

1 **PMRT1, a *Plasmodium* specific parasite plasma membrane transporter is essential for asexual
2 and sexual blood stage development**

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43 **Abstract**

44 Membrane transport proteins perform crucial roles in cell physiology. The obligate intracellu-
45 lar parasite *Plasmodium falciparum*, an agent of human malaria, relies on membrane
46 transport proteins for the uptake of nutrients from the host, disposal of metabolic waste, ex-
47 change of metabolites between organelles and generation and maintenance of
48 transmembrane electrochemical gradients for its growth and replication within human eryth-
49 rocytes. Despite their importance for *Plasmodium* cellular physiology, the functional roles of a
50 number of membrane transport proteins remain unclear, which is particularly true for orphan
51 membrane transporters that have no or limited sequence homology to transporter proteins in
52 other evolutionary lineages. Therefore, in the current study, we applied endogenous tagging,
53 targeted gene disruption, conditional knockdown and knockout approaches to investigate the
54 subcellular localization and essentiality of six membrane transporters during intraerythrocytic
55 development of *P. falciparum* parasites. They are localized at different subcellular structures
56 – the food vacuole, the apicoplast, and the parasite plasma membrane – and four out of the
57 six membrane transporters are essential during asexual development. Additionally, the
58 plasma membrane resident transporter 1 (PMRT1, PF3D7_1135300), a unique *Plasmodium*-
59 specific plasma membrane transporter, was shown to be essential for gametocytogenesis
60 and functionally conserved within the genus *Plasmodium*. Overall, we reveal the importance
61 of four orphan transporters to blood stage *P. falciparum* development, which have diverse
62 intracellular localizations and putative functions.

63 **Importance (150 words)**

64 *Plasmodium falciparum*-infected erythrocytes possess multiple compartments with designat-
65 ed membranes. Transporter proteins embedded in these membranes do not only facilitate
66 movement of nutrients, metabolites and other molecules between these compartments, but
67 are common therapeutic targets and can also confer antimalarial drug resistance. Orphan
68 membrane transporter in *P. falciparum* without sequence homology to transporters in other
69 evolutionary lineages and divergent to host transporters may constitute attractive targets for
70 novel intervention approaches. Here, we localized six of these putative transporters at differ-
71 ent subcellular compartments and probed into their importance during asexual parasite
72 growth using reverse genetic approaches. In total, only two candidates turned out to be dis-
73 pensable for the parasite, highlighting four candidates as putative targets for therapeutic in-
74 terventions. This study reveals the importance of several orphan transporters to blood stage
75 *P. falciparum* development.

76 **Introduction**

77 *Plasmodium* spp. malaria parasites inhabit diverse intracellular niches and need to import
78 nutrients and export waste across both, host-cell and parasite membranes. Despite this,

79 there are less than 150 putative membrane transporters encoded in the genome of *Plasmo-*
80 *dium falciparum*, the most virulent malaria parasite, making up only 2.5% of all encoded
81 genes (*P. falciparum* 3D7 v3.2: 5280 genes) (1–8), which is reduced compared to other uni-
82 cellular organisms of similar genome size. The loss of redundant transporters is a typical
83 feature of many intracellular parasites (9) and, as a result, the proportion of transporters that
84 are indispensable for parasite survival increases (2), some of which have been shown to be
85 critical for the uptake of several anti-Plasmodial compounds and/or to be involved in drug
86 resistance (10–23). Moreover, the parasite's intracellular lifestyle resulted in the evolution of
87 additional specialized transporters without human homologues (1). During its
88 intraerythrocytic development, the parasite relies on the uptake of nutrients, such as amino
89 acids, pantothenate or fatty acids, from its host erythrocyte as well as from the extracellular
90 blood plasma (24–27). As *P. falciparum* resides in a parasitophorous vacuole (PV) in the
91 host erythrocyte, nutrients acquired from the extracellular milieu must traverse multiple
92 membranes: the erythrocyte plasma membrane (EPM), the parasitophorous vacuole mem-
93 brane (PVM), the parasite plasma membrane (PPM) and eventually membranes of intracellu-
94 lar organelles, such as those of the apicoplast or mitochondria (24, 28–30). The unique re-
95 quirements of malaria parasite survival have led to the evolution of a number of orphan
96 transporters, whose localization or function cannot be predicted based on sequence homolo-
97 gy to transporters in other organisms (4, 31). Despite the likely importance of uniquely
98 adapted transporters to *P. falciparum* survival, subcellular localization, essentiality, function
99 and substrate specificity for most *P. falciparum* transporters has not been directly determined
100 (2, 24, 29). The best functional evidence available for many *Plasmodium*-specific transport-
101 ers comes from a recent knockout screen of these orphan transporters in the rodent malaria
102 parasite *Plasmodium berghei* (31). However, whether observations for different transporters
103 in the *P. berghei* model are directly transferrable to *P. falciparum* have yet to be examined.
104 Therefore, in this study, we explored the localization and essentiality of four predicted orphan
105 transporters that had been partially characterised in *P. berghei* and included two additional
106 transporters with no experimental characterization available.

107 **Results**

108 To date, the predicted 'transportome' of *P. falciparum* consists of 117 putative transport sys-
109 tems (encoded by 144 genes) classified as channels (n=19), carriers (n=69), and pumps
110 (n=29) (2). Functions of the vast majority of transporter genes were inferred from sequence
111 homology to model organisms, however, given their lack of homology, 39 gene products
112 could not be associated with any functional or subcellular localization and were categorized
113 as orphan transporters accordingly (4). A subset of orphan transporters characterized in the
114 *P. berghei* malaria model was selected for further characterization in *P. falciparum*. The four
115 transporters selected were reported to be important at different stages of rodent malaria par-

116 asite growth with i) *P. berghei* drug/metabolite transporter 2 (*PfDMT2*: PF3D7_0716900)
117 found to be essential for asexual blood stage development, ii) *P. berghei* zinc transporter 1
118 (*PfZIP1*: PF3D7_0609100) was essential across transmission stages but not blood stages,
119 where there was only a slight growth defect, iii) *P. berghei* cation diffusion facilitator family
120 protein (*PfCDF*: PF3D7_0715900) knockout parasites had a defect during transmission stag-
121 es but not during asexual stages, and iv) *P. berghei* major facilitator superfamily domain-
122 containing protein (*PfMFS6*: PF3D7_1440800) was found to be essential for parasite trans-
123 mission from mosquitos to a new host, with a growth defect observed at asexual and game-
124 tocyte stages but not during mosquito stage parasite growth (31, 32). In order to confirm ex-
125 pression of these four, initially selected, transporters in *P. falciparum* asexual stages, we
126 searched the list of “Genes coding for transport proteins” included in the Malaria Parasite
127 Metabolic Pathways (MPMP) database (1, 33) for proteins with i) RNA-seq (34, 35) and ii)
128 proteomics evidence (36, 37) in asexual blood stages. During our initial searches of the
129 MPMP database but also including PlasmoDB (38) and the most recent *P. falciparum* 3D7
130 genome (v3.2) and annotations, we identified two additional putative transporters in *P. falciparum*
131 (PF3D7_0523800, PF3D7_1135300), whose *P. berghei* homologs were not targeted
132 and functionally characterized by Kenthirapalan *et al.* (31) or investigated in any other exper-
133 imental model. Given their obvious lack of sequence homology to transporter proteins in oth-
134 er evolutionary lineages and clear classification as orphan membrane transporter, both pro-
135 teins were subsequently included in our characterization of *P. falciparum* orphan transport-
136 ers, and named as ‘food vacuole resident transporter 1’ (FVRT1: PF3D7_0523800) and as
137 ‘plasma membrane resident transporter 1’ (PMRT1: PF3D7_1135300) based on their subcel-
138 lular localization. AlphaFold-based structure predictions (39) and results from structure ho-
139 mology search (40) of all six selected transporters are provided in **Figure S1**.

140 **Localization of putative *P. falciparum* transporters**

141 To determine subcellular localization, we tagged the six putative transporters endogenously
142 with GFP using the selection-linked integration (SLI) system (41) (**Figure 1A**). Additionally, a
143 glmS ribozyme sequence was included in the 3'UTR, which enabled conditional gene knock-
144 down upon addition of glucosamine (42). Correct integration of the plasmid into the respec-
145 tive genomic locus was verified by PCR and expression of the GFP-fusion protein was con-
146 firmed by Western blot for each generated cell line (**Figure S2A, B**).

147 All transgenic cell lines expressed the GFP-fusion protein, demonstrating that these trans-
148 porters are expressed in asexual blood stage parasites (**Figure 1B-G, S2A**). Expression lev-
149 els were sufficient to allow determination of subcellular localization (**Figure 1B-G**): (i)
150 PF3D7_0523800-GFP localized to the food vacuole, (ii) *PfDMT2*-GFP and *PfMFS6*-GFP
151 apicoplast localization, and (iii) *PfZIP1*-GFP and PF3D7_1135300-GFP parasite plasma
152 membrane (PPM) localization. However, *PfCDF*-GFP showed an obscure staining pattern

153 with a weak spot within the parasite cytosol in ring and trophozoite state parasites, but multi-
154 ple foci in schizont stages (Figure 1D). To pinpoint this localization, an additional cell line with
155 endogenously 3xHA-tagged *PfCDF* was generated, confirming the focal localization of
156 *PfCDF* in asexual stages (Figure S2C).

157 Except for *PfCDF*, the observed localizations of the other five transporters were confirmed by
158 co-localization studies using appropriate episomally expressed marker proteins: P40PX-
159 mCherry (43, 44) for the food vacuole, ACP-mCherry (45, 46) for apicoplast and Lyn-
160 mCherry (41, 47) for PPM. The focal distribution of *PfCDF*-GFP was co-localized with a
161 rhoptry (ARO-mCherry (48, 49)) and a micronemes (AMA1-mCherry (50, 51)) marker, but
162 *PfCDF*-GFP did not colocalize with either marker (Figure 1H). Additionally, for *PfZIP* and
163 PF3D7_1135300 the PPM localization was further confirmed in free merozoites (Figure S2D,
164 E) and by confocal microscopy-based co-localization of PF3D7_1135300-GFP with the PPM
165 marker Lyn-mCherry (Figure S2F). Accordingly, as noted above, we named PF3D7_0523800
166 as 'food vacuole resident transporter 1' (FVRT1) and PF3D7_1135300 as 'plasma mem-
167 brane resident transporter 1' (PMRT1).

168 **Targeted-gene disruption (TGD), conditional knockdown and conditional knockout of
169 putative transporters**

170 In order to test whether the putative transporters are essential for *P. falciparum* during its
171 intraerythrocytic cycle, we first tried to functionally inactivate them by targeted gene disrup-
172 tion (TGD) using the SLI system (41) (Figure S3A). TGD cell lines were successfully ob-
173 tained for *PfZIP1* and *PfCDF* (Figure S3B, C). For *PfZIP1*-TGD, the correct integration of the
174 plasmid into the genomic locus and absence of wildtype locus was verified by PCR and sub-
175 sequent growth experiments revealed no growth defect compared to *P. falciparum* 3D7
176 wildtype parasites (Figure S2B), suggesting its redundancy during asexual parasite prolifera-
177 tion. For *PfCDF*-TGD the correct integration of the plasmid into the genomic locus was also
178 verified, but wildtype DNA was still detectable and remained even upon prolonged culturing
179 under G418/WR selection and limited dilution cloning (Figure S3C). In contrast, six
180 (*PfPMRT1*, *PfDMT2*) or eight (*PfFVRT1*, *PfMFS6*) independent attempts to obtain TGD cell
181 lines for the other four transporters with the respective plasmids failed, indicating that these
182 genes have an indispensable role in blood stage parasite growth.

183 To probe into the function of the putative transporters where we were unable to generate
184 gene-disruptions, we utilized the glmS ribozyme sequence. The corresponding sequence
185 was integrated into the 3'UTR of the targeted genes. This enabled the induction of condition-
186 al degradation of respective mRNAs upon addition of glucosamine (42) and the assessment
187 of the phenotypic consequences. Upon addition of 2.5 mM glucosamine to young ring stage
188 parasites we found a 76.8% (+/- SD 3.7) reduction in GFP fluorescence intensity in *PfDMT2*-
189 GFP parasites, 72.7% (+/- SD 9.4) reduction in *PfMFS6*-GFP and a 77.7% (+/- SD 6.1) re-

190 duction in *PfPMRT1*-GFP in schizonts of the same cycle (Figure 2A–C, S4A–C). No measur-
191 able reduction in fluorescence intensity could be detected for *PfFVRT1*-GFP or *PfCDF*-GFP
192 expressing parasite lines (Figure S4D–F). Presence of the *glmS* cassette in both plasmids
193 was confirmed by PCR (Figure S4H). For parasite cell lines with a significant reduction in the
194 expression of the endogenously tagged protein, proliferation was analyzed in the absence
195 and presence of 2.5 mM glucosamine (Figure 2D, S4G). While no significant effect on growth
196 was observed for *PfMFS6*, a growth reduction of 68.5 % (+/- SD 2.1) over two cycles was
197 observed upon knockdown of *PfDMT2*. For *PfPMRT1*, a minor growth delay was measura-
198 ble, which resulted in a significantly reduced parasitemia at day 3 upon knockdown using 2.5
199 mM glucosamine (two tailed Wilcoxon rank sum test, $W = 15$, $n_1 = 5$, $n_2 = 3$, $P = 0.03$), but
200 was not significant when using 5 mM glucosamine (two tailed Wilcoxon rank sum test, $W =$
201 10, $n_1 = 4$, $n_2 = 3$, $P = 0.16$) (Figure 2E). Additionally, significantly fewer newly formed ring
202 stage parasites were observed at 84 hours post invasion (hpi) (Figure 2F), and multiple pair-
203 wise post-hoc comparisons using the Conover-Iman rank sum test and Benjamini-Hochberg
204 method to control the false discovery rates showed significant step-wise reductions of ring
205 stage parasites after induction of *GlmS*-based knockdown of *PfPMRT1* using both, 2.5 mM
206 glucosamine (adjusted $P = 0.0078$) and 5 mM glucosamine (adjusted $P = 0.0005$) in compar-
207 ison to untreated control cell cultures.

208 To better characterize the minor growth phenotype of *PfPMRT1*-GFP-*glmS* parasites that
209 might be due to incomplete knockdown, we generated a conditional *PfPMRT1* knockout cell
210 line (cond Δ PMRT1) using the Dimerizable Cre (DiCre) system (52, 53). Again using the SLI
211 system (41), the endogenous *PfPMRT1* was disrupted upstream of the region encoding the
212 N-terminal transmembrane domain, but, at the same time introducing a recodonized second
213 functional copy of *PfPMRT1* flanked by loxP sites in the genomic locus. This loxP-flanked
214 allelic copy of *PfPMRT1* encodes an additional 3x hemagglutinin (HA) tag, which can be
215 conditionally excised upon addition of a rapamycin analog (rapalog) via the enzymatic activity
216 of an episomally expressed DiCre (Figure 3A). First, correct integration of the plasmid into
217 the genomic locus was verified by PCR (Figure 3B). Second, expression and localization of
218 the recodonized HA-tagged protein at the PPM was verified by colocalization with the
219 merozoite plasma membrane marker MSP1 (54) (Figure 3C). Third, excision of the
220 recodonized gene upon rapalog addition was confirmed on genomic level by PCR (Figure
221 3D) and on protein level by Western blot analysis at 24 hpi and 48 hpi (Figure 3E). To assess
222 the effect of conditional *PfPMRT1* knockout on parasite proliferation, we determined growth
223 of the transgenic parasite cell line with and without rapalog over five days (Figure 3F, S5A).
224 In contrast to the *glmS*-based knockdown experiment, DiCre-based gene excision (induced
225 by the addition of rapalog to young ring stages of cond Δ PMRT1 parasite cell cultures) abol-
226 ished growth within the first replication cycle (Figure 3F, S5A). The specificity of the observed

227 growth phenotype was verified by gene complementation. To achieve this, we episomally
228 expressed recodonized *PfPMRT1* with TY1-epitope tag either under the constitutive *nmd3* or
229 the weaker *sf3a2* promoter (55) in the cond Δ PMRT1 cell line (Figure 3D, F, S5B, C). Correct
230 localization of the TY1-tagged *PfPMRT1* at the PPM was verified by immunofluorescence
231 assays (IFA) (Figure 3G). Notably both, complementation of the *PfPMRT1* knockout cell line
232 (cond Δ PMRT1) with recodonized *PfPMRT1* either under control of the constitutive *nmd3* or
233 the weaker *sf3a2* promoter, restored parasite growth (Figure 3F, S5B, C). The level of growth
234 restoration with low level expression of recodonized *PfPMRT1* is in line with the results from
235 glmS-knockdown experiments, which showed that a reduction of about 75% in protein ex-
236 pression resulted only in a minor growth perturbation (Figure 2C, D).

237 **Loss of the PPM-localized *PfPMRT1* leads to an arrest of parasite development at
238 trophozoite stage and the formation of PPM derived protrusions**

239 To determine, which particular parasite stages are affected by the knockout of *PfPMRT1*, we
240 added rapalog to tightly synchronized parasites at different time points (4, 20 and 32 hpi)
241 (Figure 4A) and monitored parasite growth by flow cytometry. Additionally, we quantified
242 growth perturbation by microscopy of Giemsa smears at 4, 20, 24, 32, 40, 48, 72 and 96 hpi
243 (Figure 4B, S6A, B). When adding rapalog at 4 hpi, parasite development progressed
244 through ring and early trophozoite stages up to 24 hpi with no visible abnormality. After-
245 wards, parasites with deformed and enlarged protrusions started to appear and further de-
246 velopment occurred to be stalled. At 32 hpi, almost all parasites had developed to late
247 trophozoites/early schizonts in the control, whereas these stages were completely absent in
248 *PfPMRT1*-deficient parasites. Over 50% of the parasites were pycnotic or possessed large
249 protrusions, the remaining parasites stayed arrested at the trophozoite stage. Quantification
250 of the percentage of parasites with protrusions between 20 hpi and 32 hpi revealed 94.8%
251 (+/- SD 4.0) protrusion-positive parasites (Figure 4C). The activation of gene excision at later
252 time points by adding rapalog at 20 hpi or 32 hpi resulted in no or minor growth perturbation
253 in the first cycle with successful re-invasion, but again led to parasites arresting at the
254 trophozoite stage in the second cycle with an accumulation of protrusions (Figure 4A, S6A,
255 B).

256 In order to get further insights into the morphological changes in *PfPMRT1*-deficient para-
257 sites, we incubated these parasites with dihydroethidium (DHE) to visualize the parasite cy-
258 tosol (44). We observed an absence of staining within the protrusions, suggesting they are
259 not filled with parasite cytosol (Figure 4D). Next, we transfected the cond Δ PMRT1 cell line
260 with a plasmid encoding the PPM marker Lyn-mCherry (41) and observed Lyn-mCherry-
261 positive protrusions upon knockout of *PfPMRT1* starting to become visible at 24 hpi, indicat-
262 ing that the protrusions originate from the PPM (Figure 4E). In line with this, protrusion mem-

263 branes were also stainable with BODIPY TR C5 ceramide in cond Δ PMRT1 parasites at 32
264 hpi (Figure 4F).

265 **Depletion of *PfPMRT1* results in an early arrest of gametocyte development**

266 RNA-seq data suggest *PfPMRT1* is also expressed during other developmental stages, such
267 as gametocytes (56, 57). Therefore, we assessed expression of *PfPMRT1*-GFP during
268 gametocytogenesis by re-engineering *PfPMRT1*-GFP-glmS in the inducible gametocyte pro-
269 ducer (iGP) '3D7-iGP' (58) parasite line, which allows the robust induction of sexual commit-
270 ment by conditional expression of gametocyte development 1 protein (GDV1) upon addition
271 of shield-1 (58) (Figure S7A). We show that *PfPMRT1* is indeed expressed during all stages
272 of gametocytogenesis and again localizes to the PPM, colocalizing with the PPM-marker
273 Lyn-mCherry (41) (Figure 5A, B). Conditional knockdown of *PfPMRT1* via the glmS-ribozyme
274 system (Figure S7B) resulted in a reduction in *PfPMRT1*-GFP fluorescence intensity of
275 79.4% (+/- SD 9.2%) at 7 days post induction (dpi) or 75.5% (+/- SD 23.2%) at 10 dpi, with-
276 out an effect on gametocyte development (Figure S7C–F). In order to exclude that a role of
277 *PfPMRT1* in gametocytogenesis is covered by only a partial knockdown resulting in low lev-
278 els of expressed protein and to determine if *PfPMRT1* is essential for gametocytogenesis, we
279 episomally expressed GDV1-GFP-DD in the cond Δ PMRT1 parasite line, enabling conditional
280 induction of sexual commitment upon addition of shield 1 in these parasites (59). Conditional
281 knockout of *PfPMRT1* in these transgenic parasites at day three post gametocyte induction
282 resulted in pycnotic parasites from day 5 onwards, while excision of *PfPMRT1* at day 5 post
283 induction had no effect on gametocyte development (Figure 5C, D). Excision of the
284 recodonized gene upon rapalog addition was confirmed at a genomic level by PCR for both
285 conditions (Figure 5E). Quantification of parasite stages at day 10 post induction of GDV1
286 expression revealed 77.9% (+/- SD 7.7%) gametocytes and 22.1% (+/- SD 7.7%) pycnotic
287 parasites in the control, while 100% of parasites were already pycnotic in the cultures, with
288 induced knockout by addition of rapalog at day 3 post gametocyte induction by GDV1 ex-
289 pression (Figure 5F). This data indicates that *PfPMRT1* is important for early gametocyte
290 development.

291 **PMRT1 is unique to the genus *Plasmodium* and interspecies complementation assays
292 showed partial functional conservation**

293 *PfPMRT1* shows a lack of sequence similarities with known or putative transporters and/or
294 conserved domains shared with known transporter families (2, 5). Our phylogenetic analysis
295 revealed that homologs of *PfPMRT1* are present across *Plasmodium* species with amino
296 acid sequence identities of about 90% in the subgenus *Laverania*, but about 50% outside
297 *Laverania* (Figure 6A). However, prediction of the protein structure using AlphaFold (39) indi-
298 cates two bundles of four transmembrane helices with reasonable similarity of the C-terminal

299 bundle with the photosynthetic reaction center Maquette-3 protein (60) (RMSD of 3.12) (Figure 6B, Figure S1B). In order to test for functional conservation, we expressed the *PfPMRT1* 300 homologs of *P. vivax* (PVP01_0936100) and *P. knowlesi* (PKNH_0933400) episomally as C- 301 terminal Ty-1 fusion proteins under the *nmd3* promoter in the cond Δ PMRT1 parasites. Both 302 fusion proteins are expressed. They were again localized at the PPM as shown by IFA (Figure 303 6C, Figure S8), and, importantly, were able to partially restore growth after two cycles to 304 64.8% (+/- SD 9.8%) and 65.1% (+/- SD 7.4%) compared to cond Δ PMRT1 parasites (Figure 305 6D, S8). Excision of the recodonized endogenous *Pfpmrt1* gene upon rapalog addition was 306 confirmed at a genomic level by PCR (Figure 6E). These data indicate that PMRT1 is func- 307 tionally conserved within the genus *Plasmodium*. 308

309 **Discussion**

310 In this manuscript we functionally described four so called “orphan transporter” (31) in *P. 311 falciparum*, which were partially characterized in *P. berghei*, and included two additional so 312 far uncharacterized proteins with transporter sequence signature.

313 We localized *PfFVRT1*-GFP – annotated on PlasmoDB (38) as putative divalent metal trans- 314 porter – at the food vacuole of the parasite, which is in line with a previously predicted food 315 vacuole association (1) and its reported homology (1, 61) to the conserved eukaryotic 316 endosomal/lysosomal natural resistance-associated macrophage protein (NRAMP) trans- 317 porter (62) in our structure similarity search. Repeated attempts to generate a TGD cell line 318 failed, indicating an important role of this transporter during asexual blood stage develop- 319 ment which is in agreement with data from a *P. falciparum* genome wide essentiality screen 320 (63).

321 In concordance with recently published data identifying *PbDMT2* and *PbMFS6* as leaderless 322 apicoplast transporters (32), we localized GFP-fusion proteins of *PfDMT2* and *PfMFS6* at the 323 apicoplast. Successful knockdown of *PfDMT2* resulted in a growth defect in the second cycle 324 after induction, resembling the described delayed death phenotype of other apicoplast genes 325 that were functionally inactivated (32, 64–66). It suggests an essential role of *PfDMT2* in 326 apicoplast physiology, as observed by Sayers *et al.* (32) for the rodent malaria *P. berghei*. 327 This is further supported by our failed attempts to disrupt this gene using the SLI system.

328 We also failed to disrupt the *PfMFS6* locus, which is in agreement with the gene knockout 329 studies in *P. berghei* that led to a markedly decreased multiplication rate (31, 32, 67). Never- 330 theless, glmS-based knock-down, although comparable to *PfDMT2*-GFP knockdown (72.7% 331 versus 76.8% reduction in GFP fluorescence, respectively) had no effect on parasite prolif- 332 eration in our study. This might indicate that these reduced levels of *PfMFS6*, in contrast to 333 reduced levels of *PfDMT2*, are sufficient for normal asexual replication *in vitro*.

334 Another candidate, *PfCDF*, annotated as putative cation diffusion facilitator family protein, 335 showed multiple cytosolic foci within the parasite with no co-localization with apical organelle

336 markers. The homologue in *Toxoplasma gondii*, *TgZnT* (TgGT1_251630) shows a similar
337 cellular distribution (68). It has recently been shown to transport Zn²⁺, to localize to vesicles
338 at the plant-like vacuole in extracellular tachyzoites and to be present at dispersed vesicles
339 throughout the cytoplasm of intracellular tachyzoites (68). The essentiality of *PfCDF* for *in*
340 *vitro* blood stage growth is debatable. We were not able to generate a clonal wild-type free
341 TGD cell line although correct integration of the plasmid into the genomic locus could be veri-
342 fied (Figure S3C). This points towards its dispensability for *in vitro* blood stage growth, which
343 is supported by i) its high (1.0) mutagenesis index score in a *P. falciparum* genome-wide mu-
344 tagenesis screen (63) and ii) gene deletion experiments in rodent malaria species showing
345 that CDF proteins are non-essential for *in vivo* blood stage development in *P. yoelii* (69) and
346 *P. berghei* (31, 67).

347 Finally, two putative transporters, *PfZIP1* and *PfPMRT1*, localized to the PPM. We show that
348 *PfZIP1* is non-essential for *P. falciparum* *in vitro* blood stage development, in line with a high
349 (0.7) mutagenesis index score in a *P. falciparum* genome-wide mutagenesis screen (63).
350 However, this is in contrast to the reported strong fitness loss in *P. berghei* (67) knockout
351 mutants and failed knockout attempts in *P. yoelii* and *P. berghei* *in vivo* mouse models (32,
352 69). These observations may reflect differences between *Plasmodium* species or differing
353 requirements for *in vitro* and *in vivo* growth conditions.

354 *PfPMRT1* is annotated as a conserved *Plasmodium* membrane protein with unknown func-
355 tion. It has been described as a protein showing structural characteristics of a transporter,
356 without sharing sequence similarities with known or putative transporters and/or conserved
357 domains of known transporter families (2, 5). It encompasses 410 amino acids with eight
358 predicted (70) transmembrane domains (TM) (Figure S1). The N- and C-terminal parts of
359 *PfPMRT1* are both predicted (71) to be facing the cytosolic side of the parasite. Surface elec-
360 trostatics indicate a clear polarity of *PfPMRT1* with negative charges facing the
361 parasitophorous vacuole (PV) lumen and positive charges inside the parasite cytosol (Figure
362 S8F). The loops protruding into the PV lumen of *PfPMRT1* are generally larger than the cyto-
363 solic loops and possess stretches of negatively charged amino acids likely relevant for its
364 transport function. Further functional characterization of *PfMRT1* will deliver insight into its
365 transporter capabilities and its physiological role.

366 Our phylogenetic analysis confirmed PMRT1 as unique for *Plasmodium* species with high
367 sequence conservation only within the *Laverania* subgenus (72). In line with data from ge-
368 nome-wide mutagenesis screens (63, 67) and reported failed knockout attempts in *P. yoelii*
369 (69), we found that *PfPMRT1* is essential for parasite growth, as its functional inactivation
370 resulted in growth arrest at the trophozoite stage accompanied by the accumulation of PPM-
371 derived protrusions within the parasite. In contrast, conditional knockdown resulted only in a
372 growth delay, indicating that minor residual *PfPMRT1* protein levels appear to be sufficient to

373 promote parasite growth. This finding was validated by episomal expression of an allelic copy
374 under the control of the weak *sf3a2* promoter (55) in the *PfPMRT1* knockout parasites. Additionally,
375 we found that *PfPMRT1* is essential for early gametocytogenesis. Interestingly, the
376 induction of the knockout at stage II–III had no effect on gametocytogenesis. This might be
377 due to sufficient amounts of *PfPMRT1* already present at the PPM, but could also indicate
378 that the function of the transporter is not required for later stage gametocyte maturation.
379 For future work, further functional and pharmacological characterization of this transporter
380 will provide insights into its biological role in different stages of the parasites life cycle, as
381 transcriptomic data indicates – along with expression in blood stages (34, 35) – *PfPMRT1* is
382 expressed in oocysts of *P. falciparum* (73, 74) and *P. berghei* (75).

383 **Material and methods**

384 **Cloning of plasmid constructs for parasite transfection**

385 For endogenous tagging using the SLI system (41) a 889 bp (for *PfPMRT1*;
386 PF3D7_1135300), 905 bp (*PfFVRT1*; PF3D7_0523800), 827bp (*PfZIP1*; PF3D7_0609100),
387 873 bp (*PfDMT2*; PF3D7_0716900), 877 bp (*PfMFS6*; PF3D7_1440800), 785 bp (*PfCDF*;
388 PF3D7_0715900) long homology region (HR) was amplified using 3D7 gDNA and cloned
389 into pSLI-GFP-glmS (76) (derived from pSLI-GFP (41)) using the NotI/MluI restriction site. In
390 order to generate *PfPMRT1*-2xFKBP-GFP a 1000 bp long HR was amplified using 3D7
391 gDNA and cloned into pSLI-2xFKBP-GFP (41).

392 For SLI-based targeted gene disruption (SLI-TGD) (41) a 501 bp (*PfPMRT1*), 378 bp
393 (*PfFVRT1*), 511 bp (*PfZIP1*), 399 bp (*PfDMT2*), 396 bp (*PfMFS6*), 741 bp (*PfCDF*) long ho-
394 mology region was amplified using 3D7 gDNA and cloned into the pSLI-TGD plasmid (41)
395 using NotI and MluI restriction sites.

396 For conditional deletion of *PfPMRT1*, the first 492 bp of the *PfPMRT1* gene were PCR ampli-
397 fied to append a first loxP site and a recodonized T2A skip peptide. The recodonized full-
398 length coding region of *PfPMRT1* was synthesized (GenScript, Piscataway, NJ, USA) and
399 PCR amplified with primers to add a second loxP site after the gene to obtain a second
400 fragment. Both fragments were cloned into pSLI-3xHA (55), using NotI/Spel and AvrII/XmaI
401 sites. This resulted in plasmid pSLI-*PfPMRT1*-loxP and the resulting transgenic cell line after
402 successful genomic modification was transfected with pSkip-Flox (41) using 2 µg/ml
403 Blasticidin S to obtain a line expressing the DiCre fragments (cond Δ PMRT1).

404 For complementation constructs, the recodonized *PfPMRT1* gene was PCR amplified using
405 primers to append the TY1 sequence and cloned via Xhol and AvrII or KpnI into pEXP1comp
406 (55) containing yDHODH as a resistance marker and different promoters (*nmd3*
407 (PF3D7_0729300), *sf3a2* (PF3D7_0619900)) driving expression of the expression cassette.
408 This resulted in plasmids c-^{nmd3}*PfPMRT1*-ty1 and c-^{sf3a2}*PfPMRT1*-ty1.

409 *PfPMRT1* homologues of *P. vivax* (PVP01_0936100) (77) and *P. knowlesi* (PKNH_0933400)
410 (78) were amplified from parasite gDNA and cloned into p^{nmd3}EXP1comp (55) via the
411 Xhol/AvrII restriction site. For co-localization experiments the plasmids pLyn-FRB-mCherry
412 (41), P40PX-mCherry (44), pARL-^{crt}ACP-mCherry (46), pARL-^{ama1}ARO-mCherry (49) and
413 pARL-^{ama1}AMA1-mCherry (51) were used. For conditional gametocyte induction yDHODH
414 was amplified by PCR from pARL-^{ama1}AMA1-mCherry-yDHODH (51) and cloned into GDV1-
415 GFP-DD-hDHFR(59)(59) using the Xhol/Xhol restriction site.
416 Oligonucleotides and plasmids used in this study are listed in Table S1A and S1B.

417 ***P. falciparum* culture and transfection**

418 Blood stages of *P. falciparum* 3D7 were cultured in human erythrocytes (O+). Cultures were
419 maintained at 37°C in an atmosphere of 1% O₂, 5% CO₂ and 94% N₂ using RPMI complete
420 medium containing 0.5% Albumax according to standard protocols (79). To maintain syn-
421 chronized parasites, cultures were treated with 5% sorbitol (80).
422 Induction of gametocytogenesis was done as previously described (58, 59). Briefly, GDV1-
423 GFP-DD expression was achieved by addition of 4 μM shield-1 to the culture medium and
424 gametocyte cultures were treated with 50 mM N-acetyl-D-glucosamine (GlcNAc) for five days
425 starting 72 hours post shield-1 addition to eliminate asexual parasites(81). Alternatively,
426 asexual ring stage cultures with >10% parasitemia were synchronized with Sorbitol (80)
427 cultured for 24 hours and treated with 50 mM N-acetyl-D-glucosamine (GlcNAc) (81) for five
428 days.
429 For transfection, Percoll-purified (82) late-schizont-stage parasites were transfected with 50
430 μg of plasmid DNA using Amaxa Nucleofector 2b (Lonza, Switzerland) as previously de-
431 scribed(83). Transfectants were selected either using 4 nM WR99210 (Jacobus Pharmaceu-
432 ticals), 2 μg/ml Blasticidin S (Life Technologies, USA), or 0.9 μM DSM1 (84) (BEI Resources;
433 <https://www.beiresources.org>). In order to select for parasites carrying the genomic modifica-
434 tion using the SLI system (41), G418 (Sigma-Aldrich, St. Louis, MO) at a final concentration
435 of 400 μg/ml was added to 5% parasitemia culture. The selection process and testing for
436 integration were performed as previously described (41).
437 For SLI-TGD, a total of six (*PfPMRT1*, *PfDMT2*, *PfZIP1*, *PfCDF*) or eight (*PfFVRT1*, *PfMFS6*)
438 independent 5 ml cultures containing the episomal plasmid were selected under G418 for at
439 least eight weeks.

440 **Imaging and immunofluorescence analysis (IFA)**

441 Fluorescence images of infected erythrocytes were observed and captured using a Zeiss
442 Axioskop 2plus microscope with a Hamamatsu Digital camera (Model C4742-95), a Leica
443 D6B fluorescence microscope equipped with a Leica DFC9000 GT camera and a Leica Plan
444 Apochromat 100x/1.4 oil objective or an Olympus FV3000 with a x100 MPLAPON oil objec-

445 tive (NA 1.4). Confocal microscopy was performed using a Leica SP8 microscope with laser
446 excitation at 405 nm, 490 nm, and 550 nm for DAPI, GFP, and mCherry excitation, respec-
447 tively. An HC PL APO 63x NA 1.4 oil immersion objective was used and images were ac-
448 quired with the Hyvolution mode of the LASX microscopy software. After recording, images
449 were deconvolved using Huygens (express deconvolution, setting 'Standard').
450 Microscopy of unfixed IEs was performed as previously described (85). Briefly, parasites
451 were incubated in RPMI1640 culture medium with Hoechst-33342 (Invitrogen) for 15 minutes
452 at 37°C prior to imaging. 7 μ l of IEs were added on a glass slide and covered with a cover
453 slip. Control images of 3D7 wild type parasites across the IDC are included in Figure S8D, E.
454 BODIPY TR C5 ceramide (Invitrogen) staining was performed by adding the dye to 32 hours
455 post invasion parasites in a final concentration of 2.5 μ M in RPMI as previously described
456 (85). For DHE staining of the parasite cytosol (44), 80 μ l of resuspended parasite culture
457 were incubated with DHE at a final concentration of 4.5 μ g/ml in the dark for 15 minutes prior
458 to imaging.
459 IFAs were performed as described previously (86). Briefly, IEs were smeared on slides and
460 air-dried. Cells were fixed in 100% ice cold methanol for 3 minutes at -20°C. Afterwards, cells
461 were blocked with 5% milk powder for 30 minutes. Next primary antibodies were diluted in
462 PBS/3% milk powder and incubated for 2 hours, followed by three washing steps in PBS.
463 Secondary antibodies were applied for 2 hours in PBS/3% milk powder containing 1 μ g/ml
464 Hoechst-33342 (Invitrogen) or DAPI (Roche) for nuclei staining, followed by 3 washes with
465 PBS. One drop of mounting medium (Mowiol 4-88 (Calbiochem)) was added and the slide
466 sealed with a coverslip for imaging.
467 To assess the localisation of the endogenously HA-tagged *PfPMRT1* IFAs were performed in
468 suspension with Compound 2-stalled schizonts (87) to distinguish protein located at the PPM
469 from that located at the PVM as previously done (55, 88). For this, trophozoite stages were
470 treated with Compound 2 (1 μ M) overnight, and arrested schizonts were harvested, washed
471 in PBS, and fixed with 4% paraformaldehyde/0.0075% glutaraldehyde in PBS. Cells were
472 permeabilized with 0.5% Triton X-100 in PBS, blocked with 3% BSA in PBS, and incubated
473 overnight with primary antibodies diluted in 3% BSA in PBS. Cells were washed 3 times with
474 PBS and incubated for 1 hour with Alexa 488 nm or Alexa 594 nm conjugated secondary
475 antibodies specific for human and rat IgG (Invitrogen) diluted 1:2,000 in 3% BSA in PBS and
476 containing 1 μ g/ml DAPI. Cells were directly imaged after washing 5 times with PBS
477 Antisera used: 1:200 mouse anti-GFP clones 7.1 and 13.1 (Roche), 1:500 rat anti-HA clone
478 3F10 (Roche), 1:1000 human anti-MSP1 (89), 1:10000 mouse anti-TY1 (ThermoFischer Sci-
479 entific Cat.No: MA5-23513). Contrast and intensities were linear adjusted if necessary and
480 cropped images were assembled as panels using Fiji (90), Corel Photo-Paint X6 and
481 Adobe Photoshop CC 2021.

482 **Immunoblots**

483 For immunoblotting parasites were released from erythrocytes by incubation with 0.03%
484 saponin in PBS for 10 minutes on ice followed by three wash steps with D-PBS. Proteins
485 were then extracted with lysis buffer (4 % SDS, 0.5 % Triton X-100, 0.5x D-PBS in dH₂O) in
486 the presence of protease cocktail inhibitor (Roche) and 1 mM PMSF followed by addition of
487 reducing SDS sample buffer and 5 minutes incubation at 55°C. Parasite proteins were sepa-
488 rated on a 10% SDS-PAGE gel using standard procedures and transferred to a nitrocellulose
489 membrane (Amersham™Protran™ 0.45 µm NC, GE Healthcare) using a transblot device
490 (Bio-Rad) according to manufacturer's instructions or to a nitrocellulose membrane (Licor) in
491 a tankblot device (Bio-Rad) using transfer buffer (0.192 M glycine, 0.1% SDS, 25 mM Tris-
492 HCl pH = 8.0) with 20% methanol.

493 Rabbit anti-aldolase (91) and anti-SBP1 (91) antibodies were diluted 1:2,000, mouse anti-
494 GFP clones 7.1 and 13.1 (Roche) antibody was diluted 1:500 or 1:1,000, mouse anti-Ty1
495 (Sigma) was diluted 1:20000, rabbit anti-BIP (92) was diluted 1:2500 and rat anti-HA clone
496 3F10 (Roche) antibody was diluted 1:1,000.

497 The chemiluminescent signal of the HRP-coupled secondary antibodies (Dianova) was visu-
498 alized using a Chemi Doc XRS imaging system (Bio-Rad) and processed with Image Lab
499 Software 5.2 (Bio-Rad). To perform loading controls and ensure equal loading of parasite
500 material anti-aldolase antibodies were used. The corresponding immunoblots were incubated
501 two times in stripping buffer (0.2 M glycine, 50 mM DTT, 0.05% Tween 20) at 55°C for 1 hour
502 and washed 3 times with TBS for 10 minutes. For Western blots shown in Figure
503 S8C fluorescent signals of secondary goat anti-rabbit IgG coupled to IRDye® 680CW and
504 goat anti-mouse IgG coupled to IRDye® 800CW were visualized using Odyssey Fc Imager
505 by LI-COR Biosciences.

506 **Growth Assay**

507 A flow cytometry-based assay adapted from previously published assays (44, 93) was per-
508 formed. For this, parasite cultures were resuspended and 20 µl samples were transferred to
509 an Eppendorf tube. 80 µl RPMI containing Hoechst-33342 and dihydroethidium (DHE) was
510 added to obtain final concentrations of 5 µg/ml and 4.5 µg/ml, respectively. Samples were
511 incubated for 20 minutes (protected from UV light) at room temperature, and parasitemia was
512 determined using an LSRII flow cytometer by counting 100,000 events using the FACSDiva
513 software (BD Biosciences) or using an ACEA NovoCyte flow cytometer.

514 **Stage distribution assay**

515 In order to obtain tightly synchronized parasite cultures, percoll purified schizonts (82) were
516 cultured for four hours together with fresh erythrocytes, followed by sorbitol synchronization
517 and resulting in a four-hour age window of parasites. Next, the culture was divided in four

518 dishes and rapalog was added at a final concentration of 250 nM immediately to one dish
519 and at 20 hours post invasion (hpi) and 32 hpi to the respective dishes. Giemsa smears and
520 samples for flow cytometry were collected at the indicated timepoints. The parasitemia was
521 determined using a flow cytometry assay and the stages were determined microscopically
522 counting at least 50 infected erythrocytes per sample and timepoint.

523 **Gametocyte stage distribution assay**

524 Giemsa-stained blood smears 10 days post induction of GDV1 expression were obtained
525 and at least 10 fields of view were recorded using a 63x objective per treatment and time
526 point. Erythrocyte numbers were then determined using the automated Parasitemia software
527 (<http://www.gburri.org/parasitemia/>) while the number of gametocytes, pycnotic and asexual
528 parasites was determined manually in >1800 erythrocytes per sample. This assay was done
529 blinded.

530 **GImS-based knockdown**

531 GImS based knockdown assay was adapted from previously published assays (42, 76). To
532 induce knockdown 2.5 or 5 mM glucosamine was added to highly synchronous early rings
533 stage parasites. As a control, the same amount of glucosamine was also added to 3D7
534 wildtype parasites. For all analyses, the growth medium was changed daily, and fresh glu-
535 cosamine were added every day.

536 Knockdown was quantified by fluorescence live cell microscopy at day 1 and 3 of the growth
537 assay. Parasites with similar size were imaged, and fluorescence was captured with the
538 same acquisition settings to obtain comparable measurements of the fluorescence intensity.
539 Fluorescence intensity (integrated density) was measured with Fiji(90), and background was
540 subtracted in each image. The data were analyzed with Graph Pad Prism version 8.

541 GImS based knockdown experiments in gametocytes were performed as described previ-
542 ously (94). Briefly, synchronized ring stage cultures were induced by the addition of shield-1.
543 At day 3 post induction the culture was spilt into two dishes and one dish was cultured in the
544 presence of 2.5 mM glucosamine for the remaining ten days. Knockdown was quantified by
545 fluorescence live cell microscopy at day 7 and 10 post induction, as described above and
546 gametocyte parasitemia was determined at day 10 post induction using the automated
547 Parasitemia software (<http://www.gburri.org/parasitemia/>).

548 **DiCre mediated conditional knockout**

549 The parasites containing the integrated pSLI-*PfPMRT1*-loxP construct were transfected with
550 pSkip-Flox (41) using 2 µg/ml Blasticidin S to obtain a line expressing the DiCre fragments.
551 To induce excision, the tightly synchronized parasites (detailed description see growth as-
552 say) were split into 2 dishes and rapalog was added to one dish (Clontech, Mountain View,
553 CA) to a final concentration of 250 nM. The untreated dish served as control culture. Excision

554 was verified at genomic level after 24 and 48 hours of cultivation by PCR and on protein level
555 by Western blot using anti-HA antibodies.

556 **Phylogenetic analysis**

557 A blastp search of the PMRT1 sequence (PlasmoDB (38): PF3D7_1135300; UniProt:
558 Q8II12) was performed against the nr database (9 May 2021) using Geneious Prime
559 2021.2.2 (<https://www.geneious.com>) and an E-value of 10e-0 (BLOSUM62 substitution ma-
560 trix). Blast hits were filtered for sequences from taxa represented in the currently favored
561 haemosporidian parasite phylogeny (95). The phylogeny derived from an amino acid align-
562 ment using Bayesian framework with a partitioned supermatrix and a relaxed molecular clock
563 (18_amino_acid_partitioned_BEAST_relaxed_clock_no_outgroup.tre; (95)) was visualized
564 with associated data using the R package ggtree v3.3.0.900 (96, 97). A multiple protein se-
565 quence alignment of PMRT1 and homologous sequences was performed using MAFFT
566 v7.490 (98) using the G-INS-I algorithm to obtain a highly accurate alignment. Protein statis-
567 tics were calculated using Geneious Prime 2021.2.2 (<https://www.geneious.com>) and EM-
568 BOSS pepstats v6.6.0.0 (99).

569 **Prediction of protein structures**

570 AlphaFold structure predictions (39) were retrieved from <https://alphafold.ebi.ac.uk> and the
571 PDB used for DALI protein structure homology search (40). PyMOL Molecular Graphics Sys-
572 tem, Version 2.5.2 Schrödinger was used for visualization of all structures, generation of fig-
573 ures and the calculation of the root mean square deviation (RMSD) between the predicted
574 crystal structure of *PfPMRT1* and the Maquette-3 protein (PDB: 5vjt (60)) by cealign. The
575 Adaptive Poisson-Boltzmann Solver (APBS) within PyMOL was used to predict the surface
576 electrostatics of *PfPMRT1*.

577 Parasite icons were generated using BioRender (biorender.com), plasmids and oligonucleo-
578 tides were designed using ApE (100) and statistical analysis was performed using GraphPad
579 Prism version 8 (GraphPad Software, USA).

580

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599 **Author contribution**

600 Conceptualization: JSW, TWG, AB, JSTR
601 Methodology: GN, SG
602 Investigation: JSW, PMR, JY, GF; JSTÄ, HVT, AA, IH, BL
603 Formal Analysis: JSTR
604 Writing original manuscript: JSW, AB, JSTR, DW
605 Review & Editing: JSW, PMR, TWG, AB, JSTR, DW
606 Funding Acquisition: DW, CL, TWG, AB, JSTR
607 Resources: TWG
608 Project Administration: TWG, AB, JSTR
609 Supervision: DW, TS, SG, TWG, AB
610 All authors read and approved the manuscript.

611

612 **Figures**

613 **Figure 1: Subcellular localization of six putative *P. falciparum* transporters during**
614 **asexual blood stage development.**

615 **(A)** Schematic representation of endogenous tagging strategy using the selection-linked in-
616 tegration system (SLI). pink, human dihydrofolate dehydrogenase (hDHFR); grey, homology
617 region (HR); green, green fluorescence protein (GFP) tag; dark grey, T2A skip peptide; blue,
618 neomycin resistance cassette; orange, glmS cassette. Stars indicate stop codons, and ar-
619 rows depict primers (P1 to P4) used for the integration check PCR. **(B–G)** Localization of (B)
620 *PfVRT1*-GFP-glmS, (C) *PfZIP1*-GFP-glmS, (D) *PfCDF*-GFP-glmS, (E) *PfDMT2*-GFP-glmS,
621 (F) *PfMFS6*-GFP-glmS and (G) *PfPMRT1*-GFP-glmS by live-cell microscopy in ring,
622 trophozoite and schizont stage parasites. Nuclei were stained with Hoechst-33342. **(H)** Co-
623 localization of the GFP-tagged putative transporters with marker proteins P40PX-mCherry
624 (food vacuole), ACP-mCherry (apicoplast), Lyn-mCherry (parasite plasma membrane), ARO-

625 mCherry (rhoptry) and AMA1-mCherry (microneme) as indicated. Nuclei were stained with
626 Hoechst- 33342. Scale bar, 2 μ m.
627

628 **Figure 2: Conditional knockdown of putative transporter indicate importance of**
629 ***PfDMT2* and *PfPMRT1* for parasites fitness.**

630 **(A–C)** Live cell microscopy and quantification of knockdown by measuring mean fluores-
631 cence intensity (MFI) density and size (area) of (A) *PfDMT2*-GFP-glmS (B) *PfMFS6*-GFP-
632 glmS and (C) *PfPMRT1*-GFP-glmS parasites 40 hours after treatment without (control) or
633 with 2.5 mM glucosamine. Scale bar, 2 μ m. Statistics are displayed as mean +/- SD of three
634 (A–B) or four (C) independent experiments and individual data points are color-coded by ex-
635 periments according to Superplots guidelines (101). P-values displayed were determined
636 with two-tailed unpaired t-test. **(D)** Growth of parasites treated without (control) or with 2.5
637 mM glucosamine determined by flow cytometry is shown as relative parasitemia values after
638 two cycles. Shown are means +/- SD of three (*PfPMRT1*-GFP-glmS, *PfDMT2*-GFP-glmS,
639 *PfMFS6*-GFP-glmS) and five (3D7 wild type parasites) independent growth experiments. P-
640 values displayed were determined with unpaired t test with Welch correction and Benjamin-
641 Hochberg for multiple testing correction. Individual growth curves are shown in **Figure S4G**.
642 **(E)** Growth of *PfPMRT1*-glmS and 3D7 parasites after treatment with 2.5 mM (left panel) and
643 5 mM glucosamine (right panel) compared to untreated control parasites over five consecu-
644 tive days. P-values displayed were determined for comparison between *PfPMRT1*-glmS and
645 3D7 parasites at day 3 using two-tailed Wilcoxon rank sum test. **(F)** Mean +/- SD distribution
646 of ring and schizont stage parasites in *PfPMRT1*-glmS and 3D7 cell lines treated without
647 (control), with 2.5 mM or 5 mM glucosamine at 84 hpi (80 hours post addition of glucosa-
648 mine) of three independent experiments. P-values displayed were determined using the
649 Conover-Iman rank sum test and Benjamini-Hochberg method for multiple testing correction
650 after Kruskal-Wallis testing.

651 **Figure 3: *PfPMRT1* is essential for asexual blood stage development.**

652 **(A)** Simplified schematic of DiCre-based conditional *PfPMRT1* knockout using selection-
653 linked integration (SLI). Pink, human dihydrofolate dehydrogenase (hDHFR); grey, homology
654 region (HR); green, T2A skip peptide; light blue, recodonized *PfPMRT1*; dark blue, 3xHA tag,
655 yellow, neomycin phosphotransferase resistance cassette; orange, loxp sequence. Scissors
656 indicate DiCre mediated excision sites upon addition of rapalog. Stars indicate stop codons,
657 and arrows depict primers (P1 to P5) used for the integration check PCR and excision PCR.
658 **(B)** Diagnostic PCR of unmodified wildtype and transgenic cond Δ PMRT1 knock-in (KI) cell
659 line to check for genomic integration using Primer P1-P4 as indicated in (A). **(C)** Immunofluo-
660 rescence assay (IFA) of cond Δ PMRT1 late stage schizont parasites showing localization of

661 *PfPMRT1*-3xHA at the parasite plasma membrane (PPM) co-localizing with the merozoite
662 surface protein 1 (MSP1). **(D)** Diagnostic PCR to verify the excision at genomic level at 24
663 hpi / 20 hours post rapalog addition for cond Δ PMRT1 and at 48 hpi for cond Δ PMRT1, c-
664 $nmd3$ *PfPMRT1*-ty1 and c- $sf3a2$ *PfPMRT1*-ty1 parasites using Primer P1-P5 as indicated in (A).
665 Black arrow head, original locus; red arrow head, excised locus. **(E)** Western blot using α -HA
666 to verify knockout of *PfPMRT1* on protein level 4, 24 and 48 hours post invasion. Expected
667 molecular weight of *PfPMRT1*-3xHA: 53.3 kDa. Antibodies detecting Aldolase and SBP1
668 were used as loading controls. **(F)** Growth curves of cond Δ PMRT1, c- $nmd3$ *PfPMRT1*-ty1 and
669 c- $sf3a2$ *PfPMRT1*-ty1 parasites +/- rapalog monitored over five days by flow cytometry. One
670 representative growth curve is depicted (replicates in Figure S5). Summary is shown as relative
671 parasitemia values, which were obtained by dividing the parasitemia of rapalog treated
672 cultures by the parasitemia of the corresponding untreated ones. Shown are means +/- SD of
673 three (cond Δ PMRT1, c- $nmd3$ *PfPMRT1*-ty1) or four (c- $sf3a2$ *PfPMRT1*-ty1) independent growth
674 experiments. **(G)** IFA of cond Δ PMRT1 complemented with C-terminal TY1-tagged *PfPMRT1*
675 constructs expressed either under the constitutive *nmd3* or the weak *sf3a2* promoter to verify
676 PPM localization. Scale bar, 2 μ m.
677

678 **Figure 4: Knockout of *PfPMRT1* results in accumulation of PPM-derived protrusions**

679 and growth arrest at the trophozoite stage.

680 **(A)** Parasite stage distribution in Giemsa smears displayed as heatmap showing percentage
681 of parasite stages for tightly synchronized (+/- 2 h) 3D7 control and cond Δ PMRT1 (rapalog
682 treated at 4 hpi, 20 hpi or 32 hpi) parasite cultures over two consecutive cycles. A second
683 replicate is shown in Figure S6A **(B)** Giemsa smears of control and at 4 hpi rapalog treated
684 cond Δ PMRT1 parasites over two cycles. Scale bar, 5 μ m. **(C)** Live cell microscopy of 4 hour
685 window synchronized 3D7 control and cond Δ PMRT1 parasites +/- rapalog stained with
686 dihydroethidium (DHE) at 20–32 hpi. **(D)** Quantification of parasites displaying protrusions
687 (green) for 4 hour window synchronized 3D7 control and rapalog treated cond Δ PMRT1 para-
688 sites. Shown are percentages of normal parasites versus parasites displaying protrusions as
689 means +/- SD of three independent experiments. **(E)** Live cell microscopy of 8 hour window
690 synchronized 3D7 control and rapalog treated cond Δ PMRT1 parasites, episomally express-
691 ing the PPM marker Lyn-mCherry at 24–40 hpi. **(F)** Live cell microscopy of 3D7 control and
692 cond Δ PMRT1 parasites +/- rapalog stained with BODIPY TR C5 ceramide at 32 hpi. Scale
693 bar, 2 μ m.
694

695 **Figure 5: *PfPMRT1* is essential for early gametocyte development.**

696 **(A)** Live cell microscopy of 3D7-iGP-*Pf*PMRT1-GFP parasites across the complete gameto-
697 cyte development. White arrow heads indicate remaining GDV1-GFP signal observed in
698 close proximity to the Hoechst signal, as previously reported (59, 94, 102, 103). **(B)** Live cell
699 microscopy of *Pf*PMRT1-GFP parasites expressing the PPM marker Lyn-mCherry. Nuclei
700 were stained with Hoechst-33342. Scale bar, 2 μ m. **(C)** Experimental setup of gametocyte
701 induction upon GDV1-GFP-DD expression (+shield-1) and conditional *Pf*PMRT1 knockout
702 (+rapalog) and elimination of asexual blood stage parasites (+GlcNac). **(D)** Gametocyte de-
703 velopment over 12 days of cond Δ PMRT1/GDV1-GFP-DD or 3D7-iGP parasites without (con-
704 trol) or with rapalog addition at day 3 (3 dpi) or day 5 (5 dpi) after induction of sexual com-
705 mitment by conditional expression of GDV1-GFP upon addition of shield-1. Scale bar, 5 μ m.
706 **(E)** Diagnostic PCR to verify the excision on genomic level at 5 dpi and 12 dpi. Black arrow
707 head, original locus; red arrow head, excised locus. **(F)** Representative Giemsa smears and
708 quantification of parasite stage distribution at day 10 post induction for parasites treated
709 without (control) or with rapalog at day 3 post induction. For each condition parasitemia and
710 parasite stages distribution in (Δ PMRT1: $n_{\text{control}} = 3370, 2304, 2759$ and $n_{\text{rapalog}} = 3010, 1830,$
711 2387; 3D7-iGP: $n_{\text{control}} = 4985, 4685, 5206$ and $n_{\text{rapalog}} = 4930, 4332, 5384$) erythrocytes of
712 three independent experiments were determined and are displayed as percentage. Nuclei
713 were stained with Hoechst-33342. Scale bar, 10 μ m.
714

715 **Figure 6: PMRT1 is a genus-specific transporter with conserved function.**
716 **(A)** Phylogenetic tree of haemosporidian parasites (modified from (95)) containing PMRT1
717 homologous sequences associated with data on pairwise amino acid sequence identity to
718 *Pf*PMRT1. The phylogeny is derived from Bayesian Inference using BEAST using a fully par-
719 titioned amino acid dataset and lognormal relaxed molecular clock (95). Silhouettes depict
720 representatives of the vertebrate hosts for each lineage and white filled bars indicate pair-
721 wise identities of PMRT1 homologs used for subsequent complementation assays. **(B)** Struc-
722 tural alignment of predicted *Pf*PMRT1 structure with Maquette-3 protein (PDB: 5vjt) (60).
723 Both structures have a root mean square deviation (RMSD) over the aligned α -carbon posi-
724 tion of 3.12 over 184 residues calculated in PyMol. **(C)** IFA of c- nmd3 *Pk*-ty1 and c- nmd3 *Pv*-ty1
725 parasites to verify correct localization of the expressed complementation fusion proteins at
726 the parasite plasma membrane. Nuclei were stained with Hoechst-33342. Scale bar, 2 μ m.
727 **(D)** Growth of cond Δ PMRT1 parasites complemented with *Pf*PMRT1 homologs from *P. vivax*
728 (PVP01_0936100) and *P. knowlesi* (PKNH_0933400). Shown are relative parasitemia val-
729 ues, which were obtained by dividing the parasitemia of rapalog treated cultures by the
730 parasitemia of the corresponding untreated controls together with means +/- SD from three c-
731 nmd3 *Pf*-ty1 (\triangleq c- nmd3 *Pf*PMRT1-ty1 Figure 3D, S5B) and six (c- nmd3 *Pk*-ty1, c- nmd3 *Pv*-ty1) inde-
732 pendent growth experiments. One sample t-test **(E)** Diagnostic PCR to verify the excision of

733 *PfPMRT1* on genomic level at 48 hpi for c-^{nmd3}*Pf*-ty1, c-^{nmd3}*Pk*-ty1 and c-^{nmd3}*Pv*-ty1 para-
734 sites. Black arrow head, original locus; red arrow head, excised locus.
735

736 **Figure S1: Structure predictions and structure homology search of candidate proteins**

737 **(A)** AlphaFold structure predictions of the six selected orphan transporters visualized in
738 PyMol. **(B)** Results from protein structure comparison server Dali using the AlphaFold-
739 generated PDB files of the selected transporters as input structure. Shown are the top five
740 non-redundant hits with Z score (significance estimate), msd (difference between the root-
741 mean-square-deviation (rmsd) value associated with a protein structure pair and the rmsd
742 value that would have been observed in the case that the two structures had the same crys-
743 tallographic resolution), lali (number of aligned positions), nres (number of residues in the
744 matched structure) and %id (the percentage sequence identity in the match).

745

746 **Figure S2: Validation of generated transgenic cell lines by PCR and Western blot.**

747 **(A)** Confirmatory PCR of unmodified wildtype (WT) and transgenic knock-in (KI) cell lines
748 (PF3D7_0523800-GFP-glmS (*Pf*FVRT1), PF3D7_0609100-GFP-glmS (*Pf*ZIP1),
749 PF3D7_0715900-GFP-glmS (*Pf*CDF), PF3D7_0716900-GFP-glmS (*Pf*DMT2),
750 PF3D7_1440800-GFP-glmS (*Pf*MFS6) and PF3D7_1135300-GFP-glmS (*Pf*PMRT1)) to
751 check for genomic integration at the 3'- and 5'-end of the locus. Position of the primer used
752 are indicated with numbered arrows in **Figure 1A**. **(B)** Western Blot analysis of wildtype (3D7)
753 and knock-in (KI) cell lines using mouse anti-GFP to detect the tagged full-length protein
754 (upper panel) and rabbit anti-alcohol dehydrogenase to control for equal loading (lower panel). Protein size
755 is indicated in kDa. Expected molecular weight for GFP fusion proteins: *Pf*FVRT1 (107.5
756 kDa), *Pf*ZIP1 (69.0 kDa), *Pf*DMT2 (66.4 kDa), *Pf*MFS6 (98.8 kDa), *Pf*PMRT1 (77.5 kDa),
757 *Pf*CDF (91.6 kDa) **(C)** Localization of *Pf*CDF-3xHA by IFA in ring, trophozoite and schizont
758 parasites. Nuclei were stained with Hoechst. Diagnostic PCR of unmodified wildtype (WT)
759 and transgenic knock-in (KI) cell line. **(D)** Localization of *Pf*PMRT1_2xFKBP-GFP across the
760 IDC. Nuclei were stained with DAPI. Scale bar, 2 μ m. Diagnostic PCR of unmodified wildtype
761 (WT) and transgenic knock-in (KI) cell line. **(E)** Localization of *Pf*ZIP1-GFP in merozoites.
762 Nuclei were stained with DAPI. Scale bar, 2 μ m. **(F)** Confocal microscopy of *Pf*PMRT1-GFP
763 co-expressing the PPM marker Lyn-mCherry. Scale bar, 1 μ m. Nuclei were stained with
764 Hoechst.

765

766 **Figure S3: Targeted gene disruption (TGD) of *Pf*ZIP1 and *Pf*CDF.**

767 **A)** Schematic representation of TGD strategy using the selection-linked integration system
768 (SLI). pink, human dihydrofolate dehydrogenase (hDHFR); grey, homology region (HR);

769 green, green fluorescence protein (GFP) tag; dark grey, T2A skip peptide; blue, neomycin
770 resistance cassette. Stars indicate stop codons, and arrows depict primers (P1 to P4) used
771 for the integration check PCR. **(B)** Localization of *PfZIP1*-TGD-GFP in ring, trophozoite and
772 schizont parasites. Nuclei were stained with Hoechst-33342. Scale bar, 2 μ m. Confirmatory
773 PCR of unmodified wildtype (WT) and transgenic targeted gene disruption (TGD) cell line.
774 Growth curves of *PfZIP1*-TGD vs. 3D7 parasites monitored over five days by FACS. Three
775 independent growth experiments were performed and a summary is shown as percentage of
776 growth compared to 3D7 parasites. **(C)** Localization of *PfCDF*-TGD in ring, trophozoite and
777 schizont parasites. Nuclei were stained with DAPI. Confirmatory PCR of unmodified wildtype
778 (WT) and transgenic targeted gene disruption (TGD) cell line. Scale bar, 1 μ m.
779

780 **Figure S4: Conditional knockdown via glmS system.**

781 Live cell microscopy of **(A)** *PfFVRT1*-GFP-glmS, **(B)** *PfCDF*-GFP-glmS, **(C)** *PfZIP1*-GFP-
782 glmS **(D)** *PfDMT2*-GFP-glmS, **(E)** *PfMFS6*-GFP-glmS and **(F)** *PfPMRT1*-GFP-glmS parasites
783 40 hours after treatment without (control) or with 2.5 mM Glucosamine. Nuclei were stained
784 with Hoechst-33342. Scale bar, 2 μ m. **(G)** Individual growth curves of the growth assays
785 shown in [Figure 2D](#). **(H)** PCR using a GFP forward and glmS reverse primer confirming the
786 presence of the GFP and glmS sequence in the pSLI-*PfFVRT1*-GFP-glmS and *PfCDF*-GFP-
787 glmS plasmids. pSLI-PF3D7_0631900-GFP (35) was used as negative control.
788

789 **Figure S5: Conditional knockout of *PfPMRT1* via DiCre-based system**

790 Replicates of growth curves of cond Δ PMRT1, c^{-nmd3}*PfPMRT1*-ty1 and c^{-sf3a2}*PfPMRT1*-ty1
791 parasites +/- rapalog monitored over five days by FACS shown in [Figure 3](#).
792

793 **Figure S6: Conditional knockout of *PfPMRT1***

794 **(A)** Parasite stage distribution in Giemsa smears displayed as heatmap showing percentage
795 of stages for control, 4 hpi, 20 hpi or 32 hpi rapalog treated 4 hour window synchronized
796 cond Δ PMRT1parasite cultures over one cycle. **(B)** Giemsa smears of control and 4 hpi, 20
797 hpi or 32 hpi rapalog treated parasites at 4, 16, 20, 24, 32, 40 and 48 hpi. Scale bar, 5 μ m.
798

799 **Figure S7: Conditional knockdown of *PfPMRT1* has no effect during gametocyte de-
800 velopment.**

801 **(A)** Confirmatory PCR of unmodified wildtype (WT) and transgenic 3D7-iGP-*PfPMRT1*-GFP-
802 glmS to check for genomic integration at the 3'- and 5'-end of the locus. Position of the pri-
803 mer used are indicated with numbered arrows in [Figure 1A](#). **(B)** Schematic representation of

804 the experimental setup. **(C)** Live cell microscopy of 3D7-iGP-*PfPMRT1*-GFP stage I – V ga-
805 metocytes. Scale bar, 2 μ m. **(D)** Giemsa smears of stage I – V gametocytes cultured either
806 without (control) or with 2.5 mM glucosamine. Scale bar, 5 μ m. **(E)** Quantification of knock-
807 down by measuring mean fluorescence intensity (MFI) density and size (area) of parasites at
808 day 7 and day 12 post induction of gametocytogenesis cultured either without (control) or
809 with 2.5 mM glucosamine. Scale bar, 2 μ m. Statistics are displayed as mean +/- SD of four
810 independent experiments and individual data points are displayed as scatterplot color-coded
811 by experiments according to Superplots guidelines(101)(101). P-values displayed were de-
812 termined with two-tailed unpaired t-test. **(F)** For each condition gametocytemia at day 10 post
813 gametocyte induction was determined by counting between 1256-2653 (mean 2147) cells
814 per condition in Giemsa-stained thin blood smears. Displayed are means +/- SD of inde-
815 pendent growth experiments with the number of experiments (n) indicated. P-values dis-
816 played were determined with two-tailed unpaired t-test.

817

818 **Figure S8:** Individual growth curves of c-^{nmd3}*Pk*-ty1 **(A)** and c-^{nmd3}*Pv*-ty1 **(B)** parasites +/-
819 rapalog monitored over two IDCs by FACS shown in **Figure 6**. **(C)** Western Blot analysis of c-
820 ^{nmd3}*Pf*-ty1, c-^{nmd3}*Pk*-ty1 and c-^{nmd3}*Pv*-ty1 cell lines using mouse anti-ty1 to detect the tagged
821 full-length protein (upper panel) and rabbit anti-BIP to control for loading (lower panel). Pro-
822 tein size is indicated in kDa. **(D)** and **(E)** 3D7 wild type parasites imaged across the IDC to
823 establish autofluorescence levels with Zeiss Axioskop 2plus microscope (D) or Leica D6B
824 fluorescence microscope (E). **(F)** Surface electrostatics of the predicted *PfPMRT1* structure
825 generated by APBS within PyMol.

826

827 **Table S1: Oligonucleotides and plasmids used in this study**

828

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Figure 1

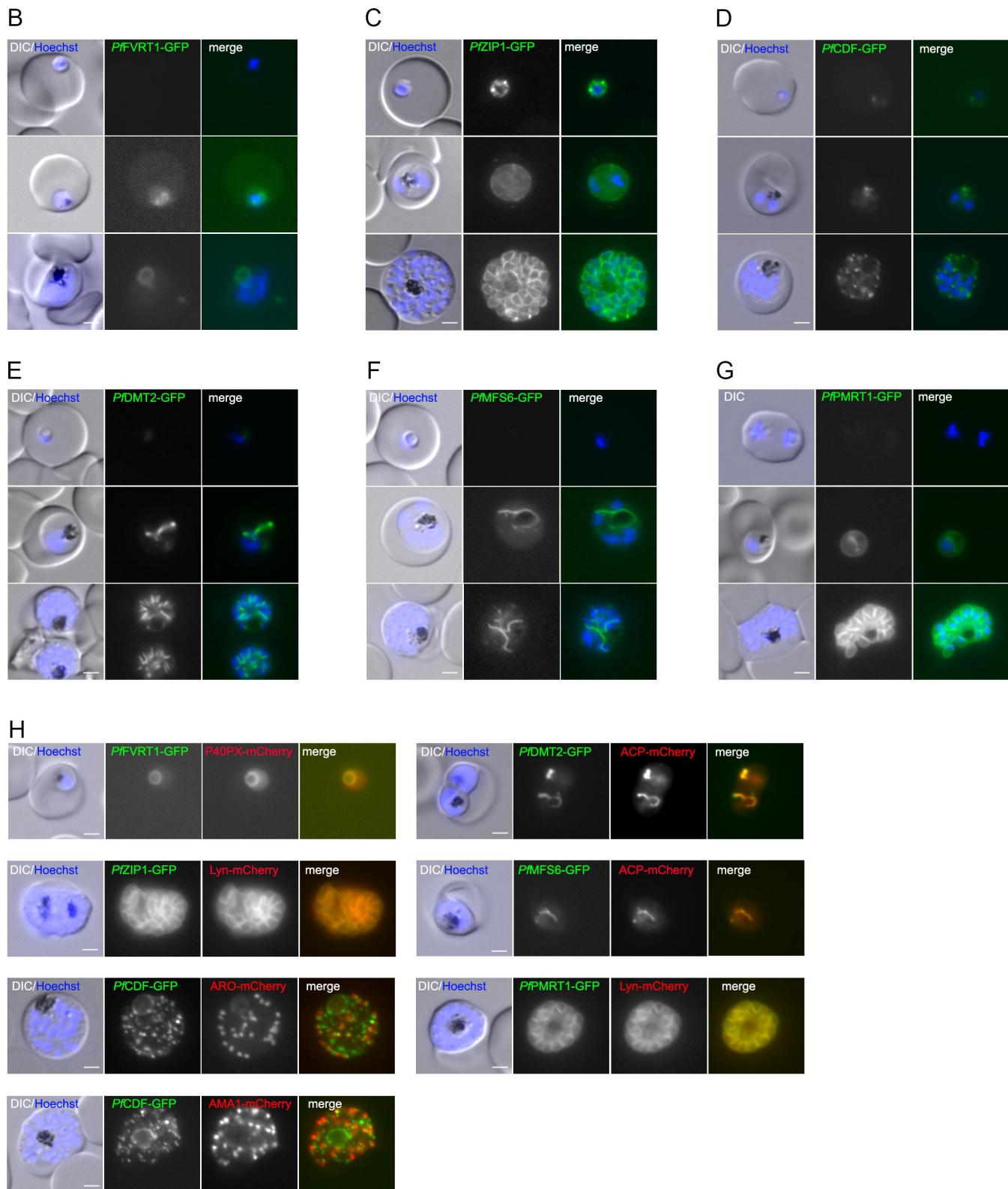
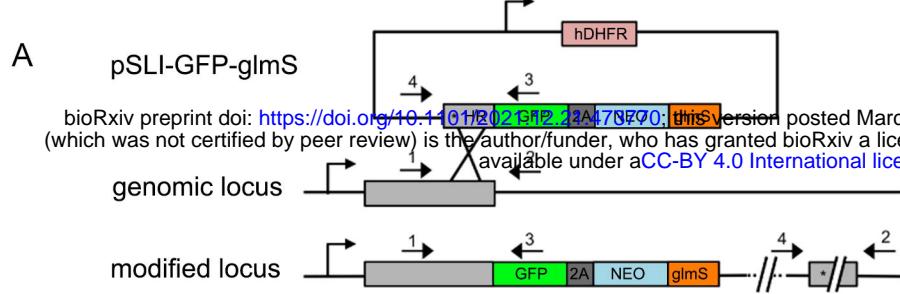


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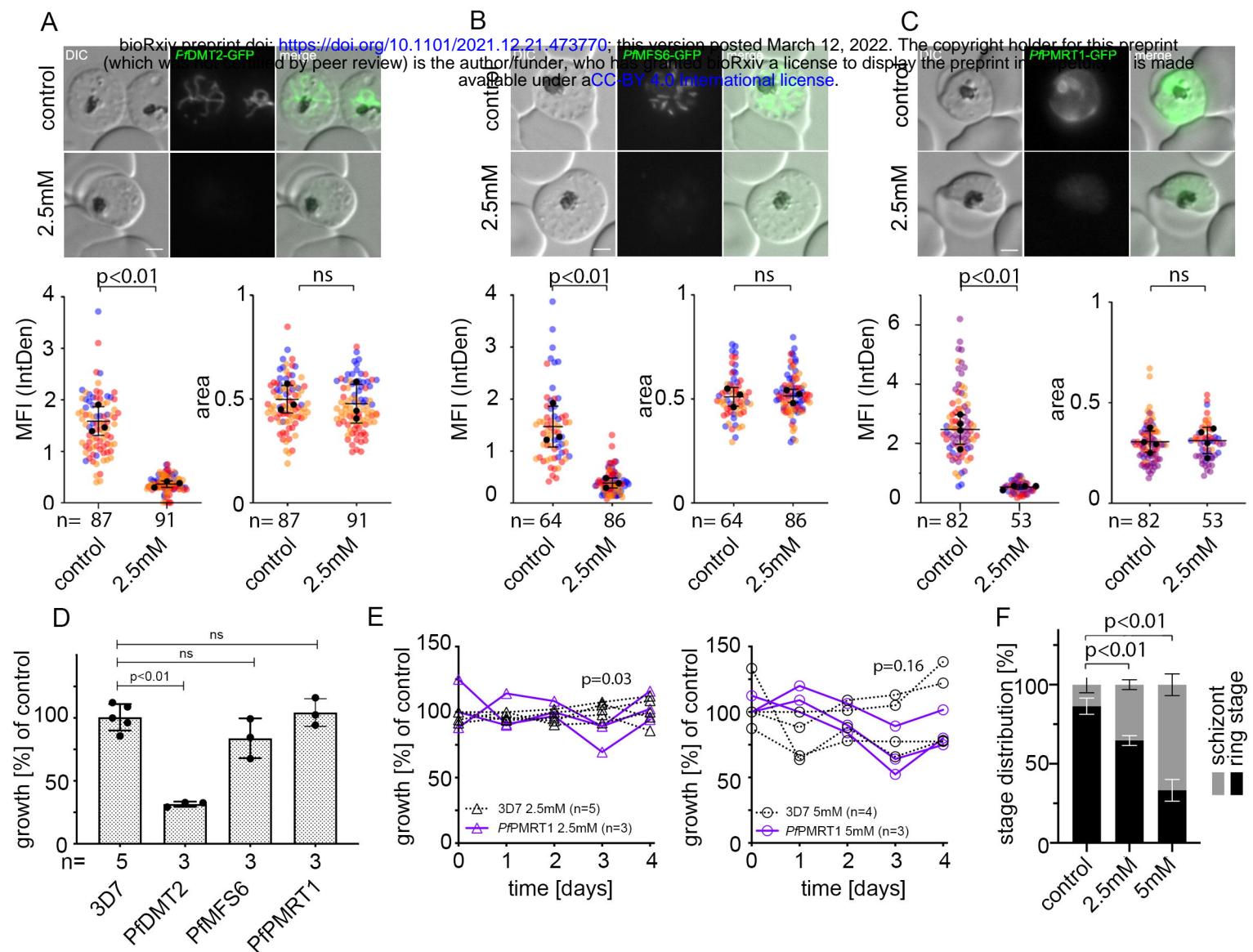


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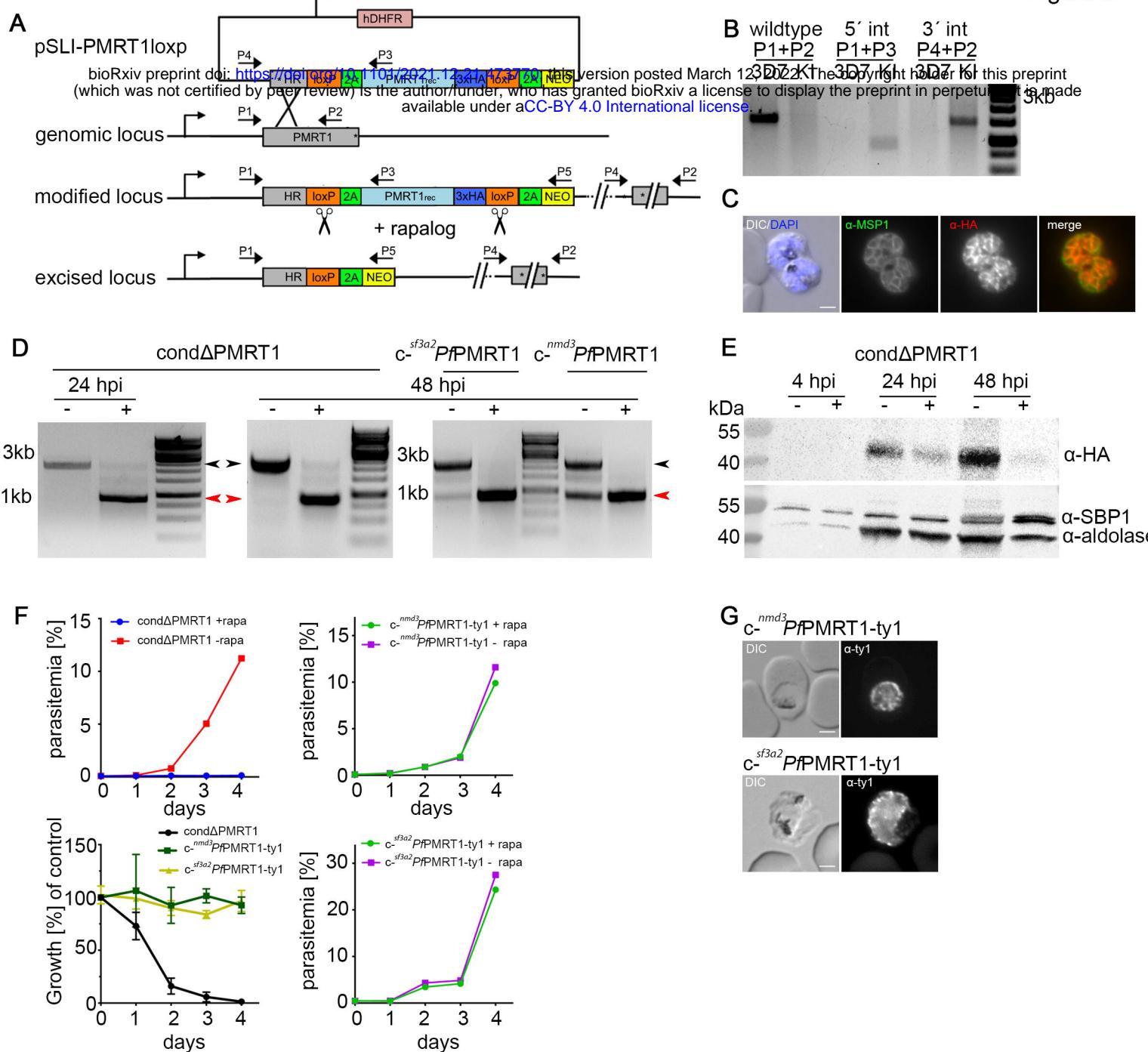
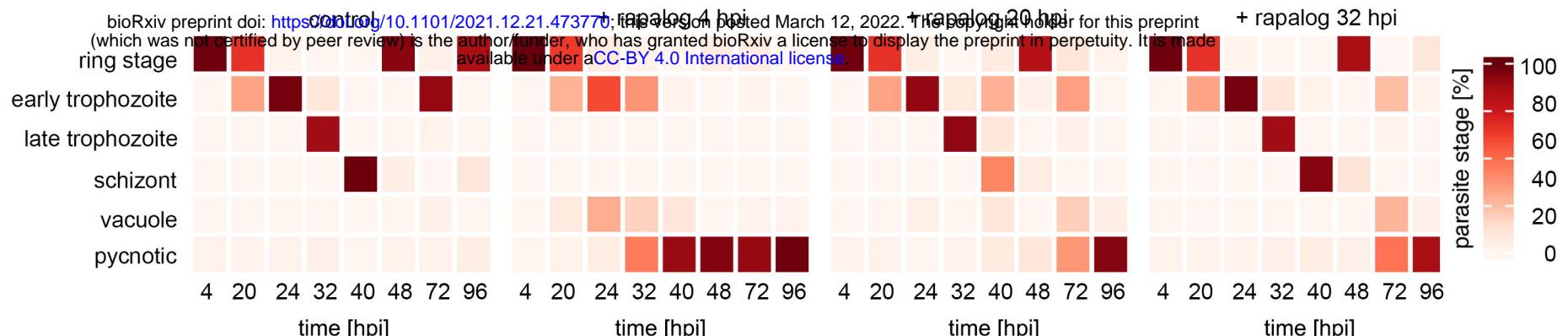
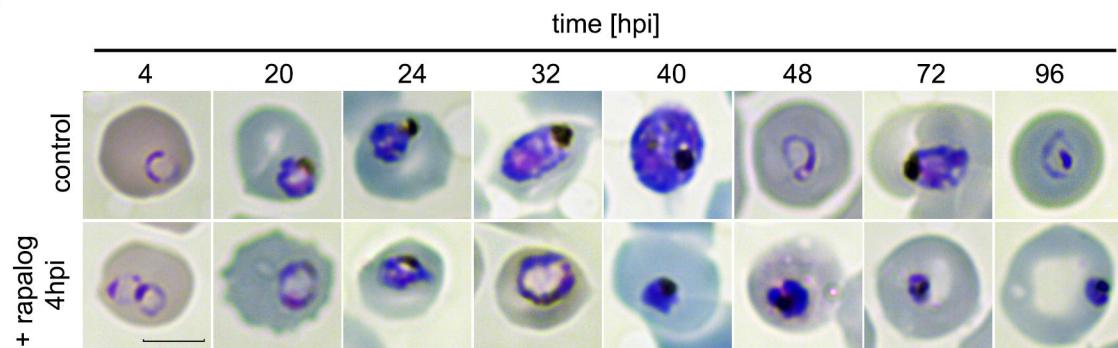


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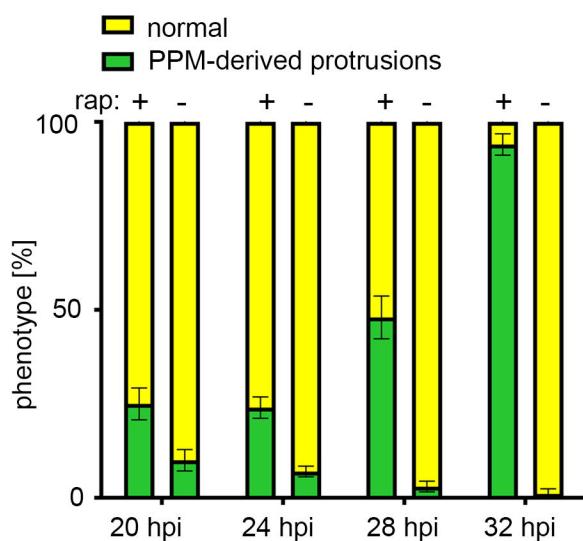
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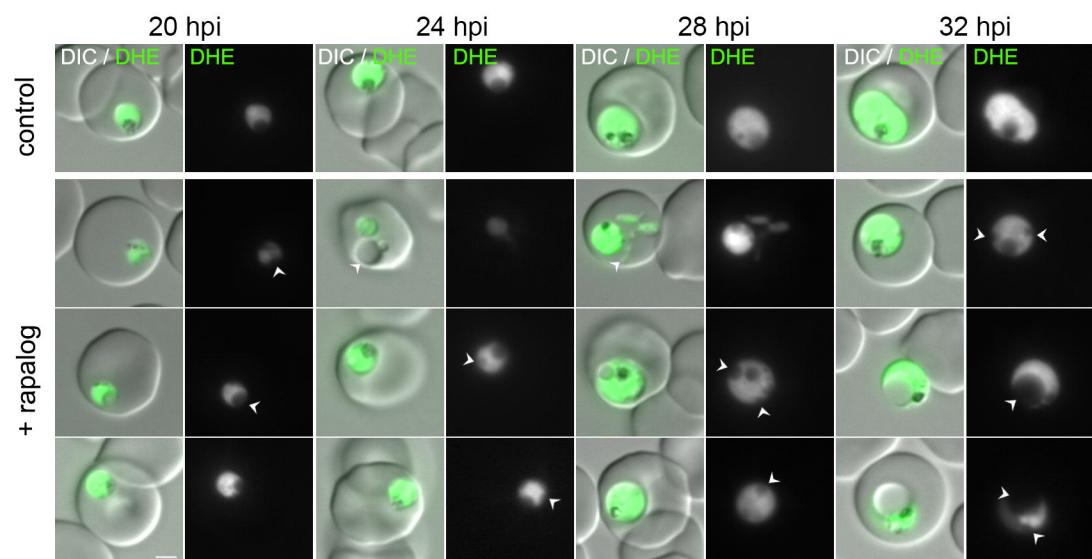
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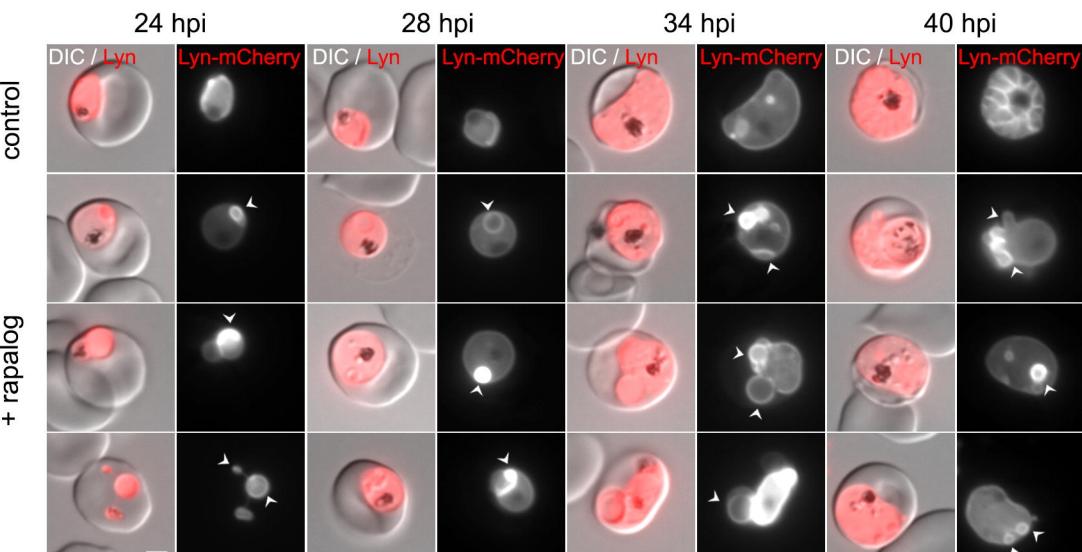
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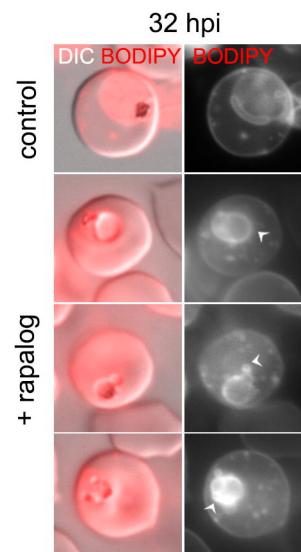
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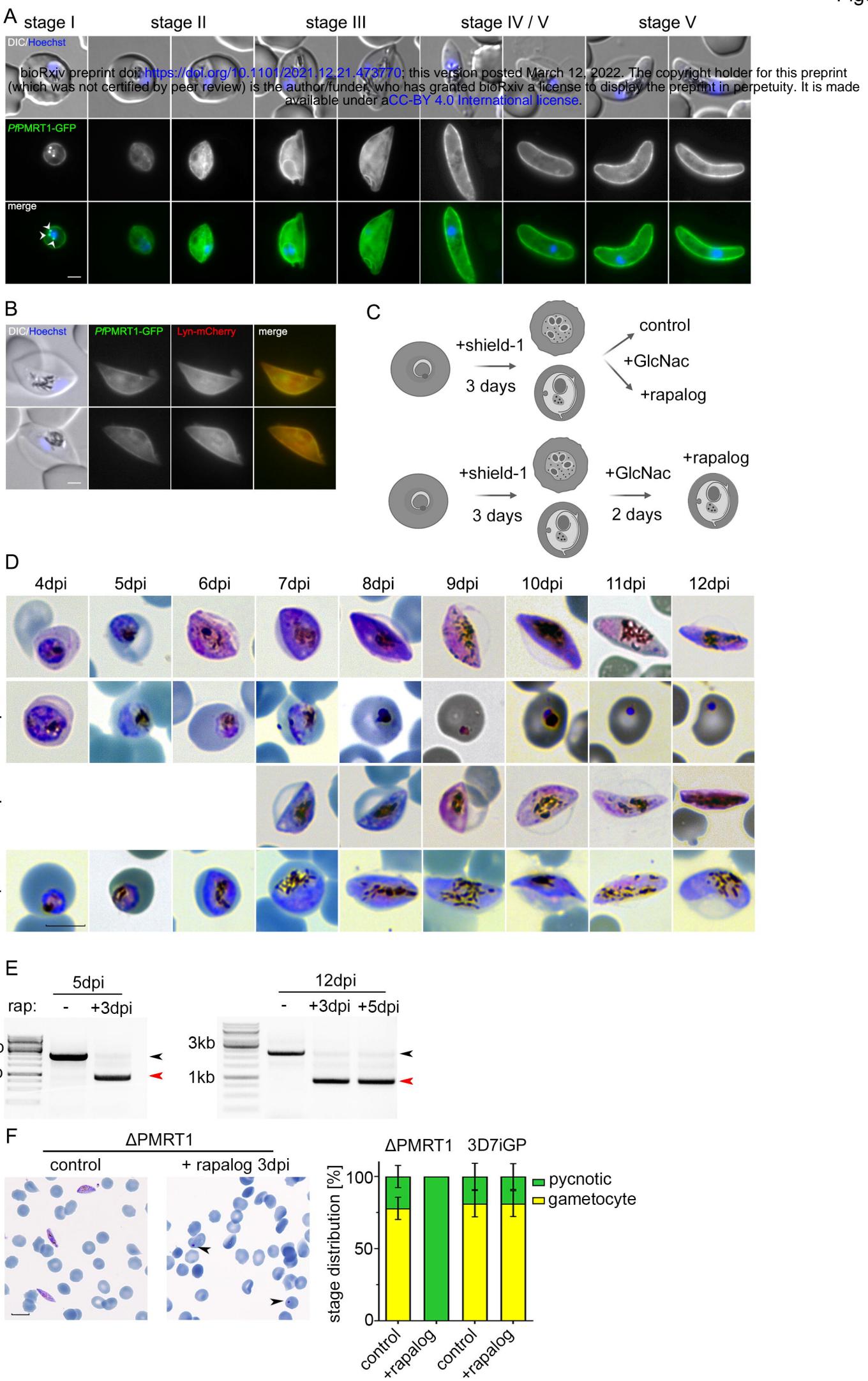
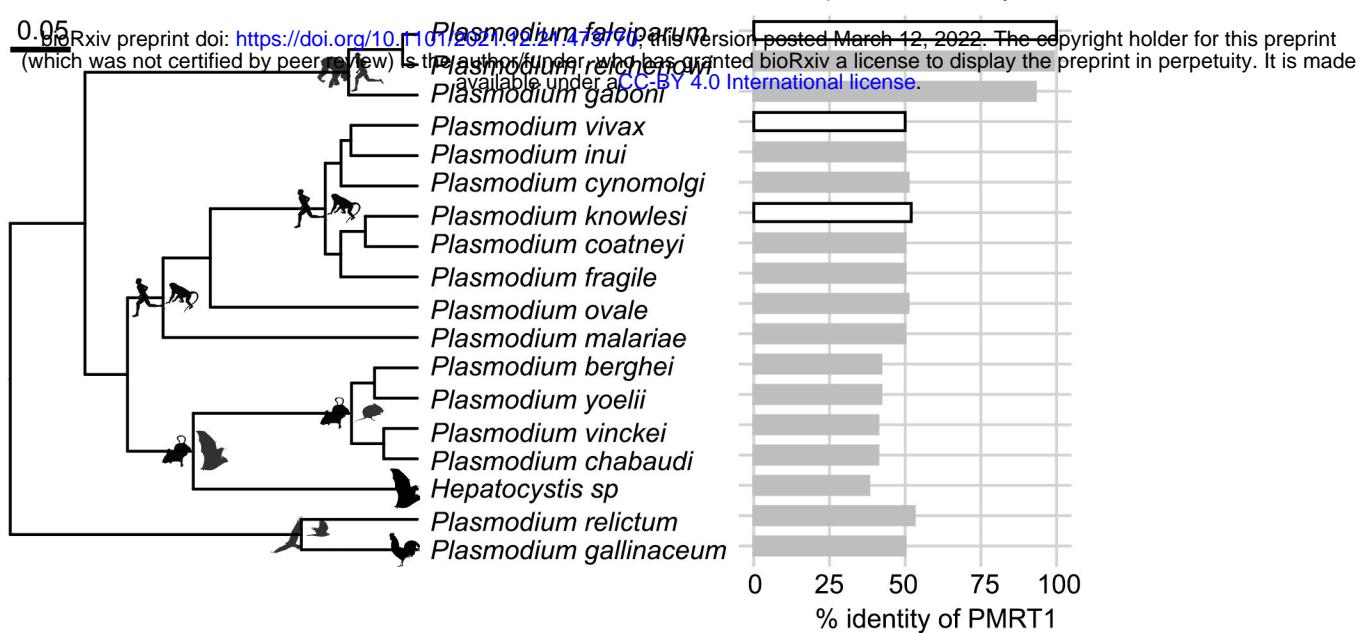
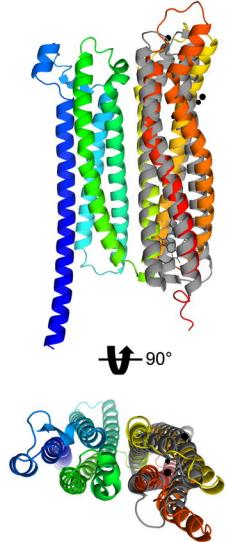


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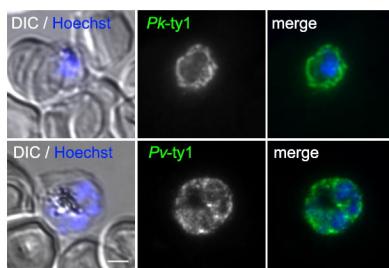
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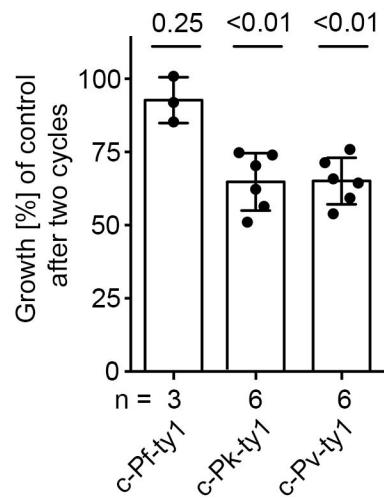
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