1 **Title: Formin-2 drives intracellular polymerisation of actin filaments enabling correct segregation**

2 **of apicoplasts in *Plasmodium falciparum* and *Toxoplasma gondii*.**

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21   **Abbreviations:**

22   C2, Compound 2; FV, food vacuole; F-actin, filamentous actin; PfACT1, *P. falciparum* actin-1; PfFRM1,

23   *P. falciparum* Formin-1; PfFRM2, *P. falciparum* Formin-2; TgFRM1, Toxoplasma gondii Formin-1;

24   TgFRM2, Toxoplasma gondii Formin-2; PV, parasitophorous vacuole; RAP, rapamycin; RON4, rhoptry

25   neck protein-4; TJ, tight junction, IMC, inner membrane complex. PCR, Polymerase chain reaction;

26   IFA, (indirect) Immunofluorescence assay; HA, Haemagglutinin; YFP, yellow fluorescent protein; RT,

27   room temperature.

28   **Abstract**

29   Pathogenic obligate-intracellular apicomplexan parasites possess an essential chloroplast-like

30   organelle called the apicoplast that undergoes division and segregation during replication. Parasite

31   actin is essential during intracellular development, implicated in vesicular transport, parasite

32   replication and apicoplast inheritance. However, the inability to visualise live actin dynamics in

33   apicomplexan parasites limited functional characterisation of both filamentous-actin (F-actin) and

34   actin regulatory factors. Apicomplexans possess at least two distinct formins, Formin-1 and Formin-

35   2, predicted to serve as actin-nucleating factors, and previously implicated in regulating gliding

36   motility and host cell invasion. Here, we expressed chromobodies and validated them as F-actin-

37   binding sensors in *Plasmodium falciparum* and characterised the *in vivo* dynamics of the F-actin

38   network. The F-actin network could be modulated chemically and disrupted by conditionally deleting

39   the *actin-1* gene. In a comparative approach, we demonstrate that Formin-2 is closely associated

40   with apicoplasts and with the F-actin network in *P. falciparum* and *Toxoplasma gondii*.

41   Consequently, disruption of Formin-2 resulted not only in an apicoplast segregation defect, but also

42   in complete abrogation of F-actin dynamics in intracellular parasites. Together, our results strongly

43   indicate that Formin-2-mediated filament formation is the common primary mechanism for F-actin

44   nucleation during apicomplexan intracellular growth effecting apicoplast segregation.

45 **Introduction**

46   The phylum Apicomplexa includes a variety of obligate intracellular parasites, which invade into and

47   replicate inside mammalian cells, causing immense disease burden in humans and in commercially

48   important livestock. One of its notorious members, the malaria parasite *Plasmodium falciparum*, is a

49   major health concern in developing nations, causing ~500,000 deaths annually (White,

50   Pukrittayakamee et al. 2014). Another member, *Toxoplasma* is a highly successful parasite infecting

51   almost a third of the global human population and can be fatal in immunocompromised patients

52   (Torgerson and Mastroiacovo 2013). There is limited success in the development of vaccines against

53   these parasites and the current drugs are associated with drug resistance, making it crucial to

54   investigate novel biological targets.

55   Actin is one of the most abundant proteins in eukaryotic cells. Due to its ability to form polymers,

56   this cytoskeletal protein is involved in numerous processes such as cell motility, cytokinesis,

57   organellar and vesicular transport, secretion and endocytosis (Svitkina 2018). Actins encoded by

58   apicomplexan parasites are highly divergent compared to canonical actins from other eukaryotes

59   (Douglas, Nandekar et al. 2018). *In vitro*, apicomplexan actins form only short, unstable polymers

60   due to different polymerisation kinetics, caused by variation of certain key amino acids, otherwise

61   conserved in metazoans (Kumpula and Kursula 2015). However, until recently an analysis of F-actin

62   localisation and dynamics in apicomplexan parasites was hindered by the unavailability of F-actin

63   sensors (Tardieux 2017), a limitation recently overcome by the expression of F-actin binding

64   chromobodies in *T.gondii* (Periz, Whitelaw et al. 2017). Intriguingly, in this parasite, F-actin can form

65   an extensive intra-vacuolar network that appears to be involved in material exchange and

66   synchronisation of parasite division (Periz, Whitelaw et al. 2017).

67   Until recently, studies on apicomplexan F-actin focused on its critical role during host cell invasion

68   and gliding motility (Soldati, Foth et al. 2004, Baum, Gilberger et al. 2008), where it is believed to

69   provide the force for both processes (Frenal, Dubremetz et al. 2017). However, recent studies using

70 conditional mutants for *actin-1* in two apicomplexans, *P. falciparum* and *T. gondii* highlight

71 additional critical roles of F-actin during intracellular parasite development (Das, Lemgruber et al.

72 2017, Periz, Whitelaw et al. 2017, Whitelaw, Latorre-Barragan et al. 2017). Intriguingly, some

73 functions, such as inheritance of the chloroplast-like organelle, the apicoplast, appears to be

74 conserved (Andenmatten, Egarter et al. 2013, Egarter, Andenmatten et al. 2014, Das, Lemgruber et

75 al. 2017, Whitelaw, Latorre-Barragan et al. 2017), while differences for the dependency of F-actin

76 can be observed for other critical steps of the asexual life cycle. For example, host cell invasion is

77 possible without *actin-1* (albeit at highly reduced levels) in case of *T. gondii* (Andenmatten, Egarter

78 et al. 2013, Egarter, Andenmatten et al. 2014, Whitelaw, Latorre-Barragan et al. 2017), while it is

79 completely blocked in case of *P. falciparum* (Das, Lemgruber et al. 2017)*. In contrast, *P. falciparum*

80 does not require actin dynamics for egress from the host cell (Das, Lemgruber et al. 2017, Perrin,

81 Collins et al. 2018)*, while it is essential for *T.gondii.*

82 Of the two actin genes present in *P. falciparum* (Gardner, Hall et al. 2002), only *actin-1* (*pfact1*) is

83 expressed in all life-cycle stages and is the only actin expressed during asexual replicative stages,

84 whereas *actin-2* expression is confined to the sexual gametocyte and insect stages (Vahokoski,

85 Bhargav et al. 2014). *P. falciparum* undergoes a 48h asexual replicative cycle in the intermediate

86 human host where it invades into, grows and replicates within erythrocytes, causing all clinical

87 manifestations of the disease. After invasion, the merozoite form of the parasite (similar to *T. gondii*

88 tachyzoites) establishes itself within a parasitophorous vacuole (PV), loses its ovoid shape to become

89 amoeboid and feeds on host haemoglobin creating a food vacuole (FV) where haem is detoxified

90 (Gruring, Heiber et al. 2011). The parasite then replicates by a process best described as internal

91 budding, where daughter parasites develop within the mother (Francia and Striepen 2014). In the

92 case of *T. gondii*, only two daughters are formed at a time in a process called endodyogeny. In

93 contrast, malaria replication within the erythrocyte, termed schizogony, results in the formation of

94 16-32 merozoites at once. Towards the end of a replicative cycle the parasite *de novo* forms its

95 invasion-related organelles: the inner membrane complex (IMC), micronemes and rhoptries. In

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96   contrast, parasite mitochondria and the apicoplast undergo growth and division and are trafficked

97   into each daughter cell (Bannister, Hopkins et al. 2000). Although endodyogeny and schizogony

98   appear very different, it is believed that both processes are very similar and use conserved molecular

99   machinery. Indeed, independent studies identified the same factors to be critical for both replicative

100   modes (Francia and Striepen, 2014).

101   Despite this, it could be assumed that differences, especially with respect to vesicular transport

102   processes such as endocytosis and intravacuolar parasite communication, exist to adapt to different

103   replication modes. This puts F-actin in the spotlight, since it plays a central role in these processes, as

104   is the case in other eukaryotes (Svitkina 2018). We recently characterised a conditional mutant of

105   PfACT1 and observed that in good agreement with the function of actin in *T. gondii* (Andenmatten,

106   Egarter et al. 2013), inheritance of the apicoplast is compromised during schizogony (Das, Lemgruber

107   et al. 2017). While the phenotypic analysis of conditional mutants is useful to identify conserved and

108   unique functions of F-actin in apicomplexans, the inability to visualise F-actin in these parasites led

109   to models, sometimes conflicting with each other and with the canonical behaviour of F-actin in

110   other eukaryotes.

111   Common actin-labelling probes such as Phalloidin do not label apicomplexan actin and LifeAct could

112   not be successfully expressed in these parasites (Periz, Whitelaw et al. 2017). Recently, actin-binding

113   single-domain nanobodies tagged to fluorescent probes, called chromobodies were successfully

114   expressed in *T. gondii* and shown to have minimal effect on actin dynamics (Periz, Whitelaw et al.

115   2017), as also demonstrated in other eukaryotic cells (Rocchetti, Hawes et al. 2014, Panza, Maier et

116   al. 2015, Melak, Plessner et al. 2017).

117   Here we adapted this technology to *P. falciparum* and demonstrate for the first time the localisation,

118   dynamics and role of F-actin dynamics for parasite development in asexual stages. Interestingly, we

119   find F-actin closely associated with the apicoplast throughout intracellular growth, leading to the

120   question of which actin regulatory proteins are involved in this process. Most actin nucleation

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121   proteins such as the Arp2/3 complex and the WAVE/WASP complex, and actin cross-linkers such as

122   α-actinin and fimbrin are missing in apicomplexans (Baum, Papenfuss et al. 2006, Schuler and

123   Matuschewski 2006). Two conserved nucleators found in *P. falciparum* are the formins, Formin-1

124   (PfFRM1) and Formin-2 (PfFRM2) which localise to distinct compartments in the cell (Baum, Tonkin

125   et al. 2008). Orthologs of both formins have been implicated in host cell invasion in *T.gondii* (Daher,

126   Plattner et al. 2010), with *T. gondii* Formin-2 (TgFRM2) also being implicated in apicoplast

127   maintenance (Jacot, Daher et al. 2013) – leading to inconsistencies in reports and questions whether

128   the two formins have conserved or divergent functions in both parasites.

129   Here we reanalysed the role of Formin-2 in *P. falciparum* and *T. gondii* and, in contrast to previous

130   reports, demonstrate that it localises adjacent to apicoplasts in both parasites. Conditional

131   disruption of Formin-2 not only results in a complete abrogation of actin dynamics in *P. falciparum*

132   and *T. gondii,* it also leads to loss of the apicoplast. Together our study highlights a highly conserved

133   role of Formin-2 in the intracellular development of apicomplexan parasites. Importantly, apicoplast

134   loss appears to be not the only critical phenotype caused by Formin-2 depletion, since in *P.*

135   *falciparum* the loss of fitness due to the deletion of PfFRM2 cannot be complemented by addition of

136   isopentenyl pyrophosphate (IPP), the only essential metabolite produced by apicoplasts (Yeh and

137   DeRisi 2011), suggesting other critical roles of Formin2-mediated actin nucleation in these parasites.

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139   **Results**

140   **1. Cellular expression of chromobodies in *P. falciparum* enables the visualisation of an actin**

141   **network throughout the asexual development of *P. falciparum* and in gametocytes.**

142   **a. Chromobodies label F-actin structures in *P. falciparum* asexual stages and in gametocytes.**

143   Chromobodies were expressed under the *heat shock protein 86 (hsp86)* promoter (Crabb and

144   Cowman 1996) to obtain expression throughout the 48h asexual life cycle. We succeeded in

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145   generating parasites stably expressing chromobodies tagged either with the emerald tag (CB-EME)

146   or the halo tag (CB-HALO), indicating that the expression of these constructs does not have a major

147   deleterious impact on the fitness of *P. falciparum* **(Fig. 1A)**, as previously reported for *Toxoplasma*

148   (Periz, Whitelaw et al. 2017) and other eukaryotes . The halo tag allowed visualisation of F-actin in

149   live parasites by use of the red ligand Halo-TMR. Dynamic filamentous structures were evident in

150   both CB-EME and CB-HALO expressing parasites **(Fig 1B)** throughout the 48 hour life cycle **(Videos**

151   **V1, V2, V3 and V4).** These structures could be completely disrupted by adding the F-actin

152   destabilising drug cytochalasin-D **(Fig. 1B and Video V3)**, demonstrating that chromobodies bind F-

153   actin structures in *P. falciparum*. However, while both CB-versions labelled similar structures, we

154   found that expression of CB-EME resulted in a better signal-to-noise ratio, probably because no

155   permeable, fluorescent ligand needs to be added **(Fig.1B)**. Therefore, for the rest of this study results

156   for parasites expressing CB-EME are presented. Some F-actin structures were highly dynamic,

157   changing within a time-scales of seconds, while other structures appeared stable over tens of

158   seconds **(Video V3)**. For better visualisation of F-actin structures, we used super-resolution

159   microscopy (SR-SIM) which enabled us to observe a complex F-actin network in these parasites **(Figs.**

160   **1C and S1),** similar to a network observed for *T.gondii* (Periz, Whitelaw et al. 2017). Since CB-EME

161   was expressed from an episome, we quantified mean intensity of fluorescence in different cells and

162   found it to be within a narrow range **(Fig. S1B)**. Interestingly, F-actin was most prominent around the

163   FV of the parasite **(Figs. 1C and S1)**. When we co-stained the chromobody-labelled network with an

164   antibody which specifically recognises parasite actin, we observed a rather cytosolic distribution

165   with the antibody, although similar structures as seen with chromobody were apparent. This

166   indicates that the antibody recognises monomeric globular actin (G-actin) along with F-actin, while

167   the chromobody recognises primarily filamentous structures **(Fig. 1C and Fig S1)**. In contrast to

168   asexual parasites, gametocytes express both PfACT1 and actin-2, and exhibit F-actin staining along

169   the length of the parasite and at the tips (Hliscs, Millet et al. 2015). Upon expression of CB-EME,

170   gametocytes showed intense dynamic F-actin structures at their tips and running along the whole

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171   body of the cell changing in seconds **(Fig. 1D and Video V5)**. Importantly, this dynamic network

172   appears very similar to the one reported by Hlisc et al., 2015, which has been shown to lie beneath

173   the IMC of the gametocyte. It is important to note that chromobodies do not distinguish between

174   PfACT1 and actin-2 and therefore the observed filaments could be built from both proteins.

175   Together, our data show that expression of chromobodies does not cause significant phenotypic

176   effects and allow reliable labelling of the F-actin cytoskeleton. We also confirm previous data

177   obtained with antibodies directed against F-actin and show that during the gametocyte stage, F-

178   actin forms a dynamic and extensive network that passes through the whole cell and is enriched at

179   the tips of the parasite.

180   **b. Highly dynamic F-actin-rich filopodia-like structures extend outward from the periphery of**

181   **growing parasites.**

182   After erythrocyte invasion, the parasite immediately loses its ovoid zoite structure and becomes an

183   amoeboid ring-stage parasite. These young parasites are highly dynamic and can switch between

184   various shapes forming multi-lobed structures, possibly mediated by their cytoskeletal networks

185   (Gruring, Heiber et al. 2011). On observing chromobody-expressing parasites during ring and early

186   trophozoite stages we noted F-actin rich projections at the periphery of ~60% parasites (n=50) **(Fig.**

187   **2A, B and Video V2)**. These F-actin projections are highly dynamic, changing in orders of seconds.

188   Upon treatment with the F-actin depolymerising drug cytochalasin-D the peripheral dynamic F-actin

189   protrusions disappeared **(Fig. 2A lower panel)**, but the multilobed structures of the parasite were

190   not disrupted **(Video V6)**. The F-actin stabilising drug jasplakinolide also disrupted these filopodia-

191   like extensions and resulted in formation of thick filaments **(Fig 4A lower panel)**, implying the

192   requirement of dynamic regulation of F-actin for maintenance of these projections. The physiological

193   relevance of these filopodia is unclear at this point and further research into this phenomenon is

194   required.

195   **c. Apical polymerisation of F-actin in merozoites following egress.**

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196   Next, we wished to analyse the fate of the observed F-actin network upon parasite egress. We

197   synchronised parasites with a 2-step Percoll and sorbitol treatment and harvested schizonts at 44h

198   post-invasion. Reversible inhibitors of protein kinase G, Compound-1 and -2, stall schizont

199   development at very mature stages without allowing them to undergo egress (Collins, Hackett et al.

200   2013). We treated highly mature schizonts with Compound-2 to allow them to fully mature without

201   undergoing egress for 4h. Upon washing away Compound-2, the parasites egressed normally with

202   the concomitant appearance of F-actin accumulation at the apical tip of the parasite **(Fig. 2C, Video**

203   **V4)**. Cytochalasin-D treatment allowed normal egress of parasites, as previously observed (Video S8

204   of (Weiss, Gilson et al. 2015)), but completely abrogated F-actin polymerisation at the apical tip **(Fig.**

205   **2C)**. Finally, we performed IFA on invading merozoites using rhoptry neck protein 4 (RON4) as

206   junctional marker. We verified that F-actin accumulates just behind the RON4 ring **(Fig. 2D)**

207   confirming previous observations (Riglar, Richard et al. 2011, Angrisano, Riglar et al. 2012).

208   **d. Chromobody labelled F-actin structures disappear upon disruption of PfACT1.**

209   Although *P. falciparum* parasites possess two actin genes *pfact1* and *pfact2*, PfACT1 is the only

210   protein expressed during the asexual life cycle (Vahokoski, Bhargav et al. 2014). In order to confirm

211   that chromobodies label authentic F-actin structures based on polymerisation of PfACT1, we

212   transfected the chromobody constructs CB-EME **(Fig. 3A)** and CB-HALO **(Fig. S2B, C)** into a

213   conditional mutant of PfACT1 (loxPACT1) (Das, Lemgruber et al. 2017) **(Fig 3A)**. Upon activation of

214   DiCre with rapamycin, the *pfact1* locus is excised together with loss of PfACT1 protein within 35h

215   (Das, Lemgruber et al. 2017). Upon disruption of *pfact1* in 1h-old ring stages, CB-EME **(Fig. 3B, D, E,**

216   **Video V7)** and CB-HALO **(Fig. S2)** labelled F-actin structures completely disappeared in late

217   trophozoites and schizonts and closely resembled parasites treated with cytochalasin-D **(Fig. 1B)**. As

218   previously reported (Das, Lemgruber et al. 2017), PfACT1-disrupted parasites could not invade new

219   erythrocytes **(Fig. 3C)**.

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220   We previously found that apicoplast inheritance depends on PfACT1 (Das, Lemgruber et al. 2017). In

221   order to determine the localisation of F-actin during apicoplast segregation we used deconvolution

222   microscopy on fixed parasites stained with the apicoplast marker CPN60, which revealed a close

223   apposition of apicoplasts with F-actin structures **(Fig 3F DMSO)**. Upon disruption of PfACT1, a defect

224   in apicoplast segregation was apparent **(Fig 3F RAP)** recapitulating the phenotype observed

225   previously (Das, Lemgruber et al. 2017). Super-resolution microscopy confirmed that actin filaments

226   labelled by CB-EME are closely placed next to migrating apicoplasts **(Fig S1, lower panel)**. In contrast,

227   no obvious defects in mitochondria segregation could be detected in PfACT1-disrupted parasites

228   **(Fig. 3E and Video V7)** as previously reported (Das, Lemgruber et al. 2017), implying that unlike

229   apicoplasts, mitochondria do not require F-actin for migration into daughter cells. IMC markers

230   GAP45 and MTIP showed normal staining in CB-EME expressing parasites **(Fig S1)**.

231   We reasoned that since most canonical actin filament stabilising and nucleating proteins are absent

232   in Apicomplexa, the parasite must depend on formins for F-actin assembly. Previously, PfFRM1 has

233   been localised to the invasion junction and PfFRM2 to the cytosol (Baum, Tonkin et al. 2008). Since

234   we observed the intracellular F-actin network in the cytosol, we speculated that Formin-2 is the

235   main nucleator of F-actin during intracellular parasite development, even though it has been

236   implicated in host cell invasion in the case of *T.gondii* (Daher, Plattner et al. 2010).

237   **2. Apicomplexan Formin-2 sequences contain a PTEN-C2-like domain found usually in plant**

238   **formins**

239   Formins possess a formin homology (FH) 1 and an FH2 domain, which nucleate actin monomers as

240   well as elongate unbranched F-actin by continuous processive binding to the barbed end of the

241   filament (Courtemanche 2018). In a previous report (Baum, Tonkin et al. 2008), only FH1/FH2

242   domains were described for apicomplexan formins. Here, we queried for presence of known PFAM

243   domains using NCBI conserved domain search and in addition to FH1/FH2, found tetratricopeptide

244   repeat (TPR) domains in both PfFRM1 and TgFRM1, while a PTEN C2-like domain was recognised in

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245   PfFRM2 and TgFRM2 **(Fig. 4A)**. This led us to hypothesise that Formin-1 and Formin-2 with different

246   N-terminal domains diverged early in evolution and different domain organisations have been

247   retained for different functions. We queried for various FH2-domain containing proteins from

248   Apicomplexans and found that Formin-2-like sequences are found in a different clade from Formin-

249   1-like sequences **(Fig. 4B)**, as also previously noted (Baum, Tonkin et al. 2008). Strikingly, the PTEN-

250   C2-domain (or a diverged PTEN-C2 domain) was found only in Formin-2-like sequences **(Fig. 4B)**.

251   Interestingly, PTEN-C2 domains are important for membrane recruitment (Das, Dixon et al. 2003)

252   and it has been shown to be recruited to rice chloroplast membranes (Zhang, Zhang et al. 2011),

253   leading us to hypothesise that a similar mechanism operates for apicoplast recruitment of Formin-2

254   sequences in apicomplexans.

2. *Plasmodium* and *Toxoplasma* Formin-2 localise adjacent to apicoplasts

256   In order to characterise the role of Formin-2 in detail, we decided to perform a comparative analysis

257   in both *T.gondii* and *P. falciparum.* Therefore, we epitope tagged Formin-2 in both parasites. For

258   tagging in *T.gondii* we used a CRISPR/Cas9-based strategy to introduce a 3x hemagglutinin (3HA) tag

259   at the TgFRM2 C-terminus **(Fig. 4C)** and confirmed correct integration by diagnostic PCR **(Fig 4D).**

260   Upon colocalisation with the anti-apicoplast antibody G2-Trx (Biddau and Sheiner, unpublished), we

261   found TgFRM2 to be localised adjacent to apicoplasts **(Fig, 4E)**, which was confirmed by super-

262   resolution microscopy **(Fig. 4F)**. For localisation of PfFRM2, we simultaneously epitope tagged and

263   floxed PfFRM2 by single cross-over homologous recombination in a DiCre-expressing parasite strain

264   **(Fig. 4G)** and confirmed integrants by diagnostic PCR **(Fig. 4H)**. Integrants were cloned by limiting

265   dilution and two distinct clones of 'LoxPpfformin2' were used for phenotypic characterisation.

266   PfFRM2 showed a punctate pattern in context of PfACT1 antibody staining **(Fig 4I)**. Next, we checked

267   for PfFRM2 localisation in relation to the apicoplast and observed a close apposition of the

268   apicoplasts with the PfFRM2 staining throughout the 48h malaria life-cycle **(Fig. 4J),** which was

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269   confirmed by super-resolution microscopy **(Fig. 4K)**. In conclusion, both *Toxoplasma* and *P.*

270   *falciparum* Formin-2 localises adjacent to apicoplasts.

271   **3. DiCre-mediated conditional disruption of Formin-2 causes a defect in apicoplast segregation in**

272   *P. falciparum*

273   Next, we wished to evaluate the fate of *P. falciparum* upon conditional DiCre-mediated disruption of

274   the *pffrm2* gene **(Fig 5A)**. 1h old tightly synchronised ring stage parasites were divided into two

275   flasks and either pulse-treated with rapamycin (RAP) or DMSO (control) for 4h and their phenotype

276   determined at 44h post RAP-treatment. Excision was determined by diagnostic PCR of the genomic

277   locus **(Fig 5B)** and fitness of the PfFRM2 conditional knock out (KO) was measured by a growth curve

278   which showed significant loss of viability **(Fig. 5C)**. Loss of protein was ~90% as determined by

279   Western blot **(Fig. 5D)** and was confirmed by IFA **(Fig. 5E)**, which indicated a loss of protein in ~95%

280   parasites (N=350). Giemsa stained PfFRM2 KO parasites were dysmorphic with apparent inclusions

281   of haemoglobin **(Fig. 5F, red arrows).** In order to determine the morphological defects in FRM2 KO

282   parasites, we co-stained PfFRM2 KO parasites with several organellar markers and were unable to

283   see significant differences (not shown), except for apicoplast segregation **(Fig. 5G).** The number of

284   parasites with normally segregated apicoplasts was significantly reduced, with a high percentage of

285   cells showing collapsed or morphologically aberrant apicoplasts **(Fig 5G, H)**. A range of apicoplast

286   phenotypes was evident, from totally collapsed, intermediate to apparently normal **(Fig S3A)**. To

287   determine if the loss of viability of the PfFRM2 KO parasites was solely due to loss of the apicoplast,

288   we attempted to rescue the phenotype with 200μM isopentenyl pyrophosphate (IPP) which has

289   been previously shown to complement growth in parasites lacking apicoplasts (Yeh and DeRisi 2011).

290   However, we did not see any improvement in viability, indicating that the loss of fitness is due to

291   additional defects caused by abrogation of F-actin dynamics in the parasite. We determined number

292   of nuclei in 44h PfFRM2 KO parasites and found a significant decrease in the number of nuclei **(Fig.**

293   **S3B)**, indicating a developmental defect. Since PfACT1 is required for normal cytokinesis (Das,

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294   Lemgruber et al. 2017), we interrogated if the IMC is normally formed in PfRM2 KO parasites. We

295   purified mature schizonts on a 70% Percoll cushion and determined by IFA that IMC formation was

296   compromised in these parasites, with normal IMC staining dropping from 58±8% in WT to 19±8% in

297   PfRM2 KOs **(Fig. S3C, D)**. When we allowed PfRM2 KO parasites to egress and compared them to

298   control parasites, we found conjoined merozoites in PfRM2 KOs, a defect previously seen in PfACT1

299   KO parasites (Das, Lemgruber et al. 2017), indicating that PfRM2 and PfACT1 coordinate cytokinesis

300   in *P .falciparum*.

301

302   **4. DiCre-mediated disruption of Formin-2 abrogates the actin network in *P. falciparum***

303   **trophozoites**

304   Next, we wished to determine whether F-actin assembly and dynamics is interfered upon deletion of

305   PfRM2 and if this directly affects apicoplast segregation. We expressed CB-EME in LoxPpfformin2 to

306   generate the line LoxPpfformin2/CBEME **(Fig. 6A)** and visualised actin filaments **(Fig 6B, DMSO).** As

307   observed in wild-type parasites (**Fig 1),** F-actin was decorated with punctate PfRM2-HA staining **(Fig**

308   **S4, Pf)**. Upon DiCre-mediated excision in ring stages, we saw a complete abrogation of the dynamic

309   F-actin network in mature schizont stage parasites **(Fig 6B RAP)**, which dropped from exhibiting an F-

310   actin network in 92±5% cells in WT to 5±4% cells in PfRM2 KO **(Fig. 6C)**. Furthermore, we confirmed

311   the apicoplast phenotype in parasites expressing CB-EME **(Fig 6D)**.

312   Interestingly, we observed that actin-rich filopodia formed at the cell-periphery (see also **Fig. 2A, B)**

313   disappeared upon PfRM2 disruption **(Fig. 6E)**, providing a mechanism for this novel phenomenon.

314   Since PfRM1 was localised to the parasite apex/ invasion junction during host cell entry (Baum,

315   Tonkin et al. 2008), we reasoned that apical polymerisation of F-actin should not be affected in

316   PfRM2 KO parasites, if indeed the two formins perform distinct functions in their distinct

317   localisations. To this end, we allowed mature schizonts to egress and release free merozoites and

13

318   subsequently imaged them by live fluorescence microscopy. Consistent with this hypothesis, we

319   found that the ability of F-actin polymerisation at the parasite apex was not compromised in PfFRM2

320   KO parasites **(Fig. 6F)**, strongly indicating distinct functions of PfFRM1 and PfFRM2.

321

322   **5. Conditional deletion of Formin-2 in *Toxoplasma* disrupts apicoplast segregation and F-actin**

323   **dynamics**

324   Finally, in order to assess if the function of Formin-2 is conserved in apicomplexan parasites, we

325   analysed its role in *T.gondii.* We first checked the localisation of TgFRM2 with respect to the F-actin

326   network. Similar to *P. falciparum*, TgFRM2-HA formed puncta on CB-EME labelled F-actin network

327   within the parasite, where it appeared to co-localise with a polymerisation centre **(Fig. S4 Tg)**. Next,

328   we simultaneously floxed *tgfrm2* together with addition of C-terminal YFP tag to create the

329   LoxPTgFRM2 line **(Fig. 7A).** This enabled us to confirm localisation of Formin-2 and determine the

330   comparative effect of a conditional KO in *Toxoplasma*. Integration of the C-terminal YFP-tag and

331   *LoxP* sites was confirmed by diagnostic PCR, as was excision of the *tgfrm2* locus upon RAP-treatment

332   **(Fig S5A)**. For the localisation of TgFRM2 it was necessary to stain fixed parasites with a YFP-

333   antibody, suggesting low expression levels of TgFRM2. We confirmed localisation of TgFRM2

334   adjacent to the apicoplast **(Fig 7B upper panel)**. Upon RAP-treatment, excision of TgFRM2 was

335   apparent in 14% (clone A) or 33% (clone B) of parasites, as assessed by quantification of parasites

336   where no TgFRM2 could be detected by IFA. Importantly, loss of TgFRM2 staining correlated with an

337   apicoplast segregation phenotype in the majority of parasites **(Fig 7B lower panel, 42.4 % [n=66] in**

338   **clone A and 70% [n=100] in clone B)**. A baseline apicoplast segregation phenotype was observed in

339   2% (clone A) or 1% (clone B) of vacuoles in the control population. Transient expression of CB-EME in

340   LoxPTgFRM2 parasites enabled us to image F-actin and demonstrated that, in good agreement with

341   data from *P. falciparum*, intracellular F-actin was adjacent to the apicoplast **(Fig 7C control).**

342   Intriguingly, excision of TgFRM2 **(Fig. 7C RAP)** led to the disappearance of intracellular F-actin, while

14

343   (in contrast to *P. falciparum*), the intravacuolar F-actin network was still present (**Fig.7C**), indicating

344   that another formin, potentially Formin-3 (Daher, Klages et al. 2012), which is not present in

345   *P.falciparum*, contributes to its formation.

346

347   **Discussion**

348   Due to the unconventional behaviour of apicomplexan actin, the visualisation of actin filaments in *P.*

349   *falciparum* was hampered by the lack of reagents and F-actin sensors that do not interfere

350   significantly with F-actin polymerisation and depolymerisation. Therefore, previous attempts to use

351   established indicators such as Life-Act from other eukaryotic systems failed (Tardieux 2017). In a

352   recent study it was shown that actin-binding nanobodies fused to epitope tags could be expressed in

353   *Toxoplasma gondii,* allowing for the first time to analyse F-actin localisation and dynamics in living

354   parasites (Periz, Whitelaw et al. 2017). Here we successfully adapted this technology to *P. falciparum*

355   using two different epitope tags, the halo tag and the emerald tag. This allowed us to visualise F-

356   actin throughout the asexual life-cycle of *P. falciparum* and in gametocytes without causing any

357   aberrant phenotypes, suggesting that this reagent, as in the case of *T.gondii* (Periz, Whitelaw et al.

358   2017) and all other eukaryotes tested so far (Melak, Plessner et al. 2017) does not significantly

359   interfere with F-actin dynamics. Importantly, validation of this reagent using either F-actin

360   modulating drugs or a conditional mutant for PfACT1 led to expected results and phenotypes,

361   demonstrating that F-actin dynamics is finely balanced in the parasite. Since expression of

362   chromobodies does not interfere with parasite viability, it can be assumed that its influence on F-

363   actin dynamics is at best minor, potentially leading to slightly altered F-actin dynamics. Similar

364   influences of F-actin sensors have been observed and discussed in other eukaryotes (Melak, Plessner

365   et al. 2017). As a general rule, any F-actin sensor will influence F-actin dynamics or might not be able

366   to stain all F-actin structures. In this respect, F-actin binding nanobodies are a novel tool and appear

367   to have only minimal effects on F-actin dynamics compared to other sensors (Melak, Plessner et al.

368   2017), which might explain that they are well tolerated in Apicomplexans, where rapid F-actin

369   dynamics appears to be critical for parasite viability. Using this novel tool, we not only demonstrate

370   here that F-actin can be found in very close proximity of apicoplasts, we also demonstrate a novel

371   function of F-actin during the intracellular development of *P. falciparum*: formation of highly

372   dynamic, filopodia-like structures. While the role of this process is currently unknown, we show that

373   interference with F-actin dynamics, either by addition of actin-modulating drugs of conditional

374   mutants for PfACT1 or PfFRM2, led to a complete blockade in the formation of these structures.

375   Super-resolution imaging revealed a complex F-actin network in *P. falciparum*, similar to that

376   observed in *T.gondii* (Periz, Whitelaw et al. 2017) with extensive filaments around the FV.

377   Importantly, our previous characterisation of a conditional mutant for PfACT1 highlighted the

378   diverse functions of actin during the asexual life cycle of the parasite (Das, Lemgruber et al. 2017),

379   which perfectly correlates to the localisation and dynamics found here using chromobody-expressing

380   parasites. PfACT1 is essential for *P. falciparum* invasion into erythrocytes and we show for the first

381   time the temporal and spatial dynamics of actin polymerisation by live microscopy prior to invasion.

382   Despite a growing body of evidence suggesting the importance of calcium signalling and

383   phosphorylation of IMC proteins by kinases such as CDPK1 and PKA (Baker, Drought et al. 2017,

384   Kumar, Kumar et al. 2017) during invasion, what triggers the polymerisation of actin is largely

385   unknown. Our data suggest that early signalling events just after egress are a trigger for actin-

386   polymerisation at the apical end. This is likely to be mediated by an apically resident nucleator of F-

387   actin, a likely candidate being Formin-1, since PfFRM2 KO parasites could still polymerise actin at the

388   apical end, as demonstrated in this study.

389   Therefore, the expression of chromobodies in *P. falciparum* allows us to phenotypically probe the

390   state of the F-actin network *in vivo* in a rapid and robust manner. F-actin can be clearly visualised

391   during growth, in invading merozoites and in gametocytes – opening up many avenues for further

392   research. Using this novel tool, combined with powerful reverse genetics made possible by the DiCre

393   system (Collins, Das et al. 2013) we show here that Formin-2 in both *Toxoplasma* and *Plasmodium* is

394   required for the intracellular polymerisation of F-actin, a mechanism employed by the parasite for

395   correct segregation of apicoplasts. Using extensive bioinformatic searches within alveolates, we

396   found the presence of a PTEN-C2-like domain only in apicomplexan Formin-2 sequences. This

397   domain has been demonstrated in rice to be responsible for Formin-2 targeting to chloroplast

398   membranes (Zhang, Zhang et al. 2011). It is therefore likely that the apicomplexan PTEN-C2-like

399   domain is used for apicoplast recruitment of apicomplexan Formin-2.

400   Interestingly, while we found that the role of Formin-2 appears to be conserved in *T.gondii* and *P.*

401   *falciparum* for intracellular F-actin dynamics and apicoplast inheritance **(Fig. 8)**, in the case of *T.*

402   *gondii* the intravacuolar F-actin network is still formed, suggesting that *T.gondii* and potentially other

403   coccidia have additional, compensatory mechanisms at their disposal to form this network, which

404   appears to be critical for material exchange, synchronised replication of parasites and host cell

405   egress (Periz, Whitelaw et al. 2017).

406   In conclusion, we show here that chromobodies can be used to determine F-actin dynamics in

407   apicomplexan parasites and will form the basis for functional *in vivo* studies of other actin regulatory

408   proteins found in apicomplexans. Furthermore, they can be used as an efficient tool to probe for

409   drugs specifically interfering with F-actin dynamics in apicomplexans.

410

411   **Experimental procedures**

412   **1. Culture and transfection of *P. falciparum***

413   *P. falciparum* was cultured in RPMI 1640 with Albumax (Invitrogen) and schizonts were purified on a

414   bed of 70% Percoll as described previously (Blackman 1994). About 10 µg of plasmid was ethanol

415   precipitated and resuspended in 10 µL sterile buffer TE (Qiagen). The Amaxa™ P3 primary cell 4D

416   Nucleofector™ X Kit L (Lonza) was used for transfections. The input DNA was added to 100 µL P3

17

417 primary cell solution, mixed with 10-20 µL of packed synchronous mature schizonts and added to the

418 cuvette, which was electroporated in a 4D-Nucleofector machine (Lonza) using the program FP158.

419 The transfected schizonts were rapidly added to 2 mL of complete medium (RPMI with Albumax

420 supplemented with glutamine) containing erythrocytes at a haematocrit of 15%, and left shaking in a

421 shaking incubator at 37°C for 30 min. Finally the cultures were supplemented with 7 mL of complete

422 RPMI medium to obtain a final haematocrit of 3% and incubated overnight at 37°C in a small angle-

423 necked flask (Nunc™). Parasites were selected by use of appropriate drug medium. The culture

424 medium was subsequently exchanged every day for the next 4 days to remove cell debris which

425 accumulates during electroporation and then twice a week until parasites were detected by Giemsa

426 smear. Drug-resistant parasites were generally detectable in thin blood films 2-3 weeks post-

427 transfection. After this, parasite stocks (at ~5% ring parasitaemia) were cryopreserved in liquid

428 nitrogen. Lines were then cloned by limiting dilution using a simple plaque assay (Thomas, Collins et

429 al. 2016).

430 **2. Cloning and expression of chromobodies in *P. falciparum***

431 The CB-HALO and CB-EME plasmid consists of a sequence encoding actin chromobody from

432 Chromotek followed downstream by an in frame sequence encoding Halo (Promega) or the emerald

433 tag. CB-EME and CB-HALO sequences were amplified by PCR and cloned into the vector pB-

434 map2gfpdd (Nicholas Brancucci, unpublished) via restriction sites NheI and HindIII to remove the

435 map2gfpdd sequence and put the CB-sequences under the *hsp86* promoter. The resulting plasmids

436 pB-CBEME and pB-CBHALO were sequenced on both strands to confirm correct nucleotide

437 sequences. These constructs were transfected as described into the loxPACT1 parasite clone B2 (Das,

438 Lemgruber et al. 2017) to obtain parasite lines LoxPPfACT1/CBEME, LoxPPfACT1/CBHALO and into

439 the parental 1G5DiCre clone (Collins, Das et al. 2013) to obtain the line CBEME/1G5DiCre and

440 CBHALO/1G5DiCre. Lines were selected with 2.5 µg/mL blasticidin. CB-EME expression was visible by

441 fluorophore excitation/emission in the green range and the HALO ligand was made visible by use of

18

442   the ligand HALO-TMR at 1:40,000 with excitation/emission in the red range. Alternatively antibodies

443   were used against the HALO tag to stain for CB-HALO.

444   **3. *P. falciparum* IFA**

445   Thin blood films were made on glass slides and fixed in 4% paraformaldehyde in PBS for 20 min. The

446   slides were then permeabilised with 0.1% Triton-X/PBS for 10 min, washed and blocked overnight in

447   4% BSA/PBS. Antigens were labelled with suitable primary and secondary antibodies in 4% BSA/PBS

448   with 5 min PBS washes in between. Slides were finally air dried and mounted with DAPI-Fluormount-

449   G® (SouthernBiotech).

450   Staining of the RON4 junction in CB-EME expressing was performed by fixation and immunostaining

451   in solution as described previously (Riglar, Richard et al. 2011).

452   For image acquisition, z–stacks were collected using a UPLSAPO 100× oil (1.40NA) objective on a

453   Deltavision Core microscope (Image Solutions – Applied Precision, GE) attached to a CoolSNAP HQ2

454   CCD camera. Deconvolution was performed using SoftWoRx Suite 2.0 (Applied Precision, GE).

455   An Elyra S1 microscope with Superresolution Structured Illumination (SR-SIM) (Zeiss) was used for

456   super-resolution imaging.

457   **4. Time lapse microscopy of live *P. falciparum***

458   Video microscopy of *P. falciparum* schizont egress was performed as described previously (Collins,

459   Hackett et al. 2013). Synchronised schizonts were Percoll® purified and treated with 1 µM C2 in

460   RPMI medium with Albumax® (Gibco) for 4h. Microscopy chambers (internal volume ~80 µl) for

461   observing live schizonts were built by adhering 22×64 mm borosilicate glass coverslips to microscope

462   slides with strips of double-sided tape, leaving ~4 mm gaps at each end. C1 was washed off before

463   video microscopy and the schizonts were immediately resuspended into warm (37°C) RPMI (with

464   Albumax) and introduced by capillary action into the pre-warmed chamber. The chamber was

465 transferred to a temperature-controlled microscope stage at 37°C on a Deltavision Core microscope

466 (Image Solutions – Applied Precision, GE). Images were routinely collected at 5 s intervals, beginning

467 6 min 30 sec after washing off C1, over a total of 30 min.

468 Other than during egress, CB-EME and CB-HALO expressing parasites were imaged at intervals of 1

469 sec.

470 **5. Bioinformatics**

471 Proteomes of interest (Table T2) were downloaded from the UniProt-KB website (www.uniprot.org).

472 These were concatenated into a single proteome sequence dataset. All sequence identifiers and

473 annotations referred to are from UniProt Hidden Markov Models (PFAM profiles) PF02181.23

474 (FH2.hmm, Formin Homology 2 Domain) and PF10409.9 (PTEN_C2.hmm, C2 domain of PTEN

475 tumour-suppressor protein) were downloaded from Pfam (El-Gebali, Mistry et al. 2018). These

476 profiles were used with the HMMER package (HMMER 3.1b1 (May 2013); http://hmmer.org/) to

477 search the proteome sequences (hmmsearch), and to align sequences of interest (hmmalign). The

478 proteome sequence dataset was searched for FH2 domains (FH2.hmm) with hmmsearch, and

479 sequences with regions scoring >28bits recorded. These sequences were retrieved from the dataset,

480 and subjected to alignment against the FH2.hmm. The profile conformant subsequences were

481 extracted from the alignment and this sequence set subjected to alignment using:

482 1) hmmalign to FH2.hmm, 2) clustalw (Thompson, Higgins et al. 1994) 3) muscle (Edgar 2004) and 4)

483 T_Coffee. These multiple sequence aligments were combined and evaluated in T_coffee (Keller,

484 Kollmar et al. 2011) using the -aln and -special_mode evaluate options of T_coffee and the

485 alignment edited to remove columns of avgerage quality <4 and occupancy <30% (T_coffee -

486 other_pg seq_reformat option). Rooted neighbour-joining trees of Formin Homology type 2 domains

487 (FH2) was contructed from this alignment (or subsets of it) using the SplitsTree program [version

488 1.14.8,*]. The proteome dataset was searched for the presence of PTEN_C2 conformant sequences.

489 As only an inconsistent subset of sequences were found in both PTEN_C2 and FH2 selected

20

490   sequences; one such subsequence (A0A1A7VGT3_PLAKH, residues 1096-1238) was used as the

491   query of an iterative psi-blast [@], (E-value cutoff =10) using the proteome data set as the database.

492   The program converged after 3 iterations. The sequences flagged by psi-blast as having PTEN_C2-like

493   sequence were compared with the sequences flagged by hmmsearch as having FH2 domains, and

494   such sequences annotated on the phylogenetic tree.

495   **6. Creation of LoxPPfFRM2-HA and LoxPPfFRM2/CBEME strains**

496   To obtain conditional truncation of the *pffrm2* gene we used silent *loxP* sites within a heterologous

497   *P. falciparum* intron loxPint (Jones, Das et al. 2016). We ordered from Geneart® a ~800 bp targeting

498   sequence followed by the LoxPint module in the context AATTGTAG-LoxPint-ATAGCTTT followed by

499   a recodonised version of rest of the 3' region of the gene together with a C-terminal 3HA tag. This

500   ordered synthetic fragment was cloned into the pHH1-loxPMSP1 plasmid (Das, Hertrich et al. 2015)

501   via restriction sites AflII and ClaI, replacing the msp1 sequence with *pffrm2*, giving rise to the plasmid

502   pHH1-LoxPintFormin2 **(Fig. 4G).** This was transfected into the DiCre expressing strain B11 (Perrin,

503   Collins et al. 2018) and integrants selected by cycling on and off the drug WR99210 (Jacobus

504   Pharmaceuticals, New Jersey, USA). The integrant line LoxPPfFRM2 was cloned by limiting dilution

505   and two clones used for phenotypic characterisation. The strain LoxPPfFRM2/CBEME was created by

506   transfecting the pB-CBEME plasmid into a LoxPPfFRM2-HA clone line and transfectants selected

507   using the drug blasticidin (Sigma).

508   **7. Conditional truncation of *pfact1* and *pffrm2***

509   Various floxed parasite strains were synchronised by Percoll and sorbitol as previously described

510   (Collins, Hackett et al. 2013). Briefly, schizonts were purified on a bed of 66% Percoll and allowed to

511   reinvade into fresh erythrocytes for 1-2h. The remainder of the schizonts were removed by Percoll

512   and the freshly invaded rings were subjected to 5% sorbitol for 7 min at 37°C to lyse any remaining

513   schizonts. The tightly synchronised rings were divided into two flasks and pulse-treated for 4h at

21

514   37°C with 100 nM rapamycin or with 1% DMSO as control. The rings were then washed and returned

515   to culture. Phenotypic analysis was performed primarily 44h post RAP-treatment unless stated

516   otherwise.

517

518   **8. Culturing of *Toxoplasma* parasites and host cells**

519   Human foreskin fibroblasts (HFFs) (RRID: CVCL_3285, ATCC) were grown on tissue culture-treated

520   plastics and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%

521   foetal bovine serum, 2 mM L-glutamine and 25 mg/mL gentamycin. Parasites were cultured on HFFs

522   and maintained at 37° C and 5% CO_2. Cultured cells and parasites were regularly screened against

523   mycoplasma contamination using the LookOut Mycoplasma detection kit (Sigma) and cured with

524   Mycoplasma Removal Agent (Bio-Rad) if necessary.

525   **9. Microscopy for *Toxoplasma***

526   Widefield images were acquired in z-stacks of 2 µm increments and were collected using an Olympus

527   UPLSAPO 100× oil (1.40NA) objective on a Delta Vision Core microscope (AppliedPrecision, GE)

528   attached to a CoolSNAP HQ2 CCD camera. Deconvolution was performed using SoftWoRx Suite 2.0

529   (AppliedPrecision, GE). Further image processing was performed using ImageJ software.

530   Super-resolution microscopy (SR-SIM) was carried out using an ELYRA PS.1 microscope (Zeiss) as

531   described previously (Periz et al., 2017). Images were acquired using a Plan Apochromat 63×, 1.4 NA

532   oil immersion lens, recorded with a CoolSNAP HQ camera (Photometrics), and analysed using ZEN

533   Black software (Zeiss) and ImageJ software.

534   **10. *Toxoplasma* IFA**

535   For immunofluorescence analysis, HFF monolayers infected with *Toxoplasma* parasites were grown

536   on coverslips and fixed at the indicated time points in 4% paraformaldehyde for 20 min at RT.

22

537   Afterwards coverslips were permeabilised in 0.2% Triton X–100 in 1× PBS for 20 min, followed by

538   blocking (3% BSA & 0.2% Triton X–100 in 1x PBS) for at least 30 min. The staining was performed

539   using indicated combinations of primary antibodies for 1 h and followed by secondary Alexa Fluor

540   488 or Alexa Fluor 594 conjugated antibodies (1:3000, Invitrogen–Molecular Probes) for another 45

541   min, respectively. The primary antibodies used in this studies are anti-G2Trx (1:500, rabbit,

542   apicoplast, Dr Lilach Sheiner), anti-HSP60/CPN60 (1:2000, rabbit, apicoplast, Dr Lilach Sheiner), anti-

543   Atrx1 (1:500, mouse, apicoplast, Prof Peter Bradley), anti-HA (1:500, rat, Roche [cat# 1187431001])

544   and anti-YFP (1:500, rabbit, Abcam [ab6556

11. Generation of the TgFRM2-HA and loxPTgFRM2-YFP strains in RHΔku80DiCre parasites

546   Guide RNAs targeting the upstream region of TgFRM2 and the C-terminal region were designed

547   using EuPaGDT (Ref: Duo Peng and Rick Tarleton. EuPaGDT: a web tool tailored to design CRISPR

548   guide RNAs for eukaryotic pathogens. Microbial Genomics. 2015. doi: 10.1099/mgen.0.000033).

549   These were cloned into a vector expressing a Cas9-YFP fusion as well as the specific gRNAs as

550   previously described (Curt-Varesano, Braun et al. 2016). The designed gRNAs

551   ACTTTTCATAGTATAGGAGG CGG and AATAGGGGTCTGTAGGTTAA GGG bind 989 bp upstream of the

552   start codon and 12 bp upstream of the stop codon of TgFRM2 respectively. To introduce the

553   upstream LoxP site, the LoxP sequence ATAACTTCGTATAGCATACATTATACGAAGTTAT flanked with

554   respective 33 bp homology on each side was ordered as a 100 bp primer (ThermoFischer Scientific).

555   The repair template for the C-terminal tag (HA or YFP) was generated by PCR using Q5 polymerase

556   (New England Biolabs) from template plasmids with 50 bp of target-specific homology introduced via

557   the primer. All tags are flanked by the same sequence, the upstream linker sequence

558   GCTAAAATTGGAAGTGGAGGA encoding for the amino acid sequence AKIGSGG, the tag itself, a stop

559   codon and the LoxP sequence. The YFP tag is superfolder YFP 2, and was sub-cloned from pSYFP2-C1

560   (gift from Dorus Gadella (Addgene plasmid # 22878; http://n2t.net/addgene:22878;

561   RRID:Addgene_22878) (Kremers, Goedhart et al. 2006). All C-terminal repair templates were pooled,

562   purified using a PCR purification Kit (Blirt). Together with 10 μg Cas9 vector encoding the respective

563   gRNA, 1 x 107 of freshly released RHΔku80DiCre tachyzoites (an improved version created by Dr

564   Moritz Treeck from the original (Andenmatten, Egarter et al. 2013)) were transfected using 4D

565   AMAXA electroporation. 24 hours after transfection, parasites were mechanically released, filtered

566   and sorted for transient YFP expression into 96 well plates using a FACS sorter (FACSARIA III, BD

567   Biosciences). Individual plaques were screened by PCR and the C-terminus of TgFRM2 was

568   sequenced (Eurofins Genomics). Into a clone with TgFRM2-YFP-LoxP, the upstream LoxP was

569   introduced as described. Screening for upstream LoxP integration was performed by PCR with a

570   primer binding at the junction of gRNA binding sequence and LoxP site. Using a different set of

571   primers, the complete upstream LoxP site was amplified via PCR and verified by sequencing. Two

572   distinct clones were obtained for LoxPTgFRM2 (clone A and B) and used for phenotypic

573   characterisation.

574   **12. Induction of the conditional TgFRM2 KO**

575   To obtain TgFRM2 KO parasites, the loxPTgFRM2-YFP parental line was grown in 50nM rapamycin

576   containing media as described above until fixing. In IFA, TgFRM2 KO parasites were always compared

577   to a control population of untreated loxPTgFRM2-YFP.

578   To quantify TgFRM2-YFP excision 48h post inoculation, 100 vacuoles were counted for each clone

579   (clone A and B) and condition (RAP treated vs control population). The vacuoles were assessed with

580   regards to their loss of FRM2-YFP signal. A single IFA was counted for each clone. To assess the

581   apicoplast segregation phenotype in FRM2-YFP negative vacuoles, 66 (clone A) and 100 vacuoles

582   (clone B) were counted. For this, quantification was achieved by using the same IFA that was used

583   for the excision rate quantification.

584   **13. Transient transfection of CB-EME into *Toxoplasma* parasites**

585   To have parasites transiently expressing CB-EME, 1 x 107 of freshly released TgFRM2-HA or

586   loxPTgFRM2-YFP parasites were transfected with 20 µg DNA by AMAXA electroporation.

587   Subsequently, parasites were grown on HFFs as described above and fixed with 4%

588   paraformaldehyde after 48h or 72h.

589   **Table T1. Antibodies used in this study**

Antibody	Reference
Anti actin	(Angrisano, Riglar et al. 2012), RRID: AB_2665920
Anti CPN60 (apicoplast)	(Agrawal, van Dooren et al. 2009)
Anti RON4	(Richard, MacRaild et al. 2010)
Anti GAP45	(Jones, Kitson et al. 2006)
Anti MTIP	(Jones, Kitson et al. 2006)
Anti-enolase	(Dutta, Tewari et al. 2018)
Anti-HA	Roche (Cat #118743100)
Anti-YFP	Abcam (Cat #ab6556)
Anti-Atrx1	(DeRocher, Coppens et al. 2008)
Anti-G2Trx	Biddau and Sheiner, unpublished.

590

Author contributions

592   SD performed *P. falciparum* experiments. JFS performed *T. gondii* experiments. MS produced the

593   TgFRM2-HA and LoxPTgFRM-YFP strains. JW performed bioinformatic analyses. SD and MM

594   conceived the project. SD, MM and JFS wrote the manuscript.

25

Figure and Movie Legends

Figure 1, Chromobody constructs with different epitope tags label F-actin structures throughout the *P. falciparum* lifecycle. **A.** Chromobody constructs used in this study under the *hsp86* promoter with an emerald tag (CB-EME) and halo tag (CB-HALO). Blasticidin-S-deaminase (BSD) confers resistance to blasticidin. **B.** CB-EME and CB-HALO label actin filaments (DMSO), which disappear upon cytochalasin-D treatment (+CytoD). White arrows denote examples of F-actin structures. Also see **Video V3**. **C.** Super resolution imaging reveals an actin network in *P. falciparum* (CBEME), which stains partially similar to an actin antibody (actin). DAPI labels nuclei. See also **Fig. S1**. **D.** The F-actin network and dynamics can be visualised in gametocytes (see also **Video V5**). Brightfield images provided in greyscale alongside. Scale bar 5μm.

Figure 2, Rapid actin dynamics are visible during intracellular growth, egress and invasion. A. Filopodia-like protrusions from the parasite cell body extend into the host cell cytosol (white arrows), which are disrupted by cytochalasin-D (+CytoD) and jasplakinolide (+JAS). **B.** Time lapse images show rapid changes in filopodia-like protrusions (white arrows). See also **Video V2**. CB-EME visible in the green channel, and brightfield images have been provided below in greyscale. **C.** Time lapse images show actin polymerising at a polar end of the merozoite, post egress (CB-EME). Corresponding brightfield images have been presented. Cytochalasin-D treatment prohibits the polar polymerisation of actin (+CytoD). **D.** IFA of invading merozoites with the junction marker RON4 shows CB-EME staining close to the RON4 stain, implying that F-actin polymerises at the apical end prior to invasion.

Figure 3, CB-EME staining of the F-actin network disappears upon genetic disruption of PfACT1: A. Schematic of transfection of CB-EME into the loxPpfact1 strain and PfACT1 loss upon DiCre-activation using rapamycin. **B.** The F-actin network (CB-EME, green and anti-PfACT1, red DMSO) is lost upon DiCre-mediated disruption of PfACT1 (RAP). DAPI labels nuclei. **C.** Giemsa-stained parasites showing invasion is abrogated in PfACT1 KOs. **D.** Live microscopy of CB-EME expressing parasites and

625 the loss of fluorescence intensity upon RAP-treatment. Right panel shows quantification of

626 fluorescence intensities. **E.** Stills from live imaging of CB-EME expressing parasites co-stained with

627 Mitotracker (mitochondria). The branched mitochondrial structure (DMSO) is not disrupted upon

628 loss of PfACT1 (RAP). See also **Video V7**. **F.** IFA showing apicoplasts (red) colocalised with branches of

629 the F-actin network (CB-EME, DMSO) and the disruption of the network together with apicoplasts

630 when PfACT1 is deleted (RAP). Scale bar 5μm.

631 **Figure 4, Apicomplexan formins have distinct protein domains, with Formin-2 localising to**

632 **apicoplasts in *Toxoplasma* and *P. falciparum*: A.** Other than the conserved FH1/FH2 domains, Pf/Tg

633 Formin-1 contain tetratricopeptide repeat domains, while Pf/Tg Formin-2 contain a PTEN C2-like

634 domain. **B.** Rooted neighbour-joining tree of FH2 domains detected in apicomplexan sequences

635 flagged by hmmsearch and extracted from alignments produced by hmmalign, both using the PFAM

636 profile PF02181.23. Proteins with sub-sequences similar to PTEN-C2 domains (detected by psi-Blast)

637 are indicated with circular leaf symbols (and shaded blue). Those sequences annotated as Formin-1

638 (#) and Formin-2 (*) are indicated. Colour coding of the leaf nodes: Red: Plasmodium, Green:

639 Eimeria, Magenta: Sarcocystidae, Cyan: Piroplasmida, Black: Cryptosporidium, Grey: Gregarinidae **C.**

640 Strategy depicting endogenous C-terminal HA tagging of TgFRM2 in Toxoplasma. CRISPR/Cas9 was

641 exploited to introduce a double-stranded DNA break and repair DNA amplified by PCR with

642 homologous DNA regions coding for 3xHA. **D.** Diagnostic PCR confirming integration of DNA

643 described in C into the RH_Δku80_DiCre line. **E.** IFA showing localisation of TgFRM2-HA (green) at

644 the vicinity of the apicoplast staining (anti-G2Trx, red). Nuclei are stained with DAPI (blue). White

645 dotted line depicts the parasite vacuole outline. Toxoplasma parasites were fixed 24h after

646 inoculation. **F.** Super-resolution microscopy confirming the close apposition of TgFRM2-HA (green)

647 to the apicoplast (anti-G2Trx, orange). Toxoplasma parasites were fixed 24h after inoculation. Scale

648 bar is 2.5μm. **G.** Strategy showing simultaneous floxing and C-terminal HA tagging of the *pfformin2*

649 locus using single cross over recombination into a DiCre expressing strain to give rise to the

650 LoxPpfformin2 strain (modified). Primers for diagnostic PCR have been annotated as half arrows. **H.**

27

651   Diagnostic PCR confirming integration in one of the two transfected lines (integrant). **I.** IFA showing

652   localisation of PfFRM2-HA (green) in the context of a PfACT1-antibody staining (red). Nuclei are

653   stained with DAPI (blue). **J.** IFA showing localisation of PfFRM2-HA adjacent to the apicoplast using a

654   CPN60 antibody (red) throughout *P. falciparum* intracellular development (20, 40, 48h). **K.** Super-

655   resolution image confirming the tight apposition of PfFRM2 (green) with apicoplasts (orange). Scale

656   bars are 5µm, except where stated otherwise.

657   **Figure 5, Conditional deletion of PfFRM2 disrupts apicoplast segregation and causes a severe**

658   **fitness defect: A.** Strategy showing the DiCre-mediated genomic excision of the LoxPpfFRM2 locus.

659   Primers for diagnostic PCR have been annotated as red/blue half arrows. **B.** Diagnostic PCR

660   confirming genomic excision of the *pffrm2* locus upon rapamycin treatment (+). **C.** A growth curve

661   showing the relative fitness of RAP-treated PfFRM2 KO parasites in comparison to DMSO controls.

662   Various time points from the pulse treatment of 1h-old rings at time 0 in the 1st, 2nd and 3rd growth

663   cycles have been measured. **D.** left panel, Western blot showing the loss of PfFRM2-HA upon RAP-

664   treatment, enolase has been used as a control. Right panel, Quantification of PfFRM2-HA from three

665   different blots shows at least a 10-fold drop in protein levels, Error bars depict SD. **E.** IFA showing

666   loss of PfFRM2-HA staining (green) upon RAP-treatment. Levels of PfACT1-staining (red) do not

667   change. **F.** Giemsa stained images of RAP-treated parasites reveal dysmorphic parasites. **G.**

668   Apicoplast segregation (red) is affected to various degrees in RAP-treated parasites as compared to

669   DMSO controls. See also **Figs. S3A** and **6D**. **H.** Quantification of phenotypes seen in **G.** Error bars

670   depict SD. **I.** Isopentenyl pyrophosphate (IPP) cannot rescue the fitness defect (RAP+IPP) in PfFRM2

671   KO parasites (RAP) as compared to parasitemia to the DMSO controls. Error bars depict SD. Scale

672   bars 5 µm.

673   **Figure 6, Conditional deletion of PfFRM2 abrogates the intracellular F-actin network but not the**

674   **apical polymerisation of F-actin prior to invasion: A.** Strategy showing episomal expression of CB-

675   EME in the LoxPpffrm2 background. **B.** Upper panels: Stills from a video showing loss of normal

676   intracellular F-actin fluorescence (green). Brightfield images have been provided. See also **Video V8**.

677   Lower left panel: Zoomed images of indicated parasites in upper panels showing loss of the actin

678   network in RAP (none, red arrows) as compared to DMSO controls (normal). **C.** Graph showing loss

679   of normal F-actin fluorescence in ~95% RAP-treated parasites. >90% of DMSO controls show

680   presence of the network. **D.** IFA staining of the apicoplast with a CPN60 antibody (red) on top of the

681   fluorescent F-actin network (green) shows a defect in apicoplast segregation in RAP treated

682   parasites (white arrows). Nuclei are stained in blue and brightfield images are provided alongside.

683   Examples of normally segregated apicoplasts, intermediate and severely disrupted apicoplasts have

684   been provided. **E.** Loss of filopodia in RAP-treated young trophozoites imaged 72h post-RAP

685   treatment. Stills from a video have been shown, where time points are depicted in white. Normal

686   filopodia are marked with white arrows in DMSO controls. **F.** Post-schizont egress, merozoites from

687   DMSO controls and RAP-treated group show similar propensity to polymerise apical F-actin (CB-EME

688   fluorescence shown in green). Red arrows show apical F-actin in zoomed images (lower panels).

689   Scale bars 5µm.

690   **Figure 7. Conditional deletion of TgFRM2 disrupts normal segregation of apicoplasts together with**

691   **abrogation of the intracellular F-actin polymerisation centre. A.** Strategy to generate LoxPTgFRM2-

692   YFP, a floxed and C-terminal YFP-tagged *TgFRM2* locus in the RH_Δku80_DiCre line. For this purpose,

693   CRISPR/Cas9 was exploited to introduce DNA double-strand breaks in the 5' UTR and C-terminus of

694   the *TgFRM2* gene. Integration was confirmed by PCR (see **Fig. S5A**). Arrows represent PCR primers

695   used in **Fig. S5A**. **B.** IFA staining with anti-YFP (TgFRM2-YFP) and anti-Atrx1 (apicoplast) shows an

696   apicoplast segregation defect in TgFRM2-YFP KO parasites. In control parasites, TgFRM2-YFP

697   localises to the vicinity of the apicoplast (upper panel). The loss of TgFRM2-YFP causes an apicoplast

698   segregation defect (lower panel, white arrow). The lower panel depicts a TgFRM2-YFP KO vacuole

699   together with a TgFMR2-YFP positive vacuole for comparison. Scale bars are 5µm. **C.** IFA depicting

700   CB-EME and apicoplast (anti-CPN60) in control and RAP-treated LoxPTgFRM2-YFP parasites. In

701   untreated parasites, the apicoplast localises to intracellular actin polymerisation centres (control,

29

702 white arrows in zoom). Parasites exhibiting TgFRM2 KO-specific apicoplast phenotype lack

703 intracellular actin polymerisation centres. Zoomed images depict indicted areas. Scale bars are 5µm.

704 IFAs depicted in this figure were performed for the LoxPTgFRM2 clone B.

705 **Figure 8. A model showing the dependence of apicoplast segregation on the intracellular F-actin**

706 **network in *P. falciparum* and *Toxoplasma*.** In apicomplexan parasites, despite using different

707 replication modes (endodyogeny, schizogony), the function of actin and Formin-2 is highly conserved

708 with regards to intracellular F-actin dynamics and apicoplast inheritance. One difference appears to

709 be the formation of an intravacuolar network that can still occur in the case of *T.gondii* but not *P.*

710 *falciparum* upon disruption of Formin-2.

711 **Figure S1, Super-resolution of the CB-EME-labelled F-actin network: A.** Super-resolution imaging of

712 schizonts show the F-actin network (CB-EME, yellow) co-stained in magenta with IMC-markers

713 GAP45, MTIP and the apicoplast marker CPN60. Note the very close apposition of the apicoplasts

714 with the F-actin network. DAPI stains nuclei (cyan). **B.** Mean fluorescence intensity of the CB-EME

715 channel of each of the schizonts has been presented, N=40. Error bars show SD.

716 **Figure S2**: **CB-HALO labels the F-actin network similar to CB-EME**. **A.** IFA of CB-HALO expressing

717 parasites showing staining with anti-PfACT1 and with an anti-HALO antibody. Note that the anti-

718 HALO antibody shows background staining in the parental strain. **B.** Intensity of CB-HALO expression

719 and filament structures (red) are lost upon PfACT1 disruption (RAP). **C.** IFA with anti-GAP45

720 antibodies show normal formation of merozoites in PfACT1-disrupted parasites (RAP) expressing CB-

721 HALO.

722 **Figure S3. Additional defects in merozoite formation/ cytokinesis in PfFRM2 KO parasites. A.** IFA

723 image showing schizonts with varying degrees of apicoplast (red) segregation defect 44h post-RAP-

724 treatment of LoxPpffrm2. An example of an intermediate apicoplast defect has been shown (white

725 arrow). Nuclei are stained in blue. **B.** Number of nuclei in the IFA from A were binned to <5, 5 to 16

30

726   and 16+ in DMSO controls and RAP-treated parasites. The graph shows a general reduction in the

727   number of nuclei in RAP-treated parasites. **C.** LoxPpffrm2/CBEME parasites were stained with an

728   anti-GAP45 antibody (red) 44h post DMSO/RAP-treatment and examples of the IMC defect have

729   been provided. CBEME fluorescence is shown in green. **D.** Upon quantification of C, normally formed

730   merozoites reduced significantly in PfFRM2 KOs. **E.** When DMSO/RAP-treated schizonts were

731   allowed to egress, conjoined merozoites were apparent in the RAP treated controls (red arrows) but

732   not in DMSO controls.

733   **Figure S4. Formin-2 localises to the F-actin network in *P. falciparum* and *T.gondii*: Upper panel.** IFA

734   depicting PfFRM2-HA localisation using an anti-HA antibody in *P. falciparum* expressing CB-EME

735   (green). PfFRM2-HA localises to F-actin (merge, white arrows). Nuclei were stained with DAPI (blue).

736   **Lower panel.** IFA showing staining for TgFRM2-HA using an anti-HA antibody in *Toxoplasma*

737   transiently expressing CB-EME (green). TgFRM2-HA localised in close proximity to intracellular actin

738   polymerisation events (merge, white arrows). Parasites were transiently transfected with CB-EME

739   and grown for 48h. Scale bars are 5µm**.**

740   **Figure S5. Loss of TgFRM2-YFP upon RAP-treatment**

741   **A.** Integration PCR for TgFRM2-YFP parasites described in **Fig. 7** confirming 5' integration (i) and 3'

742   integration (ii). While the amplification of YFP indicates the presence of non-induced parasites in the

743   rapamycin-treated population (iii), excision of TgFRM2-YFP could only be shown for parasites

744   growing under rapamycin (iv). For the excision PCRs (iii, iv) parasites were grown with or without

745   50nM Rapamycin and mechanically lysed after 48h prior to gDNA collection. Coloured arrows refer

746   to **Fig. 7A** and indicate the sequence amplified by PCR. **B.** IFA depicting apicoplast (anti-CPN60) and

747   background fluorescence in LoxPTgFRM2-YFP parasites (clone B), which outlines the mitochondria.

748   In the control population (without rapamycin treatment), the YFP-tagged FRM2 was not detectable

749   in the absence of an YFP-antibody. RAP-treated parasites show the characteristic mislocalisation of

31

750   apicoplast material into the residual body, while mitochondria appear unaffected. Parasites were

751   grown for 72h before fixation. Scale bars are 5µm.

752   **Movie V1, Rapid shape changes of ring stages of *P. falciparum* expressing CB-EME (green).**

753   Acquisition time is shown in seconds. Scale bar 5µm.

754   **Movie V2, Dynamic Filopodia-like F-actin extensions from the parasite edges into the RBC cytosol.**

755   Acquisition time is shown in seconds. Scale bar 5µm.

756   **Movie V3, Dynamic actin filaments in CB-EME expressing parasites (DMSO) are disrupted upon**

757   **addition of cytochalasin-D.** The green channel shows CB-EME expression. Brightfield images also

758   shown. Acquisition time is shown in seconds. Scale bar 5µm**.**

759   **Movie V4, Polar polymerisation of F-actin at the merozoite tip following egress.** Time lapse images

760   of a representative schizont which undergoes egress, followed by polymerisation of F-actin at the

761   merozoite edge (white arrows appearing). Images (green channel, CBEME) and brightfield

762   (greyscale) were acquired every 5 sec. Acquisition time is shown in seconds. Scale bar 5µm.

763   **Movie V5, F-actin dynamics in gametocytes.** Two representative examples of gametocytes

764   expressing CB-EME show dynamic filaments running along the parasite length and enriched at the

765   tips. Acquisition time is shown in seconds. Scale bar 5µm.

766   **Movie V6, Multilobular structures of trophozoites are not lost upon addition of cytochalasin-D.**

767   **Movie V7, CB-EME staining disappears upon conditional genetic deletion of *pfact1*.** Ring stage

768   LoxPpfACT1/CBEME parasites were pulse treated with DMSO or RAP for 4h and imaged after 40

769   hours. CB-EME was imaged in the green channel and shows a disappearance of F-actin upon RAP-

770   treatment. Mitochondria were stained with Mitotracker® (red channel). Acquisition time is shown in

771   seconds. Scale bar 5µm.

772 **Movie V8, Actin filaments disappear upon genetic deletion of *pffrm2.*** Ring stage

773 LoxPpfFRM2/CBEME parasites were DMSO- or RAP-treated for 4h and imaged 40 hours later. CB-

774 EME was imaged in the green channel and shows a disappearance of intracellular F-actin upon RAP-

775 treatment. Acquisition time is shown in seconds. Scale bar 5μm.

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937

Figure 1

A

B

C

D

CB-EME   brightfield

CB-HALO +ligand   brightfield

DMSO

+CytoD

DAPI   CB-EME   actin   merge   composite

CB-EME   brightfield   CB-EME   brightfield

gametocyte

Figure 2

Figure 3

A

loxP-pfact1

target | syn | *pbdt3´*

+ *hsp86* chromo EME BSD

↓ rapamycin

hsp86 chromo EME BSD

target

disrupted loxPpfact1

+

B

| | DAPI | CB-EME | anti-PfACT1 | merge | brightfield |

DMSO

RAP

C

CB-EME DMSO | CB-EME RAP

D

CB-EME | brightfield

DMSO

RAP

Mean Intensity (AU)

DMSO RAP

E

CB-EME | mitochondria | brightfield

DMSO

RAP

F

DAPI | CB-EME | apicoplast | merge 1 | merge 2 | brightfield

DMSO

RAP

Figure 4

A
Pf/Tg Formin-1
TPR TPR — FH1 FH2

Pf/Tg Formin-2
PTEN — FH1 FH2

B
annotated Formin-2
Piroplasmida
Plasmodia
Eimeridae
Sarcocystidae
Gregarinidae
Cryptosporidium
annotated Formin-1
Arabidopsis

C
5'UTR — Tgfrm2 — STOP — 3'UTR
STOP
HA
STOP
5'UTR — Tgfrm2 — HA — 3'UTR

D
-ve / RH_Δku80_DiCre / TgFRM2_HA
bp
3000
1500
500

E
DAPI | TgFRM2-HA | apicoplast | anti-HA/DAPI | anti-HA/apicoplast

F
DAPI | TgFRM2-HA | apicoplast | merge
super-resolution

G
pfformin2
target
loxPint
target syn HA
pHH1-LoxPintformin2
WR
B11/DiCre
target syn HA WR target
modified pfformin2 locus

H
Integrant | Endogen.
1 2 | 1 2
bp
2000
1000
700
300
200

I
Integrant
DAPI | PfFRM2-HA | anti-PfAct1 | merge

J
DAPI | PfFRM2-HA | apicoplast | anti-HA/apicoplast | merge
24h 2um
40h
48h

K
super-resolution
nucleus | PfFRM2-HA | apicoplast

Figure 5

Figure 6

Figure 7

Figure 8

Formin-2 WT   Formin-2 cKO

T. gondii

intercellular F-actin network

intercellular F-actin network unaffected

apicoplasts disrupted

intracellular F-actin

intracellular F-actin abrogated

P. falciparum

apicoplasts disrupted

intracellular F-actin

intracellular F-actin abrogated

● nucleus   ● apicoplast   ○ Formin-2   ⌇ F-actin

Figure S1

A

| DAPI | CB-EME | GAP45 | merge |

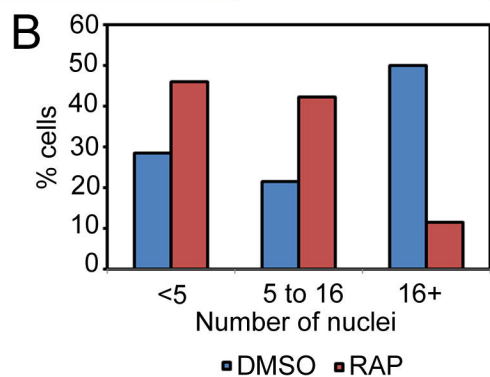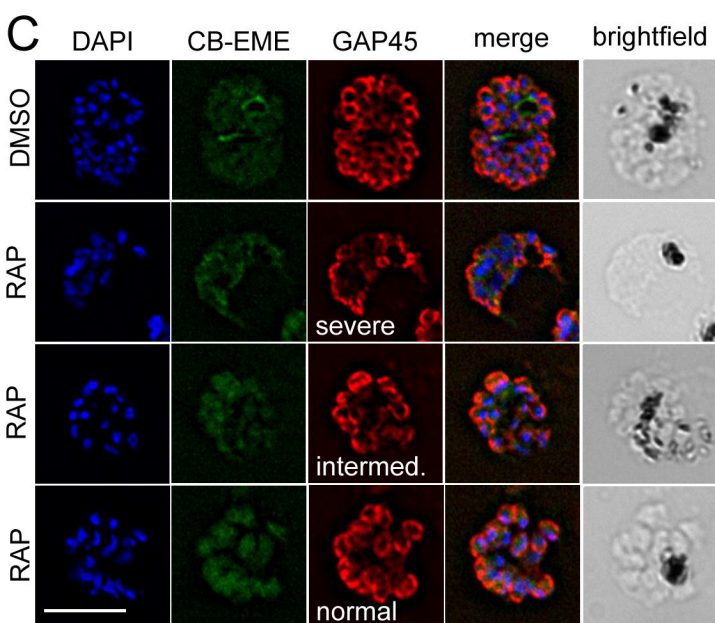| DAPI | CB-EME | MTIP | merge |

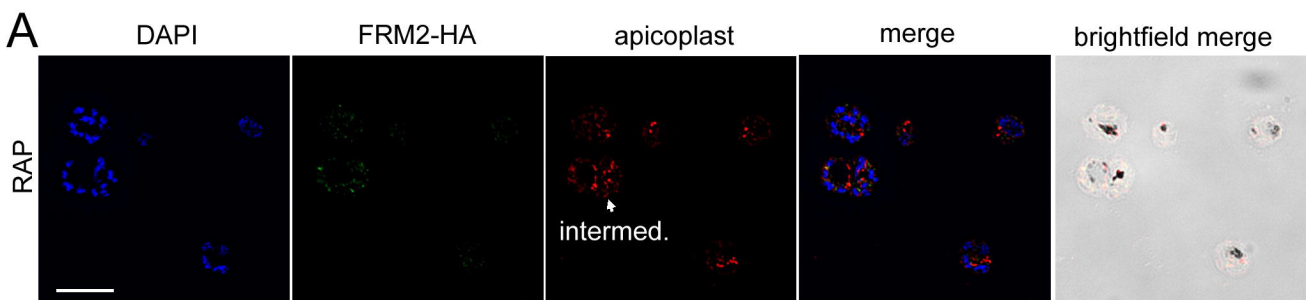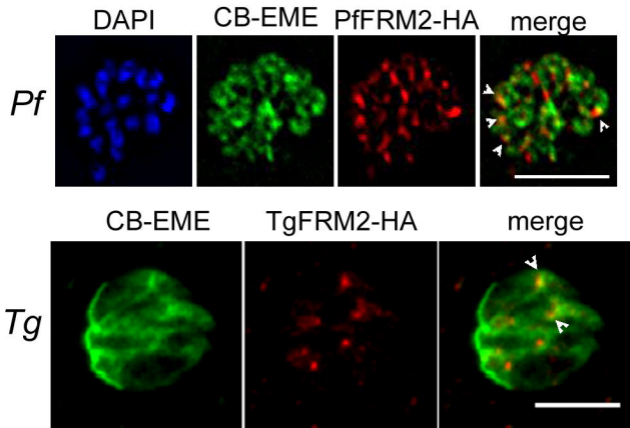| DAPI | CB-EME | apicoplast | merge |

B

Mean intensity (AU)
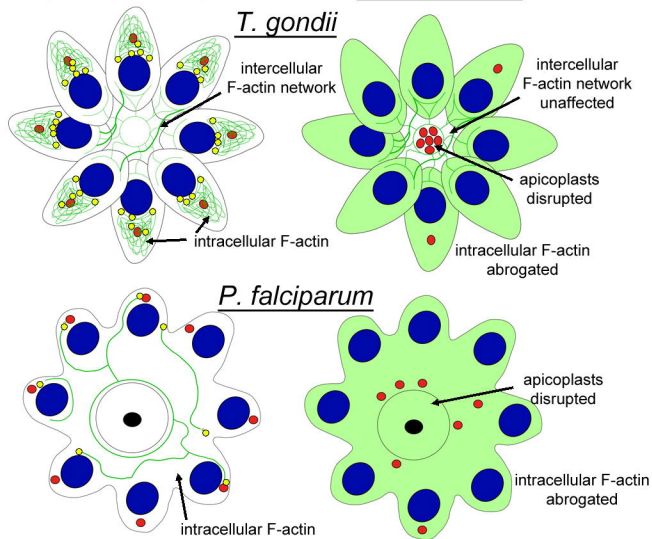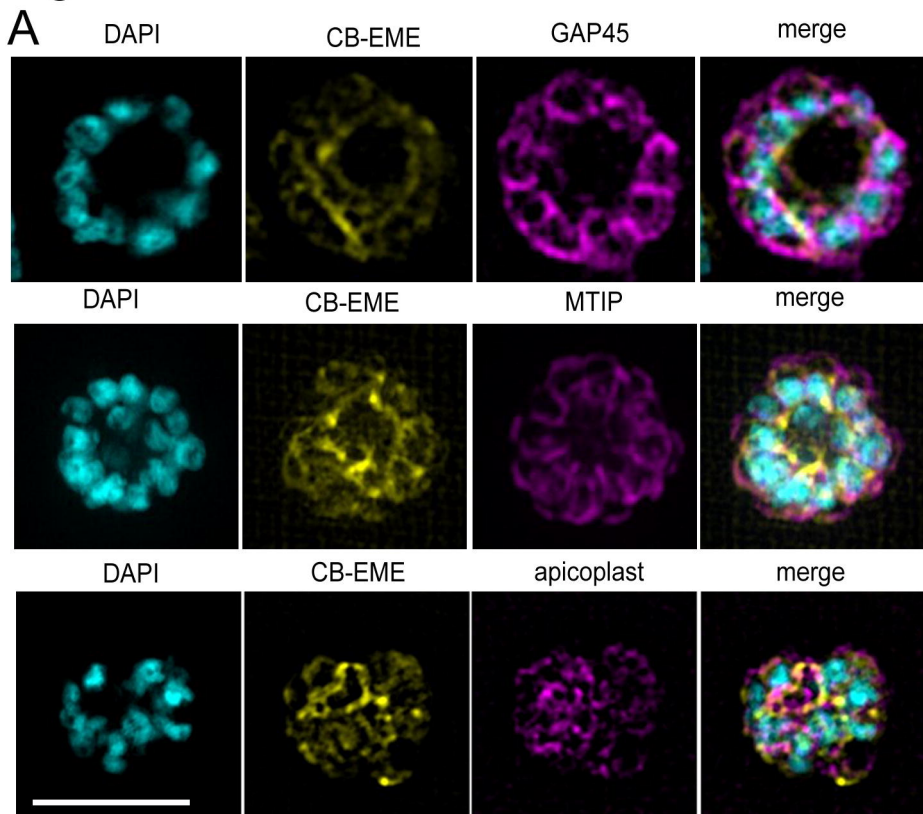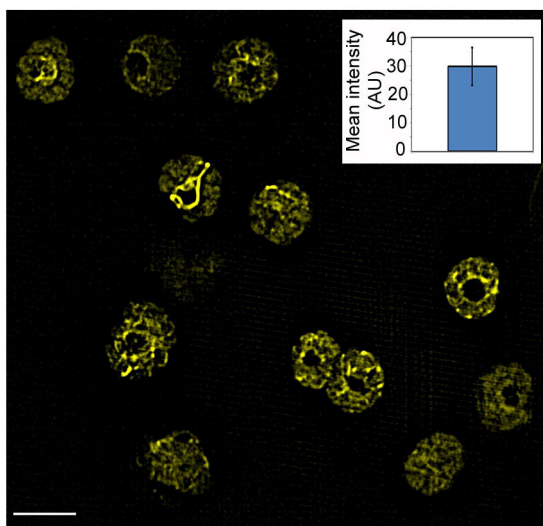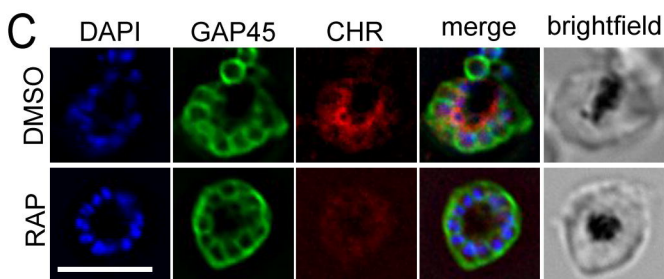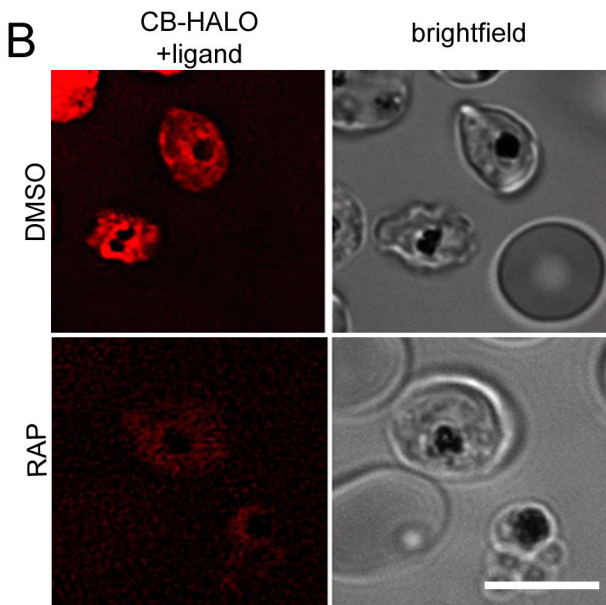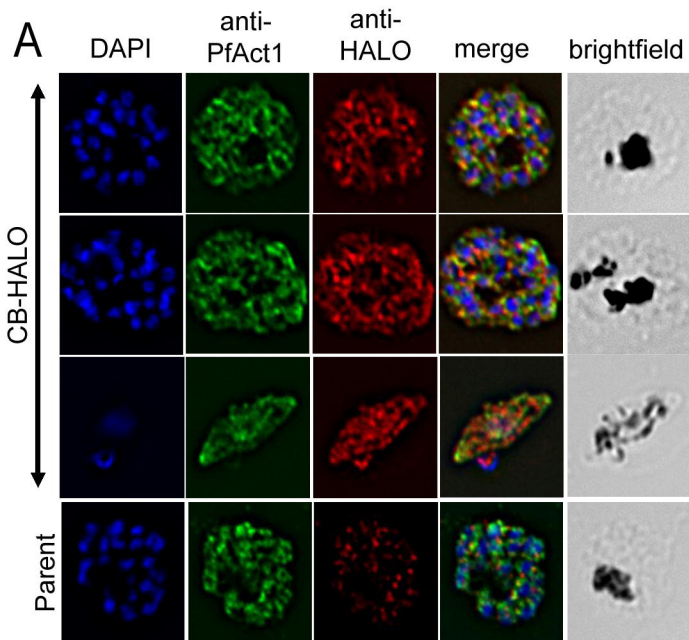
Figure S2

Figure S3

Figure S4

Figure S5

A

(i)

bp — 1 2 3 4
3000
500

(ii)

bp — 1 2 3 4
1500
1000
500

(iii)

bp — 1 | 3 | 4 | RAP
              − + | − +
3000
1500
1000

(iv)

bp — 1 | 3 | 4 | RAP
              − + | − +
2000
1000
300
200

1: -ve; 2: RH_Δku80_DiCre
3,4: loxP_TgFRM2_YFP_loxP clones A,B

B

DAPI | background (green) | apicoplast | brightfield/apicoplast

control

RAP