ISP1-Anchored Polarization of GCβ/CDC50A Complex Initiates Malaria Ookinetes Gliding Motility

Graphical Abstract

Highlights

- GCβ polarization coincides with gliding initiation of mature ookinete
- GCβ polarization elevates cGMP level and activates PKG signaling
- CDC50A binds to and stabilizes GCβ during ookinete development
- Polarization of GCβ/CDC50A complex is anchored by ISP1 at the IMC

Authors
Han Gao, Zhenke Yang, Xu Wang, ..., Xin-zhuan Su, Huiting Cui, Jing Yuan

Correspondence
yuanjing@xmu.edu.cn

In Brief
The upstream mechanism of how the malaria parasites activate cGMP signaling for ookinete gliding remains unknown. Gao et al. reveal that Plasmodium GCβ polarization at "ookinete extrados site" in a precise spatial-temporal manner is the trigger for elevating cGMP level and activating PKG signaling for initiating ookinete gliding motility.
ISP1-Anchored Polarization of GCβ/CDC50A Complex Initiates Malaria Ookinete Gliding Motility

Han Gao, 1,3 Zhenke Yang, 1,3 Xu Wang, 1,3 Pengge Qian, 1 Renjie Hong, 1 Xin Chen, 1 Xin-zhuan Su, 2 Huiting Cui, 1 and Jing Yuan 1,4,*

1State Key Laboratory of Cellular Stress Biology, Innovation Center for Cell Signal Network, School of Life Sciences, Xiamen University, Xiamen, Fujian 361102, China
2Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD 20892, USA
3These authors contributed equally
4Lead Contact
*Correspondence: yuanjing@xmu.edu.cn

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SUMMARY

Ookinete gliding motility is essential for penetration of the mosquito midgut wall and transmission of malaria parasites. Cyclic guanosine monophosphate (cGMP) signaling has been implicated in oookinete gliding. However, the upstream mechanism of how the parasites activate cGMP signaling and thus initiate oookinete gliding remains unknown. Using real-time imaging to visualize Plasmodium yoelii guanylate cyclase β (GCβ), we show that cytoplasmic GCβ translocates and polarizes to the parasite plasma membrane at “oookinete extrados site” (OES) during zygote-to-ookinete differentiation. The polarization of enzymatic active GCβ at OES initiates gliding of matured oookinete. Both the P4-ATPase-like domain and guanylate cyclase domain are required for GCβ polarization and oookinete gliding. CDC50A, a co-factor of P4-ATPase, binds to and stabilizes GCβ during oookinete development. Screening of inner membrane complex proteins identifies ISP1 as a key molecule that anchors GCβ/CDC50A complex at the OES of mature oookinetes. This study defines a spatial-temporal mechanism for the initiation of oookinete gliding, where GCβ polarization likely elevates local cGMP levels and activates cGMP-dependent protein kinase signaling.

INTRODUCTION

The spread of a malaria parasite relies on its successful development in a mosquito vector. Upon entering the mosquito midgut from a blood meal, gametocytes are activated to gametes that fertilize to form round-shaped immotile zygotes. Within 12–20 hr, the zygotes further differentiate into crescent-shaped motile oookinetes that penetrate the midgut epithelium and develop into oocysts, each containing hundreds of sporozoites. Mature sporozoites then invade the salivary glands and infect a new vertebrate host when the mosquito bites again [1]. Gliding motility of malaria parasites is essential for ookinete penetration of mosquito midgut wall and sporozoite migration to salivary gland for transmission from mosquito to vertebrate host. Ookinetes gliding is achieved via a multiple-component protein complex called the glideosome located between parasite plasma membrane (PPM) and the underside of the inner membrane complex (IMC) [2, 3]. The IMC complex consists of flattened vesicles underlying the plasma membrane interconnected with the cytoskeleton and is known to play roles in motility and cytokinesis [2, 3]. A secreted transmembrane adhesion protein, CTRP, connected to actin, serves as an anchor for host cell ligand or extracellular matrix [4]. Mechanical force produced by the actomyosin motor is converted to backward movement of CTRP, generating forward gliding motility that acts as a driving force for invasion of host cells [5]. 3’-5’-cyclic guanosine monophosphate (cGMP), cGMP-dependent protein kinase G (PKG), phosphodiesterase delta (PDEδ), and guanylate cyclase beta (GCβ) have been shown to be crucial for oookinete motility in the rodent malaria parasite Plasmodium berghei [6–8]. Coordinated activities of GCβ (synthesizes cGMP) and PDEδ (hydrolyzes cGMP) regulate cGMP levels that activate PKG, leading to phospholipase C (PLC)/inositol triphosphate (IP3)-mediated Ca2+ release, phosphorylation of multiple proteins in the glideosome, and initiation of oookinete gliding [7–9]. However, how the parasite initiates cGMP signaling upstream of PKG and regulates oookinete gliding remains unknown.

The Plasmodium yoelii parasite encodes two large guanylate cyclases (GCα, 3,850 amino acids [aas] and GCβ, 3,015 aas; Figure S1A) that contain 22 transmembrane (TM) helixes spanning an N-terminal P4-ATPase-like domain (ALD) and a C-terminal guanylate cyclase domain (GCD) [10–12]. The GC enzymes possessing this ALD/GCD structure are observed in many protozoan species (Figure S1B). Whereas the GCD is responsible for cGMP synthesis, the function of the ALD is still obscure [12]. In this study, we show that GCβ is expressed in oookinetes, and its polarization at the oookinete extrados site (OES) is essential for oookinete gliding. Both ALD and GCD are indispensable for GCβ polarization. We also identify a co-factor (CDC50A) that shows OES polarization and may function to stabilize GCβ during oookinete development and gliding. Screening of IMC-related proteins identifies another protein (IMC sub-compartment protein 1 [ISP1]) that anchors GCβ at the OES. This study defines a spatial-temporal mechanism for the initiation of oookinete gliding motility.
Figure 1. Dynamics of GCβ Polarization to a Unique OES and Initiation of Ookinete Gliding

(A) Diagrams of GCβ tagged with a sextuple HA epitope (6HA) (red) at three different locations. GCβ possesses a P4-type ATPase-like domain (ALD) (blue) and a guanylate cyclase domain (GCD) (green). The 6HA is inserted at the C terminus (gcβ::6HAc), between ALD and GCD (gcβ::6HAm), and at the N terminus (gcβ::6HAn), respectively.

(B) Western blotting of tagged GCβ protein in ookinetes. P28 protein as loading control is shown.

(C) IFA detection of GCβ during the life cycle of the gcβ::6HAc parasite. Nuclei are labeled with Hoechst 33342. The scale bars represent 5 μm.

(D) Co-localization of GCβ with proteins of known cellular localizations in ookinetes. ARA1 (apical collar), apical ring associated protein 1; MTIP (glideosome), myosin A tail domain interacting protein; MyoB (apical ring), myosin B; P28, ookinete plasma membrane protein. The scale bar represents 5 μm. The right panel shows the diagram of apical structure of Plasmodium ookinete.

(E) Western blotting of GCβ, P28, and IMC1i (inner membrane complex protein 1i) proteins of the gcβ::6HA/imc1i::4Myc ookinetes treated with PBS, trypsin (Try), or heat-inactivated (HI) trypsin. The left panel shows the predicted topology of GCβ.

(F) IFA of GCβ in ookinetes of three tagged parasite lines with or without Triton X-100 permeabilization. The scale bars represent 5 μm.

(G) IFA of GCβ protein in the ookinete treated with PBS or trypsin. The scale bars represent 5 μm.
RESULTS

GCβ Is Polarized at a Unique Extrados Site in Mature Ookinetes

To dissect the roles of GC proteins in oocYTE gliding, we first investigated the expression of GCα and GCβ in ookinetes. We tagged both GCα and GCβ with a sextupel hemagglutinin (HA) epitope (6HA) (Data S1), using the Cas9 method described previously [13]. GCα was expressed in asexual blood stages and gametocytes, but not in ookinetes, and was not pursued further in this study (Figures S1C and S1D). We tagged GCβ with 6HA at the C or N terminus as well as at the region between the ALD and GCD domains (Figure 1A). Successful tagging was confirmed by both genotypic PCR (Data S1) and western blotting (Figure 1B). All of the gcβ::6HA parasites showed normal progression throughout the life cycle (Table S1). Immunofluorescence assay (IFA) indicated that GCβ protein was expressed in gametocytes, zygotes, and ookinetes and could not be detected in asexual blood stage parasites (Figure 1C). Interestingly, GCβ was localized in the cytoplast of both gametocytes and zygotes but was concentrated at a site posterior to the apical structure in mature ookinetes (Figure 1D). Because of its unique location in ookinetes, we designate the specific location as OES.

To further investigate GCβ localization in ookinetes relative to proteins known to be expressed within specific organelles or locations, we engineered parasite clones with additional proteins tagged with quadruple Myc epitope (4Myc) from the gcα::6HA parasite (Data S1). These proteins included MTIP (glideosome) [14], IMC1i (IMC) [2], ARA1 (apical collar) [15], myosin B (apical ring) [16], and DHHC10 (crystalloid body) [17] (Figures S1E and S1F). GCβ was localized at the extrados area behind the apical collar defined by ARA1 (Figure 1D). Only P28 (plasma membrane) and MTIP showed overlapping localization with GCβ in mature ookinete (Figure 1D). Additionally, GCβ did not co-localize with proteins in cellular organelles, including endoplasmic reticulum (ER), Golgi apparatus, and apicoplast through double staining using antisera targeting BIP, ERD2, and ACP proteins, respectively (Figure S1G). These data show that GCβ is expressed in the cytoplast of gametocytes and zygotes but is polarized at a unique position in mature ookinetes.

GCβ Is Expressed on the PPM of Mature Ookinete with N and C Termini Facing the IMC

The ALD and GCD domains, as well as the inter-domain linker, are predicted to be intracellular (Figure S1A). However, whether GCβ is localized at PPM or IMC remains to be determined. We treated the gcα::6HA/imc1i::4Myc ookinetes with trypsin to digest the extracellular parts of GCβ if it was localized on the PPM surface. Western blotting analysis detected a protein band of ∼240 kDa from PBS- or heat-inactivated trypsin-treated ookinetes, but not in trypsin-treated ookinetes, suggesting surface exposure (Figure 1E). As a control, we also detected digestion of the PPM protein P28, but not the IMC protein IMC1i (Figure 1E). These results indicate that GCβ is localized on the PPM. Additionally, all three 6HA-tagged GCβ could be detected using the anti-HA antibody only after Triton X-100 treatment (Figure 1F), which supports the predicted topology of GCβ (Figure 1E). Interestingly, trypsin treatment did not alter GCβ polarization (Figure 1G), suggesting the existence of other proteins or structures acting to stabilize GCβ at OES.

GCβ Polarization at OES Coincides with Initiation of Ookinete Gliding

The round-shaped immotile zygotes undergo significant morphological changes (stages I–V) to differentiate into crescent-shaped motile oocyttes (Figure 1H, upper panel). To investigate the GCβ’s localization dynamics during oocYTE matura- tion and its relationship with oocYTE gliding, we analyzed GCβ expression from zygote to mature oocytte using in-vitro-cultured gcβ::6HAc parasites. GCβ was distributed in the cytoplast and localized with BIP from zygote (stage I) to retort (stage II; Figure S1H), started to cluster at OES in stage IV retort, and completely polarized to OES of mature oocyttes (stages V; Figure 1H, middle panel). We also isolated parasites from infected mosquito midguts and observed a similar dynamic distribution of GCβ (Figure 1H, lower panel), confirming the in vitro observations. Indeed, the rates of GCβ polarization at OES were almost identical in oocyttes either from mosquitoes or in vitro cultures (Figure 1I). We next isolated the heavy (including plasma membrane and cytoskeleton) and light (including cytoplast) fractions from extracts of retort and mature oocyttes after hydropenic lysis and showed that GCβ could be detected in both fractions of the retorts but only in heavy fraction of mature oocyttes (Figure 1J), supporting GCβ association with plasma membrane in mature oocyttes.

We next quantified GCβ polarization level by calculating fluorescent signals at OES over the whole cell at different stages of oocYTE development (Figure S1I) and measured oocYTE gliding using a Matrigel-based assay [7, 18]. We showed that oocYTE gliding was highly correlated with GCβ polarization at OES (Figure 1K). No stage II and III retorts had gliding motility; stage IV retorts showed some motility (1–3 μm/min) and initial GCβ

See also Figures S1 and S2, Tables S1 and S2, and Video S1.
polarization; and mature ookinetes (stage V) with clear GCβ polarization had acquired normal gliding (5–12 μm/min; Figure 1K).

To capture the dynamics of GCβ polarization and the timing of ookinete gliding initiation, we generated a parasite, gcβ::mScarlet (Data S1), with GCβ C-terminally tagged with mScarlet that had enhanced red fluorescence [19] and allowed tracking GCβ expression in real time. The mScarlet-tagged protein was expressed at OES of mature ookinetes (Figure S2A), and the tagging modification did not affect ookinete gliding (Figure S2B). Real-time tracking the GCβ::mScarlet signals of the developing retorts and ookinetes showed cytoplasmic distribution of GCβ in both the protrusion and the zygote remnant of an immotile stage IV retort (Figure 1L; Video S1). Strikingly, as soon as the majority (~60%) of the GCβ were polarized at OES of mature ookinete, the parasites started gliding (Figure 1L; Video S1), suggesting that accumulation of GCβ to a required level at the OES is the trigger for gliding. Additionally, GCβ polarization at OES was always present as long as an ookinete was moving spirally (Figures S2C and S2D). These observations directly link GCβ polarization at the OES to initiation of ookinete gliding.

**Ookinete Gliding Depends on cGMP Synthesis Activity of GCβ**

We disrupted the gcβ gene in wild-type (WT) and the gcβ::6HAc parasites (Figures S3A–S3C). Parasites without GCβ could develop into ookinetes with normal morphology (Figure S3D) but lost gliding (Figure S3E), oocyst and sporozoite formation in the mosquito, and infectivity to mouse (Figures S3F and S3G). These results confirm that GCβ is essential for ookinete gliding and mosquito transmission, which is consistent with findings in gcβ disrupted P. berghei parasites [5, 8].

To test whether cGMP synthesis activity of polarized GCβ at OES is required for ookinete gliding, we generated GCβ mutant parasites that maintained GCβ OES polarization but lost the ability to synthesize cGMP. Sequences analysis reveal the conserved residues Asn-Thr-Ala-Ser-Arg (NTASR) in the α4 helix of catalytic domain 1 (C1) of GC, which are likely critical for the cyclase to bind its substrate guanosine triphosphate (GTP) [20], and mutations in these residues may reduce or abolish the cyclase activity (Figure S3H). Accordingly, we introduced mutations by replacing “NTASR” with “NKASR” or “AKASA” in the gcβ::6HA parasite, generating GCDm1 and GCDm2 parasites, respectively (Figure S3I). Both mutants showed normal GCβ polarization and expression levels similar to that of gcβ::6HA parasite (Figures 2A and 2B) but had severely impaired ookinete motility (Figure 2C), resembling the phenotype of gcβ disruption (Figure S3E). To further test whether the GC activity for cGMP synthesis results in a loss in ookinetes of these mutants, we utilized a recently developed probe (Green cGull) that for cGMP synthesis results in a loss in ookinetes of these mutants [21]. We episomally expressed a plasmid containing the gene encoding Green cGull and observed basal levels of fluorescent signal in the cytoplasm of WT, Δgcβ, and GCDm2 ookinetes when treated with DMSO (Figures 2D and 2E). The fluorescent signals in WT ookinetes significantly increased after a 20-min treatment with zaprinast, an inhibitor active against Plasmodium PDEs, which degrade cGMP [7], but not in Δgcβ and GCDm2 ookinetes (Figures 2D and 2E). These data not only demonstrate loss of cGMP synthesis activity in mature ookinetes of the Δgcβ and GCDm2 parasites but also show that ookinete gliding depends on the cGMP synthesis activity of GCβ enriched at the OES.

**GCβ Polarization Elevates cGMP Levels and Activates PKG Signaling**

cGMP signals in malaria parasites exert their function via directly binding and activating the master effector, PKG, and thus, transducing signaling downstream [7, 22]. We tagged the endogenous PKG protein with 4Myc and found that PKG maintains evenly cytoplasmic distribution during zygote to ookinete development of both single-tagged pkg::4myc and double-tagged gcβ::6HA/pkg::4myc parasites (Figures 2F and S3J). To test whether PKG is required for GCβ polarization and ookinete gliding, we treated the gcβ::6HA ookinetes with a potent Plasmodium PKG inhibitor, compound 2 (C2) [7]. As expected, C2 treatment completely inhibited ookinete gliding (Figure 2G), confirming the essential role of PKG in ookinete gliding as previously reported in P. berghei [7]. However, C2 treatment had no influence on GCβ polarization in mature ookinetes (Figure 2H).

Balanced activities of GCβ and PDEs are critical for maintaining appropriate cGMP concentration, and changes in protein expression or localization in one of them may affect cGMP levels and downstream PKG signaling. To investigate PDEs expression and localization relative to GCβ, we tagged PDEs with 4Myc to generate pdeδ::4myc parasite (Table S1) and observed the cytoplasmic distribution of PDEδ during the zygote to ookinete differentiation (Figure S3J). Furthermore, we generated a doubly tagged parasite, gcβ::6HA/pdeδ::4myc, by tagging the endogenous PDEδ with 4Myc in the gcβ::6HAc parasite (Data S1). At zygote and retort stages, both proteins were distributed at both zygote remnant and protrusion and mostly co-localized (Figure 2I). In mature ookinetes, PDEδ remained relatively evenly distributed throughout the cytoplasm, whereas GCβ polarized at the OES (Figure 3I). The re-distribution of these two proteins led to local enrichment of GCβ, with higher levels of GCβ over PDEδ at OES (Figure 2J), which could probably create an elevated cGMP level at the OES and drive PKG activation locally (Figure 2K).

**Both ALD and GCD Domains Are Required for GCβ Polarization**

To analyze the role of ALD in GCβ expression or localization, we generated a modified parasite, gcβ::T2A, by introducing the “ribosome skip” T2A peptide (EGRGSLTCDVEENPGP) into the middle linker region in the gcβ::6HAc parasite (Figure 3A). The T2A peptide allows expression of the ALD (residues 1–1,248) and GCD peptides (residues 1,249–3,015) separately. Western blotting detected a protein band (GCD::6HA) smaller than that of GCβ::6HAc protein (Figure 3B), resembling the phenotype of gcβ disruption (Figure S3E). To further test whether the GC activity for cGMP synthesis results in a loss in ookinetes of these mutants, we utilized a recently developed probe (Green cGull) that emits EGFP fluorescence when binding to cGMP [21]. We episomally expressed a plasmid containing the gene encoding Green cGull and observed basal levels of fluorescent signal in the cytoplasm of WT, Δgcβ, and GCDm2 ookinetes when treated with DMSO (Figures 2D and 2E). The fluorescent signals in WT ookinetes significantly increased after a 20-min treatment with zaprinast, an inhibitor active against Plasmodium PDEs, which degrade cGMP [7], but not in Δgcβ and GCDm2 ookinetes (Figures 2D and 2E). These data not only demonstrate loss of cGMP synthesis activity in mature ookinetes of the Δgcβ and GCDm2 parasites but also show that ookinete gliding depends on the cGMP synthesis activity of GCβ enriched at the OES.
and normal gliding (Figure S3E). To further confirm the T2A-mediated separation of ALD and GCD, we generated another parasite gcβ::T2An (Figure S4A), in which ALD and GCD were tagged with the triple V5 epitope (3V5) and 6HA, respectively. Separate expression of ALD and GCD was confirmed on western blot using anti-V5 and anti-HA antibodies, respectively (Figure S4B).

IFTA analysis revealed cytoplasmic distribution for both ALD and GCD with little co-localization (Figure S4C). Like gcβ::T2A, this gcβ::T2An also displayed a defect in ookinete gliding (Figure S4D). Together, these results show that expression of both ALD and GCD together in a single protein is required for GCβ polarization and ookinete gliding.

![Figure 2. GCβ Polarization Elevates cGMP Levels and Activates PKG](image)

(A) IFA analysis of GCβ in mature ookinetes of the GCDm1 and GCDm2 parasites. The upper panel shows the mutations (red) introduced in the GCD. The scale bars represent 5 μm.

(B) Western blotting of GCβ expression in ookinetes of the GCDm1 and GCDm2 parasites. (C) Gliding motility of the GCDm1 and GCDm2 ookinetes. n is the number of ookinetes expressing the Green cGull probe reporter. The fluorescent signals were microscopically monitored in ookinetes without treatment (−) or with DMSO or Zap treatment (+) for 20 min.

(E) Quantification of the fluorescent intensity change (F/F₀) in (D). n is the number of ookinetes tested in each group. The horizontal line shows the mean values.

(F) Two-colored IFA analysis of GCβ and PKG proteins during ookinete development of the gcβ::6HA/pkg::4Myc parasite. The scale bars represent 5 μm.

(G) Ookinete gliding motility of wild-type and parasites treated with DMSO or a potent Plasmodium PKG inhibitor, compound 2 (C2).

(H) IFA analysis of GCβ proteins in mature ookinetes of the gcβ::6HA parasites treated with DMSO or C2. The scale bars represent 5 μm.

(I) Two-colored IFA analysis of GCβ and PDE8 proteins during ookinete development of the gcβ::6HA/pde8::4Myc parasite. The scale bars represent 5 μm.

(J) Protein polarization rate of GCβ and PDE8 at OES of retort and ookinete in (I).

(K) A proposed model of GCβ polarization at OES and initiation of cGMP and PKG-dependent ookinete gliding. In mature ookinetes, GCβ polarizes at OES, and PDE8 remains in the cytoplasm, which breaks cGMP synthesis-hydrolysis balance and increases cGMP levels, activates PKG, and initiates ookinete gliding.

See also Figure S3 and Tables S1 and S2.
P4-ATPase Co-factor CDC50A Co-localizes and Interacts with GCβ

The ALD of GCβ is structurally related to the P4-ATPase proteins, which function as flipases translocating phospholipids, such as phosphatidylserine (PS) from exofacial to cytofacial leaflets of membranes in eukaryotic cells [23, 24]. However, sequence analysis revealed that ALD contains mutations in several conserved functional motifs (Figure S4A–S4H). In addition, saponin treatment, which is expected to impair the PS-lipid component in the membrane via depleting cholesterol [25], did not affect GCβ polarization (Figure S4I). These data suggest that PS-lipid is unlikely the mediator for GCβ polarization.

P4-ATPase interacts with the co-factor protein, CDC50, which is required for trafficking of the complex from ER to plasma membrane and for flipase activity [26] (Figure 4A). A search of the Plasmodium genomes identified three paralogs of cdc50 genes: cdc50a (PY17X_0619700); cdc50b (PY17X_0916600); and cdc50c (PY17X_0514500; Figure S5A). To determine which CDC50 associates with GCβ, we generated parasites with individual CDC50 protein tagged with 6HA: cdc50a::6HA; cdc50b::6HA; and cdc50c::6HA (Figure 4B). Of the three proteins, only CDC50A has polarization at OES similar to GCβ in mature ookinetes (Figure 4B). Notably, CDC50A is exclusively expressed in gametocytes, zygotes, and ookinetes during the parasite life cycle (Figures S5B and S5C) and, similar to GCβ, polarized at OES during zygote to ookinete development (Figure S5D). These observations were reproduced in another independent mScarlet-tagged parasite, 50a::mScarlet (Figure S5E).

Next, we generated two doubly tagged parasites, gcβ::6HA/50a::mCherry and gcβ::6HA/50a::3V5, from the gcβ::6HA parasite (Table S1). Results from these parasites show that GCβ and CDC50A were completely co-localized at the cytoplasm of female gametocytes, zygotes, and retorts and at ookinete OES (Figures 4C and 4D). Furthermore, results from immunoprecipitation using anti-HA antibody indicate that GCβ binds to CDC50A in ookinete lysates of the gcβ::6HA/cdc50a::mCherry parasite (Figure 4E). These data demonstrate that CDC50A co-localizes and binds to GCβ during ookinete development.

Deletion of CDC50A Phenocopies GCβ Deficiency in Ookinete Gliding

We next genetically disrupted the cdc50a gene and showed that, similar to gcβ disruption, Δcdc50a parasites displayed normal asexual blood stage growth, gametocyte formation, and ookinete differentiation (Figures S6A–S6D) but had severe defect in ookinete gliding (Figure 4F). Parasites with gliding defect cannot penetrate the mosquito midgut and produce no oocysts; indeed, no midgut oocyst (day 7) or salivary gland sporozoite (day 14) was detected in the mosquitoes infected with Δgcβ or Δcdc50a parasites (Figures 4G and 4H). To further confirm the phenotype, we deleted gcβ or cdc50a gene in a parasite strain expressing mCherry-labeled P28, 17XNL/P28mCh [27], to investigate early oocyst development (Table S1). Again, these mutant parasites lost ookinete gliding (Figure S6E) and produced no oocyst in mosquitoes (Figure S6F). In mosquitoes infected with these parasites, no early midgut parasites were observed at as early as 36 hr post-blood feeding (Figure S6G). To rule out that the phenotype defects were caused by Cas9 off-target effects, we re-introduced a cdc50a gene with sequence encoding an N-terminal Flag tag back to the endogenous cdc50a locus in the Δcdc50a parasite (Data S1). This complemented parasite (Δ50a/50a) showed proper CDC50A protein expression driven...
Figure 4. CDC50A Mimics GCβ Function in Ookinete Gliding

(A) Diagram of P4-ATPase (green) and CDC50 (red) protein complex in eukaryotes.

(B) Topology and IFA analysis of three CDC50 proteins in ookinetes of P. yoelii: CDC50A (50A); CDC50B (50B); and CDC50C (50C). These endogenous proteins were tagged with a 6HA tag (red rectangle) C-terminally. The scale bars represent 5 μm.

(C) Two-colored IFA analysis of CDC50A and GCβ proteins during gametocyte to ookinete development of the double-tagged gcβ::6HA/cdc50a::mCherry parasite using anti-HA and anti-mCherry antibodies. The scale bars represent 5 μm.

(D) Two-colored IFA analysis of CDC50A and GCβ proteins in ookinetes of the double-tagged gcβ::6HA/cdc50a::3V5 parasite. The scale bar represents 5 μm.

(E) Co-immunoprecipitation assay of GCβ and CDC50A proteins in ookinetes of the gcβ::6HA/cdc50a::mCherry strain (double modified strain [DMS]).

(F) Ookinete gliding motility of the wild-type, Δgcβ, Δ50a, and the complemented Δ50a/50a parasites.

(G) Number of oocysts in mosquito midgut 8 days post-blood feeding. n is the number of mosquitoes tested in each group. The horizontal line shows the mean value of each group. Right panel shows the dissected mosquito midguts stained with 0.5% mercurochrome. The scale bars represent 50 μm.

(H) Western blot of the Flag-tagged CDC50A expression in ookinetes of the complemented Δ50a/50a parasite.

(i) Ookinete gliding motility of the parasites with various combinations of double deletions of gcβ, 50a, pdeδ, and cdpk3 genes.

(j) A proposed model depicting positions of GCβ and CDC50A in cGMP signaling for ookinete gliding.

See also Figures S5 and S6 and Tables S1 and S2.
Figure 5. CDC50A Stabilizes GCβ during Sexual Development
(A) RT-PCR analysis of gcβ and 50a transcripts in gametocytes and ookinetes of the gcβ::6HA and gcβ::6HA/Δ50a parasites. 18s rRNA gene as control is shown.
(B) Western blot of GCβ expression in gametocytes and ookinetes of the gcβ::6HA and gcβ::6HA/Δ50a parasites. The right panel is the quantifications of GCβ band intensity in the blot from three independent experiments.
(C) IFA analysis of GCβ protein in gametocyte (left) and ookinete (right) of the gcβ::6HA and 50a-deleted parasites. Two independent modified strains, gcβ::6HA/Δ50a and Δ50a/gcβ::6HA, were tested. The right panel is quantifications of the fluorescent signal of GCβ.
(D) Western blot of 50a expression in the 50a::6HA and 50a::6HA/Δgcβ parasites. Right panel is the quantification of the results from three independent experiments.
(E) IFA of 50a protein during in vitro ookinete development of 50a::6HA and 50a::6HA/Δgcβ parasites.
(F) Western blot of GCβ and 50a proteins in ookinete of the gcβ::6HA/Δ50a parasite complemented with 3V5-tagged 50a gene from either P. yoelii or P. falciparum.
(G) IFA analysis of GCβ proteins in ookinete of complemented parasites.
(H) Ookinetes gliding motility of the complemented parasites.
(I) A proposed model of CDC50A binding and stabilizing GCβ.

In (C), (E), and (G), scale bars represent 5 μm. See also Tables S1 and S2.

by the endogenous promoter (Figure 4F) and displayed normal ookinete gliding (Figure 4F), oocyst counts (Figure 4G), and infectivity of mice (Figure 4H). Together, these results confirm that loss of the CDC50A protein causes ookinete gliding defect and mosquito transmission blocking.

Four genes (gcβ, cdc50a, pdeδ, and cdpk3) have been shown to affect ookinete gliding. To further investigate the functional relationships of these genes, we generated double knockout (DKO) parasites of gcβ/50a, gcβ/cdpk3, 50a/cdpk3, gcβ/pdeδ, and 50a/pdeδ (Table S1) and compared the effects of these DKO s on ookinete motility with single-gene deletion. The gcβ/50a DKO displayed the similar level of gliding defect with single gene deletion (Figure 4J). Both gcβ/cdpk3 and 50a/cdpk3 DKO showed slight reductions in gliding than the Δcdpk3 (Figure 5J). The Δpdeδ had higher gliding than that of WT, probably due to increased motility with elevated cGMP level; however, DKO parasites (gcβ/pdeδ and 50a/pdeδ) almost completely abolished ookinete gliding (Figure 4J), suggesting that GCβ and CDC50A may function similarly in the signaling upstream of cGMP (without cGMP synthesis, there will be no cGMP for hydrolysis).
Consistent with these observations, zaprinast (Zap) treatment boosted gliding of WT ookinetes, but not with either Δgcβ or Jcdc50a parasite (Figure 4K). Together, these results show that CDC50A serves as a GCβ co-factor, having a similar expression pattern and deletion phenotype to those of GCβ, to regulate cGMP levels in ookinete gliding (Figure 4L).

**CDC50A Stabilizes GCβ during Ookinete Development**

To investigate how CDC50A regulates GCβ, we deleted the cdc50a gene in the gcβ::6HA parasite and generated the gcβ::6HA/Δ50a parasite (Data S1). CDC50A depletion did not affect gcβ mRNA levels in either gametocytes or ookinetes (Figure 5A), ruling out an effect of CDC50A on gcβ transcription. However, an approximately 90% reduction in GCβ protein abundance was observed in both gametocytes and ookinetes of the gcβ::6HA/Δ50a compared to the parental line in both IFA and western blotting analyses (Figures 5B and 5C). As expected, no OES polarization of GCβ occurred in these parasites (Figure 5C). In addition, we generated another parasite, Δ50a/gcβ::6HA, by tagging GCβ in the Δcdc50a parasites (Data S1) and observed the same results (Figure 5C). In contrast, deleting gcβ had no impact on CDC50A protein abundance in gametocytes or ookinetes of the 50a::6HA/Δgcβ line (Figure 5D). Interestingly, CDC50A protein alone did not polarize at OES in the 50a::6HA/Δgcβ ookinete (Figure 5E). These data indicate that CDC50A stabilizes GCβ during gametocyte-zygote-ookinete development, which may explain the similar phenotypic defects in Δgcβ and Δcdc50a parasites but does not carry the signal for directing the protein complex to the OES. Instead, the polarization signal is likely within GCβ as shown above.

CDC50A amino acid sequences display high homology (75% identity) between P. yoelii and human malaria parasite P. falciparum, suggesting conserved functions. To test this, we complemented the gcβ::6HA/Δ50a parasite with the cdc50a gene from the P. falciparum Pfcdc50a or P. yoelii Pycdc50a as control) by episomal expression of the Pfcdc50a or Pycdc50a. CDC50A protein expression was detected in ookinetes of the parasites complemented with either Pfcdc50a or Pycdc50a C-terminally tagged with 3V5 (Figure 5F). Importantly, both proteins successfully restored GCβ expression and polarization in ookinetes (Figures 5F and 5G) and ookinete gliding comparable to that of WT parasite (Figure 5H). Together, these data show that CDC50A may stabilize GCβ protein or play a role in the translation of GCβ mRNA during sexual development and its functions are evolutionarily conserved between P. yoelii and P. falciparum (Figure 5I).

**ISP1 Polarizes and Interacts with GCβ at OES of Mature Ookinete**

GCβ is likely anchored by the molecules at the IMC of mature ookinetes because (1) GCβ polarizes at a curved region of the ookinete (Figure 1D) that is mostly maintained by the IMC [28, 29] and (2) PPM-residing GCβ remains polarized at OES even after trypsin digestion (Figure 1Q). Therefore, we searched putative IMC proteins expressed in ookinetes identified previously [30] and selected 10 genes for protein localization analyses by tagging the protein with 6HA or 4Myc (Figure S7A). Out of 10 proteins, only the ISP1 displayed OES polarization as well as some distribution along the cell periphery in the isp1::6HA ookinete (Figure 6A). We observed the same location of ISP1 in the ookinetes of another tagged parasite—isp1::3V5 (Figure S7B). ISP3, another member of the ISP proteins, distributes along the periphery of ookinete (Figure 6A).

We generated doubly tagged gcβ::6HA/isp1::3V5 parasites by tagging endogenous isp1 with 3V5 in the gcβ::6HA parasite to investigate GCβ and ISP1 expression in the same parasite (Figure 6B; Table S1). ISP1 was expressed and polarized as an elongated dot in early zygotes, became two branches lining the future apical in the retort, and polarized at the OES in mature ookinete (Figure 6B), which is consistent with the observations in P. berghei [31]. Using stochastic optical reconstruction microscopy (STORM), we overlaid GCβ and ISP1 signals at OES and observed overlapping signals at the middle (Figure 6C). Furthermore, we detected the interaction between GCβ and ISP1 in ookinete lysates of the gcβ::6HA/isp1::3V5 parasite using immunoprecipitation (Figure 6D), indicating that GCβ and ISP1 interact with each other.

**GCβ Polarization Is Maintained by ISP1 at the IMC**

ISP1 was reported as an essential gene refractory to deletion in P. berghei asexual blood stages [31]. However, we were able to disrupt the isp1 gene in P. yoelii 17XL using the Cas9 method and obtained three mutant clones from two independent transfections (Data S1). isp1 parasites showed normal asexual blood stages and gametocyte development in mouse, male gametocyte activation, and mature ookinetes with normal morphology (Figures S7C–S7F). However, isp1 disruption caused a slight decrease in conversion rate to mature ookinete (25% in isp1; 51% in WT; Figure S7G). Importantly, the Δisp1 ookinetes with normal morphology showed significantly reduced ookinete gliding (Figure 7A) and oocyst counts in mosquito (Figure 7B).

ISP1 may play a role in anchoring GCβ at the OES. To test this, we deleted the isp1 gene in the gcβ::6HA parasite generating the gcβ::6HA/Δisp1 parasite (Data S1). ISP1 depletion did not affect GCβ protein abundance (Figure S7H) but disrupted GCβ polarization in ~93% of the ookinetes (Figures 7C and 7D); GCβ appeared to be randomly distributed in cytoplasm, at cell periphery, or at the apical region (Figure 7C). Indeed, gcβ::6HA/Δisp1 ookinetes also displayed a severe defect in gliding compared with those of parental gcβ::6HA (Figure 7E). To further confirm the defect, we performed complementation to rescue the defect of the gcβ::6HA/Δisp1 parasite by episomal expression of the 3V5-tagged PyISP1 (from P. yoelii) and PfISP1 (from P. falciparum; Data S1). Both tagged PyISP1 and PfISP1 protein expression were detected in ookinetes of the complemented parasites (Figure 7F), and these complementations successfully restored GCβ polarization (Figures 7D, 7G, and 7H) and ookinete gliding to the gcβ::6HA/Δisp1 ookinetes (Figure 7E), consistent with the high homology (90% identity) in ISP1 protein sequence between P. falciparum and P. yoelii (Figure S7I). In contrast, GCβ depletion in the isp1::3V5 parasite had no impact on the ISP1 dynamics during maturation and final OES polarization during ookinete differentiation (Figure 7I), suggesting that ISP1 itself contains a GCβ-independent signal for OES polarization at mature ookinete.

The ISP1 protein bears two N-terminal cysteine residues for palmitoyl-transferase-mediated palmitoylation modification
DISCUSSION

Using *P. yoelii* as a model, here, we show that GCβ polarization at the ookinete OES is essential for the initiation of ookinete gliding. Why does the GCβ polarization occur only after ookinete maturation? A previous study showed that PDEβ deletion led to a defect in ookinete development and gliding, which could be rescued by additional GCβ disruption or PKG inhibition in *P. berghei* [8]. Premature activation of cGMP and PKG signal caused by PDEβ disruption before ookinete maturation could interfere with the programmed development of ookinetes. These observations not only suggest both GCβ and PDEβ are constitutively active for synthesizing and hydrolyzing cGMP, respectively, during the ookinete development but also suggest that strictly spatial-temporal regulation of cGMP and PKG signaling is required for coordinating ookinete development and gliding. Consistent with this speculation, our results showed that both GCβ and PDEβ were distributed in cytoplasmic membrane structures (mostly ER) and largely co-localized in zygotes and retorts, which likely allow maintenance of a balanced and low level of cGMP throughout the cytoplasm, assuming that all the enzymes are constitutively active. In mature ookinetes, GCβ is polarized at OES but PDEβ remains cytoplasmic. GCβ polarization generates a higher protein ratio of GCβ over PDEβ at OES and likely a higher rate of cGMP synthesis than hydrolysis locally. This locally elevated cGMP may activate the PKG signaling and then initiate the ookinete gliding. The sequential events in this process are supported by direct observations of GCβ polarization at OES and OES localization of both ISP1 and GCβ, indicating that ISP1, with signal for tracking to OES and residing at the IMC, could anchor GCβ at the OES of mature ookinetes.

(Figure S7L), which is critical for its docking to the IMC [32]. We attempted to complement the gcβ::6HA/Δisp1 parasite by episomal expression of the 3V5-tagged ISP1-bearing C7A/C8A mutations (cysteine changed to alanine in both amino acid 7 and 8 positions). The ISP1CTA/CBA::3V5 protein lost palmitoylation modification compared with ISP1WT::3V5 protein (Figure 7J). Consistently, ISP1CTA/CBA::3V5 localized evenly at cytoplasm instead of polarizing at OES (Figure 7K) and failed to rescue the GCβ polarization in the ookinetes of complemented gcβ::6HA/Δisp1 parasite (Figure 7L). Furthermore, treating the developing ookinete of the gcβ::6HA/isp1::3V5 parasite with 2-BMP, a potent inhibitor of protein palmitoylation [33], impaired ookinete differentiation and maturation (Figure S7J) as well as OES localization of both ISP1 and GCβ in ookinetes with abnormal morphology (Figure S7K). Again, these abnormal ookinetes displayed no gliding (Figure S7L). Together, these data indicate that ISP1, with signal for tracking to OES and residing at the IMC, could anchor GCβ at the OES of mature ookinetes (Figure 7M).

By real-time capturing mScarlet-tagged GCβ signals, we clearly showed that ookinetes start to move only when the majority (>60%) of GCβ is clustered at the OES (Figure 1L), providing a mechanism for the initiation of ookinete gliding motility. In addition, we demonstrated that CDC50A, an essential component of P4-ATPase trafficking and activity in other organisms [26], plays an important role in GCβ protein expression and ISP1, an IMC protein, contributes to anchoring GCβ at OES of mature ookinetes.

![Figure 6. ISP1 Polarizes and Interacts with GCβ at OES of Mature Ookinete](image-url)
See also Figure S7 and Tables S1 and S2.

Figure 7. GCβ Polarization Is Maintained by ISP1 at the IMC
(A) Ookinete gliding motility of wild-type and Δisp1 parasites. 
(B) Number of oocysts in the mosquito midguts 7 days post-blood feeding.
(C) IFA analysis of GCβ localization in ookinetes of the gcβ::6HA/Δisp1 parasite. The scale bar represents 5 μm.
(D) Percentage of oocysts showing different localization of GCβ in (C). More than 100 oocysts were analyzed in each group from three independent tests.
(E) Ookinete gliding motility of gcβ::6HA, gcβ::6HA/Δisp1, and the complemented parasites: Pyisp1::3V5 (P. yoelii isp1) and Pfisp1::3V5 (P. falciparum isp1).
(F) Western blot detecting the 3V5-tagged PyISP1 or PfISP1 proteins expression in the complemented parasites.
(G) IFA analysis of GCβ and ISP1 proteins in ookinetes of the complemented parasites. The scale bars represent 5 μm.
(H) Quantification of GCβ polarization rate at OES of ookinetes in (G).
(I) Western blot detection of expression and palmitoylation of ISP1 in the gcβ::6HA/Δisp1 parasite complemented with the 3V5-tagged wild-type ISP1 (ISP1WT::3V5) or ISP1 bearing C7A/C8A mutations (ISP1C7AC8A::3V5). BiP as the loading control is shown.
(J) Two-colored IFA analysis of ISP1 and GCβ proteins in ookinetes of the gcβ::6HA/Δisp1 parasite complemented with ISP1C7AC8A::3V5. The scale bar represents 5 μm.
(K) Percentage of oocysts with GCβ polarization at OES from the gcβ::6HA/Δisp1 parasites complemented with ISP1WT::3V5 or ISP1C7AC8A::3V5. The value is means ± SEM of three independent tests analyzing more than 150 oocysts. Two-tailed t test was used.
(L) A proposed model of the IMC-residing protein ISP1 in anchoring GCβ at OES of mature oocites.

See also Figure S7 and Tables S1 and S2.
and the initiation of ookinete gliding (Figure 1L), although we were not able to detect elevated level of cGMP at OES directly using a cGMP probe reporter Green cGull developed recently [21]. This is likely due to either the extremely fast diffusion property of cytoplasmic cGMP inside the ookinete [34, 35] or limited sensitivity of the probe in detecting cGMP. Further investigation using more sensitive methods is necessary to prove that locally elevated cGMP concentration drives ookinete gliding motility.

In many organisms, from yeast to mammals, CDC50 is a co-factor or chaperon of P4-ATPase proteins that mediates the complex’s cellular trafficking [36]. Disruption of cdc50a dramatically reduced Gcb protein levels in gametocytes and ookinetes and abolished ookinete gliding. Interestingly, the CDC50A protein level is not affected after Gcb deletion, and it alone cannot polarize to OES. These results imply that CDC50A may not contain the signal for trafficking the complex to OES, as reported in other organisms [36, 37]; instead, it may function as a chaperon stabilizing Gcb in Plasmodium, although we cannot rule out that CDC50A could also regulate Gcb at the translational level.

IMC-residing protein ISP1 co-localizes and interacts with Gcb at OES of mature ookinetes, with Gcb distributed at the PPM and ISP1 at the IMC, functioning as an anchor pulling the Gcb complex to OES in mature ookinetes. Consistently, the 93% of ookinetes lost Gcb polarization after ISP1 depletion. However, approximately 7% of ookinetes still maintained Gcb polarization, suggesting that other proteins may participate in anchoring Gcb/CDC50A complex at OES. It is still unknown how the Gcb is “pulled” to ISP1 at OES of mature ookinete, although ISP1 already polarizes in zygote stage (Figure 6B); it is possible that some specific proteins are expressed and direct Gcb/CDC50A to the OES when ookinete is mature or about to mature. Previous studies have shown that biogenesis of the IMC is dependent on vesicular transport by the alveolate-specific GTPase protein, Rab11A and Rab11B, in apicomplexans [38, 39]. Whether Rab11A and Rab11B play a role in translocating the Gcb/CDC50A to the OES requires further investigation.

We propose a model for Gcb polarization-directed cGMP signaling and the initiation of ookinete gliding. (1) From zygote to retort stages, cytoplasmic-distributed Gcb/CDC50A complex and PDE5 maintain a sub-threshold cGMP level precluding PKG activation in the cytoplasm throughout the whole cell, assuming that all the enzymes are constitutively active. (2) Upon ookinete maturation, the Gcb/CDC50A complex translocates to the PPM and is anchored by the IMC-residing ISP1 at OES. (3) The Gcb polarization presumably increases the local cGMP concentration that drives PKG activation and initiates ookinete gliding. Mosquito midgut traversal by ookinetes is a critical limiting step during the malaria transmission, and elucidating the mechanism involved in ookinete gliding could assist the development of interventions for blocking disease transmission.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **CONTACT FOR REAGENT AND RESOURCE SHARING**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Mouse usage and ethics statement
  - Genotypic analysis of transgenic parasites
  - Housing conditions of mosquitoes
  - Culture conditions for in vitro systems
- **METHOD DETAILS**
  - Plasmid construction and parasite transfection
  - Parasite negative selection with 5-Fluorouracil
  - Gametocyte induction in mouse
  - In vitro ookinete culture and purification
  - Mosquito feeding and transmission assay
  - Ookinete motility assay
  - Chemical treatment of ookinetes and gliding motility
  - Plasmid transfection for protein transient expression in ookinetes
  - Antibodies and antiserum
  - Immunofluorescence assays
  - Imaging of live ookinetes using confocal fluorescence microscopy
  - Cellular cGMP detection in ookinetes
  - Cellular phosphatidylserine detection in ookinetes
  - Protein extraction and western blotting
  - Cellular fractionation
  - Immunoprecipitation
  - Detection of protein palmitoylation
  - Bioinformatic searches and tools
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures, two tables, one video, and one data file and can be found with this article online at https://doi.org/10.1016/j.cub.2018.06.069.

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**AUTHOR CONTRIBUTIONS**

H.G. and J.Y. designed the study. H.G., Z.Y., and X.W. generated the modified parasites. X.C. performed the STORM imaging. J.Y. and H.C. supervised the work. X.S., H.G., and J.Y. analyzed the data and wrote the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE                      | SOURCE                                               | IDENTIFIER        |
|------------------------------------------|------------------------------------------------------|-------------------|
| **Antibodies**                           |                                                      |                   |
| Rabbit anti-Flag                          | Sigma-Aldrich                                       | cat# F2555; RRID:AB_796202 |
| Rabbit anti-HA                           | Cell Signaling Technology                           | cat#3724S; RRID:AB_1549585 |
| Mouse anti-HA                            | Cell Signaling Technology                           | cat#2367S; RRID:AB_10691311 |
| Rabbit anti-Myc                          | Cell Signaling Technology                           | cat#2272S; RRID:AB_10692100 |
| Mouse anti-Myc                           | Cell Signaling Technology                           | cat#2276S; RRID:AB_331783 |
| Rabbit anti-mCherry                      | Abcam                                               | cat# ab183628; RRID:AB_2650480 |
| Mouse anti-α Tubulin II                  | Sigma-Aldrich                                       | cat#T6199; RRID:AB_477583 |
| Mouse anti-V5                            | Genescript                                           | cat#A01724-100    |
| Alexa 488 conjugated goat anti-mouse IgG antibody | ThermoFisher Scientific                           | cat#A11001; RRID:AB_2534069 |
| Alexa 488 conjugated goat anti-rabbit IgG antibody | ThermoFisher Scientific                           | cat# A31566; RRID:AB_10374301 |
| Alexa 555 conjugated goat anti-mouse IgG antibody | ThermoFisher Scientific                           | cat#A21422; RRID:AB_141822 |
| Alexa 555 conjugated goat anti-rabbit IgG antibody | ThermoFisher Scientific                           | cat#A21428; RRID:AB_141784 |
| Alexa 555 conjugated goat anti-rat IgG antibody | ThermoFisher Scientific                           | cat#A21434; RRID:AB_141733 |
| Goat anti-mouse IgG HRP-conjugated       | Abcam                                               | cat#ab6789; RRID:AB_955439 |
| Goat anti-rabbit IgG HRP-conjugated      | Abcam                                               | cat#ab6721; RRID:AB_955447 |
| Rabbit anti-P28 serum                    | Prepared in our lab [27]                            | N/A               |
| Rabbit anti-BiP serum                    | Prepared in our lab                                 | N/A               |
| Rabbit anti-ERD2 serum                   | Prepared in our lab                                 | N/A               |
| Rat anti-ACP serum                       | Prepared in our lab                                 | N/A               |
| Rabbit anti-Hep17 serum                  | Prepared in our lab                                 | N/A               |
| **Experimental models: parasite strains**|                                                      |                   |
| P.yoelii 17XNL strain                    | [13]                                                 | N/A               |
| P.yoelii 17XNL/P28M strain               | [27]                                                 | N/A               |
| **Plasmids and vectors**                 |                                                      |                   |
| pYCm Cas9 plasmid                        | [27]                                                 | N/A               |
| PL0019                                   | Malaria Research and Reference Reagent Resource Center | Cat#MRA-788       |
| PL0019-Pysoap-mScarlet                   | This manuscript                                     | N/A               |
| PL0019-Pysoap-BeCyClope::mScarlet        | This manuscript                                     | N/A               |
| PL0019-Pysoap-AnnexinV::mScarlet         | This manuscript                                     | N/A               |
| PL0019-Pycdc50a::3V5 rescue              | This manuscript                                     | N/A               |
| PL0019-Pyisp1::3V5 rescue                | This manuscript                                     | N/A               |
| PL0019-PFisp1::3V5 rescue                | This manuscript                                     | N/A               |
| PL0019-Pysoap-GreencGull                 | This manuscript                                     | N/A               |
| **Chemicals, Peptides, and Recombinant Proteins** |                                                      |                   |
| RPMI 1640 medium liquid                   | Hyclone                                             | cat#SH30809.01B   |
| Fetal Bovine Serum                       | GIBCO                                               | cat#16000044      |
| Xanthurenic acid                         | Sigma-Aldrich                                       | cat#D120804       |
| Matrigel                                 | BD                                                  | cat#356234        |
| Nycodenz                                 | Axis-shield                                         | cat#66108-95-0    |
| Giemsa solution                          | Sigma-Aldrich                                       | cat#GS80          |
| Trypsin                                  | Sigma-Aldrich                                       | cat#T1426         |
| Hoechst 33342                            | ThermoFisher Scientific                             | cat#23491-52-3    |
| Protease inhibitor cocktail              | Medchem Express                                     | cat#HY-K0010      |

(Continued on next page)
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse usage and ethics statement
All animal experiments were performed in accordance with approved protocols (XMULAC20140004) by the Committee for Care and Use of Laboratory Animals of Xiamen University. The ICR mice (female, 5 to 6 weeks old) were purchased from the Animal Care Center of Xiamen University and used for parasite propagation, drug selection, parasite cloning, and mosquito feedings.

Genotypic analysis of transgenic parasites
All transgenic parasites were generated from *P. yoelii* 17XNL strain and are listed in Table S1. Parasite infected blood samples from infected mice were collected from the mouse orbital sinus, and mouse blood cells were lysed using 1% saponin in PBS. Parasite genomic DNAs were isolated from transfected blood stage parasite populations using DNeasy Blood kits (QIAGEN) after washing off hemoglobin and subjected to diagnostic PCR. For each modification, both the 5' and 3' homologous recombination was detected by diagnostic genotype PCR (see Data S1), confirming successful integration of the homologous templates. All the primers used in this study are listed in Table S2. Parasite clones with targeted modifications were obtained after limiting dilution. At least two clones of each gene-modified parasite were used for phenotype analysis.
Housing conditions of mosquitoes
The Anopheles stephensi mosquito (strain Hor) was reared at 28°C, 80% relative humidity and at a 12h light/dark cycle in the standard insect facility. Mosquito adults were maintained on a 10% sucrose solution.

Culture conditions for in vitro systems
Parasite ookinetes were prepared using in vitro culture. 100 μL of infected blood containing gametocytes was obtained from the orbital sinus of infected mouse and mixed immediately with 1 mL ookinite culture medium (RPMI 1640 medium containing 25 mM HEPES, 10% FCS, 100 μM xanthurenic acid, and pH 8.0). The mixture was incubated at 22°C for 12–24 h to allow gametogenesis, fertilization, and ookinite differentiation. Ookinite formation was monitored by Giemsa staining of smears of the cultured cells.

METHOD DETAILS

Plasmid construction and parasite transfection
CRISPR/Cas9 plasmid pYCm was used for parasite genomic modification. To construct the vectors for gene deleting, we amplified the 5' and 3' genomic sequence (400 to 700 bp) of target genes as left and right homologous arms using specific primers (Table S2) and inserted into the restriction sites in pYCm. Oligonucleotides for guide RNAs (sgRNAs) were annealed and ligated into pYCm. For each gene, two sgRNAs were designed to target the coding region of gene (Table S2) using the online program Zifit [46]. To construct the vectors for gene tagging and T2A insertion, we first amplified the C- or N-terminal segments (400 to 800 bp) of the coding regions as left or right arm and 400 to 800 bp from 5'UTR or 3'UTR following the translation stop codon as left and right arm, respectively. A DNA fragment (encoding mCherry, mScarlet, 6HA, 4Myc, or 3V5 tag) was inserted between the left and right arms in frame with the gene of interest. For each gene, two sgRNAs were designed to target sites close to the C- or N-terminal part of the coding region. To construct vectors for site-directed nucleotide mutations, the substitution sites were designed with a restriction site for modification detection and placed in the middle of the homologous arms. Parasite-infected red blood cells (RBC) were electroporated with 5 μg purified circular plasmid DNA using the Lonza Nucleotector. Transfected parasites were immediately intravenously injected into a new mouse and placed under pyrimethamine pressure (provided in drinking water at concentration 6 μg/ml) from day 2 post-transfection. Parasites with transfected plasmids usually appear 5 to 7 days during drug selection.

Parasite negative selection with 5-Fluorouracil
Modified parasites subject for sequential modification were negatively selected to remove episomal pYCm plasmid. 5-Fluorouracil (5FC, Sigma, P6627) was prepared in water at a final concentration of 2.0 mg/ml and was provided to the mice in a dark drinking bottle. A naive mouse receiving parasites with residual plasmid from previous pyrimethamine selection was subjected to SFC pressure for 8 days, with a change of drug at day 4. To estimate the amount of plasmid in the parasite populations, we used two independent primer pairs from the plasmid backbone to amplify the DNAs. All PCR primers used are listed in Table S2.

Gametocyte induction in mouse
ICR mice were treated with phenylhydrazine (80 μg/g mouse body weight) through intraperitoneal injection. Three days post treatment, the mice were infected with 2.0 × 10^6 parasites through tail vein injection. Peaks of gametocytemia usually were observed three days post infection. Male and female gametocytes were counted via Giemsa staining of thin blood smears. Gametocytemia was calculated as the ratio of male or female gametocyte over parasitized erythrocyte. All experiments were repeated three times independently.

In vitro ookinite culture and purification
In vitro culture for ookinite development was prepared as described previously [45]. Briefly, mouse blood with 4%-6% gametocytemia was collected in heparin tubes and immediately added to ookinite culture medium. Parasites were cultured in the medium with a blood/medium volume ratio of 1:10 at 22°C. After 12–24 h culture, the ookinite culture was Giemsa-stained and analyzed for ookinite morphology. Ookinite conversion rate was calculated as the number of ookinetes (both normal and abnormal morphology) per 100 female gametocytes. Ookinetes were purified using ACK lysing method as described previously [46]. Briefly, the cultured ookinetes were collected by centrifugation and transferred into ACK lysing buffer (ThermoFisher Scientific, A1049201) on ice for 8 min. After erythrocytes lysis, the remaining ookinetes were isolated via centrifugation and washed twice with PBS. The ookinetes were examined on the hemocytometer under 40× objective lens for purity and counted. Only the samples with > 80% ookinite purity were used for further biochemical analysis.

Mosquito feeding and transmission assay
For mosquito transmission, thirty female Anopheles stephensi mosquitoes were allowed to feed on an anesthetized mouse carrying 4%-6% gametocytemia for 30 min. For oocyst formation assay, mosquito midguts were dissected on day 7 or 8 post blood-feeding and stained with 0.1% mercurochrome for oocyst counting. For salivary gland sporozoite counting, salivary glands from 20–30 mosquitoes were dissected on day 14 post blood-feeding, and the number of sporozoites per mosquito was calculated.
For sporozoite infection of mice, 15–20 infected mosquitoes were allowed to bite one anesthetized naive mouse for 30 min. The time for parasite emerging in mouse peripheral blood circulation after the bite was considered as prepatent time.

**Ookinete motility assay**

Ookinete gliding motility was evaluated as previously described [8]. All procedures were performed in a temperature-controlled room with 22°C. Briefly, 20 μL of the suspended ookinete cultures were mixed with 20 μL of Matrigel (BD, #356234) on ice. The mixtures were transferred onto a slide, covered with a coverslip, and sealed with nail varnish. The slide was placed at 22°C for 30 min before observation under microscope. After tracking a gliding ookinete under microscopic field, time-lapse videos (1 frame per 20 s, for 20 min) were taken to monitor ookinete movement using a 40× objective lens on a Nikon ECLIPSEE100 microscope fitted with an ISH500 digital camera controlled by ISCapture v3.6.9.3N software (Tucson). Time-lapse movies were analyzed with Fiji software and the Manual Tracking plugin. Motility speed was calculated by dividing the distance an ookinete moved by the time it took. All experiments were repeated three times independently.

**Chemical treatment of ookinetes and gliding motility**

To evaluate the effects of chemical treatment on ookinete development and GCji protein localization, chemicals were added to developing ookinete cultures at variable times, and the cultures were collected for Giemsa staining or IFA analysis. Compound 2 (5 μM C2) targeting *Plasmodium* PKG [7], 2-BMP (100 μM) inhibiting *Plasmodium* DHHCs, or 0.1% saponin were used in this study. For the effects of chemical treatment on ookinete gliding motility, 5 μM C2 or 100 μM zaprinast (zap) inhibiting *Plasmodium* PDEs were added to the mixture containing both ookinete culture and Matrigel before gliding motility assay. All experiments were repeated three times independently.

**Plasmid transfection for protein transient expression in ookinetes**

Transient expression of proteins in ookinetes via plasmid episome was as described with minor modifications [45]. Coding sequence of target proteins with appropriate 5’- and 3’-UTR regulatory regions were inserted into the pL0019-derived vector with human dhfr marker for pyrimethamine selection. Briefly, blood stage parasites were electroporated with 10 μg plasmid DNA and selected with pyrimethamine (70 μg/ml) for 7 days. Meanwhile, another group of ICR mice were treated with phenylhydrazine for 3 days through intraperitoneal injection. The phenylhydrazine-treated mice were infected with 2.0 × 10^6 drug-selected parasites through intravenous injection and further selected for another 3–4 days until peak gametocytemia was reached. The high-level gametocytemia blood was collected for ookinete culture and further tests.

**Antibodies and antiserum**

The primary antibodies used were: rabbit anti-HA (western, 1:1000 dilution, IFA, 1:500 dilution), mouse anti-HA(IFA, 1:500), rabbit anti-MyC (western, 1:1000), and mouse anti-MyC (IFA, 1:500) from Cell Signaling Technology, mouse anti-αTubulin II (Sigma-Aldrich) (IFA, 1:1000), mouse anti-V5 (Genescript)(western, 1:1000, IFA, 1:500), rabbit anti-mCherry (Abcam) (western, 1:1000, IFA, 1:500), Rabbit anti-Flag (Sigma-Aldrich,) (western, 1:1000). The secondary antibodies used were: goat anti-rabbit IgG HRP-conjugated and goat anti-mouse IgG HRP-conjugated secondary antibody from Abcam (1:5000), the Alexa 555 goat anti-rabbit IgG, Alexa 488 goat anti-rabbit IgG, Alexa 555 goat anti-mouse IgG, Alexa 488 goat anti-mouse IgG, and Alexa 555 goat anti-rat IgG secondary antibody from ThermoFisher Scientific(1:500). The anti-serums, including the rabbit anti-Hep17(western, 1:1000), rabbit anti-P28(western, 1:1000, IFA, 1:1000), rabbit anti-BiP(western, 1:1000, IFA, 1:500), rat anti-ACP(IFA, 1:100), and rabbit anti-ERD2(IFA, 1:500) were prepared in the Lab.

**Immunofluorescence assays**

Purified parasites were fixed using 4% paraformaldehyde and transferred to a Poly-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, and incubated with the primary antibodies diluted in 3% BSA-PBS at 4°C for 12 h. The coverslip was incubated with fluorescent conjugated secondary antibodies for 1 h. 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. Stochastic optical reconstruction microscopy (STORM) imaging was acquired using a Nikon N-STORM 5.0 Super-Resolution Microscope System.

**Imaging of live ookinetes using confocal fluorescence microscopy**

Developing ookinetes (20 μl) of gcji:mScarlet parasite from 8 to 12 hr cultures were mixed with 20 μL of Matrigel thoroughly. The mixtures were transferred onto a slide, covered with a coverslip, and sealed with nail varnish. The developing ookinetes were monitored under a Zeiss LSM 780 confocal microscope. Stage IV live ookinetes were monitored and fluorescent signals were tracked and recorded.

**Cellular cGMP detection in ookinetes**

Cellular cGMP detection was conducted using the Green-cGull probe as described previously [21] with minor modifications. The coding region of Green-cGull protein driven by 1.5 kb Pysoap 5’-UTR and 1.0 kb Pbdhfr 3’-UTR was inserted to pL0019-derived plasmid containing human dhfr marker for pyrimethamine selection. Briefly, blood stage parasites were electroporated with 10 μg...
plasmid DNA and selected with pyrimethamine (70 μg/ml) for 7 days. Ookinetes from 12 to 24 hr in vitro cultures were enriched by centrifugation and resuspended in 1% low-melting agarose (Sigma-Aldrich, A9414) to avoid cell movement during detection. The mixtures were transferred to the bottom of 15 mm glass-bottom cell culture dish (Corning, #801002) and overlaid with RPMI 1640 medium. Using a Zeiss LSM 780 confocal microscope, the fluorescent signals of Green-cGull were monitored in 30 randomly chosen ookinetes for their basal fluorescence (F0) (collected before treatment) and enhanced fluorescence (F) collected 20 min post zaprinast treatment respectively. cGMP response was calculated as the ratio of F/F0.

**Cellular phosphatidylserine detection in ookinetes**
To detect the phosphatidylserine (PS) on the outer leaflet of plasma membrane of ookinetes, Annexin V-FITC assay kit (Abcam, ab14085) was used according to the manufacturer’s instructions. To detect the PS on the inner leaflet of plasma membrane of ookinetes, a sequence encoding human Annexin V tagged with mScarlet driven by 1.5 kb Pysoap 5'-UTR and 1.0 kb Pbdhfr 3'-UTR was inserted to pL0019-derived plasmid containing human dhfr marker for pyrimethamine selection. Briefly, blood stage parasites were electroporated with 10 μg plasmid DNA and selected with pyrimethamine (70 μg/ml) for 7 days. Ookinetes from transfected parasites were prepared from in vitro culture. Both Annexin V-mScarlet and mScarlet expressed ookinetes were treated with 1 μM A23187, and the cytoplasmic distribution and intensity of the fluorescent signal was monitored using a Zeiss LSM 780 confocal microscope.

**Protein extraction and western blotting**
Protein extraction from asexual blood parasites, gametocytes, zygotes, retorts, and ookinetes was performed using buffer A (0.1% SDS, 1mM DTT, 50 mM NaCl, 20 mM Tris-HCl; pH 8.0) containing protease inhibitor cocktail and PMSF. After ultrasonication, the protein solution was incubated on ice for 30 min before centrifugation at 12,000 g for 10 min at 4°C. The supernatant was lysed in Laemml sample buffer. GCβ was separated in 4.5% SDS-PAGE and transferred to PVDF membrane (Millipore, IPVH00010). The membrane was blocked in 5% skim milk TBST buffer and incubated with primary antibodies. After incubation, the membrane was washed three times with TBST and incubated with HRP-conjugated secondary antibodies. The membrane was washed four times in TBST before enhanced chemiluminescence detection.

**Cellular fractionation**
Cellular fractionation was conducted as described previously with minor modifications [47]. The purified retorts and ookinetes were ruptured in the hypotonic buffer (10 mM HEPES, 10 mM KCl, pH 7.4) after passing through a 1 mL syringe needle gently ten times. Total cell lysate were centrifuged for 15 min at 1,000 g, and the supernatant (light fraction, including cytoplasm and cytosol vesicles) and the pellet (heavy fraction, including plasma membrane, IMC, and cytoskeleton) were collected respectively and solubilized in Laemmli buffer for 10 min on ice. The solubilized protein samples were analyzed by western blotting.

**Immunoprecipitation**
For immunoprecipitation analysis, 1.0-2.0 x 10⁶ ookinetes 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) and centrifuged at 12,000 g for 10 min at 4°C before collecting the supernatant solution. Rabbit anti-HA antibody (1 μg, CST, #3724S) was added to the protein solution and incubated at 4°C for 12 h on a vertical mixer. After incubation, 20 μL buffer A plus pre-balanced protein A/G beads (Pierce, #20423) was added and incubated for 2 h. The beads were washed three times with buffer A plus before elution with Laemmli buffer.

**Detection of protein palmitoylation**
The palmitoylation modification of ISP1 protein was performed using Acyl-RAC assay described previously [48]. Ookinetes were lysed in DHHC Buffer B (2.5% SDS, 1 mM EDTA, 100 mM HEPES, pH 7.5) containing protease inhibitor cocktail and PMSF and incubated on ice for 30 min. After centrifugation at 12,000 g for 10 min, supernatant was collected and treated with 0.1% methyl methanethiosulfonate (MMTS) at 42°C for 15 min. MMTS was removed by acetone precipitation followed by washing with 70% acetone three times. Protein samples were solubilized in DHHC Buffer C (1% SDS, 1 mM EDTA, 100 mM HEPES, pH 7.5) and were captured on thiopropyl Sepharose 6B (GE Healthcare, 17-0402-01) in the presence of 2 M hydroxyamine or 2 M NaCl (negative control) by agitating for 3 h at room temperature. Loading controls (Input) were collected before addition of thiopropyl Sepharose 6B beads. After five times washing with urea DHHC Buffer (1% SDS, 1 mM EDTA, 100 mM HEPES, 8 M urea, pH 7.5), the captured proteins were eluted from thiopropyl Sepharose 6B beads in 60 μl urea DHHC Buffer supplemented with 50 mM DTT, and mixed with Laemmli sample buffer for further western blot analysis.

**Bioinformatic searches and tools**
The genomic sequences of target genes were downloaded from PlasmoDB database. The transmembrane domains of proteins were identified using the TMHMM Server (http://www.cbs.dtu.dk/services/TMHMM/) [41]. The phylogeny tree and protein amino acid sequence alignment was analyzed using MEGA5.0 [42].
QUANTIFICATION AND STATISTICAL ANALYSIS

For quantification of protein expression in western blot, protein band intensity was quantified using Fiji software from three independent experiments. The signals of target proteins were normalized with that of control proteins. For quantification of protein expression in IFA, confocal fluorescence microscopy images were acquired under identical parameters. Fluorescent signals were quantified using Fiji software [43]. More than 30 cells were randomly chosen in each group. Protein expression was expressed as the relative percentage compared to control group. Protein polarization rate was calculated as the ratio of the protein fluorescent signal at OES over the fluorescent signal from the whole cell. Statistical analysis was performed using GraphPad Software 5.0 [44]. Two-tailed Student’s t test or Whitney Mann test was used to compare differences between treated groups and their paired controls. n represents the number of mosquitos or parasite cells tested in each group, or experimental replication. The exact value of n was indicated within the figures. p value in each statistical analysis was also indicated within the figures.