The *Plasmodium falciparum* rhoptry protein RhopH3 plays essential roles in host cell invasion and nutrient uptake

Emma S. Sherling[1,2], Ellen Knuepfer[1], Joseph A. Brzostowski[3], Louis H. Miller[2], Michael J. Blackman[1,4]*, Christiaan van Ooij[1]*

[1]The Francis Crick Institute, 1 Midland Road, London, NW1 1AT, United Kingdom
[2]Laboratory of Malaria and Vector Research and [3]Laboratory of Immunogenetics Imaging Facility, National Institute of Allergy and Infectious Disease, National Institute of Health, Rockville, MD 20852, USA
[4]Department of Pathogen Molecular Biology, London School of Hygiene & Tropical Medicine, London WC1E 7HT, United Kingdom

Correspondence and requests for materials should be addressed to M.J.B. (email: Mike.Blackman@crick.ac.uk) or C.v.O. (email: Christiaan.vanOoij@crick.ac.uk)

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52 **Abstract**

53 Merozoites of the protozoan parasite responsible for the most virulent form

54 of malaria, *Plasmodium falciparum,* invade erythrocytes. Invasion involves

55 discharge of rhoptries, specialized secretory organelles. Once intracellular,

56 parasites induce increased nutrient uptake by generating new permeability

57 pathways (NPP) including a *Plasmodium* surface anion channel (PSAC).

58 RhopH1/Clag3, one member of the three-protein RhopH complex, is

59 important for PSAC/NPP activity. However, the roles of the other members

60 of the RhopH complex in PSAC/NPP establishment are unknown and it is

61 unclear whether any of the RhopH proteins play a role in invasion. Here we

62 demonstrate that RhopH3, the smallest component of the complex, is

63 essential for parasite survival. Conditional truncation of RhopH3

64 substantially reduces invasive capacity. Those mutant parasites that do

65 invade are defective in nutrient import and die. Our results identify a dual

66 role for RhopH3 that links erythrocyte invasion to formation of the

67 PSAC/NPP essential for parasite survival within host erythrocytes.

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70 **Introduction**

71 Parasites of the genus *Plasmodium* are the causative agents of malaria, a

72 disease that claims nearly 600,000 lives each year (1). Of the five

73 *Plasmodium* species that infect humans, *Plasmodium falciparum* is

74 responsible for nearly all the mortality associated with malaria. The disease

75 is the result of asexual replication of the parasite in erythrocytes. For

76 approximately the first half of the 48 h *P. falciparum* intraerythrocytic life

77 cycle, the parasite exists in a mononuclear trophozoite form (the earliest

78 stages of which are generally referred to as ring stages), during which the

79 parasite grows rapidly. During this phase, *P. falciparum*-infected

80 erythrocytes gain the capacity to adhere to host vascular endothelium, a

81 process that depends on the export of parasite proteins to form adhesive

82 structures called knobs at the host erythrocyte surface. Nuclear division

83 then commences, initiating differentiation into a schizont (a process called

84 schizogony). This multinucleated form eventually undergoes segmentation

85 to form invasive merozoites that egress upon rupture of the infected

86 erythrocyte to invade new erythrocytes.

87   Egress and erythrocyte invasion involves the regulated discharge of

88 several sets of apical merozoite secretory organelles that are unique to

89 apicomplexan parasites. The largest of these organelles, called rhoptries,

90 contain several proteins involved in adhesion to the host cell. Rhoptries are

91 also thought to mediate formation of the nascent parasitophorous vacuole

92 (PV), a membranous compartment that surrounds the parasite after entry

93 has been completed (2,3). Despite the importance of rhoptries in invasion

94 and subsequent host cell remodeling, a detailed understanding of the

95   function of many rhoptry proteins is lacking. Rhoptries comprise at least two

96   subdomains(3) referred to as the rhoptry neck and the bulb. The contents of

97   these subdomains likely mediate different functions, as reflected by

98   evidence suggesting that they are released sequentially during invasion (4).

99   Proteins of the rhoptry neck are well conserved between *Plasmodium spp.*

100 and the related apicomplexan parasite *Toxoplasma gondii*, suggesting

101 conserved functions (3,5). In contrast, proteins of the rhoptry bulb appear

102 to be genus-specific, perhaps reflecting functions unique to each parasite

103 (3). A function for several *Plasmodium* rhoptry bulb proteins has been

104 proposed, such as a role for a protein called RAMA in transport of proteins

105 to the rhoptry (6), but the inability to produce mutants lacking these

106 proteins has precluded conclusive assignments of function (7). Hence, the

107 molecular functions of most rhoptry proteins remain unknown.

108   One component of the *P. falciparum* rhoptry bulb that has received

109 particular attention is the so-called high molecular weight (HMW) rhoptry or

110 RhopH complex, which consists of three proteins called RhopH1/Clag,

111 RhopH2, and RhopH3 (8,9). Whilst RhopH2 and RhopH3 are each encoded by

112 single-copy genes, RhopH1/Clag, the largest component of the complex,

113 exists in 5 isotypes encoded by separate genes entitled *clag2*, *clag3.1*,

114 *clag3.2*, *clag8* and *clag9* (10,11). RhopH1/Clag3.1 and RhopH1/Clag3.2 are

115 nearly identical proteins that are expressed in a mutually exclusive manner

116 (12-14). Each RhopH complex contains only one form of RhopH1/Clag (11),

117 so each parasite has the potential to produce four different RhopH

118 complexes, differentiated by the particular RhopH1/Clag isotype bound. All

119 members of the RhopH complex are expressed late in the intraerythrocytic

120   cycle (8). The complex is then released during invasion (15) and inserted

121   into the nascent PV membrane (PVM) soon after parasite entry (16,17).

122   Genetic and chemical genetic investigation has revealed a role for

123   the RhopH1/Clag3 proteins in the function of the *Plasmodium* surface anion

124   channel (PSAC), a new permeability pathway (NPP) induced in host

125   erythrocytes following parasite entry and involved in nutrient acquisition by

126   the intracellular parasite (18). Pharmacological inhibition of

127   RhopH1/Clag3.2 function was found to block PSAC/NPP activity, and

128   selection for drug-resistant mutants revealed that part of the protein is

129   exposed at the surface of the erythrocyte and that it may form the channel

130   itself (19,20). However, parasites that do not produce either

131   RhopH1/Clag3.1 or RhopH1/Clag3.2 display only a small growth

132   disadvantage (13) and inhibition of the function of these proteins has only a

133   small effect on parasite growth rates *in vitro* (21). Parasites lacking

134   RhopH1/Clag9 are viable, and an early report suggested that loss of the

135   *clag9* gene resulted in loss of binding to CD36 (22). However, this has been

136   disputed (23), as a subsequent study identified another gene closely linked

137   to the *clag9* gene that is important for CD36 binding (24). Hence, whilst the

138   function of RhopH1/Clag9 remains to be determined, like RhopH1/Clag3.1

139   and RhopH1/Clag 3.2, it is not essential. There are no reports describing a

140   deletion, or attempted deletion, of *clag2* or *clag8*.

141   Much less is known of the function of the RhopH2 and RhopH3

142   components of the complex. There is no report of attempted disruption of

143   the *rhopH2* gene, but the *rhopH3* gene is refractory to deletion in the

144   haploid blood stages (25), suggesting an essential role. Hints that this might

145   include a function in invasion derive from studies showing that antibodies to

146   RhopH3 can inhibit invasion (8,26). However, whether RhopH3 plays other

147   essential roles that involve all forms of the RhopH complex is unknown.

148   Here we use a conditional mutagenesis approach to modify the

149   *rhopH3* gene in a manner that blocks formation of the RhopH complex. The

150   resulting mutant parasites show two distinct phenotypes: a significant

151   decrease in the level of erythrocyte invasion and a complete block in

152   intracellular development at the trophozoite stage. Our findings reveal that

153   RhopH3 and the RhopH complex have essential roles in two distinct stages of

154   the erythrocytic lifecycle.

155

156   **Results**

157   **Efficient conditional truncation of the *rhopH3* gene.** Previous attempts to

158   disrupt the *P. falciparum rhopH3* gene using conventional genetic

159   techniques were unsuccessful (25), suggesting an indispensable role in

160   asexual blood stages. To gain insights into this role we therefore adopted

161   the DiCre conditional recombinase system recently adapted to *P. falciparum*

162   (27) to examine the consequences of functional inactivation of RhopH3. For

163   this, we used Cas9-mediated genome editing (28) to introduce synthetic

164   introns containing *loxP* sites (29) into the *rhopH3* gene such that they

165   flanked an internal region spanning exons 4-6, the region of the gene that

166   shows the highest level of conservation across *Plasmodium rhopH3* orthologs

167   (Figure 1A, Figure 1 – figure supplement 1). This genomic modification was

168   made in the DiCre-expressing *P. falciparum* 1G5DC parasite clone (27) in

169   order that excision of the floxed sequence could be induced by treatment of

170   the transgenic parasites with rapamycin. DiCre-mediated excision was

171   predicted to generate an internally-truncated mutant form of *rhopH3*

172   lacking its most highly conserved region.

173   Successful modification of the *rhopH3* gene in the transfected

174   parasite population following introduction of the targeting vector was

175   confirmed by diagnostic PCR (not shown). Subsequent limiting dilution

176   cloning of the modified parasites resulted in the isolation of parasite clones

177   *rhopH3-loxP* 5F5 and *rhopH3-loxP* 4B11, which were derived from

178   independent transfections using different guide RNAs. Modification of the

179   native *rhopH3* locus in the expected fashion was confirmed in both parasite

180   clones by diagnostic PCR and Southern blot (Figure 1B and Figure 1C). Both

181   clones displayed RhopH3 expression levels and *in vitro* replication rates

182   indistinguishable from the parental 1G5DC parasites (Figure 1 – figure

183   supplement 2), indicating that the modified *rhopH3* gene generated wild

184   type levels of RhopH3 and that the modifications had no impact on parasite

185   viability. The clones were therefore used for all subsequent experiments.

186   To examine the efficiency of conditional excision of the floxed

187   sequence in the *rhopH3-loxP* clones, tightly synchronized ring stage cultures

188   of both clones were divided into two and treated for 4 h with either

189   rapamycin or DMSO (vehicle control). Following washing and further

190   incubation for ~44 h to allow maturation of the parasites to schizont stage

191   (at which peak expression of RhopH3 occurs (8)), genomic DNA from the

192   clones was examined by PCR and Southern blot. This revealed highly

193   efficient excision of the floxed *rhopH3* sequence (Figure 1D and Figure 1E).

194   DiCre-mediated site-specific recombination between the introduced

195   *loxP* sites in the modified *rhopH3* locus of the *rhopH3-loxP* parasites was

196   expected to reconstitute a functional, albeit chimeric, intron. Upon splicing

197   of this intron exons 3 and 7 are placed in frame, producing a modified

198   RhopH3 gene product (called RhopH3Δ4-6) that retains wild type N-terminal

199   and C-terminal segments but lacking residues encoded by exons 4-6.

200   Extracts of the rapamycin-treated and control parasites were analyzed by

201   immunoblot ~44 h following treatment using antibody anti-Ag-44, which

202   recognizes an epitope within the C-terminal segment of RhopH3 encoded by

203   exon 7 (30). As shown in Figure 1F and Figure 1 – figure supplement 3,

204   rapamycin treatment produced the expected change in mass, converting the

205   ~110 kDa wild-type RhopH3 to a ~70 kDa RhopH3Δ4-6 product. This

206   conversion was highly efficient, with no residual full-length protein

207   detected in the rapamycin-treated schizonts. These results confirmed the

208   excision data and demonstrated essentially complete conditional truncation

209   of RhopH3 within a single erythrocytic cycle in the *rhopH3-loxP* parasite

210   clones.

211

212   **Truncation of *rhopH3* leads to mislocalization of other components of the**

213   **RhopH complex.** We next aimed to determine the effects of RhopH3

214   truncation on its subcellular localization within the parasite, as well as on

215   the trafficking of other members of the RhopH complex.

216   Immunofluorescence analysis (IFA) showed that, as expected, RhopH3

217   colocalized with the rhoptry marker RAP2 (31,32) in mature schizonts of

218   control *rhopH3-loxP* parasites (Figure 2A). However, in rapamycin-treated

219 (RhopH3Δ4-6) parasites, this colocalization was lost, although RAP2 was still

220 detected in a punctate, apically-disposed pattern typical of rhoptries

221 (Figure 2A). To determine the effects of this mistrafficking on localization of

222 the other two RhopH complex proteins, control and rapamycin-treated

223 *rhopH3-loxP* parasites were probed with anti-RAP2 as well as either anti-

224 RhopH1/Clag3.1 (11) or anti-RhopH2 antibodies (9). This showed that, as in

225 the case of RhopH3, rhoptry localization of both RhopH1/Clag3.1 and

226 RhopH2 was lost in rapamycin-treated parasites (Figure 2A). These results

227 indicated that truncation of RhopH3 to the RhopH3Δ4-6 form resulted in

228 mistrafficking of at least some components of the RhopH complex. To

229 determine whether the mistrafficked rhoptry proteins all localized to the

230 same parasite compartment, the parasites were co-stained with various

231 combinations of antibodies against two of the three complex proteins. This

232 showed that neither RhopH2 nor RhopH1/Clag3.1 colocalized with

233 RhopH3Δ4-6 in the mutant parasites (Figure 2B). The RhopH2 and

234 RhopH1/Clag3.1 signals were also distinct in the mutant parasites, although

235 in this case some limited colocalization of these proteins was apparent

236 (bottom images, Figure 2B).

237   To better define the fate of the mistrafficked RhopH3Δ4-6 in the

238 mutant parasites, rapamycin-treated mature schizonts were probed with

239 antibodies to the merozoite plasma membrane surface marker MSP1. This

240 indicated that the mutant protein was expressed in a location surrounding

241 (and so likely external to) the plasma membrane of individual segmented

242 intracellular merozoites (Figure 2 – figure supplement 1). In confirmation of

243 this, IFA of naturally released free merozoites showed that the truncated

244   RhopH3Δ4-6 was often largely undetectable in merozoites of the mutant

245   parasites (Figure 2C).

246   One interpretation of these results was that truncation of RhopH3

247   interfered with formation of the RhopH complex. To test this notion, we

248   used a monoclonal antibody (mAb) specific for RhopH2 to

249   immunoprecipitate the complex from extracts of schizonts of *rhopH3-loxP*

250   clone 5F5. As shown in Figure 2D, both RhopH3 and RhopH1/Clag3.1 were

251   precipitated as expected from lysates of control parasites. In contrast,

252   RhopH3Δ4-6 was undetectable in the precipitate from lysates of rapamycin-

253   treated parasites, although RhopH1/Clag3.1 could still be detected. This

254   showed that truncation of RhopH3 ablates its association with RhopH2,

255   although it does not appear to affect the interaction between RhopH2 and

256   RhopH1/Clag3.1. Collectively, these results suggested that truncation of

257   RhopH3 caused mistrafficking of other components of the complex, probably

258   due to loss of the association between RhopH3 and these other proteins.

259

260   **Loss of the RhopH complex is a lethal** event. The above results showed

261   that whilst truncation of RhopH3 affected trafficking of the RhopH complex,

262   it did not prevent schizont development in the erythrocytic growth cycle in

263   which the parasites were treated with rapamycin (henceforth referred to as

264   cycle 1). To evaluate the effects of RhopH3 modification and mistrafficking

265   on longer-term parasite viability, we first exploited a recently developed

266   assay in which parasite replication is assessed in 96-well microplates over a

267   period of 5-7 erythrocytic cycles by visualization of the localized lysis of

268   host erythrocytes in static cultures in 96-well microplates. Under these

269 conditions, successful parasite growth results in formation of

270 microscopically discernible zones of clearance of erythrocytes referred to as

271 plaques (33). As shown in Table 1, in 3 separate assays DMSO-treated

272 *rhopH3-loxP* parasites seeded at ~10 parasites per well produced plaques in

273 nearly every well, with a mean average of ~8 plaques per well for clone 5F5

274 and ~5 plaques per well for clone 4B11 (Table 1). In contrast, in the plates

275 seeded with an identical density of rapamycin-treated parasites, only ~10%

276 of the wells contained plaques and no well contained more than one plaque

277 (Figure 3A, Table 1). Analysis by diagnostic PCR of several parasite clones

278 isolated from individual plaques that appeared in plates seeded with

279 rapamycin-treated parasites revealed that in all cases they derived from

280 parasites that possessed an intact *rhopH3-loxP* gene, indicating that these

281 corresponded to a small subpopulation of parasites in which excision of the

282 floxed sequence had not taken place (Figure 3B). Further analysis by PCR of

283 one of these non-excised clones showed that the DiCre cassette had been

284 lost (Figure 3C) probably due to a genomic rearrangement. This parasite

285 clone (named RhopH3 NE) served as a useful control for subsequent

286 experiments.

287   To further examine the effects of RhopH3 truncation on long-term

288 parasite viability, low parasitaemia cultures of the *rhopH3-loxP* clones 5F5

289 and 4B11 were divided equally into two flasks, treated with either DMSO or

290 rapamycin, then the parasites simply maintained in continuous culture,

291 monitoring increase in parasitaemia at 48 h intervals as well taking samples

292 for analysis by diagnostic PCR. Cultures of the parental 1G5DC parasites as

293 well as the DiCre-defective RhopH3 NE clone were similarly treated and

11

294   monitored in parallel. As shown in Figure 3D, whilst replication of the

295   1G5DC and RhopH3 NE parasites was unaffected by rapamycin treatment,

296   the rapamycin-treated 5F5 and 4B11 clones showed a dramatic decrease in

297   growth rate. However in both clones the appearance of replicating parasites

298   was evident by cycle 3, suggesting that these might correspond to a minor

299   population of normally-replicating non-excised parasites. Diagnostic PCR

300   analysis of the 5F5 culture supported this notion. At the end of cycle 1, PCR

301   using primers that distinguish between the excised and non-excised locus

302   showed the expected highly efficient excision of the floxed *rhopH3-loxP*

303   sequence in the rapamycin-treated culture, with the non-excised locus

304   undetectable. However, periodic examination of the parasites by diagnostic

305   PCR over the ensuing 6 erythrocytic cycles showed a time-dependent

306   increase in the proportion of non-excised parasites in the rapamycin-treated

307   culture, suggesting that the initially undetectable population of non-excised

308   parasites gradually overgrew the cultures. This occurred likely as a result of

309   a selective advantage conferred on them by the replication defect displayed

310   by the RhopH3Δ4-6 parasites. By cycle 5, the excised locus was hardly

311   detectable in the rapamycin-treated culture (Figure 3E). Together with the

312   results of the plaque assay, these results allowed us to conclude that

313   truncation of RhopH3 results in complete loss of long-term parasite viability.

314

315   **Loss of the RhopH complex leads to an invasion defect**. The severe growth

316   defect displayed in the plaque and growth assays could result from an

317   inability of mutant parasites to egress from the host erythrocyte, a block in

318   invasion, or a developmental arrest during intracellular growth. We

319   therefore next investigated the capacity of RhopH3Δ4-6 parasites to undergo

320   egress. For this, we used time-lapse differential interference contrast (DIC)

321   microscopy to observe the egress of merozoites from highly mature,

322   synchronized schizonts at the end of cycle 1 (i.e. ~45 h following treatment

323   of ring-stage *rhopH3-loxP* parasites with rapamycin or DMSO). This revealed

324   no gross differences in the efficiency or morphology of egress

325   (Supplementary video 1), indicating that the absence of the RhopH complex

326   from rhoptries does not affect egress.

327   To investigate a potential invasion phenotype resulting from RhopH3

328   truncation, a synchronized culture of *rhopH3-loxP* parasites at early ring

329   stage was divided into two, treated with either DMSO or rapamycin and then

330   allowed to mature to schizont stage before purifying the mature schizonts

331   and adding them to fresh erythrocytes. After incubation for a further 4 h to

332   allow the *rhopH3-loxP* schizonts to undergo merozoite egress and invasion,

333   the percentage of erythrocytes infected with cycle 2 ring-stage parasites

334   was quantified. The results consistently showed that the ring-stage

335   parasitemia values in cultures derived from the rapamycin-treated *rhopH3-*

336   *loxP* parasites was only ~50% of that in their DMSO-treated counterparts

337   (Figure 3F). Importantly, invasion by the control RhopH3 NE and the

338   parental 1G5DC parasites was unaffected by rapamycin treatment. Taken

339   together with the other results, these data showed that the absence of the

340   RhopH complex from parasite rhoptries significantly affects the ability of

341   the parasite to invade new host cells.

342

13

343 **The RhopH complex is required for intracellular parasite development.**

344 Although the results of the above experiments pointed to a severe invasion

345 defect in parasites lacking the RhopH complex, it was unclear whether this

346 could be sufficient to explain the results of the plaque assay, which

347 indicated a complete lack of long-term viability in the RhopH3Δ4-6 mutants.

348 To explore this further, we examined growth and development of the

349 mutants using microscopic examination of Giemsa-stained cultures. This

350 showed that whereas rapamycin-treated *rhopH3-loxP* parasites appeared

351 morphologically normal at the end of the cycle 1 as well as at the ring stage

352 of cycle 2, development of the mutant parasites stalled at trophozoite stage

353 in cycle 2 (Figure 4A) and the parasites did not develop into schizonts. To

354 confirm this developmental block we used flow cytometry to monitor the

355 DNA content of the parasites in cycle 2. This confirmed that rings derived

356 from rapamycin-treated *rhopH3-loxP* parasites did not increase their DNA

357 content during cycle 2 (Figure 4B), i.e. they did not progress to the

358 multinuclear schizont stage. Taken together, these data indicated that the

359 RhopH complex is essential for the trophozoite to schizont developmental

360 transition of the intracellular parasite.

361

362 **Protein export occurs normally in the RhopH3Δ4-6 mutants.** Export of

363 parasite proteins into the host erythrocyte is important for parasite

364 virulence and for the uptake of nutrients; blocking export prevents

365 modification of the erythrocyte surface with the knob structures that play a

366 role in cytoadhesion, and also prevents development of the parasite beyond

367 the trophozoite stage (34,35). Since we observed a similar growth

368 phenotype in cycle 2 in the RhopH3Δ4-6 parasites, we decided to determine

369 whether the developmental arrest was the result of a generalized defect in

370 protein export. To do this, we examined the subcellular localization of

371 KAHRP and MAHRP1, parasite proteins that are established markers for

372 protein export and Maurer's clefts (intraerythrocytic membranous structures

373 of parasite origin) respectively, in cycle 2 trophozoites derived from

374 rapamycin-treated *rhopH3-loxP* parasites (36,37). This revealed no

375 discernible alterations in protein export and Maurer's cleft formation in the

376 RhopH3Δ4-6 mutants (Figure 5A). This conclusion was corroborated by

377 electron microscopy, which revealed the formation of knobs on the surface

378 of erythrocytes infected with rapamycin-treated *rhopH3-loxP* parasites

379 (Figure 5B). We concluded that protein export from the intracellular

380 parasite can take place normally in the absence of the RhopH complex.

381

382 **Import pathways are defective in *rhopH3* mutant parasites**. The

383 developmental arrest observed in cycle 2 trophozoites of the RhopH3Δ4-6

384 parasites was strikingly reminiscent of the effect of isoleucine starvation on

385 *P. falciparum* (38) Isoleucine is transported into the parasitized cell via the

386 PSAC/NPP, the parasite-induced uptake pathway responsible for enhanced

387 nutrient uptake in parasite-infected erythrocytes (39). The PSAC/NPP is also

388 responsible for the permeability of parasite-infected erythrocytes to the

389 alcohol sugar sorbitol (18), leading to the capacity of sorbitol solutions to

390 mediate osmotic lysis of infected erythrocytes. This lysis can be readily

391 quantified by measuring levels of host cell hemoglobin released following

392 treatment of parasitized cells with a sorbitol solution (40). To determine

393   whether the PSAC/NPP was functional in the RhopH3Δ4-6 mutants, their

394   resistance to sorbitol-mediated lysis was assessed. As shown in Figure 6A,

395   erythrocytes infected with parental 1G5DC parasites or the non-excised

396   RhopH3 NE clone displayed the expected sensitivity to sorbitol, as did

397   erythrocytes infected with control (DMSO-treated) *rhopH3-loxP* parasites. In

398   contrast, erythrocytes infected with cycle 2 rapamycin-treated *rhopH3-loxP*

399   parasites were insensitive to sorbitol; the amount of hemoglobin released

400   upon sorbitol treatment was not significantly different from the amount

401   released by treatment of the infected erythrocytes with an isotonic control

402   buffer (PBS).

403   To further investigate the activity of the PSAC/NPP in the RhopH3Δ4-

404   6 mutants, erythrocytes infected with cycle 2 rings were incubated with 5-

405   aminolevulinic acid (5-ALA). This compound is excluded from uninfected

406   erythrocytes but is taken up by infected erythrocytes and converted to

407   fluorescent protoporphyrin IX (PPIX) (41). Import of 5-ALA has previously

408   been shown to be sensitive to furosemide, a small molecule inhibitor of

409   PSAC/NPP, and is also blocked upon downregulation of parasite export and

410   PSAC/NPP activity in transgenic *P. falciparum* (34,41,42). Import of 5-ALA

411   therefore acts as a convenient reporter for PSAC/NPP activity. Examination

412   by fluorescence microscopy (Figure 6B and 6C) and FACS (Figure 6D) showed

413   that erythrocytes infected with DMSO-treated *rhopH3-loxP* clone 5F5 and

414   4B11 parasites readily took up 5-ALA, whereas no fluorescence was observed

415   in erythrocytes infected with rapamycin-treated *rhopH3-loxP* parasites

416   following incubation with 5-ALA. In contrast, rapamycin-treatment had no

417   effect on the capacity of the parental 1G5DC or DiCre-deficient RhopH3 NE

418   parasites to take up 5-ALA (Figure 6B-D). Combined, these results

419   convincingly indicate that the PSAC/NPP is defective in the *rhopH3* mutants.

420

421   **Discussion**

422   In this study we have shown that RhopH3 plays a central role in the

423   formation of a functional RhopH complex and that mutation of RhopH3

424   results in two severe, but very distinct, phenotypes: 1) a ~50% decrease in

425   host erythrocyte invasion; and 2) a block in the development in the early

426   trophozoite stage of those parasites that do invade. This block in

427   development is a lethal event; no parasites carrying the mutant form of the

428   *rhopH3-loxP* gene were recovered in a plaque assay and parasites with an

429   intact *rhopH3-loxP* gene quickly outgrew the mutant parasites after

430   rapamycin treatment. These results represent the first published evidence

431   that RhopH3 is essential and mark the first time a rhoptry protein has been

432   shown to have two separate, seemingly unrelated functions at different

433   stages of the erythrocytic life cycle. RhopH3 is also the first rhoptry bulb

434   protein to be directly assigned a role in invasion; other rhoptry proteins

435   previously experimentally implicated in invasion are located in the rhoptry

436   neck. The release of rhoptry neck proteins is considered the step at which

437   the parasite commits to host cell entry (43), so the discovery of an

438   important invasion factor that is presumably released later in the invasion

439   pathway is important.

440   Whilst it was surprising that loss of the function of the RhopH

441   complex leads to two different, seemingly unrelated, phenotypes, previous

442   results had hinted at a role for RhopH3 and the complex in both processes.

443 RhopH3, and proteolytic fragments of RhopH3, can bind to erythrocytes and

444 to liposomes (44,45). This appears to occur even in the absence of other

445 members of the RhopH complex, indicating that, for its role in invasion,

446 RhopH3 may not require the function of the other proteins of the complex.

447 Further supporting a role for RhopH3 in invasion is the finding that anti-

448 RhopH3 antibodies can block invasion (26,45). Nonetheless it is curious that

449 ~50% of the parasites still enter the erythrocyte in the absence of full-length

450 RhopH3. RhopH3Δ4-6 may retain sufficient activity in 50% of the parasites to

451 allow invasion to take place. Alternatively, these parasites could use a

452 RhopH3-independent pathway. Invasion by *P. falciparum* relies on several

453 redundant pathways and there is precedent for a partial reduction of

454 invasion by mutant parasites (46). It will be of interest to determine

455 whether invasion pathways that are currently thought to be redundant

456 become essential in the absence of wild type RhopH3. A third possibility is

457 that RhopH3 is involved in a post-invasion process. However, the invasion

458 assay used here would detect all intracellular parasites that have completed

459 invasion. We therefore favor the interpretation that the observed decrease

460 in the number of newly invaded *rhopH3-loxP* parasites indicates that the

461 invasion process is not completed in the mutants.

462   The other phenotype displayed by the RhopH3Δ4-6 mutants, the block

463 in development during the early trophozoite stage, is likely the result of a

464 defect in nutrient intake owing to improper trafficking ablating the function

465 of RhopH1/Clag. The loss of sorbitol sensitivity of erythrocytes infected with

466 the RhopH3Δ4-6 mutants and their impermeability to 5-ALA indicate that

467 RhopH1/Clag3.1 (the RhopH1/Clag3 isotype expressed in these 3D7-derived

468   parasites) and RhopH1/Clag3.2 are not functioning at the erythrocyte

469   surface. RhopH1/Clag3 proteins are transported to the erythrocyte surface

470   in a PEXEL-independent manner (34) and are exposed on the surface of the

471   erythrocyte (19), but the mechanism by which these proteins are

472   transported from the rhoptry, beyond the PVM and to the erythrocyte

473   plasma membrane is unknown. RhopH3 and RhopH2 are have been detected

474   in the PVM immediately after invasion, (17)(47), as well as in the

475   erythrocyte at later developmental stages of the intraerythrocytic parasite

476   (34,48). It is likely that mislocalization of RhopH3Δ4-6 in merozoites

477   prevents the proper, or properly timed, release of the complex during

478   invasion and prevents RhopH1/Clag from being delivered to its correct

479   location. As the RhopH1/Clag3.1 and RhopH1/Clag 3.2 in the trophozoite

480   stage parasite derives entirely from protein that is introduced during

481   invasion (15), mislocalization at the merozoite stage likely cannot be

482   corrected by additional subsequent protein synthesis.

483   The complete arrest in development of the RhopH3Δ4-6 mutants in

484   the cycle following gene modification (cycle 2) also provides insight into the

485   potential roles of the RhopH1/Clag proteins. Little is known about these

486   proteins other than the importance of the RhopH1/Clag3.1 and RhopH1/Clag

487   3.2 proteins in the PSAC (18). However, chemical inhibition of

488   RhopH1/Clag3.2 function and PSAC activity in parasites cultured in rich

489   medium (RPMI 1640, the same medium used in this study) leads to only a

490   small decrease in parasite viability (21). Similarly, parasites that do not

491   produce RhopH1/Clag3.1 or RhopH1/Clag 3.2 have only a minimal growth

492   disadvantage compared to wildtype parasites in a competition assay (13).

493   Parasites lacking RhopH1/Clag9 have no apparent growth phenotype *in vitro*

494   (the gene is absent from the D10 and T9-96 laboratory strains that lack part

495   of chromosome 9) (49,50). In contrast, parasites lacking RhopH1/Clag2 or

496   RhopH1/Clag8 have not been reported so the essentiality of these proteins

497   is unknown. We speculate that the RhopH3Δ4-6 mutants very likely transport

498   none of the RhopH1/Clag proteins to the erythrocyte surface. If so, the

499   observed growth phenotype is therefore essentially that of a disruption of

500   all the *clag* genes. The striking growth phenotype of this mutant is in stark

501   contrast to the mild phenotype of RhopH1/Clag3.2 inhibition (21) or the

502   absence of both the RhopH1/Clag3 proteins (13) when the parasites are

503   grown in RPMI. This may indicate that RhopH1/Clag2 and RhopH1/Clag8 play

504   important roles in nutrient uptake as well, as previously suggested (21), and

505   that RhopH complexes containing several different RhopH1/Clag proteins

506   together mediate the uptake of the nutrients required for parasite growth in

507   the infected erythrocyte. Interestingly, most *Plasmodium* species encode

508   fewer RhopH1/Clag proteins than *P. falciparum*; some species possess only

509   two *clag* genes, comprising a *clag9* orthologue and a second orthologue more

510   closely related to the other *P. falciparum clag* genes (3,10). In conclusion,

511   our results raise the intriguing possibility that, in *P. falciparum*,

512   RhopH1/Clag proteins in addition to RhopH1/Clag3.1 and RhopH1/Clag3.2

513   function in nutrient import.

514   Together the results presented in this study provide new insights into

515   the role of the rhoptry in the malarial blood stages and reveal that rhoptry

516   proteins can function in multiple, distinct processes. They furthermore show

517   that the functions of rhoptry proteins extend beyond the initial invasion of

518   the erythrocyte and can affect parasite growth throughout the blood stage

519   life cycle.

520

521   **Materials and methods**

522   **Reagents and antibodies,** Oligonucleotide primers were from Sigma-Aldrich,

523   as was rapamycin (cat# R0395), which was prepared as a 10 μM stock in

524   DMSO. 5-aminoleuvlinic acid (5-ALA) from Sigma-Aldrich (cat# A3785) was

525   prepared as a 1 mM stock in DMSO. Restriction enzymes were from New

526   England Biolabs. The antifolate drug WR99120 (Jacobus Pharmaceuticals,

527   New Jersey, USA), was stored as a 20 μM stock in DMSO. Polyclonal

528   antiserum α-Ag44, which recognizes the C-terminal 134 amino acid residues

529   of RhopH3 (30), was a kind gift of Ross Coppel (Monash University,

530   Australia). A polyclonal antiserum against *P. falciparum* AMA1 has been

531   previously described (51), as have polyclonal antibodies against *P.*

532   *falciparum* MSP1 and the anti-MSP1 mAb 89.1 (52). Other antibodies were

533   kindly provided by Osamu Kaneko, Nagasaki University Japan (rabbit anti-

534   RhopH1/Clag3.1A), John Vakonakis, University of Oxford UK (rabbit anti-

535   MAHRP1), Ross Coppel at Monash University Australia (anti-KAHRP), Tony

536   Holder, the Francis Crick Institute UK (anti-RhopH2 mAb 61.3). Monoclonal

537   antibody 7.7 (anti-EXP2) was from The European Malaria Reagent

538   Repository, contributed by Jana McBride and the mouse anti-RAP2 mAb

539   MRA-876 was obtained from BEI resources, National Institute of Allergy and

540   Infectious Disease (NIAID), National Institutes of Health (NIH), contributed

541   by Allan Saul. Use of these antibodies in immunoblot and IFA analyses have

542   been described elsewhere (9,11,30,51,53-56).

543

P. falciparum **culture, transfection and growth analysis.** All *P. falciparum*

transgenesis work described used the 3D7-derived DiCre-expressing clone

1G5DiCre (27), here referred to as 1G5DC. Asexual blood stage parasites

were continuously cultured in RPMI 1640 medium containing Albumax

(Gibco) as a serum substitute and synchronised using established procedures

(57). For introduction of transfection plasmids, mature schizonts were

enriched using Percoll (GE Healthcare) and electroporated using an Amaxa

4D electroporator and P3 Primary cell 4D Nucleofector X Kit L (Lonza) using

programme FP158 as described (27).

Long-term parasite growth as measured by plaque-forming ability was

determined by diluting trophozoite-stage cultures to a density of 10

parasites per well in complete medium with human erythrocytes at a

haematocrit of 0.75% and plating 200 μL of this suspension into flat

bottomed 96 well microplates, as previously described (33). Plates were

incubated for 10 days in gassed humidified sealed modular chambers before

plaque formation was assessed by microscopic examination using a Nikon

TMS inverted microscope (40x magnification) and documented using a

Perfection V750 Pro scanner (Epson).

Growth characteristics of mutant parasites were determined by

microscopy of Giemsa-stained thin films. Long-term growth was also

measured using flow cytometry of hydroethidine-stained trophozoite-stage

parasites, as described (58). Cultures adjusted to a parasitaemia of 0.1%

were monitored every 48 h for up to 7 intraerythrocytic cycles. All

experiments were carried out in triplicate, data analysed using GraphPad

568 Prism and presented as the mean ± standard error of the mean (SE). In

569 addition, cells 92 h post-rapamycin treatment were fixed in 4%

570 formaldehyde and 0.1% glutaraldehyde and stained with 2 μM Hoechst 33342

571 (Invitrogen) before detection of the Hoechst emission (a measure of DNA

572 replication) by the 355 nm laser of a LSR II (BD Biosciences), through a

573 440/40 nm bandpass filter. Doublet cells were excluded using a FCS-A versus

574 FCS-H display. Samples were analysed using FlowJo software.

575 Erythrocyte invasion assays were carried out using a modification of a

576 method described previously (59). Highly synchronous, mature schizonts of

577 the parasite clones under examination were enriched by centrifugation over

578 Percoll cushions then added at a parasitaemia of 1% to fresh erythrocytes .

579 After incubation for 4 h, parasites were stained with SYBR Green-I and the

580 percentage of newly ring-infected erythrocytes was determined by flow

581 cytometry using a BD FACS Calibur flow cytometer (BD Biosciences). SYBR

582 Green-I was excited by a 488 nm 20 mW blue laser and detected by a

583 530/30 filter. BD CellQuest Pro (BD Biosciences, UK) was used to collect

584 100,000 events per sample. Experiments were done in triplicate, data

585 analysed using GraphPad Prism and presented as the mean ± SE.

586

587 **Generation of *rhopH3-loxP* parasites and conditional RhopH3 truncation.**

588 Parasites harbouring a floxed segment of the genomic *rhopH3* gene were

589 generated by Cas9-mediated replacement of *rhopH3* endogenous introns 3

590 and 6 as well as the intervening sequence. The repair plasmid, called

591 pESS_R3_E46_loxP (synthesised by GENEWIZ) comprised synthetic

592 heterologous *loxP*-containing *SERA2* and *sub2* introns(29) flanking a

593 recodonized form of *rhopH3* exons 4-6. The complete native sequences of

594 exons 3 and 7 were included on either side of this central module to act as

595 flanking regions for homology-directed repair. Protospacer Workbench(60)

596 was used to identify 20 bp protospacer sequences specifically targeting

597 *rhopH3*. To generate pSgRNA plasmids expressing suitable sgRNAs, InFusion-

598 based cloning (Clontech) was used to replace the BtgZI adaptor sequence of

599 pL6-X(28) with annealed oligos encoding a sgRNA targeting *rhopH3* exon 4

600 (RHOPH3_sgRNA_E4_F and RHOPH3_sgRNA_E4_R, generating pSgRNA1), 5

601 (RHOPH3_sgRNA_E5_F and RHOPH3_sgRNA_E5_R, generating pSgRNA2) or 6

602 (RHOPH3_sgRNA_E6_F and RHOPH3_sgRNA_E6_R, generating pSgRNA3) (see

603 Table 2 for sequences of all oligonucleotide primers used in this study).

604 Schizonts of *P. falciparum* clone 1G5DC were transfected with 20 µg Cas9-

605 expressing pUF(28), 20 µg pESS_R3_E46_loxP repair plasmid and 8 µg of

606 sgRNA-containing pSgRNA1, pSgRNA2 or pSgRNA3. Twenty-four hours post-

607 transfection, the electroporated parasites were treated with 2.5 nM

608 WR99210 for 48 h to select for transfectants harbouring pUF1 before

609 returning the cultures to medium without drug. Integrant parasites generally

610 reached parasitaemia levels suitable for cryopreservation within 2-5 weeks.

611   Detection of integration of pESS_R3_E46_loxP in the parasite

612 population was performed by diagnostic PCR using primers

613 RHOPH3_exon2_F1 plus RHOPH3_exon5_WT_R (producing a product specific

614 to the wild type *rhopH3* locus), or RHOPH3_exon2_F1 plus RHOPH3_exon4-

615 6rec_R and RHOPH3_exon4-6rec_F plus RHOPH3_3UTR_R (producing

616 products specific to the *rhopH3-loxP* modified locus). Integrant parasite

617 clones *rhopH3-loxP* 5F5 and *rhopH3-loxP* 4B11 were then isolated by limiting

618   dilution. Persistence of the integrated DiCre locus in these clones was

619   confirmed by PCR analysis using primers +27 plus -11 producing a 1900 bp

620   product specific to the integrated DiCre cassette in 1G5DC parasites, or +27

621   plus -25 producing an amplicon of 1700 bp specific to the unmodified *SERA5*

622   locus.

623   Recombination between the *loxP* sites was induced in tightly

624   synchronised ring-stages of parasite clones *rhopH3-loxP* 5F5 and *rhopH3-*

625   *loxP* 4B11 by incubation for 4 h in the presence of 100 nM rapamycin in 1%

626   (v/v) DMSO; mock treatment was with 1% (v/v) DMSO only (vehicle control).

627   DiCre-mediated excision of the floxed *rhopH3* exons 4-6 was detected by

628   PCR analysis of schizont stage genomic DNA (harvested ~40 h following mock

629   or rapamycin treatment) using primers RHOPH3_exon2_F1 and

630   RHOPH3_exon7_R. Truncation of RhopH3 was evaluated by immunoblot

631   analysis of SDS extracts of mature Percoll-enriched schizonts, probing with

632   anti-Ag44 antibodies (or anti-AMA1 as a loading control) followed by

633   horseradish-peroxidase secondary antibodies as described(61).

634

635   **Southern blot**. For Southern blot analysis, a 738 bp probe corresponding to

636   part of *rhopH3* exon 3 was produced by PCR amplification from *P.*

637   *falciparum* IG5DC genomic DNA with primers RHOPH3_exon3_SB_F and

638   RHOPH3_exon3_SB_R (Table 2). Probe radiolabelling and hybridisation to

639   SacI/BsgI/XmnI-digested gDNA from clones of interest was as previously

640   described (62).

641

642 **Immunoprecipitation and immunoblot analysis**. For analysis of RhopH

643 complex formation, mature Percoll-enriched schizont-stage parasites (42 h

644 post rapamycin or mock treatment) were harvested and stored at -80˚C.

645 Frozen parasite pellets were thawed into 100 µL NP-40 lysis buffer (1%

646 Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCL, 5 mM EDTA, 5 mM EGTA pH

647 8.0) containing a complete protease inhibitor cocktail (Roche). Samples

648 were clarified by centrifugation at 14,000 rpm at 4˚C and the supernatant

649 passed through a 0.22 µm cellulose acetate Spin-X centrifuge tube filter

650 (Corning). 100 µL Protein G-Sepharose beads (Abcam) were added to the

651 resulting supernatant and pre-clearing carried out at 4°C overnight.

652 Following addition of mAb 61.3 (9), samples were incubated at 4°C overnight

653 before antigen-antibody complexes were precipitated using Protein G-

654 Sepharose beads overnight at 4˚C. The beads were washed five times in

655 wash buffer I (50 mM Tris-HCl pH 8.2, 5 mM EDTA, 0.5% Nonidet P-40, 1

656 mg/mL bovine serum albumin, 0.5 M NaCl) and twice in wash buffer II (50

657 mM Tris-HCl pH 8.2, 5 mM EDTA, 0.5% Nonidet P-40) before antigen-antibody

658 complexes were eluted using NuPAGE LDS Sample Buffer (Life Technologies)

659 and proteins resolved using precast NuPAGE Novex 3-8% Tris-Acetate protein

660 gels (Life Technologies). Following electrophoresis, samples were evaluated

661 by immunoblot analysis probing with anti-Ag44 or anti-RhopH1/Clag3.1A

662 antibodies followed by horseradish-peroxidase secondary antibodies as

663 described (61).

664

665 **Immunofluorescence microscopy**. Immunofluorescence microscopy was

666 performed on formaldehyde-fixed thin blood smears, permeabilised with

26

667   0.1% (v/v) Triton X-100. Monoclonal anti-RAP2 (MRA-876), directly labeled

668   with Alexa Fluor 594 using the Alexa Fluor 594 Antibody Labelling Kit (Life

669   Technologies), was used at a dilution of 1:300. Samples were probed with

670   primary antibodies used at the following dilutions: anti-Ag44 (1:2000), mAb

671   61.3 (1:100), anti-CL3.1A (1:100), anti-MAHRPI (1:2000), anti-KAHRP

672   (1:250), rabbit anti-MSP1 (1:1000), mAb 89.1 (1:1000), and mAb 7.7 (1:100).

673   Bound primary antibodies were detected using Alexa Fluor 488-, 566- or 594-

674   conjugated anti-rabbit or anti-mouse secondary antibodies (Life

675   Technologies), diluted 1:8000. Slides were mounted in ProLong Gold

676   Antifade Mountant with DAPI (Life Technologies) and trophozoite images

677   captured using a Nikon Eclipse Ni-E widefield microscope with a

678   100x/1.45NA objective and a Hamamatsu C11440 digital camera. Schizont

679   stage images were captured with a Zeiss LSM 880 using a 63x/1.4 NA

680   objective equipped with an Airyscan detector to improve the optical

681   resolution of the scanned images. The DAPI, Alexa Fluor 488 and Alexa Fluor

682   594 channels were imaged sequentially over the axial dimension and

683   processed using the integrated Zeiss software to enhance the optical

684   resolution isometrically ~1.8 fold. All images were processed using either

685   the Zen 2012 or FIJI software packages. For display purposes, linear

686   adjustments were made to the intensity scale of each channel to equalize

687   the intensity output to enhance areas of co-localization. Relative intensities

688   between samples are not comparable.

689

690   **Transmission electron microscopy**. Parasite cultures 92 h following

691   rapamycin (or mock) treatment were fixed at 37ºC in 8% formaldehyde in

692   0.2 M phosphate buffer pH 7.4 (PB) for 15 min by adding 1 volume of

693   fixative solution to 1 volume of culture. The cells were pelleted, then

694   further incubated in 2.5% glutaraldehyde, 4% formaldehyde in 0.1 M PB at

695   room temperature for a further 30 min. Cells were washed in 0.1 M PB

696   before being embedded in 4% (w/v) low-melting point agarose in distilled

697   water. The agarose-embedded samples were cut into 1 mm^3 blocks, post-

698   fixed in 1% (w/v) OsO_4 and 1.5% (w/v) potassium ferrocyanide for 60 min at

699   4ºC then incubated sequentially in 1% (w/v) tannic acid in 0.05 M PB for 45

700   min and 1% (w/v) sodium sulphate in 0.05 M PB for 5 min. The samples were

701   washed in water and dehydrated through a graded series of ethanol before

702   being embedded in Epon resin (Taab 812). Blocks were trimmed and

703   ultrathin 70 nm sections cut using a diamond knife on a UC6 Ultramicrotome

704   (Leica Microsystems), picked up on 150 hexagonal mesh copper grids and

705   post stained with lead citrate before being imaged using a Tecnai G2 Spirit

706   120 kV transmission electron microscope (FEI Company) with an Orius

707   camera (Gatan Inc.).

708

709   **Analysis of erythrocyte membrane permeability**. Sorbitol sensitivity of

710   parasites was determined 72 h following rapamycin or DMSO treatment (i.e.

711   in cycle 2). Cultures at equal parasitaemia were incubated in osmotic lysis

712   buffer (280 mM sorbitol, 20 mM Na-HEPES, 0.1 mg/mL BSA, pH 7.4) for 7

713   min, then hemoglobin release determined by measuring the absorbance of

714   the cell supernatants at 405 nm, as previously described(63,64).

715   5-ALA uptake was determined by incubating cultures of synchronous

716   cycle 2 ring-stage parasites overnight in phenol red-free RPMI 1640 medium

717   (K-D Medical) supplemented with 200 μM 5-ALA. Just prior to analysis,

718   parasite nuclei were stained by treatment with 2 μM Hoechst. PPIX and

719   Hoechst fluorescence were captured using a Zeiss LSM 880 equipped with a

720   63x/1.4 NA objective in standard confocal detection mode. Images were

721   captured with the same acquisition setting so that measurements of

722   intensity are directly comparable. Co-occurrence of PPIX and Hoechst was

723   quantified using the MetaMorph software "Cell Scoring" application. Cells

724   were also analyzed on a SORP LSRFortessa, detecting PPIX emission with a

725   532 nm laser through a 605/40 nm bandpass filter and Hoechst emission

726   with the 406 nm laser through a 440/40 nm bandpass filter. Erythrocyte

727   doublets were excluded using a FCS-A versus FCS-H display and data

728   analyzed by BD FACSDiva software.

729

730   **Time-lapse video microscopy**. *P. falciparum* egress was imaged as

731   previously described (27,65), using 1 μM (4-[7-[(dimethylamino)methyl]-2-(4-

732   fluorphenyl)imidazo[1,2-*a*]pyridine-3-yl]pyrimidin-2-amine (compound 2) to

733   tightly synchronise egress. Following removal of compound 2 by washing,

734   parasites were suspended in fresh pre-warmed medium and introduced into

735   a pre-warmed microscopy chamber on a temperature controlled microscope

736   stage at 37°C. Beginning 6 min after washing off the compound 2, DIC

737   images were collected at 5 s intervals for 30 min using a Nikon Eclipse Ni

738   Microscope fitted with a Hamamatsu C11440 digital camera and converted

739   to QuickTime movies using Nikon NIS-Elements software.

740

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Author contributions

1040 E.S.S., M.J.B. and C.v.O. conceived and designed the experiments. E.S.S.

1041 performed the experiments and analysed the data. E.K. designed the

1042 Southern blotting strategy and J.A.B. assisted with image acquisition.

1043 L.H.M., M.J.B. and C.v.O. supervised the project. E.S.S., M.J.B. and C.v.O.

1044 wrote the manuscript.

1045

1046 **Competing financial interest**

1047 The authors declare no competing financial interest.

1048

1049 **Corresponding authors**

1050 Correspondence and requests for materials should be addressed to M.J.B.

1051 (Mike.Blackman@crick.ac.uk) or C.v.O. (Christiaan.vanOoij@crick.ac.uk)

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1059 Table 1. Conditional truncation of RhopH3 results in decreased parasite
1060 survival as determined by plaque assay.
1061

[a] Plaque assay no.	Treatment	[b] Proportion of wells containing plaques (%)	Mean number of plaques/well
1 (clone 5F5)	DMSO	98.88	7.7
	Rapamycin	10.56	0.11
2 (clone 5F5)	DMSO	100	9.1
	Rapamycin	8.89	0.09
3 (clone 4B11)	DMSO	99.44	5.24
	Rapamycin	9.44	0.1

1062
1063 [a] Three independent plaque assays were set up on different days.
1064 [b] A total of 180 wells were used for each treatment (+/-RAP) in each assay.
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1072 **Table 2. Oligonucleotide primers used in this study.** Guide sequences
1073 shown in bold.
1074

Primer name	Sequence (5'-3')
RHOPH3_sgRNA_E4_F*	taagtatataatatt**TTCTTCGTTTTTAAAAAAAG**gttttagagctagaa
RHOPH3_sgRNA_E4_R*	ttctagctctaaaac**CTTTTTTTAAAAACGAAGAA**aatattatatactta
RHOPH3_sgRNA_E5_F*	taagtatataatatt**CACCGATTTTAGCTTTAAAG**gttttagagctagaa
RHOPH3_sgRNA_E5_R*	ttctagctctaaaac**CTTTAAAGCTAAAATCGGTG**aatattatatactta
RHOPH3_sgRNA_E6_F*	taagtatataatatt**ACATTCTTATCATTATATTT**gttttagagctagaa
RHOPH3_sgRNA_E6_R*	ttctagctctaaaac**ACATTCTTATCATTATATTT**aatattatatactta
RHOPH3_exon2_F1	AGGAAATGGCCCAGACGC
RHOPH3_exon5_WT_R	TCTTTAAAGCTAAAATCGGTGATATTATGGCTC
RHOPH3_exon4-6rec_R	CAGGAAGTTACCTTTCAGCAGGG
RHOPH3_exon4-6rec_F	CCCTGCTGAAAGGTAACTTCCTG
RHOPH3_3UTR_R	CGAATATGTAATCAGTTGTATTTTTTCTCTAAAAGTTCATAG
+27	CAATATCATTTGAATCAAACAGTGGT
-11	CTTTGCCATCCAGGCTGTTC
-25	CCATTGGACTAGAACCTTCAT
RHOPH3_exon7_R	CATAAAGAACGTCTTGTTTTCTGTATCCAATACC
RHOPH3_exon3_SB_F	CAAATATGCTATATGTGTAGGTACTCAATTTAAC
RHOPH3_exon3_SB_R	CATATAACTTTGGAGATGTAGAACCACAAGG

1075

1076 **Figure legends**

1077 **Figure 1** Conditional truncation of the *rhopH3* gene. A) The *rhopH3* gene

1078 comprises seven exons (numbered grey boxes) and six introns (blue lines).

1079 Using Cas9-mediated recombination, the region spanning introns 3 through 6

1080 was replaced with two *loxP*-containing (purple open arrowhead) *P.*

1081 *falciparum* introns (*SERA2* (orange line) and *sub2* (green line)) flanking a

1082 recodonized and fused version of exons 4 through 6 (exon 4-6, green box).

1083 Integration of this sequence by homologous recombination was promoted by

1084 the addition sequences of exon 3 and 7 to either side of the introns. Colored

1085 arrowheads, primer binding sites. B, S and X, BsgI, SacI and XmnI restriction

1086 sites. Dotted line, probe used for Southern blotting. Rapamycin-induced

1087 site-specific recombination between the *loxP* sites removes the recodonized

1088 exon 4-6. B) PCR analysis of *rhopH3-loxP* clones 5F5 and 4B11 confirms the

1089 expected gene modification event. Genomic DNA from parental 1G5DC (WT)

1090 parasites or the clones was used as template for PCR using the indicated

1091 primers (see panel A). Numbers between the arrowheads indicate the

1092 expected size of the amplicon. C) Southern blot analysis of parental 1G5DC

1093 (WT) and the *rhopH3-loxP* parasite clones confirms the expected

1094 modification of the *rhopH3* locus. Genomic DNA was digested with BsgI, SacI

1095 and XmnI and hybridized with a radiolabeled probe that binds to part of

1096 exon 3 (dotted line in panel A). Expected fragment sizes are 3016 bp for the

1097 WT *rhopH3* locus and 3349 bp for the *rhopH3-loxP* locus. D) Efficient

1098 rapamycin-induced truncation of the *rhopH3* gene. Clones *rhopH3-loxP* 5F5

1099 and 4B11 were analyzed by PCR ~44 h after treatment with DMSO (D) or

1100 rapamycin (R) using the indicated primers (see panel A). Excision decreases

1101 the amplicon from 2760 bp to 1755 bp. E) Southern blot showing efficient

1102 rapamycin-induced truncation of the *rhopH3* gene. Genomic DNA extracted

1103 from control or rapamycin-treated *rhopH3-loxP* clones 5F5 and 4B11 was

1104 digested and probed as described in panel C. Expected fragment sizes are

1105 3349 bp for the non-excised locus and 4784 bp for the excised locus. F)

1106 Immunoblot analysis of mature schizonts of *rhopH3-loxP* clone 5F5,

1107 examined ~44 h following treatment at ring stage with DMSO (D) or

1108 rapamycin (R). The blots were probed with an antibody against RhopH3 (left

1109 panel) or the merozoite protein AMA1 (right panel) as a loading control. The

1110 expected molecular masses of WT RhopH3 and RhopH3Δ4-6 are 110 kDa and

1111 ~70 kDa, respectively. In panels B-F, positions of relevant molecular mass

1112 markers are indicated.

1113

1114 **Figur**e 2 Truncation of RhopH3 leads to mistrafficking of components of the

1115 RhopH complex and loss of complex formation. A) IFA showing colocalization

1116 of RhopH3, RhopH2 and RhopH1/Clag3.1 with the rhoptry marker RAP2 in

1117 schizonts of control (DMSO) *rhopH3-loxP* parasites but loss of colocalization

1118 following rapamycin (Rapa) treatment. Parasite nuclei were visualized by

1119 staining with 4,6-diamidino-2-phenylindole (DAPI). Scale bar, 5 μm. B)

1120 Colocalization of the members of the RhopH complex. RhopH3, RhopH2 and

1121 RhopH1/Clag3.1 colocalize in *rhopH3-loxP* parasites treated with DMSO, but

1122 this colocalization is lost in parasites treated with rapamycin. C)

1123 Mislocalisation and reduced levels of RhopH3 in naturally released free

1124 merozoites of *rhopH3-loxP* parasites treated with rapamycin. Samples were

1125 probed with a monoclonal antibody to the merozoite surface marker MSP1

1126　as well as anti-RhopH3 antibodies. Scale bar, 2 μm D) Immunoprecipitation

1127　reveals disruption of the RhopH complex in rapamycin-treated *rhopH3-loxP*

1128　parasites. RhopH2 was immunoprecipitated from extracts of control or

1129　rapamycin-treated *rhopH3-loxP* parasites. Subsequent immunoblotting with

1130　antibodies against RhopH3 or RhopH1/Clag3.1 revealed the absence of

1131　RhopH3 from the immunoprecipitates derived from the rapamycin-treated

1132　parasites, although RhopH2 and RhopH1/Clag3.1 still showed association.

1133　Arrowheads indicate the expected position of migration of the full-length

1134　(WT) and truncated RhopH3, and RhopH1/Clag3.1. The *rhoph3-loxP* clone

1135　5F5 was used throughout for these experiments.

1136

1137　**Figur**e 3 Loss of long-term viability in parasites lacking the RhopH complex.

1138　A) Representative wells seeded with identical concentrations (10 parasitised

1139　cells/well) of DMSO-treated or rapamycin-treated *rhopH3-loxP* clone 5F5

1140　parasites, showing formation of plaques only in the wells seeded with DMSO-

1141　treated parasites. Two of the plaques are indicated by white arrowheads. B)

1142　PCR analysis of the *rhopH3-loxP* locus in the small number of clones isolated

1143　from wells seeded with rapamycin-treated *rhopH3-loxP* parasites. The size

1144　of the PCR product indicates excision of the floxed sequence had not taken

1145　place in these seven clones (numbered 1-7), whereas rapamycin induced

1146　efficient excision in the parent 5F5 clone (left-hand two tracks). For PCR

1147　strategy, see Figure 1D. C) PCR analysis of the modified *SERA5* locus in

1148　parasite clone RhopH3 NE, showing loss of the DiCre cassette in this clone.

1149　D) Growth curves showing replication of parasites of the indicated clones

1150　over the course of 5 erythrocytic cycles. Data were averaged from 3

1151 biological replicate experiments and presented as the mean ± standard error

1152 of the mean. E) Non-excised parasites quickly outgrow RhopH3Δ4-6

1153 parasites. The relative abundance of parasites harboring the excised or

1154 intact *rhopH3-loxP* locus in a population of rapamycin-treated *rhopH3-loxP*

1155 clone 5F5 parasites was determined by diagnostic PCR over the course of 7

1156 erythrocytic growth cycles (indicated, where cycle 1 indicates that in which

1157 treatment occurred). F) Decreased erythrocyte invasion by rapamycin-

1158 treated *rhopH3-loxP* parasites. Parasites of the indicated clones were

1159 treated with DMSO or rapamycin and allowed to invade fresh erythrocytes.

1160 Ring-stage parasitemia levels were determined 4 h later. Data were

1161 averaged from 3 biological replicate experiments. Error bars depict standard

1162 error of the mean. Statistical significance was determined by a two-tailed t-

1163 test where $p \leq 0.0001$ (indicated by asterisks) and $p > 0.05$, non-significant

1164 (ns).

1165

1166 **Figur**e 4 Loss of the RhopH complex results in developmental arrest. A)

1167 Developmental block in rapamycin-treated *rhopH3-loxP* parasites. Giemsa-

1168 stained images showing intracellular development of DMSO-treated and

1169 rapamycin-treated *rhopH3-loxP* clone 5F5 parasites from the end of cycle 1

1170 to the end of cycle 2. A clear developmental block was evident in the

1171 rapamycin-treated parasites in cycle 2. The number of hours following the

1172 beginning of cycle 1 is indicated, as well as its relation to the time point of

1173 rapamycin treatment (indicated in the schematic timeline). B) Flow

1174 cytometry analysis of DMSO-treated and rapamycin-treated *rhopH3-loxP*

1175 clones 5F5 and 4B11. Analysis was performed at the end of the cycle 2 (92 h

1176 after rapamycin-treatment). The intensity of Hoechst 33342 staining

1177 provides a measure of the DNA content of the parasites, reflecting parasite

1178 development.

1179

1180 **Figur**e 5 Loss of the RhopH complex does not ablate parasite protein export.

1181 Cycle 2 (72 h post rapamycin treatment) DMSO-treated and rapamycin-

1182 treated *rhopH3-loxP* clone 5F5 trophozoite-stage parasites were probed with

1183 antibodies against the parasitophorous vacuole membrane marker EXP2 to

1184 delineate the parasite in the infected erythrocyte, as well as antibodies

1185 specific for either the Maurer's cleft marker MAHRP1 (top panels) or the

1186 export marker KAHRP (bottom panels). Scale bar, 5 µm. B) Transmission

1187 electron micrograph showing a comparison between cycle 2 parasites of

1188 DMSO-treated or rapamycin-treated *rhopH3-loxP* clone 5F5 parasites ~ 92 h

1189 following rapamycin treatment. The developmental block in the RhopH3Δ4-6

1190 parasite is clearly evident, as is the presence of knobs (arrowed) on the

1191 surface of the erythrocyte in both cases. Components of the mutant parasite

1192 labelled are the digestive vacuole (DV), haemozoin (H), nucleus (N),

1193 parasitophorous vacuole membrane (PVM), cytostomes (C) and parasite

1194 plasma membrane (PPM). The mutant parasites displayed no obvious

1195 ultrastructural differences from wild-type trophozoites at a similar

1196 developmental stage (not shown). Scale bar, 1 µm

1197

1198 **Figur**e 6 Loss of the RhopH complex results in reduced sorbitol sensitivity

1199 and reduced uptake of exogenous small molecules. A) Synchronous cycle 2

1200 parasites of the indicated clone (parasitaemia ~5%) treated 72 h previously

1201 with DMSO or rapamycin in cycle 1 were suspended in osmotic lysis buffer

1202 containing 280 mM sorbitol or in PBS, and the resulting cell lysis determined

1203 by measuring the absorbance of the supernatant at 405 nm. An equal

1204 volume of parasite culture was lysed in 0.15% (w/v) saponin to give a value

1205 for 100% lysis and all other absorbance values normalized to this. Data were

1206 averaged from 3 biological replicate experiments. Statistical significance

1207 was determined by a two-tailed t-test; significance levels are indicated:

1208 $p \leq 0.001$, ***; $p \leq 0.01$, **; $p \leq 0.05$, *; and $p > 0.05$, non-significant (ns). B)

1209 Uptake of 5-ALA by erythrocytes infected with either DMSO-treated or

1210 rapamycin-treated *rhopH3-loxP* clone 5F5 parasites at cycle 2. Cultures

1211 were incubated overnight with 200 µM 5-ALA and uptake of the compound

1212 and its subsequent conversation to PPIX in infected erythrocytes visualized

1213 by fluorescence microscopy. Infected erythrocytes were visualized by

1214 staining with Hoechst 33342. Top panels show fields of view containing

1215 multiple infected erythrocytes of the indicated strain. Scale bar, 50 µm

1216 Bottom panels show individual infected erythrocytes. Scale bar, 5 µm. C)

1217 Quantitation of the levels of uptake of 5-ALA by infected erythrocytes. For

1218 each condition, a total of 1300 Hoechst-positive cells were analyzed for

1219 intensity of PPIX fluorescence using MetaMorph (Molecular Devices) and a

1220 statistical significance was determined by a two-tailed t-test. Significance

1221 levels are indicated: $p \leq 0.0001$, **** and $p > 0.05$, non-significant (ns). D) FACS

1222 analysis of 5-ALA-treated parasites. Uptake of 5-ALA and its subsequent

1223 conversation to PPIX in cycle 2 parasites following treatment in cycle 1 with

1224 rapamycin or DMSO was determined by flow cytometry of Hoechst stained

1225 parasites. Gating was applied to distinguish Hoechst negative cells (red

1226   population), Hoechst positive/PPIX negative cells (green population) and

1227   Hoechst positive/PPIX-positive cells (purple population). For the 1G5DC

1228   parental and RhopH3-loxP NE parasite clones, most of the parasites were

1229   positive for both Hoechst and PPIX fluorescence regardless of their

1230   treatment with rapamycin or DMSO. In contrast, for rapamycin-treated

1231   *rhopH3-loxP* clones 5F5 and 4B11, most of the parasites were Hoechst

1232   positive/PPIX negative indicating a defect in 5-ALA uptake.

1233
1234
1235

1236 **Supplemental figure legends.**

1237

1238 **Figure 1 – figure supplement 1.**Multiple alignment of predicted primary

1239 sequences of *rhopH3* orthologues from *P. falciparum* (PF3D7_0905400), *P.*

1240 *chabaudi* (PCHAS_0416900) and *P. vivax* (PVX_098712). The portion of the

1241 protein encoded by exon 4-6 in the *P. falciparum* orthologue is underlined.

1242 Note that this region includes some of the most highly conserved regions of

1243 the protein. Sequence data were obtained from PlasmoDB (1) and aligned

1244 using Clustal Omega (2). '*'indicates positions of identity, ':' indicates

1245 conservation of residues with strongly similar chemical properties and '.'

1246 indicates conservation of residues of weakly similar properties.

1247

1248 **Figure 1 – figure supplement 2.** Modification (floxing) of the *rhoph3* gene

1249 does not impact on gene expression or parasite growth. A) Immunoblot

1250 analysis of untreated mature schizonts of *rhopH3-loxP* clones 5F5 and 4B11,

1251 as well as the RhopH3 NE clone and the parental 1G5DC parasites. The blots

1252 were probed with an antibody against RhopH3 (top), RhopH1/Clag3.1

1253 (middle) or the merozoite protein EBA175 (3) (bottom) as a loading control.

1254 B) Growth curves showing similar replication rates of parasites of the

1255 indicated clones (not treated with rapamycin) over the course of 4

1256 erythrocytic cycles. Data were averaged from 3 biological replicate

1257 experiments and presented as the mean ± standard error of the mean.

1258 Linear regression analysis showed that all the slopes fall within the same

1259 95% confidence interval range.

1260

1261 **Figure 1 – figure supplement 3**. Conditional truncation of RhopH3 in both

1262 the 5F5 and 4B11 *rhopH3-loxP* clones. Immunoblot analysis of mature

1263 schizonts of the indicated clones ~44 h following treatment at ring stage

1264 with DMSO (D) or rapamycin (R). The blots were probed with an antibody

1265 against RhopH3 (top) or the mAb 89.1 against the merozoite surface protein

1266 MSP1 (bottom) as a loading control. The expected molecular masses of WT

1267 RhopH3 and RhopH3Δ4-6 are 110 kDa and ~70 kDa, respectively. Positions of

1268 relevant molecular mass markers are indicated.

1269

1270 **Figure 2 – figure supplement 1**. Truncation of RhopH3 leads to

1271 mistrafficking of components of the RhopH complex. IFA of mature schizonts

1272 of control (DMSO) and rapamycin-treated rhopH3-loxP parasites, probed

1273 with MSP1-specific antibodies (either mAb 89.1 or rabbit polyclonal anti-

1274 MSP1 antibodies; red) and antibodies to the three indicated RhopH

1275 components (green). Mis-localisation of the RhopH proteins was observed in

1276 all cases, and in the case of RhopH3 the protein often appeared to reside

1277 external to the plasma membrane of intracellular merozoites. Parasite

1278 nuclei were visualized by staining with DAPI. Note that, for clarity, the

1279 merge panels do not include the DAPI signal. Scale bar, 5 μm.

1280

1281 **Supplementary figure references**

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1296

1297

1298 **Supplementary Video 1** Parasite egress is unaffected by loss of the RhopH

1299 complex. Synchronized parasites of *rhopH3-loxP* clone 4B11 were treated

1300 with DMSO or rapamycin at ring stage, then allowed to mature to schizont

1301 stage and further synchronised by incubation for 3-5 h in the presence of 1

1302 µM (4-[7-[(dimethylamino)methyl]-2-(4-fluorphenyl)imidazo[1,2-*a*]pyridine-

1303 3-yl]pyrimidin-2-amine (compound 2), which reversibly stalls egress. Egress

1304 of the parasites was then monitored by time-lapse DIC video microscopy

1305 following removal of the compound 2, as described previously[1]. DMSO-

1306 treated samples are shown on the left, rapamycin-treated are samples

1307 shown on the right.

1308

1309 **Supplementary video reference**
1310
1311 1.   Das, S., Hertrich, N., Perrin, A. J., Withers-Martinez, C., Collins, C.
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1317

A

Native *rhopH3* locus

Targeting region

Integration

Floxed *rhopH3* locus

Rapamycin-induced recombination

Excised *rhopH3* locus

B

—1939 bp— —1979 bp— —1694 bp—

C Southern blot

D

Unexcised ►——2760 bp——◄
Excised ►——1755 bp——◄

E Southern blot

F Probed anti-RhopH3   Probed anti-AMA1
Immunoblot

```
P.falciparum   MRSKHLVTLFIITFLSFSTVKVWGKDVFAGFVTKKLKTLLDCNFALYYNFKGNGPDAGSF
P.chabaudi     MPTNSLTKLFLASLSTFSVSQVWGKDYFSGSLNQNLTDILKCNFISYYNSKPGEPDPNAF
P.vivax        MRSKLFVTLFITCLVTLSSVQVLGREYFSGFVNKKLKNLLQCNLAAYYNLRDNGPDPNAF
               * ::  :..**:    : ::*   :* *:: *:* :.::*. :*.**:   ***  :    **  :*

P.falciparum   LDFVDEPEQFYWFVEHFLSVKFRVPKHLKDKNIHNFTPCLNRSWVSEFLKEYEEPFVNPV
P.chabaudi     LDFVGEPEQFYWFVENYLSVPFTVPQHLSSNSGHNWKSCLKKKWVYEFLKEYEEPEVSYL
P.vivax        LDFVGEPEQFYWFIEHYLSVPFSIPRNLKNNKSHSYWSCINRSWVSEFLRKYEEPDINEL
               **** ********:*.:*** *  :*:.*..:. *.:   *:::.** ***::**** :. :

P.falciparum   MKFLDKEQRLFFTYNFGDVEPQGKYTYFPVKEFHKYCILPPLIKTNIKDGESGEFLKYQL
P.chabaudi     ASVLDKEQRHYYKDTFGDKEPVAKYTIFEVKEFDSYCLLPPFVKTNIRSKNSKENLSFQM
P.vivax        MKFLDKEQRVYFSYTFQNIEPVAKYTSFPIKEFHKYCVLPPLIETNIKQKDKGGNLSFQL
               ..****** ::. .* : ** .*** * :***..**:***::***:. :.   *.:*:

P.falciparum   NKEEYKVFLSSVGSQMTAIKNLYSTVEDEQRKQLLKVIIENESTNDISVQCPTYNIKLHY
P.chabaudi     DKTDYQTYLEVPDNKYAAMKHLSTNMEYGEKQEIVKAIVESNNENSFQLICPSYYIKIHY
P.vivax        NQAEYKIYLSSVGTPTSALKNLYLNMEDGERKNIVKTIVENERSNNVFVNCPVYDLKLHY
               :: :*: :*.      . .  :*:*.*  .:*   :::::::*.*:*.: *.. : ** * :*:**

P.falciparum   NTECKPNSNILTCIDDYIKNTCIDAVKND-EDPTICDHLLNLFNSLKDFQIESFQKFLTA
P.chabaudi     TKECANSNNILKCIDEFLRKTCEKKTESKHPSADLCEHLQFLFESLKNPYLDNFKKFMTN
P.vivax        NKECGSQPNVLKCLDDYIKHLCERRIAHR-EKGTFCDDLLFLFDALKEPYVENFKKFLSR
               ..**   . *:*.*:*:::. *       .   :*:.*   **::**:    ::.*:**::

P.falciparum   SDFTLIKPQSVWNVPIFDIYKPKNYLDSVQNLDTECFKKLNSKNLIFLSFHDDIPNNPYY
P.chabaudi     DELQLTKPRGGWTHPLFHTYSRKDYSNPKMKILPDAFKEFSSENPIFFSFSAEIPEKYSY
P.vivax        DDVHLVKPQSVWGIPLFTTYKPRDLKNPKNNVPLNVFKVLNSKNKLFLSFFDEIPKSPYY
               .:. * **:. *  *:*  *. ::   :    ::   : ** :.*:* :*:**  :**:.   *

P.falciparum   NVELQEIVKLSTYT-YSIF̲D̲K̲L̲Y̲N̲F̲F̲F̲V̲F̲K̲K̲S̲G̲A̲P̲I̲S̲P̲V̲S̲V̲K̲E̲L̲S̲H̲N̲I̲T̲D̲F̲S̲F̲K̲E̲D̲N̲S̲E̲I̲
P.chabaudi     VDNVSIYFKLRNYSSSSVFDTFQNIFSVFKKKTPAIAPVSVKEVSQDIDNFEFKAPKKPI
P.vivax        MEESQPLVKLSDFA-SSIFDKLHRFFYVFKKKGNQISPVSVKELSHNISDFSFKHDTSNI
                :  .  .**  ::   *:**.:  .:* ****.   *:******:*::* :*.** .. *

P.falciparum   Q̲C̲Q̲N̲V̲R̲K̲S̲L̲D̲L̲E̲V̲D̲V̲E̲T̲M̲K̲G̲I̲A̲A̲E̲K̲L̲C̲K̲I̲I̲E̲K̲F̲I̲L̲T̲K̲DDAS---K-----PEKSDIHRGF
P.chabaudi     QCIGVKKSLDLSIDVDIFKVASVEKICSVIDKYALTEDSDFNKVPTEKKMKKLDKLKKGF
P.vivax        ECKKVKKSLNLELEVEVAKGVAAEKICNLIEGFVLTKGKKDKTIR-----GEVDDVHKGF
               :*  *:***:*.::*:   *  :.**:*.:*: : **: .           :  ..::.:**

P.falciparum   R̲I̲L̲C̲I̲L̲I̲S̲T̲H̲V̲E̲A̲Y̲N̲I̲V̲R̲Q̲L̲L̲N̲M̲E̲S̲M̲I̲S̲L̲T̲R̲Y̲T̲S̲L̲Y̲I̲H̲K̲F̲F̲K̲S̲V̲T̲L̲L̲K̲G̲N̲F̲L̲Y̲K̲N̲N̲K̲A̲I̲R̲
P.chabaudi     HIDCILVSTYVEGYNLIRQFLNLENVLSLIRYTSLYTHKFLKSVTTLKEHFLYGQKNAIG
P.vivax        RIQCILIATHVEALNIVRQLLNMESMLSLTRYTSLYLHKFFKSVTALKGSFLYENPSAIR
               :* ***::*:**. *:;**:**:*.::** ****** ***:**** **  ***  : .**

P.falciparum   Y̲S̲R̲A̲C̲S̲K̲A̲S̲L̲H̲V̲P̲S̲V̲L̲Y̲R̲R̲N̲I̲Y̲I̲P̲E̲T̲F̲L̲S̲L̲Y̲L̲G̲L̲S̲N̲L̲V̲S̲S̲N̲P̲S̲S̲P̲F̲F̲E̲Y̲A̲I̲I̲E̲F̲L̲V̲T̲Y̲Y̲N̲
P.chabaudi     YAKSCGSAVLYIPSVMYRRNLYVPETFLSLYLGLSNLVSSNPSSPFFEYSIIDFLITYFN
P.vivax        HARACGRAVLHVPAVLYRRNIYLAETFLSLYLGLSNLVSSNPSSPFFEYAIIEFLVSYFN
               :::::*. * *:*:*:*:****:*: ***************************:**:**::*:*

P.falciparum   KGSEKFVLYFISIISVLYINEYYYEQLSCFYPKEFELIKSRMIHPNIVDRILKGIDNLMK
P.chabaudi     KGTKKFLYYFISIISILHINRYYYEQIYCHHNNHFDTLKSKMIHPDIVKGIMKKLRSILN
P.vivax        KGSEKFLLYLFSIVSVLYINIYYYEQLYCHHREQFELLRSKMIHPNIADRILENIKTLIK
               **:**: *:*:**:*:*:** *****: *.: :.*: ::*:****:*.. *:: : .:::

P.falciparum   S̲T̲R̲Y̲D̲K̲M̲R̲T̲M̲Y̲L̲D̲F̲E̲S̲S̲DIFSR̲E̲K̲VFTALYNFDSFIKTNEQLKKKNLEEISEIPVQLETS
P.chabaudi     KPKYSKMMELYKKLESETLFNYDEMAKILFEFDEFAQNKDVQEKAQKTIDEEEKPEVNTL
P.vivax        NPRYMKMRTFYMKFDNEDMFDKRKVFEVLYSFDEFLSSSDAQQKAKMQDISDESVDLDTT
               . :* **  :*  .::.. .:*.  :;     *:.**.*  ...:  :*  :      .:   :::*

P.falciparum   NDGIGYRKQDVLYETDKPQTMDEASYEETVDEDA--HHV-NEKQHSAHFLDAIAEKDILE
P.chabaudi     EEMANYNE-EWLPKLTRP--------------TIPHDPYDEPNPNIKFDENVRSDGNLT
P.vivax        NDGIGLRKEDALFESEQQGSMESIED---IDEGLEGVDP-NQQKKTAEYLKMVPDEDKAN
               ::    .: : * : :         .  :: : . .: . ..

P.falciparum   EKTKDQDLEIELYKYMGPLKEQSKSTSAAST-SDEISG-------SEGPSTESTSTGNQG
P.chabaudi     LADRDKELELSLFKYIGSLPADKTASTTEDHTASPTEDHAASTTEDHAASTTEDHAGSTT
P.vivax        LTDRNKELELDLYKYIGTLNETSAGVGTVST-HSPATAARMGLNAGGGRAVRPAA---KG
               :::::**:.*:**:* *   .. :.  .       . :.

P.falciparum   EDKTTDNTYKEMEELEEAEGTSNLKKGLEFYKSSLKLDQLDKEKPKKKKSKRKKKRDSSS
P.chabaudi     ES----QTDLAFDENVKPTDAEEMKT-----ASKEHLNTLDTEDTNNDERRSSYT-----
P.vivax        LHQRMGQMGKDLK-RMKGKDTSKLRKGVDFYESSTSLNQISPGESATEGKGSST------
               :    :.    :  :.::::.     *.  *: :.   .  ..      .

P.falciparum   DRILLEESKTFTSENEL-------------
P.chabaudi     ----NEDDRSEISEPEFQHTAPEFYQDENL
P.vivax        -NVSTEG--TSPSET---------------
                    *    :   **
```

A

Probed: anti-RhopH3

Probed: anti-RhopH1/Clag3.1

Probed: anti-MSP1

B

Probed: anti-RhopH3

Probed: anti-EBA175

A

B

A

DAPI | MAHRP1 | EXP2 | Merge | DIC

DMSO

Rapa

DAPI | KAHRP | EXP2 | Merge | DIC

DMSO

Rapa

B

DMSO | Rapa