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A splitCas9 phenotypic screen in *Toxoplasma gondii* identifies proteins involved in host cell egress and invasion

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20   **Abstract**

21   Apicomplexan parasites, such as Toxoplasma gondii, have specific adaptations that enable

22   invasion and exit from the host cell. Owing to the phylogenetic distance between

23   apicomplexan parasites and model organisms, comparative genomics has limited capacity to

24   infer gene functions. Further, although CRISPR/Cas9-based screens have assigned roles to

25   some toxoplasma genes, the functions of encoded proteins have proven difficult to assign.

26   To overcome this problem, we devised a conditional Cas9-system in T. gondii that enables

27   phenotypic screens. Using an indicator strain for F-actin dynamics and apicoplast

28   segregation, we screened 320 genes to identify those required for defined steps in the

29   asexual life-cycle. The detailed characterization of two genes identified in our screen,

30   through the generation of conditional knockout parasites using the DiCre system, revealed

31   that signalling linking factor (SLF) is an integral part of a signalling complex required for early

32   induction of egress, and a novel conoid protein (conoid gliding protein, CGP) functions late

33   during egress and is required for the activation of gliding motility. Establishing different

34   indicator lines and applying our conditional Cas9 screen could enable the identification of

35   genes involved in organellar biogenesis, parasite replication or maintenance of the

36   endosymbiotic organelles in future.

Main

Apicomplexans are early branching eukaryotes related to ciliates and dinoflagellates, with unique adaptations to an intracellular, parasitic existence. The huge phylogenetic distance to well established model organisms is also reflected by the fact that many genes are unique and annotated as hypothetical. With the advancement of the CRISPR/Cas9 technology in *T. gondii,* genome-wide screens allowed the identification of genes that are important during the asexual stage of the parasite[1] with many hypothetical genes being critical for the survival of the parasite. While pooled screens allow the identification of fitness conferring genes, downstream assays are required to define functional groups based on the specific phenotype caused by their deletion.

A prerequisite for phenotypic screens in *T. gondii* is the use of conditional regulation systems in a high-throughput manner. We previously attempted to implement a conditional Cas9 system based on ddFKBP, which suffered from inefficient regulation, prohibiting tight temporal control of Cas9 activity[2]. This resulted in non-essential genes being rapidly lost in a transfected pool (unpublished).

Here, we adapted a tight control system for Cas9, based on splitCas9 (sCas9)[3] and generated indicator parasites, expressing chromobodies directed against F-actin[4] and a marker for the apicoplast (FNR-RFP; a plastid like organelle) that allowed the identification of specific phenotypes in an image-based screen for parasites with abnormal invasion, replication and host cell egress and in parallel the characterisation of changes in F-actin dynamics and apicoplast segregation. We screened a curated library of 320 genes, with more than 40% annotated as hypothetical and conserved within the phylum of Apicomplexa[1] and grouped them into relevant phenotypes. Focusing on genes required for host cell egress, we identified and characterised a novel conoid associated protein (TGGT1_240380) and a putative neurotransmitter symporter (TGGT1_208420). Detailed analysis, using time lapse demonstrates that these genes are critical for distinct, independent steps that act at different times during host cell egress.

Results

Adaptation of a conditional Cas9-system in T. gondii

In order to achieve tight, temporal control of Cas9, we chose the sCas9 system[3], where the Cas9 enzyme is split into two subunits (N- and C-terminus), which are fused to a FKBP and FRB domain. Upon addition of rapamycin these subunits interact and Cas9 activity is restored (Fig.1a). We generated transgenic RH*ΔHX* parasites expressing both sCas9 subunits (Extended DataFig.1a,b), resulting in the recipient strain RHsCas9.

To test efficiency and specificity of this system, we generated vectors for stable expression of small guide RNAs (sgRNA) against previously described essential (gap40) and non-essential genes *(sag1*[5]). The critical function of the gliding associated protein 40 (GAP40) in assembly of the inner membrane complex (IMC) has been described previously[6]. The gRNA expression constructs were randomly integrated into the genome of RHsCas9 and into RH*ΔHX* parasites (Supplementary Table1; Extended DataFig.1c,d,e). Subsequently, parasites were induced with 50nM rapamycin for 1h or 48h before they were analysed. Induction of RHsCas9-gap40, but not RH-gap40 or RHsCas9 parasites resulted in a severe replication defect (Fig.1b,c). Notably, deletion of *gap40* using the DiCre-system causes a similar phenotype, caused by the incomplete assembly of the IMC[6]. No difference in induction rate was observed upon 1 or 48 hours of rapamycin treatment, demonstrating that the sCas9 system allows efficient and rapid activation (Fig.1c).

A second control experiment targeted the non-essential surface antigen 1 gene (*sag1*)[5]. While >95% of parasites demonstrated loss of SAG1, we found that up to 50% of parasites also showed aberrant morphology of their nuclei in addition to loss of SAG1 (Fig.1d,e). This phenotype was only present in rapamycin-treated parasites co-expressing sCas9 and *sag1* sgRNA. No impact on parasite morphology was observed in RHsCas9 parasites (cultured with or without rapamycin), indicating that this phenotype is not caused by general toxicity of sCas9 (Fig.1e). To assess this phenotype further, we induced RHsCas9-sag1 parasites for 1h, mechanically released them from the host cell 48h later and allowed them to infect fresh HFF cells, thus representing the 2nd lytic cycle. Most of these parasites were negative for

93 SAG1 (79% ±4.8), and no aberrant morphology or nuclei were detectable, confirming that

94 *sag1* is not critical for parasite survival *in vitro* (Fig.1e). Additional experiments were

95 performed and demonstrated that the introduction of a double strand break (DSB) in the

96 genome leads to a replication defect in a subpopulation of induced parasites (see

97 Supplementary Text; Extended DataFig.2). Based on these results, we reasoned that the

98 identification of specific defects in invasion, egress or other distinct phenotypes is straight

99 forward, while genes involved in nuclear stability and/or replication can be identified by the

100 significant increase in the percentage of parasites showing a defect in morphology and/or

101 nuclear shape (Extended DataFig.2). Consequently, if the parasite population demonstrates

102 a nuclear phenotype to less than 50% and parasites are still capable of invasion and egress,

103 the corresponding candidate is likely not critical and clonal parasites can be isolated after the

104 second lytic cycle, as shown for *sag1* (Fig.1d,e).

105 Furthermore, the use of specific indicator strains for parasite organelles and structures

106 improves the readout of screens and allows identification of specific phenotypes

107 (Supplementary Text).

108

109 *An indicator line for a screen of F-actin and egress mutants.*

110 To determine if this system could be used in phenotypic screens, we attempted to find

111 candidates involved in the dynamics of F- actin with a special attention to the stabilisation

112 and/or disassembly of the intravacuolar network (IVN) during egress. As previously

113 described, actin and actin binding proteins significantly affect the dynamics of the IVN and

114 therefore the synchronisation of parasite replication, segregation of the apicoplast and

115 egress[4].

116 To determine if candidates can be identified with high confidence in such a screen, we

117 generated an indicator strain expressing the actin Chromobody CB-EmeraldFP (CbEm)[4] and

118 the apicoplast marker FNR-RFP[7] (Fig.1f). Next, we assessed if disruption of previously

119 described genes reproduces previously published phenotypes. At this end, we chose *sag1*,

120 as negative control, dynamin related protein (*drpA*[8]), which should only affect apicoplast

121 division, actin-1 (*act1*[4,9])*,* actin depolymerisation factor *(adf*[10]*)* and *formin 2* (*frm2)*[11] (Fig.1g,h

122 and Extended DataFig.1g), which should cause different effects on F-actin dynamics and

123 apicoplast division. Since these previous studies demonstrated that depletion of proteins

124 involved in F-actin dynamics (including ACT1) or apicoplast segregation did not result in

125 abnormal morphology of nuclei, it is straight forward to exclude the non-specific phenotypes

126 caused by a DSB.

127 Stable transfection with the respective sgRNA expression vectors and disruption of the target

128 gene was validated by sequencing of the respective loci after gene disruption (Extended

129 DataFig.1g). Parasitophorous vacuoles (PV) containing parasites with abnormal nuclei,

130 representing the non-specific phenotype were quantified, but excluded from detailed analysis

131 (Extended DataFig.2f). As expected, disruption of *sag1* or *drpA* had no detectable effect on

132 the formation of the intravacuolar F-actin network (Fig.1h) and a strong defect in apicoplast

133 segregation was observed in the case of *drpA*-disruption, as previously described[8]. In

134 contrast, disruption of *act1* and *adf* led to disintegration and stabilisation of the intravacuolar

135 network respectively (Fig.1h) as described[4,9,12]. In addition, disruption of the actin nucleator

136 *frm2* also led to a more prominent staining of the intravacuolar network and abrogation of the

137 cytosolic polymerisation close to the Golgi, replicating the effect observed when excising the

138 locus using a DiCre strategy[11]. In all cases, apicoplast inheritance was affected as expected.

139 Therefore, we concluded that the indicator strain allows the identification of specific

140 phenotypes related to apicoplast maintenance, actin dynamics, or both.

141 We also assessed if delays in parasite egress can be identified to screen for genes required

142 for host cell egress (Extended DataFig.3). In good agreement with the literature, disruption of

143 *adf* and *act1* led to a significant delay in egress, while the behaviour of parasites with

144 disrupted *sag1* was similar to WT parasites (Extended DataFig.3a,b,c).

145

146 *Phenotypic screening using a custom-designed sgRNA library.*

147 For the phenotypic screen, we curated a library containing genes without annotated signal

148 peptides that are conserved only in apicomplexans, according to the annotation in the

149   ToxoDB 30 release, and have been hypothesised to be fitness conferring (phenotypic score

150   <-1.5)[1]. In addition, some genes were included that were of interest for independent projects,

151   resulting in 320 candidates that were targeted (Supplementary Table2). As internal controls,

152   sgRNAs targeting *gap40* (parasite replication[6]) and *profilin* (*prf;* F-actin dynamics and

153   egress[13]) were included (Extended DataFig.4a,b) in the library. Synthesised sgRNAs were

154   cloned into a vector containing the DHFR selection cassette as previously described[14]. The

155   number of bacteria in the recovered sample carrying ligated plasmid was estimated to be 3 ×

156   10^6 cfu, exceeding the number recommended to maintain complexity[14]. Library complexity

157   was confirmed by sequencing sgRNA from 35 colonies picked at random (not shown), out of

158   which we identified 29 unique gRNAs (83%).

159   Next, the library was transfected into the indicator strain, followed by drug selection, and

160   sorting of single parasites into ten 96-well plates (Fig.2a).

161   We obtained 608 clonal parasites that were inoculated onto three replica plates. Two sets

162   were induced with rapamycin for 48 and 72h, followed by fixation and automated imaging.

163   Images were independently analysed twice by different authors and graded by the relative

164   strength of phenotypes (Fig.2b). Using the decision tree shown in Fig.2b, we grouped

165   phenotypes into "nuclear stability and replication" , "F-actin phenotype" and apicoplast

166   segregation (Extended DataFig. 4c-h). It is important to mention that "nuclear stability and

167   replication" phenotypes were selected based on the observed, significant increase of the

168   ratios of PVs containing morphological aberrant parasites that are arrested in replication

169   (>70%) compared to controls that show the non-specific nuclear phenotype (<50%).

170   Therefore, screening for genes involved in replication and/or nuclear stability is still possible

171   using sCas9 (see also Supplementary Text), as evidenced by the fact that we identified our

172   internal control, *gap40* (for replication) and *prf* (for F-actin) multiple times (Supplementary

173   Table3, Extended DataFig.4a,b).

174   We dismissed clones with integration of multiple different sgRNAs, which caused "super-

175   aberrant" phenotypes (Extended DataTable3, Extended DataFig.4i). This procedure resulted

176   in a total of 99 candidate genes (Supplementary Table3) that were prioritised by re-analysing

177   the obtained images. We focused on clones with the highest induction rates and excluded

178   candidates with a strong replication defect (see above; Fig.2b). In summary, disruption of 42

179   genes showed detectable differences in F-actin formation, apicoplast segregation and/or host

180   cell egress (Extended DataTable4). However, it should be noted that F-actin changes were

181   relatively minor, when compared to the internal control (*prf*) (Extended Data Fig.4a) or the

182   effects seen upon disruption of *adf or frm2* (Fig.1h).

183   We succeeded in tagging 12 of the 16 candidates classified as F-actin and/or apicoplast

184   phenotype (Extended DataFig.5; See Extended DataFig.7a for tagging strategy).

185   Interestingly, one of these genes, TGGT1_208420, localised to the intravacuolar network and

186   the apical pole of the parasite and was also found as important for natural egress (see below;

187   Fig.3a and Extended DataFig.6a).

188

189   *Selection of candidates involved in parasite egress.*

190   Next, parasite egress was analysed 72h after inoculation and induction of sCas9. At this time

191   point, most initially infected host cells were lysed and parasites reinvaded neighbouring host

192   cells (Fig.2b). We identified 33 clones with a potential delay in host cell egress

193   (Supplementary Table 4). Upon a second round of visual inspection, parasites forming

194   smaller or aberrant PVs, indicating obvious replication defects, were excluded, resulting in

195   the identification of candidates where conditional disruption resulted in a delayed egress

196   phenotype (Supplementary Table 4).

197   Analysis of stimulated egress using calcium ionophore A23187 (Ci A23187) revealed that

198   disruption of 4 candidate genes resulted in significantly delayed egress. In contrast, control

199   parasites with disrupted *act1* and *adf*, were inhibited in egress (Extended DataFig.3a and

200   Fig.6a,b). Since disruption of the gene TGGT1_252465, resulted in slower replication, it was

201   excluded from additional analysis (Extended DataFig.6c). Finally, the 3 remaining candidates

202   also demonstrated a significant reduction in host cell invasion rates when compared to

203   controls (Extended DataFig.6d). One candidate (TGGT1_248640) has recently been

204   described as one of the non-discharge proteins, TgNd6[15].

8

205 Here we focus our analysis on the remaining 2 candidates, TGGT1_240380 and

206 TGGT1_208420, named conoid gliding protein (CGP) and signalling linker factor (SLF)

207 respectively (Fig.2b).

208

209 *CGP and SLF are required for egress and invasion*

210 Database research did not reveal much information regarding the putative function of these

211 hypothetical proteins. Both are highly conserved within the phylum apicomplexans and

212 predicted to be critical for the parasite, based on their phenotypic scores[16]. SLF is predicted

213 to be a putative sodium: neurotransmitter symporter with 12 transmembrane domains.

214 Previously it was colocalised with other members of a Signalling Platform important for

215 microneme secretion[17]. However, AID-based knockdown showed no phenotype, contrary to

216 what we observed after Cas9-mediated disruption of *slf*. Regarding CGP, this protein is

217 annotated in ToxoDB as a hypothetical protein and contains a CLU-central domain and a

218 tetratricopeptide (TPR)-like domain. It is also predicted to localise at the plasma membrane

219 based on hyperLOPID data[18]. However, no interaction partners nor potential function is

220 predicted for this protein.

221 We tagged both genes C-terminally with Halo-Tag (*cgp*-Halo) or mCherry (*slf*-mCherry or *slf*-

222 Halo) (Fig.3a, Extended DataFig.7a). Both proteins showed an apical localisation, as

223 evidenced via colocalisation with the apical IMC-marker ISP1[19]. In addition, SLF also

224 accumulated in the intravacuolar network that connects individual parasites within the PV[4]

225 (Extended DataFig.8a).

226 To validate the phenotype seen with sCas9, genes were floxed in the RHDiCreΔ*Ku80* strain[20]

227 (Fig.3b; Extended DataFig.7, Fig.8a and Fig.9a) to generate conditional null mutants (cKO).

228 Induction of DiCre using rapamycin led to a severe growth defect, as evidenced by plaque

229 assays, confirming their critical role during the asexual life cycle of the parasite (Fig.3c).

230 Further analysis fully confirmed the phenotype obtained using sCas9 and demonstrated a

231 crucial function of both proteins in host cell egress and invasion, while parasite replication,

232 morphology, apicoplast and secretory organelles were not affected (Fig.3d,e; Extended

233 DataFig.8 and Fig.9).

234 Gliding motility was significantly reduced in both mutants (Fig.3f; Extended DataFig.8e,

235 Fig.9c; Movie_S1). Interestingly, in the case of parasites lacking *slf* (*slf*-cKO) gliding motility

236 and egress (Fig.3e,f; Extended DataFig.8e,f) could be partially rescued upon addition of Ci

237 A23187 (Extended DataFig.8f; Movie_S2), indicating that it is involved in a signalling

238 cascade, upstream of intracellular calcium release. Indeed, *slf*-cKO showed a significantly

239 reduced secretion of micronemes, that was less severe when Ci A23187 was used as an

240 inducer, compared to BIPPO[21] (54.5% versus 74.3% microneme secretion reduction; Fig.3g;

241 Extended DataFig.8). Therefore, the phenotype of *slf*-cKO appeared similar to the

242 phenotypes observed upon deletion of components of the phosphatidic acid (PA) signalling

243 platform, such as diacylglycerol kinase 2 (DGK2), cell division control 50 related protein

244 (CDC50.1), guanylate cyclase (GC) or unique GC organiser (UGO)[17].

245 In the case of *cgp* knockout parasites (*cgp*-cKO), egress was blocked irrespective of addition

246 of Ci A23187, BIPPO or Propanolol (Fig.3e). Furthermore, no defects of microneme

247 secretion could be observed (Fig.3h; Extended DataFig.9), placing this protein into a different

248 functional category.

249

250 *CGP and SLF act at two distinct, temporally controlled steps.*

251 We then characterised the sequential action of CGP and SLF during host cell egress. The

252 intravacuolar network is rapidly disintegrated early in the egress process[4], thereby acting as

253 an early indicator for initiation of host cell egress. Therefore, we introduced CbEm[4] into the

254 UPRT locus of both mutants to analyse localisation and dynamics of F-actin (Fig.4a;

255 Extended DataFig.7k,l; Movies_S3, S4 and S5).

256 When analysing F-actin dynamics upon induction of egress, the following steps can be

257 differentiated in WT parasites: 1) initiation of intravacuolar network disassembly, 2) reduction

258 of F-actin nucleation close to the Golgi, probably caused by FRM2[4] and 3) strong posterior

259 accumulation of F-actin and activation of motility (Fig.4a,b). In the case of *slf*-cKO parasites,

260 none of these steps could be observed indicating that initiation of egress is completely

261 blocked and that the initial signals leading to the induction of microneme secretion are

262 identical with regulation of F-actin dynamics during host cell egress (Fig.4a,b). This

263 phenotype was partially rescued upon addition of Ci A23187 (Extended Data Fig.8 and

264 Fig.10a,b). Interestingly, parasites which did not egress after induction with Ci A23187 were

265 still able to efficiently disassemble the IVN in most cases, and F-actin accumulation at the

266 posterior pole was initiated. Despite this, motility was not initiated at the time of fixation or

267 after 10 mins of recording. (Movie_S3. Extended DataFig.8).

268 Intriguingly, induction with propranolol led to a different egress phenotype, when compared to

269 induction with BIPPO. Here the parasites that remained within the PVM were able to

270 disassemble the filaments but appeared unable to initiate motility (Fig.4a; Movie_S3).

271 Furthermore, these parasites changed their shape and appeared rounded up, which was

272 only observed upon induction of *slf-c*KO parasites with propranolol.

273 In contrast, deletion of *cgp* led to a block in a later stage during egress. While disassembly of

274 the intravacuolar network and reduction of F-actin nucleation close to the Golgi occurred

275 normally, neither posterior accumulation of F-actin nor parasite motility appears to be

276 initiated (Fig.4a,b; Movie_S4).

277 In summary, this analysis highlights that SLF and CGP act at two temporally different steps

278 during host cell egress. While SLF acts at the initiation step, CGP acts downstream, after the

279 intravacuolar F-actin network has been disassembled.

280 Finally, we were interested to know if the PV-membrane (PVM) is dissolved. Therefore, we

281 expressed *sag1*ΔGPI-dsRed, which is secreted into the PV. Upon lysis of the PV this protein

282 diffuses into the cytoplasm of the host cell, as seen in case of control parasites (Fig.4c, top

283 row). Deletion of CGP had no influence on lysis of the PV, since dsRed signal diffused at a

284 similar time as seen in case of controls. In contrast, upon deletion of SLF, the PVM remained

285 intact and dsRed signal is trapped within the PV (Fig.4c; Movie_S5).

286

287

288 *SLF is required for integrity of the PA signalling complex*

289 Bioinformatic predictions of SLF place this protein into the family of sodium neurotransmitter

290 symporters and demonstrates that it is highly conserved in all apicomplexan parasites.

291 This protein was previously identified as a putative and dispensable interaction partner of the

292 signalling platform, since knockdown using the auxin-inducible degron (AID) system had no

293 effect on the parasite lytic cycle[17]. However, disruption and excision of *slf* demonstrated one

294 of the strongest phenotypes obtained in this screen, suggesting that the AID system is

295 insufficient to knockdown protein levels of SLF. Indeed, SLF colocalised with other members

296 of the PA signalling pathway such as GC, CDC50.1 and UGO (Fig.5a) at the apical tip of the

297 parasite and the intravacuolar network. Importantly, deletion of SLF results in mislocalisation

298 of other components of this signalling complex and vice versa (Fig.5b,c), indicating that this

299 complex is assembled early in the secretory pathway, probably the ER, and only reaches its

300 final destination if all partners are present. This was seen in 100% of vacuoles, where one

301 component is missing. While this confirms an important structural role of SLF for functional

302 assembly of the signalling complex, future studies are required to determine if this protein

303 also acts as a sodium neurotransmitter as predicted in ToxoDB. In a first attempt, we focused

304 on GABA (γ-aminobutyric acid) as a potential substrate for this putative symporter, since it

305 was demonstrated that *T. gondii* synthesises high levels of GABA[22] and modulates host cell

306 migration using GABA as messenger[23]. However, we were unable to either complement the

307 phenotype by adding increasing concentrations of GABA or to mimic the phenotype by

308 addition of GABA analogues (Extended DataFig.10c,d). In conclusion, SLF is a critical

309 component for the integrity of the PA signalling platform required for egress. Future analysis

310 will reveal if this protein is directly involved in the signalling cascade by acting as a

311 symporter.

312

313 *CGP is a novel component of the conoid.*

314 STED imaging showed a localisation of CGP anterior to the conoid markers RNG2, which

315 localises to the second apical polar ring, and SAS6-like (SAS6L), a marker of the conoid

12

316   body[24]. This apical localisation was detected in retracted and protruded conoids (Fig.6a).

317   Importantly, conoid structure appeared unaffected upon deletion of *cgp* (Fig.6b) indicating

318   that this protein does not play a key role for the integrity of the conoid itself. Similarly, the

319   secretory organelles (micronemes and rhoptries) were not affected by deletion of *cgp* and

320   secretion of micronemes occurred normally (Fig.3h; Extended DataFig.9e,f).

321   While bioinformatic analysis of this protein suggests that it is highly conserved within

322   apicomplexan parasites (not shown), no clear orthologue could be identified in other

323   eukaryotes. Therefore, future studies are required to identify potential interaction partners

324   and the mechanistic action during host cell egress. Moreover, it would be interesting to

325   determine if their function is also conserved in other apicomplexan parasites.

326

327   **Discussion**

328   In this study, we adapted an efficient and tightly regulated conditional Cas9-system based on

329   sCas9[3]. Our detailed analysis of this system demonstrated that repair of DSB introduced by

330   Cas9 is occasionally inefficient, leading to aberrant, non-specific phenotypes in a

331   subpopulation of parasites. While these effects need to be considered when conducting a

332   phenotypic screen, they can easily be deducted from the readout of the screen, especially

333   when modern, automated image analysis methods are used that are now also available to

334   determine the phenotype of *Toxoplasma*-Host interactions[25]. Computational image analysis

335   tools should allow to not only exclude but to differentiate between the non-specific nuclear

336   and specific replication phenotypes. In order to do so, a huge dataset of known phenotypes

337   needs to be created in order to train the algorithms[26], an effort well worthwhile to undertake,

338   especially when larger genome-wide screens for multiple phenotypes are planned.

339   Here, screening of an indicator strain that co-expresses fluorescent actin binding

340   chromobodies (CbEm)[4] and an apicoplast marker[7] allowed the identification of mutants with

341   defects in apicoplast maintenance and F-actin dynamics in addition to mutants with inhibited

342   host cell egress. Therefore, we curated a sgRNA library targeting 320 candidate genes that

343   are conserved among apicomplexans. Based on the phenotypic characterisation of identified

344 mutants, we did not identify a novel factor that is directly involved in regulation and

345 organisation of F-actin dynamics during intracellular replication. Instead, several factors were

346 identified, where F-actin dynamics was only slightly altered, when compared to positive

347 controls (Prf, ADF, ACT1). In contrast, two novel genes identified here, *slf* and *cgp,* are

348 crucially involved in the regulation of actin dynamics during host cell egress where they play

349 distinct roles.

350 Time-lapse microscopy analysis suggests that disassembly of the intravacuolar network

351 precedes the initiation of motility and egress of parasites from the PV[4] (Fig4a; Movie_S3,

352 S4). Indeed, stabilisation of F-actin by depletion of ADF results in an egress phenotype,

353 where parasites were able to initiate motility, but remain connected by the intravacuolar

354 network (Fig.6c, Extended DataFig.3).

355 When we applied this analysis to conditional mutants for SLF and CGP, we observed

356 important differences in the behaviour during host cell egress. While deletion of SLF caused

357 an early block in host cell egress, where neither the intravacuolar network nor the PVM is

358 disassembled, deletion of CGP caused a late block in egress, where parasites disassembled

359 both the intracellular F-actin network and PVM but are incapable of initiating motility and

360 leaving the host cell. While CGP is not involved in microneme secretion, it is likely that it

361 directly or indirectly regulates gliding motility by regulating F-actin dynamics, since deletion of

362 CGP resulted in clear differences in F-actin dynamics, as evidenced by missing posterior

363 accumulation of F-actin.

364 In the case of SLF, we found that this protein is an important component of a previously

365 described PA signalling platform required for host cell egress[17], since deletion of SLF

366 resulted in mislocalisation of the remaining components, CDC50.1, GC or UGO within the ER

367 of the parasite. Together this indicates that the signalling complex might need to be

368 assembled within the ER before being transported to the apical tip and intravacuolar network,

369 where it fulfils its critical function.

370 These findings highlight the robustness of this approach for finding essential genes based on

371 their function. In future screens, establishment of different indicator lines expressing markers

372   for the secretory organelles, IMC or mitochondria could be used to specifically identify genes

373   involved in organellar biogenesis, parasite replication or maintenance of the endosymbiotic

374   organelles, to name a few examples for future applications of this technology

375   (Supplementary Text and Extended DataFig.10e,f,g).

376   Concurrently with our work, Smith and colleagues developed an alternative strategy, based

377   on high-throughput CRISPR-mediated tagging of candidate genes with the AID-system and

378   demonstrate the efficiency of their strategy by labelling and downregulation of the *T. gondii*

379   kinome, resulting in the characterisation of kinases, involved in diverse functions. As with all

380   technologies, both approaches have their advantages and disadvantages. While the mAID-

381   based screening approach allows direct localisation and rapid regulation of the protein of

382   interest, there is a potential risk for incomplete protein knock-down, the difficulty to

383   functionally tag some proteins, and limitation to proteins that are accessible to the

384   degradation machinery. The sCas9-based screen on the other hand acts directly on the

385   DNA-level by introduction of indel mutations in the gene of interest. The kinetics of protein

386   depletion is probably comparable to the Tet-inducible and DiCre-system [27] and significantly

387   slower compared to AID. Therefore, screens based on sCas9 have no restriction regarding

388   the gene of interests and can in principle be performed with all gRNAs-libraries designed

389   previously for other projects. Indeed, we are currently performing a genome wide screen

390   using sCas9 (Jimenez-Ruiz et al., in preparation). A drawback of sCas9 (as with all cas9-

391   based approaches that rely on NHEJ repair of the introduced DSB) is the occurrence of

392   aberrant, nuclear phenotypes in a subpopulation of parasites, making it somewhat difficult to

393   clearly interpret nuclear and replication phenotypes without further downstream analysis (see

394   also Supplementary Text for more details). In conclusion, both strategies are highly

395   complementary and have a huge potential to screen the *T. gondii* genome in search for key

396   candidates based on their function and resulting phenotypes.

397

398   **Materials and Methods**

399   *Cloning DNA constructs*

400 The N- and C-termini of the Cas9 enzyme (split4 variant) were amplified from the original

401 plasmids provided by Zetsche and colleagues[3] via PCR. The PCR amplicons were ligated

402 into the pGEM®-T Easy vector and sequenced. Subsequently, they were cloned into the

403 *Toxoplasma* expression vector p5RT70-HX[28] via the restriction enzymes EcoRI (NEB;

404 R0101S) and PacI (NEB; R0547S). For the C-term-Cas9 vector, the *hx* selection marker was

405 removed by restriction with SacII (NEB: R0157S). The resulting plasmids were confirmed by

406 sequencing.

407 pU6-*sag1* gRNA-scaffold and pU6-*gap40* gRNA-scaffold sequence was synthesised and

408 cloned into a backbone vector containing the DHFR resistance cassette by GeneScript. The

409 Q5® Site-Directed Mutagenesis Kit (New England Biolabs) was used to insert sgRNAs

410 targeting control genes of interest (*act1*, *adf*, *frm2* and *drpA*) into the synthesised sgRNA

411 plasmid according to the manufacturer's instructions. Importantly, a universal reverse primer

412 was used together with a forward primer to which the whole sgRNA sequenced was

413 attached, see also[29]. Further sgRNAs used in this study were cloned into the universal pU6

414 vector via BsaI digestion, primers annealing and standard ligation using T4 Ligase (see oligo

415 sequences Supplementary Table 5) as previously described[29]. All sgRNA-plasmids were

416 confirmed by sequencing.

417 To generate mutated *sag1**, genomic DNA was amplified and inserted via EcoRI and PacI

418 into p5RT70-HX[28]. Mutations at the sgRNA binding sequence were introduced via

419 mutagenesis using Q5 Site Mutagenesis following manufacturer's protocol (NEB; E0554S)

420 using primers described in Supplementary Table 5.

421

422 *Culturing of T. gondii and host cells*

423 *T. gondii* tachyzoites were passaged onto Human foreskin fibroblasts (HFFs; ATCC, SCRC-

424 1041) monolayers at 37 ˚C and 5% CO_2 in DMEM (Sigma, D6546) supplemented with 10%

425 FBS (BioSell FBS.US.0500), 4mM L-Glutamate (Sigma, G7513) and 20 µg/ml gentamicin

426 (Sigma G1397).

427

Generation of transgenic parasites

Freshly lysed *Toxoplasma* tachyzoites were transfected with Amaxa 4D-Nucleofector system (Lonza; AAF-1003X). ~1×10^6 parasites were centrifuged and resuspended in 50 µl P3 buffer. Up to 20 µg of DNA for transfection, including vectors, donor DNA and/or single stranded DNA, was ethanol precipitated and resuspended in another 50 µl of P3 Buffer. Both resuspensions were mixed in a 100 µl cuvette (P3 Primary cells 4D-Nucleofector X kit L, V4XP-3024, Lonza). The programme FI-158 was used for electroporation. Immediately after transfection, parasites were resuspended in fresh complete DMEM and added onto a dish with confluent HFF cells.

For generation of sCas9-parasites, a total of 20 µg of the vectors containing the sCas9 subunits were linearised with NotI (NEB; R3189S) and transfected simultaneously in RHΔ*hxgprt* (RHΔHX), adding NotI restriction enzyme to the transfection mix. Integrants were selected with 25 mg/ml mycophenolic acid (MPA; Sigma; M3536) and 40 mg/ml of xanthine (Sigma; X3627)[30]. After isolating a clone containing both functional subunits, sgRNAs against the *hxgprt* cassette were transfected and the parasites were selected with 80 mg/ml 6-Thioxanthine (Sigma-Aldrich; 852570)[30]. The *hxgprt* cassette was then sequenced to confirm the introduction of indels.

Vectors containing sgRNAs targeting control genes (*gap40*, *sag1*, *act1*, etc) were linearised via NotI and transfected into RHΔHXsCas9 as described above. To select for the *dhfr* resistance marker, parasites were treated with 1 µM pyrimethamine (Sigma 46706; Donald and Roos 1993). Insertion of sgRNAs were confirmed by PCR of the sgRNA cassette (Extended DataFig.1f).

For the generation of RHsCas9-CbEm-FNR-RFP, RHsCas9 parasites were first transfected with the vector containing the CbEm cassette[4]. Transfectants were enriched via Fluorescence Assisted Cell Sorting (FACS; S3 BioRad), and a clone was isolated via limiting dilution. Secondly, the vector containing FNR-RFP was linearised via NotI and transfected into RHsCas9-CbEm strain and selected with 20 µg/ml chloramphenicol [5]. After selection, clones were isolated by limiting dilution.

sgRNA library generation and transfection

A list of guide RNAs was obtained after searching in ToxoDB (release 30. February 2017) for genes annotated as apicomplexan specific, without signal peptide and with a phenotypic score ≤ -1.5. Control genes, such as *gap40* and *prf,* were included to this list. 1 gRNA per gene was selected from Sidik et al. (2016) genome wide library (Supplementary Table 2). The resulting 320 gRNAs were synthesised (CustomArray, Inc. USA), amplified by PCR, and cloned as a pool into a plasmid using Gibson Assembly (NEB E26115) as performed by Sidik et al. (2018)[14]. The vector plasmid carries a pU6 promoter, and a DHFR cassette. Assembled plasmids were transformed in two batches, and bacteria allowed to grow to log phase. The plasmids were extracted, purified, pooled, and 200 ng retransformed into the bacteria for further library amplification. Library complexity was estimated based on 1) the number of independent colonies obtained after transforming bacteria with 200 ng of library DNA (as described in Sidik et al. 2018[14]) and 2) random isolation of individual colonies and sequencing of the respective sgRNA. From the colony picking, 10 μg of different isolated gRNAs were transfected individually into RHsCas9-CbEm-FNR-RFP and analysed in parallel to the screen described below.

Following the generation of the vector library, 60 μg of pooled plasmid library was transfected into RHsCas9-CbEm-FNR-RFP line specifically created to be used as an indicator strain for this project. This transfection gave rise to a pool of parasites which were collectively carrying the guide RNA library. A minimum of 1×10^7 parasites were passaged each time. To obtain clonal populations, transfectants were subjected to 3 weeks of pyrimethamine selection followed by FACS (BD FACSAriaIII) into ten 96-well plates, one event per well.

Phenotypic screening of sCas9 mutants

Conditional mutagenesis via sCas9, was induced using 50 nM rapamycin (Sigma; R0395-1MG) for 48 and 72 h, and the plates were imaged using the LasX navigator on a Leica DMi8 widefield microscope attached to a Leica DFC9000 GTC camera, using a 20x objective. After

484 choosing the correct carrier on the navigator, the plates were aligned and three random

485 images were taken from each well, using the 'On demand' adaptive autofocus setting. The

486 images obtained were independently screened by eye by two investigators before

487 subsequent selection of the candidate clones. Clones which were seen to exhibit aberrant

488 organellar morphologies or altered F-actin dynamics, and egress defects were then selected

489 and the guide RNAs present in the clonal populations, which exhibited phenotypes deemed

490 relevant to the project, were then sequenced to identify the gene disrupted. To prioritise the

491 candidates for further characterisation, the phenotypes observed were graded from 1 (least

492 severe) to 4 (most severe) (Supplementary Table 3).

493

494 *Generation of tagged and floxed strains*

495 Guide RNAs for cleavage upstream and downstream of the genes of interest were designed

496 using EuPaGDT[31]. Sequences of all sgRNAs employed in this study are detailed in

497 Supplementary Table 1. The sgRNAs were ligated into a vector coding for Cas9-YFP

498 expression, as has been previously described[32].

499 Repair templates for integration of a loxP sequence and a tag were generated as in Stortz et

500 al. (2019)[11]. Briefly, repair templates carrying the upstream loxP sequence were ordered as

501 ssDNA oligos (ThermoFischer Scientific), the loxP sequences being flanked by 33 bp of

502 homology. The repair templates carrying tags, such as 3xHA, SYFP2, Halo and SNAP, were

503 generated by PCR, where the 50 bp of homology flanking the tags were introduced via the

504 primer. The repair templates were pooled according to the gene and purified using a PCR

505 purification kit (Blirt; EM26.1) (Extended DataFig.7a,b).

506 Parasite transfection, sorting and screening for positive mutants was done according to

507 Stortz *et al.* (2019)[11]. Briefly, newly released RHDiCreΔ*ku80* tachyzoites[33] were transfected

508 with the repair templates and 10-12 µg of vectors (encoding Cas-YFP and the respective

509 sgRNAs) as described above. The parasites were mechanically egressed 24 to 48 h after

510 transfection, passed through a 3 µm filter, and those transiently expressing Cas9-YFP

511 enriched via FACS (FACSARIA III, BD Biosciences) into 96-well plates (a minimum of 3

512 events per well). Resultant clonal lines were screened by PCR and repair template

513 integration confirmed by sequencing (Eurofins Genomics).

514 For the insertion of CbEm into tagged lines, a specific sgRNA targeting the uracil

515 phosphoribosyltransferase (UPRT) locus was designed and cloned into a Cas9-

516 YFP-expressing vector. CbEm cassette from the original plasmid used in Periz et al.[4], was

517 PCR amplified and integrated into the UPRT locus (Extended DataFig.7k).

518

519 *Immunofluorescence assays*

520 Parasites were fixed in 4% paraformaldehyde for 15-20 minutes at room temperature.

521 Samples were blocked and permeabilised in phosphate-buffered saline (PBS) with 2% BSA

522 and 0.2% Triton X–100 for at least 20 minutes. Antibody labelling was performed using the

523 indicated combinations of primary antibodies for 1 h, followed by the incubation with

524 secondary antibodies for another 45 min. All antibodies are listed in Supplementary Table 6.

525 α-GFP-ATTO 488 (1:500, Nano Tag Biotechnologies, N0304-At488-L) was directly used for

526 1h after permeablisation. Parasites containing Halo or SNAP tags were incubated with

527 specific dyes for 1 h and washed away, followed by incubation with media for 1 h before

528 fixation unless specifically indicated elsewhere (see Supplementary Table 6). Images were

529 taken using Leica DMi8 Widefield microscope or an Abberior 3D STED microscope. The

530 library parasite pictures obtained using the Abberior 3D STED microscope were taken using

531 the confocal setting for FNR-RFP and Hoechst, and STED for the CbEm imaging.

532

533 *Invasion/replication assays*

534 24 h invasion/replication assays were performed using sCas9 parasite clones isolated as

535 previously described, with some changes[34]. 5×10^6 parasites were used to infect covers and

536 were left to settle down on ice before allowed invasion for 20 min. Parasites were stained

537 with α-SAG1 antibody without permeabilisation and α-GAP45 antibody after permeabilisation.

538 For invasion, the number of vacuoles in 10 randomly selected fields of view were counted for

539 each parasite line and condition. For replication, the number of parasites per vacuoles were

540 counted. At least 100 vacuoles were counted.

541 In the case of RHDiCreΔ*ku80* floxed strains, loxP*cgp*-Halo parasites were used for

542 invasion/replication assays, loxP*slf*-Halo were used for invasion assays, and loxP*slf*-mCherry

543 were used for replication assays. Parasites were pre-induced for 96 h ± 50 nM rapamycin.

544 Parasites were then mechanically egressed, and 5×10^6 parasites (for invasion assays) or

545 4×10^6 (for replication assays) were inoculated in each well and left to invade for 1 h.

546 For invasion assays, parasites were allowed to settle on ice for 10 min, and then allowed to

547 invade for 1 h at 37 ˚C before fixation. Subsequent IFAs were done following the same

548 protocol as the invasion assays done on the sCas9 parasite strains. A minimum of 150

549 parasites were counted to calculate the percentage of invaded parasites.

550 For replication assays, samples were washed 3 times with DMEM to remove non-attached

551 parasites and left at 37 ˚C for another 24 h. Samples were fixed with 4% PFA and labelled

552 with α-GAP45 (loxP*cgp*-Halo) or IMC1 and α-RFP (loxP*slf*-mCherry).

553 LoxP*cgp*-Halo parasites were pre-incubated with HaloTag Oregon Green dye (0.2 µM) for 1

554 h, whereas loxP*slf*-Halo parasites were incubated with Halo Janelia 646 (20 nM) for 15 h

555 prior to the start of the invasion assay.

556 These experiments were carried out in triplicate and a minimum of 100 parasites/ vacuoles

557 were counted (n = >100). In case of rapamycin induced floxed parasites (cKO), only

558 parasites/ vacuoles lacking signal for SLF or CGP were included in the counting.

559

560 *Plaque assay*

561 A total of 500-1000 parasites per well were inoculated into confluent HFFs in 6 well-plates

562 and incubated for 6 days ± 50 nM rapamycin[34].

563 In case of GABA (Tocris, 0344) or Gabapentin (Sigma-Aldrich, G154) plaque assays, media

564 was supplemented with different concentrations of GABA (100 mM stock concentration) or

565 Gabapentin (50 mM stock concentration).

566   Images were taken using the LAS X Navigator software and a Leica DMi8 Widefield

567   microscope using 10× objective (Microsystems). Starting in the middle of the well, an area of

568   12 x 12 fields was imaged. Focus maps were created and autofocus controls were applied

569   for taking the final images. After acquisition of the images, "mosaic merge" processing tool in

570   LAS X software was used for merging the pictures into one big final image.

571

572   *Egress assays*

573   For egress assays depicted in Extended DataFig. 3, induced (50 nM Rapamycin) and non-

574   induced RHsCas9-CbEm-adf/sag1 parasites were grown for 48 h. Egress was then induced

575   by incubating parasites with 2 µM Ci A23187 for 8min under normal culturing conditions.

576   Subsequently, parasites were fixed with 4% PFA for 20min and α-SAG1 or α-GAP45 were

577   used for parasite visualisation.

578   1×10^5 sCas9 background parasites were grown on HFF cells incubated with ± 50 nM

579   rapamycin for 4 h. They were then washed with DMEM three times to remove rapamycin and

580   any extracellular parasites. Parasites were maintained for another 44 h in the incubator at 37

581   ˚C before inducing egress.

582   In case of parasites floxed in the DiCre strain, parasites were pre-treated ± 50 nM rapamycin

583   for 24 h and 3×10^5 were inoculated onto confluent HFF cells, incubated for 1 h at 37 ˚C,

584   then washed 3 times with PBS and maintained at 37 ˚C for 32 h before inducing egress. Halo

585   tagged parasites were pre-incubated with HaloTag Oregon Green (0.2 µM) or Janelia Fluor

586   646 (20 nM) for 1 h and washed three times with PBS before inducing egress. To induce

587   parasite egress, media was exchanged with pre-warmed DMEM without serum but

588   supplemented with various inducers for different lengths of time (2 µM Ci A23187 (Sigma-

589   Aldrich, C7522-1mg) for 5 min, 50 µM BIPPO (a phosphodiesterase inhibitor that stimulates

590   microneme secretion[21]; Thompson Lab) for 5 min, or 125 µM Propranolol hydrochloride

591   (Merck, 40543) for 7 min).

592   After induction, sCas9 expressing parasites were fixed with 4% PFA and the number of

593   egressed and non-egressed vacuoles counted. DiCre-expressing parasites were fixed with

either 4% PFA or 100% methanol. α-SAG1 or α-GAP45 antibodies were used for the visualisation of parasites. In case of rapamycin induced floxed parasites, only vacuoles lacking signal of the respective protein was considered in the counting (cKO). At least 100 vacuoles were counted for each condition and replicate and the percentage of the egressed vacuoles was calculated.

For time-lapse images, floxed parasites expressing CbEm (loxP*cgp*-Halo/CbEm and loxP*slf*-Halo/CbEm) were treated ± 50 nM rapamycin for 24 h and then mechanically released and inoculated onto glass-bottom live cell dishes, and cultured for a minimum of 32 h before inducing egress. Halo-tagged parasites were pre-incubated with Janelia Fluor 646 (20 nM) for around 5 h followed by washing 3 times with PBS and then incubated with normal media for at least 1 h before egress induction.

Dishes were placed in the pre-warmed chamber of Leica DMi8 microscope and media was exchanged with complete Fluorobrite DMEM (ThermoFischer Scientific, A1896701) containing the respective inducers. Videos were taken with a 63x oil objective at 0.33 frames per second (FPS). Videos were recorded in triplicate per condition as a minimum. In case of conditional knockouts, only vacuoles lacking the signal for SLF or CGP were recorded (cKO). For calculating the dynamics of CbEm after stimulating egress chemically, regions of interest (ROI) were drawn around the apicoplast region, the region between basal CbEm labelling and the apicoplast (termed nuclear region), the *T. gondii* cell, and a background region outside the vacuole. Relative intensity of the CbEm in apicoplast region was then determined as:

$$relative\ intensity = \frac{(mean\ apicoplast - mean\ nuclear\ region) \times apicoplast\ area}{(mean\ total\ Toxoplasma - mean\ background) \times total\ toxoplasma\ area}$$

Relative intensity of the CbEm in basal pole region was then determined as:

$$relative\ intensity = \frac{(mean\ basal\ part - mean\ nuclear\ region) \times basal\ part}{(mean\ total\ Toxoplasma - mean\ background) \times total\ toxoplasma\ area}$$

where mean was defined as:

$$Mean = \frac{RawIntDen}{area}$$

620

621 To check PVM integrity, loxP*cgp*-Halo/CbEm and loxP*slf*-Halo/CbEm were treated with ± 50

622 nM rapamycin for 24 h and transfected with the vector pTub- *sag1*ΔGPI-dsRed. 48 hours

623 later, egress was induced with 50 µM BIPPO and recorded as described above. Over 10

624 egress events were recorded for each condition.

625 To check the F-actin IVN network upon induction of egress with Ci A23187, assays were

626 performed as described above for loxP*slf*-Halo/CbEm strain infecting HFF on coverslips with

627 2.5×10^5 parasites. After induction with 2 µM A23187 for 5 min, parasites were fixed with

628 cytoskeleton buffer (CBs) [35] for 25 min and followed by 50 mM NH4CL for 10 min. Anti-GFP

629 Atto-488 antibody was used to amplify the CbEm signal. CB buffer was previously reported in

630 Periz et al. (2019) and is the result of a mixture of CB1 and CB2 (4:1). CB1: MES pH 6.1 10

631 mM, KCL 138 mM, $MgCl_2$ 3 mM, EGTA 2 mM, 5% PFA. CB2 :MES pH6.1 10 mM, KCL

632 163.53 mM, MgCl 3.555 mM, EGTA 2.37 mM, Sucrose 292 mM.

633

634 *Trail deposition assay and live gliding assay*

635 For trail deposition assays, induced (50 nM rapamycin, 72 h for loxP*cgp*-Halo and 96 h for

636 loxP*slf*-mCherry) and non-induced (72 h for loxP*cgp*-Halo and 96 h for loxP*slf*-mCherry)

637 parasites were washed, mechanically egressed via 26-gauge needles and filtered through 3

638 µm filters. Parasites were then centrifuged at 1000 x g for 5 min at room temperature and the

639 pellet was resuspended in pre-warmed endo buffer (44.7 mM K_2SO_4, 10 mM $MgSO_4$, 100

640 mM sucrose, 5 mM glucose, 20 mM Tris, 0.35% w/v BSA, pH 8.2) at a concentration of

641 2×10^6 parasites/ml. 1 ml of this mixture was added to a FCS coated glass-bottom live cell

642 dish and incubated for 15 min at room temperature. Endo buffer was gently replaced with

643 1ml of pre-warmed sterile gliding buffer (1mM EGTA and 100mM HEPES in HBSS solution).

644 Parasites were incubated for 20 min at 37 ℃ and then fixed with 4% PFA for 20 min.

645 Parasites were stained with α-*Toxoplasma gondii* antibody (Abcam; see Supplementary

Table 6) without permeabilisation. 15 random fields of view were imaged and the total number of trails parasites left were counted.

For live gliding assays, to measure parasite gliding kinetics, time-lapse videos were taken with a 63× objective at 2 FPS using a Leica DMi8 microscope using DIC. After 20 minutes of recording per condition, a Z-stack image of the fluorescence channel targeting the protein of interest was taken to distinguish the cKO from the non-induced parasites. Only cKO were considered for the analysis for rapamycin induced parasites. Halo tagged parasites were pre-incubated with Janelia Fluro 646 dye (20 nM) or HaloTag TMR (500 nM) at least 2 hours as described above before performing live gliding assays. Parasite motility was analysed by manual tracking plugin tool by Icy software. All the assays were done in Ca^{2+}-free gliding buffer unless otherwise indicated.

For trail deposition and gliding assays with 2 µM Ci A23187, compounds were added to the gliding buffer described above.

Microneme secretion assay

Microneme secretion assay protocol was adapted from Bisio *et al.* (2019)[17]. Parasites treated for 72 h ± 50 nM rapamycin were mechanically released by 26-gauge needles and washed twice with cold intracellular buffer and then being resuspended in pre-warmed intracellular buffer (5 mM NaCl, 142 mM KCl, 1 mM $MgCl_2$, 2 mM EGTA, 5.6 mM glucose, 25 mM HEPES, pH 7.2) containing 2 µM A23187, 5 µM BIPPO or DMSO and then incubated for 30 min at 37 ℃. Afterwards, the supernatant was collected and further centrifuged, followed by Western blot analysis (WB). For WB, 4-20% precast polyacrylamide gel (BioRad, 4561096) were used. GRA1 and Mic2 antibody was used to label the membranes, summarised in Supplementary Table 6. Stained membranes were imaged using Odyssey CLX-1849 (LI-COR).

For quantification of microneme secretion assays, this experiment was made in triplicates and run on independent membranes. Measurements were done by Image J. MIC2 secretion

673 was related to GRA1 secretion. Graphs in Fig. 3g and h are percentages of MIC2/GRA1

674 relative to the WT in each condition.

675

676

677 *Imaging processing*

678 LasX software (v. 3.4.2.183668) from Leica was used to obtain parasite imaging data and LI-

679 COR Image Studio Software for WB images. All images and movies were processed using

680 Fiji (ImageJ) software v.2.1.0 and/or Icy Image Processing Software 1.8.6.0. With the

681 exception of images of parasites expressing CbEm and time-lapse videos, all widefield

682 images were deconvolved using Huygens essential v.18.04.

683 Schemes were created using Adobe InDesign or Microsoft PowerPoint.

684

685 *Software*

686 *In silico* cloning was performed using pDraw32 (AcaClone software. v. 1.1.120) and ApE. A

687 plasmid editor (by M. Wayne Davis. V.2.0.53c) software. Sequencing results were analysed

688 using BioEdit v.7.2. Generation of gRNAs for tagging was done in EuPaGDT[31].

689

690 *Statistics and reproducibility*

691 All data were plotted by Excel (Microsoft 365) or Graphpad Prism 8.2.1. Statistical analysis

692 were carried out using Graphpad Prism. One-tail ANOVA and multiple comparison T-test

693 were performed or unpaired two-tailed Student *t*-test as required.

694 All images presented in this manuscript are representative images. All IFAs were repeated at

695 least 2 times with same results. In case of the screen, 3 images of every well were taken and

696 selected candidates for further analysis were isolated and induce at least one more time to

697 verify the phenotype observed.

698

699 **Acknowledgement**

Author contributions

706 W.L. identified egress mutants and performed phenotypic assays for SLF and CGP. J.G.

707 designed and performed the phenotypic screen, identified and tagged candidate genes and

708 assisted with phenotyping. J.F.S. established and characterised the sCas9-system in *T.*

709 *gondii*, designed the sgRNA library and analysed egress for ADF. M.G. assisted with

710 phenotyping and analysis of parasites. J.P. assisted in experiment design and data analysis.

711 M.M. designed and coordinated the project and experiments, analysed the data, contributed

712 resources and wrote the paper. E.J-R. designed and coordinated the project and

713 experiments, analysed the data, and wrote the paper.

714

Competing interests

716 The authors declare no competing interests.

717

Figures Captions

719 **Figure 1. Adaptation and characterisation of the splitCas9-system in *T. gondii*. a,** Schematic of
720 the splitCas9-system. Transgenic parasites are generated co-expressing the two splitCas9 subunits
721 together with a single-guide RNA (sgRNA). **b,** Analysis of RHsCas9-gap40 parasites that were treated
722 with 50 nM rapamycin for 48h. Scale bars are 5µm. Three distinct phenotypes can be observed: the
723 gap40 phenotype, as described previously[6] (top panel, asterisk); an aberrant phenotype (bottom
724 panel); and parasites with normal IMC and GAP40 localisation (top panel, arrow). **c,** Quantification of
725 gap40 phenotypes shown in (b) in indicated parasite strains. Parasites were induced with or without
726 rapamycin for the indicated time and fixed 48 h post infection. Data represents three independent
727 experiments. For each condition 100 vacuoles were counted (total n=300). Mean and standard
728 deviation (SD) are represented. **d,** Analysis of RHsCas9-sag1 parasites that were treated with 50 nM
729 rapamycin for 48h before fixation using indicated antibodies. Scale bars are 5µm. Two distinct
730 phenotypes can be observed: healthy vacuoles lacking SAG1 (bottom panel, arrow); and parasites
731 lacking SAG1 while displaying aberrant nuclear and cellular morphology (bottom panel, asterisk). **e,**
732 Quantification of the phenotypes shown in (d). Parasites were treated with rapamycin for 1h or for the
733 whole growth period of 48h as indicated. Data represent three independent experiments. For each
734 condition, 100 vacuoles were counted (total n=300). Mean and SD are represented. **f,** Depiction of
735 indicator parasites co-expressing CbEm, FNR-RFP and the sCas9-subunits. Scale bar is 5 µm. **g,**
736 Analysis of indicator parasites expressing sgRNA targeting *sag1*. Scale bars are 5 µm. **h,** Analysis of

737 indicator parasites expressing indicated sgRNAs. IFA depicting the effect of *drpA* (RHsCas9-*drpA*), *adf*
738 (RHsCas9-*adf*), *act1* (RHsCas9-*act1*) and *frm2* (RHsCas9-*frm2*) disruption on actin network and
739 apicoplast segregation. Scale bars are 5 μm. Statistics: one-sided ANOVA with Turkey's multiple test
740 for comparison was used to analyse quantification data. ns: non-significant p > 0.9999 ; colour in p
741 values represent the condition analysed. Data presented as Mean + SD.

742 **Figure 2. Phenotypic screen for actin dynamics, apicoplast segregation and egress mutants. a,**
743 Scheme of the experimental design. Indicator parasites, RHsCas9-CbEm-FNR-RFP, were transfected
744 with the sgRNA library and grown in human foreskin fibroblasts (HFFs). Parasites were selected with
745 pyrimethamine and sorted into 96-well plates. After 7 days, they were split into replica plates. Two
746 replica plates were induced for 48h or 72h with rapamycin, before automated imaging was performed.
747 Candidate clones were subsequently picked from non-induced replica plates. **b,** Representation of the
748 selection of candidates in the screen. **i,** Representative images of parasites after 48h post induction
749 (hpi). Parasites were evaluated based on their aberrant morphology or nucleus. Clones presenting
750 aberrant phenotype in more than 70% of the screen were classified as replication phenotype. These
751 clones were not followed up in this study. From the clones that presented a maximum of 50% of
752 aberrant vacuoles, those presenting a change in filamentous network or in apicoplast morphology
753 were selected for further analysis. Scale bars are 25 μm. **ii,** Representative images of wells at 72 hpi
754 showing potential delay or blockage in egress are shown. Top row shows clones without egress
755 phenotype. Bottom row shows two examples of clones with a strong egress phenotype. Only clones
756 presenting a partial or total block in egress where selected. Scale bars, 30 μm. Finally, 2 candidates
757 were selected for further characterisation. **iii,** Images showing the phenotype of the two identified
758 egress mutants 48 hpi. Note that the apicoplast appears normal in both cases, while slight differences
759 in the F-actin network, which appears more prominent and condensed, can be observed in case of
760 TGGT1_208420. Parasites were induced for 48 h with rapamycin before fixing and imaging. Scale
761 bars, 5 μm.

762 **Figure 3. Analysis of *cgp* and *slf*. a,** Endogenously tagged TGGT1_240380 (*cgp*-Halo) localised at
763 the apical tip, while TGGT1_208420 (*slf*-Halo or *slf*-mCherry) demonstrated a dual localisation at the
764 apical region and the residual body. Scale bar: 5 μm. **b,** Analysis of conditional knockouts for *cgp* and
765 *slf* using the DiCre-system. IFA depicts floxed *cgp*-Halo (loxP*cgp*-Halo) and floxed *slf*-mCherry
766 (loxP*slf*-mCherry) induced or not with 50 nM rapamycin (Rapa). Dual labelling was performed to stain
767 the IMC (GAP45 or IMC1) of the parasite. Scale bar, 5 μm. **c,** Plaque assays of loxP*cgp*-Halo and
768 loxP*slf*-mCherry parasites confirm a severe growth defect upon deletion of *slf* or *cgp*. Scale bar,1.5
769 mm. **d,** Invasion-attachment assay for the indicated parasites lines. Parasites were pre-treated ± 50
770 nM rapamycin for 96 hours. Results were normalised to DiCreΔ*ku80* strain. For each condition, a
771 minimum of 150 vacuoles were counted per experiment in 3 biologically independent experiments. **e,**
772 Induced egress assay in the presence or absence of different inducers: Calcium ionophore (Ci)
773 A23187 (2 μM) for 5 min, BIPPO (50 μM) for 5 min, and Propranolol (125 μM). For each condition, a
774 minimum of 100 vacuoles were counted per experiment in 3 biologically independent experiments. **f,**
775 Quantification of trail deposition. Parasites were pretreated ± 50 nM rapamycin for 72 or 96 hours as
776 indicated. Results were normalised to DiCreΔ*ku80* strain. Number of trails were counted in 3
777 biologically independent replicates. **g and h,** Quantification of microneme secretion assay performed
778 on wildtype (WT) parasites, loxP*slf*-mCherry (**g**) and loxP*cgp*-Halo (**h**) after 72 hours treatment ± 50
779 nM rapamycin. This assay was performed in 3 biologically independent replicates. Values shown (%
780 secretion) are the ratio MIC2/GRA1 relative to the secretion in WT parasites. Representative images
781 are shown in Extended Data fig. 8j and 9g. Statistics in this figure: unpaired two tailed Student's *t*-test
782 were calculated in all graphs except in 3e where one-way analysis of variance (one-way ANOVA) with
783 Turkey's multiple comparison test was applied. Colour-coded *P* values represent the condition
784 analysed. Data are presented as Mean + SD. Dots represent the mean for each replica (n = 3).

785 **Figure 4. SLF and CGP act at different times during egress. a,** Egress of parasites expressing
786 CbEm labelling F-actin was induced with 50 μM BIPPO and imaged with an interval of 3 seconds
787 between frames. Yellow boxes show F-actin in intravacuolar network. Pink boxes show F-actin
788 polymerisation centre close to the apicoplast/Golgi region (insets have enhanced contrast for better
789 visualisation). Upper panel showing non-induced parasites as control (see movie_S3 and S4). Yellow
790 arrows: F-actin accumulation at the basal pole. Middle panel depicting F-actin in loxP*slf*-Halo parasites
791 previously induced with 50 nM rapamycin. Only parasites lacking signal for SLF were recorded (see

792 movie_S3). Bottom panel depicting F-actin in loxP*cgp*-Halo parasites previously induced with 50 nM
793 rapamycin. Only parasites lacking signal for CGP were recorded (see movie_S4). Blue arrow: no
794 relocalisation of F-actin to the basal pole. Time is displayed in minutes : seconds. Scale bar, 5 µm. **b,**
795 Quantification of the average relative fluorescence intensity of indicated parasites after induction of
796 egress with 50 µM BIPPO. Graphs show the average of indicated individual measurements ± SD. Red
797 arrows indicate the time where the F-actin network started to depolymerise. This time was set as t = 0
798 s for all movies evaluated. Time interval between each frame is 3 seconds. Each video analysed
799 represent a biologically independent replica (non-rapamycin induced n = 11, *cgp*-iKO n = 10 and *slf*-
800 iKO n = 9) and a representative image is shown in panel a. **c,** Egress of parasites expressing CbEm
801 (yellow) and SAG1ΔGPI-dsRed (pink) was induced with 50 µM BIPPO. Upper row images are
802 showing non-induced LoxP*cgp*-Halo parasites, upon induction, dsRed signal diffused into the host cell,
803 indicating lysis of parasitophorous vacuole membrane (PVM). Middle row depicting parasites lacking
804 CGP (LoxP*cgp*-Halo + Rapa) signal after rapamycin induction. Although the PVM lysed, parasites did
805 not move out of the cell. Bottom row depicting parasites lacking SLF signal after rapamycin induction
806 (LoxP*slf*-Halo + Rapa), where dsRed signal is kept within the PV suggesting intact PVM. Time is
807 displayed in minutes : seconds. Scale bar, 5 µm. See also movie_S5. This assay was performed in at
808 least 3 biologically independent experiments.

809 **Figure 5. SLF is a crucial part of the signalling platform. a,** GC, UGO and CDC50.1 colocalise with
810 SLF. Scale bar: 5 µm. **b,** Localisation of indicated components of the signalling platform upon deletion
811 of *slf*. Deletion of *slf-mCherry* results in mislocalisation of GC/UGO/CDC50.1. Analysis was performed
812 96 hours post induction. Scale bar, 5 µm. **c,** Deletion of *gc/ ugo/cdc50.1* (see also Extended Data Fig.
813 7 for generation of conditional KOs for individual components) results in mislocalisation of SLF at 96
814 hours post induction. Scale bar: 5 µm. These assays were performed at least twice with same results
815 and a representative image is here shown.

816 **Figure 6. CGP localises to the preconoidal rings and egress models. a,** STED microscopy of
817 indicated parasites. Colocalisation of CGP-Halo with RNG2 or SAS6L proteins in intracellular parasites
818 or extracellular parasites following conoid protrusion stimulation with 2 µM calcium ionophore A23187
819 for 10 min before fixation. White dash lines indicate parasite periphery. Scale bar, 5 µm for intracellular
820 parasites and 3 µm for extracellular parasites. Scale bar of insets: 0.5 µm. **b,** Absence of CGP did not
821 affect the localisation of RNG2 and SAS6L. Scale bar, 5 µm. Images in a and b were taken in at least
822 3 biologically independent experiments with same results and a representative image of each is
823 shown. **c,** Block of egress caused by deletion of SLF, CGP and ADF (see Extended Data Fig.3) is
824 intrinsically linked to the disassembly of the F-actin network. **1-3)** Natural egress process in a wildtype
825 parasite. **1)** At the start of the egress the signalling platform consisting of CDC50.1, GC, UGO and
826 SLF, localised at the plasma membrane of the apical tip and the intravacuolar network (IVN), initiates
827 a cascade leading to egress. Zoom in boxes: yellow arrows indicate the position of the signalling
828 platform at the apical tip and residual body. Purple arrow indicates the position of CGP at the conoid of
829 the parasites. **2)** As a first step towards egress, the IVN disassembles, which coincides with lysis of
830 the PVM. **3)** F-actin accumulates at the basal end of the parasite and motility is initiated. **4)** In the
831 absence of SLF, the signalling platform is mislocalised, resulting in early block of egress. No
832 disassembly of the intravacuolar network or PVM lysis occurs. **5)** Depletion of actin regulatory proteins
833 results in stabilisation of the network. Although parasites lyse the PVM and initiate motility, the network
834 keeps connecting individual parasites resulting in delayed or blocked egress (see Figure S3 and [11]). **6)**
835 Deletion of *cgp* results in a late block of egress. The signalling cascade appears intact, leading to
836 disassembly of the intravacuolar network and lysis of the PVM. Instead, no relocation of F-actin to the
837 basal pole can occur and motility is not initiated.

838

839

840

Data availability

All imaging data generated and used for this paper are available from the authors on reasonable request. The source data are provided as a Source Data file. Expression constructs and parasite strains have been deposited in Addgene (https://www.addgene.org/). Information on *T.gondii* genes and proteins were obtained in ToxoDB (release 30 to 56). Data obtained from sequencing have been deposited in SRA (https://www.ncbi.nlm.nih.gov/sra): Bioproject ID: PRJNA821386

References

1   Sidik, S. M. *et al.* A Genome-wide CRISPR Screen in Toxoplasma Identifies Essential Apicomplexan Genes. *Cell* **166**, 1423-1435.e1412, doi:10.1016/j.cell.2016.08.019 (2016).

2   Serpeloni, M. *et al.* UAP56 is a conserved crucial component of a divergent mRNA export pathway in Toxoplasma gondii. *Molecular microbiology* **102**, 672-689, doi: 10.1111/mmi.13485. (2016).

3   Zetsche, B., Volz, S. E. & Zhang, F. A split-Cas9 architecture for inducible genome editing and transcription modulation. *Nature biotechnology* **33**, 139-142, doi:10.1038/nbt.3149 (2015).

4   Periz, J. *et al.* Toxoplasma gondii F-actin forms an extensive filamentous network required for material exchange and parasite maturation. *eLife* **6**, doi:10.7554/eLife.24119 (2017).

5   Kim, K., Soldati, D. & Boothroyd, J. C. Gene replacement in Toxoplasma gondii with chloramphenicol acetyltransferase as selectable marker. *Science (New York, N.Y.)* **262**, 911-914, doi:10.1126/science.8235614 (1993).

6   Harding, C. R. *et al.* Gliding Associated Proteins Play Essential Roles during the Formation of the Inner Membrane Complex of Toxoplasma gondii. *PLoS pathogens* **12**, e1005403, doi:10.1371/journal.ppat.1005403 (2016).

7   Striepen, B. *et al.* The plastid of Toxoplasma gondii is divided by association with the centrosomes. *The Journal of cell biology* **151**, 1423-1434, doi:10.1083/jcb.151.7.1423 (2000).

8   van Dooren, G. G. *et al.* A novel dynamin-related protein has been recruited for apicoplast fission in Toxoplasma gondii. *Current biology : CB* **19**, 267-276, doi:10.1016/j.cub.2008.12.048 (2009).

9   Andenmatten, N. *et al.* Conditional genome engineering in Toxoplasma gondii uncovers alternative invasion mechanisms. *Nature methods* **10**, 125-127, doi:10.1038/nmeth.2301 (2013).

10   Del Rosario, M. *et al.* Apicomplexan F-actin is required for efficient nuclear entry during host cell invasion. *EMBO Rep*, e48896, doi:10.15252/embr.201948896 (2019).

11   Stortz, J. F. *et al.* Formin-2 drives polymerisation of actin filaments enabling segregation of apicoplasts and cytokinesis in Plasmodium falciparum. *eLife* **8**, doi:10.7554/eLife.49030 (2019).

12   Mehta, S. & Sibley, L. D. Actin depolymerizing factor controls actin turnover and gliding motility in Toxoplasma gondii. *Molecular biology of the cell* **22**, 1290-1299, doi:10.1091/mbc.E10-12-0939 (2011).

13   Plattner, F. *et al.* Toxoplasma profilin is essential for host cell invasion and TLR11-dependent induction of an interleukin-12 response. *Cell host & microbe* **3**, 77-87, doi:10.1016/j.chom.2008.01.001 (2008).

14   Sidik, S. M., Huet, D. & Lourido, S. CRISPR-Cas9-based genome-wide screening of Toxoplasma gondii. *Nature protocols* **13**, 307-323, doi:10.1038/nprot.2017.131 (2018).

886   15   Aquilini, E. *et al.* An Alveolata secretory machinery adapted to parasite host cell invasion. *Nat*
887   *Microbiol* **6**, 425-434, doi:10.1038/s41564-020-00854-z (2021).
888   16   Sidik, S. M. *et al.* A Genome-wide CRISPR Screen in Toxoplasma Identifies Essential
889   Apicomplexan Genes. *Cell* **166**, 1423-1435 e1412, doi: 10.1016/j.cell.2016.08.019. (2016).
890   17   Bisio, H., Lunghi, M., Brochet, M. & Soldati-Favre, D. Phosphatidic acid governs natural egress
891   in Toxoplasma gondii via a guanylate cyclase receptor platform. *Nature microbiology* **4**, 420-
892   428, doi:10.1038/s41564-018-0339-8 (2019).
893   18   Barylyuk, K. *et al.* A Comprehensive Subcellular Atlas of the Toxoplasma Proteome via
894   hyperLOPIT Provides Spatial Context for Protein Functions. *Cell host & microbe* **28**, 752-
895   766.e759, doi:10.1016/j.chom.2020.09.011 (2020).
896   19   Beck, J. R. *et al.* A novel family of Toxoplasma IMC proteins displays a hierarchical
897   organization and functions in coordinating parasite division. *PLoS pathogens* **6**, e1001094,
898   doi:10.1371/journal.ppat.1001094 (2010).
899   20   Andenmatten, N. *et al.* Conditional genome engineering in Toxoplasma gondii uncovers
900   alternative invasion mechanisms. *Nature methods* **10**, 125-127, doi: 10.1038/nmeth.2301.
901   (2013).
902   21   Bullen, H. E., Bisio, H. & Soldati-Favre, D. The triumvirate of signaling molecules controlling
903   Toxoplasma microneme exocytosis: Cyclic GMP, calcium, and phosphatidic acid. *PLoS Pathog*
904   **15**, e1007670, doi:10.1371/journal.ppat.1007670 (2019).
905   22   MacRae, J. I. *et al.* Mitochondrial metabolism of glucose and glutamine is required for
906   intracellular growth of Toxoplasma gondii. *Cell Host Microbe* **12**, 682-692,
907   doi:10.1016/j.chom.2012.09.013 (2012).
908   23   Fuks, J. M. *et al.* GABAergic signaling is linked to a hypermigratory phenotype in dendritic
909   cells infected by Toxoplasma gondii. *PLoS Pathog* **8**, e1003051,
910   doi:10.1371/journal.ppat.1003051 (2012).
911   24   Koreny, L. *et al.* Molecular characterization of the conoid complex in Toxoplasma reveals its
912   conservation in all apicomplexans, including Plasmodium species. *PLoS biology* **19**, e3001081,
913   doi:10.1371/journal.pbio.3001081 (2021).
914   25   Fisch, D. *et al.* Defining host-pathogen interactions employing an artificial intelligence
915   workflow. *eLife* **8**, doi:10.7554/eLife.40560 (2019).
916   26   Moen, E. *et al.* Deep learning for cellular image analysis. *Nat Methods* **16**, 1233-1246,
917   doi:10.1038/s41592-019-0403-1 (2019).
918   27   Jimenez-Ruiz, E., Wong, E. H., Pall, G. S. & Meissner, M. Advantages and disadvantages of
919   conditional systems for characterization of essential genes in Toxoplasma gondii.
920   *Parasitology* **141**, 1390-1398, doi:10.1017/s0031182014000559 (2014).
921   28   Meissner, M., Schluter, D. & Soldati, D. Role of Toxoplasma gondii myosin A in powering
922   parasite gliding and host cell invasion. *Science (New York, N.Y.)* **298**, 837-840,
923   doi:10.1126/science.1074553 (2002).
924   29   Sidik, S. M., Hackett, C. G., Tran, F., Westwood, N. J. & Lourido, S. Efficient genome
925   engineering of Toxoplasma gondii using CRISPR/Cas9. *PloS one* **9**, e100450,
926   doi:10.1371/journal.pone.0100450 (2014).
927   30   Donald, R. G., Carter, D., Ullman, B. & Roos, D. S. Insertional tagging, cloning, and expression
928   of the Toxoplasma gondii hypoxanthine-xanthine-guanine phosphoribosyltransferase gene.
929   Use as a selectable marker for stable transformation. *The Journal of biological chemistry* **271**,
930   14010-14019, doi:10.1074/jbc.271.24.14010 (1996).
931   31   Peng, D. & Tarleton, R. EuPaGDT: a web tool tailored to design CRISPR guide RNAs for
932   eukaryotic pathogens. *Microb Genom* **1**, e000033, doi:10.1099/mgen.0.000033 (2015).
933   32   Curt-Varesano, A., Braun, L., Ranquet, C., Hakimi, M. A. & Bougdour, A. The aspartyl protease
934   TgASP5 mediates the export of the Toxoplasma GRA16 and GRA24 effectors into host cells.
935   *Cell Microbiol* **18**, 151-167, doi:10.1111/cmi.12498 (2016).
936   33   Hunt, A. *et al.* Differential requirements for cyclase-associated protein (CAP) in actin-
937   dependent processes of Toxoplasma gondii. *eLife* **8**, doi:10.7554/eLife.50598 (2019).

938   34   Egarter, S. *et al.* The toxoplasma Acto-MyoA motor complex is important but not essential for
939   gliding motility and host cell invasion. *PloS one* **9**, e91819, doi:10.1371/journal.pone.0091819
940   (2014).
941   35   Periz, J. *et al.* A highly dynamic F-actin network regulates transport and recycling of
942   micronemes in Toxoplasma gondii vacuoles. *Nature communications* **10**, 4183,
943   doi:10.1038/s41467-019-12136-2 (2019).

944

945

a

p5RT70 | N-term Cas9 | FRB // HX
p5RT70 | FKBP | Cas9 C-term
pU6 | sgRNA // DHFR

N

- Rapamycin
+ 50 nM Rapamycin

N-terminus Cas9
sgRNA
C-terminus Cas9
GOI

DSB — NHEJ — INDEL

b

RHsCas9-*gap40*

DIC | DAPI | α-IMC | α-GAP40 | Merged

c

p < 0.0001 / ns / p < 0.0001
p < 0.0001 / ns / p < 0.0001
p < 0.0001 / p = 0.0008 / p < 0.0001
ns / p = 0.0056 / p = 0.0003
ns / p = 0.0056 / p = 0.0003
ns / ns / ns
ns / ns / ns

48h | 1h | No Rapa | 48h | No Rapa | 48h | No Rapa
50nM Rapa | 50nM Rapa | 50nM Rapa | 50nM Rapa
RHsCas9_gap40sgRNA | RH_gap40sgRNA | RHsCas9

■ gap40 phenotype ■ partial gap40 phenotype ■ no gap40 phenotype

d

RHsCas9
RHsCas9-*sag1*
-Rapa
+Rapa

DIC | DAPI | α-SAG1 | α-MIC8 | Merged

e

p < 0.0001 / p < 0.0001 / p < 0.0001
ns / ns / ns
p < 0.0001 / p < 0.0001 / p = 0.0044
ns / ns / ns
p < 0.0001 / p < 0.0001 / p < 0.0001

48h | 1h | No Rapa | 1h | 48h | No Rapa
50nM Rapa | No Rapa | 50nM Rapa | 50nM Rapa
1st lytic cycle | 2nd lytic cycle
RHsCas9_sag1sgRNA1 | RHsCas9

■ SAG1 negative ■ aberrant nuclei and cellular morphology ■ SAG1 positive

f

CbEm | FNR-RFP | Merged/DAPI

g

RHsCas9-sag1
- Rapa
+Rapa

DAPI | CbEm | α-SAG1 | Merged

h

wt
RHsCas9-*act1*
RHsCas9-*frm2*
RHsCas9-*adf*
RHsCas9-*drpA*

DAPI | CbEm | Apicoplast | Merged

a

in culture

Fixation
48hpi / 72hpi   - rapamycin

Automated imaging
20x, N.A. of 0.75   + rapamycin

x10

drug

FACS

x2

x2

b

Isolated clones (608)

48 hours

72 hours

i

> 70 % aberrant morphology or nuclei

FNR-RFP   CbEm   Hoechst   Merged

Nuclear / Replication Phenotype

9B11   16.9 %

≤ 50 % aberrant morphology or nuclei

FNR-RFP   CbEm   Hoechst   Merged

No relevant Phenotype

4A2   61.2 %

ii

No egress phenotype

6A4   5B8   92.2 %

Apicoplast phenotype

7F9   6.3 %

F-actin phenotype

9F10   15.6 %

Egress phenotype

4H10   5D11   7.8 %

2 candidates for characterisation

iii

RHsCas9 strains

FNR-RFP   CbEm   Hoechst   Merged

- Rapa   RHsCas9

+ Rapa   TGGT1_240380

TGGT1_208420

a

cgp-Halo:
- Janelia 646 | α-ISP1 | Merged
- Janelia 646 | α-IMC1 | Merged

slf-Halo:
- Oregon Green | α-ISP1 | Merged

slf-mCherry:
- α-RFP | α-IMC1 | Merged

b

loxP*cgp*-Halo:
- Oregon Green | α-GAP45 | Merged (−Rapa, +Rapa)

loxP*slf*-mCherry:
- α-RFP | α-IMC1 | Merged (−Rapa, +Rapa)

c

loxP*cgp*-Halo: − Rapa | + Rapa
loxP*slf*-mCherry: − Rapa | + Rapa

d

Invasion% normalised

| Rapa | + | − | − | + | + |
| DiCreΔKu80 | loxP*cgp*-Halo | loxP*slf*-Halo | loxP*cgp*-Halo | loxP*slf*-Halo |

P < 0.0001
P < 0.0001

e

Legend: No inducer | Bippo | Ci A23187 | Propranolol

Egressed vacuoles %

P = 0.0005, < 0.0001, 0.0004, < 0.0001
P = 0.0010, < 0.0001, < 0.0001, < 0.0001
P = 0.0002, < 0.0001, < 0.0001, < 0.0001
P = 0.0002, < 0.0001, 0.0012, < 0.0001

P = 0.8221
P = 0.0447
P = 0.1509

P = 0.9313
P < 0.0001
P < 0.0001

Rapa: + + + + / − − − − / − − − − / + + + + / + + + +

DiCreΔKu80 | loxP*cgp*-Halo | loxP*slf*-mCherry | loxP*cgp*-Halo | loxP*slf*-mCherry

f

Gliding % normalised

72 hpi | 96 hpi

Legend: −Rapa | +Rapa

P = 0.0083
P = 0.0006
P = 0.0004
P = 0.0087

DiCreΔKu80 | loxP*cgp*-Halo | DiCreΔKu80 | loxP*slf*-mCherry

g

% normalised microneme secretion

P = 0.0052
P = 0.0117
P < 0.0001
P = 0.0177

−Rapa +Rapa | −Rapa +Rapa | −Rapa +Rapa | −Rapa +Rapa
DicreΔku80 | loxPslf-mCherry | DicreΔku80 | loxPslf-mCherry
A23187 | BIPPO

h

% normalised microneme secretion

P = 0.7049
P = 0.8489

−Rapa +Rapa | −Rapa +Rapa
DicreΔku80 | loxPcgp-Halo
A23187

a

LoxP*slf*-Halo -Rapa + BIPPO

LoxP*slf*-Halo (cKO) +Rapa +BIPPO

LoxP*cgp*-Halo (cKO) +Rapa +BIPPO

b

LoxP*cgp*-Halo -Rapa + BIPPO

LoxP*cgp*-Halo (cKO) +Rapa +BIPPO

LoxP*slf*-Halo (cKO) +Rapa +BIPPO

Relative fluorescence intensity

seconds

Apicoplast region   Basal part

c

LoxP*cgp*-Halo -Rapa + BIPPO

LoxP*cgp*-Halo (cKO) +Rapa +BIPPO

LoxP*slf*-Halo (cKO) +Rapa +BIPPO

a

loxP*slf*-mCherry/*gc*-3HA

α-RFP α-HA Merged

loxP*slf*-mCherry/*ugo*-3HA

α-RFP α-HA Merged

loxP*slf*-mCherry/*cdc50.1*-SYFP2

α-RFP α-GFP Merged

b

loxP*slf*-mCherry/*gc*-3HA

α-RFP α-HA Merged/Hoechst

-Rapa

+Rapa

loxP*slf*-mCherry/*ugo*-SYFP2

α-RFP α-GFP Merged/Hoechst

-Rapa

+Rapa

loxP*slf*-mCherry/*cdc50.1*-SYFP2

α-RFP α-GFP Merged/Hoechst

-Rapa

+Rapa

c

loxP*gc*-SYFP2/*slf*-Halo

α-GFP Janelia 646 Merged/Hoechst

-Rapa

+Rapa

loxP*ugo*-SYFP2/*slf*-Halo

α-GFP Janelia 646 Merged/Hoechst

-Rapa

+Rapa

loxP*cdc50.1*-SYFP2/*slf*-Halo

α-GFP Janelia 646 Merged/Hoechst

-Rapa

+Rapa

a

loxP*cgp*-Halo/*rng2*-6HA Intracellular parasites

Merged | Janelia 549 | α-HA | Merged

loxP*cgp*-Halo/*rng2*-6HA Extracellular parasites

Merged | Janelia 549 | α-HA | Merged

loxP*cgp*-Halo/*sas6l*-eGFP Intracellular parasites

Merged | Janelia 549 | α-GFP | Merged

loxP*cgp*-Halo/*sas6l*-eGFP Extracellular parasites

Merged | Janelia 646 | α-GFP | Merged

b

Janelia 646 | α-HA | Merged/ GAP45

loxP*cgp*-Halo/*rng2*-6HA

-Rapa

+Rapa

Janelia 646 | eGFP | Merged/ GAP45

loxP*cgp*-Halo/*sas6l*-eGFP

-Rapa

+Rapa

c

4 — *slf*-cKO

1

SLF | UGO | CDC50.1
GC
Plasma membrane

Egress signalling

2

IVN disassembly and PVM rupture

3

Movement initiation

5 — *adf*-cKO

6 — *cgp*-cKO

a

p = 0.0004 / p = 0.0001 / p = 0.939

120

100

80

60

40

20

0

RHΔKu80   RH

■ Lack of SAG1
■ aberrant nuclei and morphology (no SAG1)
■ SAG1 (no phenotype)

b

sag1 wt   AAGGCAGTGAGACGCGCCGTCAC−GGCAGGGGT

RHsCas9-Δhx-sag1-1-KO AAGGCAGTGAGACGCGCCGTCACGGGCAGGGGT

c

sag1   p5RT70 sag1*
WT   TGAGACGCGCCGTCACGGCA
Mut   TcAGgCGgCgGTgACGGCt
Amino AlaValArgArgAlaValTheAlaGly

sag1KO-sag1*

sag1   p5RT70 sag1*
WT   TGAGACGCGCCGTCACGGCA
Mut   TcAGgCGgCgGTgACGGCt
Amino AlaValArgArgAlaValTheAlaGly

sag1wt-sag1*

RHsCas9-sag1

d

DAPI   α-SAG1   α-GAP45   Merge

sag1KO-sag1*
No Rapa
50 nM Rapa

sag1wt-sag1*
No Rapa
50 nM Rapa

e

+ Rapa   − Rapa

100

80

60

40

20

0

p < 0.0001

p < 0.0001

sag1wt-sag1*   sag1KO-sag1*

Aberrant nuclei and cellular morphology (%)

f

120

100

80

60

40

20

0

p < 0.0001
p < 0.0001
p < 0.0001
p < 0.0001

RHsCas9-sag1   RHsCas9-gap40   RHsCas9-act1   RHsCas9-frm2   RHsCas9-drpA

■ Normal nuclear and cell morphology   ■ Abnormal nuclear or cell morphology

a

RHsCas9-adf

intracellular | egress

CbEm/α-GAP45 | CbEm/α-GAP45

+Rapa

−Rapa

- / + / - / +

intravacuolar filaments

+Rapa / −Rapa

- / + / - / +

Remains of intravacuolar filaments after egress

RHsCas9-sag1

intracellular | egress

CbEm/α-SAG1 | CbEm/α-SAG1

−Rapa

+Rapa

−Rapa

+Rapa

Remains of intravacuolar filaments after egress

b

+ 50 nM Rapa

% Egress

p < 0.0001
p < 0.0001
p = 0.0003

RHsCas9-sag1 | RHsCas9-adf | RHsCas9-act-1

+ 0 nM Rapa

% Egress

p = 0.6043
p = 0.5057
p = 0.982

RHsCas9-sag1 | RHsCas9-adf | RHsCas9-act-1

c

RHsCas9-sag1

p = 0.0008
p > 0.9999
p = 0.9995
p = 0.7997

intracellular | egress | intracellular | egress | intracellular

no A23187, no Rapa | 2µM A23187, no Rapa | 2µM A23187, 50nM Rapa

■ intravacuolar filaments present   ■ intravacuolar filaments absent

RHsCas9-adf

p = 0.0007
p < 0.0001
p = 0.6907
p = 0.664

intracellular | egress | intracellular | egress | intracellular

no A23187, no Rapa | 2µM A23187, no Rapa | 2µM A23187, 50nM Rapa

■ intravacuolar filaments present   ■ intravacuolar filaments absent

a

| FNR-RFP | CbEm | Hoechst | Merged |

b

| FNR-RFP | CbEm | Hoechst | Merged |

c DAPI F-actin Apicoplast

d DAPI F-actin Apicoplast

e DAPI F-actin Apicoplast

f DAPI F-actin Apicoplast

g DAPI F-actin Apicoplast

h DAPI F-actin Apicoplast

i

| FNR-RFP | CbEm | Hoechst | Merged |

a

RHsCas9 strains + rapamycin | Tagged strains

FNR-RFP | CbEm | Hoechst | Merged | DIC | Tag

248490 — 3HA
229460 — YFP
301410 — mCherry
286790 — 3HA
255920 — 3HA
210490 — 3HA

b

294930 — YFP

c

269700 — mCherry
249970 — 3HA
263680 — 3HA
259720 — mCherry
226320 — SNAP

a

Egressed vacuoles % normalised

RHsCas9
RHsCas9-sag1 — *P* = 0.5398
RHsCas9-act1 — *P* < 0.0001
RHsCas9-240380 — *P* = 0.0117
RHsCas9-208420 — *P* = 0.0169
RHsCas9-214790 — *P* = 0.0511
RHsCas9-273100 — *P* = 0.2668
RHsCas9-248640 — *P* = 0.0488
RHsCas9-252465 — *P* = 0.0210
RHsCas9-216000 — *P* = 0.1552
RHsCas9-305340 — *P* = 0.0296
RHsCas9-209100 — *P* = 0.9693
RHsCas9-294930 — *P* = 0.4464

b

+Rapa+Ci   -Rapa+Ci

RHsCas9
RHsCas9-sag1
RHsCas9-act1
RHsCas9-252465
RHsCas9-248640
RHsCas9-208420
RHsCas9-240380

c

% of vacuoles

Legend: 2 (grey), 4 (pink), 8 (blue), ≥16 (green)

P = 0.8568, 0.7048, 0.6574, 0.6634
P = 0.0225, 0.0275, 0.0084, 0.0459
P = 0.0152, 0.0045, 0.0320, 0.3474
P = 0.0459, 0.0866, 0.0600, 0.5665
P = 0.6596, 0.1596, 0.4619, 0.8279

Rapa: − + (RHsCas9), − + (RHsCas9-248640), − + (RHsCas9-252465), − + (RHsCas9-208420), − + (RHsCas9-240380)

d

Invasion% normalised

RHsCas9-248640 — *P* = 0.0273
RHsCas9-252465 — *P* = 0.6536
RHsCas9-208420 — *P* = 0.0002
RHsCas9-240380 — *P* < 0.0001

a

loxP*slf*-Halo/CbEm

Janelia 646   α-GFP   Merged

b

KO efficiency %

P < 0.0001
P < 0.0001
P = 0.0005
P = 0.0407
P = 0.0107

24h   48h   72h   96h

c

≥16   8
4   2

Vacuoles %

P = 0.3384, 0.4613, 0.0460, 0.5854
P = 0.3329, 0.7148, 0.0373, 0.7968

Rapa   −   −   +

DiCreΔ*Ku80*   LoxP*slf*-mCherry

d

Invasion normalised %

P = 0.0005
P = 0.0004

Rapa   +   −   +

DiCreΔ*Ku80*   loxP*slf*-Halo

e

i)

loxP*slf*-Halo

Total displacement (µm)

P = 0.1563

Rapa   −   +

ii)

loxP*slf*-Halo

Average speed (µm/s)

P = 0.7689

Rapa   −   +

f

−Rapa   +Rapa

−A23187

+A23187

g

α-RFP   MIC2   Merged

−Rapa

+Rapa

α-RFP   AMA1   Merged

−Rapa

+Rapa

h

α-RFP   ROP1   Merged   Hoechst

−Rapa

+Rapa

α-RFP   ROP2-4   Merged   Hoechst

−Rapa

+Rapa

i

mCherry   HSP60   Merged   Hoechst

−Rapa

−Rapa

j

Ci A23187   BIPPO   DMSO

WT   loxP*slf*-mCh   WT   loxP*slf*-mCh   WT   loxP*slf*-mCh

kDa   Rapa   − +   − +   − +   − +   − +   − +

90 —   MIC2

25 —   GRA1

a

b

P = 0.1570, P = 0.0757, P = 0.1956, P = 0.6193
P = 0.4861, P = 0.1582, P = 0.2474, P = 0.5107

≥16   8
4   2

Vacuoles %

Rapa -   -   +

DiCreΔKu80   LoxPcgp-Halo

c

i)   ii)

loxPcgp-Halo   loxPcgp-Halo

Total displacement (μm)   Average gliding speed (μm/s)

P < 0.0001   P < 0.0001

Rapa -   +   Rapa -   +

d

CGP   HSP60   Merged

-Rapa

+Rapa

e

CGP   MIC2   Merged   CGP   MIC6   Merged   CGP   MIC8   Merged

-Rapa   -Rapa   -Rapa

+Rapa   +Rapa   +Rapa

f

CGP   ROP1   Merged   CGP   ROP2-4   Merged

-Rapa   -Rapa

+Rapa   +Rapa

g

              Ci A23187   DMSO
                    loxPcgp   loxPcgp
        WT   -Halo   WT   -Halo
kDa Rapa -   +   -   +   -   +   -   +

90-   MIC2

25-   GRA1

a

Intact IVN

IVN disassembled

No posterior accumulation

Posterior accumulation

b

■ Intact IVN
■ No accumulation at basal pole
■ Accumulation at basal pole

P = 0.0009, 0.3963, 0.0003

Vacuoles %

Rapa − + +
A23187 − − +

c

GABA

0 mM 100 nM 1 μM 500 μM 1 mM 10 mM 15 mM

H₂O vehicle control

Vol. = 10mMGABA Vol. = 15mMGABA

GABA no parasites

10 mM 15 mM

−Rapa

+Rapa

d

Gabapentin

0 nM 100 nM 1 μM 500 μM 1 mM 5 mM

H₂O

5 mM

Gabapentin no parasites

5 mM

−Rapa

+Rapa

e

IMC MIC8 5 μm

CbEm mChTub

CbEm HSP60

f

DAPI CbEm mChTub Merged DIC

−Rapa 48h

+Rapa 48h

g

P = 0.9805

P < 0.0001

P < 0.0001

P < 0.0001

− Rapa + Rapa
sCa9CbEm-mChTUB-αtub gRNA

− Rapa + Rapa
sCa9CbEm-mChTUB-sag1 gRNA

■ Aberrant morphology or nuclei ■ No phenotype