An intracellular membrane protein GEP1 regulates xanthurenic acid induced gametogenesis of malaria parasites

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Gametocytes differentiation to gametes (gametogenesis) within mosquitos is essential for malaria parasite transmission. Both reduction in temperature and mosquito-derived XA or elevated pH are required for triggering cGMP/PKG dependent gametogenesis. However, the parasite molecule for sensing or transducing these environmental signals to initiate gametogenesis remains unknown. Here we perform a CRISPR/Cas9-based functional screening of 59 membrane proteins expressed in the gametocytes of Plasmodium yoelii and identify that GEP1 is required for XA-stimulated gametogenesis. GEP1 disruption abolishes XA-stimulated cGMP synthesis and the subsequent signaling and cellular events, such as Ca\textsuperscript{2+} mobilization, gamete formation, and gametes egress out of erythrocytes. GEP1 interacts with GCα, a cGMP synthesizing enzyme in gametocytes. Both GEP1 and GCα are expressed in cytoplasmic puncta of both male and female gametocytes. Depletion of GCα impairs XA-stimulated gametogenesis, mimicking the defect of GEP1 disruption. The identification of GEP1 being essential for gametogenesis provides a potential new target for intervention of parasite transmission.

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Male and female gametocytes are sexual precursor cells essential for malaria parasite transmission. Within 10–15 min after being taken up by a mosquito, gametocytes differentiate into gametes in mosquito midgut, a process known as gametogenesis. A female gametocyte forms a rounded female gamete, whereas a male gametocyte undergoes three mitotic divisions, assembles eight intracytoplasmic axonemes, and produces eight flagellated male gametes. Both male and female gametes egress from their residing erythrocytes via an inside-out mechanism, during which the parasitophorous vacuole membrane (PVM) ruptures prior to the opening of the erythrocyte membrane (EM). After the release from erythrocytes, the male and female gametes fertilize to produce zygotes and then the motile ookinete that penetrates mosquito midgut wall to develop into oocysts each containing thousands of sporozoites. The sporozoites then migrate to mosquito salivary glands and are injected into a new host when the mosquito bites again.

Gametogenesis is triggered by two stimuli, a drop in temperature of approximately 5 °C and the presence of xanthurenic acid (XA) that is a metabolite of tryptophan from mosquito. An additional signal reported to induce gametogenesis is an increase in pH from 7.4 to 8. Since the groundbreaking discovery of XA as a trigger for Plasmodium gametogenesis in mosquitoes, studies have shown that XA can enhance parasite guanylyl cyclase (GC) activity on gametocyte membrane fraction, leading to increased level of second messenger 3′–5′-cyclic guanosine monophosphate (cGMP). Two integral membrane GC proteins (GCα and GCβ) are found in Plasmodium parasites. GCα has been implicated to be responsible for cGMP synthesis during gametogenesis because disruption of GCβ has no effect on XA-induced gametogenesis. The increased level of cGMP activates cGMP-dependent protein kinase G (PKG) that functions as a master regulator of the downstream signaling events during gametogenesis. Inhibition of PKG using Compound 2 (C2) prevented gametocytes rounding up, gamete formation of both sexes, and gametes egress from erythrocytes in P. falciparum and P. berghei. PKG-dependent Ca2+ mobilization was also observed in the cytosol of P. falciparum and P. berghei gametocytes 10–15 s after addition of XA. PKG activates the synthesis of inositol (1,4,5)-trisphosphate (IP3) via phosphoinositide metabolism and triggers cytosolic mobilization of Ca2+ that likely originates from the endoplasmic reticulum. Unfortunately, the molecule(s) responsible for sensing XA or transducing the XA-stimulated signal to activate the cGMP-PKG signaling remain unknown.

Membrane proteins are known to play critical roles in sensing, transporting, and/or transducing environmental signals to initiate cellular responses. To identify potential molecules involved in sensing or transducing XA signal during gametogenesis, we perform CRISPR/Cas9-mediated genetic deletion screens of 59 candidate genes encoding integral membrane proteins expressed in gametocytes of the rodent malaria parasite P. yoelii. We identify a multiple-spanning membrane protein GEPl (gametogenesis essential protein 1) that was essential for XA-stimulated gametogenesis. Disruption of GEPl completely abolishes XA-stimulated gametogenesis of both sexes. Parasites deficient of GEPl show no synthesis of XA-stimulated cGMP and no downstream cellular and signaling events such as Ca2+ mobilization, parasite egress out of PVM and EM, genome replication and axoneme assembly in male gametocytes, and release of translational repression in female gametocytes. GEPl interacts with GCa in gametocytes, and GCα depletion also impairs XA-stimulated gametogenesis, mimicking the effects of GEPl disruption. This study identifies a molecule essential for the initiation of gametogenesis and a potential target for blocking parasite transmission.

Results

GEPl is essential for XA-stimulated gametogenesis. To identify membrane proteins critical in sensing XA or transducing XA-induced signal during gametogenesis, we identified 59 P. yoelii genes that are expressed in gametocytes and encode proteins with 1 to 22 predicted transmembrane domains (TMs) from the Plasmodb database (Supplementary Table 1). We designed single guide RNA (sgRNA) to disrupt each of these genes using CRISPR/Cas9 methods and were able to successfully knock-out (KO) 45 (76%) of the genes in the P. yoelii 17XNL strain, obtaining at least two cloned lines for each mutant (Supplementary Fig. 1a, c, d, i). The remaining 14 genes (24%) were refractory to repeated deletion attempts using three independent sgRNA sequences, suggesting their essential roles for asexual blood-stage growth.

The 45 gene deletion mutants proliferated asexually in mouse blood normally and were able to produce both male and female gametocytes although the gametocytemia level varied among these mutants (Supplementary Fig. 2, Supplementary Fig. 3a). Next we measured the gametogenesis of male gametocyte by counting exflagellation centers (ECs) formed in vitro after stimulation with 50 μM XA at 22 °C. Only one mutant (PY17X_1116300 disruption) showed complete deficiency in EC formation and male gamete release (Fig. 1a–c). The PY17X_1116300 gene contains four exons (Fig. 1d) encoding a putative amino acid transporter protein that is essential for gametogenesis; we therefore name the gene gep1 for gametogenesis essential protein 1. As controls, disruption of P. yoelii cdk4 or map2 also caused defect in EC formation (Fig. 1a), confirming the phenotypes observed in P. berghei. Consequently, the Agep1 mutant parasite produced no ookinite in vitro culture (Supplementary Fig. 3b), oocyst in Anopheles stephensi midgut (Fig. 1f), or sporozoite in mosquito salivary gland (Supplementary Fig. 3c).

To further confirm the phenotype of Agep1, we generated three additional gep1 mutant parasites (Agep1in1, Agep1fl, and Agep1msscarlet) (Fig. 1d, Supplementary Fig. 1c–e). The Agep1in parasite had a 464 bp deletion at the 5′ coding region, causing a frameshift for the remaining coding region. The Agep1fl parasite had the whole gep1 coding region deleted, and the Agep1msscarlet parasite had its gep1 coding regions replaced with a gene encoding red fluorescent protein mScarlet. These mutations were confirmed by PCR and DNA sequencing (Supplementary Fig. 1j, k), and the mutant parasites displayed developmental phenotypes similar to those of Agep1 in both mouse and mosquito stages (Fig. 1e, f, Supplementary Fig. 3a–c). We also reintroduced the 558 bp deleted segment plus a sxt6p HA epitope (6HA) into the Agep1 parasite to rescue the gene function using Cas9-mediated homologous replacement (Fig. 1d, Supplementary Fig. 1b, j). Two clones of the rescued parasite (Agep1/gep1::6HAc1 and Agep1/gep1::6HAc2) showed expression of the GEPl::6HA protein in both Western blotting and immunofluorescence analysis (IFA) (Supplementary Fig. 3d, e). Importantly, both clones produced wild type (WT) levels of EC in vitro (Fig. 1e) and midgut oocyst in mosquitoes (Fig. 1f). The GEPl protein is well-conserved among P. yoelii, P. berghei, and the human P. falciparum parasites (Supplementary Fig. 4), suggesting conserved function. Deletion of P. berghei gep1 gene (PBANKA_1115100) resulted in parasite clones that failed to form XA-stimulated ECs in vitro and midgut oocyst in mosquitoes (Supplementary Fig. 1l, m, Supplementary Fig. 3f–h). Together, these results demonstrate that GEPl depletion completely block male gametogenesis and mosquito transmission of malaria parasites.

GEPl is expressed in cytosol puncta of gametocytes. GEPl is a Plasmodium-specific protein with 905 residues and 14 predicted
TM (Fig. 2a). Previous transcriptomic study indicated the gep1 gene is transcribed in gametocytes and ookinetes, but not in asexual blood stages of *P. falciparum* and *P. berghei*. To investigate protein expression and localization, we tagged the endogenous MEP1 with 6HA at N-terminus (Supplementary Fig. 1g, j), generating 6HA:gep1 parasite that had normal development throughout the life cycle (Supplementary Fig. 5a). The MEP1 protein is expressed in gametocytes and ookinetes, but not in asexual blood stages and other mosquito stages of the 6HA:gep1 parasite (Fig. 2b, c). We also tagged the GEPI protein with four Myc (4Myc) (Supplementary Fig. 1j, Supplementary Fig. 5b) and observed similar expression pattern in the 4Myc:gep1 parasite (Fig. 2d). In addition, mScarlet fluorescent signals driven by the endogenous gep1 promoter were detected only in
Fig. 2 GEP1 is essential for gametogenesis of both sexes. a Predicted GEP1 protein structure with 14 TM domains (green bar) and cytoplasmic N-termini and C-termini. b IFA analysis of GEP1 expression in asexual blood stages (ABS), gametocytes, ookinetes, oocysts, and sporozoites of the 6HA::gep1 parasite using anti-HA antibody. Hoechst 33342 (Blue) is used for nuclear acid stain for all images in this figure. c Western blot analysis of GEP1 in ABS and gametocytes of the 6HA::gep1 parasite. BiP as loading control. d IFA analysis of GEP1 in the 4Myc::gep1 parasite using anti-Myc antibody. mScarlet fluorescence protein expression driven by the endogenous gep1 promoter in ABS and gametocytes of the Δgep1mScarlet parasite. f Co-staining of GEP1 and α-Tubulin (male gametocyte specific) in the non-activated (NAG) 6HA::gep1 gametocytes. x/y in the figure is the number of cell displaying signal/the number of cell tested. g and h, P28 expression during in vitro gametocyte to ookinete differentiation. P28 expression is detected in female gametes, fertilized zygotes, and ookinetes in IFA (g) and western blot (h). mpa: minute post activation; hpa, hour post activation. i Day 7 midgut oocyst counts from mosquitoes infected with parasites, including 17XNL, Δgep1, Δnek4, or Δmap2 parasite alone, as well as mixtures of Δgep1/Δnek4, Δgep1/Δmap2, or Δmap2/Δnek4 parasites. Δnek4 and Δmap2 are female and male gamete-defect parasites, respectively. x/y on the top is the number of mosquito containing oocyst/the number of mosquito dissected; Mosquito infection prevalence is shown above. Scale bar = 5 μm for all images in this figure. Experiments were independently repeated three times in b, c, d, e, f, g, and two times in i. Two-tailed unpaired Student’s t test in i.
gametocytes, but not in asexual blood stages of the Δgep1mScarlet parasite (Fig. 2e). Co-staining H6A::gep1 gametocytes with anti-α-Tubulin (male gamocyte specific) and anti-HA antibody showed that GEP1 was expressed in both male and female gametocytes (Fig. 2f). Interestingly, GEP1 is not expressed in plasma membrane, but in punctate dots in the cytoplasm of gametocytes and ookinetes (Fig. 2b, d, f).

GEP1 regulates both male and female gametogenesis. Because GEP1 is expressed in both male and female gametocytes, we asked whether GEP1 also regulates the gametogenesis of female gametocytes. P28 protein, a marker for female gamete, is expressed in female gametes, fertilized zygotes, and ookinetes of 17XNL parasite, but not in the Δgep1 parasite 2 h after XA-stimulation (Fig. 2g, h), indicating that GEP1 depletion also causes defect in female gametogenesis. We next performed genetic crosses between Δgep1 and Δmap2 (male gamete-deficient) or Δnek4 (female gamete-deficient) parasites (Supplementary Fig. 1j, Supplementary Fig. 6d, e). No midgut oocyst was observed in mosquitoes from crosses between Δgep1 and Δmap2 parasites (from 8.4% to 7.6%) after XA stimulation (Fig. 3a). Strikingly, agellated male gametes, but not agellated gametes within 10–15 s post stimulation of gametocytes (Fig. 4e), indicating no PVM lysis in stimulated Δgep1 gametocytes (Fig. 3e), while intact PVM was maintained in the Δgep1 gametocytes (Fig. 3b, Supplementary Fig. 1j, k). These results demonstrate that GEP1 depletion affects EM lysis.

XA triggers a cytosolic Ca2+ mobilization event within 10–15 s post stimulation of gametocytes, which is essential for gametocyte formation and EM rupture. We next examined XA-stimulated Ca2+ mobilization in the Δgep1 gametocytes using Fluo-8 probe as described. Fluo-8 did not affect the gametogenesis since WT gametocytes pre-loaded with Fluo-8 could form XA-stimulated ECs (Supplementary Fig. 7a) and responded to A23187, a Ca2+ ionophore, in a dose-dependent manner (Supplementary Fig. 7b). As expected, XA triggered a sharp increase in cytosolic Ca2+ signal in WT gametocytes, reaching maximal levels 10–15 s post stimulation, which resembled the observations in P. berghei using luminescence-based GFP::Aequorin sensor. However, no Ca2+ response was detected in XA stimulated Δgep1 gametocytes (Fig. 3d). Ca2+ mobilization occurred in the Δmap2 gametocytes as MAP2 functions downstream of Ca2+ signal (Supplementary Fig. 7d).

Different from Ca2+-dependent EM rupture, PVM rupture is controlled by a Ca2+-independent mechanism. To study PVM lysis, a parasite line sep1::4Myc was generated by C-terminally tagging a PVM protein SEP1 with 4Myc (Supplementary Fig. 1j). This parasite line developed normally throughout the life cycle (Supplementary Fig. 5e), indicating intact protein function of SEP1::4Myc. We next deleted the gep1 gene in the sep1::4Myc parasite, generating sep1::4Myc/Δgep1 mutant (Supplementary Fig. 1j). IFA showed lysis of Sep1::4Myc-labeled PVM in the sep1::4Myc gametocytes (Fig. 3e), while intact PVM was maintained in the sep1::4Myc/Δgep1 gametocytes 8 min post XA stimulation (Fig. 3e), indicating no PVM lysis in stimulated Δgep1 gametocytes. Together, these results suggest that GEP1 functions upstream of PKG in XA-stimulated signaling cascade (Fig. 3f).

Impaired cGMP synthesis in GEP1 deficient parasite. Because cGMP is the direct upstream signal activating PKG in XA-stimulated gametogenesis, we examined intracellular cGMP synthesis during gametogenesis. Purified gametocytes were stimulated with XA for 2 min, and cGMP levels were measured using an enzyme immunoassay. Strikingly, XA induced a significant increase in cGMP level in WT gametocytes (Fig. 4a), consistent with previous observation in P. falciparum. In contrast, the Δgep1 gametocytes failed to increase cGMP in response to XA stimulation (Fig. 4a). As a control, cGMP response occurred in Δmap2 gametocytes because MAP2 functions downstream of both cGMP and Ca2+ signaling. These results indicate that GEP1 regulates cGMP level, the most upstream intracellular signal known in Plasmodium gametogenesis.

cGMP level is tightly regulated by the opposing actions of cGMP-synthesizing GC and cGMP-hydrolyzing phosphodiesterase (PDE). Inhibition of PDE activity by specific inhibitor Zaprinast (Zap) has been shown to trigger P. falciparum gametogenesis in the absence of XA. Indeed, treatment of WT gametocytes with 100 μM Zap also induced EC counts comparable to those induced by 50 μM XA (Fig. 4b), and gametogenesis stimulated by either XA or Zap could be blocked.

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indeed induce gametogenesis although the underlying mechanism is not
XA and Zap, increasing pH from 7.4 to 8.0 has been reported to
stimulate or Zap inhibition of PDE activity. In addition to
therefore no elevation of cGMP in the
suggest that the GC activity for cGMP synthesis is impaired, and
observed in the control
but not in the
detected signifi-
cGMP level in gametocytes treated with Zap for 2 min and
rin both Ca2+-dependent and Ca2+-independent cellular events of gametogenesis. EM: erythrocyte membrane, PVM: parasitophorus vacuole
membrane, PPM: parasite plasma membrane. x/y in a, c, and e are the number of cell displaying representative signal/the number of cell analyzed. Scale bar = 5 μm for all images in this figure. All experiments in this figure were repeated three times independently with similar results.

by a Plasmodium PKG protein inhibitor C2 (Fig. 4b), consistent
with the established cGMP-PKG signal cascade of
gametogenesis14,15. In contrast, the Δgep1 gametocytes failed to
form ECs after treatment with Zap (Fig. 4b). No EC were
observed in the control Δmap2 gametocytes treated in either XA
or Zap (Fig. 4b). Consistently, we examined the intracellular
cGMP level in gametocytes treated with Zap for 2 min and
detected significant increase in both WT and Δmap2 gametocytes,
but not in the Δgep1 gametocytes (Fig. 4c). Together, these results
suggest that the GC activity for cGMP synthesis is impaired, and
therefore no elevation of cGMP in the Δgep1 gametocytes after
XA stimulation or Zap inhibition of PDE activity. In addition to
XA and Zap, increasing pH from 7.4 to 8.0 has been reported to
induce gametogenesis although the underlying mechanism is not
clear23,24. Treating WT gametocytes with pH 8.0 at 22 °C indeed
induced comparable number of ECs to those induced by XA or
Zap (Fig. 4b), and gametogenesis could be blocked by C2
treatment (Fig. 4b)15, indicating that the signaling stimulated by
pH 8.0 is also cGMP/PKG-dependent. However, pH 8.0
treatment could not induce gametogenesis of the Δgep1
gametocytes, further suggesting impaired activity of cGMP
synthesis in GEP1 deficient parasite (Fig. 4d).

GEP1 interacts and co-localizes with GCa. We next carried out
immunoprecipitation and mass spectrometry experiments to
identify molecules that may interact with GEP1 in gametocytes.
By comparison of peptide signals (hits) between WT and 6HA::
gep1 gametocyte samples from three biological replicates, we
obtained 308 proteins that might interact with GEP1 (Supple-
mental Table 2), including GCa protein that is the enzyme

Fig. 3 GEP1 acts upstream of PKG in the cGMP-PKG-Ca2+ signaling cascade. a α-Tubulin expression and distribution in differentiating male gametocytes from 17XNL, Δgep1 and Δmap2 parasites after XA stimulation. mpa: minute post XA activation. b Flow cytometry analysis of genomic DNA content in XA-stimulated male gametocytes of 17XNL, Δgep1 and Δcdpk4 parasites. The parasites were fixed with 4% paraformaldehyde at indicated time and stained with Hoechst. c Representative images of gametocytes stained by anti-mouse TER119 antibody 0 and 30 min post XA stimulation (mpa). d Flow cytometry detection of cytosolic Ca2+ in gametocytes using Fluo-8 probe. Purified gametocytes were preloaded with Fluo-8, and signals were collected 30 s before addition of XA or DMSO. Black arrows indicate the time for DMSO or XA addition. e Representative IFA images of the sep1::4Myc and sep1::4Myc/Δgep1
gametocytes stained by anti-Myc antibody. f Proposed location of GEP1 in the XA-PKG-Ca2+ signal cascade of gametogenesis. GEP1 depletion causes defect in both Ca2+-dependent and Ca2+-independent cellular events of gametogenesis. EM: erythrocyte membrane, PVM: parasitophorus vacuole membrane, PPM: parasite plasma membrane. x/y in a, c, and e are the number of cell displaying representative signal/the number of cell analyzed. Scale bar = 5 μm for all images in this figure. All experiments in this figure were repeated three times independently with similar results.

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presumably responsible for cGMP synthesis during gametogenesis (Fig. 5a, b)\textsuperscript{8-10}. The \textit{P. yoelii} GC\textalpha has a large protein (3850 amino acids) with 22 TM domains in an N-terminal P4-ATPase-like domain (ALD) and a C-terminal guanylate cyclase domain (GCD)\textsuperscript{34,35}. To study the expression of GC\textalpha in gametocytes, we generated two parasite lines (\textit{gca::6HA and gca::4Myc}) with endogenous GC\textalpha C-terminally tagged with 6HA and 4Myc, respectively (Supplementary Fig. 1j). These parasites developed normally in mouse and mosquito hosts (Supplementary Fig. 5c, d). Similar to GEP1, GC\textalpha was also expressed as cytoplasmic puncta in both male and female gametocytes of the \textit{gca::6HA and gca::4Myc} parasites (Supplementary Fig. 8a). To further confirm the interaction between GEP1 and GC\textalpha, we generated a doubly tagged parasite line, \textit{4Myc::gep1/gca::6HA (DTS1)}, by tagging the endogenous GEP1 with 4Myc in the \textit{gca::6HA} parasite (Supplementary Fig. 1j, Supplementary Fig. 5f-h). Results from immunoprecipitation using anti-Myc antibody indicated that GC\textalpha interacted with GEP1 in cell lysate of the \textit{DTS1} gametocytes (Fig. 5c). We next generated another independent doubly tagged parasite, \textit{6HA::gep1/gca::4Myc (DTS2)} by tagging GC\textalpha with 4Myc in the \textit{6HA::gep1} parasite (Supplementary Fig. 1j, Supplementary Fig. 5f-h) and detected similar interaction between GEP1 and GC\textalpha (Fig. 5d). As a control, no interaction between GEP1 and GC\textbeta was detected in gametocytes of the \textit{4Myc::gep1/gc::6HA (DTS3)} parasite (Supplementary Fig. 8b). These data demonstrate that GEP1 interacts with GC\textalpha in gametocytes. In addition, IFA results from the \textit{DTS1} parasite showed that GEP1 and GC\textalpha are co-localized at cytosome puncta in non-activated gametocytes (Fig. 5e, f). Together, these data suggest that GEP1 co-localizes and binds to GC\textalpha in gametocytes.

**GC\textalpha depletion causes defect in \textit{XA}-stimulated gametogenesis.**

GC\textalpha has been implicated in cGMP synthesis during gametogenesis\textsuperscript{8-10}; however, there has been no direct evidence to support the speculation. We attempted to disrupt the \textit{gca} gene but failed to obtain a GC\textalpha mutant parasite, indicating an essential function in asexual blood stage development, as reported in \textit{P. falciparum} and \textit{P. berghei} previously\textsuperscript{10}. We used a promoter swap method described previously\textsuperscript{36} to replace 1322 bp of endogenous \textit{gca} promoter region with that (1626 bp) of \textit{seral} gene (\textit{PY17X_0305700}) (Fig. 6a, Supplementary Fig. 1h), whose transcripts are expressed in asexual stages, but absent in gametocytes and mosquito stages\textsuperscript{37}. In this editing, a 6HA tag was inserted in frame at the N-terminus of the GC\textalpha coding sequence. Correct modification in two parasite clones of the resulting mutant
parasite gcakd was confirmed by PCR (Supplementary Fig. 1j). The promoter replacement allowed expression of the GCα protein in asexual blood stages at a level comparable with that of another parallelly modified parasite 6HA::gcα (Supplementary Fig. 1j), but significantly reduced GCα protein expression in gametocytes (Fig. 6b, c). Notably, the gcakd parasite completely lost the ability to synthesize cGMP and form ECs after XA stimulation in vitro (Fig. 6d, e). In mosquitoes fed with gcakd parasite-infected mouse blood, no oocyst was detected in mosquito midgut (Fig. 6f). These results support that GCα is the GC responsible for XA-stimulated cGMP synthesis in gametogenesis (Fig. 6g). In addition, the phenotype caused by GCα knockdown in gametocytes resembles that of GEP1 defect.

Compared to the expression of GCα in both male and female gametocytes, GCβ expression was detected in gcβ::6HA female gametocytes only (Supplementary Fig. 8a, lower panel). In

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**Table:**

| Gene_ID   | Unique peptide | Description                                      |
|-----------|----------------|--------------------------------------------------|
| PY17X_1347900 | 21            | Conserved plasmodium protein, unknown function  |
| PY17X_1226000 | 19            | Tyrosine--tRNA ligase, putative                  |
| PY17X_0911700 | 15            | Guanylyl cyclase alpha                            |
| PY17X_1109100 | 15            | Conserved protein, unknown function              |
| PY17X_0404000 | 13            | HAD superfamily protein, putative                |
| PY17X_1114400 | 10            | Deoxyribodipyrimidine photo-lyase, putative      |
| PY17X_0922400 | 9             | Conserved plasmodium protein, unknown function   |
| PY17X_0706700 | 9             | Conserved plasmodium protein, unknown function   |
| PY17X_1221300 | 9             | Oocyst capsule protein cap380, putative          |

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**Fig. 5 GEP1 interacts with GCα in gametocytes.** a Top 10 GEP1 interacting proteins in the gametocytes of the 6HA::gep1 parasite detected by immunoprecipitation and mass spectrometry (MS), including guanylyl cyclase α (GCα) with 15 peptides detected. b MS2 spectrum of a representative peptide of the GCα protein. c Co-immunoprecipitation of Myc::GEP1 and GCα::HA proteins in gametocytes of the double tagged parasite 4Myc::gep1/gcα::6HA (DTS1). IP-Myc, anti-Myc antibody was used. d Co-immunoprecipitation of HA::GEP1 and GCα::Myc proteins in gametocytes of the double tagged parasite 6HA::gep1/gcα::4Myc (DTS2). IP-Myc, anti-Myc antibody was used. e Two-colored IFA of GEP1 and GCα proteins in the DTS1 gametocytes using anti-HA (GCα) and anti-Myc (GEP1) antibodies (left panel). Cross sections (white dash line) of the cells show the co-localization of GEP1 and GCα (right panel). Scale bar = 5 μm. f Pearson coefficient analysis for GEP1 and GCα co-localization shown in e, data are shown as mean ± SD from n = 10 cells measured. Experiments in c, d, and e were repeated three times independently with similar results.
addition, GCβ depletion had no effect on XA-stimulated elevation of cGMP (Supplementary Fig. 8c) and in vitro EC formation (Supplementary Fig. 8d) in gametocytes of the ΔGCβ parasite\(^6\), in agreement with previous reports in *P. falciparum* and *P. berghei*\(^9,33\). These results exclude the involvement of GCβ in XA-stimulated cGMP signaling and gametogenesis.

**GEP1 depletion has no effect on GCα expression and localization.** As GCα and GEP1 interacted with each other and functioned upstream of cGMP signaling, we investigated whether GEP1 depletion would affect the expression and cellular localization of GCα in gametocytes. We deleted gep1 gene in the gca::6HA parasite, generating a gca::6HA/Δgep1 mutant parasite (Supplementary Fig. 1j, Supplementary Fig. 5i, j). GEP1 depletion had no effect on gca mRNA level or GCα protein abundance in gametocytes of the gca::6HA/Δgep1 parasite compared to the parental parasite (Fig. 7a, b). As a control, depletion of CDPK4 had no effect on both mRNA and protein level of GCα either because CDPK4 functions downstream of cGMP signal (Fig. 7a, b). In addition, XA stimulation had no effect on protein abundance of both GEP1 and GCα in gametocytes of the DTS1 parasite (Fig. 7c).

Next, we investigated the effect of XA stimulation in cellular localization of GEP1 and GCα proteins in gametocytes of the 6HA::gep1 or gca::6HA parasite, respectively. Two minutes post XA stimulation, both GEP1 and GCα were expressed as cytoplasmic puncta in activated female gametocytes (Fig. 7d, e). Even 8 min post XA stimulation, both GEP1 and GCα still maintained in cytoplasmic puncta in activated female gametocytes (Supplementary Fig. 9a, b). Strikingly, both proteins were redistributed from cytoplasm to the cell periphery of activated male gametocytes (Supplementary Fig. 9c, d), repeating the results from single color IFA. In activated male gametocytes, eight axonemes are assembled in the cytoplasm and coiled around the enlarged nucleus containing octoploid genome, likely pushing the cytosolic puncta to cell periphery. However, no redistribution of GCα was detected from cytoplasm to cell periphery in the stimulated gca::6HA/Δgep1 male gametocytes (Fig. 7e), which could be explained by no
initiation of gametogenesis caused by GEP1 depletion. To further confirm the observations above, we treated the gametocytes with PKG inhibitor C2 to block the initiation of XA-stimulated gametogenesis. Indeed, no redistribution of either GEP1 or GCα was observed from cytoplasm to the cell periphery in the stimulated male gametocytes of the 6HA::gep1 parasite when both anti-Myc and anti-HA primary antibodies were present (Fig. 7f), indicative of GEP1 and GCα interaction. As a control, no PLA signal was detected in gametocytes of the single tagged gcα::6HA parasite. 2 min post XA stimulation, the PLA signals were detected in cytoplasm of activated female gametocytes but in cell periphery of activated male gametocytes (Fig. 8a), which is consistent with the protein localization in IFA analysis (Fig. 7d, e, Supplementary Fig. 9c). Quantifying the number of PLA signal dots in each cells of gametocytes showed no difference between non-activated and activated gametocytes (Fig. 8b). However, the fluorescence intensity of PLA signal in the XA-activated

Fig. 7 GCα expression and localization in the GEP1-depleted gametocytes. a RT-PCR analysis of gcα transcript in gametocytes of the 17XNL, Δgep1, and Δcdpk4 parasites. b Western blotting detecting GCα protein in gametocytes of the 17XNL, gcα::6HA, gcα::6HA/Δgep1, and gcα::6HA/Δcdpk4 parasites. c Western blotting detecting GEP1 (Myc) and GCα (HA) proteins expression in gametocytes of DTS1 parasite 2 min post XA stimulation. Ctl are control groups without XA stimulation. d Co-staining of GEP1 and α-Tubulin expressions in gametocytes of the 6HA::gep1 parasite 2 min post XA stimulation. NAG: non-activated, AG: XA stimulation. e Co-staining of GCα and α-Tubulin expressions in the gcα::6HA and 6HA::gcα::gep1 gametocytes 2 min post XA stimulation. NAG: non-activated, AG: XA stimulation. f Co-staining of α-Tubulin and HA-tagged GEP1 or GCα expressions in the 6HA::gep1 (upper panel) and gcα::6HA (lower panel) gametocytes 2 min post XA stimulation plus C2 treatment. x/y in d, e, and f are the number of cell displaying representative signal/the number of cell analyzed. Scale bar = 5 μm for all images in this figure. All experiments in this figure were repeated three times independently.

XA stimulation likely enhances the GEP1/GCα interaction. Lastly we asked whether XA stimulation could enhance the interaction between GEP1 and GCα in gametocytes. Proximity Ligation Assay (PLA) is a homogeneous immunohistochemical tool that couples the specificity of ELISA with the sensitivity of PCR, which allows in situ detection of endogenous proteins interaction with high specificity and sensitivity. We performed the PLA to investigate the protein interaction in both non-activated gametocytes and activated gametocytes 2 min post XA stimulation. Robust PLA signals were detected in cytoplasm of the non-activated gametocytes of DTS1 parasite when both anti-Myc and anti-HA primary antibodies were present (Fig. 7f), indicative of GEP1 and GCα interaction. As a control, no PLA signal was detected in gametocytes of the single tagged gcα::6HA parasite. 2 min post XA stimulation, the PLA signals were detected in cytoplasm of activated female gametocytes but in cell periphery of activated male gametocytes (Fig. 8a), which is consistent with the protein localization in IFA analysis (Fig. 7d, e, Supplementary Fig. 9c). Quantifying the number of PLA signal dots in each cells of gametocytes showed no difference between non-activated and activated gametocytes (Fig. 8b). However, the fluorescence intensity of PLA signal in the XA-activated...
The cytosolic cGMP level is balanced by the activities of cGMP-synthesizing GC and cGMP-hydrolyzing PDE10,11,33. That inhibition of PDE activity by inhibitor Zap could trigger gametogenesis in the absence of XA suggests the existence of low and sub-threshold endogenous cGMP level precluding PKG activation and gametogenesis defect, we investigated the position where GEP1 exerts its function in the XA-stimulated signaling cascade during gametogenesis. Previous studies have shown that cGMP enhances exflagellation of *P. berghei* and *P. falciparum*11,41. In addition, XA was shown to increase cGMP synthesis by GC from isolated membrane preparations of *P. falciparum* gametocytes7, suggesting that XA-stimulated gametogenesis is mediated by elevated GC activity and cGMP synthesis. Consistent with these observations, we detected significant increases in cytosolic cGMP level in WT gametocytes 2 min after XA stimulation, but not in Δgep1 gametocytes. GEP1 depletion resulted in impaired cGMP production in response to XA, indicating that GEP1 locates upstream of cGMP in the XA-cGMP-PKG-Ca2+ cascade. Compared with the 10–15 min required for whole process of gametogenesis, XA rapidly triggers a cytosolic Ca2+ mobilization within 10–15 s post stimulation, which was also observed in other studies13. These results suggest that GEP1 functions at an early or initiating step of gametogenesis. Consistently, disruption of gep1 causes defects in all PKG-downstream cellular and signaling events during gametogenesis, including Tubulin polymerization for axoneme assembly, genome replication in male gametocytes, release of P28 translational repression in female gametocytes, PVM and EM rupture for egressing of both male and female gametes from erythrocytes, and Ca2+ mobilization. These results suggest that GEP1 functions upstream of cGMP-PKG-Ca2+ cascade in XA-stimulated gametogenesis.

The cytosolic cGMP level is balanced by the activities of cGMP-synthesizing GC and cGMP-hydrolyzing PDE10,11,33. That inhibition of PDE activity by inhibitor Zap could trigger gametogenesis in the absence of XA suggests the existence of low and sub-threshold endogenous cGMP level precluding PKG activation in gametocytes11,33. Strikingly, the Δgep1 gametocytes not only
failed to initiate XA-stimulated gametogenesis, but also could not undergo Zap-induced gametogenesis. Consistently, we detected no significant Zap-induced elevation of cytosolic cGMP level in the Δgep1 gametocytes as seen in WT gametocytes. These results suggest that GEPI is an essential component of the GC synthesis machinery, and its depletion completely impairs parasite ability to synthesize cGMP, resulting in no accumulation of basal level cGMP in gametocytes.

Two large guanylyl cyclases (GCα and GCβ) for cGMP synthesis are found in *Plasmodium* parasites. GCα and GCβ in *P. yoelii* consist of 3850 and 3015 amino acids, respectively, and both proteins are predicted to have 22 TMs distributed in an N-terminal P4-ATPase-like domain (ALD) and a C-terminal guanylyl cyclase domain (GCD). GC enzymes possessing the ALD/GCD structure are observed in many protozoan species. Whereas the GCD is responsible for cGMP synthesis, the function of the ALD is still obscure. Both *P. berghei* and *P. falciparum* parasites without GCβ can produce functional male gametes. Consistent with these reports, our study also showed deletion of gcb did not affect XA-stimulated cGMP elevation and male gamete formation, confirming that GCβ is not the enzyme for cGMP synthesis during gametogenesis. Using unbiased immunoprecipitation and mass spectrometry analysis, we found that GEPI interacted with GCα and this interaction was confirmed by co-immunoprecipitation and co-localization analyses. Furthermore, we attempted to disrupt the gca gene, but were not able to obtain a viable mutant parasite, consistent with previous reports in other *Plasmodium* species. Alternatively, we generated a mutant parasite with decreased GCα expression in gametocytes. Specific knockdown of GCα in gametocytes blocked XA-stimulated cGMP elevation and the consequent gametogenesis, mimicking the defect of GEPI disruption. These results indicate that GCα is the enzyme for cGMP synthesis in gametogenesis.

Interestingly, GEPI and GCα proteins were expressed as cytoplasmic puncta in female gametocytes either before or after XA stimulation. In the contrast, both proteins were redistributed from cytoplasm to the cell periphery of male gametocytes post XA stimulation. Once gametogenesis is initiated after XA stimulation, eight axonemes are assembled and coiled around the enlarged nucleus containing octaploid genome, possibly occupying most cytoplasmic space and pushing cytoplasmic vesicles, including the GEPI/GCα residing puncta or possible membrane vesicle, to the periphery of the stimulated male gametocytes. Consistent with our observations, Carucci et al. also revealed that GCα displayed a peripheral localization in the *P. falciparum* stimulated gametocytes using immunoelectron microscopy. In addition, these results also suggest that GEPI likely exerts its function in controlling cGMP synthesis by directly binding GCα and regulating GCα conformation because GEPI depletion had no effect in the expression and cellular localization of GCα in gametocytes.

GEPI possesses 14 predicted TM domains, encoding a possible sodium-neurotransmitter symporter or amino acid transporter family protein. Three independent studies recently revealed that the *Toxoplasma gondii* another Apicomplexan parasite, regulates natural egress of tachyzoites from host cell via a guanylate cyclase receptor platform. Similar to *Plasmodium* GCα and GCβ, *T. gondii* guanylate cyclase (TgGC) also possesses the atypical ALD/GCD structure. By crosslinking experiment coupled to immunoprecipitation and mass spectrometry, 55 TgGC-interacting proteins were identified, including a top 5th hit encoding a putative sodium-neurotransmitter symporter family protein. Notably, TGGT1_208420 displays some similarity in protein sequence with GEPI. These results suggest the interaction between GC and sodium-neurotransmitter symporter family protein is conserved in *Plasmodium* and *T. gondii*. Similar to *P. yoelii* GEPI, depletion of this protein does not cause tachyzoite growth defect, suggesting a dispensable role in asexual lytic cycle of *T. gondii* although its function in sexual cycle is unknown. In addition, these studies also identified another *T. gondii* GC-interacting protein UGO that is believed to act as a chaperone. Whether the *Plasmodium* UGO ortholog protein (PY17X_1204500) plays a similar role in the GC machinery remains to be determined.

Based on our results, we proposed a model for GEPI/GCa-mediated cGMP signaling in XA-stimulated gametogenesis. The membrane protein GEPI acts as a binding partner of GCα. In the absence of XA, GEPI supports a functional conformation of GCα that maintains its basal catalytic activity and synthesizes low and sub-threshold endogenous cGMP level precluding PKG activation. In the presence of XA, the stimulation enhances the interaction of GEPI/GCa, leading to enhanced GC activity of GCα and increased cGMP level for PKG activation. In the GEPI-deficient gametocytes, GCα loses catalytic activity of cGMP synthesis and therefore fails to elevate cGMP level in response to XA, post-termination, or environmental pH. Currently, we cannot exclude the possibility that there is an unknown molecule as the XA sensor residing in cytoplasm or plasma membrane and functioning upstream of GEPI/GCa complex. XA-stimulated gametocyte to gamete differentiation in the midgut is the first and essential step for mosquito transmission of malaria parasites, and elucidating the mechanisms involved may facilitate development of measures to block disease transmission.

### Methods

#### Usage and ethics statement
Animal experiments were performed in accordance with the approved protocols (XMULAC20140004) by the Committee for Care and Use of Laboratory Animals of Xiamen University. ICR mice (female, 5 to 6 weeks old) were purchased and housed in the Animal Care Center of Xiamen University and kept at room temperature under a 12 h light/dark cycle at a constant relative humidity of 45%.

#### Mosquito maintenance.
The *Anopheles stephensi* mosquito (strain Hor) was reared at 28 °C, 80% relative humidity and at a 12 h light/dark cycle. Mosquitoes were fed on a 10% sucrose solution.

#### Plasmid construction and parasite transfection.
CRISPR/Cas9 plasmid pYCM was used for all the genetic modifications. For gene deleting, 5′- and 3′-genomic segments (400 to 700 bp) of the target genes were amplified as left and right homologous arms, respectively, using gene specific primers (Supplementary Table 3). The PCR products were digested with appropriate restriction enzymes, and the digested products were inserted into matched restriction sites of pYCM. Oligonucleotides were annealed and ligated into pYCM. For gene deleting, two sgRNAs were designed to disrupt the coding region of a target gene (Supplementary Table 3) using the online program ZiFiTR. For gene tagging, a 400 to 800 bp segment from N-terminal or C-terminal of the coding region and 400 to 800 bp sequences from 5′UTR or 3′UTR of a target gene were amplified and fused with a DNA fragment encoding 6HA or 4MYC in frame at N-terminal or C-terminal of the gene. For each tagging modification, two sgRNAs were designed to target sites close to the C-terminal or N-terminal of the gene coding region. Infected red blood cells (irBC) were electroprogrammed with 5 μg circular plasmid DNA using Lonza Nucleofector. Transfected parasites were immediately injected i.v. into a naive mouse and treated with pyrimethamine (6 μg/ml) in drinking water. Parasites with transfected plasmids usually appear 5 to 7 days post drug selection.

#### Genotype analysis of transgenic parasites.
All transgenic parasites were generated from *P. yoelii* 17XNL strain or *P. berghei* ANKA strain. The schematic for different genetic modifications and the results of parasite transfection, single cloning and genetic verification of modified strains are summarized in Supplementary Fig. 1. Blood samples from infected mice were collected from the orbital sinus, and blood cells were lysed using 1% saponin in PBS. Parasite genomic DNAs were isolated from blood stage parasites using DNeasy Blood kits (QIAGEN). For each parasite, both 5′ and 3′ homologous recombination events were detected using specific PCR primers (Supplementary Fig. 1). PCR products from some modified oligonucleotides were sequenced. All the primers used in this study are listed in Supplementary Table 3. Parasite clones with targeted modifications were obtained after limiting dilution. At least two clones for each gene-modified parasite were
used for phenotype analysis. Parasite growth characteristics in mouse and in mosquito for the modified parasite strains are shown in Supplementary Fig. 5.

**Negative selection with 5-fluorouracil.** Parasites subjected to sequential modifications were negatively selected with 5-Fluorouracil (SFC, Sigma, F6627) to remove episomal plasmid. SFC (2 mg/ml) in drinking water was provided to mice in a dark bottle for 8 days with a change of drug on day 4. Clearance of episomal plasmid in parasites after negative selection was confirmed by checking the parasite survival after reapplying pyrimethamine pressure (6 μg/ml) in new infected mice.

**Gametocyte induction.** ICR mice were treated with phenylhydrazine (80 μg/g, mouse body weight) through intraperitoneal injection. Three days post treatment, the mice were infected with 3.0 × 10⁶ parasites through tail vein injection. Gametocytoma usually peaks at day 3 post infection. Male and female gametocytes were counted via Giemsa staining of thin blood smears. Gametocytoma was calculated as the ratio of male or female gametocyte over parasitized erythrocytes. All experiments were repeated three times independently.

**Male gametocyte exflagellation assay.** Two and a half microliters of mouse tail blood with 4–6% gametocytoma were added to 100 μl exflagellation medium (RPMI 1640 supplemented with 10% fetal calf serum and 50 μM XA, pH 7.4) containing 1 μl of 200 units/ml heparin. After 10 min of incubation at 22 °C, the numbers of EC and RBC were counted in a hemocytometer under a light microscope. The percentage of RBCs containing male gametocytes was calculated from Giemsa-stained smears, and the number of ECs per 100 male gametocytes was then calculated as exflagellation rate. Compound 2 (5 μM) and Zaprinast (100 μM) were added to exflagellation medium with or without XA (for Zaprinast) to evaluate their effects on exflagellation.

**In vitro ookinet elongation.** In vitro culture for ookinet elongation was prepared as described previously. Briefly, mouse blood with 4–6% gametocytoma was collected in heparin tubes and immediately added to ookinet culture medium (RPMI 1640 medium containing 25 mM HEPES, 10% fetal calf serum, 100 μM XA, and pH 8.0) in a blood/medium volume ratio of 1:10. The cultures were incubated at 22 °C for 12 h to allow gametogenesis, fertilization, and ookinet elongation. Ookinet elongation was monitored by Giemsa-staining of culture smears. Ookinet elongation rate was calculated as the number of ookinetes (including mature and immature) per 100 female gametocytes.

**Mosquito feeding and transmission assay.** Thirty female mosquitoes were allowed to feed on an anaesthetized mouse with 4–6% gametocytoma for 30 min. Mosquito midguts were dissected on day 7 post blood-feeding and stained with 0.1% mercurochrome for detection of oocyst. Salivary glands from 20–30 mosquitoes were dissected on day 14 post blood-feeding, and the number of sporozoites in mosquito salivary glands was counted.

**Parasite genetic cross.** Genetic crosses between two different parasite lines were performed by infecting phenylhydrazine pre-treated mice with equal numbers of both parasites. Day 3 pi, 50 female mosquitoes were allowed to feed on mice carrying gametocytes for 30 min. Mosquito midguts were dissected on day 7 post blood-feeding and stained with 0.1% mercurochrome for oocyst counting.

**Gametocyte purification.** Gametocytes were purified using the method described previously. Briefly, mice blood with 4–6% gametocytoma was collected in heparin tubes and immediately added to ookinet culture medium (RPMI 1640 medium containing 25 mM HEPES, 10% fetal calf serum, 100 μM XA, and pH 8.0) in a blood/medium volume ratio of 1:10. The cultures were incubated at 22 °C for 12 h to allow gametogenesis, fertilization, and ookinet elongation. Ookinet elongation was monitored by Giemsa-staining of culture smears. Ookinet elongation rate was calculated as the number of ookinetes (including mature and immature) per 100 female gametocytes.

**Trypan blue staining.** Purified gametocytes were prepared in PBS and mixed with 0.4% trypan blue solution at a 1:9 volume ratio. The mixtures were incubated at room temperature for 5 min and examined under a light microscope.

**Propidium iodo staining.** Purified gametocytes were prepared in PBS and stained with Propidium iodo (PI) at a final concentration of 50 μg/ml. The mixtures were incubated at room temperature for 10 min, washed with PBS twice, and then examined under a fluorescence microscope.

**Flow cytometry analysis.** For measuring DNA content in gametocytes, half of purified gametocytes were immediately fixed and half were transferred to exflagellation medium for gametogenesis for 8 min before fixation. Cells were fixed in 4% paraformaldehyde (PFA) for 20 min, washed in PBS and stained with Hoechst 33342 (0.5 μg/ml) for 30 min. Hoechst fluorescence signal of gametocytes was collected using Novocye 3130 flow cytometer. For detecting GFP and mCherry in gametocytes, the gametocytes were stained with Hoechst 33342 and washed with PBS twice. GFP and mCherry fluorescence signal of gametocytes was collected using BD LSR Fortessa flow cytometer. Cell gating strategies are provided in Supplementary Fig. 11.

**Ca²⁺ mobilization assay using flow cytometry.** Purified gametocytes were washed three times with Ca²⁺ free buffer (CFB, 137 mM NaCl, 4 mM KCl, 20 mM glucose, 20 mM HEPES, 4 mM NaHCO₃, pH 7.2–7.3, 0.1% BSA) and then incubated in CFB containing 5 μM Fluo-8 at 37 °C for 20 min. Fluo-8 loaded gametocytes were washed twice with CFB and suspended in RPMI 1640 for flow cytometer analysis. Fluo-8 fluorescence signal reflecting calcium (Ca²⁺) content in gametocytes was collected using BD LSR Fortessa flow cytometer. Signals were consecutively collected at 30 s before until 90 s post addition of XA (100 μM) or A23187 (0.1 and 1 μM). Cell gating strategies are provided in Supplementary Fig. 11.

**Detection of cellular CGMP.** The assay for measuring cGMP levels in gametocytes was performed using a cyclic cGMP enzyme immunoassay kit (Cayman Chemical, #381021). For each test, more than 1.5 × 10⁶ gametocytes were collected and maintained in GMB buffer on ice. After treatment with 100 μM XA or 100 μM Zap for 2 min, cells were immediately lysed by 0.2 M cold hydrochloric acid on ice for 10 min, vortexed, and passed through a 22-gauge needle. For each replicate, three equal volumes of cell extract from each parasite preparation were parallel tested according to manufacturer’s instructions.

**Antibodies and antisera.** The primary antibodies used were: rabbit anti-HA (Western blot, 1:1000 dilution, IFA, 1:500 dilution) and rabbit anti-Myc (Western blot, 1:1000 dilution) from Cell Signaling Technology; mouse anti-HA (IFA, 1:200) and mouse anti-Myc (IFA, 1:200) from Santa Cruz; mouse anti-a-Tubulin II from Sigma-Aldrich (IFA, 1:1000). The secondary antibodies used were: goat anti-rabbit IgG HRP-conjugated and goat anti-mouse IgG HRP-conjugated secondary antibodies from Abcam (1:5000); the Alexa 555 labeled goat anti-rabbit IgG, Alexa 555 labeled goat anti-mouse IgG, and Alexa 488 labeled goat anti-mouse IgG secondary antibodies from Thermo Fisher Scientific (1:500); Alexa 488 labeled anti-mouse-TER-191 IgG antibody from BioLegend (IFA, 1:1000), biotinylated anti-rabbit IgG (H+L) antibody from Cell Signaling Technology (IFA, 1:1000); Streptavidin-APC from Bioscience (IFA, 1:500). The anti-sera, including rabbit anti-Hep17 (Western blot, 1:1000), rabbit anti-P28 (Western blot, 1:1000, IFA, 1:1000), rabbit anti-BiP (Western blot, 1:1000) were prepared by immunization of synthetic peptides or recombinant protein as described previously.

**Immunofluorescence assays.** Purified parasites or chemical-treated parasites were fixed in 4% PFA and transferred onto a pol-y-L-Lysine pre-treated coverslip. The fixed cells were permeabilized with 0.1% Triton X-100 PBS solution for 7 min, blocked in 5% BSA solution for 60 min at room temperature or 4 °C overnight, and incubated with the primary antibodies diluted in PBS with 3% BSA at 4 °C for 12 h. The coverslip was incubated with fluorescein-conjugated secondary antibodies. Cells were stained with Hoechst 33342, mounted in 90% glycerol solution, and sealed with nail polish. All images were captured and processed using identical settings on a Zeiss LSM 780 confocal microscope.

**Proximity ligation assay.** The PLA assay detecting in situ protein interaction was performed using the kit (Sigma-Aldrich: DUO92008, DUO92001, DUO92005, and DUO82049). Non-activated and activated gametocytes were fixed with 4% PFA for 30 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with a blocking solution overnight at 4 °C. The primary antibodies were diluted in the Duolink Antibody Diluent, added to the cells and then incubated in a humidity chamber overnight at 4 °C. The primary antibodies were removed and the slides were washed with Wash Buffer A twice. The PLUS and MINUS PLA probe were diluted in Duolink Antibody Diluent, added to the cells and incubated in a preheated humidity chamber for 1 h at 37 °C. Next, cells were washed with Wash Buffer A and incubated with the ligation solution for 30 min at 37 °C. Then, cells were washed with Wash Buffer A twice and incubated with the amplification solution for 100 min at 37 °C in the dark. Cells were washed with 1× Wash Buffer B and fixed with 0.1× Wash Buffer B once. Finally, cells were incubated with Hoechst 33342 and washed with PBS. Images were captured and processed using identical settings on a Zeiss LSM 780 confocal microscope.

**Protein extraction and western blotting.** Proteins were extracted from asexual blood parasites and gametocytes using buffer A (0.1% SDS, 1 mM DTT, 50 mM NaCl, 20 mM Tris-HCl, pH 8.0) containing protease inhibitor cocktail and PMSF. After ultrasonication, the protein solution was kept on ice for 15 min before centrifugation at 14,000 × g for 10 min at 4 °C. The supernatant was lysed in Laemmli sample buffer. GE1 protein was separated in 9% SDS-PAGE and transferred to PVDF membrane (Millipore, IPVH00010). GCa and GCp proteins were separated in 4.5% SDS-PAGE.
After incubation, the membrane was washed three times with TBST and incubated with HRP-conjugated secondary antibodies. The membrane was washed five times in TBST before enhanced chemiluminescence detection.

**Immunoprecipitation.** For immunoprecipitation analysis, 6.0 × 10^7 gametocytes were lysed in 1 ml protein extraction buffer A plus (0.01% SDS, 1 mM DTT, 50 mM NaCl, 20 mM Tris-HCl, pH 8.0). After ultrasonication, the protein solution was incubated on ice for 15 min before centrifugation at 14,000 × g for 10 min. Rabbit anti-Myc antibody (1 μg, CST, #2272 s) or Rabbit anti-HA antibody (1 μg, CST, F3724 s) was added to the supernatant, and the solution was incubated on a vertical mixer at 4°C for 15 h. After incubation, 20 μl buffer A plus pre-balanced protein A/G beads (Pierce, #20423) was added and incubated for 5 h. The beads were washed three times with buffer A plus elution with Laemmli buffer.

**Mass spectrometry.** After immunoprecipitation as described above, proteins were eluted twice with 0.3% SDS in 20 mM Tris-HCl (pH 8.0). Eluted proteins were precipitated using 20% trichloroacetic acid (TCA), washed twice with 1 ml cold acetone, and dried in centrifugation vacuum. The protein pellets were dissolved in buffer containing 1% DMSO, 10 mM TCEP, 40 mM CAA, Tris-HCl pH 8.5 and were digested with trypsin (1:100 ratio) at 37°C for 12–16 h after dilution with water to reduce SDS content to 0.5%. Peptides were desalted using SDB-RPS StageTips. For mass spectrometry analysis, tryptic peptides were desalted using C18 ZipTips and dried under vacuum. The dried peptides were reconstituted in 0.1% formic acid and analyzed using Orbitrap Velos Q-TOF mass spectrometer. The data were processed using Proteome Discoverer 1.4, which searched against the Plasmodium falciparum database.

**Bioinformatics analysis and tools.** The genomic sequences of Plasmodium genes were downloaded from the Plasmodium database of PlasmoDB (http://plasmodb.org). Transmembrane domains of proteins were identified using the TMHMM Server [http://www.cbs.dtu.dk/services/TMHMM/]. Multiple sequence alignments were performed by ClustalW in MEGA7.0 [41]. Flow cytometry data were analyzed using FlowJo v10.

**Quantification and statistical analysis.** Statistical analysis was performed using GraphPad Software 8.0. Two-tailed Student’s t-test or Whitney Mann test was used to compare differences between treated groups. P-value in each statistical analysis was indicated within the figures.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The data supporting the findings of this study are available within the paper and its Supplementary Information files or are available from the corresponding author on reasonable request. The source data underlying Figs. 1a, e, f, 3a, 4a–c, 5e–f, 6c–f, 8b–c and Supplementary Figs. 2a, 3a–c, S 2–3, j, 5a–j, 6d–e, 8–d, 9–d, c–d, and 10b–c are provided as a Source Data file.

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Competing interests
The authors declare no competing interests.

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