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, *]

[1] State Key Laboratory of Cellular Stress Biology, Innovation Center for Cell Signal Network, School of Life Sciences, Xiamen University, Xiamen, Fujian 361102, China

[2] Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

[3] These authors contributed equally

[4] Lead Contact

* Correspondence: yuanjing@xmu.edu.cn

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 ookinete gliding. However, the upstream mechanism of how the parasites activate cGMP signaling and thus initiate ookinete 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 "ookinete extrados site" (OES) during zygote to ookinete differentiation. The polarization of enzymatic active GCβ at OES initiates gliding of matured ookinete. Both the P4-ATPase-like domain and guanylate cyclase domain are required for GCβ polarization and ookinete gliding. CDC50A, a co-factor of P4-ATPase, binds to and stabilizes GCβ during ookinete development. Screening of inner membrane complex proteins identifies ISP1 as a key molecule that anchors GCβ/CDC50A complex at the OES of mature ookinetes. This study defines a spatial-temporal mechanism for the initiation of ookinete 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 hours, the zygotes further differentiate into crescent-shaped motile ookinetes 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

51 is essential for ookinete penetration of mosquito midgut wall and sporozoite migration
52 to salivary gland for transmission from mosquito to vertebrate host. Ookinete gliding is
53 achieved via a multiple-component protein complex called the glideosome located
54 between parasite plasma membrane (PPM) and the underside of the inner membrane
55 complex (IMC)[2, 3]. The IMC complex consists of flattened vesicles underlying the
56 plasma membrane interconnected with the cytoskeleton and is known to play roles in
57 motility and cytokinesis [2, 3]. A secreted trans-membrane adhesion protein, CTRP,
58 connected to actin, serves as an anchor for host cell ligand or extracellular matrix[4].
59 Mechanical force produced by the actomyosin motor is converted to backward
60 movement of CTRP, generating forward gliding motility that acts as a driving force for
61 invasion of host cells[5]. 3'-5'-cyclic guanosine monophosphate (cGMP), cGMP-
62 dependent protein kinase G (PKG), phosphodiesterase delta (PDEδ), and guanylate
63 cyclase beta (GCβ) have been shown to be crucial for ookinete motility in the rodent
64 malaria parasite *Plasmodium berghei*[6-8]. Coordinated activities of GCβ (synthesizes
65 cGMP) and PDEδ (hydrolyzes cGMP) regulate cGMP levels that activate PKG, leading
66 to PLC/IP3-mediated Ca^{2+} release, phosphorylation of multiple proteins in the
67 glideosome, and initiation of ookinete gliding[7-9]. However, how the parasite initiates
68 cGMP signaling upstream of PKG and regulates ookinete gliding remains unknown.
69
70 The *Plasmodium yoelii* parasite encodes two large guanylate cyclases (GCα, 3850 aa
71 and GCβ, 3015 aa) (Fig. S1A) that contain 22 transmembrane (TM) helixes spanning
72 an N-terminal P4-ATPase-like domain (ALD) and a C-terminal guanylate cyclase
73 domain (GCD)[10-12]. The GC enzymes possessing this ALD/GCD structure are
74 observed in many protozoan species (Fig. S1B). While the GCD is responsible for
75 cGMP synthesis, the function of the ALD is still obscure[12].

76

77 In this study, we show that GCβ is expressed in ookinetes and its polarization at the
78 ookinete extrados site (OES) is essential for ookinete gliding. Both ALD and GCD are
79 indispensable for GCβ polarization. We also identify a co-factor (CDC50A) that shows
80 OES polarization and may function to stabilize GCβ during ookinete development and
81 gliding. Screening of IMC-related proteins identifies another protein (IMC sub-
82 compartment Protein 1, ISP1) that anchors GCβ at the OES. This study defines a
83 spatial-temporal mechanism for the initiation of ookinete gliding motility.

84

85 **Results**

86 **GCβ is polarized at a unique extrados site in mature ookinetes**

87 To dissect the roles of GC proteins in ookinete gliding, we first investigated the
88 expression of GCα and GCβ in ookinetes. We tagged both GCα and GCβ with a sextuple
89 HA epitope (6HA) (Data S1), using the Cas9 method described previously [13]. GCα
90 was expressed in asexual blood stages and gametocytes, but not in ookinetes, and was
91 not pursued further in this study (Fig. S1C and S1D). We tagged GCβ with 6HA at the
92 C- or N-terminus as well as at the region between the ALD and GCD domains (Fig.
93 1A). Successful tagging was confirmed by both genotypic PCR (Data S1) and western
94 blotting (Fig. 1B). All of the *gcβ::6HA* parasites showed normal progression throughout
95 the life cycle (Table S1). Immunofluorescence assay (IFA) indicated that GCβ protein
96 was expressed in gametocytes, zygotes, and ookinetes and could not be detected in
97 asexual blood stage parasites (Fig. 1C). Interestingly, GCβ was localized in the
98 cytoplasm of both gametocytes and zygotes, but was concentrated at a site posterior to
99 the apical structure in mature ookinetes (Fig. 1D). Because of its unique location in
100 ookinetes, we designate the specific location as ookinete extrados site (OES).

101

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

115

116 **GCβ is expressed on the PPM of mature ookinete with N- and C-termini facing the**
117 **IMC**

118 The ALD and GCD domains, as well as the inter-domain linker, are predicted to be
119 intracellular (Fig. S1A). However, whether GCβ is localized at PPM or IMC remains
120 to be determined. We treated the *gcβ::6HA/imc1i::4Myc* ookinetes with trypsin to
121 digest the extracellular parts of GCβ if it was localized on the PPM surface. Western
122 blotting analysis detected a protein band of ~240 kD from PBS- or heat-inactivated
123 trypsin-treated ookinetes, but not in trypsin treated ookinetes, suggesting surface
124 exposure (Fig. 1E). As a control, we also detected digestion of the PPM protein P28,
125 but not the IMC protein IMC1i (Fig. 1E). These results indicate that GCβ is localized

126 on the PPM. Additionally, all three 6HA-tagged GCβ could be detected using the anti-
127 HA antibody only after Triton X-100 treatment (Fig. 1F), which supports the predicted
128 topology of GCβ (Fig. 1E). Interestingly, trypsin treatment did not alter GCβ
129 polarization (Fig. 1G), suggesting the existence of other proteins or structures acting to
130 stabilize GCβ at OES.
131
132 **GCβ polarization at OES coincides with initiation of ookinete gliding**
133 The round-shaped immotile zygotes undergo significant morphological changes (stages
134 I to V) to differentiate into crescent-shaped motile ookinetes (Fig. 1H, upper panel). To
135 investigate the GCβ's localization dynamics during ookinete maturation and its
136 relationship with ookinete gliding, we analyzed GCβ expression from zygote to mature
137 ookinete using *in vitro* cultured *gcβ::6HAc* parasites. GCβ was distributed in the
138 cytoplasm and localized with BiP from zygote (stage I) to retort (stage III) (Fig. S1H),
139 started to cluster at OES in stage IV retort, and completely polarized to OES of mature
140 ookinetes (stages V) (Fig. 1H, middle panel). We also isolated parasites from infected
141 mosquito midguts and observed a similar dynamic distribution of GCβ (Fig. 1H, lower
142 panel), confirming the *in vitro* observations. Indeed, the rates of GCβ polarization at
143 OES were almost identical in ookinetes either from mosquitoes or *in vitro* cultures (Fig.
144 1I). We next isolated the heavy (including plasma membrane and cytoskeleton) and
145 light (including cytoplasm) fractions from extracts of retort and mature ookinetes after
146 hypotonic lysis, and showed that GCβ could be detected in both fractions of the retorts,
147 but only in heavy fraction of mature ookinetes (Fig. 1J), supporting GCβ association
148 with plasma membrane in mature ookinetes.
149
150 We next quantified GCβ polarization level by calculating fluorescent signals at OES

151 over the whole cell at different stages of ookinete development (Fig. S1I) and measured
152 ookinete gliding using a matrigel-based assay [7, 18]. We showed that ookinete gliding
153 was highly correlated with GCβ polarization at OES (Fig. 1K). No stage II and III
154 retorts had gliding motility; stage IV retorts showed some motility (1–3 μm/min) and
155 initial GCβ polarization; and mature ookinetes (stage V) with clear GCβ polarization
156 had acquired normal gliding (5–12 μm/min) (Fig. 1K).
157
158 To capture the dynamics of GCβ polarization and the timing of ookinete gliding
159 initiation, we generated a parasite, *gcβ::mScarlet* (Data S1), with GCβ C-terminally
160 tagged with mScarlet that had enhanced red fluorescence[19] and allowed tracking GCβ
161 expression in real time. The mScarlet-tagged protein was expressed at OES of mature
162 ookinetes (Fig. S2A), and the tagging modification did not affect ookinete gliding (Fig.
163 S2B). Real-time tracking the GCβ::mScarlet signals of the developing retorts/ookinetes
164 showed cytoplasmic distribution of GCβ in both the protrusion and the zygote remnant
165 of an immotile stage IV retort (Fig. 1L and Video S1). Strikingly, as soon as the majority
166 (~60%) of the GCβ were polarized at OES of mature ookinete, the parasites started
167 gliding (Fig. 1L and Video S1), suggesting that accumulation of GCβ to a required level
168 at the OES is the trigger for gliding. Additionally, GCβ polarization at OES was always
169 present as long as an ookinete was moving spirally (Fig. S2C and S2D). These
170 observations directly link GCβ polarization at the OES to initiation of ookinete gliding.

171 **Ookinete gliding depends on cGMP synthesis activity of GCβ polarized at OES**

172 We disrupted the *gcβ* gene in wild type (WT) and the *gcβ::6HAc* parasites (Fig. S3A-
173 S3C). Parasites without GCβ could develop into ookinetes with normal morphology
174 (Fig. S3D); but lost gliding (Fig. S3E), oocyst and sporozoite formation in the mosquito,
175 and infectivity to mouse (Fig. S3F and S3G). These results confirm that GCβ is essential

176 for ookinete gliding and mosquito transmission, which is consistent with findings in
177 *gcβ* disrupted *P. berghei* parasites [6, 8].
178
179 To test whether cGMP synthesis activity of polarized GCβ at OES is required for
180 ookinete gliding, we generated GCβ mutant parasites that maintained GCβ OES
181 polarization, but lost the ability to synthesize cGMP. Sequences analysis reveal the
182 conserved residues Asn-Thr-Ala-Ser-Arg (NTASR) in the α4-helix of catalytic domain
183 1 (C1) of GC, which are likely critical for the cyclase to bind its substrate GTP[20], and
184 mutations in these residues may reduce or abolish the cyclase activity (Fig. S3H).
185 Accordingly, we introduced mutations by replacing "NTASR" with "NKASR" or
186 "AKASA" in the *gcβ::6HA* parasite, generating *GCDm1 and GCDm2* parasites,
187 respectively (Fig. S3I). Both mutants showed normal GCβ polarization and expression
188 levels similar to that of *gcβ::6HA* parasite (Fig. 2A and 2B), but had severely impaired
189 ookinete motility (Fig. 2C), resembling the phenotype of *gcβ* disruption (Fig. S3E). To
190 further test if the GC activity (cGMP synthesis) loss in ookinetes of these mutants, we
191 utilized a recently developed probe (Green cGull) that emits enhanced GFP
192 fluorescence when binding to cGMP[21]. We episomally expressed a plasmid
193 containing the gene encoding Green cGull protein and observed basal levels of
194 fluorescent signal in the cytoplasm of WT, Δ*gcβ*, and *GCDm2* ookinetes when treated
195 with DMSO (Fig. 2D and 2E). The fluorescent signals in WT ookinetes significantly
196 increased after a 20 min treatment with Zaprinast, an inhibitor active against
197 *Plasmodium* PDEs which degrade cGMP [7], but not in Δ*gcβ* and *GCDm2* ookinetes
198 (Fig. 2D and 2E). These data not only demonstrate loss of cGMP synthesis activity in
199 mature ookinetes of the Δ*gcβ* and *GCDm2* parasites, but also show that ookinete gliding
200 depends on the cGMP synthesis activity of GCβ enriched at the OES.

201

202 **GCβ polarization elevates cGMP levels and activates PKG signaling**

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

214

215 Balanced activities of GCβ and PDEδ are critical for maintaining appropriate cGMP
216 concentration, and changes in protein expression or localization in one of them may
217 affect cGMP levels and downstream PKG signaling. To investigate PDEδ expression
218 and localization relative to GCβ, we tagged PDEδ with 4Myc to generate *pdeδ::4myc*
219 parasite (Table S1) and observed the cytoplasmic distribution of PDEδ during the
220 zygote to ookinete differentiation (Fig. S3J). Furthermore, we generated a doubly-
221 tagged parasite, *gcβ::6HA/pdeδ::4myc,* by tagging the endogenous PDEδ with 4Myc in
222 the *gcβ::6HAc* parasite (Data S1). At zygote and retort stages, both proteins were
223 distributed at both zygote remnant and protrusion and mostly co-localized (Fig. 2I). In
224 mature ookinetes, PDEδ remained relatively evenly distributed throughout the
225 cytoplasm, whereas GCβ polarized at the OES (Fig. 3I). The re-distribution of these

226 two proteins led to local enrichment of GCβ, with higher levels of GCβ over PDEδ at
227 OES (Fig. 2J), which could probably create an elevated cGMP level at the OES and
228 drive PKG activation locally (Fig. 2K).
229

230 **Both ALD and GCD domains are required for GCβ polarization**

231 To analyze the role of ALD in GCβ expression or localization, we generated a modified
232 parasite, *gcβ::T2A*, by introducing the "ribosome skip" T2A peptide
233 (EGRGSLLTCGDVEENPGP) into the middle linker region in the *gcβ::6HAc* parasite
234 (Fig. 3A). The T2A peptide allows expression of the ALD (residues 1–1248) and GCD
235 peptides (residues 1249–3015) separately. Western blotting detected a protein band
236 (GCD::6HA) smaller than the full-length protein (Fig. 3B), indicating separated ALD
237 and GCD expression in the *gcβ::T2A* ookinetes. Notably, the GCD lost OES
238 polarization with cytoplasmic distribution (Fig. 3C and 3D). As expected, this parasite
239 had severely impaired gliding (Fig. 3E). As a control, we replaced a key proline at
240 position 17 of the T2A peptide with arginine to abrogate its function (Fig. 3A). The
241 resulting *gcβ::T2Am* parasite expressed a full-length protein with a molecular weight
242 comparable to that of GCβ::6HA protein (Fig. 3B). The *gcβ::T2Am* ookinetes also
243 maintained GCβ polarization (Fig. 3C and 3D) and normal gliding (Fig. 3E). To further
244 confirm the T2A-mediated separation of ALD and GCD, we generated another parasite
245 *gcβ::T2An* (Fig. S4A), in which ALD and GCD were tagged with the triple V5 epitope
246 (3V5) and 6HA, respectively. Separate expression of ALD and GCD was confirmed on
247 western blot using anti-V5 and anti-HA antibodies, respectively (Fig. S4B). IFA
248 analysis revealed cytoplasmic distribution for both ALD and GCD with little co-
249 localization (Fig. S4C). Like *gcβ::T2A*, this *gcβ::T2An* also displayed a defect in
250 ookinete gliding (Fig. S4D). Together, these results show that expression of both ALD

251 and GCD together in a single protein is required for GCβ polarization and ookinete

252 gliding.

253

254 **P4-ATPase co-factor CDC50A co-localizes and interacts with GCβ**

255 The ALD of GCβ is structurally related to the P4-ATPase proteins, which functions as

256 flippase translocating phospholipids such as phosphatidylserine (PS) from exofacial to

257 cytofacial leaflets of membranes in eukaryotic cells[23, 24]. However, sequence

258 analysis revealed that ALD contains mutations in several conserved functional motifs

259 (Fig. S4E), including the critical DKTGT motif, suggesting a pseudo P4-ATPase. To

260 investigate whether PS is enriched at OES and thus mediates GCβ polarization, we

261 stained the living WT ookinetes with the Annexin V-FITC probe and detected no

262 enrichment of PS molecule at either exofacial or cytofacial leaflets of plasma

263 membranes at the OES (Fig. S4F-S4H). In addition, saponin treatment, which is

264 expected to impair the PS-lipid component in the membrane via depleting

265 cholesterol[25], did not affect GCβ polarization (Fig. S4I). These data suggest that PS-

266 lipid is unlikely the mediator for GCβ polarization.

267

268 P4-ATPase interacts with the co-factor protein, CDC50, which is required for

269 trafficking of the complex from ER to plasma membrane and for flippase activity [26]

270 (Fig. 4A). A search of the *Plasmodium* genomes identified three paralogs of *cdc50*

271 genes: *cdc50a* (PY17X_0619700), *cdc50b* (PY17X_0916600), and *cdc50c*

272 (PY17X_0514500) (Fig. S5A). To determine which CDC50 associates with GCβ, we

273 generated parasites with individual CDC50 protein tagged with 6HA: *cdc50a::6HA*,

274 *cdc50b::6HA*, and *cdc50c::6HA* (Fig. 4B). Of the three proteins, only CDC50A has

275 polarization at OES similar to GCβ in mature ookinetes (Fig. 4B). Notably, CDC50A

276 is exclusively expressed in gametocytes, zygotes, and ookinetes during the parasite life
277 cycle (Fig. S5B and S5C), and similar to GCβ, polarized at OES during zygote to
278 ookinete development (Fig. S5D). These observations were reproduced in another
279 independent mScarlet-tagged parasite, *50a::mScarlet* (Fig. S5E). Next we generated
280 two doubly-tagged parasites, *gcβ::6HA/50a::mCherry* and *gcβ::6HA/50a::3V5,* from
281 the *gcβ::6HA* parasite (Table S1). Results from these parasites show that GCβ and
282 CDC50A were completely co-localized at the cytoplasm of female gametocytes,
283 zygotes, and retorts, and at ookinete OES (Fig. 4C and 4D). Furthermore, results from
284 immunoprecipitation using anti-HA antibody indicate that GCβ binds to CDC50A in
285 ookinetes lysate of the *gcβ::6HA/cdc50a::mCherry* parasite (Fig. 4E). These data
286 demonstrate that CDC50A co-localizes and binds to GCβ during ookinete development.
287

288 **Deletion of CDC50A phenocopies GCβ deficiency in ookinete gliding**

289 We next genetically disrupted the *cdc50a* gene and showed that, similar to *gcβ*
290 disruption, Δ*cdc50a* parasites displayed normal asexual blood stage growth,
291 gametocyte formation, and ookinete differentiation (Fig. S6A-D), but had severe defect
292 in ookinete gliding (Fig. 4F). Parasites with gliding defect cannot penetrate the
293 mosquito midgut and produce no oocysts; indeed, no midgut oocyst (day 7) or salivary
294 gland sporozoite (day 14) was detected in the mosquitoes infected with Δ*gcβ* or
295 Δ*cdc50a* parasites (Fig. 4G and 4H). To further confirm the phenotype, we deleted *gcβ*
296 or *cdc50a* gene in a parasite strain expressing mCherry-labeled P28, 17XNL/P28mCh
297 [27], to investigate early oocyst development (Table S1). Again, these mutant parasites
298 lost ookinete gliding (Fig. S6E) and produced no oocyst in mosquitoes (Fig. S6F). In
299 mosquitoes infected with these parasites, no early midgut parasites were observed at as
300 early as 36 h post blood feeding (Fig. S6G). To rule out that the phenotype defects were

301 caused by Cas9 off-target effects, we re-introduced a *cdc50a* gene with sequence
302 encoding an N-terminal Flag tag back to the endogenous *cdc50a* locus in the Δ*cdc50a*
303 parasite (Data S1). This complemented parasite (Δ*50a/50a*) showed proper CDC50A
304 protein expression driven by the endogenous promoter (Fig. 4I) and displayed normal
305 ookinete gliding (Fig. 4F), oocyst counts (Fig. 4G), and infectivity of mice (Fig. 4H).
306 Together, these results confirm that loss of the CDC50A protein cause ookinete gliding
307 defect and mosquito transmission blocking.
308
309 Four genes (*gcβ*, *cdc50a*, *pdeδ*, and *cdpk3*) have been shown to affect ookinete gliding.
310 To further investigate the functional relationships of these genes, we generated double
311 knockout (DKO) parasites of *gcβ/50a*, *gcβ/cdpk3*, *50a/cdpk3*, *gcβ/pdeδ*, and *50a/pdeδ*
312 (Table S1), and compared the effects of these DKOs on ookinete motility with single
313 gene deletion. The *gcβ/50a* DKO displayed the similar level of gliding defect with
314 single gene deletion (Fig. 4J). Both *gcβ/cdpk3* and *50a/cdpk3* DKO showed slight
315 reductions in gliding than the Δ*cdpk3* (Fig. 5J). The Δ*pdeδ* had higher gliding than that
316 of WT, probably due to increased motility with elevated cGMP level; however, DKO
317 parasites (*gcβ/pdeδ* and *50a/pdeδ*) almost completely abolished ookinete gliding (Fig.
318 4J), suggesting that GCβ and CDC50A may function similarly in the signaling upstream
319 of cGMP (without cGMP synthesis, there will be no cGMP for hydrolysis). Consistent
320 with these observations, Zap treatment boosted gliding of WT ookinetes, but not with
321 either Δ*gcβ* or Δ*cdc50a* parasite (Fig. 4K). Together, these results show that CDC50A
322 serves as a GCβ co-factor, having a similar expression pattern and deletion phenotype
323 to those of GCβ, to regulate cGMP levels in ookinete gliding (Fig. 4L).
324
325 **CDC50A stabilizes GCβ during ookinete development**

326 To investigate how CDC50A regulates GCβ, we deleted the *cdc50a* gene in the
327 *gcβ::6HA* parasite and generated the *gcβ::6HA/Δ50a* parasite (Data S1). CDC50A
328 depletion did not affect *gcβ* mRNA levels in either gametocytes or ookinetes (Fig. 5A),
329 ruling out an effect of CDC50A on *gcβ* transcription. However, an approximately 90%
330 reduction in GCβ protein abundance was observed in both gametocytes and ookinetes
331 of the *gcβ::6HA/Δ50a,* compared to the parental line in both IFA and western blotting
332 analyses (Fig. 5B and 5C). As expected, no OES polarization of GCβ occurred in these
333 parasites (Fig.5C). In addition, we generated another parasite, *Δ50a/gcβ::6HA*, by
334 tagging GCβ in the *Δcdc50a* parasite (Data S1) and observed the same results (Fig. 5C).
335 In contrast, deleting *gcβ* had no impact on CDC50A protein abundance in gametocytes
336 or ookinetes of the *50a::6HA/Δgcβ* line (Fig. 5D). Interestingly, CDC50A protein alone
337 did not polarize at OES in the *50a::6HA/Δgcβ* ookinete (Fig. 5E). These data indicate
338 that CDC50A stabilizes GCβ during gametocyte-zygote-ookinete development, which
339 may explain the similar phenotypic defects in *Δgcβ* and *Δcdc50a* parasites, but does not
340 carry the signal for directing the protein complex to the OES. Instead, the polarization
341 signal is likely within GCβ as shown above.
342
343 CDC50A amino acid sequences display high homology (75% identity) between *P. yoelii*
344 and human malaria parasite *P. falciparum*, suggesting conserved functions. To test this,
345 we complemented the *gcβ::6HA/Δ50a* parasite with the *cdc50a* gene from the *P.*
346 *falciparum* (*Pfcdc50a*) or *P. yoelii* (*Pycdc50a* as control) by episomal expression of the
347 *Pfcdc50a* or *Pycdc50a*. CDC50A protein expression was detected in ookinetes of the
348 parasites complemented with either *Pfcdc50a* or *Pycdc50a* C-terminally tagged with
349 3V5 (Fig. 5F). Importantly, both proteins successfully restored GCβ expression and
350 polarization in ookinetes (Fig. 5F and 5G) and ookinete gliding comparable to that of

351 WT parasite (Fig. 5H). Together, these data show that CDC50A may stabilize GCβ
352 protein or play a role in the translation of GCβ mRNA during sexual development and
353 its functions are evolutionarily conserved between *P. yoelii* and *P. falciparum* (Fig. 5I).
354

355 **ISP1 polarizes and interacts with GCβ at OES of mature ookinete**

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

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

376 (Fig. 6D), indicating that GCβ and ISP1 interact with each other.

377

378 **GCβ polarization is maintained by ISP1 at the IMC**

379 ISP1 was reported as an essential gene refractory to deletion in *P. berghei* asexual blood

380 stages[31]. However, we were able to disrupt the *isp1* gene in *P. yoelii* 17XNL using

381 the Cas9 method and obtained three mutant clones from two independent transfections

382 (Data S1). Δ*isp1* parasites showed normal asexual blood stages and gametocyte

383 development in mouse, male gametocyte activation, and mature ookinetes with normal

384 morphology (Fig. S7C–F). However, *isp1* disruption caused a slight decrease in

385 conversion rate to mature ookinete (25% in Δ*isp1*; 51% in WT) (Fig. S7G). Importantly,

386 the Δ*isp1* ookinetes with normal morphology showed significantly reduced ookinete

387 gliding (Fig. 7A) and oocyst counts in mosquito (Fig. 7B).

388

389 ISP1 may play a role in anchoring GCβ at the OES. To test this, we deleted the *isp1*

390 gene in the *gcβ::6HA* parasite generating the *gcβ::6HA/*Δ*isp1* parasite (Data S1). ISP1

391 depletion did not affect GCβ protein abundance (Fig. S7H), but disrupted GCβ

392 polarization in ~93% of the ookinetes (Fig. 7C and 7D); GCβ appeared to be randomly

393 distributed in cytoplasm, at cell periphery, or at the apical region (Fig. 7C). Indeed,

394 *gcβ::6HA/*Δ*isp1* ookinetes also displayed a severe defect in gliding compared with

395 those of parental *gcβ::6HA* (Fig. 7E). To further confirm the defect, we performed

396 complementation to rescue the defect of the *gcβ::6HA/*Δ*isp1* parasite by episomal

397 expression of the 3V5-tagged PyISP1 (from *P. yoelii*) and PfISP1 (from *P. falciparum*)

398 (Data S1). Both tagged PyISP1 and PfISP1 protein expression were detected in

399 ookinetes of the complemented parasites (Fig. 7F), and these complementations

400 successfully restored GCβ polarization (Fig. 7D, 7G and 7H) and ookinete gliding to

401 the *gcβ::6HA/Δisp1* ookinetes (Fig. 7E), consistent with the high homology (90%

402 identity) in ISP1 protein sequence between *P. falciparum* and *P. yoelii* (Fig. S7I). In

403 contrast, GCβ depletion in the *isp1::3V5* parasite had no impact on the ISP1 dynamic

404 localization and final OES polarization during ookinete differentiation (Fig. 7I),

405 suggesting that ISP1 itself contains a GCβ-independent signal for OES polarization at

406 mature ookinete.

407

408 The ISP1 protein bears two N-terminal cysteine residues for palmitoyl

409 transferase-mediated palmitoylation modification (Fig. S7I), which is critical for its

410 docking to the IMC[32]. We attempted to complement the *gcβ::6HA/Δisp1* parasite by

411 episomal expression of the 3V5-tagged ISP1 bearing C7A/C8A mutations (cysteine

412 changed to alanine in both amino acid 7 and 8 positions). The $ISP1^{C7A/C8A}$::3V5 protein

413 lost palmitoylation modification compared with $ISP1^{WT}$::3V5 protein (Fig. 7J).

414 Consistently, $ISP1^{C7A/C8A}$::3V5 localized evenly at cytoplasm instead of polarizing at

415 OES (Fig. 7K) and failed to rescue the GCβ polarization in the ookinetes of

416 complemented *gcβ::6HA/Δisp1* parasite (Fig. 7L). Furthermore, treating the developing

417 ookinete of the *gcβ::6HA/isp1::3V5* parasite with 2-BMP, a potent inhibitor of protein

418 palmitoylation[33], impaired ookinete differentiation and maturation (Fig. S7J) as well

419 as OES localization of both ISP1 and GCβ in ookinetes with abnormal morphology (Fig.

420 S7K). Again, these abnormal ookinetes displayed no gliding (Fig. S7L). Together, these

421 data indicate that ISP1, with signal for tracking to OES and residing at the IMC, could

422 anchor GCβ at the OES of mature ookinetes (Fig. 7M).

423

424 Discussion

425 Using *P. yoelii* as a model, here we show that GCβ polarization at the ookinete OES is

426 essential for the initiation of ookinete gliding. By real-time capturing mScarlet-tagged
427 GCβ signals, we clearly showed that ookinetes start to move only when the majority
428 (>60%) of GCβ is clustered at the OES (Fig. 1L), providing a mechanism for the
429 initiation of ookinete gliding motility. In addition, we demonstrated that CDC50A, an
430 essential component of P4-ATPase trafficking and activity in other organisms [26],
431 plays an important role in GCβ protein expression, and ISP1, an IMC protein,
432 contributes to anchoring GCβ at OES of mature ookinetes.
433

434 Why does the GCβ polarization occur only after ookinete maturation? A previous study
435 showed that PDEδ deletion led to a defect in ookinete development and gliding, which
436 could be rescued by additional GCβ disruption or PKG inhibition in *P. berghei* [8].
437 Premature activation of cGMP/PKG signal caused by PDEδ disruption before ookinete
438 maturation could interfere with the programmed development of ookinetes. These
439 observations not only suggest both GCβ and PDEδ are constitutively active for
440 synthesizing and hydrolyzing cGMP, respectively, during the ookinete development,
441 but also suggest that strictly spatial-temporal regulation of cGMP/PKG signaling is
442 required for coordinating ookinete development and gliding. Consistent with this
443 speculation, our results showed that both GCβ and PDEδ were distributed in
444 cytoplasmic membrane structures (mostly ER) and largely co-localized in zygotes and
445 retorts, which likely allow maintenance of a balanced and low level of cGMP
446 throughout the cytoplasm assuming that all the enzymes are constitutively active. In
447 mature ookinetes, GCβ is polarized at OES, but PDEδ remains cytoplasmic. GCβ
448 polarization generates a higher protein ratio of GCβ over PDEδ at OES and likely a

449 higher rate of cGMP synthesis than hydrolysis locally. This locally elevated cGMP may
450 activate the PKG signaling and then initiate the ookinete gliding. The sequential events
451 in this process are supported by direct observations of GCβ polarization at OES and the
452 initiation of ookinete gliding (Fig. 1L), although we were not able to detect elevated
453 level of cGMP at OES directly using a cGMP probe reporter Green cGull developed
454 recently[21]. This is likely due to either: the extremely fast diffusion property of
455 cytoplasmic cGMP inside the ookinete [34, 35] or limited sensitivity of the probe in
456 detecting cGMP. Further investigation using more sensitive methods is necessary to
457 prove that locally elevated cGMP concentration drives ookinete gliding motility.
458
459 In many organisms, from yeast to mammals, CDC50 is a co-factor or chaperon of P4-
460 ATPase proteins that mediates the complex's cellular trafficking [36]. Disruption of
461 *cdc50a* dramatically reduced GCβ protein levels in gametocytes and ookinetes, and
462 abolished ookinete gliding. Interestingly, the CDC50A protein level is not affected after
463 GCβ deletion, and it alone cannot polarize to OES. These results imply that CDC50A
464 may not contain the signal for trafficking the complex to OES, as reported in other
465 organisms [36, 37]; instead, it may function as a chaperon stabilizing GCβ in
466 *Plasmodium*, although we cannot rule out that CDC50A could also regulate GCβ at the
467 translational level.
468
469 IMC-residing protein ISP1 co-localizes and interacts with GCβ at OES of mature
470 ookinetes, with GCβ distributed at the PPM and ISP1 at the IMC, functioning as an
471 anchor pulling the GCβ complex to OES in mature ookinetes. Consistently, the majority
472 (93%) of ookinetes lost GCβ polarization after ISP1 depletion. However, approximately

473 7% of ookinetes still maintained GCβ OES polarization, suggesting that other proteins
474 may participate in anchoring GCβ/CDC50A complex at OES. It is still unknown how
475 the GCβ is 'pulled' to ISP1 at OES of mature ookinete, although ISP1 already polarizes
476 in zygote stage (Fig. 6B); it is possible that some specific proteins are expressed and
477 direct GCβ/CDC50A to OES when ookinete is mature or about to mature. Previous
478 studies have shown that biogenesis of the IMC is dependent on vesicular transport by
479 the alveolate specific GTPase protein, Rab11A and Rab11B, in apicomplexans [38, 39].
480 Whether Rab11A and Rab11B play a role in translocating the GCβ/CDC50A to the OES
481 requires further investigation.
482
483 We propose a model for GCβ polarization-directed cGMP signaling and the initiation
484 of ookinete gliding. 1. From zygote to retort stages, cytoplasmic-distributed
485 GCβ/CDC50A complex and PDEδ maintain a sub-threshold cGMP level precluding
486 PKG activation in the cytoplasm throughout the whole cell, assuming that all the
487 enzymes are constitutively active. 2. Upon ookinete maturation, the GCβ/CDC50A
488 complex translocates to the PPM and is anchored by the IMC-residing ISP1 at OES. 3.
489 The GCβ polarization presumably increases the local cGMP concentration that drives
490 PKG activation and initiates ookinetes gliding. Mosquito midgut traversal by ookinetes
491 is a critical limiting step during the malaria transmission, and elucidating the
492 mechanism involved in ookinete gliding could assist the development of interventions
493 for blocking disease transmission.
494

495 ## Acknowledgments

496 We thank Dr. David Baker (London School of Hygiene & Tropical Medicine) for his
497 comments on this manuscript. This work was supported by the National Natural Science
498 Foundation of China (81522027, 31772443, and 31501912), the Fundamental Research

499 Funds for the Central Universities (20720160069, 20720150165, and 2013121033), the
500 China's 1000 Young Talents Program, the "111" Project of the Ministration of
501 Education of China (B06016), and the Division of Intramural Research, National
502 Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH,
503 X-z.S.). The authors thank Cindy Clark, NIH Library Writing Center, for manuscript
504 editing assistance.

505

506 Author contributions

507 G.H. and Y.J. designed the study. G.H., Y.ZK, and W.X. generated the modified
508 parasites, conducted the phenotype analysis, IFA assay, image analysis, mosquito
509 experiments, ookinete motility assay, and biochemical experiments. H.J. and Q.PG.
510 generated the modified parasites. C.X. performed the STORM imaging. Y.J. and C.HT,
511 supervised the work. X-z.S, G.H., and Y.J. analyzed the data and wrote the manuscript.

512

513 Declaration of Interests

514 The authors declare no competing interests

515

516 References

517 **1. Bennink, S., Kiesow, M.J., and Pradel, G. (2016). The development of malaria parasites in**
518 **the mosquito midgut. Cell Microbiol *18*, 905-918.**
519 **2. Al-Khattaf, F.S., Tremp, A.Z., and Dessens, J.T. (2015). Plasmodium alveolins possess**
520 **distinct but structurally and functionally related multi-repeat domains. Parasitol Res *114*,**
521 **631-639.**
522 **3. Keeley, A., and Soldati, D. (2004). The glideosome: a molecular machine powering motility**
523 **and host-cell invasion by Apicomplexa. Trends Cell Biol *14*, 528-532.**
524 **4. Dessens, J.T., Beetsma, A.L., Dimopoulos, G., Wengelnik, K., Crisanti, A., Kafatos, F.C.,**
525 **and Sinden, R.E. (1999). CTRP is essential for mosquito infection by malaria ookinetes.**
526 **EMBO J *18*, 6221-6227.**
527 **5. Boucher, L.E., and Bosch, J. (2015). The apicomplexan glideosome and adhesins -**
528 **Structures and function. J Struct Biol *190*, 93-114.**
529 **6. Hirai, M., Arai, M., Kawai, S., and Matsuoka, H. (2006). PbGCbeta is essential for**
530 **Plasmodium ookinete motility to invade midgut cell and for successful completion of**
531 **parasite life cycle in mosquitoes. J Biochem *140*, 747-757.**
532 **7. Brochet, M., Collins, M.O., Smith, T.K., Thompson, E., Sebastian, S., Volkmann, K.,**
533 **Schwach, F., Chappell, L., Gomes, A.R., Berriman, M., et al. (2014). Phosphoinositide**

534 **metabolism links cGMP-dependent protein kinase G to essential Ca2+ signals at key**
535 **decision points in the life cycle of malaria parasites. PLoS Biol *12*, e1001806.**
536 **8. Moon, R.W., Taylor, C.J., Bex, C., Schepers, R., Goulding, D., Janse, C.J., Waters, A.P.,**
537 **Baker, D.A., and Billker, O. (2009). A cyclic GMP signalling module that regulates gliding**
538 **motility in a malaria parasite. PLoS Pathog *5*, e1000599.**
539 **9. Ishino, T., Orito, Y., Chinzei, Y., and Yuda, M. (2006). A calcium-dependent protein kinase**
540 **regulates Plasmodium ookinete access to the midgut epithelial cell. Mol Microbiol *59*,**
541 **1175-1184.**
542 **10. J U.Linder, P.E., Andreas Reimer, Thomas Kru¨ ger, Helmut Plattner1, Anita Schultz and**
543 **Joachim E.Schultz (1999). Guanylyl cyclases with the topology of mammalian adenylyl**
544 **cyclases and an N-terminal P-type ATPase-like domain in Paramecium, Tetrahymena and**
545 **Plasmodium. EMBO J *18*, pp. 4222-4232.**
546 **11. Baker, D.A., Drought, L.G., Flueck, C., Nofal, S.D., Patel, A., Penzo, M., and Walker, E.M.**
547 **(2017). Cyclic nucleotide signalling in malaria parasites. Open Biol *7*.**
548 **12. Carucci, D.J., Witney, A.A., Muhia, D.K., Warhurst, D.C., Schaap, P., Meima, M., Li, J.L.,**
549 **Taylor, M.C., Kelly, J.M., and Baker, D.A. (2000). Guanylyl cyclase activity associated with**
550 **putative bifunctional integral membrane proteins in Plasmodium falciparum. J Biol Chem**
551 ***275*, 22147-22156.**
552 **13. Zhang, C., Xiao, B., Jiang, Y., Zhao, Y., Li, Z., Gao, H., Ling, Y., Wei, J., Li, S., Lu, M., et**
553 **al. (2014). Efficient editing of malaria parasite genome using the CRISPR/Cas9 system.**
554 **MBio *5*, e01414-01414.**
555 **14. Bergman, L.W., Kaiser, K., Fujioka, H., Coppens, I., Daly, T.M., Fox, S., Matuschewski,**
556 **K., Nussenzweig, V., and Kappe, S.H. (2003). Myosin A tail domain interacting protein**
557 **(MTIP) localizes to the inner membrane complex of Plasmodium sporozoites. J Cell Sci**
558 ***116*, 39-49.**
559 **15. Kaneko, I., Iwanaga, S., Kato, T., Kobayashi, I., and Yuda, M. (2015). Genome-Wide**
560 **Identification of the Target Genes of AP2-O, a Plasmodium AP2-Family Transcription**
561 **Factor. PLoS Pathog *11*, e1004905.**
562 **16. Yusuf, N.A.G., J. L. Wall, R. J. Knuepfer, E. Moon, R. W. Schulte-Huxel, C. (2015). The**
563 **Plasmodium Class XIV Myosin, MyoB, Has a Distinct Subcellular Location in Invasive**
564 **and Motile Stages of the Malaria Parasite and an Unusual Light Chain. J Biol Chem *290*,**
565 **12147-12164.**
566 **17. Santos, J.M.D., N. Kehrer, J. Ramesar, J. Avramut, M. C. Koster, A. J. Dessens, J. T. (2016).**
567 **Maternally supplied S-acyl-transferase is required for crystalloid organelle formation and**
568 **transmission of the malaria parasite. Proc Natl Acad Sci U S A *113*, 7183-7188.**
569 **18. Frenal, K., Dubremetz, J.F., Lebrun, M., and Soldati-Favre, D. (2017). Gliding motility**
570 **powers invasion and egress in Apicomplexa. Nat Rev Microbiol *15*, 645-660.**
571 **19. Bindels, D.S., Haarbosch, L., van Weeren, L., Postma, M., Wiese, K.E., Mastop, M.,**
572 **Aumonier, S., Gotthard, G., Royant, A., Hink, M.A., et al. (2017). mScarlet: a bright**
573 **monomeric red fluorescent protein for cellular imaging. Nat Methods *14*, 53-56.**
574 **20. Winger, J.A., Derbyshire, E.R., Lamers, M.H., Marletta, M.A., and Kuriyan, J. (2008).**
575 **The crystal structure of the catalytic domain of a eukaryotic guanylate cyclase. BMC**
576 **Struct Biol *8*, 42.**
577 **21. Matsuda, S., Harada, K., Ito, M., Takizawa, M., Wongso, D., Tsuboi, T., and Kitaguchi, T.**

578 (2017). Generation of a cGMP Indicator with an Expanded Dynamic Range by
579 Optimization of Amino Acid Linkers between a Fluorescent Protein and PDE5alpha. ACS
580 Sens *2*, 46-51.
581 22. Deng, W., and Baker, D.A. (2002). A novel cyclic GMP-dependent protein kinase is
582 expressed in the ring stage of the Plasmodium falciparum life cycle. Mol Microbiol *44*,
583 1141-1151.
584 23. Andersen, J.P., Vestergaard, A.L., Mikkelsen, S.A., Mogensen, L.S., Chalat, M., and
585 Molday, R.S. (2016). P4-ATPases as Phospholipid Flippases-Structure, Function, and
586 Enigmas. Front Physiol *7*, 275.
587 24. Lopez-Marques, R.L., Theorin, L., Palmgren, M.G., and Pomorski, T.G. (2014). P4-
588 ATPases: lipid flippases in cell membranes. Pflugers Arch *466*, 1227-1240.
589 25. Raghupathy, R., Anilkumar, A.A., Polley, A., Singh, P.P., Yadav, M., Johnson, C.,
590 Suryawanshi, S., Saikam, V., Sawant, S.D., Panda, A., et al. (2015). Transbilayer lipid
591 interactions mediate nanoclustering of lipid-anchored proteins. Cell *161*, 581-594.
592 26. Paulusma, C.C., Folmer, D.E., Ho-Mok, K.S., de Waart, D.R., Hilarius, P.M., Verhoeven,
593 A.J., and Oude Elferink, R.P. (2008). ATP8B1 requires an accessory protein for
594 endoplasmic reticulum exit and plasma membrane lipid flippase activity. Hepatology *47*,
595 268-278.
596 27. Zhang, C., Gao, H., Yang, Z., Jiang, Y., Li, Z., Wang, X., Xiao, B., Su, X.Z., Cui, H., and
597 Yuan, J. (2017). CRISPR/Cas9 mediated sequential editing of genes critical for ookinete
598 motility in Plasmodium yoelii. Mol Biochem Parasitol *212*, 1-8.
599 28. Raibaud, A., Lupetti, P., Paul, R.E., Mercati, D., Brey, P.T., Sinden, R.E., Heuser, J.E., and
600 Dallai, R. (2001). Cryofracture electron microscopy of the ookinete pellicle of Plasmodium
601 gallinaceum reveals the existence of novel pores in the alveolar membranes. J Struct Biol
602 *135*, 47-57.
603 29. Morrissette, N.S., and Sibley, L.D. (2002). Cytoskeleton of apicomplexan parasites.
604 Microbiol Mol Biol Rev *66*, 21-38; table of contents.
605 30. Kono, M., Herrmann, S., Loughran, N.B., Cabrera, A., Engelberg, K., Lehmann, C., Sinha,
606 D., Prinz, B., Ruch, U., Heussler, V., et al. (2012). Evolution and architecture of the inner
607 membrane complex in asexual and sexual stages of the malaria parasite. Mol Biol Evol *29*,
608 2113-2132.
609 31. Poulin, B., Patzewitz, E.M., Brady, D., Silvie, O., Wright, M.H., Ferguson, D.J., Wall, R.J.,
610 Whipple, S., Guttery, D.S., Tate, E.W., et al. (2013). Unique apicomplexan IMC sub-
611 compartment proteins are early markers for apical polarity in the malaria parasite. Biol
612 Open *2*, 1160-1170.
613 32. Fung, C., Beck, J.R., Robertson, S.D., Gubbels, M.J., and Bradley, P.J. (2012). Toxoplasma
614 ISP4 is a central IMC sub-compartment protein whose localization depends on
615 palmitoylation but not myristoylation. Mol Biochem Parasitol *184*, 99-108.
616 33. Jennings, B.C., Nadolski, M.J., Ling, Y., Baker, M.B., Harrison, M.L., Deschenes, R.J.,
617 and Linder, M.E. (2009). 2-Bromopalmitate and 2-(2-hydroxy-5-nitro-benzylidene)-
618 benzo[b]thiophen-3-one inhibit DHHC-mediated palmitoylation in vitro. J Lipid Res *50*,
619 233-242.
620 34. Allbritton, N.L., Meyer, T., and Stryer, L. (1992). Range of messenger action of calcium
621 ion and inositol 1,4,5-trisphosphate. Science *258*, 1812-1815.

622 **35.** **Chen, C., Nakamura, T., and Koutalos, Y. (1999). Cyclic AMP diffusion coefficient in frog**
623 **olfactory cilia. Biophys J *76*, 2861-2867.**
624 **36.** **Azouaoui, H., Montigny, C., Jacquot, A., Barry, R., Champeil, P., and Lenoir, G. (2016).**
625 **Coordinated Overexpression in Yeast of a P4-ATPase and Its Associated Cdc50 Subunit:**
626 **The Case of the Drs2p/Cdc50p Lipid Flippase Complex. Methods Mol Biol *1377*, 37-55.**
627 **37.** **Bryde, S., Hennrich, H., Verhulst, P.M., Devaux, P.F., Lenoir, G., and Holthuis, J.C. (2010).**
628 **CDC50 proteins are critical components of the human class-1 P4-ATPase transport**
629 **machinery. J Biol Chem *285*, 40562-40572.**
630 **38.** **Agop-Nersesian, C., Egarter, S., Langsley, G., Foth, B.J., Ferguson, D.J., and Meissner, M.**
631 **(2010). Biogenesis of the inner membrane complex is dependent on vesicular transport by**
632 **the alveolate specific GTPase Rab11B. PLoS Pathog *6*, e1001029.**
633 **39.** **Agop-Nersesian, C., Naissant, B., Ben Rached, F., Rauch, M., Kretzschmar, A., Thiberge,**
634 **S., Menard, R., Ferguson, D.J., Meissner, M., and Langsley, G. (2009). Rab11A-controlled**
635 **assembly of the inner membrane complex is required for completion of apicomplexan**
636 **cytokinesis. PLoS Pathog *5*, e1000270.**
637 **40.** **Wright, D.A., Thibodeau-Beganny, S., Sander, J.D., Winfrey, R.J., Hirsh, A.S., Eichtinger,**
638 **M., Fu, F., Porteus, M.H., Dobbs, D., Voytas, D.F., et al. (2006). Standardized reagents and**
639 **protocols for engineering zinc finger nucleases by modular assembly. Nat Protoc *1*, 1637-**
640 **1652.**
641 **41.** **Billker, O., Dechamps, S., Tewari, R., Wenig, G., Franke-Fayard, B., and Brinkmann, V.**
642 **(2004). Calcium and a calcium-dependent protein kinase regulate gamete formation and**
643 **mosquito transmission in a malaria parasite. Cell *117*, 503-514.**
644 **42.** **Rodriguez, M.C., Margos, G., Compton, H., Ku, M., Lanz, H., Rodriguez, M.H., and**
645 **Sinden, R.E. (2002). Plasmodium berghei: routine production of pure gametocytes,**
646 **extracellular gametes, zygotes, and ookinetes. Exp Parasitol *101*, 73-76.**
647 **43.** **Harris, L.A., Shew, T.M., Skinner, J.R., and Wolins, N.E. (2012). A single centrifugation**
648 **method for isolating fat droplets from cells and tissues. J Lipid Res *53*, 1021-1025.**
649 **44.** **Howie, J., Reilly, L., Fraser, N.J., Vlachaki Walker, J.M., Wypijewski, K.J., Ashford, M.L.,**
650 **Calaghan, S.C., McClafferty, H., Tian, L., Shipston, M.J., et al. (2014). Substrate**
651 **recognition by the cell surface palmitoyl transferase DHHC5. Proc Natl Acad Sci U S A**
652 ***111*, 17534-17539.**
653 **45.** **Bernsel, A., Viklund, H., Hennerdal, A., and Elofsson, A. (2009). TOPCONS: consensus**
654 **prediction of membrane protein topology. Nucleic Acids Res *37*, W465-468.**
655 **46.** **Zhang, Y., Zhuang, Y.L., Nie, X.M., Liu, Y., Song, Y.H., Wang, G.C., and Zhu, C.F. (2012).**
656 **[Analysis of HLA-A, B, and DRB1 polymorphism and genetic distance in Han population**
657 **living in Yantai and Weihai regions of Shandong province]. Zhonghua Yi Xue Yi Chuan**
658 **Xue Za Zhi *29*, 229-233.**
659 **47.** **Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T.,**
660 **Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source**
661 **platform for biological-image analysis. Nat Methods *9*, 676-682.**
662 **48.** **Abou-Zeid, K.A., Yoon, K.S., Oscar, T.P., Schwarz, J.G., Hashem, F.M., and Whiting, R.C.**
663 **(2007). Survival and growth of Listeria monocytogenes in broth as a function of**
664 **temperature, pH, and potassium lactate and sodium diacetate concentrations. J Food Prot**
665 ***70*, 2620-2625.**

666
667
668
669
670
671
672
673
674
675
676
677
678
679 ## Figure legend

680 **Figure 1. Dynamics of GCβ polarization to a unique ookinete extrados site (OES)**
681 **and initiation of ookinete gliding.**

682 **(A)** Diagrams of GCβ tagged with a sextuple HA epitope (6HA, red) at three different
683 locations. GCβ possesses a P4-type ATPase like domain (ALD, blue) and a guanylate
684 cyclase domain (GCD, green). The 6HA is inserted at the C-terminus (*gcβ::6HAc*),
685 between ALD and GCD (*gcβ::6HAm*), and at the N-terminus (*gcβ::6HAn*), respectively.
686 **(B)** Western blotting of tagged GCβ protein in ookinetes. P28 protein as loading control.
687 **(C)** IFA detection of GCβ during the life cycle of the *gcβ::6HAc* parasite. Nuclei are
688 labeled with Hoechst 33342. Scale bar = 5 μm.
689 **(D)** Co-localization of GCβ with proteins of known cellular localizations in ookinetes.
690 P28, ookinete plasma membrane protein; MTIP (glideosome), myosin A tail domain
691 interacting protein; MyoB (apical ring), myosin B; ARA1 (apical collar), apical ring

692 associated protein 1. Scale bar = 5 μm. The right panel shows the diagram of apical
693 structure of *Plasmodium* ookinete.
694 **(E)** Western blotting of GCβ, P28, and IMC1i (inner membrane complex protein 1i)
695 proteins of the *gcβ::6HA/imc1i::4Myc* ookinetes treated with PBS, trypsin (Try), or
696 heat inactivated (HI) trypsin. The left panel shows the predicted topology of GCβ.
697 **(F)** IFA of GCβ in ookinetes of three tagged parasite lines with or without Triton X-100
698 permeabilization. Scale bar = 5 μm.
699 **(G)** IFA of GCβ protein in the ookinete treated with PBS or trypsin. Scale bar = 5 μm.
700 **(H)** IFA showing GCβ localization during ookinete development *in vitro* and *in vivo*.
701 Upper panel, diagrams depicting morphological changes from zygote (stage I) to
702 crescent-shaped mature ookinete (stage V). IFA images of tagged-GCβ expression from
703 *in vitro* cultured parasites (middle panel) or *in vivo* infected mosquito midgut (bottom
704 panel). Black arrow indicates the apical of ookinetes. Scale bar = 5 μm.
705 **(I)** Quantification of GCβ polarization level at the OES during ookinete development
706 obtained from mosquitoes or *in vitro* culture as in (**H**). Polarization rates are means ±
707 SEM of at least 30 ookinetes in each group.
708 **(J)** Western blotting of GCβ from the isolated cellular fractions (total protein, light
709 fraction, and heavy fraction) of retorts and ookinetes.
710 **(K)** Relationship of GCβ polarization rate (red) at OES and gliding speed (blue) of
711 ookinetes in different stages. Polarization rates are means ± SEM of at least 30
712 ookinetes. The range of whisker plots for ookinete gliding speeds indicates the 2.5 and
713 97.5 percentiles, the box includes 50% of all values, and the horizontal line shows
714 median values obtained for the tested number (n) of ookinetes in each group.
715 **(L)** Real-time capturing of fluorescent signals with mScarlet-tagged GCβ in developing
716 ookinete and initiation of gliding motility. Percentage number (lower left) is the GCβ
717 polarization rate (signal at the OES over signal from the whole cell). Note, at 69 min or
718 60%, the parasite started moving as reference to the nearby cells. Scale bar = 5 μm.
719 See also Figure S1 and S2, Table S1 and S2, and Video S1.
720

721

722 **Figure 2. GCβ polarization elevates cGMP levels and activates PKG**

723 **(A)** IFA analysis of GCβ in mature ookinetes of the *GCDm1* and *GCDm2* parasites. The
724 upper panel shows the mutations (red) introduced in the GCD. Scale bar = 5 μm.
725 **(B)** Western blotting of GCβ expression in ookinetes of the *GCDm1* and *GCDm2*.
726 **(C)** Gliding motility of the *GCDm1* and *GCDm2* ookinetes. n is the number of ookinetes
727 tested in each group.
728 **(D)** Detection of endogenous cGMP in ookinetes of wildtype and *GCDm2* parasites
729 expressing the Green cGull probe reporter. The fluorescent signals were
730 microscopically monitored in ookinetes without treatment (-) or with DMSO or Zap
731 treatment (+) for 20 min.
732 **(E)** Quantification of the fluorescent intensity change (F/F_0) in (**D**). n is the number of
733 ookinetes tested in each group. The horizontal line shows the mean values.
734 **(F)** Two-colored IFA analysis of GCβ and PKG proteins during ookinete development
735 of the *gcβ::6HA/pkg::4Myc* parasite. Scale bar = 5 μm.
736 **(G)** Ookinete gliding motility of wildtype parasites treated with DMSO or a potent
737 *Plasmodium* PKG inhibitor, Compound 2 (C2).
738 **(H)** IFA analysis of GCβ proteins in mature ookinete of the *gcβ::6HA* parasites treated
739 with DMSO or C2. Scale bar = 5 μm.
740 **(I)** Two-colored IFA analysis of GCβ and PDEδ proteins during ookinete development
741 of the *gcβ::6HA/pdeδ::4Myc* parasite. Scale bar = 5 μm.
742 **(J)** Protein polarization rate of GCβ and PDEδ at OES of retort and ookinete in (**I**).
743 **(K)** A proposed model of GCβ polarization at OES and initiation of cGMP/PKG
744 dependent ookinete gliding. In mature ookinetes, GCβ polarizes at OES, while PDEδ
745 remains in the cytoplasm, which breaks cGMP synthesis-hydrolysis balance and
746 increases cGMP levels, activates PKG, and initiates ookinete gliding.
747 See also Figure S3, and Table S1 and S2.

748

749

750 **Figure 3. Expression of ALD and GCD in a single peptide is required for GCβ**
751 **polarization and ookinete gliding**
752 **(A)** Diagrams of the endogenous GCβ protein modification. The viral "ribosome skip"
753 T2A peptide was inserted into the region between the ALD and GCD domains in the
754 *gcβ::T2A* parasite, leading to expression of the two domains separately. In the
755 *gcβ::T2Am* parasite, replacing a proline with arginine in the T2A abrogated the peptide
756 function, resulting in expression both ALD and GCD in one peptide.
757 **(B)** Western blotting of GCβ protein using anti-HA antibody in the modified strains.
758 **(C)** IFA analysis of labeled GCβ proteins in ookinetes of modified strains. Scale bar =
759 5 μm.
760 **(D)** Quantification of GCβ polarization rate at OES of the ookinetes in (**C**).
761 **(E)** Gliding motility of ookinetes from different modified strains.
762 See also Figure S4, and Table S1 and S2.
763
764
765 **Figure 4. CDC50A mimics GCβ function in ookinete gliding**
766 **(A)** Diagram of P4-ATPase (green) and CDC50 (red) protein complex in eukaryotes.
767 **(B)** Topology and IFA analysis of three CDC50 proteins in ookinetes of *P. yoelii*:
768 CDC50A (50A), CDC50B (50B), and CDC50C (50C). These endogenous proteins were
769 tagged with a 6HA tag (red rectangle) C-terminally. Scale bar = 5 μm.
770 **(C)** Two-colored IFA analysis of CDC50A and GCβ proteins during gametocyte to
771 ookinete development of the double-tagged *gcβ::6HA/cdc50a::mCherry* parasite using
772 anti-HA and anti-mCherry antibodies. Scale bar = 5 μm.
773 **(D)** Two-colored IFA analysis of CDC50A and GCβ proteins in ookinete of the double-
774 tagged *gcβ::6HA/cdc50a::3V5* parasite. Scale bar = 5 μm.
775 **(E)** Co-immunoprecipitation assay of GCβ and CDC50A proteins in ookinetes of the
776 *gcβ::6HA/cdc50a::mCherry* strain (Double modified strain, DMS).
777 **(F)** Ookinete gliding motility of the wildtype, Δ*gcβ*, Δ*50a*, and, the complemented
778 Δ*50a*/*50a* parasites.

779 **(G)** Number of oocysts in mosquito midgut 8 days post blood feeding. n is the number
780 of mosquitoes tested in each group. The horizontal line shows the mean value of each
781 group. Right panel shows the dissected mosquito midguts stained with 0.5%
782 mercurochrome. Scale bar = 50 μm.
783 **(H)** Formation and infectivity to mouse of salivary gland sporozoites in the mosquitoes
784 14 days post blood feeding. In each group, ten mosquitoes were fed on one mouse and
785 the prepatent time was measured.
786 **(I)** Western blot of the Flag-tagged CDC50A expression in ookinetes of the
787 complemented Δ*50a/50a* parasite.
788 **(J)** Ookinete gliding motility of the parasites with various combinations of double
789 deletions of *gcβ*, *50a*, *pdeδ*, and *cdpk3* genes.
790 **(K)** Ookinete gliding motility of the parasites with or without the *Plasmodium* PDE
791 inhibitor Zaprinast (Zap, 100 μM) treatment.
792 **(L)** A proposed model depicting positions of GCβ and CDC50A in cGMP signaling for
793 ookinete gliding.
794 See also Figure S5 and S6, and Table S1 and S2.
795
796
797 **Figure 5. CDC50A stabilizes GCβ during sexual development**
798 **(A)** RT-PCR analysis of *gcβ* and *50a* transcripts in gametocytes and ookinetes of the
799 *gcβ*::*6HA* and *gcβ*::*6HA*/Δ*50a* parasites. *18s rRNA* gene as control.
800 **(B)** Western blot of GCβ expression in gametocytes and ookinetes of the *gcβ*::*6HA* and
801 *gcβ*::*6HA*/Δ*50a* parasites. The right panel is the quantifications of GCβ band intensity
802 in the blot from three independent experiments.
803 **(C)** IFA analysis of GCβ protein in gametocyte (left) and ookinete (right) of the
804 *gcβ*::*6HA* and *50a*-deleted parasites. Two independent modified strains, *gcβ*::*6HA*/Δ*50a*
805 and Δ*50a*/*gcβ*::*6HA*, were tested. The right panel is quantifications of the fluorescent
806 signal of GCβ.
807 **(D)** Western blot of 50A expression in the *50a::6HA* and *50a::6HA*/Δ*gcβ* parasites.

808 Right panel is the quantification of the results from three independent experiments.

809 **(E)** IFA of 50A protein during *in vitro* ookinete development of *50a::6HA* and

810 *50a::6HA*/Δ*gcβ* parasites. Scale bar = 5 μm.

811 **(F)** Western blot of GCβ and 50A proteins in ookinete of the *gcβ*::*6HA*/Δ*50a* parasite

812 complemented with 3V5-tagged *50a* gene from either *P. yoelii* or *P. falciparum*.

813 **(G)** IFA analysis of GCβ proteins in ookinete of complemented parasites.

814 **(H)** Ookinete gliding motility of the complemented parasites.

815 **(I)** A proposed model of CDC50A binding and stabilizing GCβ.

816 See also Table S1 and S2.

817

818

819 **Figure 6. ISP1 polarizes and interacts with GCβ at OES of mature ookinete**

820 **(A)** Protein polarization levels at OES based on IFA signals for IMC-related proteins in

821 mature ookinetes. The localization of the tested proteins is indicated in Fig. S7A.

822 Polarization rates are means ± SEM of at least 30 cells, and indicated at the top of each

823 column. Lower panel is the IFA images of three selected proteins: IMC1i, and IMC sub-

824 compartment protein 1 and 3 (ISP1 and ISP3, respectively).

825 **(B)** IFA analysis of ISP1 and GCβ proteins from zygote to ookinete development in the

826 parasite *gcβ::6HA/isp1::3V5*. Scale bar = 5 μm.

827 **(C)** Stochastic optical reconstruction microscopy (STORM) imaging of GCβ and ISP1

828 proteins in mature ookinete. Scale bar = 0.5 μm.

829 **(D)** Co-immunoprecipitation assay of GCβ and ISP1 proteins in ookinetes of the

830 *gcβ::6HA*/*isp1::3V5* parasite.

831 See also Figure S7, and Table S1 and S2.

832

833

834 **Figure 7. GCβ polarization is maintained by ISP1 at the IMC**

835 (A) Ookinete gliding motility of wildtype and Δ*isp1* parasites.

836 (B) Number of oocysts in the mosquito midguts 7 days post blood feeding.

837 **(C)** IFA analysis of GCβ localization in ookinetes of the *gcβ::6HA/Δisp1* parasite. Scale
838 bar = 5 μm.
839 **(D)** Percentage of ookinete types showing different localization of GCβ in (**C**). More
840 than 100 ookinetes were analyzed in each group from three independent tests.
841 **(E)** Ookinete gliding motility of *gcβ::6HA*, *gcβ::6HA/Δisp1*, and the complemented
842 parasites: *Pyisp1::3V5* (*P. yoelii isp1*) and *Pfisp1::3V5* (*P. falciparum isp1*).
843 **(F)** Western blot detecting the 3V5-tagged PyISP1 or PfISP1 proteins expression in the
844 complemented parasites.
845 **(G)** IFA analysis of GCβ and ISP1 proteins in ookinetes of the complemented parasites.
846 Scale bar = 5 μm.
847 **(H)** Quantification of GCβ polarization rate at OES of ookinetes in (**G**).
848 **(I)** IFA analysis of ISP1 protein from zygote to ookinete development of the
849 *isp1::3V5/Δgcβ* parasites. Scale bar = 5 μm.
850 **(J)** Western blot detection of expression and palmitoylation of ISP1 in the
851 *gcβ::6HA/Δisp1* parasite complemented with the 3V5-tagged wildtype ISP1
852 ($ISP1^{WT}$::3V5) or ISP1 bearing C7A/C8A mutations ($ISP1^{C7A/C8A}$::3V5). BiP as the
853 loading control.
854 **(K)** Two-colored IFA analysis of ISP1 and GCβ proteins in ookinetes of the
855 *gcβ::6HA/Δisp1* parasite complemented with $ISP1^{C7A/C8A}$::3V5. Scale bar = 5 μm.
856 **(L)** Percentage of ookinetes with GCβ polarization at OES from the *gcβ::6HA/Δisp1*
857 parasites complemented with $ISP1^{WT}$::3V5 or $ISP1^{C7A/C8A}$::3V5. The value is means ±
858 SEM of three independent tests analyzing more than 150 ookinetes. Two-tailed *t* test
859 was used.
860 **(M)** A proposed model of the IMC-residing protein ISP1 in anchoring GCβ at OES of
861 mature ookinetes.
862 See also Figure S7, and Table S1 and S2.
863
864
865

866
867
868
869
870
871
872
873
874
875
876 **CONTACT FOR RESOURCE AND REAGENT SHARING**

877 Further information and requests for resources and reagents should be directed to and
878 will be fulfilled by the Lead contact, Jing Yuan (yuanjing@xmu.edu.cn).

879

880 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

881 **Mouse usage and ethics statement**

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

887 **Genotypic analysis of transgenic parasites**

888 All transgenic parasites were generated from *P. yoelii* 17XNL strain and are listed in
889 Table S1. Parasite infected blood samples from infected mice were collected from the

890 mouse orbital sinus, and mouse blood cells were lysed using 1% saponin in PBS.
891 Parasite genomic DNAs were isolated from transfected blood stage parasite populations
892 using DNeasy Blood kits (Qiagen) after washing off hemoglobin and subjected to
893 diagnostic PCR. For each modification, both the 5' and 3' homologous recombination
894 was detected by diagnostic genotype PCR (see Data S1), confirming successful
895 integration of the homologous templates. All the primers used in this study are listed in
896 Table S2. Parasite clones with targeted modifications were obtained after limiting
897 dilution. At least two clones of each gene-modified parasite were used for phenotype
898 analysis.

899 **Housing conditions of mosquitos**

900 The *Anopheles stephensi* mosquito (strain Hor) was reared at 28°C, 80% relative
901 humidity and at a 12h light/dark cycle in the standard insect facility. Mosquito adults
902 were maintained on a 10% sucrose solution.

903 **Culture conditions for *in vitro* systems**

904 Parasite ookinetes were prepared using *in vitro* culture. 100 µl of infected blood
905 containing gametocytes was obtained from the orbital sinus of infected mouse and
906 mixed immediately with 1 ml ookinete culture medium (RPMI 1640 medium
907 containing 25 mM HEPES, 10% FCS, 100 µM xanthurenic acid, and pH 8.0). The
908 mixture was incubated at 22 °C for 12–24 h to allow gametogenesis, fertilization, and
909 ookinete differentiation. Ookinetes formation was monitored by Giemsa staining of
910 smears of the cultured cells.

911

912 **METHOD DETAILS**

913 **Plasmid construction and parasite transfection**

914 CRISPR/Cas9 plasmid pYCm was used for parasite genomic modification. To construct
915 the vectors for gene deleting, we amplified the 5'- and 3'- genomic sequence (400 to
916 700 bp) of target genes as left and right homologous arms using specific primers (Table
917 S2) and inserted into the restriction sites in pYCm. Oligonucleotides for guide RNAs
918 (sgRNAs) were annealed and ligated into pYCm. For each gene, two sgRNAs were
919 designed to target the coding region of gene (Table S2) using the online program ZiFit
920 [40]. To construct the vectors for gene tagging and T2A insertion, we first amplified the
921 C- or N-terminal segments (400 to 800 bp) of the coding regions as left or right arm
922 and 400 to 800 bp from 5'UTR or 3' UTR following the translation stop codon as left
923 and right arm, respectively. A DNA fragment (encoding mCherry, mScarlet, 6HA,
924 4Myc, or 3V5 tag) was inserted between the left and right arms in frame with the gene
925 of interest. For each gene, two sgRNAs were designed to target sites close to the C- or
926 N-terminal part of the coding region. To construct vectors for site-directed nucleotide
927 mutations, the substitution sites were designed with a restriction site for modification
928 detection and placed in the middle of the homologous arms. Parasite-infected red blood
929 cells (RBC) were electroporated with 5 μg purified circular plasmid DNA using the
930 Lonza Nucleotector. Transfected parasites were immediately intravenously injected
931 into a new mouse and placed under pyrimethamine pressure (provided in drinking water
932 at concentration 6 μg/ml) from day 2 post-transfection. Parasites with transfected
933 plasmids usually appear 5 to 7 days during drug selection.

934 **Parasite negative selection with 5-Fluorouracil**

935 Modified parasites subject for sequential modification were negatively selected to
936 remove episomal pYCm plasmid. 5-Fluorouracil (5FC, Sigma, F6627) was prepared in
937 water at a final concentration of 2.0 mg/ml and was provided to the mice in a dark
938 drinking bottle. A naïve mouse receiving parasites with residual plasmid from previous
939 pyrimethamine selection was subjected to 5FC pressure for 8 days, with a change of
940 drug at day 4. To estimate the amount of plasmid in the parasite populations, we used
941 two independent primer pairs from the plasmid backbone to amplify the DNAs. All
942 PCR primers used are listed in Table S2.

943 **Gametocyte induction in mouse**

944 ICR mice were treated with phenylhydrazine (80 μg /g mouse body weight) through
945 intraperitoneal injection. Three days post treatment, the mice were infected with
946 2.0×10^6 parasites through tail vein injection. Peaks of gametocytemia usually were
947 observed three days post infection. Male and female gametocytes were counted via
948 Giemsa staining of thin blood smears. Gametocytemia was calculated as the ratio of
949 male or female gametocyte over parasitized erythrocyte. All experiments were repeated
950 three times independently.

951 ***In vitro* ookinete culture and purification**

952 *In vitro* culture for ookinete development was prepared as described previously [41].
953 Briefly, mouse blood with 4–6% gametocytemia was collected in heparin tubes and
954 immediately added to ookinete culture medium. Parasites were cultured in the medium
955 with a blood/medium volume ratio of 1:10 at 22°C. After 12–24 h culture, the ookinete
956 culture was Giemsa-stained and analyzed for ookinetes morphology. Ookinete

957 conversion rate was calculated as the number of ookinetes (both normal and abnormal
958 morphology) per 100 female gametocytes. Ookinetes were purified using ACK lysing
959 method as described previously[42]. Briefly, the cultured ookinetes were collected by
960 centrifugation and transferred into ACK lysing buffer (ThermoFisher Scientific,
961 A1049201) on ice for 8 min. After erythrocytes lysis, the remaining ookinetes were
962 isolated via centrifugation and washed twice with PBS. The ookinetes were examined
963 on the hemocytometer under 40× objective lens for purity and counted. Only the
964 samples with >80% ookinete purity were used for further biochemical analysis.

965 **Mosquito feeding and transmission assay**

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

975 **Ookinete motility assay**

976 Ookinete gliding motility was evaluated as previously described[8]. All procedures
977 were performed in a temperature-controlled room with 22°C. Briefly, 20 μl of the
978 suspended ookinete cultures were mixed with 20 μl of Matrigel (BD, #356234) on ice.

979 The mixtures were transferred onto a slide, covered with a cover slip, and sealed with
980 nail varnish. The slide was placed at 22°C for 30 min before observation under
981 microscope. After tracking a gliding ookinete under microscopic field, time-lapse
982 videos (1 frame per 20 s, for 20 min) were taken to monitor ookinete movement using
983 a 40× objective lens on a Nikon ECLIPSEE100 microscope fitted with an ISH500
984 digital camera controlled by *ISCapture v3.6.9.3N* software (Tucsen). Time-lapse
985 movies were analyzed with Fiji software and the Manual Tracking plugin. Motility
986 speed was calculated by dividing the distance an ookinete moved by the time it took.
987 All experiments were repeated three times independently.

988 **Chemical treatment of ookinetes and gliding motility**

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

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

998 Transient expression of proteins in ookinetes via plasmid episome was as described
999 with minor modifications [41]. Coding sequence of target proteins with appropriate 5'-
1000 and 3'-UTR regulatory regions were inserted into the pL0019-derived vector with

1001 human *dhfr* marker for pyrimethamine selection. Briefly, blood stage parasites were
1002 electroporated with 10 μg plasmid DNA and selected with pyrimethamine (70 μg/ml)
1003 for 7 days. Meanwhile, another group of ICR mice were treated with phenylhydrazine
1004 for 3 days through intraperitoneal injection. The phenylhydrazine-treated mice were
1005 infected with 2.0×10^6 drug-selected parasites through intravenous injection and further
1006 selected for another 3–4 days until peak gametocytemia was reached. The high-level
1007 gametocytemia blood was collected for ookinete culture and further tests.

1008 **Antibodies and antiserum**

1009 The primary antibodies used were: rabbit anti-HA (western, 1:1000 dilution, IFA, 1:500
1010 dilution), mouse anti-HA(IFA, 1:500), rabbit anti-Myc (western, 1:1000), and mouse
1011 anti-Myc (IFA, 1:500) from Cell Signaling Technology, mouse anti-αTubulin II (Sigma-
1012 Aldrich) (IFA, 1:1000), mouse anti-V5 (Genescript)(western, 1:1000, IFA, 1:500),
1013 rabbit anti-mCherry (Abcam) (western, 1:1000, IFA, 1:500), Rabbit anti-Flag (Sigma-
1014 Aldrich,) (western, 1:1000). The secondary antibodies used were: goat anti-rabbit IgG
1015 HRP-conjugated and goat anti-mouse IgG HRP-conjugated secondary antibody from
1016 Abcam (1:5000), the Alexa 555 goat anti-rabbit IgG, Alexa 488 goat anti-rabbit IgG,
1017 Alexa 555 goat anti-mouse IgG, Alexa 488 goat anti-mouse IgG, and Alexa 555 goat
1018 anti-rat IgG secondary antibody from ThermoFisher Scientific(1:500). The anti-serums,
1019 including the rabbit anti-Hep17(western, 1:1000), rabbit anti-P28(western, 1:1000, IFA,
1020 1:1000), rabbit anti-BiP(western, 1:1000, IFA, 1:500), rat anti-ACP(IFA, 1:100), and
1021 rabbit anti-ERD2(IFA, 1:500) were prepared in the Lab.

1022 **Immunofluorescence assays**

1023 Purified parasites were fixed using 4% paraformaldehyde and transferred to a Poly-L-
1024 Lysine pre-treated coverslip. The fixed cells were permeabilized with 0.1% Triton X-
1025 100 PBS solution for 7 min, blocked in 5% BSA solution for 60 min at room
1026 temperature, and incubated with the primary antibodies diluted in 3% BSA-PBS at 4°C
1027 for 12 h. The coverslip was incubated with fluorescent conjugated secondary antibodies
1028 for 1 h. Cells were stained with Hoechst 33342, mounted in 90% glycerol solution, and
1029 sealed with nail polish. All images were captured and processed using identical settings
1030 on a Zeiss LSM 780 confocal microscope. Stochastic optical reconstruction microscopy
1031 (STORM) imaging was acquired using a Nikon N-STORM 5.0 Super-Resolution
1032 Microscope System.

1033 **Imaging of live ookinetes using confocal fluorescence microscopy**

1034 Developing ookinetes (20 µl) of *gcβ::mScarlet* parasite from 8 to 12 hour cultures were
1035 mixed with 20 µl of Matrigel thoroughly. The mixtures were transferred onto a slide,
1036 covered with a cover slip, and sealed with nail varnish. The developing ookinetes were
1037 monitored under a Zeiss LSM 780 confocal microscope. Stage IV live ookinetes were
1038 monitored and fluorescent signals were tracked and recorded.

1039 **Cellular cGMP detection in ookinetes**

1040 Cellular cGMP detection was conducted using the Green-cGull probe as described
1041 previously[21] with minor modifications. The coding region of Green-cGull protein
1042 driven by 1.5 kb *Pysoap* 5'-UTR and 1.0 kb *Pbdhfr* 3'-UTR was inserted to pL0019-
1043 derived plasmid containing human *dhfr* marker for pyrimethamine selection. Briefly,
1044 blood stage parasites were electroporated with 10 µg plasmid DNA and selected with

1045 pyrimethamine (70 μg/ml) for 7 days. Ookinetes from 12 to 24 hour *in vitro* cultures
1046 were enriched by centrifugation and resuspended in 1% low-melting agarose (Sigma-
1047 Aldrich, A9414) to avoid cell movement during detection. The mixtures were
1048 transferred to the bottom of 15 mm glass-bottom cell culture dish (Corning, #801002)
1049 and overlaid with RPMI 1640 medium. Using a Zeiss LSM 780 confocal microscope,
1050 the fluorescent signals of Green-cGull were monitored in 30 randomly chosen ookinetes
1051 for their basal fluorescence (F_0) (collected before treatment) and enhanced fluorescence
1052 (F) collected 20 min post zaprinast treatment respectively. cGMP response was
1053 calculated as the ratio of F/F_0.

1054 **Cellular phosphatidylserine detection in ookinetes**

1055 To detect the phosphatidylserine (PS) on the outer leaflet of plasma membrane of
1056 ookinetes, Annexin V-FITC assay kit (Abcam, ab14085) was used according to the
1057 manufacturer's instructions. To detect the PS on the inner leaflet of plasma membrane
1058 of ookinetes, a sequence encoding human Annexin V tagged with mScarlet driven by
1059 1.5 kb *Pysoap* 5'-UTR and 1.0 kb *Pbdhfr* 3'-UTR was inserted to pL0019-derived
1060 plasmid containing human *dhfr* marker for pyrimethamine selection. Briefly, blood
1061 stage parasites were electroporated with 10 μg plasmid DNA and selected with
1062 pyrimethamine (70 μg/ml) for 7 days. Ookinetes from transfected parasites were
1063 prepared from *in vitro* culture. Both Annexin V-mScarlet and mScarlet expressed
1064 ookinetes were treated with 1μM A23187, and the cytoplasmic distribution and
1065 intensity of the fluorescent signal was monitored using a Zeiss LSM 780 confocal
1066 microscope.

1067 **Protein extraction and western blotting**

1068 Protein extraction from asexual blood parasites, gametocytes, zygotes, retorts, and
1069 ookinetes was performed using buffer A (0.1% SDS, 1mM DTT, 50 mM NaCl, 20 mM
1070 Tris-HCl; pH8.0) containing protease inhibitor cocktail and PMSF. After
1071 ultrasonication, the protein solution was incubated on ice for 30 min before
1072 centrifugation at 12,000 g for 10 min at 4°C. The supernatant was lysed in Laemmli
1073 sample buffer. GCβ was separated in 4.5% SDS-PAGE and transferred to PVDF
1074 membrane (Millipore, IPVH00010). The membrane was blocked in 5% skim milk
1075 TBST buffer and incubated with primary antibodies. After incubation, the membrane
1076 was washed three times with TBST and incubated with HRP-conjugated secondary
1077 antibodies. The membrane was washed four times in TBST before enhanced
1078 chemiluminescence detection.

1079 **Cellular fractionation**

1080 Cellular fractionation was conducted as described previously with minor
1081 modifications[43]. The purified retorts and ookinetes were ruptured in the hypotonic
1082 buffer (10 mM HEPES, 10 mM KCl, pH 7.4) after passing through a 1 ml syringe
1083 needle gently ten times. Total cell lysate were centrifuged for 15 min at 1,000g, and the
1084 supernatant (light fraction, including cytoplasm and cytosol vesicles) and the pellet
1085 (heavy fraction, including plasma membrane, IMC, and cytoskeleton) were collected
1086 respectively and solubilized in Laemmli buffer for 10min on ice. The solubilized
1087 protein samples were analyzed by western blotting.

1088 **Immunoprecipitation**

1089 For immunoprecipitation analysis, 1.0-2.0×10^6 ookinetes were lysed in 1 ml protein
1090 extraction buffer A plus (0.01% SDS, 1 mM DTT, 50 mM NaCl, 20 mM Tris-HCl; pH
1091 8.0) and centrifuged at 12,000 g for 10 min at 4°C before collecting the supernatant
1092 solution. Rabbit anti-HA antibody (1 μg, CST, #3724S) was added to the protein
1093 solution and incubated at 4°C for 12 h on a vertical mixer. After incubation, 20 μl buffer
1094 A plus pre-balanced protein A/G beads (Pierce, #20423) was added and incubated for
1095 2 h. The beads were washed three times with buffer A plus before elution with Laemmli
1096 buffer.

1097 **Detection of protein palmitoylation**

1098 The palmitoylation modification of ISP1 protein was performed using Acyl-RAC assay
1099 described previously[44]. Ookinetes were lysed in DHHC Buffer B (2.5% SDS, 1 mM
1100 EDTA, 100 mM HEPES, pH 7.5) containing protease inhibitor cocktail and PMSF and
1101 incubated on ice for 30 min. After centrifugation at 12,000 g for 10 min, supernatant
1102 was collected and treated with 0.1% methyl methanethiosulfonate (MMTS) at 42°C for
1103 15 min. MMTS was removed by acetone precipitation followed by washing with 70%
1104 acetone three times. Protein samples were solubilized in DHHC Buffer C (1% SDS, 1
1105 mM EDTA, 100 mM HEPES, pH 7.5 and were captured on thiopropyl sepharose 6B
1106 (GE Healthcare, 17-0402-01) in the presence of 2 M hydroxylamine or 2 M NaCl
1107 (negative control) by agitating for 3 h at room temperature. Loading controls (Input)
1108 were collected before addition of thiopropyl sepharose 6B beads. After five times
1109 washing with urea DHHC Buffer (1% SDS, 1 mM EDTA, 100 mM HEPES, 8 M urea,
1110 pH 7.5), the captured proteins were eluted from thiopropyl sepharose 6B beads in 60μl

1111 urea DHHC Buffer supplemented with 50mM DTT, and mixed with Laemmli sample

1112 buffer for further western blot analysis.

1113 **Bioinformatic searches and tools**

1114 The genomic sequences of target genes were downloaded from PlasmoDB database.

1115 The transmembrane domains of proteins were identified using the TMHMM Server

1116 (http://www.cbs.dtu.dk/services/TMHMM/) [45]. The phylogeny tree and protein

1117 amino acid sequence alignment was analyzed using MEGA5.0 [46].

1118

1119 **QUANTIFICATION AND STATISTICAL ANALYSIS**

1120 For quantification of protein expression in western blot, protein band intensity was

1121 quantified using Fiji software from three independent experiments. The signals of target

1122 proteins were normalized with that of control proteins. For quantification of protein

1123 expression in IFA, confocal fluorescence microscopy images were acquired under

1124 identical parameters. Fluorescent signals were quantified using Fiji software [47]. More

1125 than 30 cells were randomly chosen in each group. Protein expression was expressed

1126 as the relative percentage compared to control group. Protein polarization rate was

1127 calculated as the ratio of the protein fluorescent signal at OES over the fluorescent

1128 signal from the whole cell. Statistical analysis was performed using GraphPad Software

1129 5.0 [48]. Two-tailed Student's t-test or Whiney Mann test was used to compare

1130 differences between treated groups and their paired controls. n represents the number

1131 of mosquitos or parasite cells tested in each group, or experimental replication. The

1132 exact value of n was indicated within the figures. P value in each statistical analysis was

1133 also indicated within the figures.

1134

1135

1136

1137

1138

1139 Legend for Video S1 and Data S1.

1140

1141 **Video S1. Real-time tracking GCβ::mScarlet signals within a developing**

1142 **retort/ookinete of *gcβ::mScarlet* parasite. Related to Figure 1.**

1143

1144 **Data S1. Genotyping results of the genetic modified parasite strains in this study,**

1145 **Related to the STAR Methods.**

1146 **(A)** Schematic representation for CRISPR/Cas9 mediated gene deletion via double

1147 cross homologous recombination. **(B and C)** Schematic representation for

1148 CRISPR/Cas9 mediated N-terminal **(B)** or C-terminal **(C)** tagging of endogenous genes

1149 with epitope tag or fluorescence protein via double cross homologous recombination.

1150 Primers used for diagnostic PCR are indicated and listed in the Table S2. **(D to J)** For

1151 each modification, both the 5' and 3' homologous recombination was detected by

1152 diagnostic PCR, confirming successful integration of the homologous templates. For

1153 most modification, at least two single clones (sc) with targeted modifications were

1154 obtained after limiting dilution and were used for phenotype analysis.

1155