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Plasmodium berghei serine/threonine protein phosphatase PP5 plays a critical role in male gamete fertility

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ABSTRACT

Sexual development in malaria parasites involves multiple signal transduction pathways mediated by reversible protein phosphorylation. Here, we functionally characterised a protein phosphatase, Ser/Thr protein phosphatase 5 (PbPP5), during sexual development of the rodent malaria parasite *Plasmodium berghei*. The recombinant protein phosphatase domain displayed obvious protein phosphatase activity and was sensitive to PP1/PP2A inhibitors including cantharidic acid (IC_{50} = 122.2 nM), cantharidin (IC_{50} = 74.3 nM), endothall (IC_{50} = 365.5 nM) and okadaic acid (IC_{50} = 1.3 nM). PbPP5 was expressed in both blood stages and ookinetes with more prominent expression during sexual development. PbPP5 was localised in the cytoplasm of the parasite and highly concentrated beneath the parasite plasma membrane in free merozoites and ookinetes. Targeted deletion of the *pbpp5* gene had no influence on asexual blood-stage parasite multiplication or the survival curve of the infected hosts. However, male gamete formation and fertility were severely affected, resulting in almost complete blockade of ookinete conversion and oocyst development in the *Δpbpp5* lines. This sexual development defect was rescued by crossing *Δpbpp5* with the female defective *Δpbs47* parasite line, but not with the male defective *Δpbs48/45* line, thus confirming the essential function of the *pbpp5* gene in male gamete fertility. Furthermore, the aforementioned PP1/PP2A inhibitors all had inhibitory effects on exflagellation of male gametocytes and ookinete conversion. In particular, endothall, a selective inhibitor of PP2A, completely blocked exflagellation and ookinete conversion at ~548.3 nM. This study elucidated an essential function of PbPP5 during male gamete development and fertility.

1. Introduction

Despite decades of effort to control malaria, it remains a global health burden, especially for the tropical and subtropical countries. The 2017 World Malaria Report showed that there were approximately 216 million malaria cases in 2016, resulting in an estimated 445,000 deaths (WHO, 2018). Current malaria control efforts rely heavily on vector-based approaches such as insecticide-treated nets and indoor residual sprays, and effective artemisinin-based combination therapy (ACT) (Bhatt et al., 2015). However, the emergence and spread of insecticide-resistant vectors and artemisinin-resistant *Plasmodium falciparum* parasites have compromised the effectiveness of these control measures. Integrated and novel approaches targeting vulnerable steps in the life cycle of the malaria parasites are needed. Transmission of malaria from the vertebrate host to the mosquito vector is dependent on the formation of gametocytes, a process termed gametocytogenesis (Liu et al., 2011). Strategies to interfere with these developmental processes by using gametocytocidal drugs and transmission blocking vaccines are actively pursued. Currently, transmission blocking vaccines are still in development (Wu et al., 2015). Most of the commonly used antimalarial drugs only target asexual stages and have no effect on gametocyte stages (Kiszewski, 2011; Cui et al., 2015). Only the 8-aminoquinoline drug primaquine is effective on gametocytes, and to interrupt *P. falciparum* transmission a single dose of primaquine (0.25 mg/kg) is recommended by the World Health Organization (WHO) to be added to the standard ACT (Graves et al., 2018). However, there is still concern about the hemolytic risk of primaquine in people with glucose-6-phosphate

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dehydrogenase deficiency. Thus, there is an urgent need for new transmission blocking drugs, and it is hoped that better understanding of the transmission stages will yield novel drug targets.

Transmission of malaria to a susceptible mosquito is initiated when she ingests the arrested sexual precursor stage, the gametocyte, from the vertebrate host. Inside the mosquito midgut, a drop in temperature, change in pH, and mosquito factors such as xanthurenic acid induce the gametocyte to undergo gametogenesis to form male and female gametes (Billker et al., 1997). Microgametes then fertilise macrogametes to form zygotes, which transform into motile and invasive ookinetes. To orchestrate these form changes, the parasites utilise multiple signaling pathways, which involve reversible protein phosphorylation through kinases and phosphatases (Billker et al., 2004; Doerig et al., 2008; McRobert et al., 2008; Miliu et al., 2017). It is thus conceivable that a better understanding of kinases and phosphatases involved in sexual development of the parasites will help identify viable targets for transmission blocking drug discovery (Doerig and Meijer, 2007; Lucet et al., 2012).

Malaria parasites encode approximately 85 putative protein kinases and 30 protein phosphatases (Ward et al., 2004; Wilkes and Doerig, 2008; Guttery et al., 2014). Advances in functional genomics tools allowed large-scale functional analyses of *Plasmodium* kinases and phosphatases. Studies in the human and rodent *Plasmodium* parasites revealed that approximately half of the protein kinases are essential for asexual blood stages, while a further 14 protein kinases are needed for sexual development (Tewari et al., 2010; Solyakov et al., 2011). Similarly, functional studies of the 30 predicted phosphatases of the *Plasmodium berghei* phosphatome showed that 14 of them are dispensable for asexual stages, among which six show essential functions during sexual development (Guttery et al., 2014). Specifically, among the metal-dependent protein phosphatase (PPM) members, *ppm1* is essential for exflagellation of the microgametocytes, *ppm2* deletion reduces macrogamete number and ookinete conversion, whereas *ppm5* deletion severely affects oocyst development. Two unique phosphatase family members, the protein phosphatase containing kelch-like domains (ppkl) and the Shewannella-like protein phosphatase (shlp1), involve ookinete differentiation and oocyst development, respectively (Guttery et al., 2012b; Patzewitz et al., 2013). The Protein Tyrosine Phosphatases (PTP)-like A homologue is essential for sporogony, as its knockout produces similar numbers of oocysts to the wild type (WT) but no sporozoites.

Malaria parasites have homologues of all major human phosphoprotein phosphatase (PPP) subfamilies PP1–PP7 (Kutuzov and Andreeva, 2008). Functional studies showed that all of them are indispensable for asexual blood stages (Guttery et al., 2014). Of the PPP subfamilies, PP5 differs from other phosphatases in possessing three N-terminal tetratricopeptide repeat (TPR) domains, which are auto-inhibited and responsible for protein–protein interactions. Being expressed in virtually all mammalian systems, PP5 interacts with a number of proteins through its TPR domains to function as a modulator in multiple signaling pathways (Becker et al., 1994; Chinkers, 1994; Hinds and Sanchez, 2008). In protozoan parasites, down-regulation of PP5 in *Eimeria tenella* leads to apoptosis in second generation merozoites (Zhou et al., 2013). Over-expression of PP5 in *Trypanosoma brucei* rendered the parasite more resistant to geldanamycin treatment, whereas its knockdown reduced cell growth (Jones et al., 2008). PP5 in *P. falciparum* shares a similar structure with other protozoan PP5 proteins and it interacts with Heat Shock Protein 90 (Hsp90) (Dobson et al., 2001; Lindenthal and Klinkert, 2002). Although its function is not known, PP5 is highly expressed in male gametocytes in *P. berghei*, suggesting an important role in sexual development (Guttery et al., 2014). Here, we attempted to elucidate the function of PbPP5 through in vitro enzyme studies and genetic manipulation, which identified an important role of PbPP5 in regulating male gamete fertilisation.

2. Materials and methods

2.1. Sequence analysis

SMART software (http://smart.embl-heidelberg.de/) was used to analyze the conserved domains of PbPP5 (GenBank Accession number XP_673410.2) (Letunic and Bork, 2018), while TMHMM software (http://www.cbs.dtu.dk/services/TMHMM-2.0/) was used to analyze its transmembrane domain. Motif scan software (https://myhits.isb-sib.ch/cgi-bin/motif_scan) was used to analyze structural domains, and BLASTp (http://www.ncbi.nlm.nih.gov/BLAST/) was used to search for homologous proteins. Phylogenetic analysis of PbPP5 and known PPs in other species retrieved from GenBank were aligned using MUSCLE software (https://www.ebi.ac.uk/Tools/msa/muscle/). Subsequently, a phylogenetic tree was constructed using the neighbor-joining method in MEGA 7.0 (https://www.megasoftware.net/). The reliability of the tree was measured by bootstrap analysis with 1000 replicates.

2.2. Experimental animals, parasites and mosquitoes

Six- to eight-week old female BALB/c mice (Beijing Animal Institute, China) and mosquitoes were infected with WT *P. berghei* ANKA 2.34 strain and mutant parasites as previously described (Kou et al., 2016). Animal handling was conducted according to a China Medical University Animal Care and Use Committee approved protocol. Female *Anopheles stephensi* mosquitoes were fed a 10% (w/v) glucose solution and reared at 25 °C and 50–80% humidity with a 12:12 h light:dark period under standard laboratory conditions.

2.3. Generation of transgenic parasites

Targeted deletion of the *pbpp5* gene was accomplished by double-crossover homologous recombination using the vector PbGEM-301794 (kindly provided by plasmoGEM; http://plasmogem.sanger.ac.uk/). Primers used for transgenic plasmid construction are listed in Supplementary Table S1. To generate the plasmids for the *pbs47* (PlasmoDB accession number PBANKA_1359700) knockout, DNA fragments of 962 bp upstream and 766 bp downstream sequences were amplified from *P. berghei* genomic DNA (gDNA) using primers in Supplementary Table S1. To knock out *pbs48/45* (PlasmoDB accession number PBANKA_1359600), DNA fragments of 538 bp upstream and 779 bp downstream sequences were amplified (Supplementary Table S1). The amplified fragments were digested with *Hind*III/*Pst*I and *Kpn*I/*Not*I, respectively, and subsequently cloned into the pL0034 plasmid, which contains the *hdhfr* cassette conferring resistance to pyrimethamine, to generate the pL0034-pbs47 and pL0034-pbs48/45 plasmids (van Dijk et al., 1995). Before transfection, the PbGEM-30194 plasmid was linearised using *Not*I, and the pL0034-pbs47 and pL0034-pbs48/45 plasmids were linearised with *Sac*II and *Kpn*I. To generate episomally expressed full-length PbPP5 protein tagged with green fluorescent protein (GFP) at the C-terminus, specific primers PbPP5$^{\text{full lenth}}$B$_1$F and PbPP5$^{\text{full length}}$B$_2$R$_{V3}$ were used to amplify the open reading frame (ORF) of PbPP5, and PbPP5$^{5'UTR}$F and PbPP5$^{5'UTR}$R primers were used to amplify the 5′ untranslated region (UTR) region ($-1892 \sim -2$ bp) of the *pbpp5* gene (Supplementary Table S1). Gateway BP (attachment bacterial site (*att*B) and attachment phage site (*att*P)) recombination reactions of PCR product with pDONR™ 221 and pDONR™ P4-P1R (Invitrogen) were performed to generate pEN12-PbPP5$^{\text{full length}}$

and pENTR41-PbPP5-5′UTR plasmids. A Gateway MultiSite LR (attachment left site (*att*L) and attachment right site (*att*R)) recombination reaction was performed with pEN12-PbPP5$^{full\ length}$, pENTR41-PbPP5-5′UTR, pENT23-GFP and pCHDR-3/4 plasmids to generate a pCHDR-PbPP5$^{full\ length}$-GFP plasmid (van Dooren et al., 2005) according to the manufacturer's instructions. Transfection, selection and parasite cloning were performed as previously described (Janse et al., 2006a,b). Infected blood was collected to confirm the correct integration by diagnostic PCR (Supplementary Table S1).

2.4. Expression of recombinant PbPP5 and immunisation

The recombinant PbPP5 protein used in the current study was expressed using the *Pichia* expression system in the host strain GS115 his4 (auxotrophic for histidine) (Genecreate, Inc.). Briefly, the PP2Ac domain of PbPP5 (amino acid (aa) positions 407–711) was amplified, cloned into the expression vector pPIC9K plasmid and transformed into the GS115 strain. Expression of the recombinant PbPP5^{yPP2Ac} (rPbPP5 ^{yPP2Ac}) protein was induced by adding 200 µl of 5% methanol into the yeast peptone (YP) culture medium at 30 °C for 24 h. The rPbPP5^{yPP2Ac} protein was purified using a Ni-NTA column, dialyzed in 20 mM Tris–HCl (pH 7.5) and 1 mM DTT, and concentrated using Amicon®Ultra 0.5 (Millipore, USA) to yield 2 mg/ml of protein concentration.

For immunisation of mice, 6–8 weeks old female BALB/c mice (n = 10) were initially immunised by s.c. injection with 50 µg of rPbPP5^{yPP2Ac} protein emulsified in FCA (Sigma), followed by two booster injections at 2 week intervals with the same amount of protein in incomplete Freund's adjuvant. Mice in the control group (n = 10) were injected with PBS using the same immunisation procedure. Final bleeding was done 2 weeks after the last immunisation. Blood was collected from the tail vein of each mouse and allowed to clot at room temperature. The antiserum titer against PbPP5 was analyzed by ELISA as previously described (Zheng et al., 2016). Briefly, a 96-well plate was coated with the rPbPP5^{yPP2Ac} protein at 10 µg/mL overnight at 4 °C, and then blocked with 3% BSA in 1× PBS and 0.05% Tween 20 (PBS-T) for 2 h at room temperature. Mouse antiserum was diluted in PBS containing 10% calf serum albumin (pH 7.2), added to the wells, and incubated for 2 h at 37 °C. After two washes with PBS-T, horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies (Thermo Scientific) diluted 1:5000 was added and incubated for 2 h at 37 °C. After seven washes with PBS-T, the chromogenic substrate was added and developed for 5 min. The reaction was stopped by adding 50 µl of 2 mM H_2SO_4 to each well. The plate was immediately read with a plate reader at 490 nm.

2.5. Indirect immunofluorescence assay (IFA)

IFA was carried out on different developmental stages of *P. berghei* as previously described (Tonkin et al., 2004). Briefly, 100 µl of *P. berghei*-infected tail blood were collected into a 1.5 ml tube, and washed three times with PBS. Cells were fixed with 4% paraformaldehyde and 0.0075% electron microscope (EM) grade glutaraldehyde in PBS for 30 min. Fixed cells were either subjected to permeabilisation with 0.1% Triton X-100 for 5 min on ice or without permeabilisation, followed by PBS washing and rinsing with 0.1 mg/ml of sodium borohydride ($NaBH_4$)/PBS for 10 min, and blocked with 5% skimmed milk for 30 min at 37 °C. After blocking, cells were incubated with mouse anti-PbPP5 sera (1:500), mouse or rabbit anti-GFP monoclonal antibody (mAb) (abcam, USA), or rabbit anti-GAPDH (abcam, USA) at 37 °C for 1 h. After washing three times in PBS, the slides were incubated with the secondary antibodies (AlexaFluor-goat anti-mouse 488 and/or anti-rabbit 594) (Invitrogen) at 1:500 for 1 h at 37 °C. Parasite nuclei were counter-stained with 1 µg/mL of DAPI (Thermo scientific, USA). Cells were washed three times with PBS and mounted with ProLong® Gold anti-fade reagent (Thermo scientific, USA). After that, the cells were settled on a slide, covered by coverslips and visualised under a Nikon ECLIPSE 80i microscope.

2.6. Western blot

The purification of schizonts, gametocytes and ookinetes was performed as previously described (Sinden et al., 1985; Beetsma et al., 1998; Janse et al., 2006b). After collection, parasite-infected cells were treated with 0.15% saponin (Sigma) in PBS for 8 min on ice and pelleted through centrifugation at 4 °C. Parasite pellets were washed several times with PBS containing protease inhibitors (PBS-PI) and proteins were extracted with PBS-PI containing 2% SDS and 0.1% Triton-X100 for 30 min at room temperature. Equal amounts of parasite antigens (10 µg) were electrophoresed on a 12% SDS–PAGE gel and transferred to a 0.22 µm polyvinylidene fluoride (PVDF) membrane (Bio-Rad). The membrane was incubated with mouse anti-PbPP5 antiserum (1:500), anti-GFP mAb (9F9. F9, abcam), rabbit polyclonal anti-GAPDH antibodies (1:2000, abcam), or anti-Hsp70 mAb (5A5, abcam) in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 3 h at room temperature, washed three times in TBS-T, and then incubated for 2 h at 37 °C with HRP-conjugated goat anti-mouse IgG (H + L) antibodies (Thermo Scientific) diluted at 1:25,000 in TBS-T. Proteins on the blot were visualised with a Pierce ECL Western Blotting Kit (Thermo Scientific) on Tanon 4200 (Tanon). The relative molecular masses of proteins were estimated with reference to PageRuler Prestained Protein Ladder (Thermo Scientific).

2.7. Protein phosphatase assay

The phosphatase activity of the PbPP5^{yPP2Ac} protein was assayed by using the ProFluor® Ser/Thr PPase Assay kit (Promega, USA). Briefly, concentrations of PbPP5^{yPP2Ac} and His-tag protein as the control (Zhu et al., 2017) were determined by the Bradford method using BSA as the standard (TaKaRa, Japan). The rPbPP5^{yPP2Ac} and the His protein in 25 µl of phosphatase dilution solution were added into each well. An equal volume of peptide solution was added into all wells, mixed for 15 s, and incubated for 10 min at room temperature. Then, 25 µl of protease were added into each well and the plate was incubated at room temperature for 90 min. Finally, 25 µl of stabiliser solution were added into each well and the plate was read on a Biomek 2000 laboratory automation workstation (Beckman Coulter, Inc., Fullerton, CA, USA). Fluorescence emission from the product was measured with a multiwell plate reader (Cytofluor II; Applied Biosystems, Foster City, CA, USA) with excitation at 485 nm (20 nm bandwidth) and emission at 530 nm (30 nm bandwidth). Inhibition of the rPbPP5^{yPP2Ac} by several PP1/PP2A inhibitors from the Screen-Well Phosphatase Inhibitor Library (BML-2384, Enzo Life Science), including cantharidic acid, cantharidin and endothall (Supplementary Table S2), was performed as described above.

2.8. Phenotypic analysis of Δpbpp5 parasites during parasite development

To study the functions of *pbpp5* during development, 10 mice were pre-treated with phenylhydrazine. Two groups of five mice each were injected i.p. with 1×10^6 Δ*pbpp5*- and WT *P. berghei*-infected RBCs (iRBCs), respectively. Parasitemia was monitored daily by Giemsa-stained tail blood smears. On day 3 p.i., gametocytemia (mature gametocytes per 100 RBCs), gametocyte sex ratio, exflagellating male gametocytes, and interactions between male and female gametes were determined. For the exflagellation assay,

10 µl of gametocyte-infected blood were obtained from the tail vein and mixed immediately with 90 µl of complete ookinete culture medium. The mixture was placed under a Vaseline-coated coverslip at 25 °C for 15 min, the exflagellation centers were counted under a phase contrast microscope in 30 fields. The numbers of male gametocytes forming or not forming exflagellation centers were counted. An exflagellation center is defined as an exflagellating male gametocyte with more than four tightly associated red blood cells (Bialojan and Takai, 1988). For quantification of male–female interactions, 10 min after induction of gamete formation, the cell suspension was placed in a Vaseline-coated coverslip and observed for a period of 20 min under a phase contrast microscope. Attachment of males to females was scored if the male had active interaction/attachment with the female for more than 3 s. Ookinete formation was evaluated by adding 10 µl of iRBCs into 90 µl of ookinete culture medium and incubating at 19 °C for 24 h. Cultured ookinetes were labeled with a mouse anti-Pbs21 monoclonal antibody (1:500) and enumerated under a fluorescence microscope. Cross-fertilisation experiments with different parasite lines defective in either male or female gametogenesis were performed as previously described (Ponzi et al., 2009; Boisson et al., 2011). Briefly, gametes from the Δ*pbpp5* line were cross-fertilised with gametes of parasite lines that produced only fertile female gametes (Δ*p48/45*) (van Dijk et al., 2001) or fertile male gametes (Δ*p47*) (Khan et al., 2005). All the fertilisation/ookinete maturation assays were done in triplicate from independent experiments.

To determine the effect of *Δpbpp5* on sporogonic development in mosquitoes, *A. stephensi* mosquitoes (~100/mouse) starved for 6 h were allowed to feed for 30 min on three mice infected 3 days earlier with either the WT *P. berghei* or *Δpbpp5*. Fully engorged mosquitoes were maintained at 19–22 °C and in 50–80% relative humidity. Ten days after feeding, up to 40 mosquitoes were dissected in each group. The midguts of mosquitos were removed and stained with 0.5% mercurochrome (Sigma–Aldrich). Oocysts were counted to determine the prevalence (number of infected mosquitoes) and intensity of infection (number of oocysts per midgut). Mosquitoes were dissected on day 18 post blood feeding to determine the presence of salivary gland sporozoites.

2.9. Exflagellation and ookinete conversion inhibition assay

The inhibitory effects of cantharidic acid, cantharidin and endothall on *P. berghei* exflagellation and ookinete conversion were evaluated in vitro. All three compounds were prepared as 10 mM stock solutions in DMSO. They were further diluted in ookinete culture medium to a final concentration of 1× and 1.5× of their respective half maximal inhibitory concentration (IC_{50}) determined for the rPbPP5^{yPP2Ac} recombinant enzyme: cantharidic acid ($1 \times IC_{50}$ = 122.2 nM, $1.5 \times IC_{50}$ = 183.3 nM), cantharidin ($1 \times IC_{50}$ = 74.3 nM, $1.5 \times IC_{50}$ = 111.5 nM) and endothall ($1 \times IC_{50}$ = 365.5 nM, $1.5 \times IC_{50}$ = 548.3 nM). They were used for in vitro exflagellation and ookinete conversion assays as described above. Ookinete culture medium containing 0.1% DMSO was used as a control. All experiments were performed in triplicate with each replicate using the parasite-infected blood of a different mouse.

2.10. Statistical analyses

Statistical comparison between groups (IgG levels, parasitemia, gametocytemia, and ookinete numbers) was done by Student's *t* tests using the GraphPad Prism software. The intensity of infection (oocysts/midgut) was analyzed using the Mann–Whitney *U* test, while infection prevalence was assessed using the Fisher's exact test.

3. Results

3.1. Sequence analysis of PbPP5

The *pbpp5* gene encodes a protein of 711 aa. Similar to its *P. falciparum* orthologue PfPP5, PbPP5 contains a C-terminal phosphatase domain (PP2Ac, 418–694 aa) and an extended N-terminal domain containing three TPR motifs (TPR1, 186–219 aa; TPR2, 278–311 aa; and TPR3, 312–345 aa), a typical feature of PPPs (Supplementary Fig. S1A). Searching the motif database revealed 12 putative N-glycosylation sites, nine casein kinase II phosphorylation sites, 13 protein kinase C phosphorylation sites, and one tyrosine kinase phosphorylation site (Supplementary Fig. S1B). Additional biological evidence is required to validate these predictions. PbPP5 was aligned with other known PP5 protein family members, which showed significant homology in the TPR domain and the PP2Ac domain (Supplementary Fig. S1C). A phylogenetic tree constructed from the alignment showed that PbPP5 was clustered with other protozoan PP5s (Supplementary Fig. S1D).

3.2. PbPP5 enzyme activity and sensitivity to PP1/PP2A inhibitors

To examine the catalytic activity of PbPP5, the PP2Ac domain of PbPP5 protein was expressed in yeast as a His-tag fusion protein and was affinity-purified to almost homogeneity (Fig. 1A). The band size of the rPbPP5^{yPP2Ac} protein on the SDS–PAGE gel is around 36 kDa, consistent with the predicted size (Fig. 1A). The catalytic activity of rPbPP5^{yPP2Ac} protein was compared with the control His-tag protein from the pET32a (+) vector expressed and purified from *Escherichia coli* (Zhu et al., 2017) using phosphorylated S/T PPase R110 (Promega) as the substrate. As shown in Fig. 1B, rPbPP5 exhibited obvious phosphatase activity compared with the control ($P < 0.001$). Furthermore, the phosphatase activity of the rPbPP5 was sensitive to PP1/PP2A inhibitors, with IC_{50} values for cantharidic acid, cantharidin, endothall and okadaic acid of 122.2, 74.3, 365.5, and 1.3 nM, respectively (Fig. 1C–F). This result is in good accordance with the inhibitory effect of okadaic acid on recombinant PfPP5 (Dobson et al., 2001).

3.3. PbPP5 expression and localisation

To investigate the localisation of PbPP5, we generated a parasite line with episomally-expressed PbPP5 fused with GFP at its C-terminus (PbPP5-GFP) driven by its own promoter. The expression of PbPP5-GFP was confirmed by Western blot analysis of parasite lysates of mixed blood stages using the anti-GFP antibody, which showed an ~110 kDa protein band that is consistent with the predicted molecular size of the fusion protein (Fig. 2A). Additionally, we raised polyclonal mouse antibodies against rPbPP5, and the antisera recognised the rPbPP5 protein in a Western blot (data not shown). To study PbPP5 protein expression in different developmental stages, proteins extracted from different stages of the WT parasites were separated by SDS–PAGE and probed with the polyclonal anti-PbPP5 antisera. Whereas the mouse pre-immune sera did not identify any specific protein bands in a parallel Western blot (data not shown), a protein band with a molecular mass of ~82 kDa was detected in each of the developmental stages examined by the anti-PbPP5 antisera, and the protein band corresponds to the size of the endogenous PbPP5 (Fig. 2B). It is noteworthy that the PbPP5 protein was substantially more abundant in sexual stages than in asexual stages.

We used both WT and PbPP5-GFP parasites to determine the localisation of PbPP5 protein during blood stage development of the parasite. First, using mouse-anti-PbPP5 antisera and rabbit

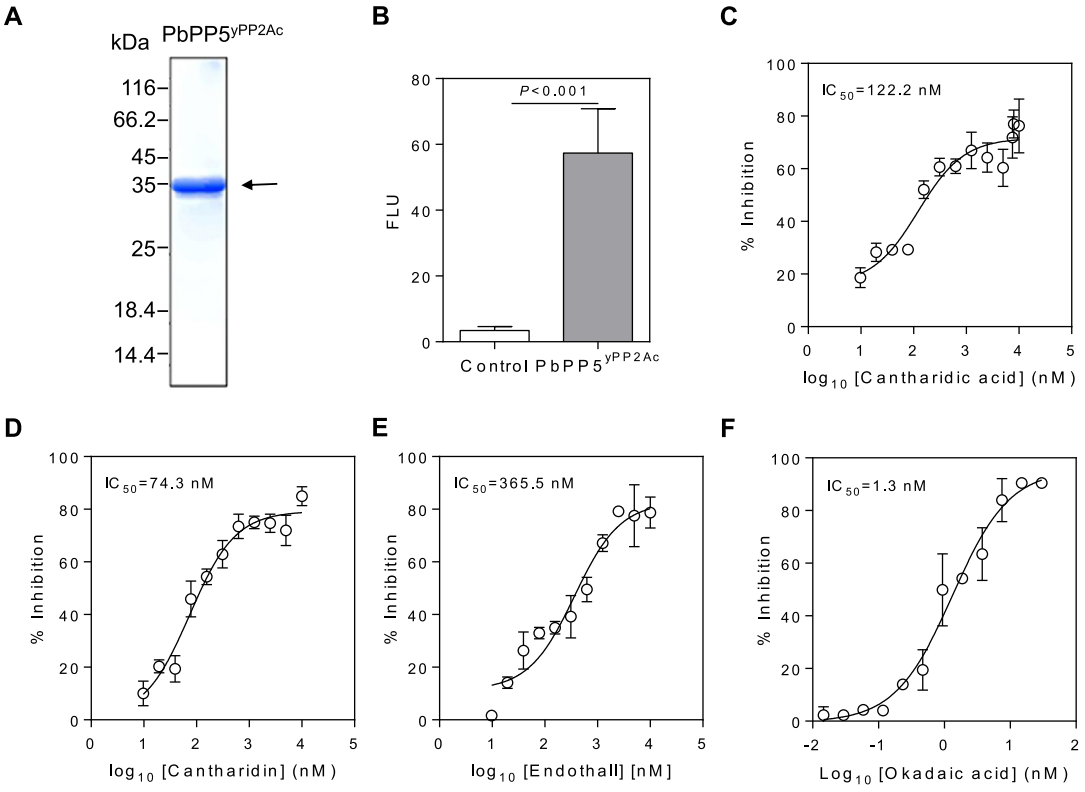

Fig. 1. The catalytic activity of rPbPP5^{yPP2Ac} (yeast system expressed Protein phosphatase 2A homologues, catalytic domain (PP2Ac) domain of recombinant *Plasmodium berghei* protein phosphatase 5) and its sensitivity to protein phosphatase-1 and -2A (PP1/PP2A) inhibitors. (A) Expression of the rPbPP5^{yPP2Ac} protein. The rPbPP5^{yPP2Ac} was purified from yeast and analyzed by SDS–PAGE under reducing conditions. Molecular weight markers are shown. The arrow indicates the expected size of rPbPP5 protein (~36 kDa). (B) Phosphatase activity of rPbPP5. Control, His-tag protein. $^{***}P < 0.001$. FLU, fluorescence light units. (C–F) Dose-response curves of different PP1/PP2A inhibitors: cantharidic acid (C), cantharidin (D), endothall (E), and okadaic acid (F). Each point represents the mean, and values are given as means and S.D. from three biological replicates.

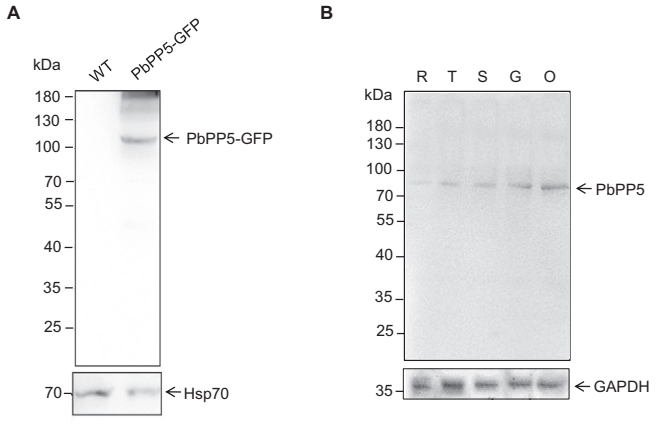

Fig. 2. Stage-specific expression of *Plasmodium berghei* protein phosphatase 5 (PbPP5). (A) Western blot of whole cell lysates of different stage PbPP5-GFP parasites with monoclonal anti-GFP antibody. (B) Western blot of wild type parasite cell lysates with anti-PbPP5 antisera. R, ring stage; T, trophozoite stage; S, schizont stage; G, gametocyte stage; O, ookinete stage. The positions of the pre-stained molecular mass markers are indicated. Western blots with mouse anti-PbHSP70 antisera or rabbit polyclonal anti-GAPDH antibody is shown as loading controls. HSP, heat shock protein.

anti-GFP antibody, we showed significant overlap of the fluorescent signals, indicating that PbPP5-GFP was correctly localised in the parasites (Supplementary Fig. S2). The fluorescence signals for GFP at ring, trophozoite, schizont and gametocyte stages were diffused in parasite cytosol, and some signals were associated with the membranes (Fig. 3A). The localisation patterns were similar to that of the PbPP5 in WT parasites detected with the anti-PbPP5 antisera (Fig. 3B). Co-localisation analysis showed that the fluorescent signals for PbPP5 and GAPDH (a cytosolic marker) partially overlapped, suggesting some of the PbPP5 proteins were cytosolic (Fig. 3B). Interestingly, in free merozoites and zygotes – ookinetes, the fluorescent signals were more intense at the rim of the parasites, which were reminiscent of membrane localisation (Fig. 3B, Supplementary Fig. S2). Yet, when the zygote to ookinete stage parasites were examined under non-permeabilised conditions, PbPP5 was not detectable, suggesting that PbPP5 was concentrated beneath the parasite plasma membrane (Supplementary Fig. S3).

3.4. PbPP5 deletion and function during asexual stages

To study the function of PbPP5 during the parasite's intraerythrocytic cycle, we generated *pbpp5* knockout (*Δpbpp5*) parasite lines. After homologous recombination, the *pbpp5* open reading frame (ORF) was replaced by the *hdhfr* selectable cassette (Supplementary Fig. S4). Correct allelic replacement was confirmed by diagnostic PCR (Supplementary Fig. S4B). Furthermore, Western blot analysis using specific antibodies against PbPP5 in mixed blood stage parasites confirmed deletion of *pbpp5*, where the PbPP5 protein of ~82.5 kDa was observed in WT but not the Δ*pbpp5* parasites (Supplementary Fig. S4C). We selected two clones, *Δpbpp5* clones k1 and k3, from two independent transfection experiments, for phenotypic analysis. In mice infected by i.v. injection of approximately the same numbers of WT, *Δpbpp5* k1, and *Δpbpp5* k3 parasites, we did not observe noticeable differences in daily parasitemias among the three groups between days 3 and

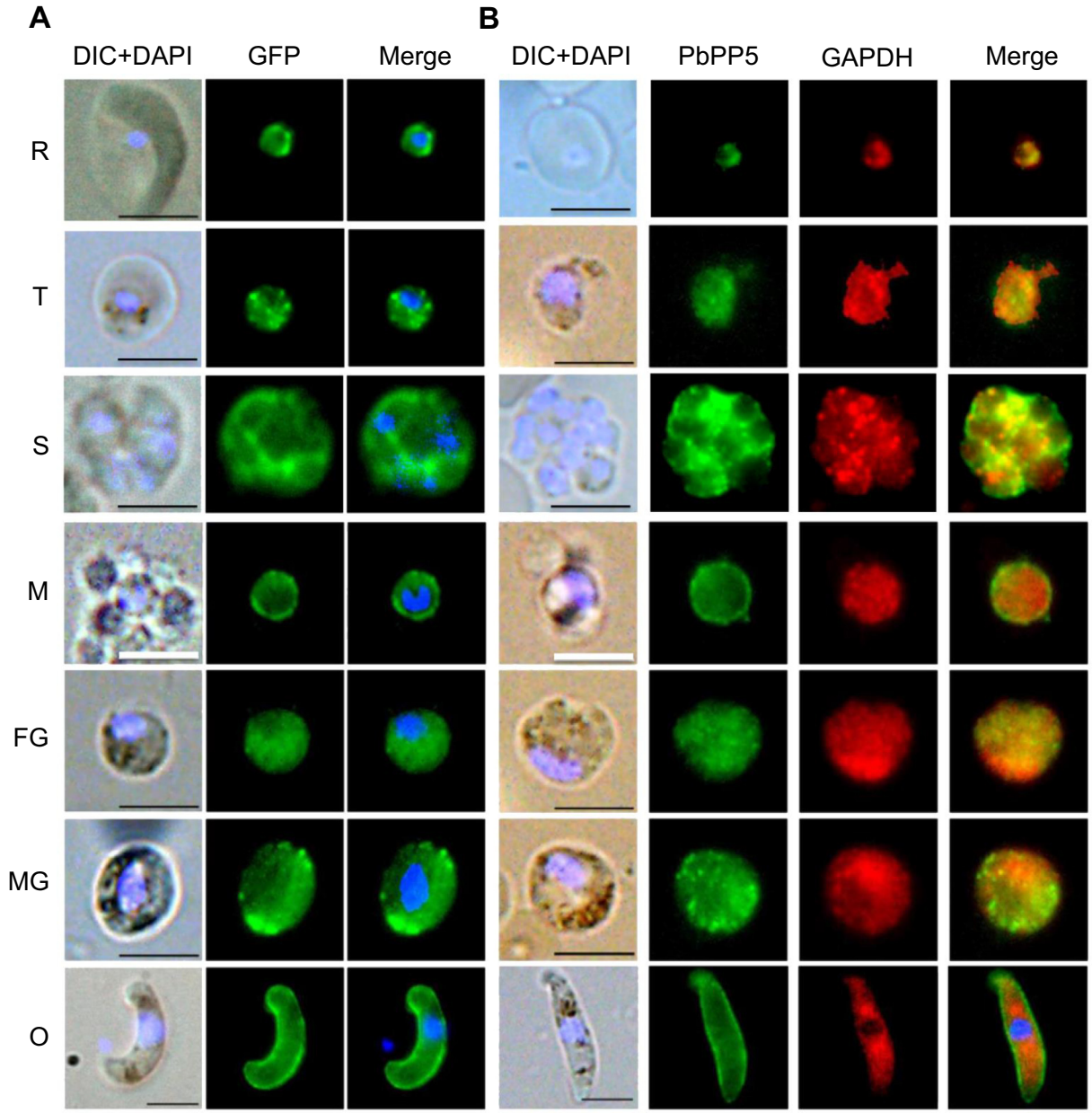

Fig. 3. Subcellular localisations of *Plasmodium berghei* protein phosphatase 5 (PbPP5). (A) IFA of *Plasmodium berghei* blood stages to localise GFP-tagged PbPP5 proteins using mouse anti-GFP antibody. (B) Co-localisation of the endogenous PbPP5 with anti-PbPP5 sera and anti-GAPDH antibodies. Different stages were observed under a Nikon fluorescence microscope with differential interference contrast, with the Alexa 488 channel (green) for detection of PbPP5, Alexa 594 channel (red) for detection of GAPDH, and with the DAPI channel (blue) to visualise DNA. Alexa 488, Alexa 594 and DAPI images were merged to show concordance. Note that exposure times for different images were different, and were not meant for quantitative comparison. Anti-GAPDH antibody was used as a marker for the parasite cytoplasm. For all the images, the black scale bar = 5 μm, and the white scale bar = 2.5 μm. R, ring stage; T, trophozoite stage; S, schizont stage. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

20 (Supplementary Fig. S5A), indicating that PbPP5 is dispensable for asexual blood stages of *P. berghei*. Furthermore, *pbpp5* deletion did not appear to affect the growth phenotype of the parasites as the survival curves of BALB/c mice infected with the WT parasites and the two Δ*pbpp5* lines were comparable (Supplementary Fig. S5B).

3.5. Functions of PbPP5 during sexual development

We next explored whether PbPP5 plays any rolesin sexual development of the parasite. Compared with the WT parasite, the *pbpp5* deletion lines did not show a defect in gametocytogenesis, as the day 3 gametocytemias were similar among the three parasite lines ($P > 0.05$, *t* test; Fig. 4A). In addition, although sex ratios of gametocytes were slightly reduced in the Δ*pbpp5* lines, the difference was not significant ($P > 0.05$, *t* test; Fig. 4B). However, when day 3 infected blood was used for in vitro culture of ookinetes, there was a complete lack of ookinetes in Δ*pbpp5* parasites (Fig. 4C).

To substantiate the in vitro findings, mosquitoes were fed on mice infected with either WT or Δ*pbpp5* k1 parasites and oocyst development was examined on day 10 after feeding. *Pbpp5* deletion almost resulted in a complete blockade of subsequent sporogonic development. In mosquitoes fed on three Δ*pbpp5*-infected mice, infection prevalences were 0% (0/33), 30.6% (11/36), and 7.5% (3/40) compared with 100% (26/26), 100% (26/26) and 100%

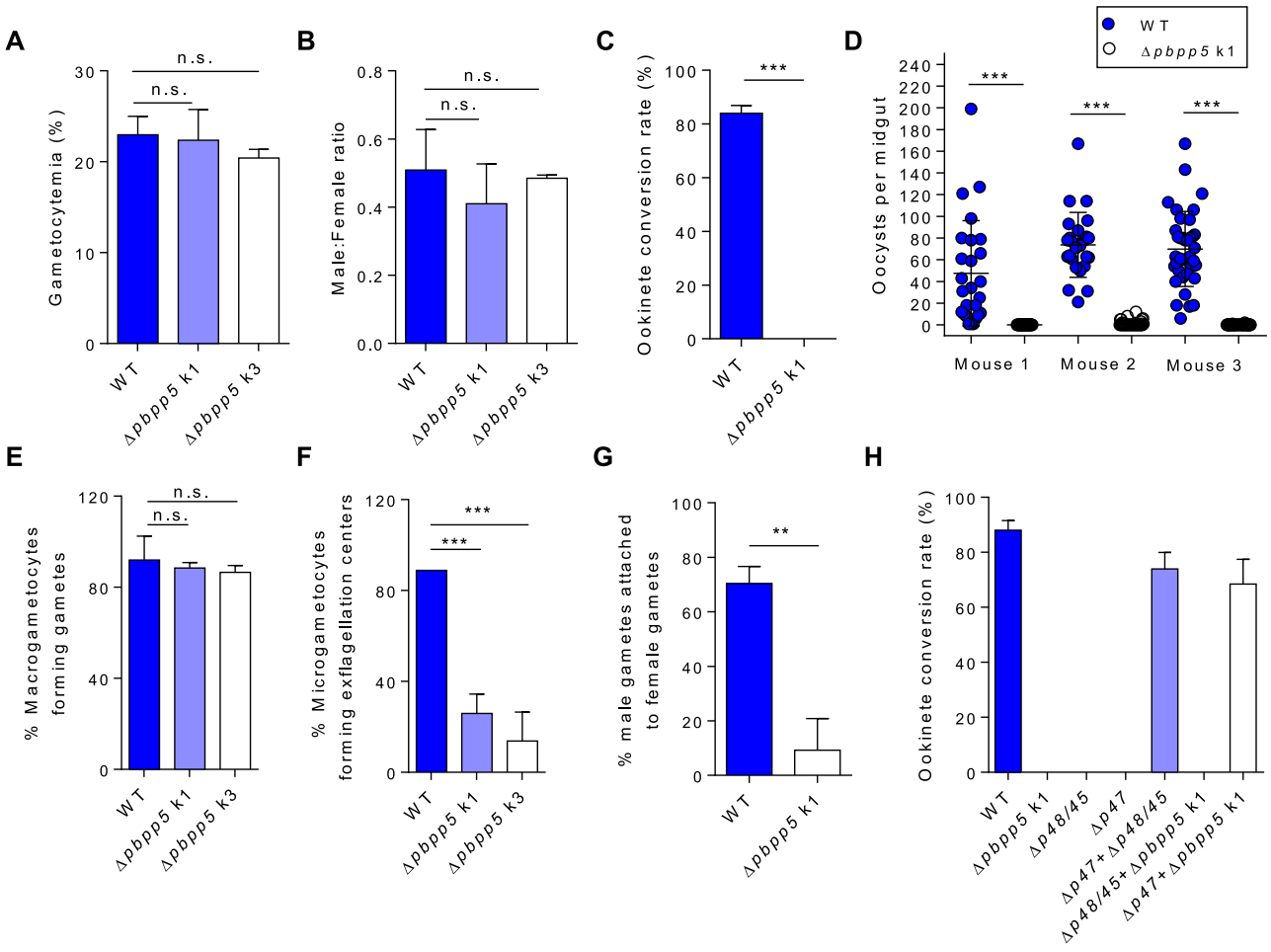

Fig. 4. Functional analysis of *Plasmodium berghei* protein phosphatase 5 (PbPP5) during sexual development. (A) Gametocytemia of wild type and Δ*pbpp5* parasites at day 3 p.i. (B) Male/female gametocyte ratios of wild type and Δ*pbpp5* parasites at day 3 p.i. (C) Absence of ookinete formation in the Δ*pbpp5* parasites. The conversion rate is the percentage of Pbs21-positive parasites (stained by anti-Pbs21 monoclonal antibody) that had successfully differentiated into elongated 'banana-shaped' ookinetes. (D) Absence of oocysts in midguts of mosquitoes infected with Δ*pbpp5* k1 parasites compared with wild type at day 10 post feeding. Circles correspond to oocyte numbers in individual mosquitoes. The panel represents one of the two or more infections performed with three infected mice per parasite clone and per infection. The number of mosquitoes analyzed per infection: wild type, $n = 29, 26, 39$; Δ*pbpp5* k1, $n = 33, 36, 40$. (E) The formation of macrogametes. (F) The interaction of Δ*pbpp5* male gametes with red blood cells (exflagellation centers). Results were obtained from five independent experiments for the comparison between the wild type and Δ*pbpp5* parasites. Two infected mice were analyzed per parasite clone. (G) The interaction of Δ*pbpp5* male gametes with female gametes. (H) Ookinete conversion after crossing Δ*pbpp5* parasites with Δ*p47* and Δ*p45/48* parasites. For all experiments, wild type parasites were used as the control and three mice were used for each group. Results were obtained from at least two independent experiments. n.s., not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

(37/37) among mosquitoes fed on WT *P. berghei*-infected mice (Fig. 4D). The mean oocyst density was 0, 1.4, and 0.1 oocysts/midgut in mosquitoes fed on the three Δ*pbpp5* k1 parasite-infected mice, significantly lower than that of 47.5, 80.8, and 69.9 oocysts/midgut in mosquitoes fed on the three WT *P. berghei*-infected mice ($P < 0.001$, Mann–Whitney *U* test; Fig. 4D). Subsequent dissection of ~35 mosquitoes 18 days after feeding on Δ*pbpp5*-infected mice did not reveal salivary gland sporozoites.

To identify which step(s) during the development of ookinetes was defective in the Δ*pbpp5* parasites, we first compared the gametogenesis process. Whereas the formation of macrogametes appeared normal in the *pbpp5* KO lines (Fig. 4E), the proportion of microgametocytes forming exflagellation centers in the Δ*pbpp5* parasite lines was severely reduced: 13.8–25% exflagellating Δ*pbpp5* male gametocytes in the Δ*pbpp5* lines compared with 88.9% in WT parasites ($P < 0.001$, *t* test; Fig. 4F). To investigate whether the male gametes lacking expression of Δ*pbpp5* could attach to female gametes, we analyzed the interactions between male and female gametes at 30 min after induction of gametogenesis under a light microscope. The results revealed that the proportions of male gametes attached to female gametes were drastically reduced in Δ*pbpp5* parasite lines (mean = 9.26%) compared with those in WT parasites (mean = 70.42%) ($P < 0.01$, *t* test; Fig. 4G).

To ascertain that the block in ookinete formation resulted from defective males, we performed genetic crosses with parasite lines defective in either males or females (Boisson et al., 2011). For this purpose, we generated *Δpbs48/45* and *Δpbs47* parasite lines that are defective in male and female gametes, respectively (Supplementary Fig. S6), using the previously described method (van Dijk et al., 2010). Crossing of Δ*pbpp5* with the *pbs48/45* deletion mutant showed no rescue of the phenotype, whereas crossing with the *pbs47* deletion parasite resulted in 78.4% ookinete formation compared with 88.0% in the WT (Fig. 4H). These results confirmed that PbPP5 was vital for the sexual development with a major effect on male gamete fertility.

3.6. Effect of phosphatase inhibitors on sexual development

To assess the potential of phosphatase inhibitors for blocking malaria transmission, we determined the activities of three PP1/PP2A inhibitors on blocking exflagellation and ookinete conversion. Previous studies identified that okadaic acid not only

inhibits PP1/PP2A, but also PP2B and PP6 (Bialojan and Takai, 1988; Cohen, 1989; Takai et al., 1992; Prickett and Brautigan, 2006), and thus was excluded from the in vitro exflagellation and ookinete conversion inhibition assay. Cantharidic acid, cantharidin and endothall all inhibited exflagellation and ookinete conversion in a concentration-dependent manner (Fig. 5). The specific PP2A inhibitor endothall completely blocked exflagellation of male gametocytes and ookinete conversion at the 1.5 × IC_{50} concentration of the recombinant rPbPP5^{yPP2Ac} protein (Fig. 5E, F).

4. Discussion

Reversible protein phosphorylation is crucial for multiple cellular processes. The protein kinases and phosphatases that catalyze protein phosphorylation and dephosphorylation are well-identified drug targets in various diseases (Cher et al., 2010; Haslbeck et al., 2015; Chen et al., 2017). Similarly, the malaria parasite kinome and phosphatome have been subjects of both bioinformatic and functional studies (Ward et al., 2004; Wilkes and Doerig, 2008; Tewari et al., 2010; Solyakov et al., 2011; Guttery et al., 2014). A recent functional study of the *P. berghei* phosphatome showed approximately half of the 30 predicted phosphatases were essential for asexual intraerythrocytic development (Guttery et al., 2014). However, the functions for phosphatases in sexual stage development are not fully understood. Among these essential phosphatases, PP5 plays important roles in protozoan parasites such as *Toxoplasma*, *Eimeria*, *Leishmania* and *Trypanosoma* (Liu et al., 2005; Golden et al., 2008; Jones et al., 2008; Han et al., 2012; Zhou et al., 2013; Figueras et al., 2014; Norris-Mullins et al., 2014; Feng et al., 2015; Wang et al., 2015). This makes PP5 protein a potential target for novel antimalarial and antiparasitic drugs.

An intriguing finding on the predominant expression of PbPP5 during sexual development and yet essential function of this gene during asation, which is consistent with the recent reports showing that both *pbpp5* and *pfpp5* are dispensable in blood stages (Bushell et al., 2017; Zhang et al., 2018). This discrepancy between the studies of Guttery et al. (2014) and those of Bushell et al. (2017), which is consistent with the recent reports showing that both *pbpp5* and *pfpp5* are dispensable in blood stages (Bushell et al., 2017; Zhang et al., 2018). This discrepancy between the studies of Guttery et al. (2014) and those of Bushell et al. (2017) and our own may be due to the use of different recombination arms. Guttery et al. (2014) used relatively short recombination arms, which may have resulted in reduced recombination efficiency during genetic manipulation. Based on these and our studies, we conclude that *pbpp5* is not essential for asexual blood stages, although we do not know whether this is due to complementation of other phosphatases expressed in asexual blood stages. Thus, the functions of PbPP5 in asexual blood stages warrant further studies to determine its targets during asexual stages and whether the *pbpp5* deletion line is more sensitive to phosphatase inhibitors.

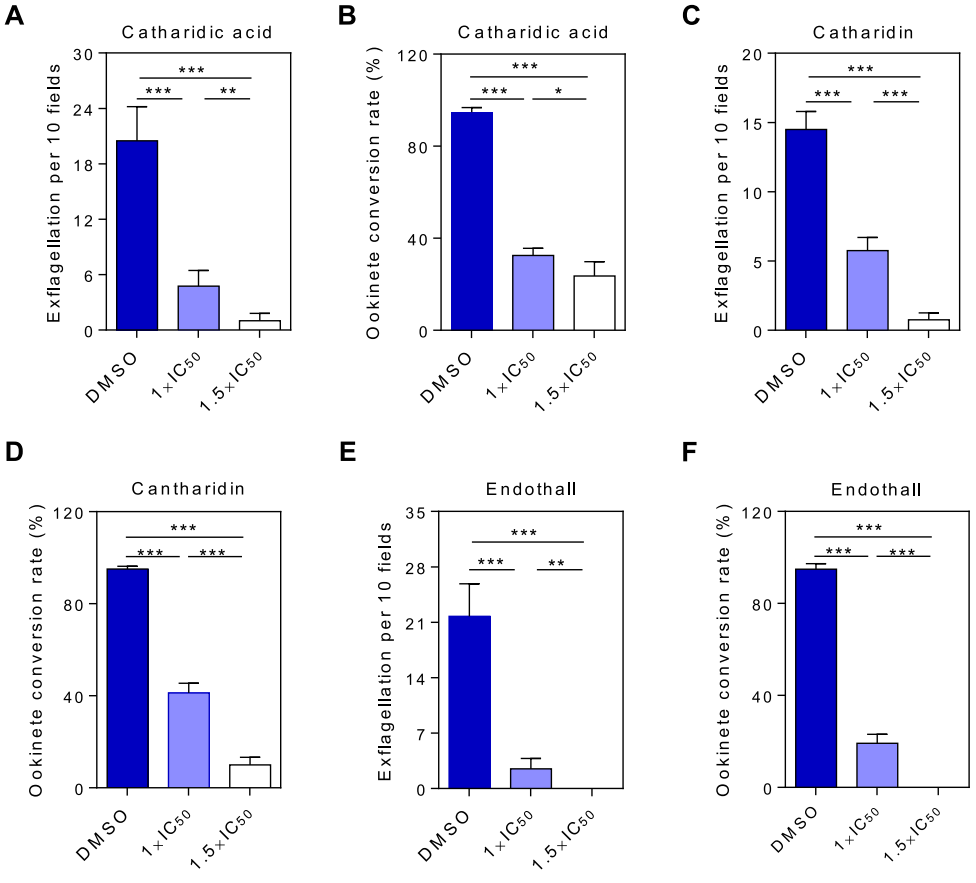

Fig. 5. Effects of protein phosphatase-1 and -2A (PP1/PP2A) inhibitors on gametocyte exflagellation (A, C, E) and ookinete formation (B, D, F). Inhibitors used include cantharidic acid (A, B), cantharidin (C, D) and endothall (E, F). Concentrations used were at 1× and 1.5× of the half maximal inhibitory concentration (IC_{50}) of each individual drug on the recombinant rPbPP5^{yPP2Ac} (yeast system expressed protein phosphatase 2A (PP2Ac) homologues, catalytic domain) domain of recombinant *Plasmodium berghei* protein phosphatase 5 enzyme activity. The conversion rate is the percentage of *Plasmodium berghei* surface protein 21 (Pbs21) -positive parasites that had successfully differentiated into elongated 'banana-shaped' ookinetes. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Interestingly, phenotypic analysis showed that *Δpbpp5* parasites exhibited severe defects in male gamete formation and subsequent interactions with the female gametes, leading to a strong reduction in ookinete formation in vitro and oocyst formation in vivo. The defect in male gametogenesis was further corroborated through genetic crosses with either the female-defective *Δpbs47* or male-defective *Δpbs48/45* lines. Whether the function of PP5 in male gametogenesis and fertility is conserved in *P. falciparum* awaits further investigation. Moreover, strong expression of PbPP5 in ookinetes and possibly sporozoites suggests that PbPP5 may have additional functions in these stages, but these are not possible to dissect with the tools used in this study.

In malaria parasites, the complex pathways of gametogenesis are still not clear, but important pieces of the puzzle have emerged. It has been shown that redundant protein phosphatases and kinases play important roles in controlling gametogenesis (Guttery et al., 2012a, 2014; Fang et al., 2017). In both *P. berghei* and *P. falciparum*, male gametocyte exflagellation is also completely blocked in parasites lacking mitogen-activated kinase-2, a gametocyte-specific mitogen-activated protein kinase (MAPK), which is not expressed in asexual stage parasites (Dorin et al., 1999; Tewari et al., 2005). The phenotypic similarity between *Δmapk2* and *Δpbpp5* knockout lines may imply that these two proteins might be in the same pathway. Although a direct link between PP5 and kinases in regulating gametogenesis has not yet been established, PfPP5 is predicted to interact with several regulatory proteins (Pandey et al., 2014). Interestingly, in mammalian cells, PP5 suppressed apoptosis of pancreatic islets and β-cells by a mechanism that involved p38 MAPK regulation (Fransson et al., 2014), suggesting a potential universal link between PP5 and MAPK. Analogously, a correlation between kinase, the GSK3α levels and spermatogenesis was noted (Bhattacharjee et al., 2018) and a phosphatase CDC14A was found to affect male fertility (Imtiaz et al., 2018). To elucidate the signaling pathways during sexual development, future work should determine the protein–protein interaction network, and substrates of the kinases and phosphatases.

Consistent with a previous study (Guttery et al., 2014), analysis of PbPP5 protein expression confirmed its ubiquitous expression during blood stages, albeit more predominant expression was noticed in sexual stages. The subcellular location of PP5 proteins varies in mammalian and protozoan parasites (Chen et al., 1994; Lindenthal and Klinkert, 2002). Even in *P. berghei*, our data demonstrated that PbPP5 retained a diffused cytosolic localisation in rings, trophozoites, schizonts and gametocytes, whereas it was concentrated at or beneath the parasite plasma membrane in merozoite and ookinete stages. From the localisation study, it is possible that PP5 might be associated with the inner membrane complex (IMC) in the zoite stages, as our protein pull-down work using the PP5-GFP parasites detected proteins present in the IMC and glideosome (data not shown). The differential localisations of PbPP5 in these stages suggest it may have different substrates and functions. Previous reports identifying the interacting proteins of protein phosphatases by using human keratinocyte (HaCaT) cells revealed that PP2Ac interacted with the regulatory subunit (MYPT1) of myosin phosphatase (Becsi et al., 2014). In *Plasmodium*, myosin is a component of the glideosome, and it is located beneath the plasma membrane, where it powers the motility of invasive stages of the parasite life cycle including merozoite, ookinete and sporozoite (Green et al., 2017). The predominant expression of PbPP5 in ookinete stages and its membrane-associated localisation pattern in merozoites and ookinetes suggest that this protein may also participate in parasite mobility other than gamete development, a question open for further investigation.

Efforts to develop new drugs have identified gametocytes as a good drug target to interrupt malaria transmission (Ross and Brancucci, 2018). Here we determined that the PP1/PP2A competitive inhibitors cantharidic acid, cantharidin, endothall and okadaic acid exhibit specific inhibition of the catalytic activity of recombinant PbPP5 (Dounay and Forsyth, 2002). These inhibitors also have significant inhibitory effects on exflagellation and ookinete conversion. Endothall, which has been reported to exhibit greater selectivity for PP2A, could completely block sexual development at 548.3 nM. The structural similarity of *Plasmodium* PP5s to other PP5 proteins suggests that newly identified inhibitors of PP5 proteins such as Ro 90–7501, aurothioglucose, and N-oleoyldopamine may also be effective on PbPP5 (Hong et al., 2017). Due to the sequence similarity between human and parasite PP5 proteins, drug development efforts targeting PP5 need to emphasise selectivity of the compounds on parasites and mammalian cells. Furthermore, due to the structural similarity of the catalytic sites of PPP family phosphatases and the common competitive inhibition mechanism the inhibitors share, the specificity of compounds toward PP5 needs to be verified by observing their inhibitory effects on the other phosphatase members of the PPP family (Swingle et al., 2004; Bertini et al., 2009). The current study may serve as the starting point for designing effective transmission blocking drugs for malaria parasites. Taken together, this study identified a critical role of the protein phosphatase PbPP5 for male gamete fertility in the rodent parasite *P. berghei*, and further emphasises signaling pathways during gametogenesis as potential targets for designing drugs targeting parasite transmission.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2019.03.007.

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