

1 **Protein Phosphatase 1 regulates atypical mitotic and meiotic**
2 **division in *Plasmodium* sexual stages**

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30 Short title: Role of PP1 during mitosis and meiosis in *Plasmodium*

31

32 **Abstract**

33 PP1 is a conserved eukaryotic serine/threonine phosphatase that regulates many
34 aspects of mitosis and meiosis, often working in concert with other phosphatases,
35 such as CDC14 and CDC25. The proliferative stages of the malaria parasite life
36 cycle include sexual development within the mosquito vector, with male gamete
37 formation characterized by an atypical rapid mitosis, consisting of three rounds of
38 DNA synthesis, successive spindle formation with clustered kinetochores, and a
39 meiotic stage during zygote to ookinete development following fertilization. It is
40 unclear how PP1 is involved in these unusual processes. Using real-time live-cell
41 and ultrastructural imaging, conditional gene knockdown, RNA-seq and proteomic
42 approaches, we show that *Plasmodium* PP1 is implicated in both mitotic exit and,
43 potentially, establishing cell polarity during zygote development in the mosquito
44 midgut, suggesting that small molecule inhibitors of PP1 should be explored for
45 blocking parasite transmission.

46

47

48 **Introduction**

49 Cell cycle progression involves sequential and highly ordered DNA replication and
50 chromosome segregation in eukaryotes^{1, 2}, which is tightly controlled and
51 coordinated by reversible protein phosphorylation catalysed by protein kinases (PKs)
52 and protein phosphatases (PPs)³. Numerous studies have highlighted the
53 importance of the phosphoprotein phosphatase (PPP) family in regulating mitosis in
54 model eukaryotic organisms, often working in conjunction with CDC25 and CDC14
55 phosphatases, as key regulators of mitotic entry and exit, respectively^{4, 5, 6, 7}.

56

57 Protein Phosphatase 1 (PP1) is a member of the PPP family and is expressed in all
58 eukaryotic cells. It plays a key role in the progression of mitosis through
59 dephosphorylation of a large variety of proteins, including mitotic kinases (such as
60 cyclin-dependent kinase 1 (CDK1)^{8, 9, 10, 11}, and regulators of chromosome
61 segregation¹². Chromosome segregation is orchestrated at the kinetochore, a protein
62 complex that assembles on the centromeres, located at the constriction point of
63 sister chromatids to facilitate and monitor attachment of the sister chromatids to
64 spindle microtubules^{13, 14}. Correct attachment of the kinetochore to spindle
65 microtubules is regulated by the KMN (KNL1, MIS12 and NDC80) protein network¹⁵,
66 which in mammalian cells integrates the activities of at least five protein kinases
67 (including MPS1, Aurora B, BUB1, PLK1, and CDK1) and two protein phosphatases
68 (PP1 and PP2A-B56). The KMN network mediates kinetochore-microtubule
69 attachments and scaffolds the spindle assembly checkpoint (SAC) to prevent
70 chromosome segregation until all sister chromatids are properly connected to the
71 spindle¹⁶. The orchestration of reversible protein phosphorylation is crucial to control
72 the spatial-temporal progression of the cell cycle and PP1 has a key role in this

73 process, in particular during mitotic exit. Throughout mitosis, PP1 is inhibited by the
74 cyclin-dependent kinase (CDK1)/cyclin B complex and Inhibitor 1^{17, 18}; however,
75 during mitotic exit concomitant destruction of cyclin B and reduced activity of CDK1
76 through dephosphorylation by CDC14 results in subsequent reactivation of PP1 via
77 autophosphorylation and completion of mitotic exit^{10, 17, 19, 20, 21}.

78

79 In *Plasmodium*, the causative organism of malaria, there is a single PP1 orthologue,
80 which is expressed throughout the parasite's complex life-cycle²² and located in both
81 nucleus and cytoplasm²³. *P. falciparum* PP1 (*PfPP1*) shares 80% identity with
82 human PP1c, and likely has a conserved tertiary and secondary structure containing
83 9 α -helices and 11 β -strands²⁴. However, *PfPP1* lacks part of the 18 amino-acid
84 motif at the C-terminus of human PP1, which contains a threonine residue (Thr320)
85 that is phosphorylated by CDC2 kinase²⁵ and dynamically regulates entry into
86 mitosis²⁴. Previous studies determined that *P. falciparum* PP1 functionally
87 complements the *Saccharomyces cerevisiae* glc7 (PP1) homologue²⁶, and
88 subsequent phylogenetic analyses revealed that homologues of the phosphatases
89 CDC14 and CDC25 are absent from *Plasmodium*²³. Genetic screens and inhibitor
90 studies have shown that PP1 is essential for asexual blood stage development^{23, 27,}
91 ^{28, 29} (also reviewed²⁴), in particular for parasite egress from the host erythrocyte³⁰.
92 Most studies in *Plasmodium* have been focused on these asexual blood stages of
93 parasite proliferation, and very little is known about the importance of PP1 for
94 transmission stages within the mosquito vector. Recent phosphoproteomic studies
95 and chemical genetics analysis have identified a number of potential *Plasmodium*
96 *falciparum* (Pf) PP1 substrates modified during egress, including a HECT E3 protein-
97 ubiquitin ligase and GC α , a guanylyl cyclase with a phospholipid transporter

98 domain³⁰. Other biochemical studies in *Plasmodium* have identified numerous PP1-
99 interacting partners (PIPs) that are structurally conserved and regulate PP1 activity,
100 including LRR1 (a human SDS22 orthologue), Inhibitor 2 and Inhibitor 3. Other PIPs
101 including eif2 β , and GEXP15 have also been identified^{9, 24, 31, 32, 33}. Synthetic
102 peptides containing the RVxF motif of Inhibitor 2 and Inhibitor 3, and the LRR and
103 the LRR cap motif of *Pf*LRR1 significantly reduce *P. falciparum* growth in vitro^{34, 35}
104 and regulate *Pf*PP1 phosphatase activity^{35, 36, 37}. However, little is known regarding
105 how PP1 is involved in regulating mitosis and meiosis in the absence of CDC14 and
106 CDC25.

107

108 The *Plasmodium* life-cycle is characterised by several mitotic stages and a single
109 meiotic stage (reviewed in^{38, 39, 40}). However, in this organism kinetochore dynamics
110 and chromosome segregation are poorly understood, especially in mitosis during
111 male gametogenesis. This is a very rapid process with three rounds of spindle
112 formation and genome replication from 1N to 8N within 12 minutes. In addition, little
113 is known about the first stage of meiosis that occurs during the zygote to ookinete
114 transition. We have followed recently the spatio-temporal dynamics of NDC80
115 throughout mitosis in schizogony, sporogony and male gametogenesis, and during
116 meiosis in ookinete development⁴¹, using approaches that offer the opportunity to
117 study the key molecular players in these crucial stages of the life cycle. Although
118 PP1 has been partially characterised and shown to have an essential role during
119 asexual blood stage development in the vertebrate host²³, the role and importance of
120 PP1 during sexual stage development in the mosquito is completely unknown.

121

122 Here, using the *Plasmodium berghei* (Pb) mouse model of malaria, we determined
123 the importance of PP1 during the sexual stages within the mosquito vector. PP1
124 expression and location were studied using the endogenous, GFP-tagged protein
125 and co-localisation with the kinetochore marker, NDC80, to follow progression
126 through chromosome segregation during male gamete formation and zygote
127 differentiation. Using a conditional gene knockdown approach we examined how
128 PP1 orchestrates atypical mitosis and meiosis, and investigated the ultrastructural
129 consequences of PP1 gene knockdown for cell morphology, nuclear pole
130 multiplication and flagella formation during male gamete formation. RNA-Seq
131 analysis was used to determine the consequence of PP1 gene knockdown on global
132 transcription, which disclosed a marked differential expression of genes involved in
133 reversible phosphorylation, motor activity and the regulation of cell polarity.
134 Proteomics studies identified motor protein kinesins as interacting partners of PP1 in
135 the gametocyte. The knockdown of PP1 gene expression blocks parasite
136 transmission by the mosquito, showing that this protein has a crucial function in
137 *Plasmodium* sexual development during both mitosis in male gamete formation and
138 meiosis during zygote to ookinete differentiation.

139

140 **Results**

141 **PP1-GFP has a diffuse subcellular location during asexual blood stage** 142 **schizogony and forms discrete foci during endomitosis.**

143 To examine the spatio-temporal expression of *Plasmodium* PP1 in real-time during
144 cell division, we generated a transgenic *P. berghei* line expressing endogenous PP1
145 with a C-terminal GFP tag. An in-frame *gfp* coding sequence was inserted at the 3'
146 end of the endogenous *pp1* locus using single crossover homologous recombination

147 **(Fig S1A)**, and successful insertion was confirmed by diagnostic PCR **(Fig S1B)**.

148 Western blot analysis of schizont protein extracts using an anti-GFP antibody

149 revealed a major 62-kDa band, the expected size for PP1-GFP protein, compared to

150 the 29 kDa WT-GFP **(Fig S1C)**.

151

152 During asexual blood stage proliferation, schizogony is characterised by multiple

153 rounds of asynchronous nuclear division without chromosome condensation or

154 cytokinesis. Nuclear division is a closed mitotic process without dissolution-

155 reformation of the nuclear envelope and with the spindle-pole body

156 (SPB)/microtubule-organising centre (MTOC) embedded within the nuclear

157 membrane⁴². It results in a multinucleated coenocyte termed a schizont, which is

158 resolved by cytokinesis at the end of schizogony into individual merozoites. Live cell

159 imaging of *P. berghei* asexual blood stages revealed a diffuse cytoplasmic and

160 nuclear distribution of PP1-GFP, together with a distinct single focus of concentrated

161 PP1-GFP in the nucleus of early trophozoite stage **(Fig 1A)**, representing the early

162 S-phase of the cell cycle when DNA synthesis starts. As schizogony proceeds, the

163 diffuse distribution of PP1-GFP remained; however, in early schizonts each cell

164 displayed two distinct PP1-GFP foci in close association with the stained nuclear

165 DNA. These pairs of PP1-GFP foci became clearer during the middle and late

166 schizont stages but following merozoite maturation and egress the intensity of the

167 foci diminished **(Fig 1A)**.

168

169 To study further the location of the PP1-GFP foci throughout mitosis, we generated

170 by genetic cross parasite lines expressing either PP1-GFP and the kinetochore

171 marker NDC80-mCherry⁴¹ or PP1-GFP and the inner membrane complex (IMC)-

172 associated myosin A (MyoA)-mCherry. Live cell imaging of these lines revealed co-
173 localisation of PP1-GFP and NDC80-mCherry foci close to the DNA of the nucleus
174 through blood stage development, and especially during late schizogony and
175 segmentation (**Fig 1B**); whereas the PP1-GFP foci at the outer periphery of the cell
176 showed a partial co-location with MyoA-mCherry, and only during mid- to late
177 schizogony (**Fig 1C**). Thus, in schizonts there is a concentration of PP1-GFP at the
178 kinetochore and at a transient peripheral location during specific stages of
179 schizogony, as well as the diffuse distribution throughout the cytoplasm (**Fig.1**).

180

181 **PP1-GFP is enriched on kinetochores during chromosome segregation**
182 **associated with putative mitotic exit in male gametogony**

183 To study PP1 expression and location through the three rounds of DNA replication
184 and chromosome segregation prior to nuclear division during male gametogony, we
185 examined PP1-GFP by live-cell imaging over a 15-minute period following male
186 gametocyte activation. Following activation with xanthurenic acid and decreased
187 temperature, male gametocytes undergo three rounds of DNA replication and mitotic
188 division followed by chromosome condensation and exflagellation, resulting in eight
189 gametes⁴³. Before activation (0 min), PP1-GFP was detected with a diffuse location
190 throughout gametocytes (**Fig 2A**). At one minute post-activation, PP1-GFP
191 accumulated at one end of the nucleus at a single focal point (**Fig 2A**). After 2-3 min
192 two distinct foci were observed on one side of the nucleus, concurrent with the first
193 round of chromosome replication/segregation (**Fig 2A**). Subsequently, four and eight
194 PP1-GFP foci were observed at 6 to 8 min and 8 to 12 min post-activation,
195 respectively, corresponding to the second and third rounds of chromosome
196 replication/segregation. These discrete PP1-GFP foci dispersed prior to karyokinesis

197 and exflagellation of the mature male gamete 15 min post-activation, leaving residual
198 protein remaining diffusely distributed throughout the remnant gametocyte and
199 flagellum (**Fig 2A**).

200

201 To investigate further the location of PP1-GFP during spindle formation and
202 chromosome segregation, the parasite line expressing both PP1-GFP and NDC80-
203 mCherry was examined by live cell imaging to establish the spatio-temporal
204 relationship of the two proteins. We found that the discrete PP1-GFP foci colocalized
205 with NDC80-mCherry at different stages of male gametogony, including when up to
206 eight kinetochores were visible (**Fig 2B**), but PP1-GFP was not associated with the
207 arc-like bridges of NDC80-mCherry representing the spindle⁴¹ at 2 min and 4 to 5
208 min post activation, suggesting that PP1-GFP is only increased at the kinetochore
209 during initiation and termination of spindle division (**Fig 2B**).

210

211 **PP1-GFP may determine apical polarity during zygote-ookinete development** 212 **and has a nuclear location on kinetochores during meiosis**

213 After fertilisation in the mosquito midgut, the diploid zygote differentiates into an
214 ookinete within which the first stage of meiosis occurs. During this process DNA is
215 duplicated to produce a tetraploid cell with four haploid genomes within a single
216 nucleus in the mature ookinete. PP1 is known to be crucial during meiotic
217 chromosome segregation¹², and therefore we analysed the spatio-temporal
218 expression of PP1-GFP during ookinete development using live cell imaging. PP1-
219 GFP was expressed in both male and female gametes with a diffuse distribution,
220 along with a single focus of intense fluorescence at one end (potentially the basal
221 body) of each male gamete (**Fig. 3A**). Initially, the zygote also had a diffuse PP1-

222 GFP distribution, but after two hours (stage I) an enriched focus developed at the
223 periphery of the zygote, marking the point that subsequently protruded out from the
224 cell body and developed into the apical end of the ookinete. A strong PP1-GFP
225 fluorescence signal remained at this apical end throughout ookinete differentiation
226 (**Fig 3A**). In addition, PP1-GFP was enriched in the nucleus at four discrete foci in
227 mature ookinetes (**Fig 3A**). Analysis of the parasite line expressing both PP1-GFP
228 and NDC80-mCherry showed that these four foci correspond to the kinetochores of
229 meiotic development in the ookinete (**Fig 3B**).

230

231 **PP1-GFP has a diffuse distribution with multiple nuclear foci during oocyst** 232 **development, representing endomitosis**

233 Upon maturation the ookinete penetrates the mosquito midgut wall and embeds in
234 the basal lamina to form an oocyst. Over the course of 21 days sporogony occurs in
235 which several rounds of closed endomitotic division produce a multiploid cell termed
236 a sporoblast⁴⁴; with subsequent nuclear division resulting in thousands of haploid
237 nuclei and concomitant sporozoite formation. We observed multiple foci of PP1GFP
238 along with diffused localization during oocyst development (**Fig 3C**). Dual colour
239 imaging showed a partial co-localisation of nuclear PP1-GFP and NDC80-mCherry
240 foci throughout oocyst development and in sporozoites (**Fig 3D**), suggesting that
241 PP1-GFP is diffusely distributed during oocyst development but also recruited to
242 kinetochores, as highlighted by multiple nuclear foci during oocyst development.

243

244 **Generation of transgenic parasites with conditional knockdown of PP1**

245 Our previous systematic analysis of the *P. berghei* protein phosphatases suggested
246 that PP1 is likely essential for erythrocytic development²³, a result which was further

247 substantiated in a recent study of *P. falciparum*³⁰. Since little is known about the role
248 of PP1 in cell division during the sexual stages of parasite development in the
249 mosquito vector, we attempted to generate transgenic, conditional knockdown lines
250 using either the auxin inducible degron (AID) or the promoter trap double
251 homologous recombination (PTD) systems. Despite successful generation of a
252 parasite line expressing PP1-AID, addition of indole-3-acetic acid (IAA) did not result
253 in PP1 depletion (**Fig S2C**) and male gametogony was not affected (**Fig S2C**).
254 Therefore, we used a promoter trap method that had been used previously for
255 functional analysis of the condensin core subunits SMC2/4 and the APC/C complex
256 component, APC3^{45, 46}. Double homologous recombination was used to insert the
257 *ama* promoter upstream of *pp1* in a parasite line that constitutively expresses GFP⁴⁷.
258 The expected outcome was a parasite expressing PP1 during asexual blood stage
259 development, but not at high levels during the sexual stages (**Fig S2D**). This strategy
260 resulted in the successful generation of two independent PP1PTD parasite clones
261 (clones 3 and 5) produced in independent transfections, and integration confirmed by
262 diagnostic PCR (**Fig S2E**). The PP1PTD clones had the same phenotype and data
263 presented here are combined results from both clones. Quantitative real time PCR
264 (qRT-PCR) confirmed a downregulation of *pp1* mRNA in gametocytes by
265 approximately 90% (**Fig 4A**).

266

267 **Knockdown of PP1 gene expression during the sexual stages blocks parasite**
268 **transmission by affecting parasite growth and development within the**
269 **mosquito vector**

270 The PP1PTD parasites grew slower than the WT-GFP controls in the asexual blood
271 stage (**Fig S2E**) but produced normal numbers of gametocytes in mice injected with

272 phenylhydrazine before infection. Gametocyte activation with xanthurenic acid in
273 ookinete medium resulted in significantly fewer exflagellating PP1PTD parasites per
274 field in comparison to WT-GFP parasites, suggesting that male gamete formation
275 was severely retarded (**Fig. 4B**). For those few PP1PTD gametes that were released
276 and viable, zygote-ookinete differentiation following fertilization was severely affected
277 (**Fig 4C**), with significantly reduced numbers of fully formed, banana-shaped
278 ookinetes. In all PP1PTD samples analysed 24 hours post-fertilisation, the vast
279 majority of zygotes were still round, and there was a significant number of abnormal
280 retort-shaped cells with a long thin protrusion attached to the main cell body (**Fig 4C,**
281 **D**).

282

283 To investigate further the function of PP1 during parasite development in the
284 mosquito, *Anopheles stephensi* mosquitoes were fed on mice infected with PP1PTD
285 and WT-GFP parasites as a control. The number of GFP-positive oocysts on the
286 mosquito gut wall was counted on days 7, 14 and 21 post-infection. No oocysts were
287 detected from PP1PTD parasites; whereas WT-GFP lines produced normal oocysts
288 (**Fig 4E, F**), all of which had undergone sporogony (**Fig 4G**). Furthermore, no
289 sporozoites were observed in the salivary glands of PP1PTD parasite-infected
290 mosquitoes, in contrast to WTGFP parasite-infected mosquitoes (**Fig 4H**). This lack
291 of viable sporozoites was confirmed by bite back experiments that showed no further
292 transmission of PP1PTD parasites, in contrast to the WTGFP lines, which showed
293 positive blood stage infection four days after mosquito feed (**Fig S2F**).

294

295

296 **Ultrastructure analysis of PP1PTD gametocytes shows defects in nuclear pole**
297 **and axoneme assembly**

298 To determine the ultrastructural consequences of reduced PP1 expression, WTGFP
299 and PP1PTD gametocytes were examined at 6- and 30-min post activation (pa) by
300 electron microscopy. At 6 min pa, the WTGFP male gametocytes exhibited an open
301 nucleus with a number of nuclear poles (**Fig 5a**). Basal bodies and normal 9+2
302 axonemes were often associated with the nuclear poles (**Fig 5b,c**). At 6 min pa, the
303 PP1PTD line exhibited a similar morphology (**Fig 5d-f**). However, quantitative
304 analysis showed that PP1PTD gametocytes were relatively less well-developed, with
305 fewer nuclear poles (0.86 WTGFP v. 0.56 PP1PTD), basal bodies (1.03 WTGFP v.
306 0.56 PP1PTD) and axonemes (2.57 WTGFP v. 1.27 PP1PTD), based on random
307 sections of fifty male gametocytes in each group.

308

309 At 30 min post activation, most WTGFP male gametocytes (83%) were at a late
310 stage of development with nuclei showing chromatin condensation (**Fig 5g**) and
311 examples of exflagellation with the flagellate gamete protruding from the surface (**Fig**
312 **5h**) and free male gametes (**Fig 5g, i**). In contrast, most PP1PTD male gametocytes
313 (85%) were stalled at an early stage of development similar to that at 6 min pa (**Fig**
314 **5j-l, Fig S3A**), based on random sections of fifty male gametocytes in each group.

315 The main morphological difference between the two parasite lines was a marked
316 increase in the number and length of the axonemes in the PP1PTD parasites at 30
317 min pa (**cf Figs 5a and j**). In summary, the development of PP1PTD male
318 gametocytes was severely retarded, although axoneme growth increased.

319

320 **PP1PTD parasites have altered expression of genes involved in cell cycle**
321 **progression, cell motility, and apical cell polarity**

322 To determine the consequences of PP1 knockdown on global mRNA transcription,
323 we performed RNA-seq in duplicate on PP1PTD and WT-GFP gametocytes, 0 min
324 and 30 min post-activation (**Fig 6A and B**). All replicates were clustered together
325 based on condition (**Fig S3B**) and totals of 13 to 32 million RNA-seq reads were
326 generated per sample for the global transcriptome (**Fig S3C**). In both 0 min and 30
327 min activated gametocytes, *pp1* was significantly down-regulated (by more than 16-
328 fold, q value < 0.05) in PP1PTD parasites compared to WT-GFP parasites (**Fig 6A**),
329 thus confirming the qPCR result (**Fig 4A**). We observed significant transcriptional
330 perturbation in both 0 min and 30 min activated gametocytes, affecting the
331 expression of 530 and 829 genes respectively (representing ~ 10% and 16% of the
332 total gene complement) (**Table S1**). Of the total perturbed genes, 344 and 581 were
333 significantly down-regulated and 186 and 248 genes were significantly up-regulated
334 in the PP1PTD parasites compared to WT-GFP controls in 0 min and 30 min
335 activated gametocytes, respectively (**Table S1**).

336

337 To explore the biological roles of the genes down-regulated following reduced *pp1*
338 expression, we performed gene-ontology (GO) based enrichment analysis. We
339 observed that many genes encoding kinases, phosphatases and motor proteins
340 were differentially expressed in either or in both 0 min or 30 mins activated
341 gametocytes (**Fig 6C**), complementing the observations from our phenotypic
342 analysis (**Fig 4C, D**). In addition, we also observed that many genes encoding
343 proteins involved in cell motility, cell-cycle progression, host-cell entry and ookinete
344 development were significantly affected in activated PP1PTD gametocytes.

345 Transcript levels measured by RNA-seq were further validated by qRT-PCR for a
346 few selected genes modulated in gametocytes at 0 min and 30 min, and involved in
347 regulation of cell cycle, cell motility, and ookinete invasion (**Fig 6D**).

348

349 **PP1-GFP interacts with similar proteins in schizonts and gametocytes, but**
350 **with a predominance of microtubule motor kinesins in gametocytes**

351 Previous studies have analysed the PP1 interactome in *P. falciparum* schizonts,
352 revealing several interacting partners^{32, 48}. Here, we analysed the PP1 interactome in
353 *P. berghei* schizonts and gametocytes to establish whether there were differences
354 that might reflect distinct functions in the two stages. We immunoprecipitated PP1-
355 GFP from lysates of schizonts following parasite culture *in vitro* for 10 hours and 24
356 hours and from lysates of gametocytes 10 to 11 min post activation because of high
357 PP1-GFP abundance at these stages (**Fig 7A, Table S2**). Mass spectrometric
358 analysis of these pulldowns identified several proteins common to both schizont and
359 gametocyte stages, suggesting similar protein complexes in both stages (**Fig 7A, B**).
360 In addition, we also identified in the pulldown from gametocyte lysates a number of
361 microtubule proteins that are associated with the spindle or axoneme, including
362 kinesin-8B, kinesin-15, kinesin-13 and PF16 (**Fig 7A, B**). These proteins are specific
363 to male gametocytes and may have important roles in axoneme assembly and male
364 gametogony^{43, 49}.

365

366 **Discussion**

367 Reversible protein phosphorylation is crucial for cell cycle progression in eukaryotes
368 and is tightly controlled by a variety of protein kinases and phosphatases^{50, 51}.
369 *Plasmodium* possesses a set of divergent protein kinases and protein phosphatases,

370 which regulate many processes during cell division and parasite development
371 throughout different stages of the life-cycle^{23, 52}, and PP1 is quantitatively one of the
372 most important protein phosphatases that hydrolyse serine/threonine linked
373 phosphate ester bonds⁵³. It is expressed in all cells and is highly conserved in
374 organisms throughout the Eukaryota including *Plasmodium*^{23, 54}, although a key
375 region at the C-terminus that is known to regulate mitotic exit is missing from the
376 parasite PP1²⁴. It forms stable complexes with several PP1-interacting proteins
377 (PIPs) that are diverse and differ in various organisms, control its location and
378 function, and assist in processes throughout the cell cycle^{8, 55, 56, 57}.

379

380 The cell cycle in *Plasmodium* differs from that of many other eukaryotes with atypical
381 mitotic and meiotic divisions throughout its life cycle^{41, 43, 58}. It lacks several classical
382 cell cycle regulators including the protein phosphatases CDC14 and CDC25²³, and
383 key protein kinases including polo-like kinases^{52, 59}. Therefore, the role of PP1, or
384 indeed other PPPs in the absence of these protein phosphatases and kinases may
385 be more crucial during cell division in *Plasmodium*. In the present study, we focused
386 on the location and functional role of PP1 in mitotic division, particularly during male
387 gametogony, and in meiosis during the zygote to ookinete transition. Both processes
388 are essential for the sexual stage of the *Plasmodium* life cycle and are required for
389 transmission by the mosquito vector. Our previous systematic functional analysis of
390 the *Plasmodium* protein phosphatome²³ showed that PP1 has an essential role
391 during asexual blood stage development; this finding was supported by a recent
392 study, which showed its role in merozoite egress from the host erythrocyte³⁰. Our
393 findings here reveal that PP1 is also expressed constitutively and co-localises with
394 the kinetochore protein NDC80 during different stages of *Plasmodium* asexual and

395 sexual development, hinting at a role during atypical chromosome segregation. Our
396 conditional PP1 gene knockdown suggests that it plays a crucial role in mitotic
397 division during male gametogony; and may regulate cell polarity in meiosis during
398 zygote to ookinete transformation.

399

400 Male gametogony in *Plasmodium* is a rapid process with DNA replication and three
401 mitotic divisions followed by karyokinesis and cytokinesis to form eight flagellated
402 male gametes within 15 min of gametocyte activation^{41, 58}. The expression and
403 localization profiles show that although the protein is diffusely distributed throughout
404 the cytoplasm and the nucleus, there is a cyclic enrichment of PP1-GFP at the
405 kinetochore, associating with NDC80-mCherry during all three successive genome
406 replication and closed mitotic divisions without nuclear division⁴¹. The accumulation
407 of PP1-GFP at the kinetochore at the start of nuclear division and subsequent
408 decrease upon completion is similar to the situation in other eukaryotes where the
409 activity of PP1 increases in G2 phase, reduces during prophase and metaphase, and
410 increases again during anaphase⁵¹. This behaviour suggests a role for PP1 in
411 regulating rapid mitotic entry and exit during male gametogony. Further analysis of
412 PP1 gene knockdown showed a significant decrease in male gamete formation (i.e.
413 during male gametogony), and ultrastructural analysis revealed fewer nuclear poles
414 and basal bodies associated with axonemes, with a concomitant absence of
415 chromosome condensation in male gametocytes of PP1PTD parasites, suggesting a
416 role for PP1 during chromosome segregation and gamete formation (i.e. flagella
417 formation). A similar phenotype was observed in our recent study of a divergent
418 *Plasmodium* cdc2-related kinase (CRK5)⁶⁰. CRK5 deletion resulted in fewer nuclear
419 poles, and no chromatin condensation, cytokinesis or flagellum formation,

420 suggesting that there may be a coordinated activity of PP1 and CRK5 in reversible
421 phosphorylation. A recent phosphoregulation study across the short time period of
422 male gametogony showed tightly controlled phosphorylation events mediated by
423 several protein kinases including ARK2, CRK5, and NEK1⁶¹; however, the reciprocal
424 protein phosphatases that reverse these events are unknown. The expression
425 pattern and cyclic enrichment of PP1 at the kinetochores suggest a key reciprocal
426 role of PP1 in reversing the protein phosphorylation mediated by these kinases. This
427 interpretation is supported by our global transcriptome analysis of the PP1PTD
428 parasite, which showed a modulated expression of several serine/threonine protein
429 kinases, protein phosphatases and motor proteins. The similar pattern of reduced
430 expression of some of these protein phosphatases and kinases suggests they have
431 a coordinated role in reversible phosphorylation. Of note, phosphoregulation of motor
432 proteins in *Plasmodium* has been described previously⁶¹ and it plays an important
433 role in spindle assembly and axoneme formation during male gametogony^{41, 43}.

434 The proteomics analysis showed that PP1 interacts with a conserved set of proteins
435 in both asexual and sexual stages of development, and with additional proteins in
436 gametocytes, implicating major motor proteins that may be required for spindle and
437 axoneme assembly during male gametogenesis. Several of the conserved
438 interacting partners also form complexes with PP1 in other eukaryotes but the
439 gametocyte specific proteins such as kinesin-8B, kinesin-15, kinesin-13, and PF16
440 are unique PIPs in *Plasmodium*^{9, 48}. These findings are consistent with our
441 transcriptomic analysis of PP1PTD showing modulated expression of motor proteins
442 in male gametocytes.

443 Analysis of PP1 during the zygote to ookinete transformation showed an additional
444 location at the nascent apical end during the early stages of ookinete differentiation,

445 which may help define the cell's polarity. This suggestion is supported by the
446 consequence of PP1PTD gene knock down in which numerous underdeveloped
447 ookinetes with a long, thin protrusion attached to the main cell body were observed.
448 This idea is also substantiated by a recent study showing that apical-basal polarity in
449 *Drosophila* is controlled by PP1-mediated, and SDS22-dependent,
450 dephosphorylation of LGL, an actomyosin-associated protein⁶². However, it is
451 important to note that the suggested role in cell polarity is based on the observation
452 that after fertilization only a few abnormal ookinetes are formed, which have the
453 morphologically distinct elongated apical end. Our transcriptomic analysis of
454 PP1PTD-gametocytes showed modulation of genes for several organelle markers
455 such as CTRP and SOAP^{63, 64}, polarity markers such as SAS6L and IMC proteins⁶⁵,
456 ⁶⁶, as well as genes involved in gliding motility including other IMC proteins, MyoA,
457 GAPs, GAPMs, and some ookinete specific proteins. These proteins are important
458 for maintenance of cell shape, gliding motility, and mosquito gut wall invasion by
459 ookinetes. These results suggest that PP1 may be involved in the phospho-
460 regulation of proteins involved in defining polarity and maintenance of ookinete
461 shape.

462 In conclusion, PP1 is a constitutively expressed phosphatase, distributed throughout
463 the cell but enriched in the nucleus, and associated with the kinetochore during
464 mitosis and meiosis, with a role in the regulation of mitosis and meiosis throughout
465 the *Plasmodium* life-cycle.

466

467 **Material and Methods**

468 **Ethics statement**

469 The animal work performed in the UK passed an ethical review process and was
470 approved by the United Kingdom Home Office. Work was carried out under UK
471 Home Office Project Licenses (30/3248 and PDD2D5182) in accordance with the
472 United Kingdom 'Animals (Scientific Procedures) Act 1986'. Six- to eight-week-old
473 female CD1 outbred mice from Charles River laboratories were used for all
474 experiments in the UK.

475 **Generation of transgenic parasites**

476 GFP-tagging vectors were designed using the p277 plasmid vector and
477 transfected as described previously²³. A schematic representation of the
478 endogenous *pp1* locus (PBANKA_1028300), the constructs and the recombined *pp1*
479 locus can be found in **Fig S1A**. For GFP-tagging of PP1 by single crossover
480 homologous recombination, a region of *pp1* downstream of the ATG start codon was
481 used to generate the construct. For the genotypic analyses, a diagnostic PCR
482 reaction was performed as outlined in **Fig. S1A**. Primer 1 (intP6tg) and primer 2
483 (ol492) were used to determine correct integration of the *gfp* sequence at the
484 targeted locus. For western blotting, purified gametocytes were lysed using lysis
485 buffer (10 mM TrisHCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA and 1% NP-40). The
486 lysed samples were boiled for 10 min at 95 °C after adding Laemmli buffer and were
487 centrifuged at maximum speed (13000g) for 5 min. The samples were
488 electrophoresed on a 4–12% SDS-polyacrylamide gel. Subsequently, resolved
489 proteins were transferred to nitrocellulose membrane (Amersham Biosciences).
490 Immunoblotting was performed using the Western Breeze Chemiluminescence Anti-
491 Rabbit kit (Invitrogen) and anti-GFP polyclonal antibody (Invitrogen) at a dilution of
492 1:1250, according to the manufacturer's instructions.

493 To study the function of PP1, we used two conditional knock down systems; a
494 promoter exchange/trap using double homologous recombination (PP1PTD) and an
495 auxin inducible degron (PP1AID) system. The PP1AID construct was derived from
496 the p277 plasmid, where the GFP sequence was excised following digestion with
497 *AgeI* and *NotI* restriction enzymes and replaced with an AID/HA coding sequence.
498 The AID-HA sequence was PCR amplified (using primers: 5'-
499 CCCCAGACGTCGGATCCAATGATGGGCAGTGTGCGAGCT-3' and 5'-
500 ATATAAGTAAGAAAAACGGCTTAAGCGTAATCTGGA-3') from the GW-AID/HA
501 plasmid (<http://plasmogem.sanger.ac.uk/>). Fragments were assembled following the
502 Gibson assembly protocol to generate the PP1-AID/HA transfection plasmid that was
503 transfected in the 615 line. Conditional degradation of PP1-AID/HA was performed
504 as described previously⁶⁷. A schematic representation of the endogenous *pp1* locus
505 (PBANKA_1028300), the constructs and the recombined *pp1* locus can be found in
506 **Fig S2A**. A diagnostic PCR was performed for *pp1* gene knockdown parasites as
507 outlined in **Fig. S2A**. Primer 1 and Primer 3 were used to determine successful
508 integration of the targeting construct at the 3' gene locus (**Fig S2B**). Primer 1 and
509 Primer 2 were used as controls (**Fig S2B**).

510 The conditional knockdown construct PP1-PTD was derived from *P_{ama1}* (pSS368))
511 where *pp1* was placed under the control of the *ama1* promoter, as described
512 previously⁶⁸. A schematic representation of the endogenous *pp1* locus, the
513 constructs and the recombined *pp1* locus can be found in **Fig S2D**. A diagnostic
514 PCR was performed for *pp1* gene knockdown parasites as outlined in **Fig. S2D**.
515 Primer 1 (5'-intPTD36) and Primer 2 (5'-intPTD) were used to determine successful
516 integration of the targeting construct at the 5' gene locus. Primer 3 (3'-intPTD) and
517 Primer 4 (3'-intPTama1) were used to determine successful integration for the 3' end

518 of the gene locus (**Fig. S2E**). All the primer sequences can be found in **Table S3**. *P.*
519 *berghei* ANKA line 2.34 (for GFP-tagging) or ANKA line 507cl1 expressing GFP (for
520 the knockdown construct) parasites were transfected by electroporation⁴⁷.

521 **Purification of schizonts and gametocytes**

522 Blood cells obtained from infected mice (day 4 post infection) were cultured for 11h
523 and 24 h at 37°C (with rotation at 100 rpm) and schizonts were purified the following
524 day on a 60% v/v NycoDenz (in PBS) gradient, (NycoDenz stock solution: 27.6% w/v
525 NycoDenz in 5 mM Tris-HCl, pH 7.20, 3 mM KCl, 0.3 mM EDTA).

526 The purification of gametocytes was achieved using a protocol described
527 previously⁶⁹ with some modifications. Briefly, parasites were injected into
528 phenylhydrazine treated mice and enriched by sulfadiazine treatment after 2 days of
529 infection. The blood was collected on day 4 after infection and gametocyte-infected
530 cells were purified on a 48% v/v NycoDenz (in PBS) gradient (NycoDenz stock
531 solution: 27.6% w/v NycoDenz in 5 mM Tris-HCl, pH 7.20, 3 mM KCl, 0.3 mM
532 EDTA). The gametocytes were harvested from the interface and activated.

533 **Live cell imaging**

534 To examine PP1-GFP expression during erythrocyte stages, parasites growing in
535 schizont culture medium were used for imaging at different stages (ring, trophozoite,
536 schizont and merozoite) of development. Purified gametocytes were examined for
537 GFP expression and localization at different time points (0, 1-15 min) after activation
538 in ookinete medium⁴³. Zygote and ookinete stages were analyzed throughout 24 h of
539 culture. Images were captured using a 63x oil immersion objective on a Zeiss Axio
540 Imager M2 microscope fitted with an AxioCam ICc1 digital camera (Carl Zeiss, Inc).

541 **Generation of dual tagged parasite lines**

542 The PP1-GFP parasites were mixed with NDC80-cherry and MyoA-cherry parasites
543 in equal numbers and injected into mice. Mosquitoes were fed on mice 4 to 5 days
544 after infection when gametocyte parasitaemia was high. These mosquitoes were
545 checked for oocyst development and sporozoite formation at day 14 and day 21 after
546 feeding. Infected mosquitoes were then allowed to feed on naïve mice and after 4 - 5
547 days, and the mice were examined for blood stage parasitaemia by microscopy with
548 Giemsa-stained blood smears. In this way, some parasites expressed both PP1-GFP
549 and NDC80-cherry; and PP1-GFP and MyoA-cherry in the resultant gametocytes,
550 and these were purified and fluorescence microscopy images were collected as
551 described above.

552 **Parasite phenotype analyses**

553 Blood containing approximately 50,000 parasites of the PP1PTD line was injected
554 intraperitoneally (i.p.) into mice to initiate infections. Asexual stages and gametocyte
555 production were monitored by microscopy on Giemsa-stained thin smears. Four to
556 five days post infection, exflagellation and ookinete conversion were examined as
557 described previously⁷⁰ with a Zeiss AxioImager M2 microscope (Carl Zeiss, Inc)
558 fitted with an AxioCam ICc1 digital camera. To analyse mosquito transmission, 30–
559 50 *Anopheles stephensi* SD 500 mosquitoes were allowed to feed for 20 min on
560 anaesthetized, infected mice with an asexual parasitaemia of 15% and a comparable
561 number of gametocytes as determined on Giemsa-stained blood films. To assess
562 mid-gut infection, approximately 15 guts were dissected from mosquitoes on day 14
563 post feeding, and oocysts were counted on an AxioCam ICc1 digital camera fitted to
564 a Zeiss AxioImager M2 microscope using a 63x oil immersion objective. On day 21
565 post-feeding, another 20 mosquitoes were dissected, and their guts crushed in a
566 loosely fitting homogenizer to release sporozoites, which were then quantified using

567 a haemocytometer or used for imaging. Mosquito bite back experiments were
568 performed 21 days post-feeding using naive mice, and blood smears were examined
569 after 3-4 days.

570 **Electron microscopy**

571 Gametocytes activated for 6 min and 30 min were fixed in 4% glutaraldehyde in 0.1
572 M phosphate buffer and processed for electron microscopy as previously
573 described⁷¹. Briefly, samples were post fixed in osmium tetroxide, treated *en bloc*
574 with uranyl acetate, dehydrated and embedded in Spurr's epoxy resin. Thin sections
575 were stained with uranyl acetate and lead citrate prior to examination in a JEOL
576 JEM-1400 electron microscope (JEOL Ltd, UK)

577 **Quantitative Real Time PCR (qRT-PCR) analyses**

578 RNA was isolated from gametocytes using an RNA purification kit (Stratagene).
579 cDNA was synthesised using an RNA-to-cDNA kit (Applied Biosystems). Gene
580 expression was quantified from 80 ng of total RNA using a SYBR green fast master
581 mix kit (Applied Biosystems). All the primers were designed using the primer3
582 software (<https://primer3.ut.ee/>). Analysis was conducted using an Applied
583 Biosystems 7500 fast machine with the following cycling conditions: 95°C for 20 s
584 followed by 40 cycles of 95°C for 3 s; 60°C for 30 s. Three technical replicates and
585 three biological replicates were performed for each assayed gene. The *hsp70*
586 (PBANKA_081890) and *arginyl-t RNA synthetase* (PBANKA_143420) genes were
587 used as endogenous control reference genes. The primers used for qPCR can be
588 found in **Table S1**.

589 **Transcriptome study using RNA-seq**

590 For RNA extraction, parasite samples were passed through a plasmodipur column to
591 remove host DNA contamination prior to RNA isolation. Total RNA was extracted
592 from activated gametocytes and schizonts of WT-GFP and PP1PTD parasites (two
593 biological replicates each) using an RNeasy purification kit (Qiagen). RNA was
594 vacuum concentrated (SpeedVac) and transported using RNA-stable tubes
595 (Biomatrica). Strand-specific 354mRNA sequencing was performed on total RNA
596 and using TruSeq stranded mRNA sample prep 355kit LT (Illumina), as previously
597 described⁷². Libraries were sequenced using an Illumina Hiseq 4000 sequencing
598 platform with paired-end 150 bp read chemistry. The quality of the raw reads was
599 assessed using FATSQC
600 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Low-quality reads and
601 Illumina adaptor sequences from the read ends were removed using Trimmomatic
602 R⁷³. Processed reads were mapped to the *P. berghei* ANKA reference genome
603 (release 40 in PlasmoDB - <http://www.plasmodb.org>) using Hisat2⁷⁴ (V 2.1.0) with
604 parameter “—rna-strandness FR”. Counts per feature were estimated using
605 FeatureCounts⁷⁵. Raw read counts data were converted to counts per million (cpm)
606 and genes were excluded if they failed to achieve a cpm value of 1 in at least one of
607 the three replicates performed. Library sizes were scale-normalized by the TMM
608 method using EdgeR software⁷⁶ and further subjected to linear model analysis using
609 the voom function in the limma package⁷⁷. Differential expression analysis was
610 performed using DeSeq2⁷⁸. Genes with a fold-change greater than two and a false
611 discovery rate corrected p-value (Benjamini-Hochberg procedure) < 0.05 were
612 considered to be differentially expressed. Functional groups shown in Figure 6C
613 were inferred from annotations available in PlasmoDB: Release 49
614 (<https://plasmodb.org/plasmo/app>).

615 **Immunoprecipitation and Mass Spectrometry**

616 Schizonts, following 11 hours and 24 hours, respectively in *in vitro* culture, and
617 male gametocytes 11 min post activation were used to prepare cell lysates.
618 Purified parasite pellets were crosslinked using formaldehyde (10 min incubation
619 with 1% formaldehyde, followed by 5 min incubation in 0.125M glycine solution
620 and 3 washes with phosphate buffered saline (PBS) (pH, 7.5).
621 Immunoprecipitation was performed using crosslinked protein and a GFP-Trap[®]_A
622 Kit (Chromotek) following the manufacturer's instructions. Proteins bound to the
623 GFP-Trap[®]_A beads were digested using trypsin and the peptides were analysed
624 by LC-MS/MS. Briefly, to prepare samples for LC-MS/MS, wash buffer was
625 removed, and ammonium bicarbonate (ABC) was added to beads at room
626 temperature. We added 10 mM TCEP (Tris-(2-carboxyethyl) phosphine
627 hydrochloride) and 40 mM 2-chloroacetamide (CAA) and incubation was performed
628 for 5 min at 70° C. Samples were digested using 1 µg Trypsin per 100 µg protein at
629 room temperature overnight followed by 1% TFA addition to bring the pH into the
630 range of 3-4 before mass spectrometry.

631 **Statistical analysis**

632 All statistical analyses were performed using GraphPad Prism 8 (GraphPad
633 Software). An unpaired t-test and two-way anova test were used to examine
634 significant differences between wild-type and mutant strains for qRT-PCR and
635 phenotypic analysis accordingly.

636

637 **Data Availability.** RNA Sequence reads have been deposited in the NCBI
638 Sequence gene expression omnibus with the accession number GSE164175.

639 "The mass spectrometry proteomics data have been deposited to the
640 ProteomeXchange Consortium with the dataset identifier PXD023571 and
641 10.6019/PXD023571.

642

643 **Author contributions.**

644 RT and MZ conceived and designed all experiments. RT, MZ, RP, DB, DSG, GK
645 performed the GFP tagging and conditional knockdown with promoter trap
646 experiments. RR and MBr generated and characterised the PP1-AID/HA line. MZ,
647 RP, GK, DB and RT performed protein pull-down experiments. ARB performed mass
648 spectrometry. AS, RN and AP performed RNA sequencing (RNA-seq). DJPF and
649 SV performed electron microscopy. RT, AAH, MZ, DSG, AS, RN, AP and DJPF
650 analyzed the data. MZ, DSG and RT wrote the original draft. RT, AAH, AP, DJPF,
651 MB edited and reviewed the manuscript and all other contributed to it.

652

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654 and other technical works, and the personnel at the Bioscience Core Laboratory
655 (BCL) in KAUST for sequencing the RNA samples and producing the raw datasets.

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669

670 **Figure legends**

671 **Fig. 1. Location of PP1 during asexual blood stage schizogony and its**
672 **association with kinetochore (Ndc80) and glideosome (MyosinA)**

673 **(A)** Live cell imaging of PP1-GFP (Green) showing its location during different stages
674 of intraerythrocytic development and in free merozoites. DIC: Differential
675 interference contrast; Hoechst: stained DNA (blue); Merge: green and blue images
676 merged. A schematic guide showing the locations of PP1GFP foci during
677 segmentation of merozoites is depicted in right hand panel. **(B)** Live cell imaging
678 showing location of PP1-GFP (green) in relation to the kinetochore marker NDC80-
679 mCherry (red) and DNA (Hoechst, blue). Merge: green, red and blue images
680 merged. A schematic guide showing the locations of PP1GFP foci with NDC80-
681 mCherry during segmentation of merozoites is depicted in right hand panel. **(C)** Live
682 imaging showing the location of PP1-GFP (green) in relation to inner membrane
683 complex marker MyoA-mCherry (red) and DNA (Hoechst, blue) during different
684 stages of intraerythrocytic development and in extracellular merozoites. A schematic
685 guide showing the locations of PP1GFP foci with NDC80-mCherry and MyoA-
686 mCherry during segmentation of merozoites is depicted in the three right-hand

687 panels. Merge: green, red and blue images merged. Sch-E (Early schizont), Sch-M
688 (Middle schizont), Sch-L (Late schizont) Sch-S (Segmented schizont). In all panels,
689 scale bar = 5 μ m.

690

691 **Fig. 2. The location of PP1 and its association with the kinetochore during**
692 **chromosome segregation in male gametogony.**

693 **(A)** Live-cell imaging of PP1-GFP during male gametogony showing an initial
694 diffused localization before activation and focal points after activation in the later
695 stages (shown as minutes post activation). Panels are DIC, Hoechst (blue, DNA),
696 PP1-GFP (green) and Merge (green and blue channels). **(B)** Live-cell imaging of
697 parasite line expressing both PP1-GFP and NDC80-mCherry showing location of
698 PP1 (green) and NDC80 (red) in male gametocytes at different time points after
699 activation. A schematic guide showing the locations of PP1GFP foci with DNA and
700 NDC80-mCherry during male gametogony is depicted in the right panel. Merge/DNA
701 is green, red and blue (Hoechst, DNA) channels. In both panels, scale bar = 5 μ m.

702

703 **Fig. 3. PP1-GFP localization during zygote formation, ookinete development,**
704 **and sporogony inside the mosquito gut**

705 **(A)** Live cell imaging showing PP1-GFP location in male and female gametes,
706 zygote and during ookinete development (stages I to V and mature ookinete). A cy3-
707 conjugated antibody, 13.1, which recognises the P28 protein on the surface of
708 zygotes and ookinetes, was used to mark these stages. Panels: DIC (differential
709 interference contrast), PP1-GFP (green, GFP), 13.1 (red), Merged: Hoechst (blue,
710 DNA), PP1-GFP (green, GFP) and P28 (red). Scale bar = 5 μ m. Insets show, at
711 higher magnification, the PP1-GFP signal on the zygote and developing apical end

712 of early stage 'retorts', and in the nucleus of late retorts and ookinete stage. **(B)** Live
713 cell imaging of PP1-GFP (green) in relation to NDC80-mCherry (red) and Hoechst
714 staining (blue, DNA) in zygote and ookinete stages. **(C)** Live cell imaging of PP1-
715 GFP in developing oocysts in mosquito guts at 7-, 14- and 21-days post-infection
716 and in a sporozoite. Panels: DIC, Hoechst (blue, DNA), PP1-GFP (green), Merged
717 (blue and green channels). **(D)** Live cell imaging of PP1-GFP in relation to NDC80 in
718 developing oocysts and in a sporozoite. Panels: DIC (differential interference
719 contrast), PP1-GFP (green), NDC80-mCherry (red), Merge (Hoechst, blue, DNA;
720 PP1-GFP, green; NDC80-mCherry, red). Scale bar = 5 μ m

721

722 **Fig. 4. PP1 has an important role during male gamete formation and zygote -**
723 **ookinete development (A)** qRT-PCR analysis of *pp1* transcription in PP1PTD and
724 WT-GFP parasites, showing the downregulation of *pp1*. Each bar is the mean of
725 three biological replicates \pm SD. **(B)** Male gametogony (exflagellation) of PP1PTD
726 line (black bar) and WT-GFP line (white bar) measured as the number of
727 exflagellation centres per field. Mean \pm SD; n=3 independent experiments. **(C)**
728 Ookinete conversion as a percentage for PP1PTD and WT-GFP parasites.
729 Ookinetes were identified using P28 antibody as a surface marker and defined as
730 those cells that differentiated successfully into elongated 'banana shaped' ookinetes.
731 Round cells show zygotes that did not start to transform and 'retorts' could not
732 differentiate successfully in ookinetes. Mean \pm SD; n=3 independent experiments.
733 **(D)** Representative images of round cells, retorts and fully differentiated ookinetes.
734 **(E)** Total number of GFP-positive oocysts per infected mosquito in PP1PTD and WT-
735 GFP parasites at 7-, 14- and 21-days post infection (dpi). Mean \pm SD; n=3
736 independent experiments. **(F)** Representative images of mosquito midguts on day 14

737 showing them full of oocysts in WTGFP and no oocyst in PP1PTD **(G)** Total number
738 of sporozoites in oocysts of PP1PTD and WT-GFP parasites at 14 and 21 dpi. Mean
739 \pm SD; n=3 independent experiments. **(H)** Total number of sporozoites in salivary
740 glands of PP1PTD and WT-GFP parasites. Bar diagram shows mean \pm SD; n=3
741 independent experiments. Unpaired t-test was performed for statistical analysis.
742 *p<0.05, **p<0.01, ***p<0.001.

743

744 **Figure 5: Ultrastructure analysis of PP1PTD gametocytes shows defects in**
745 **nuclear pole and axoneme assembly during male gametogony**

746 Electron micrographs of WTGFP (a-c, g-i) and PP1PTD (d-f, j-l) male gametocytes at
747 6 min (a-f) and 30 min (g-i) post activation (pa). Bars represent 1 μ m (a, d, g, j) and
748 100 nm in all other micrographs. **(a)** Low power micrograph of a WTGFP male
749 gametocyte with two nuclear poles (NP) associated with the nucleus (N). **(b)**
750 Enlargement of the enclosed area showing the nuclear pole (NP) with adjacent basal
751 body (B) and associated axoneme (A). **(c)** Cross section of an axoneme showing the
752 9+2 microtubule arrangement. **(d)** Low power micrograph of PP1PTD male
753 gametocyte showing the central nucleus (N) with a nuclear pole and associated
754 basal body (enclosed area). **(e)** Enlargement of the enclosed area showing the
755 nuclear pole (NP) with adjacent basal body (B) and associated axoneme (A). **(f)**
756 Cross section through an axoneme showing the 9+2 arrangement of microtubules.
757 **(g)** Low power micrograph of a 30 min pa WTGFP male gametocyte showing the
758 nucleus with areas of condensed chromatin. Note the cross sectioned free male
759 gametes (Mg). A – axoneme. **(h)** Periphery of a male gametocyte undergoing
760 exflagellation with the nucleus (N) associated with flagellum (F) protruding from the
761 surface. **(i)** Cross section of a free male gamete showing the 9+2 microtubules of the

762 flagellum (F) and electron dense nucleus (N). **(j)** Low power micrograph of a
763 PP1PTD male gametocyte at 30 min pa showing the central nucleus (N) with a
764 nuclear pole and an increased number of axoneme profiles (A) within the cytoplasm.
765 **(k)** Detail of the periphery of a nucleus showing the nuclear pole (NP), basal body
766 (B) and associated axoneme (A). **(l)** Cross section of an axoneme showing the 9+2
767 microtubule arrangement.

768

769 **Figure 6: Transcriptome of PP1PTD mutant reveals important roles of PP1 in**
770 **parasite cell cycle, motor protein function, and cell polarity during gametocyte**
771 **biology**

772 Volcano plots showing significantly down-regulated (blue Log_2 fold ≤ -1 , q value <
773 0.05) and up-regulated genes (brown Log_2 fold ≥ 1 , q value < 0.05) in PP1PTD
774 compared to wild-type lines in non (0 min)-activated gametocytes **(A)** and 30 min
775 activated gametocytes **(B)**. Non-differentially regulated genes are represented as
776 black dots. **(C)** Expression heat maps showing affected genes from specific
777 functional classes of proteins such as kinases, phosphatases, motor proteins and
778 proteins associated with parasite motility, entry into the host cell, ookinete surface
779 and cell cycle. Genes are ordered based on their differential expression pattern in
780 non-activated gametocytes. **(D)** Validation by qRT-PCR of a few genes randomly
781 selected based on the RNA-seq data. All the experiments were performed three
782 times in duplicate with two biological replicates. *p ≤ 0.05 .

783

784 **Fig. 7. Interacting partners of PP1 during asexual schizont and sexual**
785 **gametocyte stages**

786 **(A)** List of proteins interacting with PP1 during schizont and gametocyte stages. **(B)**
787 Venn diagram showing common interacting partners in schizonts and gametocytes
788 with some additional proteins specific to gametocytes.

789

790 **Supplementary figures**

791 **Fig. S1. Generation and genotypic analysis of PP1GFP parasites**

792 **(A)** Schematic representation for 3'-tagging of *pp1* gene with green fluorescent
793 protein (GFP) sequence via single homologous recombination. **(B)** Integration PCR
794 showing correct integration of tagging construct. **(C)** Western blot showing expected
795 size of PP1-GFP protein.

796

797 **Fig. S2. Generation and genotype analysis of conditional knockdown** 798 **PP1 parasites**

799 **(A)** Schematic representation of auxin inducible degron (AID) strategy to generate
800 PP1AID parasites. **(B)** Integration PCR of the PP1AID construct in the *pp1* locus.
801 Primer 1 and Primer 2 were used for control PCR while primer 1 and primer 3 were
802 used to determine successful integration of AID-HA sequence and selectable marker
803 at 3'-end of *pp1* locus **(C)** PP1AID protein expression level as measured by western
804 blotting upon addition of auxin to mature purified gametocytes; α -tubulin serves as a
805 loading control. Auxin treatment of PP1AID showed no defect in exflagellation (error
806 bars show standard deviation from the mean; technical replicates from three
807 independent infections. **(D)** Schematic representation of the promoter swap strategy
808 (PP1PTD, placing *pp1* under the control of the *ama1* promoter) by double
809 homologous recombination. Arrows 1 and 2 indicate the primer positions used to
810 confirm 5' integration and arrows 3 and 4 indicate the primers used for 3' integration.

811 **(E)** Integration PCR of the promotor swap construct into the *pp1* locus. Primer 1 (5'-
812 IntPTD36) with primer 2 (5'-IntPTD) were used to determine successful integration of
813 the selectable marker. Primer 3 (3'-intPTama1) and primer 4 (3'-IntPTD36) were
814 used to determine the successful integration of *ama1* promoter. Primer 1 (5'-
815 IntPTD36) and primer 4 (3'-IntPTD36) were used to show complete knock-in of the
816 construct and the absence of a band at 2.1 kb (endogenous) resulting in complete
817 knock-in of the construct. **(F)** Parasitaemia during blood stage schizogony showing a
818 significant slow growth of PP1PTD compared to WTGFP parasites. Experiment was
819 done with three mice each with 1000 parasites per mice injected intraperitoneally.
820 ***P<0.001 **(G)** Bite back experiments show no transmission of PP1PTD parasites
821 (black bar) from mosquito to mouse, while successful transmission was shown by
822 WT-GFP parasites. Mean \pm SD; n= 3 independent experiments.

823

824 **Fig. S3. Analysis of PP1PTD development and RNA seq analysis:**

825 **(A)** The quantification of electron microscopy data showing PP1PTD male
826 gametocytes halted at an early stage of development in comparison with WTGFP
827 male gametocytes at 30 min post activation. These data are based on analysis of
828 fifty random sections of male gametocytes per sample.

829 **(B)** Clustered dendrogram of two biological replicates of WTGFP and PP1PTD
830 mutant parasite lines during gametocyte stage using hierarchical clustering
831 algorithm. Analysis was performed on normalized count data. **(C)** RNA-seq read
832 statistics.

833

834 **Supplementary Tables**

835 **Table S1. Differentially expressed genes in PP1PTD parasites**

836 **Table S2. List of proteins pulled down by PP1GFP**

837 **Table S3. Primers used in this study**

838

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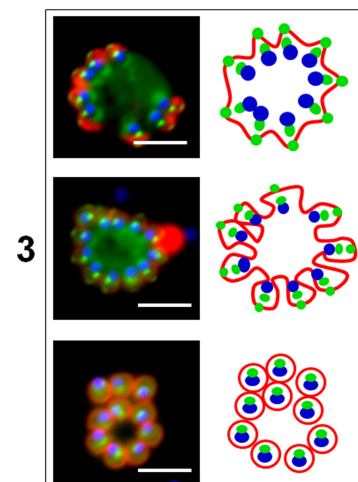
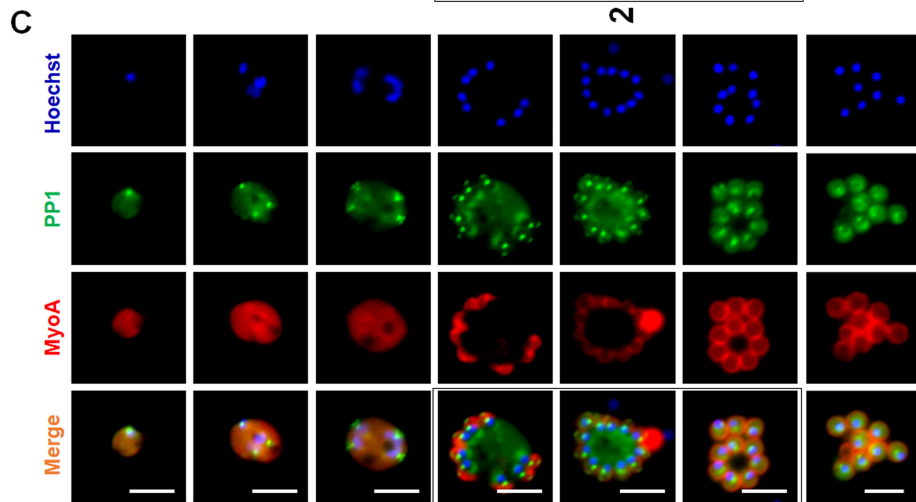
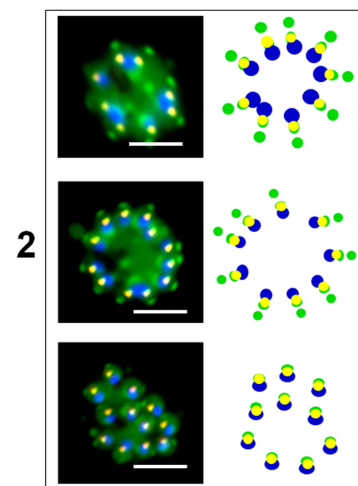
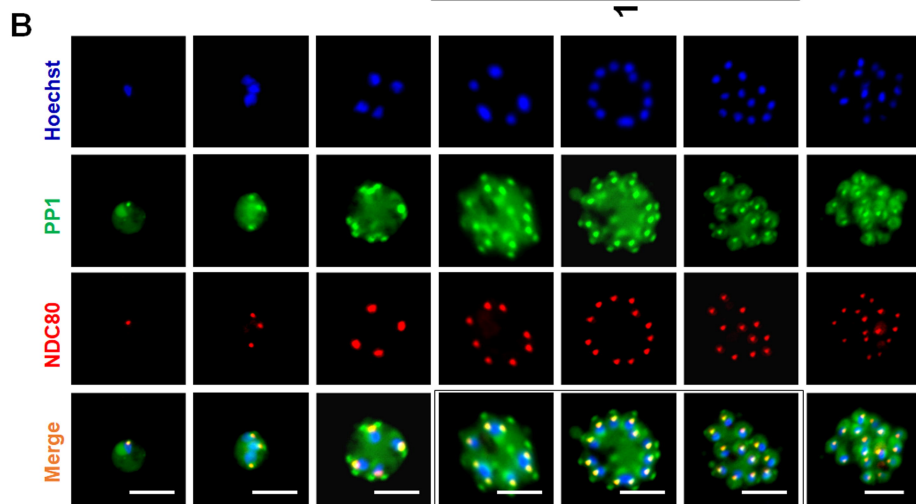
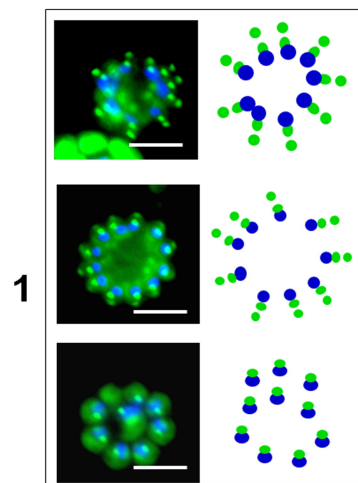
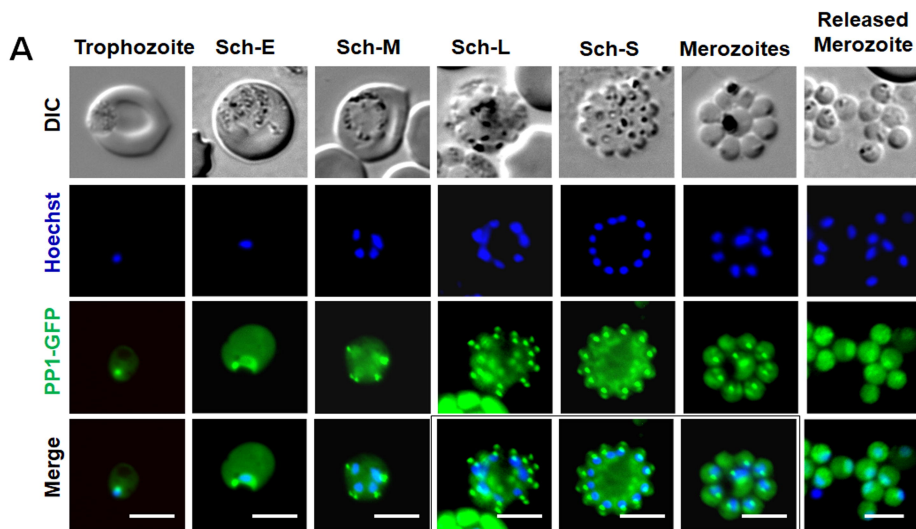
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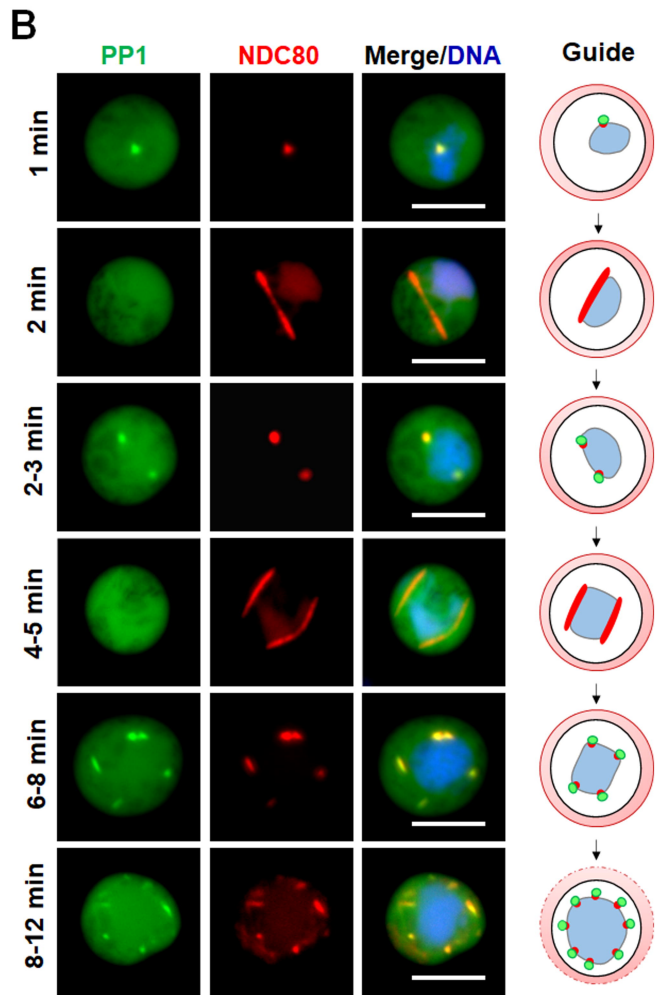
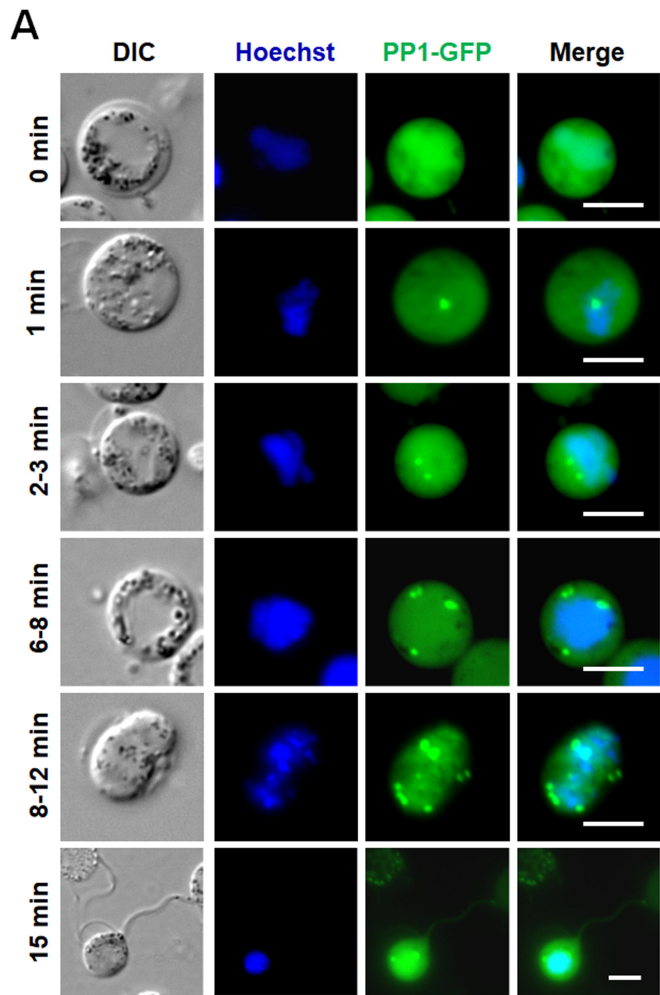
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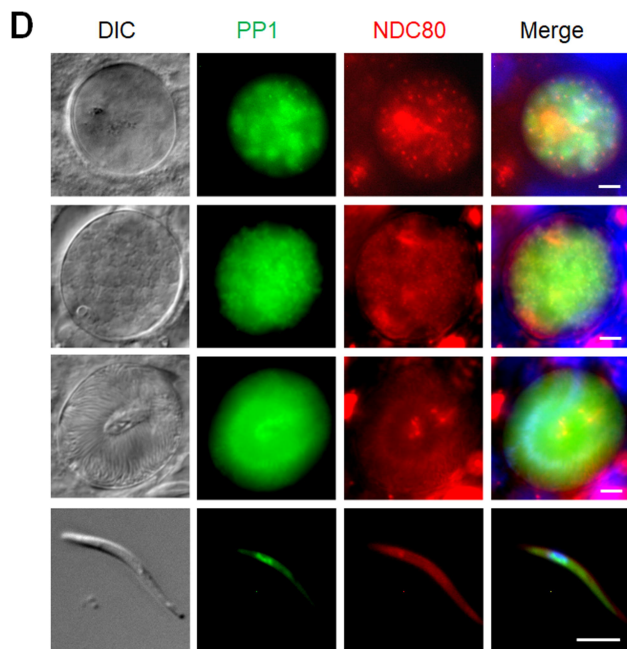
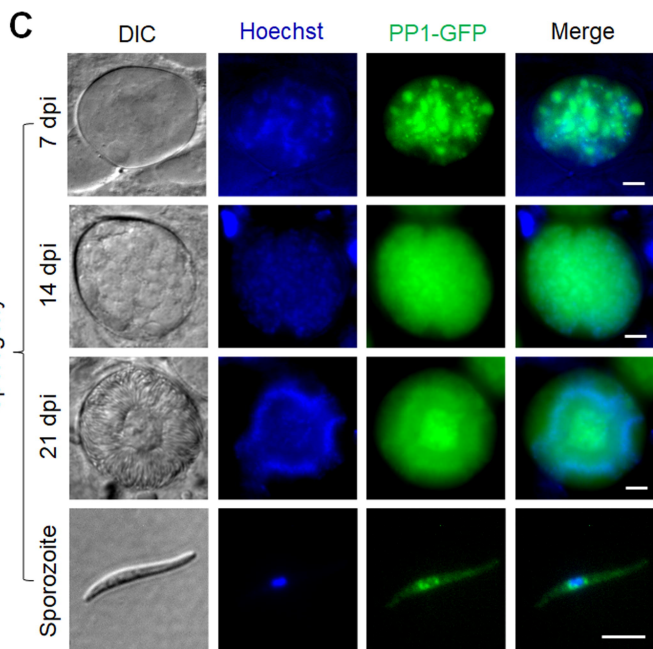
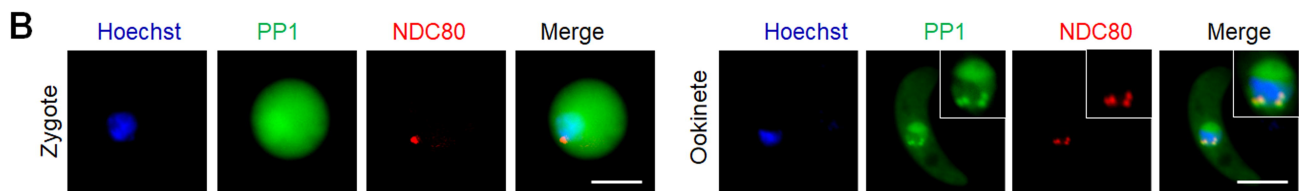
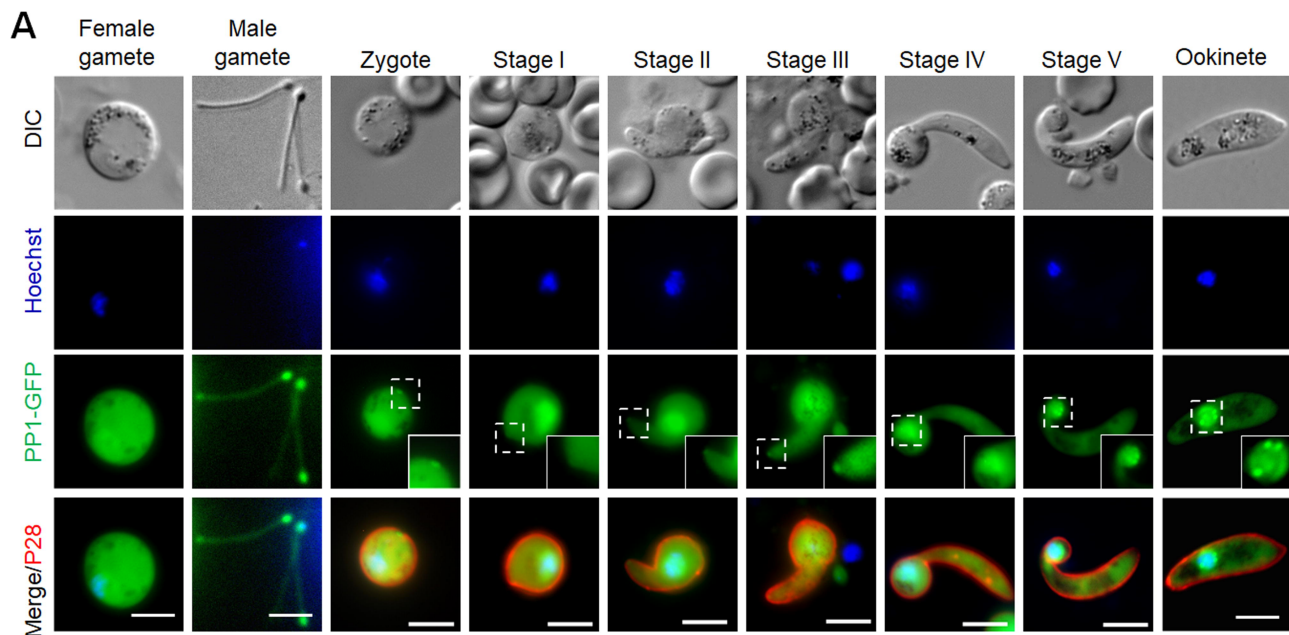


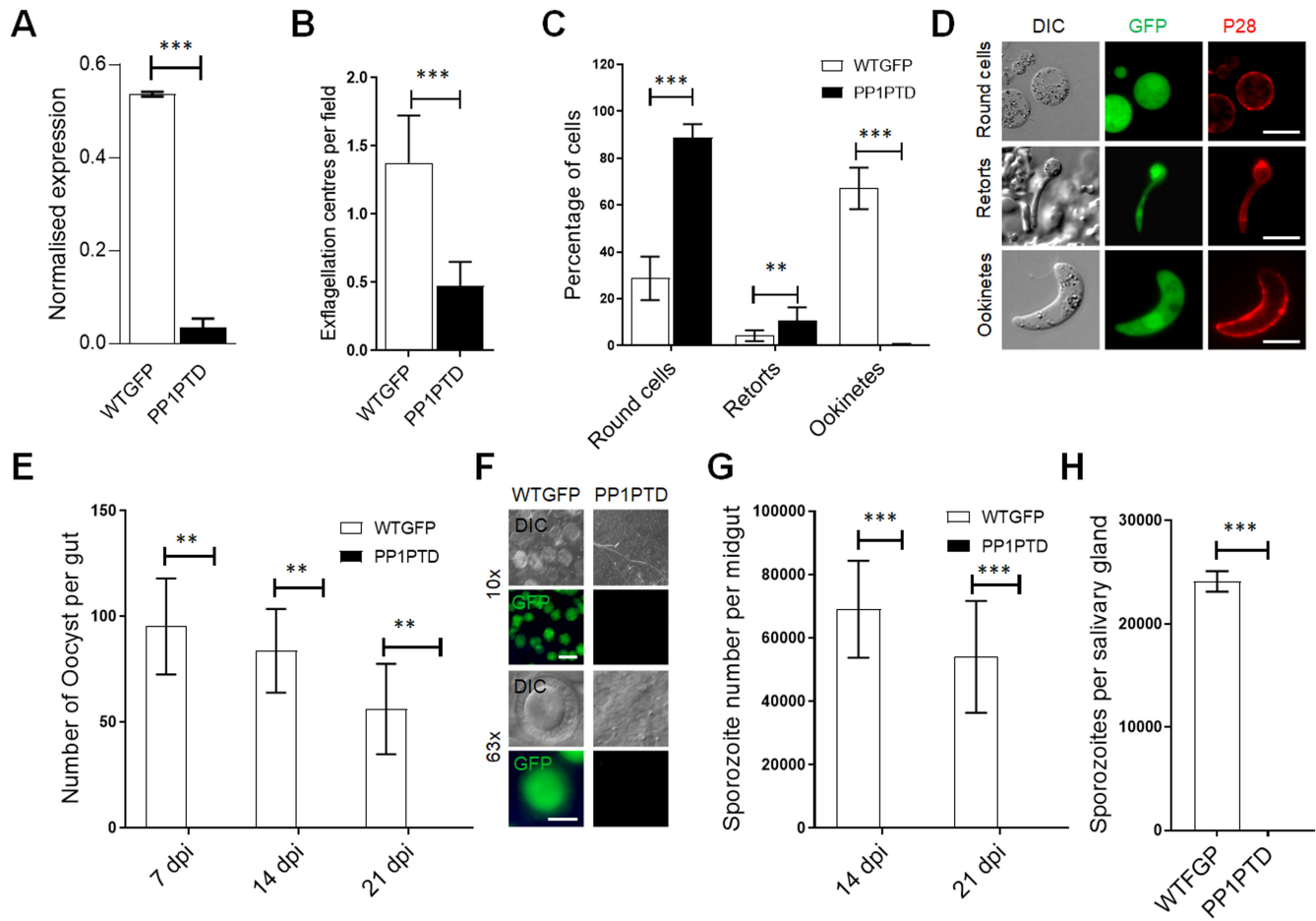
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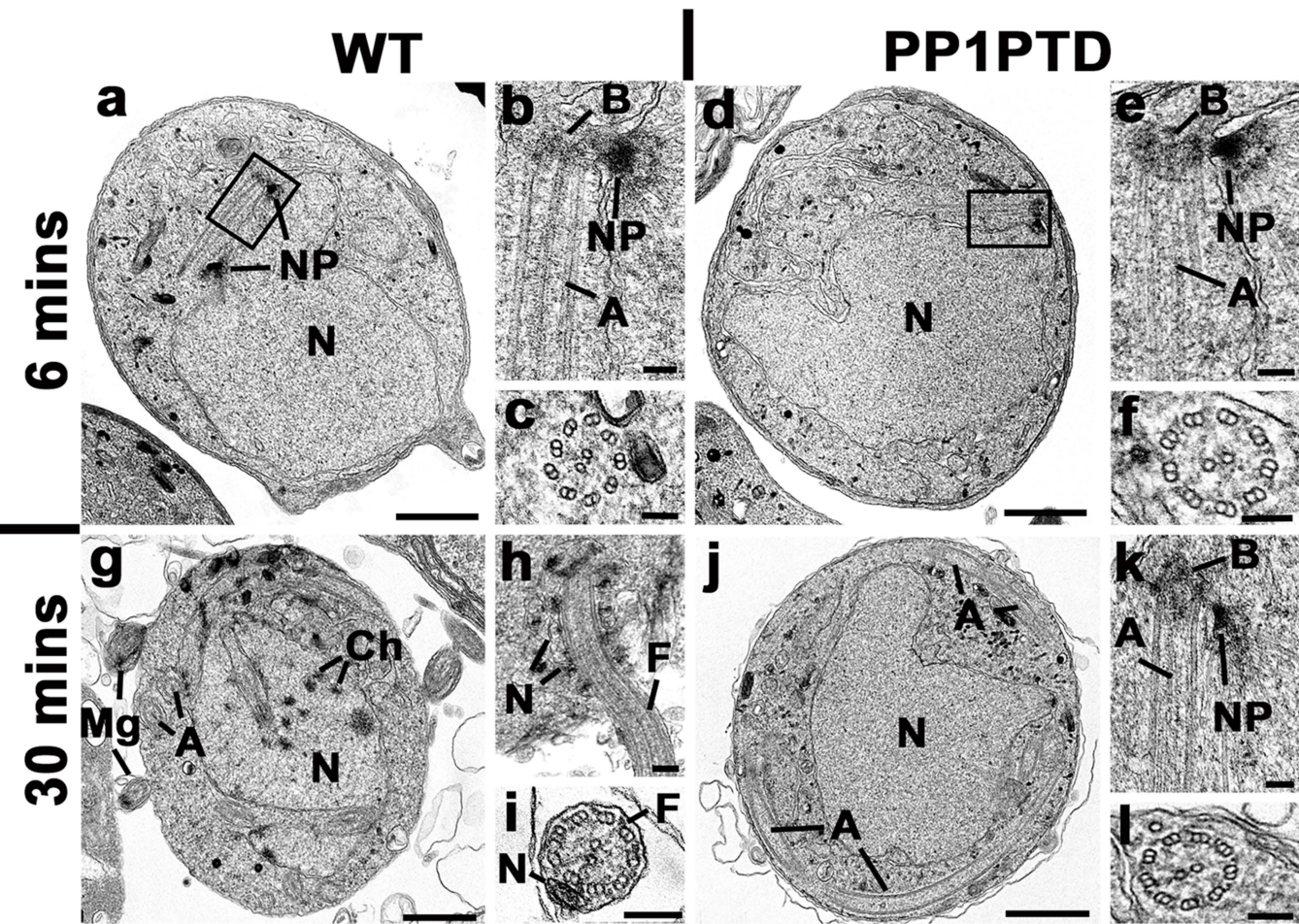
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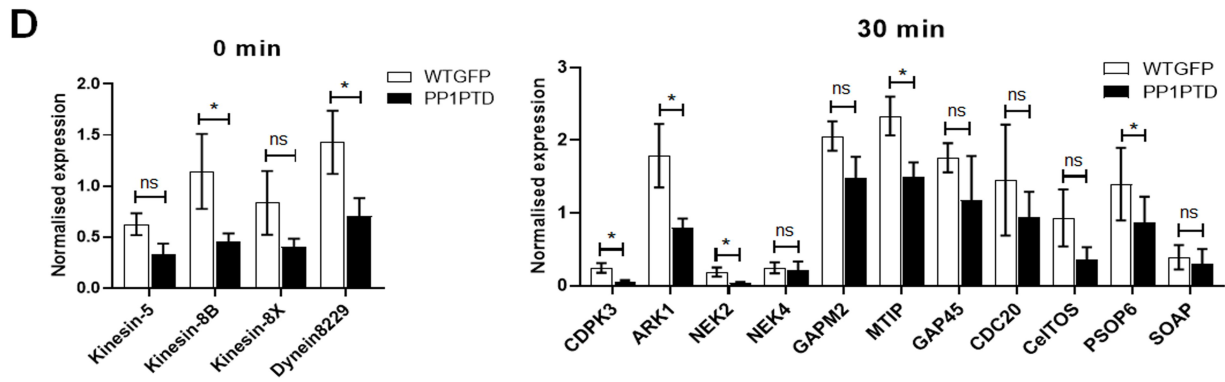
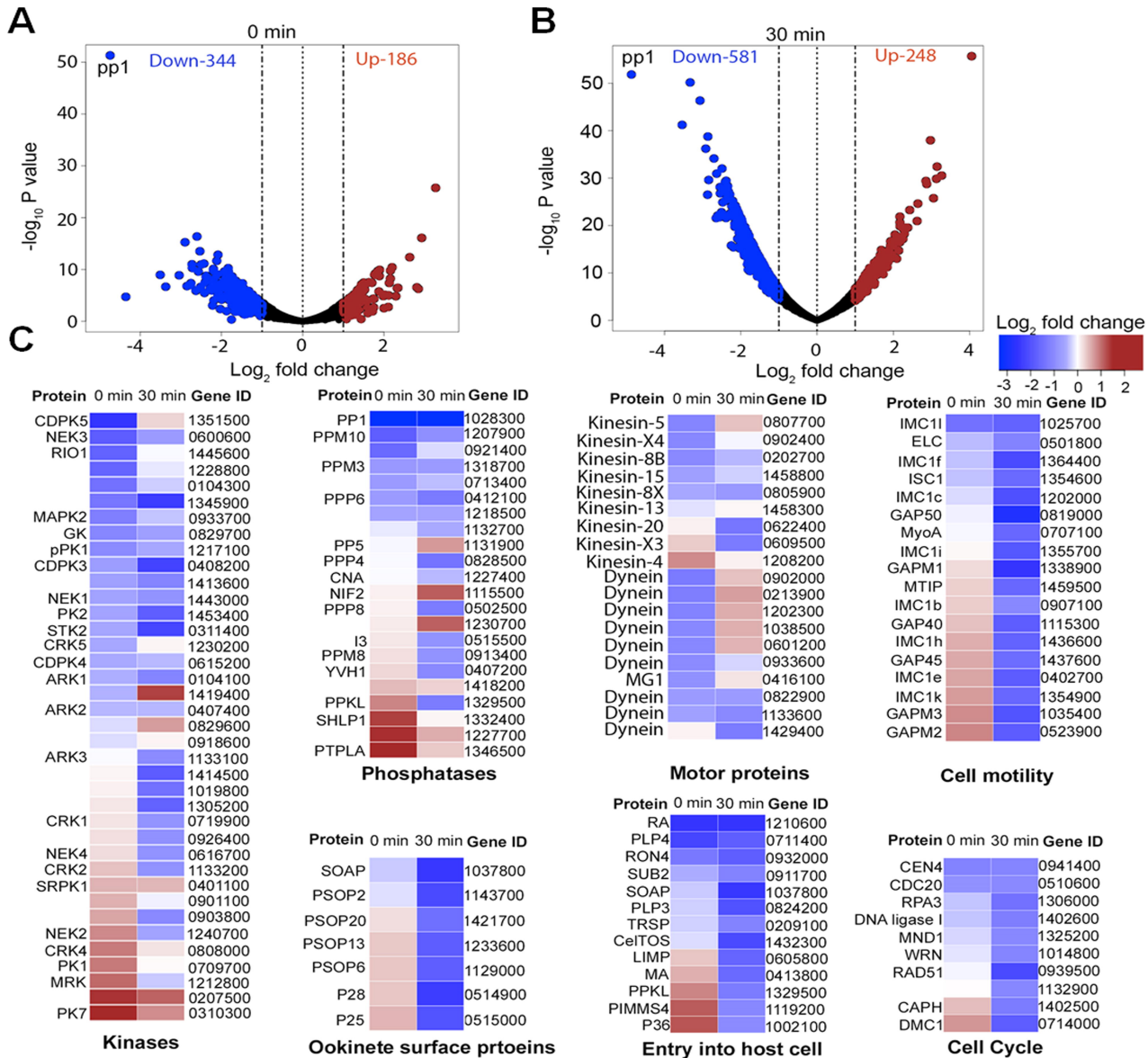
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A

<i>P. berghei</i> Gene ID	Product Description	Gene names	MW (kDa)	Schizonts- 24 h				Schizonts-10 h				Gametocytes-10 min			
				PP1-GFP		GFP		PP1-GFP		GFP		PP1-GFP		GFP	
PBANKA_1028300	serine/threonine protein phosphatase PP1	PP1	34963	31	26	23	0	37	26	25	0	23	33	26	0
PBANKA_1218500	protein phosphatase inhibitor 2, putative	PPI2	16664	11	7	9	0	9	8	7	0	10	9	9	0
PBANKA_0516600	leucine-rich repeat protein	LRR1	36735	33	26	26	0	22	29	29	0	11	22	25	0
PBANKA_0601600	conserved protein, unknown function	NEMF-like	201925	36	17	35	0	21	37	21	0	12	22	24	0
PBANKA_0515500	protein phosphatase inhibitor 3, putative	PPI3	13065	3	3	3	0	0	4	2	0	2	2	3	0
PBANKA_0515400	conserved Plasmodium protein	CD2BP2	77377	17	10	18	0	7	22	12	0	3	2	9	0
PBANKA_0310800	RTR1 domain-containing protein, putative	RTR1-like	97344	15	8	11	0	19	32	19	0	0	0	7	0
PBANKA_1458800	kinesin, putative	kinesin-15	164999	0	0	0	0	0	0	0	0	2	7	2	0
PBANKA_0202700	kinesin-8, putative	kinesin-8B	168815	0	0	0	0	0	0	0	0	2	4	2	0
PBANKA_1458300	kinesin-13, putative	kinesin-13	117181	0	0	0	0	0	0	0	0	0	3	3	0
PBANKA_0917400	armadillo repeat protein PF16	PF16	57748	0	0	0	0	0	0	0	0	0	2	2	0

B