and treated the parasites at RT with the K+/H+ ionophore nigericin, which was used as a substitute for XA. Nigericin was previously shown to trigger host cell egress of Toxo-plasma gondii as a result of a decrease in host cell potassium concentration, which then resulted in an induction of intraparasitic calcium fluxes (Fruth and Arrizabalaga, 2007). Nigericin-treated gametocyte cultures underwent gametogenesis and exflagellation was observed (data not shown). Electron microscopy revealed PMV rupture in 85% of nigericin-activated parasites (Figs 2C and S2B), indicating that changes in pH and/or potassium in the host cell have an influence on gametocyte egress.

Activation of gametocytes by XA within seconds induces an increase in intracellular calcium (Billker et al., 2004). In this context, a recent study reported that treatment of P. falciparum gametocytes with the calcium chela-tor BAPTA-AM interfered with their egress from the erythrocyte (McRobert et al., 2008). Consequently, we wanted to know, if the increase in intracellular calcium is required for PVM rupture. Treatment of gametocytes with BAPTA-AM before activation in a concentration that in pre-tests was shown to fully inhibit exflagellation resulted in no apparent blockage of PVM rupture at 30 min p.a., while in the DMSO control lacking BAPTA-AM, the gametocytes fully egressed (Fig. 2C). Ultrastructural investigations revealed that BAPTA-AM-treated activated gametocytes were often deformed (Fig. S2C). Furthermore, at 30 min p.a., the SPM and the subpellicular microtubules were sometimes observed, indicating a dys-function in morphogenesis. 65% of the activated gameto-cytes were still covered by the EM (20 gametocyte sections investigated; Fig. S2C).

In conclusion, we show that malaria gametocytes require a combination of temperature drop and XA, but not an increase in intraparasitic calcium levels to induce PVM rupture. Furthermore, PVM rupture can artificially be triggered by changes in the erythrocyte pH and/or potassium levels.

Protease inhibitors interfere with membrane rupture following gametocyte activation

In a final set of experiments we wanted to evaluate the role of parasite proteases in the egress of gametocytes from the host erythrocyte. Gametocytes were treated with protease inhibitors in concentrations that were previously shown to completely block exflagellation in P. falciparum (Rupp et al., 2008), i.e. with the cysteine/serine protease inhibitors TLCK and TPCK (100 μM), the serine protease inhibitor PMSF (1 mM), the aspartic protease inhibitor EPNP (1 mM) and the zinc metalloprotease inhibitor 1,10-phenanthroline (1,10-PH; 1 mM) before activation. We furthermore included the cysteine protease inhibitor E-64d into our investigations, which here exhibited weak inhibitory activity on exflagellation with an IC50 value of 101.4 μM (Table 1). Inhibitor treatment did not significan-tly increase the numbers of propidium iodide-positive activated gametocytes under the conditions used for the experiments, thus the protease inhibitors had no cytotoxic effect on sexual stage parasites (Fig. S3A and B). Activated gametocytes were fixed at 20 min p.a. in the presence of inhibitors and either processed for immuno-fluorescence assay or for electron microscopy.

Initially, we investigated the effect of the protease inhibi-tors on gametocyte egress via immunofluorescence assay, labelling on the one hand the activated macro- and microgametocytes, on the other hand either the PVM or the EM (Figs 3A and S4). When the EM was marked with antibodies against the transmembrane protein band 3, immunofluorescence assays revealed a significantly higher number of band 3-positive macrogametes (labelled with antibodies against the macrogamete-specific

---

**Fig. 3.** Effect of protease inhibitors on gametocyte egress. Gametocytes were pre-incubated with protease inhibitors and activated in the presence of inhibitors. Specimens were fixed 20 min p.a. and processed for immunofluorescence assay (A) or electron microscopy (B). The presence of the EM (labelled with anti-band 3 antibody) or of the PVM (labelled with anti-Pf516 antibody) was quantified in activated macrogametocytes (labelled with anti-Pf525 antibody) and activated microgametocytes (spherical anti-alpha-tubulin f-positive cells) (A). Experiments were done in triplicate, and 25, 30 or 50 sections (set to 100%) were investigated for each setting. Significances in the differences of EM- or PVM-positive cells in inhibitor-treated cells compared with untreated controls were calculated (indicated by asterisks, *P < 0.1, Student’s t-test*). The ultrastructural changes following protease inhibitor treatment were investigated in activated gametocytes (B). A, axoneme; MG, microgamete; N, nucleus. Bar, 1 μm.

© 2011 Blackwell Publishing Ltd, *Cellular Microbiology,* 13, 897–912
A Presence of EM

![Bar graph showing the presence of EM with different treatments.](image)

B Presence of PVM

![Bar graph showing the presence of PVM with different treatments.](image)

**B**

- Control
- TLCK
- TPCK
- PMSF
- E-64d
- EPNP
- 1,10-PH

### Macrogametocytes

- % band3+ cells/Pfs25+ cells total
- % band3+ cells/atubII+ cells total

### Microgametocytes

- % Pfs16+ cells/Pfs25+ cells total
- % Pfs16+ cells/atubII+ cells total
adhesion protein Pf-s25), when these were treated with TLCK, TPCK, E-64d or 1,10-PH, and significantly higher numbers of band 3-positive activated microgametocytes (labelled with antibodies against the male-specific alpha-tubulin II) after TLCK and TPCK treatment (Fig. 3A, left panel). Treatment with TLCK, TPCK or E-64d also resulted in increased numbers of activated microgametocytes positive for the gametocyte PVM-specific protein Pf-s16, with significant differences for TPCK- and E64d-treated cultures, when compared with untreated control (Fig. 3A, right panel). Surprisingly, no differences in the numbers of Pf-s16-positive cells were observed between untreated and inhibitor-treated Pf-s25-positive macrogametocytes. For all conditions tested Pf-s16-positive cells accounted for not more than 20% (Fig. 3A, right panel).

We aimed at investigating the coexpression of Pf-s16 and Pf-s25 in gametocytes in more detail using confocal laser scanning microscopy. Pf-s25 is a GPI-anchored adhesion protein with four EGF domains, which is present on the surfaces of macrogametes, zygotes and ookinetes. It belongs to class II of sexual stage proteins, hence is expressed only after the parasites have entered the mosquito midgut (reviewed in Kuehn and Pradel, 2010; Kuehn et al., 2010). The orthologous protein Pbs25 of P. berghei was previously reported to be translationally repressed in non-activated gametocytes (Paton et al., 1993; Mair et al., 2006), and repression is released upon gametocyte activation.

In accordance with these findings, no surface-associated expression of Pf-s25 was found in non-activated gametocytes (Fig. 4A, left panel), while these cells abundantly labelled for Pf-s16, indicating that the PVM is intact at this stage. Following activation, Pf-s25 was located to the surface of the activated macrogametocyte and was found mainly in areas that were negative for Pf-s16 (Fig. 4B–D, left panel). Only in macrogametes that were devoid of the Pf-s16-positive PVM, Pf-s25 was evenly distributed on the surface (Fig. 4E, left panel). Similar results were obtained, when the gametocyte PVM was labelled with antibodies against EXP-1, a PVM transmembrane protein (Günther et al., 1991). Again, Pf-s25 was detected predominantly in areas of the activated macrogametocyte surface that were negative for EXP-1 (Fig. 4, right panel). The combined data indicate that Pf-s25 was only located to the macrogametocyte surface in areas devoid of the PVM and further explain, why protease inhibitor treatment does not alter the ratio of Pf-s16-positive/Pf-s25-positive cells. While treatment with the inhibitors might result in impaired PVM rupture, these gametocytes would not label for Pf-s25.

© 2011 Blackwell Publishing Ltd, Cellular Microbiology, 13, 897–912
Because of the challenge to distinguish between intact and ruptured membranes by immunofluorescence assay, the effect of protease inhibitor treatment on gametocyte egress was investigated in more detail by electron microscopy. Ultrathin sections of protease inhibitor-treated activated gametocytes were investigated and the percentages of membranes that were either intact, ruptured or absent were quantified (Fig. 3B, Table 4). At 20 min p.a., 92% of untreated control gametocytes were devoid of the PVM and 67% of activated gametocytes had fully egressed from the EM. At this time point, the SPM had partially or fully disintegrated in 92% of parasites. The majority of activated gametocytes, however, when incubated with TLCK, TPCK or PMSF, was not able to provoke EM rupture and in consequence was not able to release the PVM fragments, which kept adhering to the parasite surface (Fig. 3B, Table 4). Treatment with E-64d, on the other hand, had a modest effect on the rupture of the PVM, and in 37% of parasites the PVM was still intact. Gametogenesis was impaired in these parasites, and 90% of E-64d-treated gametocytes also exhibited an intact EM (Table 4). Furthermore, disintegration of the SPM was impeded in 78% of parasites by treatment with EPNP or TLCK. None of the activated gametocytes formed microgametes, when treated with the above discussed protease inhibitors, and no axonemes were observed in microgametocytes, when these were treated with TLCK, TPCK and E-64d (Table 4).

In accordance with our previous findings (Rupp et al., 2008), treatment of activated gametocyte cultures with 1,10-PH did not interfere with microgamete formation. Electron microscopic studies on 1,10-PH-treated activated microgametocytes showed PVM rupture and formation of microgametes (Fig. 3B, Table 4). However, while untreated activated microgametocytes usually form microgametes radially protruding from the microgamocyte surface (e.g. see Fig. 1B of Pradel, 2007), in 73% of parasites treated with 1,10-PH, the microgametes pointed to the same direction, as evaluated by immunofluorescence assay using antibodies against alpha-tubulin II (30 microgametocytes investigated; see Fig. S4 for an example). In electron micrographs, these microgametes appeared to adhere to each other and were often covered by fragments of the EM (Fig. 3B).

In conclusion, we showed that the here tested types of protease inhibitors interfered with distinct steps of gametocyte egress from the enveloping host cell, and revealed that different types of proteases are important for the lysis and disintegration of PVM, SPM and EM. In all cases, gametogenesis was blocked because of the failure of the parasite to successfully exit the host cell.

**Discussion**

Malaria parasites hide within host cells as a strategy for immune evasion, while prolonged extracellular exposure times are avoided by the pathogen. In the human, the infective sporozoites enter liver hepatocytes within minutes after being inoculated into the dermis (Vanderberg, 1977; Vanderberg and Frevert, 2004; Medica and Sinnis, 2005), and blood stage merozoites invade new red blood cells within seconds after release from the schizont (Dvorak et al., 1975; Cowman and Crabb, 2006). Hepatocytic merozoites, on the other hand, are being released from the liver into the blood stream sheltered by membrane-enclosed vesicles, the merosomes (Sturm et al., 2006; Baer et al., 2007). Similarly, malaria parasites are encapsulated within thick-walled oocysts during most of their development in the mosquito vector. During sexual reproduction in the mosquito midgut, however, the parasites are required to emerge from the protective erythrocyte in order to prepare for fertilization and are consequently extracellularly exposed to a hostile environment for approximately 1 day.

We here describe the distinct steps of gametocyte egress following activation in the mosquito midgut and evaluate the types of protease inhibitors able to interfere with each of the steps eventually leading to the formation of fertile gametes (results summarized in Fig. S5). After entering the mosquito midgut, gametocyte emergence is initiated by external stimuli, which include a drop in temperature by approximately 5°C (Sinden, 1983; Sinden et al., 1989, 1990, 1997, 1999, 2003).

| Inhibitor | No. sections | EM | PVM | SPM |
|-----------|-------------|----|-----|-----|
|           | Intact      | Partly egressed | Fully egressed | Intact | Ruptured | Absent | Intact | Partly disint. | Fully disint. | Axonemes detectable? |
| control   | 24          | 8% | 25% | 67% | 0  | 8% | 92% | 8% | 88% | 4% | yes |
| TLCK      | 27          | 78% | 18% | 4% | 0  | 52% | 48% | 78% | 22% | 0   | no  |
| TPCK      | 27          | 85% | 0   | 15% | 4% | 18% | 78% | 30% | 66% | 4% | no  |
| PMSF      | 27          | 70% | 3%  | 22% | 11% | 33% | 56% | 37% | 63% | 0   | yes |
| E-64d     | 30          | 90% | 3%  | 7%  | 37% | 40% | 23% | 43% | 57% | 0   | no  |
| EPNP      | 32          | 41% | 28% | 31% | 0  | 22% | 78% | 78% | 22% | 0   | yes |
| 1,10-PH   | 39          | 54% | 8%  | 38% | 0  | 23% | 77% | 51% | 49% | 0   | yes |

© 2011 Blackwell Publishing Ltd, *Cellular Microbiology*, 13, 897–912
et al., 1996), and the presence of the mosquito-derived XA (Billker et al., 1998; Garcia et al., 1998). We show that the gametocytes require both signals for PVM rupture in order to initiate egress. The increase in pH from 7.4 to about 8.0 as an additional signal to induce gametocyte activation was previously reported (Nijhout and Carter, 1978; Sinden, 1983), and later discussed to be an artificial inductor of exflagellation (Alano and Billker, 2005). Our observations on XA-induced PVM rupture being dependent on an external pH above 6.6, however, shed new light on the role of the pH in gametocyte activation. In this context, Kawamoto et al. previously discussed that extracellular signals in mammals frequently result in an increase of intracellular pH as a secondary cellular response, which can be mimicked in vitro by alkaline media (Kawamoto et al., 1991). A recent study described a rise of cytosolic pH as a secondary signal in the in budding yeast Saccharomyces cerevisiae, where it activates the cAMP-dependent protein kinase A (Dechant et al., 2010). The here reported inhibitory effect of a low pH environment on XA-induced gametocyte activation strongly suggests an involvement of the pH as a secondary messenger during the pathway leading to gametogenesis. Under the conditions of low pH environment the threshold of intracellular pH might not be reached despite a potentially increased permeability for H+ In accordance with these findings, the K+/H+ ionophore nigericin was able to induce gametogenesis in P. falciparum. Nigericin was previously shown to trigger egress of T. gondii from its host cell by leading to a decrease in its potassium level, which then results in an increase of intraparasitic calcium (Fruth and Arrizabalaga, 2007), indicating that a similar mechanism might be the cause of egress in malaria gametocytes. However, a new study by Mauritiz et al. (2009) argued that infection with a malaria parasite would result in a potassium loss in the infected erythrocyte, indicating that the erythrocytic level of potassium might not play a major role in gametocyte egress. Nigericin, which is also able to transport Na+ and H+ across membranes (Henderson et al., 1969), might thus exhibit its effect by increasing the pH of the erythrocyte as a result of H+ efflux, and such H+ efflux might be impaired under low external pH.

Rupture of the PVM occurs less than a minute following perception of the external stimuli at multiple sites, while the EM is still intact. Disintegration of the gametocyte PVM before the EM was previously also reported by Quakyi et al. (1989). Such inside-out model, in which the PVM ruptures before the EM, has further been proposed for the egress of blood stage merozoites, supported by electron microscopic studies on blood stage schizonts (Aikawa, 1971) as well as by studies, in which the PV lumen was fluorescently labelled (Wickham et al., 2003). Here, the fluorescence distributed over the entire erythrocyte before schizogony, indicating that the PVM had ruptured. In analogy, in liver stage schizonts the PVM ruptures and the merozoites are released into the blood stream covered by hepatocyte-derived membranous vesicles (Sturm et al., 2006; Sturm and Heussler, 2007).

Rupture of the gametocyte PVM can in parts be inhibited by E-64d; however, high inhibitor concentrations are required. The effect of a related cysteine protease inhibitor, E-64, on membrane rupture has previously been investigated in blood stage schizonts, resulting in contradictory data. The inhibitor was either reported to block degradation of the PVM (Salmon et al., 2001; Wickham et al., 2003; Soni et al., 2005) or the EM (Glushakova et al., 2009). Worth mentioning in this context, the here reported impaired rupture of the PVM resulted in an arrest of gametogenesis, and thus the EM was also not lysed. Therefore, both membranes were present in activated gametocytes after E-64d treatment.

The gametocyte PVM ruptures at multiple sites, indicating that the PVM is actively perforated by the parasite. Five perforin-like proteins, termed PPLP1-5, as well as a newly identified perforin-like protein (PF08_0058) are encoded in the genome of P. falciparum. The proteins exhibit highly conserved MACPF (membrane attack complex/perforin) domains that are important for pore formation in target membranes (reviewed in Kafsack and Carruthers, 2010). Perforins are widespread among bacterial pathogens and mammalian immune complement components and oligomerize to form pore complexes, which insert into target membranes. The functions of some of the perforins were previously studied in P. berghei (reviewed in Roiko and Carruthers, 2009; Kafsack and Carruthers, 2010), e.g. the perforins PPLP3/MAOP and PPLP5 were postulated to be involved in the traversal of the ookinete through the midgut epithelium (Kadota et al., 2004; Ecker et al., 2007), while PPLP1/SPECT2 appears to play a role in sporozoite breaching of the liver sinusoidal cell layer before hepatocyte invasion (Ishino et al., 2005). A possible role of the perforins in host cell egress, however, is not yet known.

Parasitophorous vacuole membrane rupture in activated macrogametocytes correlates with the accumulation of osmiophilic bodies underneath the rupture sites. Osmiophilic bodies are gametocyte-specific secretory organelles first identified by electron microscopy as a result of their electron-dense features (Sinden, 1982; Aikawa et al., 1984). They appear in stage IV gametocytes, are particularly present in the macrogametocytes, and discharge their content into the PV upon activation (Sinden, 1982). Osmiophilic bodies contain the protein Pf377 (Alano et al., 1995; Severini et al., 1999). P. falciparum gametocytes lacking this protein reveal a reduced number of osmiophilic bodies and fail to egress from the host erythrocyte, pointing to a pivotal role of Pf377 in
gametocyte emergence (de Koning-Ward et al., 2008). Another protein associated with the gametocyte osmiophilic bodies, as well as with PVM-derived membranes, is MDV-1/Peg3 (Furuya et al., 2005; Silvestrini et al., 2005; Lanfranconi et al., 2007; Ponzi et al., 2009). Egress of gametocytes of both genders was impaired when lacking MDV-1/Peg3, which was suggested to play a major role in destabilizing the PVM before EM rupture (Ponzi et al., 2009). Furthermore, MDV-1/Peg3-deficient parasites show decreased ability to adhere to red blood cells (Ponzi et al., 2009), pointing at the involvement of MDV-1/Peg3 and possibly osmiophilic bodies in altering the gamete surface in preparation for fertilization.

During rupture, the PVM appears to form multiple layers by attaching to other PDMs, like the membranes of the tubular networks. Noteworthy, a new study reported a swelling of the PV and thus an extension of the PVM during egress of blood stage schizonts (Glushakova et al., 2010), and a significant swelling and enlargement of the female gamete at the onset of gametogenesis was previously shown (Sinden et al., 1978; Aikawa et al., 1984). PV swelling might also occur in emerging gametocytes, which would be in accord with the here reported undulated shape of the PVM immediately following activation. Parasitophorous vacuole membrane rupture led to the release of membrane fragments. Our immunofluorescence data indicate that rupture and release of the PVM are important for the localization of the GPI-anchored adhesion protein PfS25 to the parasite surface. Interestingly in this context, the presence of lipid rafts in the malaria parasite sexual stages has recently been reported (M. Ponzi, pers. comm.). These cholesterol-rich areas of surface membranes are supposed to be involved in protein trafficking and assembly and often contain GPI-anchored proteins (reviewed in Simons and Gerl, 2010). Noteworthy, small amounts of PfS25 are often found in vesicular structures in the maturing gametocytes, and the protein is relocated to the surface of macrogametocytes within minutes after activation (Scholz et al., 2008). It is therefore tempting to speculate that during PVM rupture and discharge of osmiophilic body and vesicle contents, temporary membrane heterogeneity of the macrogametocyte plasmalemma occurs, resulting in PfS25-positive lipid rafts.

Activation of gametocytes with XA furthermore induces within seconds an increase in intracellular calcium, which then leads to a signalling cascade resulting in DNA replication and cytokinesis of microgametes (Bilker et al., 2004; Tewari et al., 2005; reviewed in Kuehn and Pradel, 2010). The increase of intracellular calcium, however, is not required for PVM rupture, but rather appears to be involved in EM rupture and morphogenesis of the forming gametes.

Approximately 10 min following PVM rupture, the EM ruptures at one breaking point, a process sensitive to the cysteine/serine protease inhibitors TLCK and TPCK. The fact that the EM lyses at one site only, contrary to the multiple perforation sites of the PVM, allows us to hypothesize that EM rupture occurs either because of mechanical strain of the destabilized membrane during rounding up of the activated gametocyte or is caused by localized proteolysis. Proteases might thus function in destabilization of erythrocyte cytoskeletal components during EM rupture. The fact that the PVM is perforated minutes before rupture of the EM lets us further postulate that proteases are released from the PV into the erythrocyte cytosol after PVM lysis.

Another membrane, which has to be degraded during formation of gametes, is the SPM. This double membrane is part of the pellicular complex, which, besides the SPM, consists of an array of longitudinally oriented microtubules (Sinden, 1982). The complex probably gives the crescent-shaped gametocyte stability, and while the microtubules disappear within a few minutes following activation (Sinden, 1982), the SPM is present for several minutes longer and only fully disintegrates after the gametocytes have emerged. The aspartic protease inhibitor EPNP as well as the cysteine/serine protease inhibitor TLCK considerably reduce SPM disintegration, which would explain the previously described inhibitory effect of EPNP on rounding up of gametes (Table 1) (Rupp et al., 2008).

The protease inhibitors that block membrane rupture during gametocyte egress also inhibit the formation of microgametes, hence explaining why they suppress exflagellation (Rupp et al., 2008). By contrast, treatment of activated gametocytes with 1,10-PH does not interfere with microgamete formation. We previously reported that 1,10-PH renders the microgametes amotile and here provide an explanation for this phenomenon by describing a clustering of the newly formed 1,10-PH-treated microgametes. These microgametes are furthermore covered by fragments of the EM, which would prevent a contact of the microgametes with erythrocytes of the blood meal. Binding of microgametes to erythrocytes during exflagellation is mediated by the abundantly expressed surface protein PfS230, a member of the cysteine-rich motif superfamily (Ekşi et al., 2006; reviewed in Pradel, 2007). Full-length PfS230 has a molecular weight of approximately 360 kDa, but is processed during emergence, thereby releasing two peptides (Williamson et al., 1996). Noteworthy, processing of PfS230 is sensitive to 1,10-PH (Brooks and Williamson, 2000), which might be necessary for the regulation of cell–cell contacts during gametogenesis or gamete fusion (reviewed in Kuehn and Pradel, 2010).

In conclusion we report that different types of proteases are involved in the distinct steps of membrane rupture during egress of the activated gametocyte in the mosquito midgut. These proteases must be derived from the parasite and/or the red blood cell, as gametogenesis can be...
triggered in vitro without any factors of the mosquito midgut. Parasite proteases crucial for gametocyte egress play an essential role for reproduction and thus represent promising targets for transmission blocking drugs. It is our future aim to identify the parasite proteases that are involved in the distinct steps of gametocyte egress during parasite transmission from the human to the mosquito and to investigate the possible role of perforins in PVM lysis.

Experimental procedures

Compounds

The following protease inhibitors were used in this study: E-64d [(2S,3S)-trans-Epoxy succinyl-L-leucylamido-3-methylbutane ethyl ester; Sigma-Aldrich]; EPNP (1,2-epoxy-3-(p-nitrophenoxo)-propane; Acros Organics); 1,10-PH (o-phenanthroline monohydrate; Sigma-Aldrich); PMSF (Phenylmethanesulfonyl fluoride; Sigma-Aldrich); TLCK (N-o-Tosyl-L-lysine chloromethyl ketone-hydrochloride; Sigma-Aldrich); TPCK (N-o-Tosyl-L-phenylalanine chloromethyl ketone; Sigma-Aldrich).

With the exception of E-64d, which was dissolved in DMSO, all protease inhibitors were dissolved in methanol for stock solutions of 100 mM or 10 mM (TLCK, TPCK). Furthermore, the membrane-permeable calcium chelator BAPTA-AM (1,2-bis(o-Aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid, tetraacetoxymethyl ester; Sigma-Aldrich) was used and a stock solution of 10 mM in DMSO was prepared. The K+/H+ ionophore nigericin (Sigma-Aldrich) was used in a stock solution of 10 mM dissolved in methanol.

Exflagellation inhibition assay

Mature gametocyte cultures of the P. falciparum NF54 isolate were cultivated in vitro in RPMI 1640 medium complemented with 10% inactivated human serum as described (Ifediba and Vanderberg, 1981). A volume of 100 μl of mature NF54 gametocyte cultures was pre-incubated with E-64d or BAPTA-AM in concentrations ranging between 25 μM and 1 mM for 15 min at 37°C. The sample was then transferred to RT and XA was added at a concentration of 100 μM for activation. After another 15 min, the numbers of exflagellation centres were counted in 30 optical fields using a Leica DMLS microscope by 400-fold magnification. Two independent experiments were performed in duplicates and the inhibition of exflagellation was calculated as a percentage of the number of exflagellation centres in compound-treated cultures in relation to the number of exflagellation centres in untreated controls. The IC50 values were calculated from variable-slope sigmoidal dose–response curves using the GraphPad Prism program version 4.

Electron microscopy

Mature NF54 gametocyte cultures were enriched by Percoll gradient purification (Kariuki et al., 1998). A volume of 100 μl of enriched gametocytes was pre-incubated with the inhibitors or BAPTA-AM for 15 min at 37°C. Samples were then transferred to RT, activated by addition of XA at a final concentration of 100 μM, and incubated for another 2 to 30 min. In the case of nigericin, 5 μM of the ionophore in incomplete RPMI 1640 medium, pH 7.4 (without human serum), were added to the pelleted cultures for activation in the absence of XA and incubated for 30 min at RT. In experiments on the induction of PVM rupture, the cultures were kept in incomplete RPMI 1640 medium at the indicated pH and activated as described (see Fig. 2C). Exflagellating gametocyte cultures were fixed in 1% glutaraldehyde and 4% paraformaldehyde in PBS over night at 4°C. Specimens were post-fixed in 1% osmium tetroxide and 1.5% K2Fe(CN)6 in PBS for 2 h at RT, followed by incubation in 0.5% uranyl acetate for 1 h. The fixed cultures were dehydrated in increasing concentrations of ethanol and then incubated for 1 h in propylene oxide, followed by another incubation step for 1 h in a 1:1 mixture of propylene oxide and Epon (Electron Microscopy Sciences). Specimens were subsequently embedded in Epon at 60°C for 2 days. Photographs were taken with a Zeiss EM10 transmission electron microscope and scanned images were processed using Adobe Photoshop CS software.

Indirect immunofluorescence assay

Mature gametocyte cultures were pre-incubated with protease inhibitors and activated as described above. 20 min p.a., the 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 one of the following antibodies diluted in 0.01% saponin/0.5% BSA/PBS: rabbit anti-Pf s25 or anti-alpha-tubulin II antibodies (ATCC); mouse anti-Pf s16 or band 3-positive (kindly provided by Kim Williamson, Loyola University Chicago); rabbit anti-EXP-1 antibodies (Günther et al., 1991). Binding of primary antibody was visualized using fluorophore-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Alexa Fluor 488 or Alexa Fluor 596; Molecular Probes) diluted in 0.01% saponin/0.5% BSA/PBS. For labelling of erythrocyte transmembrane protein band 3, the protocol was altered as follows: The cultures were fixed in 4% paraformaldehyde and 0.0075% glutaraldehyde in PBS for 1 h at RT. After washing with PBS, specimens were blocked with 100 mM glycine/PBS and membranes were permeabilized with 0.1% Triton-X100/PBS for 15 min each, before labelling with mouse monoclonal anti-band 3 antibodies (Sigma-Aldrich) in 3% BSA/PBS and the respective Alexa Fluor 488-coupled secondary antibody. Specimens were then washed, coated on Teflon slides and processed for labelling with anti-Pf s25 or anti-alpha tubulin II antibody as described above. Specimens were examined by confocal laser scanning microscopy using a Zeiss LSM 510 or a Zeiss Axiolab microscope. Digital images were processed using Adobe Photoshop CS software. For quantitative evaluation of Pf s16 or band 3-positive cells, a total number of 25, 30 or 50 activated gametocytes (visualized by Pf s25 or alpha-tubulin II labelling) were investigated and counted in triplicate. Mean values and standard deviation were calculated. Significances in the numbers of egressed activated gametocytes were calculated using the Student’s t-test (P < 0.1).
**Cytotoxicity test**

Mature gametocyte cultures were pre-incubated with protease inhibitors and activated as described above. At 10 min p.a., anti-Pfs230 antibody was added to the cultures and incubated for 1.5 h, followed by an incubation with Alexa Fluor 488-conjugated secondary antibody for another hour. The cultures were subsequently washed with PBS. Propidium iodide (dissolved in PBS; Sigma-Aldrich) was added to the cultures at a final concentration of 5 µg ml⁻¹ and these were incubated for another 15 min at RT in the dark. Specimens were examined by confocal laser scanning microscopy using a Zeiss LSM 510. Cultures treated with 1% DMSO or 1% methanol were used for negative control and cultures treated with 50% DMSO were used for positive (death) control. For quantitative evaluation of propidium iodide-positive cells, a total number of 50 activated Pfs230-positive gametocytes were investigated for each setting and counted in triplicate. Mean values and standard deviation were calculated. Significances in the numbers of egressed activated gametocytes were calculated using the Student’s t-test (*P < 0.05*).

**Acknowledgements**

We thank Marc Kirschner (University of Würzburg) for reviewing the manuscript and Kim Williamson (Loyola University Chicago) for kindly providing anti-Pfs16 antibody. We further thank the electron microscopy team of Georg Krohne (University of Würzburg) for support. Antibodies against Pfs25 and alphatubulin II were kindly provided by MR4/ATCC. This work was supported by the MALSIG consortium of the EU 7th framework programme and the SFB479 of the Deutsche Forschungsgemeinschaft (GP). AK is associated member of the BioMedTec International Graduate School of Science ‘Lead structures of cell function’ of the Elite Network Bavaria.

**References**

Aikawa, M. (1971) Parasitological review. *Plasmodium: the fine structure of malarial parasites*. **Exp Parasitol** 30: 284–320.

Aikawa, M., Carter, R., Ito, Y., and Nijhout, M.M. (1984) New observations on gametogenesis, fertilization, and zygote transformation in *Plasmodium gallinaceum*. **J Protozool** 31: 403–413.

Alano, P., and Billker, O. (2005) Gametocytes and gametes. In *Molecular Approaches to Malaria*. Sherman, I.W. (ed.). Washington, DC: ASM Press, pp. 191–219.

Alano, P., Read, D., Bruce, M., Aikawa, M., Kaido, T., Tegoshi, T., et al. (1995) COS cell expression cloning of Plg377, a *Plasmodium falciparum* gametocyte antigen associated with osmiophilic bodies. **Mol Biochem Parasitol** 74: 143–156.

Aly, A.S., and Matuschewski, K. (2005) A malarial cysteine protease is necessary for *Plasmodium* sporozoite egress from oocysts. **J Exp Med** 202: 225–230.

Aoki, S., Li, J., Itagaki, S., Okech, B.A., Egwang, T.G., Matsuoka, H., et al. (2002) Serine repeat antigen (SERAS) is predominantly expressed among the SERA multigene family of *Plasmodium falciparum*, and the acquired antibody titers correlate with serum inhibition of the parasite growth. **J Biol Chem** 277: 47533–47540.

Arastu-Kapur, S., Ponder, E.L., Fonovic, U.P., Yeoh, S., Yuan, F., Fonovic, M., et al. (2008) Identification of proteases that regulate erythrocyte rupture by the malaria parasite *Plasmodium falciparum*. **Nat Chem Biol** 4: 203–213.

Baer, K., Klotz, C., Kappe, S.H., Schnieder, T., and Frevert, U. (2007) Release of hepatic *Plasmodium yoelii* merozoites into the pulmonary microvasculature. **PLoS Pathog** 3: e171.

Billker, O., Lindo, V., Panico, M., Etienne, A.E., Paxton, T., Dell, A., et al. (1998) Identification of xanthurenic acid as the putative inducer of malaria development in the mosquito. **Nature** 392: 289–292.

Billker, O., Dechamps, S., Tewari, R., Wenig, G., Frankel-Fayard, B., and Brinkmann, V. (2004) Calcium and a calcium-dependent protein kinase regulate gamete formation and mosquito transmission in a malaria parasite. **Cell** 117: 503–514.

Blackman, M.J. (2008) Malarial proteases and host cell egress: an ‘emerging’ cascade. **Cell Microbiol** 10: 1925–1934.

Brooks, S.R., and Williamson, K.C. (2000) Proteolysis of *Plasmodium falciparum* surface antigen, Pf230, during gametogenesis. **Mol Biochem Parasitol** 106: 77–82.

Chandramohanadas, R., Davis, P.H., Belting, D.P., Harbut, M.B., Darling, C., Velmouougane, G., et al. (2009) Apicomplexan parasites co-opt host calpains to facilitate their escape from infected cells. **Science** 324: 794–797.

Cowman, A.F., and Crabb, B.S. (2006) Invasion of red blood cells by malaria parasites. **Cell** 124: 755–766.

Dechant, R., Binda, M., Lee, S.S., Pelet, S., Winderickx, J., and Peter, M. (2010) Cytosolic pH is a second messenger for glucose and regulates the PKA pathway through V-ATPase. **EMBO J** 29: 2515–2526.

Dua, M., Raphael, P., Sijwali, P.S., Rosenthal, P.J., and Hanspal, M. (2001) Recombinant falcipain-2 cleaves erythrocyte membrane ankyrin and protein 4.1. **Mol Biochem Parasitol** 116: 95–99.

Dvorak, J.A., Miller, L.H., Whitehouse, W.C., and Shiroishi, T. (1975) Invasion of erythrocytes by malaria merozoites. **Science** 187: 748–750.

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.

Eksi, S., Czesny, B., van Gemert, G.J., Sauerwein, R.W., Eling, W., and Williamson, K.C. (2006) Malaria transmission-blocking antigen, Pf230, mediates human red blood cell binding to exflagellating male parasites and oocyst production. **Mol Microbiol** 61: 991–998.

Fruth, I.A., and Arrazabalaga, G. (2007) *Toxoplasma gondii*: induction of egress by the potassium ionophore nigericin. **Int J Parasitol** 37: 1559–1567.

Furuya, T., Mu, J., Hayton, K., Liu, A., Duan, J., Nkrumah, L., et al. (2005) Disruption of a *Plasmodium falciparum* gene linked to male sexual development causes early arrest in gametocytogenesis. **Proc Natl Acad Sci USA** 102: 16813–16818.

Garcia, G.E., Wirtz, R.A., Barr, J.R., Woolfitt, A., and Rosenberg, R. (1998) Xanthurenic acid induces gametogenesis.
in *Plasmodium*, the malaria parasite. *J Biol Chem* **273**: 12003–12005.

Glushakova, S., Mazar, J., Hohmann-Marriott, M.F., Hama, E., and Zimmerberg, J. (2009) Irreversible effect of cysteine protease inhibitors on the release of malaria parasites from infected erythrocytes. *Cell Microbiol* **11**: 95–105.

Glushakova, S., Humphrey, G., Leikina, E., Balaban, A., Miller, J., and Zimmerberg, J. (2010) New stages in the program of malaria parasite egress imaged in normal and sickle erythrocytes. *Curr Biol* **20**: 1117–1121.

Günther, K., Tummler, M., Arnold, H.H., Ridley, R., Goman, M., Scaife, J.G., and Lingelbach, K. (1991) An exported protein of *Plasmodium falciparum* is synthesized as an integral membrane protein. *Mol Biochem Parasitol* **46**: 149–157.

Hanspal, M., Dua, M., Takakuwa, Y., Chishti, A.H., and Mizuno, A. (2002) *Plasmodium falciparum* cysteine protease falcipain-2 cleaves erythrocyte membrane skeletal proteins at late stages of parasite development. *Blood* **100**: 1048–1054.

Henderson, P.J., McGivan, J.D., and Chappell, J.B. (1969) The action of certain antibiotics on mitochondrial, erythrocyte and artificial phospholipid membranes. The role of induced proton permeability. *Biochem J* **111**: 521–535.

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.

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.

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

Kariuki, M.M., Okitsu, S.L., Boato, F., Mueller, M.S., Li, D.B., Vogel, D., Westerfeld, N., et al. (2007) Antibodies elicited by a virosomal formulated *Plasmodium falciparum* serine repeat antigen-5 derived peptide detect the processed 47 kDa fragment both in sporozoites and merozoites. *Peptides* **28**: 2051–2060.

Medica, D.L., and Sinnis, P. (2005) Quantitative dynamics of *Plasmodium yoelii* sporozoite transmission by infected anopheline mosquitoes. * Infect Immun* **73**: 4363–4369.

Miller, S.K., Good, R.T., Drew, D.R., Delorenzi, M., Sanders, P.R., Hodder, A.N., et al. (2002) A subset of *Plasmodium falciparum* SERA genes are expressed and appear to play an important role in the erythrocytic cycle. *J Biol Chem* **277**: 47524–47532.

Nijhoff, M.M., and Carter, R. (1978) Gamete development in malaria parasites: bicarbonate-dependent stimulation by pH in vitro. *Parasitology* **76**: 39–53.

Oktitsu, S.L., Boato, F., Mueller, M.S., Li, D.B., Vogel, D., Westerfeld, N., et al. (2007) Egress of *Plasmodium falciparum*-infected red blood cells. *PLoS Comput Biol* **5**: e1000339.

Paton, M.G., Barker, G.C., Matsuoka, H., Ramesar, J., Janse, C.J., Waters, A.P., and Sinden, R.E. (1993) Structure and expression of a post-transcriptionally regulated malaria gene encoding a surface protein from the sexual stages of *Plasmodium berghei*. *Mol Biochem Parasitol* **59**: 263–275.

Ponnudurai, T., Lensen, A.H., Meis, J.F., and Meuwissen, J.H. (1986) Synchronization of *Plasmodium falciparum* gametocytes using an automated suspension culture system. *Parasitology* **93**: 263–274.

Porzi, M., Siden-Kiams, 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 Microbiol* **11**: 1272–1288.

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

Quakyi, I.A., Matsumoto, Y., Carter, R., Udomsangpetch, R., Sjölander, A., Berzins, K., et al. (1989) Movement of a falciparum malaria protein through the erythrocyte cyto-
plasm to the erythrocyte membrane is associated with lysis of the erythrocyte and release of gametes. *Infect Immun* **57**: 833–839.

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

Rupp, I., Bosse, R., Schirmeister, T., and Pradel, G. (2008) Effect of protease inhibitors on exflagellation in *Plasmodium falciparum*. *Mol Biochem Parasitol* **158**: 208–212.

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.

Salmon, B.L., Oksman, A., and Goldberg, D.E. (2001) Malaria parasite exit from the host erythrocyte: a two-step process requiring extraerythrocytic proteolysis. *Proc Natl Acad Sci USA* **98**: 271–276.

Schmidt-Christensen, A., Sturm, A., Horstmann, S., and Heussler, V.T. (2007) Live and let die: manipulation of host hepatocytes by exoerythrocytic *Plasmodium* parasites. *Mol Microbiol Immunol* **196**: 127–133.

Sturm, A., Heussler, V. (2007) Live and let die: manipulation of host hepatocytes by exoerythrocytic *Plasmodium* parasites. *Mol Biochem Parasitol* **158**: 208–212.

Tewari, R., Dorin, D., Moon, R., Doerig, C., and Billker, O. (2005) An atypical mitogen-activated protein kinase controls cytokinesis and flagellar motility during male gamete formation in a malaria parasite. *Mol Microbiol* **58**: 1253–1263.

Torres, J.A., Rodriguez, M.H., Rodriguez, M.C., and de la Cruz Hernandez-Hernandez, F. (2005) *Plasmodium berghei*: effect of protease inhibitors during gametogenesis and early zygote development. *Exp Parasitol* **111**: 255–259.

Vanderberg, J.P. (1977) *Plasmodium berghei*: quantitation of sporozoites injected by mosquitoes feeding on a rodent host. *Exp Parasitol* **42**: 169–181.

Vanderberg, J.P., and Frevert, U. (2004) Intravital microscopy demonstrating antibody-mediated immobilisation of *Plasmodium berghei* sporozoites injected into skin by mosquitoes. *Int J Parasitol* **34**: 991–996.

Wickham, M.E., Culvenor, J.G., and Cowman, A.F. (2003) Selective inhibition of a two-step egress of malaria parasites from the host erythrocyte. *J Biol Chem* **278**: 37658–37663.

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

Yeooh, S., O’Donnell, R.A., Koussis, K., Dluzewski, A.R., Ansell, K.H., Osborne, S.A., et al. (2007) Subcellular discharge of a serine protease mediates release of invasive malaria parasites from host erythrocytes. *Cell* **131**: 1072–1083.

**Supporting information**

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

**Fig. S1.** Ultrastructural changes in *P. falciparum* gametocytes during activation. Gametocytes were activated and processed for electron microscopy. Osmiophilic bodies (arrowheads) associate underneath the SPM at the sites of PVM rupture (indicated by asterisks) at 2 min p.a. (A). The formation of axonemes and microgametes in activated microgametocytes was investigated at 6 and 15 min p.a. (B). Detaching of the PVM and rupture of the EM were observed in gametocytes at 15 min p.a. (C). Asterisks (in C) indicate single breaking point of EM rupture. A, axoneme; EM, erythrocyte membrane; FV, food vacuole; M, mitochondrion; MG, microgamete; N, nucleus; PDM, parasite-derived membrane; PPM, parasite plasma membrane; PVM, parasitophorous vacuole membrane; SPM, subpellicular membrane. Bar, 0.5 μm (A) and 1 μm (B, C).

**Fig. S2.** PVM rupture during changes in homeostasis. Following activation, the erythrocyte cytoplasm degrades (A, left panel) and PDMs accumulate at the PVM (A, right panel) in untreated gametocytes. Treatment with nigericin at RT results in activation of gametocytes and PVM rupture (B). BAPTA-AM treatment does not impair PVM rupture, but morphogenesis of the transforming activated gametocyte (C). Asterisks indicate sites of PVM
rupture. EM, erythrocyte membrane; HZ, hemozoin; MT, microtubules; N, nucleus; PDM, parasite-derived membrane; PPM, parasite plasma membrane; PVM, parasitophorous vacuole membrane; SPM, subpellicular membrane. Bar, 1 μm.

**Fig. S3.** Propidium iodide-based cytotoxicity test on protease inhibitor-treated cultures. Gametocytes were pre-incubated with protease inhibitors and activated in the presence of inhibitors. Activated live cultures were incubated with anti-Pls230 antibody in combination with Alexa Fluor 488 secondary antibody (green) to label activated gametocytes and subsequently incubated with propidium iodide to highlight dead cells (red). Incubation with secondary antibody (2nd Ab) alone did not result in any labelling of sexual stage parasites. Bar, 25 μm. (A). No significant increase in the numbers of propidium iodide (PI)-positive dead cells was observed in a total number of 50 Pls230-positive activated gametocytes (set to 100%), when these were treated with the protease inhibitors and compared with 1% DMSO or 1% methanol (MeOH)-treated cultures (negative controls). Cultures treated with 50% DMSO were used for positive (death) control \( (P < 0.05, \text{Student's } t\text{-test}) \). Experiments were done in triplicate.

**Fig. S4.** Effect of protease inhibitors on gametocyte egress. Gametocytes were pre-incubated with protease inhibitors and activated in the presence of inhibitors. Specimens were fixed 20 min p.a. and processed for immunofluorescence assay. The presence of the EM (labelled with anti-band 3 antibody) or of the PVM (labelled with anti-Pls16 antibody) was quantified in activated macrogametocytes (labelled with anti-Pls25 antibody) and activated microgametocytes [spherical anti-alpha-tubulin II (atubII)-positive cells]. Bar, 5 μm.

**Fig. S5.** Schematic depicting the proposed inside-out mode of gametocyte egress. The inhibitors interfering with distinct steps of egress are indicated. EM, erythrocyte membrane; PPM, parasite plasma membrane; PV, parasitophorous vacuole; PVM, PV membrane; SPM, subpellicular membrane; T, temperature; XA, xanthurenic acid.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.